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Introduction

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INTRODUCTION: Immune Relevant Animal Models: Opportunities and Challenges

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Abstract

Valid interpretation of preclinical animal models in immunology-related clinical challenges is important to solve outstanding clinical needs. Given the overall complexity of the immune system and both species- and tissue-specific immune peculiarities, the selection and design of appropriate immune-relevant animal models is, however, not following a straightforward path. The topics in this issue of the *ILAR Journal* provide assessments of immune-relevant animal models used in oncology, hematopoietic-, CAR-T cell- and xenotransplantation, adjuvants and infectious diseases, and immune privileged inflammation that are providing key insights into unmet human clinical needs.

Key words: animal models; immunology; preclinical validity

Animal models are imperative for studies relating to immunology-related problems because the immune system is a highly complex network of different cell types, secreted cytokines, chemokines, and other humoral factors that function in an organ- or tissue-dependent context that is virtually impossible to emulate by in vitro studies. Given the large number of cells, transcription factors, and genetic pathways involved from activation to regulation and execution of immune-mediated effectors, the differences between animal species are highly relevant for each and every animal model used for translational studies of clinical immunology-related problems. In line with this, there is a constant debate about the relevance and predictive or translational value of immunology-related research in rodents [1–3] and reproducibility of results obtained from mice and other research models is also a well-publicized concern [4,5]. These translational challenges eventually lead to premature clinical trials with current estimates of probability of success of a clinical trial ranging from a minimum of 3.4% for oncology to a maximum of 33.4% for infectious disease vaccines [6].

In this special issue of the *ILAR Journal*, the contributors provide assessments of immune-relevant animal models from small to large that are providing key insights into unmet clinical needs for the advancement of human health. Each article outlines the current state of the art and provides deeper insights into a few selected animal models with the most promise to translate into a better understanding and treatment of clinical immunology-related problems.

Mice have been instrumental animal models for the enormous advancement of immunological understanding over the last decades. The comprehensive review by Radaelli et al [7] provides an updated summary of immune-relevant mouse strains and stocks as well as mutations and experimental interventions to induce specific perturbations of the immune system. Careful informed selection and use of the numerous mouse models will further improve the translational utility, validity, and reproducibility of research in mice.

The prospects of treating cancer by appropriate activation of the patient's own immune system is both appealing and highly promising, with cancer immunotherapy being named "breakthrough of the year" by the journal *Science* in 2013. However, relevant animal models with high translational validity for the human clinical setting are scarce. Overgaard et al [8] highlight the special opportunities in use of spontaneous tumors in dogs and experimental cancer models in pigs to study different phases of cancer immunoediting in immunocompetent large animal hosts.

Although animal models in mice and dogs have been invaluable in the development of effective treatment of malignant and nonmalignant hematological disorders by hematopoietic cell transplantation, there are still significant clinical needs to

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address. In the contribution by Graves et al [9], animal models in mice and dogs are reviewed for their potential to solve the most important areas of concern in disease relapse and graft-versus-host disease in hematopoietic cell transplantation treatment.

Adoptive transfer of engineered chimeric antigen receptor (CAR)-T cells is a promising therapy for treatment of cancers and chronic viral infections where immunity relies on cell-mediated effector mechanisms. Preclinical studies to predict efficacy and safety of new CAR-T cell developments are not easy to translate to the human clinic. In this context, Migliorini et al [10] review preclinical investigation of CAR-T cells in different humanized mouse models, in dogs where immune and nonimmune system networks are intact, and in nonhuman primate animal models with optimal translational relevance to humans for safety evaluations.

Platt et al [11] discuss successes and failures in xenotransplantation including how immunity functions as a barrier for xenograft acceptance and outlines how the new developments in genetic engineering of pigs have contributed to dramatic improvement in the outcome of experimental xenografts in nonhuman primates. The advances in stem cell research has further spurred the development of reverse xenotransplantation where human stem cells are transplanted into a pig host and undergo development and maturation to mature cells, or a tissue or an organ, before being transplanted back as an allograft into the original human donor.

Advances in vaccine development rely on the selection of antigenic targets for the adaptive immune response and how these antigens are delivered and presented for the immune system. For inactivated and subunit vaccines targeting infections where a more complex immune response than the antibody titer is needed, the adjuvant formulation of the vaccine may thus decide the efficacy of the induced antibody response. Schmidt et al [12] review methods for assessing humoral and, more difficultly, cell-mediated adjuvant efficacy and function in animal models. They further highlight examples where immune responses in mice poorly correlate with human immune responses and discuss how detailed characterization of cellular subset responses in mice may translate to humans.

To understand the interplay between infectious agents and both innate and adaptive host immune responses, appropriate animal models for the individual infectious agents must also be carefully selected. Starbæk et al [13] review virus receptor distribution and host anti-viral protein responses in different animal models for the studies of Influenza A virus with a special focus on pigs because they, in contrast to mice and ferrets, have high target and face validity being naturally infected with the same virus subtypes and with a display of similar clinical symptoms.

Another important infectious disease demonstrating the poor translational value of rodent models is enterotoxigenic *Escherichia coli* infections. Liu and Gi [14] investigate the suitability and significance of the pig model to explore mechanisms of nutritional supplements on gut health with an emphasis on resistance to enteric enterotoxigenic *Escherichia coli* infections in young children in lower income countries.

The eye, like the brain and uterus in pregnancy, is considered an immune-privileged site where immune-mediated inflammation is normally greatly reduced to protect from the consequences of inflammation. Ocular tolerance is lost in dry eye and uveitis. In the review by Gilger [15], common immune-relevant models of dry eye and uveitis are described with an overview of the immuno-pathogenesis of each disease and evaluation of models from small to large animals. For translational models of immune-mediated ocular disease in humans, there are naturally occurring large animal models, equine uveitis and canine dry eye, that have promise to translate into a better understanding and treatment of noninfectious immune-mediated ocular diseases in humans.

References

1. Handel AE, Lincoln MR, Ramagopalan SV. Of mice and men: experimental autoimmune encephalitis and multiple sclerosis. *Eur J Clin Invest*. 2011;41:1254–8.
2. Seok J, Warren HS, Cuenca AG et al. Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proc Natl Acad Sci USA*. 2013;110:3507–12.
3. Takao K, Miyakawa T. Genomic responses in mouse models greatly mimic human inflammatory diseases. *Proc Natl Acad Sci U S A*. 2015;112:1167–72.
4. Masopust D, Sivula CP, Jameson SC. Of mice, dirty mice, and men: using mice to understand human immunology. *J Immunol*. 2017;199(2):383–8 doi:10.4049/jimmunol.1700453.
5. Kafkafi N, Agassi J, Chesler EJ et al. Reproducibility and replicability of rodent phenotyping in preclinical studies. *Neurosci Biobehav Rev*. 2018;87:218–32 doi:10.1016/j.neubiorev.2018.01.003.
6. Wong CH, Siah KW, Lo AW. Estimation of clinical trial success rates and related parameters. *Biostatistics* 2019;20(2):273–86 doi: 10.1093/biostatistics/kxx069.
7. Radaelli E, Santagostino SF, Sellers RS, et al. Immune relevant and immune deficient mice: options and opportunities in translational research. *ILAR Journal* ily026 doi:10.1093/ilar/ily026. [Epub ahead of print].
8. Overgaard NH, Fan TM, Schachtschneider KM, et al. Of mice, dogs, pigs, and men: choosing the appropriate model for Immuno-oncology research. *ILAR Journal* ily014 doi:10.1093/ilar/ily014. [Epub ahead of print].
9. Graves SS, Parker MH, Storb R. Animal models for preclinical development of allogeneic hematopoietic cell transplantation. *ILAR Journal* ily006 doi:10.1093/ilar/ily006. [Epub ahead of print].
10. Migliorini D, Mason NJ, Posey AD. Keeping the engine running: the relevance and predictive value of preclinical models for CAR-T cell development. *ILAR Journal* ilz009 doi:10.1093/ilar/ilz009. [Epub ahead of print].
11. Platt JL, Cascalho M, Piedrahita JA. Xenotransplantation: progress along paths uncertain from models to application. *ILAR Journal* ily015 doi:10.1093/ilar/ily015. [Epub ahead of print].
12. Schmidt ST, Pedersen GK, Christensen D. Rational design and in vivo characterization of vaccine adjuvants. *ILAR Journal* ily018 doi:10.1093/ilar/ily018. [Epub ahead of print].
13. Starbæk SMR, Brogaard L, Dawson HD, et al. Animal models for influenza a virus infection incorporating the involvement of innate host defenses: enhanced translational value of the porcine model. *ILAR Journal* ily009 doi:10.1093/ilar/ily009 . [Epub ahead of print].
14. Liu Y, Ji P. Dietary factors in prevention of pediatric *Escherichia coli* infection: a model using domestic piglets. *ILAR Journal* ilz005 doi:10.1093/ilar/ilz005. [Epub ahead of print].
15. Gilger BC. Immune relevant models for ocular inflammatory diseases. *ILAR Journal* ily002 doi:10.1093/ilar/ily002. [Epub ahead of print].

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Review Article

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Immune Relevant and Immune Deficient Mice: Options and Opportunities in Translational Research

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Abstract

In 1989 ILAR published a list and description of immunodeficient rodents used in research. Since then, advances in understanding of molecular mechanisms; recognition of genetic, epigenetic microbial, and other influences on immunity; and capabilities in manipulating genomes and microbiomes have increased options and opportunities for selecting mice and designing studies to answer important mechanistic and therapeutic questions. Despite numerous scientific breakthroughs that have benefitted from research in mice, there is debate about the relevance and predictive or translational value of research in mice. Reproducibility of results obtained from mice and other research models also is a well-publicized concern. This review summarizes resources to inform the selection and use of immune relevant mouse strains and stocks, aiming to improve the utility, validity, and reproducibility of research in mice. Immune sufficient genetic variations, immune relevant spontaneous mutations, immunodeficient and autoimmune phenotypes, and selected induced conditions are emphasized.

Key words: biomedical research; experimental conditions; genetic background; genetic variation; immune system diseases; inbred strains; mice

Introduction

The main advantages of using mice in research include (1) their small size and very prolific nature, (2) the numerous commonalities existing between mice and humans in terms of physiology and pathobiology, (3) the well-characterized genomes and immune responses, and (4) the availability of advanced technologies for genetic and other experimental manipulations.[1,2] Despite the advantages, there is ongoing controversy surrounding the reproducibility and translatability of mouse models of disease.[3–5] Given the immune diversity within the human population, a perfect model relevant to all humans may be neither an achievable nor a reasonable expectation. However, it is possible to strive for relevant and reproducible translational models and to expect experimental designs to address specific research questions. Criticisms of mouse models (mouse blaming) are not always justified. Many factors contribute to study outcomes and reproducibility. These include genetic diversity; microbial, husbandry, and other environmental factors; experimental interventions; etc.[2] Increasing the awareness of the immunobiological variations among inbred mice and their substrains as well as other factors that may impact immune responses in mice will help improve both the validity and reproducibility of mouse-based research. Attention to these aspects is warranted in experimental design, data interpretations, and reporting of research on immunity, disease, and therapeutic interventions.[6,7]

This review aims to provide a useful compendium of resources and references for those investigators who seek to familiarize themselves with key concepts of mouse immunology

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and translate those notions into the experimental setting. The most relevant sources of immune diversity of the laboratory mouse are here emphasized with a focus on immune sufficient genetic variations, immunologically relevant spontaneous mutations, autoimmune phenotypes, and selected induced immune deficiencies.

Mouse Nomenclature

Accurate mouse nomenclature is mission critical to scientific communication.[8–10] Nomenclature "rules" for mice genes, strains, and substrains were recommended by scientists to the scientific community in the 1940s and 1950s. The first committees on standardized genetics nomenclature[11] and on standardized strain nomenclature for mice[12] included Nobel laureate George Snell. Early publications provided guidelines for gene and strain nomenclature, a list (database) of strains and substrains, and a list (database) of abbreviations for the researchers or institutions maintaining the mice.[12] The list of abbreviations became the "laboratory codes" (lab codes) that are currently curated by ILAR (http://dels.nas.edu/global/ilar/lab-codes) and are available to producers and researchers at no charge. The lab code identifies the mouse source and becomes part of its name. The 1963 revision includes a listing of named genes, including histocompatibility alleles for many of the common strains. Subsequent committees updated the guidelines and included lists of inbred strains, substrains, and known genetic variants.[13–19] These publications are enlightening regarding the history and research use of contemporary mouse strains. They indicate recognition by the scientific community of the research implications of genetic and phenotypic variations, and reflect scientists' concerns for accurate communication in published research. In 1972, a recommendation was published for standardized nomenclature for outbred stocks of laboratory animals of various species.[20] These recommendations gained traction for mice and rats, but far less for other species. Current gene nomenclature "rules" for mice (International Committee on Standardized Genetic Nomenclature for Mice: http://www.informatics.jax.org/mgihome/nomen/strains.shtml), rats (Rat Gene Nomenclature Committee: https://rgd.mcw.edu/nomen/nomen.shtml), and human genes (HUGO Gene Nomenclature Committee: http://www.genenames.org/) are available online. Guidance for mouse strains, genes, alleles/mutations as well as tutorials and assistance can be accessed from Mouse Genome Informatics Nomenclature sites (http://www.informatics.jax.org/mgihome/nomen/gene.shtml). Recommendations for reporting animal research include correct nomenclature because it communicates key research-relevant elements of the strain or substrain history and genetics, genetic modifications, backcrossing or intercrossing, and other information.[21–23]

Inbred Mouse Strains: Immune Relevant Genotypes and Phenotypes

The immune sufficient common inbred mouse strains are genetically well characterized, with genome projects on more than 30 strains.[24,25] Divergent susceptibilities of inbred strains to infections, diseases, and tumor rejection were recognized early in strain development. Characterization of these variations has exposed research-relevant Th1 or Th2 biases, diversity in major histocompatibility complex (MHC) haplotypes, natural killer (NK) cell repertoires, hemolytic complement (complement component 5 or C5) activity, and toll-like receptor (TLR) function, among others.[7,26,27] Table 1 and Supplementary Table 1 summarize some of the well-characterized immune relevant variations among immune sufficient common inbred mouse strains. Investigations on how penetrance and expressivity of immune phenotypes vary across different genetic backgrounds have enabled the discovery of key strain-related genetic modifiers that specifically enhance or suppress the manifestation of immunological disorders. This genetic source of diversity can be ultimately ascribed to a number of possible genetic alterations/variations including polymorphic alleles, unique quantitative trait loci (QTL) intervals, or specific haplotypes.[28–36] The influence of the inbred genetic background pervades many if not all the experimental contexts considered in this review.

Immune Relevant Variations Among Substrains

Substrains with quite similar names harbor important genetic (and other) variations that are increasingly recognized.[55,74–76] C57BL/6N and C57BL/6J substrains diverged in 1951, so acquisition of mutations among colonies inbreeding at different sites is unsurprising. As illustrated in Table 2, some immune relevant genetic variations among C57BL/6 substrains include a *Nlrp12* mutation in C57BL/6J mice and a *Dock2* mutation in C57BL/6NHsd mice from certain colonies.[55,77] The *Nlrp12* gene primarily controls neutrophil chemotaxis in response to bacterial invasion. C57BL/6J mice carry a missense, loss of function mutation ($Nlrp12^{C57BL/6J}$) and are more susceptible to certain bacterial infections compared with other C57BL/6 substrains harboring the wild-type *Nlrp12* allele.[55,73] More concerning may be when mutations arise within a substrain (of the same name) with colonies maintained at different sites. The $Dock2^{Hsd}$ mutation was revealed when reduced splenic marginal zone B cells and increased numbers of CD8+ T cells were identified in C57BL/6NHsd (and derived mutant mice) relative to other C57BL/6N mice.[77–79] Subsequently, Envigo tested their mice and reported that this mutation ($Dock2^{Hsd}$) was present in 6 of their 19 C57BL/6NHsd colonies (http://www.envigo.com/assets/docs/c57-customer-communication-2-final-9jun16.pdf). Many research programs maintain in-house colonies of genetically engineered animals and "wild-type" background strains that warrant genetic quality assurance (QA) testing and breeding strategies to minimize effects of random mutations and genetic drift. (https://www.jax.org/jax-mice-and-services/customer-support/technical-support/breeding-and-husbandry-support/colony-planning; https://www.taconic.com/quality/genetic-integrity/colony-management/).

Influence of Genetic Background

Influences of background strain(s) warrant consideration when working with spontaneous or genetically engineered mutations. Many genetically engineered mice (GEM) have mixed or undefined genetic backgrounds that can affect research results. When spontaneous or experimentally induced mutations are transferred congenically from the line of origin onto a different (generally inbred) background strain, penetrance and expressivity of the phenotype may be positively or negatively affected by the recipient genome as well as by remnants of the "donor" genome (i.e., chromosomal regions flanking the mutant allele included in the congenic interval).[87–90]

In immunodeficient strains, genetic and phenotypic contributions from background strains have research implications that may not be well known to those who are new to working with these mice. An internet search for commercially available immunodeficient mice bearing the $Prkdc^{scid}$ (*scid*) or $Foxn1^{nu}$ (nude or *nu*) mutations returns more than 20 strains of each on

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Table 1 Selected Immune Relevant Genetic Variations in Common Inbred Mouse Strains

	Gene symbol															
Mouse strain	***Ahr***	***Ctse***	***Hc***	***Il2***	***Il12b***	***Mx1 Mx2***	***Naip5***	***Nlrp***	***Nlrp12***	***Oas1b***	***Sirpa***	***Slamf***	***Slc11a1***	***Tcrb-v8***	***Tlr4***	**TH-bias**
A/J	*b-2*	N/A	Hc^0	N/A	N/A	Ø	S	R	N/A	Ø	N/A	N/A	R	N/A	N	2
AKR/J	*d*	N/A	Hc^0	N/A	N/A	Ø	N/A	R	N/A	Ø	N/A	N/A	R	N/A	N	1
BALB/c	*b*	N	N	N/A	N/A	Ø	R	S	N/A	Ø	L29V	2	S	N	N	2
CBA	*b-2*	N/A	N/A	N/A	N/A	Ø	N/A	S	N/A	Ø	N/A	N/A	R	N/A	N	1
C3H/HeJ	*b-2*	N	N	N/A	N/A	Ø	N/A	S	N/A	Ø	N/A	N/A	R	N/A	*Lps-d*	1
C3H/HeN	*b-2*	N/A	N	N/A	N/A	Ø	N/A	N/A	N/A	Ø	N/A	1	R	N/A	N	N/A
C57BL/6	*b-1*	Ø	N	N	N	Ø	R	R	V	Ø	N	1	S	N	N	1
C57BL/10ScCr	N/A	N/A	N	N/A	N	Ø	N/A	N/A	N/A	Ø	N/A	N/A	S	N/A	*Lps-del*	1
DBA/1J	*b*	N/A	N	N/A	N/A	Ø	N/A	N/A	N/A	Ø	N/A	N/A	S	N/A	N	1
DBA/2J	*d*	N/A	Hc^0	N/A	N/A	Ø	N/A	R	N/A	Ø	N/A	2	R	N/A	N	2
FVB/N FVB/NJ	N/A	N/A	Hc^0	N/A	N/A	Ø	N/A	S	N/A	Ø	N/A	N/A	N/A	Ø	N	N/A
MRL/MpJ	N/A	N/A	N	*m1*	N/A	Ø	N/A	N/A	N/A	Ø	N/A	2	N/A	N/A	N	N/A
NOD/ShiLtJ	N/A	N/A	Hc^0	*m1*	N/A	Ø	N/A	R	N/A	Ø	S	2	R	N/A	N	N/A
NZB	*d*	N/A	N	N/A	N/A	Ø	N/A	N/A	N/A	Ø	N/A	2	R	N/A	N	N/A
NZW	N/A	N/A	N	N/A	N/A	Ø	N/A	N/A	N/A	Ø	N/A	2	S	N/A	N	N/A
NZM2410	N/A	N/A	N	N/A	N/A	Ø	N/A	N/A	N/A	Ø	N/A	2	N/A	N/A	N	N/A
SJL/J	*d*	N/A	N	*m1*	P	Ø	N/A	N/A	N/A	Ø	N/A	N/A	R	Ø	N	1
SWR	*d*	N/A	Hc^0	N/A	N/A	Ø	N/A	S	N/A	Ø	N/A	N/A	R	Ø	N	N/A
129	*d*	N	N	N/A	N/A	Ø	R	S	N/A	Ø	N/A	N/A	R	N	N	1

Ahr (aryl hydrocarbon receptor) activates expression of phase I and II metabolizing enzymes (e.g., *Cyp450*) and is important in cellular growth and differentiation; *b1*, *b2* and *b3* alleles are considered metabolically responsive alleles not linked to autoimmunity whereas *d* alleles are metabolically nonresponsive and associated with autoimmune susceptibility.[37–40]

Ctse (cathepsin E) plays a role in antigen processing for MHC class II.[41]

Hc (hemolytic complement) plays a role in innate immune responses; Hc^0 mice are null for this allele.[42,43]

Il2 (interleukin 2) is a key immune signaling cytokine; $Il2^{m1}$ allele has a hypoactive polymorphism in the *Il2* gene.[44]

Il12b (interleukin 12b) polymorphisms (P) have been associated with autoimmune disorders in humans.[45–47]

Mx1 and Mx2 (MX dynamin-like GTPase 1 & 2) play a role in viral resistance; in most inbred mouse strains, these are not expressed.[48,49]

Naip5 (NLR family, apoptosis inhibitory protein 5) plays a key role in early innate immune responses mediated by the inflammasome; allelic polymorphism determines susceptibility to intracellular bacteria ($Naip5^{Lgn1s}$ = sensitive, $Naip5^{Lgn1r}$ = resistant).[50–52]

Nlrp (nucleotide-binding oligomerization domain-like receptors aka NOD-like receptor proteins) has a key role in pathogen-associated molecular patterns detection.[53]

Nlrp12 (NACHT, LRR and PYD domains-containing protein 12) has an important role in inflammasome and activation of caspase 1; it also controls neutrophil chemotaxis in response to bacterial invasion.[54–56]

Oas1b (2'-5' oligoA synthetase family 1b) plays a role in innate immunity to eliminate viral RNA; most inbred mouse strains carry the susceptibility allele that encodes for a nonfunctional protein.[57]

Sirpa (signal-regulatory protein alpha); in BALB/c mice it has a single polymorphism in the IgV domain (L29V), which enhances binding to human CD47, decreasing macrophage phagocytosis; in NOD mice, the increased affinity for human CD47 is driven by a deletion of 2 amino acids in domain 1.[58,59]

Slamf [signaling lymphocytic activation molecule (SLAM) family] plays a role in self-tolerance;[60] haplotype 2 is associated with autoimmune susceptibility.[61–63]

Slc11a1 [solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1] transporter that regulates iron homeostasis and impacts on the ability to control intracellular pathogens by phagocytes.[64]

Tcrb-V8 (T cell receptor beta, variable 8) plays a role in auto-immune disease susceptibility; in some strains, this is not expressed and is associated with increased susceptibility to autoimmune disease.[65–67]

Tlr4 (Toll-like receptor 4) has a role in innate immune responses, in particular responses to LPS,[68–70] the mutant alleles $Tlr4^{Lps\text{-}d}$ *and* $Tlr4^{Lps\text{-}del}$ are not functional.

Th-bias; mice have TH-1 and TH-2 biases in their immune responses.[71,72]

N/A, no data; N, wild type (normal); NOD, nonobese diabetic; Ø, not expressed nonfunctional or hypofunctional gene product; P, polymorphism; R, resistance polymorphism; S, sensitive polymorphism; V, variable.

inbred or non-inbred backgrounds, some with quite similar names but with immune variations relevant to their genetic backgrounds (and with quite different costs that can influence purchasing decisions).[91–93] Variation in "leakiness" in *scid* mice on different genetic backgrounds is a well-known example. Leakiness refers to the tendency of *scid* mice to produce some functional B and T cells as they age and are increasingly exposed to environmental antigenic stimuli. Under similar experimental

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Table 2 A Few B6 Substrains and Genetic Variations

	B6 Substrain	Source	*Dock2*	*Nlrp12*	*Nnt*	*Snca*	*Mmrn1*	*Crb1*
J	**C57BL/6J**	Jackson	N	Ø	Ø	N	N	N
	C57BL/6J[a]	Charles River	N/A	N/A	Ø	N	N	N
	C57BL/6JOlaHsd	Hsd/Envigo	N/A	N/A	N	Ø	Ø	N
	C57BL/6JRccHsd	Hsd/Envigo	N/A	N/A	N	N	N	N
	C57BL/6JBomTac	Taconic	N/A	N/A	N	N	N	N
	C57BL/6JRj	Janvier	N/A	N/A	N/A	N/A	N/A	N/A
N	**C57BL/6ByJ**	Jackson	N	N/A	N	N	N	Ø
	C57BL/6NHsd	Hsd/Envigo	Some Ø	N/A	N	N	N	Ø
	C57BL/6NRj	Janvier	N	N/A	N/A	N/A	N/A	N/A
	C57BL/6NCrl	Charles River	N	N/A	N	N	N	Ø
	C57BL/6NTac	Taconic	N	N/A	N	N	N	Ø
	C57BL/6NCr	NCI	N/A	N	N/A	N/A	N/A	N/A
References			[77–79]	[55,73]	[80,81]	[82–84]	[82–84]	[85,86]

Adapted/updated from https://www.envigo.com/resources/data-sheets/envigo-68-c57bl6-enhanced-technical-data-sheet_screen.pdf

Dock2 = the protein encoded by this gene belongs to the CDM protein family. It is specifically expressed in hematopoietic cells and is predominantly expressed in peripheral blood leukocytes. The protein is involved in remodeling of the actin cytoskeleton required for lymphocyte migration in response to chemokine signaling. It activates members of the Rho family of GTPases, for example RAC1 and RAC2, by acting as a guanine nucleotide exchange factor (GEF) to exchange bound GDP for free GTP.

Nlrp12 = This gene encodes a member of the CATERPILLER family of cytoplasmic proteins. The encoded protein, which contains an N-terminal pyrin domain, a NACHT domain, a NACHT-associated domain, and a C-terminus leucine-rich repeat region, has an important role in inflammasome and activation of caspase 1, it also controls neutrophil chemotaxis in response to bacterial invasion.

Nnt = nicotinamide nucleotide transhydrogenase; this gene encodes an integral protein of the inner mitochondrial membrane. The enzyme couples hydride transfer between NAD(H) and NADP(+) to proton translocation across the inner mitochondrial membrane.

Snca = alpha synuclein; one in a family of structurally related proteins that are prominently expressed in the brain, particularly in areas associated with learning and adaption. The exact function of alpha synuclein is not yet known.

Mmrn1 = multimerin 1; multimerin 1 is a stored platelet and endothelial cell adhesive protein that shows significant conservation. In vitro, multimerin 1 supports platelet adhesion and it also binds to collagen and enhances von Willebrand factor-dependent platelet adhesion to collagen.

Crb1 = retinal degeneration 8; the *rd-8* mutation is due to a single base pair mutation in the *CRB1* gene. This gene when mutated in humans is linked to macular degeneration and other age-related vision loss. Mice with this mutation are nearly blind by the time they are 8 weeks of age.

N/A, no data; N, wild type (normal); Ø, not expressed, nonfunctional or hypofunctional gene product.

[a]J mice distributed by Charles River in EU

conditions, leakiness is greater on the C57BL/6 and BALB/c backgrounds, low on the C3H/HeJ background, and very low on the nonobese diabetic (NOD) background.[93] Genetic factors contributing to "less sensitivity" to antigenic stimuli (and therefore less leakiness) include TRL4 deficiency in the C3H/HeJ mouse and impaired MHC-dependent antigen presentation in the NOD/ShiLtJ mouse.[94,95] Especially relevant to human xenografts, NOD mice possess a unique signal-regulatory protein alpha (*Sirpa*) polymorphism with higher affinity for the human CD47 that results in a sustained "don't-eat-me" signal and improves engraftment of human cells in NOD-*scid* and NOD-*scid*-derived mice.[58]

Autoimmune-susceptible strains develop spontaneous autoimmune disorders such as immune-mediated (Type 1-like) diabetes and systemic lupus erythematosus (SLE)-like conditions. The proclivity to develop experimentally induced autoimmune conditions, such as experimental autoimmune encephalitis (EAE) and collagen-induced arthritis (CIA), is also greatly influenced by the mouse's genetic background.[31,96–99] The NOD mouse model for Type 1 diabetes (T1D) (e.g., NOD/ShiLtJ and NOD/MrkTac mice) is characterized by the development of a T cell-mediated immune response to pancreatic islet proteins (including insulin and chromogranin) similar to humans with T1D.[100–102] Their diabetic phenotype is polygenic with a significant contribution, as in humans, by their MHC polymorphisms.[44,103–105] NOD mice have a unique MHC class II lacking expression of I-Eα and I-E surface protein, and expressing I-A^{g7}MHC class II allele that is structurally and functionally similar to the human T1D susceptibility allele, DQ8.[106,107] Other contributors to the autoimmune phenotype include a hypoactive variant of their *IL-2* gene ($Il2^{m1}$), *Sirpa* and *Cd93* polymorphisms, lack of C5 (conferred by homozygosity for Hc^{0}), and absence of complement factor H-related protein C (CFHR-C).[43,44,105,108–110] Genetic and phenotypic variations among the NOD substrains have been identified.[111]

Spontaneous lupus-like conditions in mice are associated with mutations such as Fas^{lpr} and *Yaa* and are influenced by genetic background.[28–36] Inbred strains that spontaneously develop lupus-like conditions include MRL/MpJ, BXSB/MpJ, NZB, NZW, NZBWF1 (aka NZB/W), NZM2410, and Palmerston North (PN/nBSwUmabJ).[112,63,113,114]

MRL/MpJ inbred mice are autoimmune prone and spontaneously develop an autoimmune phenotype as they age. A spontaneous mutation in the Fas^{lpr} gene in this strain resulted in the substrain MRL/MpJ-Fas^{lpr}, which develops signs of autoimmunity much earlier in life than the parent MRL/MpJ strain.[115–118] MRL/MpJ-Fas^{lpr} mice have a short lifespan (>50% mortality by 6 months old). They develop lymphoproliferative disease, immune complex glomerulonephritis, lupus-like skin disease, arthritis, and vasculitis.[115,120–123] It has been demonstrated that onset and severity of symptoms associated with the Fas^{lpr} mutation is strain dependent. For example, the Fas^{lpr} mutation results in a lymphoproliferative disease that on MRL/MpJ background is more severe than on the C57BL/6J background, but less severe than on the C3H/HeJ background.[28,29,31,124] In contrast, immune complex pathologies including glomerulonephritis, vasculitis, and arthritis are more severe and initiate earlier with the Fas^{lpr}

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mutation on the MRL/MpJ background than on either the C57BL/6J or the C3H/HeJ background. Predisposition to the development of autoimmune and/or lymphoproliferative lesions in these strains has been mapped to a number of possible other genetic variations.[28–36] Interestingly, when compared with C57BL/6J and/or C3H/HeJ mice, the MRL/MpJ strain harbors diverse polymorphic alleles, unique QTL, or specific haplotypes that render this background more susceptible to autoimmune manifestations.[28–32] As an example, low to no expression of CFHR-C in MRL/MpJ may contribute to the immune hyperresponsiveness typical of this strain.[109]

BXSB/Mp mice are a recombinant inbred (RI) strain originating from a cross between a C57BL/6J female and a SB/Le male, also developed by Murphy[125,126] in his work on autoimmune conditions (lab code Mp). They develop a lupus-like disorder that is accelerated in males and is attributed primarily to the Y-associated autoimmune accelerator locus (*Yaa*) of the SB/Le male founder. *Yaa* is a 4-mb translocated region from the X chromosome that includes multiple genes, among which *Tlr7* seems to be the major contributor to the phenotype.[127–129]

NZB mice develop a variety of autoimmune phenotypes characterized by hypergammaglobulinemia with elevated circulating autoantibodies (including anti-DNA antibodies and anti-thymocyte antibodies), Coombs positive hemolytic anemia, and immune complex glomerulonephritis. NZB mice also manifest a lymphoproliferative disorder involving the B1 subset of B cells. This condition progresses to lymphoma/leukemia, with similarities to human familial chronic lymphocytic leukemia.[130–134] NZW mice develop autoantibodies and glomerulonephritis, with a female predisposition.[135] F1 hybrid offspring of NZB females and NZW males (also referred to as NZB/W) develop a life-limiting autoimmune condition characterized by high levels of antinuclear antibodies, hemolytic anemia, proteinuria, and progressive immune complex glomerulonephritis that is more severe in females.[136–138] NZB/W autoimmune phenotypes map to multiple susceptibility loci, including *Sle*, *Lbw*, and *Wbw* loci and polymorphisms in *Tnf*, *Nkt2*, and *Cd93*, and are linked to a low to no expression of CFHR-C.[110,111,139]

NZM2410 mice (New Zealand Mixed strain 2410, e.g., NZM2410/J https://www.jax.org/strain/002676) derive from NZB/W backcrossed to NZW mice then selected for lupus-like nephritis deaths and inbred. They bear the NZW histocompatibility haplotype $H2^z$ (K^u, A^u, S^z, D^z). Males as well as females develop autoimmune glomerulonephritis at an early age, and this strain has been especially useful in mapping lupus susceptibility loci.[138,140–142]

Important Spontaneous Mutations

Supplementary Table 2 gives a comprehensive overview for most of the well-known murine immune relevant mutations that exhibit Mendelian inheritance. Historically, identification of the genetic basis for spontaneous Mendelian (monogenic) phenotypes was attained via forward genetics approaches to confirm that the heritable trait (phenotype) maps to a specific locus. Additional molecular investigations, including sequencing, are applied to define the mutation further.[88,143] An advantage of forward genetics is the relatively unbiased approach that requires no assumptions or hypotheses regarding the molecular basis of the trait or phenotype. An historical and illustrative example in immunology is the characterization of TLR4, first recognized as the main sensor for lipopolysaccharides (LPS) thanks to studies conducted on the spontaneously TLR4 deficient C3H/HeJ mice, and closely related TLR4 sufficient substrains.[95] A null mutation $Tlr4^{lps\text{-}del}$ mapping to the same site was identified later in the C57BL/10ScCr substrain of the C57BL/10 mouse and is now available as C57BL/10ScNJ.[144,68] Similarly, the role of *Foxp3* as an essential transcription factor for the development of regulatory T cell (Tregs) was first revealed via the analysis of mice with the spontaneous scurfy mutation ($Foxp3^{sf}$).[145]

Hereditary immune deficiencies related to spontaneous recessive *scid*, $Lyst^{bg}$ (*bg* or beige), and Btk^{xid} (*xid*) mutations have been valuable in the study of orthologous conditions in humans and other animals.[146] The *scid* and *nu* (nude) mutations have been especially important for their utility in studying engrafted human tissues in the context of xenotrasplantation experiments.[147]

Hereditary hyperimmune or autommune conditions related to spontaneous recessive Fas^{lpr} (*lpr*, lymphoproliferation) and $Fasl^{gld}$ (*gld*, generalized lymphoproliferative disease) mutations in an important cell death pathway have also been informative. Mice homozygous for either mutation develop lymphoproliferative and autoimmune phenotypes. The (recessive) *lpr* mutation at the *Fas* locus compromises the FAS-mediated apoptosis pathway.[115,123,148,149] The (recessive) *gld* point mutation is in the Fas ligand (*Fasl*) locus, and homozygosity for this mutation also compromises FAS-mediated apoptosis. The *gld* mutation arose spontaneously in C3H/HeJ mice, resulting in the C3H/HeJ-$Fasl^{gld}$ substrain.[150,151]

Interactions Among Mutations

Table 3 summarizes genetic and phenotypic characteristics of some of the widely used mice that carry multiple spontaneous immune relevant mutations. Before the advent of modern genetic engineering capabilities, interbreeding to combine multiple hereditary disorders was used to study phenotypic manifestations of gene interactions and to overcome limitations of the single mutation models, particularly in mice used for xenotransplantation experiments.[89] As an example, *scid*-beige mice homozygous for both the $Prkdc^{scid}$ and $Lyst^{bg}$ alleles were generated to combine the impaired B and T cell development of the $Prkdc^{scid}$ mouse with the defective NK cell function associated with the $Lyst^{bg}$ mutation. These mice are not only severely immunodeficient, but they also lack the "leaky" phenotype of the $Prkdc^{scid}$ animals. The cooperation between the 2 mutations remarkably improves xenotransplantation compared with the single mutation in the $Prkdc^{scid}$ mouse.[152,153]

Combinations of multiple mutations have proved useful in understanding the epistatic interactions among immune relevant genes. Double-mutant mice homozygous for both Fas^{lpr} and the $Foxn1^{nu}$ are an example. The congenital T cell deficiency that characterizes the $Foxn1^{nu}$ mutation is sufficient to abolish the autoimmune and lymphoproliferative phenotype associated with the Fas^{lpr} allele. This finding was consistent with the significant abrogation of the phenotype achieved by neonatal thymectomy in MRL/MpJ-Fas^{lpr}/J mice, and provided early support for the hypotheses regarding the T cell dependence of the Fas^{lpr}-associated autoimmune and lymphoproliferative condition.[89,154–157] Other important immunodeficient models featuring combinations of spontaneous and induced mutations along with specific strain-related immune variations are further discussed in a companion article by Simons and colleagues in the present issue of the *ILAR Journal* and include the well-known NSG and NOG mice. Both models carry a slightly different targeted mutation of *Il2rg* combined with the $Prkdc^{scid}$ mutation on different NOD inbred sublines.

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Table 3 Overview of Immunologically Relevant Mouse Models that Combine Multiple Spontaneous Mutations

Allelic combination	Background strain/s	Phenotype	References
Fasl^{gld}/*Fasl*^{gld} *Btk*^{xid}/*Y*	C3H/HeJ	*Btk*^{xid} decreases the severity of B cell manifestations associated with *Fasl*^{gld} including hypergammaglobulinemia, generation of anti-DNA autoantibodies and systemic immune-complex disease; no impact on T cell dependent *Fasl*^{gld} phenotype and lymphadenopathy.	89
Fas^{lpr}/*Fas*^{lpr} *Btk*^{xid}/*Y*	MRL/MpJ	*Btk*^{xid} decreases the severity of B cell manifestations associated with *Fas*^{lpr} including hypergammaglobulinemia, generation of anti-DNA autoantibodies and systemic immune-complex disease; no impact on T cell dependent *Fas*^{lpr} phenotype and lymphadenopathy.	89,158,159
Fas^{lpr}/*Fas*^{lpr} *Foxn1*^{nu}/*Foxn1*^{nu}	C57BL/6J	*Foxn1*^{nu} prevents the development of *Fas*^{lpr}-induced lymphadenopathy, unregulated B cell activation, hypergammaglobulinemia, anti-DNA autoantibodies and systemic immune-complex disease (a similar effect is obtained via neonatal thymectomy confirming the T cell dependency of *Fas*^{lpr} phenotype).	89,154–156
Fas^{lpr}/*Fas*^{lpr} *Prkdc*^{scid}/*Prkdc*^{scid}	MRL/MpJ; C.B-17	*Fas*^{lpr} rescues the developmental deficit of thymic T cells associated with *Prkdc*^{scid}; no effect on the B cell deficit caused by *Prkdc*^{scid}.	160
Fas^{lpr}/*Fas*^{lpr} *X*/*Yaa*	MRL/MpJ; C57BL/6J	*Yaa* causes accelerated onset and increased severity of *Fas*^{lpr}-induced autoimmune condition and lymphadenopathy.	161,162
Foxn1^{nu}/*Foxn1*^{nu} *Lyst*^{bg}/*Lyst*^{bg}	C57BL/6J; N:NIH(S)	*Lyst*^{bg} contributes defective NK cells to the T cell-deficient background associated with *Foxn1*^{nu}; reduced NK cell activity does not seem to impact on the engraftment rate and growth of xenotransplanted human tumor cell lines.	89,163
Foxn1^{nu}/*Foxn1*^{nu} *Btk*^{xid}/*Y* or *Btk*^{xid}/*Btk*^{xid}	N:NIH(S)	Defective T (*Foxn1*^{nu}) and B (*Btk*^{xid}) cell function and/or maturation; spectrum of the immune abnormalities is very similar to the one characterizing *Prkdc*^{scid} mutants; severe depletion of both B and T cell domains in the spleen and lymph nodes; limited production of immunoglobulins; females showing high incidence of both lymphomas and ovarian granulosa cell tumors.	89,164–166
Foxn1^{nu}/*Foxn1*^{nu} *Lyst*^{bg}/*Lyst*^{bg} *Btk*^{xid}/*Y* or *Btk*^{xid}/*Btk*^{xid}	N:NIH(S); KSN	Defective T (*Foxn1*^{nu}), NK (*Lyst*^{bg}) and B (*Btk*^{xid}) cell function and/or maturation; high incidence of multicentric lymphoblastic lymphoma; compared to single *Foxn1*^{nu} mutants, improved engraftment rate and growth of xenotransplanted human tumor cell lines.	89,167,168
Dh/*Dh*$^{+}$ *Foxn1*^{nu}/*Foxn1*^{nu}	N:NIH(S)	Combined athymia and asplenia; defective T cell maturation and function; reduced B cell number; hypogammaglobulinemia; increased incidence of spontaneous mammary tumors compared to single-mutant founder lines.	89,169
Lyst^{bg}/*Lyst*^{bg} *X*/*Yaa*	SB/Le	*Lyst*^{bg} attenuates severity and progression of *Yaa*-linked autoimmune condition resulting in prolonged survival and lack of immune complex glomerulonephritis; possible role of *Lyst* in B cell development and activation.	89
Btk^{xid}/*Y* *X*/*Yaa*	BXSB	*Btk*^{xid} prolongs survival and decreases the severity of B cell manifestations associated with *Yaa* including immune complex glomerulonephritis, hypergammaglobulinemia, autoantibody levels and lymphoid hyperplasia.	170
Prkdc^{scid}/*Prkdc*^{scid} *Lyst*$^{bg\text{-}J}$/*Lyst*$^{bg\text{-}J}$	C.B-17	Defective T, B (*Prkdc*^{scid}) and NK (*Lyst*^{bg}) cell function and/or maturation; reduced level of B cell leakiness; possible role of *Lyst* in B cell development and activation.	152,153
Prkdc^{scid}/*Prkdc*^{scid} *Hr*^{hr}/ *Hr*^{hr}	SCID Hairless Outbred (Crl:SHO)	Impaired B and T cell development (*Prkdc*^{scid}) associated with diffuse hair loss/ alopecia (*Hr*^{hr}).	171
Foxp3^{sf}/*Foxp3*^{sf} *Foxn1*^{nu}/*Foxn1*^{nu}	129/RI; BALB/c	*Foxn1*^{nu} prevents the development of *Foxp3*^{sf}-induced autoimmune disease including anemia, multisystemic immune/inflammatory cell infiltrates, hypergammaglobulinemia, lymphadenopathy and splenomegaly (a similar, but less potent, effect is obtained via neonatal thymectomy confirming the T cell dependency of *Foxp3*^{sf} phenotype).	172,173
Foxn1^{nu}/*Foxn1*^{nu} *Map3k14*^{aly}/*Map3k14*^{aly}	BALB/cAJcl; C57BL/6J	Athymia combined with lack of secondary lymphoid organs including lymph nodes, splenic white pulp, Peyer's patches and isolated lymphoid organs; severe immunodeficiency with impaired humoral and cell- mediated immune responses; preserved intestinal γδ-IEL subset; confirmation that thymus and secondary lymphoid organs are not an essential requirement for the development of γδ-IEL.	174

IEL, intraepithelial lymphocytes; NK, natural killer.

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Table 4 Induced Immunodeficiencies (Intended Experimental Interventions)

Inducers	Possible Effects on the Immune and Other Systems	References
Physical: irradiation		
γ rays and X rays	Suppression of bone marrow resulting in marrow atrophy and pancytopenia. High dose: decreased splenic and thymic weights; loss of cortical thymocytes; decreased splenic CD4+ and CD8+ T cells; decreased circulating CD3+ cells. Chronic low dose: prolonged life span in mice homozygous for the lymphoproliferation spontaneous mutation (*Fas*^{lpr}); increased CD4+ cells; suppression of IL6 and IL17, and up-regulation of Tregs in CIA mice; suppression of pro-inflammatory cytokines, reduction of CD8+ T cells, and induction of Tregs in murine EAE model. Other: acute radiation syndrome and death in *Prkdc*^{scid} mice and *Prkdc* ^{dxnph}mice (both are highly susceptible to ionizing radiations); radiation induced-thymic lymphoma in both male and female mice on a C57BL/6 background and NFS mice; radiation induced-myeloid leukemia in male RF mice (RF/J, RFM) and male CBA mice (CBA/Ca, CBA/Cne, CBA/H); induction of persistent oxidative stress in murine intestinal epithelium with potential for neoplastic transformation by heavy ion radiations; radiation-induced cataract; increased osteoclast activity and bone loss; radiation nephropathy.	182,237–244
α and β particles	Release of DAMPs; activation of DCs; systemic and long-lasting T cell-mediated antitumor response in tumor-bearing mice; efficacy of α and β emitter-labeled monoclonal antibodies against fungal infections in mice. Other: radiation nephropathy.	245–247
UVB	Immunosuppressed contact hypersensitivity (*Xpa* deficient mice); inhibited intra-tumor migration of NKs and CD8+ T cells (*Xpa* deficient mice); depressed delayed hypersensitivity in immunized mice; enhanced contact hypersensitivity and skin graft rejection in mice with dermal Langerin+ DCs. Narrowband (NB)-UVB: increased intestinal Tregs, and decreased severity of inflammatory lesions in mouse models of allogeneic GVHD.	248–254
UVA	High dose: increased IFNγ, IL12, and heme oxygenase; inhibited increment of IL10 from UVB exposure. Medium dose: NO-mediated depletion of epidermal Langerhans cells; impaired development of skin memory CD8+ T cells in a mouse model of contact hypersensitivity.	255–258
Chemical agents		
Endogenous and exogenous glucocorticoids	Direct and receptor-mediated immunosuppression: attenuated DC activity; decreased DC number (apoptosis, tissue redistribution); enhanced inflammation; thymic atrophy (decreased DP thymocytes); dampened T cell activation (interference with TCR signaling); suppressed responses of TH1 and TH17 cells; reduced immunoglobulins. Other: osteopenia, decrease in bone formation rate and mineral apposition rate in skeletally mature and young mice; osteoporosis in CD-1 mice (mouse model of glucocorticoid-induced osteoporosis); cleft palate in A/J mice.	259–265
Cyclophosphamide (CYP; Cytoxan)	Direct immunosuppression: depletion of CD8+ resident DCs in murine spleen and lymph nodes, with subsequent decrease in Treg suppressive function; neutropenia; depletion of suppressor or regulatory T cells in diabetic NOD mice. Other: enhanced antitumor efficacy by promoting proliferation/activation of adoptively transferred B and T cells after CYP-induced lymphodepletion in mice; reduced diversity of the fecal microbiota; hemorrhagic cystitis in C57BL/6 and DBA/2 mice; chronic cystitis in DBA/2 (CYP model of bladder pain syndrome); short root lengths and early apical foramen closure during molar root development in ICR mice; suppressed osteoblastogenesis and osteoclastogenesis in C57BL/6 male mice.	266–273,200
5 FU	Direct immunosuppression: depletion of MDSCs, and stimulation of TH17 cells, IL17 production by CD4+ T cells, and tumor growth; no altered levels of circulating B, T, and NK cells.	207,208
Tacrolimus (FK506)	Receptor-mediated immunosuppression: immunosuppressive effects on CD4+ T cells; marked tumor-promoting effect (topical tacrolimus) with decreased CD4/CD8 ratio; reduced inflammation in models of allergic rhinitis, conjunctivitis and arthritis. Other: nephrotoxicity.	211–215
Cyclosporin A (CsA)	Receptor-mediated immunosuppression, reversible inhibition of T cell proliferation and proinflammatory immune reactions; blockage of all the changes resulting from intercellular signaling and cross-talk between DCs to T cells.	209,210

Continued

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Table 4 Continued

Inducers	Possible Effects on the Immune and Other Systems	References
Rapamicin	Receptor-mediated immunosuppression: Inhibition of mTOR: suppressed T cell activation, proliferation, and development of FoxP3+ cells; suppression of DC maturation, B cell activation, neutrophil chemotaxis and uptake of antigen by APCs. Other: increases lifespan.	274,275
Busulfan; Treosulfan	Direct immunosuppression: Busulfan: highly myelosuppressive, minimally immunosuppressive; diminished NK cell activity; late-stage (residual) bone marrow injury; stimulation of neuroinflammation through MCP-1. Treosulfan: high persisting myeloablation in BALB/c mice; more effective depletion of splenic B and T cells.	276–280
Physical: Surgical		
Thymectomy	Thymectomy (post-natal day 2-5): autoimmune hemolytic anemia, thyroiditis, gastritis, oophoritis, orchitis, and prostatitis at puberty due to lack of Tregs.	232
Splenectomy	Systemic immune unresponsiveness; absence of tolerance after ocular injections of antigen in F4/80-deficient mice; retardation of tumor growth in melanoma-bearing mice.	281–285,236
Biological agents		
Anti-thymocyte globulin (ATG)	Depletion of naïve T cells; less effective on memory T cells in NOD mice. Prevention of autoimmune encephalomyelitis through expansion of myelin antigen-specific Foxp3+ Tregs in a murine EAE model.	283,229
β-1,3-Glucan	Increased IL2, TNFα, IL17, IFNγ, and lymphocytes in mice treated with aflatoxin B1.	284
CpG oligodeoxynucleotides	In murine models of infections: TH1 cytokine expression, activation of DCs, NK, and B cells. Combined therapy with monoclonal antibodies: increased NK cell activity.	
Bacterially derived ADP-ribosylating enterotoxins	CT toxin produced by *Vibrio cholera:* secretion of TH2 cytokines, maturation of DCs, generation of Th2 and regulatory T cells, active suppression of TH1 responses. LT enterotoxin from *E. coli*: mixed TH1/TH2 immune response.	230,285,291
Anti-lymphocyte serum (ALS)	Long-term abrogation of autoimmunity in overtly diabetic NOD mice.	286
Monoclonal antibody (mAb) therapy	Anti-mouse CD20 mAbs: depletion of mature B cells; reduction of CD4+ T cells, but maintainance of the interactions, functions, and migration of DCs and CD4+T cells; unaffected CD8+ T cell reactivity; absent release of inflammatory cytokines with effects on T cells. Anti-mouse CD4 mAbs: depletion of CD4+ T cells; expansion of CD8+ T cells with an effector phenotype and of tumor-reactive CD8+ T cells; compromised anti-tumor immune memory. Anti-mouse CD8 mAbs: depletion of CD8+ T cells; decreased infiltration of CD4+ cells, neutrophils, and macrophages; downregulation of IL1β, IL6, TNFα, CXCL1, CCL2 and up-regulation of IL4 in a mouse model of wound healing.	287–289

APCs, antigen-presenting cells; CIA, collagen-induced arthritis; CT, Cholera toxin; DAMPs, damage-associated molecular patterns; DCs, dendritic cells; DP, double positive; EAE, experimental autoimmune encephalitis; GVHD, graft-versus-host disease; LT, heat labile toxin; MCP-1, monocyte chemoattractant protein 1; MDSCs, myeloid-derived suppressor cells; NK, natural killer cell; NO, nitric oxide; Tregs, regulatory T cells.

Induced Immunodeficiencies

The mouse immune system can be modulated (regulated or disrupted) intentionally (and unintentionally) through experimental interventions such as exposures to irradiation, chemical compounds, microbial organisms (including virus, bacteria, and their toxins), or biological agents as well as through surgical manipulations. Immune suppression by these means has been especially useful in experiments of engrafted tissues or tumors and to study the immune response against specific infections or neoplasms. Examples from the major categories of intended experimental interventions to induce specific perturbations of the mouse immune system are summarized in Table 4.

Ionizing and Ultraviolet Radiation

Ionizing radiation is a historically important method to suppress or ablate immunity. The peculiar vulnerability of the hematolymphoid tissue to ionizing radiation results in extensive lymphoid depletion and sustained myeloablation. For this reason, ionizing radiation remains an important immunosuppressive intervention allowing the engraftment of xenotransplants/allotransplants, including, for example, tumors or human haematopoietic stem cells for the generation of mice with humanized immune system.[175,176] Sensitivity to irradiation has been linked to the capacity to repair radiation-induced DNA double-strand breaks. Immunodeficient mice harboring the *Prkdc*^{scid} alleles are particularly radiosensitive due to the *scid* mutation

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that affects repair of radiation-induced DNA double-strand breaks.[177,178] Susceptibility to irradiation varies among mice, with strains such as the C57BL/6, A/J, and C3H/HeMs being highly resistant and other strains such as BALB/c being highly sensitive.[177,179] A hypomorphic *Prkdc* allele (*Prkdc*^{dxnph}), identified in BALB/c strains, seems to have an important role in BALB/c susceptibility to ionizing radiation.[178,180,181] Some detail on irradiation tolerance, variations, and dosage protocols is available from the sources of mice that are commonly irradiated (https://www.taconic.com/taconic-insights/oncology-immuno-oncology/rodent-irradiation-considerations.html; https://www.jax.org/jax-mice-and-services/find-and-order-jax-mice/most-popular-jax-mice-strains/immunodeficient-mouse-and-xenograft-host-comparisons). In addition to considering strain sensitivity when determining radiation dosage, calibration of the irradiator is also important, as there is considerable decay over time and actual dosage may differ between studies or between irradiators.

Immune-suppressive effects of high-dose γ-irradiation are well known.[182] High-dose γ-irradiation differentially affects the diverse populations of mouse lymphocytes with B cells recognized as more radiosensitive than T cells.[183] Repeated low-dose gamma irradiation also has profound immunomodulatory effects and is linked to a robust Th2 skewing that may mitigate autoimmune conditions that are dependent on a Th1 response. Suppression of pro-inflammatory cytokine production, reduced CD8+ CTLs, and up-regulation of Tregs also have been demonstrated in certain experimental conditions, including CIA and EAE.[184]

Overwhelming infections remain an important cause of mortality of irradiated experimental animals and clinical patients. Mice with defective adaptive immunity including nude, *scid* and NOD *scid* mice can effectively control common opportunistic agents such as Pseudomonads, until myeloablative effects of irradiation or other interventions eliminate their innate immunity as well.[185] Effects of ionizing radiation on other tissues, and on developing or proliferating cells, influence morbidity and mortality of research mice. Radiation impact on developing brain, bone, eyes and teeth as well as on heart, lung, kidney, may complicate interpretation of disease or death related to rejection, GVHD, or other research endpoints.[186–195]

Ultraviolet (UV) radiation effects on local skin immunity are especially relevant to research on photocarcinogenesis or inflammatory skin conditions.[196–198] Effects vary with dose, duration of exposure and wavelength composition.[196–198] UV radiation primarily affects adaptive immunity, and has been used to induce and promote skin photocarcinogenesis, and to modulate the immune response in diverse experimental immunoinflammatory conditions of the skin.[196–198]

Chemicals

Experimental use of chemicals also has been and remains an important method to suppress or ablate immunity. Examples including metals, aromatic hydrocarbons and other environmental contaminants, and antimicrobial agents are summarized in Table 4. Alkylating agents that affect chromosomal DNA through formation of phosphodiesters and DNA-DNA crosslinks, are widely used. Cyclophosphamide (CYP), a cytotoxic alkylating agent used in the treatment of neoplastic and autoimmune diseases, is also exploited to induce neutropenia in the context of infectious disease studies.[199] Mice with impaired granulocyte production and/or leukocyte function secondary to CYP are more prone to develop systemic disease upon experimental infection with environmental opportunists such as *Pseudomonas aeruginosa* or *Cryptococcus neoformans*.[200,201] CYP has both immunomodulatory and immunosuppressive effects.[202] Immunosuppression in mice appears to result from the induction of apoptosis in activated B and T cells as well as NK cells.[203] At low doses, CYP may enhance immune responses to tumor antigens attributed, at least in part, to suppression of Tregs.[204] Similarly, the alkylating agent busulfan is used as conditioning regimen to enhance engraftment of xenotrasplanted hematopoietic stem cells.[205,206] Other important agents include 5-fluorouracil (5FU), which selectively depletes tumor-associated myeloid-derived suppressor cells (MDSCs) promoting the activation of tumor-specific CD8+ T cells.[207,208] Calcineurin inhibitors (CNI), such as tacrolimus and cyclosporine A, directly inhibit Tregs function, by inhibiting peripheral Tregs generation, and less directly by limiting IL2 production, in preventing transplant rejection and to treat a variety of autoimmune conditions.[209–215] Glucocorticoids are important clinically and experimentally for their anti-inflammatory and immunosuppressive effects.[216]

A variety of experimental interventions including hormones, antimicrobials, nanoparticles, etc., have immunomodulatory effects that may not be intended or expected, especially by investigators who are new to using them in mice. For example, estrogens (and synthetic estrogens such as diethylstilbestrol) and androgens have immunosuppressive effects that affect both adaptive and innate immunity.[217–220] Nanoparticles, usually studied as a drug delivery method or biomedical imaging tool (e.g., metallic nanoparticles), are typically taken up by macrophage/monocyte cells and may act either as immunostimulants or as immunosuppressants and may have additional immune effects related to imaging methods such as MRI or μCT.[221] The unique physicochemical characteristics of nanoparticles influence their interactions with host's immune system and determine the overall immunotoxicologic profile.[222,223]

Biologics

Biologics with immune modulating properties have been exploited in the experimental context to target specific functions of the mouse immune system and achieve definite preclinical endpoints.

Antibody-mediated depletion of cell lineage-specific immune effector cells has been used to delineate their roles in innate and adaptive immunity, in rejection, GVHD, and other conditions.[216,224–226] Anti-thymocyte globulin (ATG), is another important immunosuppressive agent that specifically depletes T cells from peripheral blood and lymphoid organs in NOD mice; it is also used in the modulation of graft rejection and autoimmune disorders in mice.[227,228] Glucans, CpG oligodeoxynucleotides (CpG ODN) and bacterial enterotoxins have been used as prophylactic or therapeutic interventions to modify immune responses to infections or vaccination, or to counteract effects of immunotoxic agents (see Table 4).[229,230]

Surgical

Thymectomy or splenectomy are the traditional surgical methods to alter immunity. Thymectomy in neonatal or adult animals has profound effects on T cell development and continues to be an important procedure in studies of T cell ontogeny, tolerance and education. Neonatal thymectomy experiments offered early evidence of the existence of Tregs as these mice develop autoimmune disease shortly after the removal of thymus.[231] Thymectomy is also used to investigate the dynamics

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of extrathymic T cell development.[232] However, mice exhibit a relatively high frequency of functional thymic tissue in ectopic locations, especially in close proximity to the thyroid gland (also known as cervical thymus). While ectopic thymi may be small, they can be confounding source of T cells. They are reported to be more common in NOD and BALB/c mice compared to C57BL/6 mice.[232,233]

Splenectomy has been used to study the role of the spleen in infectious disease, peripheral antigen tolerance, and tumor growth.[234] In cancer, some splenectomy studies implicate the spleen in promoting tumor antigen tolerance.[234,235] while others demonstrate a role of the spleen in maintaining an effective antitumor immune response and prevention of metastatic disease.[236]

Induced Autoimmune and Hyperimmune Conditions

Autoimmune diseases arise when there is poor control of self-reactive lymphocytes and cytokine production, or disrupted regulatory T cell and effector T cell balance. While underlying genetic polymorphisms predispose to immune hyperresponsiveness, manifestation of disease often requires additional triggers such as microbial infections, dysbiosis, or tissue damage. Once initiated, cytokines participate in disruptions of immune tolerance by altering the balance between T-effector functions and T-suppressor functions.[290–292] Strain-related variations in innate and adaptive immunity affect penetrance, onset and severity of disease.[7,27,89,293,294] Modifiers such as *Slamf*-haplotype 2 seem relevant to autoimmunity in MRL/MpJ mice and not so relevant on other backgrounds such as BALB/c.[60–62] The complexity of autoimmune conditions in mice has many parallels with human and, because of a more granular characterization of strain genetics, may have much to offer to our understanding of the human conditions and interventions for them.[295,296] Two examples are discussed here.

Rheumatoid Arthritis

Rheumatoid arthritis (RA) is an immune-mediated destruction of the synovial lining of the joints, with devastating effects on underlying cartilage and bone. Susceptibility to the induction of rheumatoid arthritis-like conditions in mice, using type II collagen-induced arthritis (CIA) or proteoglycan-(aggrecan)-induced arthritis (PGIA), depends on multiple susceptibility alleles and QTL.[96,297,298] The disease in mice and in humans is polygenic and complex. MHC H2 subtypes seem to have more impact on CIA than on PGIA susceptibility, and PGIA susceptibility is influenced by multiple genes.[96,298,299] Strains expressing the H-2^q and the H-2^r haplotypes are most susceptible to CIA. DBA/1 (H-2^q) are sensitive to CIA but insensitive to PGIA. BALB/c (H-2^d) mice are not so susceptible to CIA but are highly susceptible to PGIA.[96,297,299] In contrast, DBA/2 (H-2^d) are resistant to arthritis induction by either method, implicating roles for strain associated modifier genes.[299,300] Non-MHC QTL associated with susceptibility to CIA and/or PGIA localize to regions on mouse chromosomes 2, 3, 7, 15, and 19 that contain multiple candidate genes with known immune functions.[299]

Multiple Sclerosis

Multiple sclerosis (MS) is an inflammatory demyelinating disorder with a spectrum of disease manifestations. While disease is associated with certain genetic polymorphisms, environmental triggers as well as sex hormones have roles in disease development.[290,301] A spontaneous mouse model of MS has not been identified. But various aspects of MS are recapitulated by experimental autoimmune encephalomyelitis (EAE), classically induced "actively" by immunization with immunodominant myelin epitope components in combination with immunostimulants, or induced "passively" by adoptive transfer of preactivated myelin-specific T cells into naïve mice.[98,302–305]

EAE in mice was first reported in 1975, and the SJL/J and C3H/HeJ strains were identified as susceptible strains.[98,294,302–304,306] SJL/J mice are used to model features of relapsing-remitting MS, and their susceptibility is associated with several polymorphisms, including hyper-responsive IL12 and hypo-active IL2 and IL4.[67,306,307] Additionally, C57BL/6, DBA1, and C3H/HeJ strains also are sensitive to induction of EAE.[98,294,308]

GEM models such as transgenic mice bearing human TCR and T cells targeting myelin-specific antigens (e.g., myelin basic protein) have been informative,[309] as has immune-mediated demyelination associated with infections by Theiler's Mouse Encephalitis Virus, a Picornavirus, in susceptible SJL/J and resistant C57BL/6.[310–312] Demyelination with certain strains of Mouse Hepatitis Virus (MHV), a coronavirus, has been used to model features of MS in susceptible C57BL/6 and BALB/c mice. This is primarily a virus-mediated cytolytic phenomenon, and SJL/J resistance is attributed to their spontaneous mutation in *Ceacam1*, whose protein product is an important receptor for neurovirulent MHV strains.[313–315]

Other Immunomodulators and Unintended Experimental Consequences

Environmental Factors

Table 5 summarizes examples of immune effects of common environmental factors including husbandry conditions, microbiota, as well as effects caused by experimental or therapeutic interventions. These examples illustrate why reporting of environmental and husbandry conditions and specifics of experimental or therapeutic interventions is warranted in scientific publications. Microenvironment refers to the immediate physical environment surrounding the animal such as the cage, pen, or stall. Macroenvironment refers to the physical environment of the secondary enclosure (e.g., a room, a barn, or an outdoor habitat).[323] A multitude of factors in the microenvironment and macroenvironment can be stressors. Stressors activate the hypothalamic-pituitary-adrenal axis, in turn increasing circulating glucocorticoids. In mice, corticosterone is the primary stress-induced glucocorticoid. Corticosterone elevations (and corticosterone-mediated lymphocytolysis) are expected with stressors such as adverse environmental conditions, shipping, handling, social stresses, noise, vibration, etc.[317–319] Responses to stressors also vary with mouse strains.[320,321]

Caging

Common contemporary caging options are open top, static microisolators (filter top cages), and individually ventilated caging. Suspended wire caging is less common today but may be scientifically justified to prevent coprophagy and ingestion of drugs or metabolites in feces. Individually ventilated caging is increasingly available with advantages in terms of barrier protection of the animals, lower bioburden, and cage changing frequency and with concerns in terms of microenvironment temperature, humidity, wind, and dust. Temperature, vibration,

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Table 5 Other Immunomodulators, Including Unintended Immune Consequences of Husbandry and Environmental Factors, Clinical and Experimental Interventions

Immunomodulators	Possible Effects on the Immune and Other Systems	References
Environmental factors		
Housing conditions		
Caging	Individual ventilated cages (compared to static microisolator caging): decreased bioburden and risk of intercage infection spread; increased cold stress; decreased circulating leukocytes; decreased intracage ammonia levels and correlated nasal pathology.	322–325
Bedding	Experimentally relevant parameters influenced by the type of bedding: higher intracage ammonia levels with reclaimed wood pulp bedding; corncob bedding associated with decreased efficiency of feed conversion in mice fed a high-fat diet; hepatotoxicity associated with vermiculite and unbleached pulp from pine and eucalyptus; hepatic and mammary carcinogenesis associated with aromatic red cedar bedding; altered estrogen signaling mainly due to BPA residues; corncob bedding associated with increased aggressivity and social stress in females; drastically lower endotoxin levels and bioburden associated with paper bedding.	323,342,352–357,366
Single or group housing and social stressors	Group housing: negative social events associated with lower lymphocyte proliferation; lower level of antigen-specific IgG; granulocytosis; lymphopenia, higher predisposition to tumor development and progression, huddling associated with amelioration of cold stress. Individual housing: decreased antibody production; worsened allergic skin reaction; increased cold stress.	326–330
Environmental enrichment	Reduced stress levels; reduced oxidative stress; enhanced NK antitumor functions; enhanced macrophage chemotaxis and phagocytosis; improved capacity to clear systemic microbial infection; enhanced lymphocyte chemotaxis and proliferation; increased lifespan.	331–336
Temperature and humidity	Thermoneutral housing temperature (26°–34°C): reduced tumor formation, growth rate and metastasis due to increased CD8+ T cells; reduced myeloid-derived suppressor cells and Tregs. Sub-thermoneutral housing temperature (20°–26°C): suppressed immune responses; increased therapeutic resistance of tumor and GVHD severity; suppressed myeloid cells function; alternative activation of macrophages. Elevated humidity: increased bioburden; high ammonia levels due to expansion in urea-converting microflora.	327,328,337–341,473–476
Environmental noise and vibration	Altered tumor resistance; immunosuppression; reduced body weight; reduced fertility.	348–351,477
Inappropriate handling; untrained personnel	Increased risk of infection associated with inappropriate PPE and insufficient sterilization of equipment; pain, discomfort and stress associated with frequent/improper handling.	316
Altered light-dark cycle	Suppressed immune response; decreased splenic T cells; continuous illumination associated with decreased CD8+ and CD4+ cells in thymus and lymph nodes.	343–345
Dim lights	Elevated nighttime light exposure in male mice associated with worsened inflammation and weight gain under high-fat diet regimen.	478
Diet and water modifications		
Caloric restriction	Immune effects: reduced H_2O_2, TNF α, IL6, IL2, IL10, NO, IFNγ; decreased macrophage activation; impaired NK cell function; reduced IgA in small intestine and serum IgG. Other effects: increased lifespan; reduced age-related morbidities.	363,478–483
Protein-energy malnutrition	Impaired proliferation CD8+ T cells; modulation of intestinal IgA responses to rotavirus; increased duodenal γδ IELs; increased production of jejunal proinflammatory cytokines in response to bacteria.	484–486
Prolonged fasting (48–120 h)	Stress response due to activation of hypothalamic-pituitary-adrenal axis; thymic atrophy (apoptosis of cortical DP thymocytes).	487
High-fat diet (in C57BL/6 mice)	Suppression of delayed hypersensitivity; altered intestinal microbiota with stimulation of mucosal immunity; altered systemic metabolomes; inflammation of adipose tissue with release of adipokines, cytokines, and chemokines, and propagation of a chronic inflammatory state (inflamobesity).	488–490

Continued

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Table 5 Continued

Immunomodulators	Possible Effects on the Immune and Other Systems	References
Chlorella vulgaris supplementation	CYP-treated mice: reinstated lymphocyte proliferation and macrophage phagocytic activity; stimulation of IL2, IL12, TNFα, IFNγ, NK cell cytotoxicity; decreased splenic necrosis.	491
Polyunsaturated fatty acids supplementation	Dietary DHA and AA associated with improved allergen-induced dermatitis as consequence of increased FoxP3+ T cells, elevated IL10, and decreased TNFα.	492
Water acidification	Switch from normal tap water to acidified water associated with severe and long-lasting stress.	343
Nutritional deficiencies		
Zinc deficiency	Thymic atrophy (loss of DP thymocytes); accelerated lymphopenia with loss of antibody and cell-mediated responses; decreased number of pre-B cells, better survival for pro-T cells and mature DP and CD8+ T cells; increased myeloid lineage in bone marrow.	493–496
Vitamin A deficiency	Decreased ILC3 and antibacterial responses; compensatory expansion in IL-13-producing ILC2 and increased anti-helminth responses; intestine devoid of CD4+ and CD8+ T cells; lower salivary IgA levels and increased serum IgG response in mouse model of influenza; decreased mucosal antigen-specific IgA responses.	497–499
Vitamin D deficiency	VDR-deficient mice: increased mature DCs in skin draining lymph nodes; decreased Th1-cell responses and induction of IL10-producing Tregs.	500
Diet and water contaminations		
Estrogenic endocrine-disruptors	Isoflavones (genistein): thymic atrophy; suppression of delayed hypersensitivity; decreased splenic NK cells; decreased IFNγ in response to bacterial infection. Mycotoxins (aflatoxins, deoxynivalenol, zearalenone): elevated IgA and IgE; kidney mesangial IgA deposits; polyclonal activation of IgA secreting cells; IgA autoantibody. BPA (cages, water bottles): lupus-like syndrome (C57BL/6 mice); allergic airway disease (BALB/c mice).	365,366,501–503
Halogenated aromatic hydrocarbons (PCDFs;PCDDs)	Contaminated food and bedding: inhibited innate and adaptive immune responses; atrophy of lymphoid organs; TCDD targets thymic lymphoblasts.	364,504,505
Metals (As, Cd, Pb, Hg, Se)	Complex immune-modulating effects (immunosuppression and immunostimulation). As: decreased DCs in mediastinal lymph nodes of influenza A-infected C57BL/6 mice.	504,506
Microbial status, pathogens, and biosecurity		
MHV	MHV-3-infected C57BL/6: impairment of pre-B cells maturation and B cells functions. A59-infected BALB/c: transient lymphocyte apoptosis in the thymus. MHV-JHM-infected BALB/cByJ: functionally altered CD4+ and CD8+ T cells, and APCs.	507–509
Sendai virus	Interference with macrophage and their phagocytic activity, NK cells, and T and B cell function; increased isograft rejection.	507,510–513
MNV	Lethal infection in mice deficient for STAT1 and IFN receptors; alteration of immune/inflammatory parameters in diverse mouse models including *Mdr1a* deficient animals infected with *Helicobacter bilis* interfering with dendritic cell function and cytokine responses; infection of wild-type mice associated with mild intestinal inflammation, splenic red pulp expansion, and white pulp activation.	514–516
MuHV-1	Loss of splenic T and B cells; interference with key coordinating role of DCs; functional impairment of macrophages and loss of response to cytokines; altered responses to mitogens, antigens, increased allograft rejection, delayed type hypersensitivity responses, and clearance of other pathogens; formation of anti-cardiac autoantibodies.	440,517–520
MuHV-3	Thymic necrosis (specific targeting of CD4+ T cells in newborn mice); autoimmune gastritis in BALB/c and A strain; autoimmune oophoritis and production of antibodies to thyroglobulin.	413,440,521
MPV	Suppressed proliferation (spleen, popliteal lymph node), increased proliferation (mesenteric lymph node) in ovalbumin-primed mice; altered alloreactive T cells	522,523

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Table 5 Continued

Immunomodulators	Possible Effects on the Immune and Other Systems	References
	and abnormal CD8+ T cell rejection of tumors and skin allografts (BALB/c); rejection of syngeneic grafts.	
MVM	MVM: oncolytic, cytotoxic, replicative cancer inhibitor; deregulation of the Raf signaling cascade. MVMi: depressed myelopoeisis in neonatal BALB/c; depletion of hemopoietic precursors, leukopenia, and compensatory erythropoiesis in adult and neonate SCID mice.	415,524
Murine retroviruses	Insertional mutagenesis (with reintegration of endogenous retroviruses or transposition of retroelements): immune relevant mutation such as *Foxn1*^{nu}, *Lep*^{ob}, *Fas*^{lpr}. Endogenous retroviruses in pancreatic islets: contribution to immune-mediated insulitis NOD mice. LP-BM5-infected C57BL/6 mice: lymphadenopathy, splenomegaly; hypergammaglobulinemia; T and B cell dysfunctions; late appearance of B cell lymphomas; opportunistic infections.	439–443,448,525–529
LCMV	LCMV disease: all pathological alterations following infection are immune-mediated; prototype for virus-induced T-lymphocyte-mediated immune injury and for immune complex disease; protection from LCMV-induced disease conferred through immunesuppression; noncanonical type I IFN signaling responsible for lethality in LCMV-infected *Stat1* deficient mice.	530–532
MHV-68	Experimental infections of laboratory mice to study the pathogenesis of human lymphoproliferative disorders associated with EBV.	422,426–430
Bacteria	Mortality/morbidity (sepsis) in immune deficient mice: *Pseudomonas aeruginosa*, *Klebsiella spp*., *E coli*; potentially any bacteria in severely immunocompromised mice. Abscesses: *Staphylococci*, *Pasteurella pneumotropica*. Skin disease/morbidity: *Corynebacterium bovis*, *Staphylococci*. *Mycoplasma arginini*: suppurative arthritis in *Prkdc*^{scid} mice inoculated with contaminated cell lines.	375,378,381,440,451,533
Fungi	*Pneumocystis murina*: respiratory disease and mortality in immunodeficient mice. *Candida* spp.: recent reports associated with immune deficiency/suppression and or use of antimicrobials.	378,381,534–536
Biosecurity in immunodeficient mice	High risk of *Pneumocystis carinii* infection in T cell-deficient mice including *Foxn1*^{nu}, *Prkdc*^{scid} mice and immune impaired GEMs; immunodeficient traits in mutant mice masked by the immune/inflammatory response associated with chronic γ-herpesvirus infection; MNV infection in *Atg16l1*-deficient mice associated with Paneth cell abnormalities; murine papillomavirus associated with proliferative lesions at the mucocutaneous junctions of *Foxn1*^{nu} mice; mousepox recrudescence following immunosuppression and transmission to naïve mice.	375,400,537–540
Biosecurity: contaminated biologicals	Rodent pathogens (latent infections): contaminated serum with mousepox. Human pathogens: contaminated human cell lines (humanized mice and patient derived xenografts mice). *Mycoplasma arginini*: suppurative arthritis in *Prkdc*^{scid} mice (contaminated cell lines).	378,410,434,451,452,541
Modulation of the microbiome	SFB associated with the development of IL17 and IL22-producing CD4+ T cells (TH17 cells) in the intestinal lamina propria of germ-free mice. *Tritrichomonas muris*: associated with elevated TH1 response in the cecum of naive WT mice and accelerated colitis in *Rag1*-deficient mice after T cell transfer.	386,387,405,406
Drugs administered for clinical or experimental purposes		
Tamoxifen-inducible *Cre/loxP* system (Cre-ERT2)	Estrogen-dependent and -independent tamoxifen immunomodulatory effect; shift from a TH1- to a TH2-mediated immune response.	458,459
Tetracycline/doxycycline-inducible Tet-Off/Tet-On system	Doxycycline-dependent modulation of immune and inflammatory functions including allotransplant rejection, response to LPS, neutrophil chemotaxis; tetracycline/doxycycline-induced dysbiosis.	461,462,472
Nitrosamines, nitrates, nitrites (mutagens, carcinogens)	DMN: suppression of both humoral and cell-mediated immunity. ENU: lymphoma (AKR/J, C58/J, C57BL/6J, NOD/LtJ); myeloid malignancies (SWR/J, DBA/2J); thymic lymphoma with/without K-ras mutations.	542–544
TMP-SMX	TMP-SMX alone: no effect on hematopoiesis or immune cell functions.	545

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Table 5 Continued

Immunomodulators	Possible Effects on the Immune and Other Systems	References
Ivermectin	TMP-SMX synergized with zidovudine: anemia, thrombocytopenia, lymphopenia, and neutropenia, decreased splenic macrophages, suppressed AC-dependent T cell responses. Immunomodulation of T-helper cells; decreased recruitment of immune cells and cytokines in a model of asthma; unintended activation of tamoxifen-regulated Cre fusion protein in T cells.	460,546,547
Estrogens (for engraftment of estrogen-dependent tumors)	Increased splenic neutrophils (estrogen-treated C57BL/6 mice); enhanced IFNγ expression; thymic atrophy (DERKO mice); myelosuppression (decreased pluripotent hematopoietic stem cells). Synthetic estrogens (DES): altered thymic T cell differentiation through interference with positive and negative selection processes in prenatally exposed mice; functionally defective NK cells and increased tumor susceptibility in neonatally exposed female mice. Other: increased trabecular bone mineral density, fat reduction and increased uterine weight (DERKO mice); fibro-osseous lesions (bone marrow replacement by fibrovascular stroma (KK/HlJ and NZW/LacJ female mice).	501,548–552
Androgens (for engraftment of androgen-dependent tumors)	Androgen stimulation: thymic involution resulting from decreased colonization of bone-marrow-derived stem cells; loss of thymic epithelial cells; thymocyte apoptosis; inhibition of CD4+ T cell differentiation through upregulation of phosphate Ptpn1; erythroid hyperplasia. Castration: enhanced CD8+ T cell vaccine response to prostate-specific antigens.	553–556
Streptozotocin	Early lymphopenia in both blood and spleen; relative increased Tregs in spleen, peripheral blood, and lymph nodes; delayed islet and skin allograft rejection.	557
NPs	Suppression of systemic humoral immunity (multi wall carbon nanotubes); inhibition of T cell-mediated immunity (iron oxide NPs, fuellerene 60); myelosuppression (Sb2O3, Co, ZnO, TiO2 NPs); allergic reactions (Ag NPs); anti-inflammatory activity and inhibition of cellular responses induced by IL1B (citrate-coated gold NPs).	558–563
Other experimental interventions		
Cre/loxP	Activation of STING antiviral response by endonuclease activity of Cre recombinase.	457
CRISPR-Cas9	Adaptive immune response against Cas9.	458,459
Tetracycline/doxycycline-inducible Tet-Off/Tet-On system	Apoptotic response in activated lymphocytes resulting from DNA binding by tTA/rtTA.	464
Classical reporter molecules	Increase in the CTL response against transplanted eGFP-expressing leukemia cells in BALB/c mice; IFNγ response to the dominant CTL epitope of Luc, with consequent restricted growth and metastatic activity of the reporter-labelled tumor cells in a mouse model of mammary adenocarcinoma; antigen specific activation of T cells to the reporter gene β-galactosidase, with loss of transgene expression.	465–470,564,565

AA, arachidonic acid; AC, accessory cell; BPA, Bisphenol A; CTL, cytotoxic T lymphocyte; CYP, cyclophospharmide; DCs, dendritic cells; DERKO, double ER knockout mice; DES, diethylstilbestrol; DP, double positive; DHA, docosahexaenoic acid; DMN, dimethylnitrosamine; EBV, Epstein-Barr virus; eGFP, enhanced green fluorescent protein; ENU, N-ethyl-N-nitrosourea; GVHD, graft-versus-host disease; IBD, inflammatory bowel disease; IELs, intra-epithelial lymphocytes; ILC3, type 3 innate lymphoid cells; ILC2, type 2 innate lymphoid cells; LCMV, lymphocytic choriomeningitis virus; Luc, luciferase; MHV, mouse hepatitis virus; MHV-68, murine gammaherpesvirus 68; MNV, murine norovirus; MNM, minute virus of mice; MPV, mouse parvovirus; MuHV-1, murid herpesvirus 1 (mouse cytomegalovirus); MuHV-3, murid herpesvirus 3 (mouse thymic virus); NKs, natural killer cells; NPs, nanoparticles; PPE, personal protective equipment; rtTA, reverse tetracycline-controlled transactivator protein; SFB, segmented filamentous bacteria; TCDD, 2,3,7,8-tetrachlorodibenzodioxin; TMP-SMZ, trimethoprim/sulfamethoxazole; Tregs, regulatory T cells; tTA, tetracycline-controlled transactivator protein; VDR, vitamin D receptor.

and microbial burden (discussed further below) are among the variables with expected immune effects.[322–325]

Housing density

Co-housing or group housing of mice is practical and economical with compatible animals that do not fight and kill each other before study endpoints. Single housing can be required, especially for male mice to survive to study endpoints. Co-housing vs single housing effects on stress and immunity vary with strain, sex, and other conditions.[326–329]

Enrichment

Enrichment for shelter, nesting, and gnawing have variable effects that are often associated with strain, sex, and other conditions. In general, provision of nesting material helps to reduce the level of stress and influences positively several immune parameters including NK cell antitumor functions.[331–336]

Temperature humidity

Current temperature recommendations for mouse housing of 22–26°C are below the mouse thermoneutral zone of 30–32°C.

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The "mild" cold stress caused by standard sub-thermoneutral housing temperatures affects immune responses, tumor growth, and other experimental outcomes. Huddling and nest building are methods of behavioral thermoregulation used by mice under cold stress. Recommended relative humidity is 55% ± 10%. Humidity levels vary with type of caging, season, and geographic location. Higher humidity is associated with increased levels of ammonia and bioburden with severe impairment of respiratory mucosal immune response and increased risk of opportunistic infections, respectively.[327,328,337–342]

Illumination (Light)

Circadian and light effects on immunity are recognized in many species, including humans and mice. Albino animals have higher light sensitivity, and a number of common mouse strains are blind with retinal degeneration but still exhibit responses to light and light cycles.[343–345] Dysregulation of circadian rhythmicity in mice induces a generalized proinflammatory macrophage activation and exacerbates diet-induced systemic insulin resistance and glucose intolerance. A balanced circadian rhythm is also critical to maintain immune homeostasis via the immunoregulatory activity of the neurohormone melatonin.[346,347]

Noise vibration

While a number of common mouse strains are deaf or become deaf with age, hearing mice perceive and respond to sounds outside of human ranges. Noise and vibration are shown to cause stress, induce corticosterone, and negatively affect reproduction.[348–351]

Bedding

While contemporary commercial contact bedding materials tend to be far more standardized with more quality control and freedom from contaminants than previously, contaminants with potential effects on research outcomes can still occur in bedding material. Dust, ammonia levels, fungal spores, phytoestrogens, and endotoxins in bedding also have implications for diverse research. Regional variation among bedding material has implications for various research areas, including immunology, with corncob bedding more available in the United States than in the European Union and other sites, and with hardwoods, cellulose, or paper being other common options. The relative palatability of or preference for a bedding over the intended diet may affect consumption of the diet.[323,352–357]

Diet

Contemporary commercial research diets also are far more standardized with more quality control than previously, and nutritional deficiencies are unlikely on contemporary commercial diets. Nutritional requirements for mice, including adequate levels of nutrients,[358] minerals,[359] and vitamins,[360] exist as do guidelines for contaminants in laboratory rodent diets.[361,362] Possible contaminants with immunomodulatory effects include industrial chemicals (e.g., PCBs, PCDDs, and PCDFs), pesticides (e.g., DDT), metals, nitrosamines, endocrine-disrupting compounds, and mycotoxins. However, contaminants are identified in contemporary diets and are a concern for biomedical research and regulatory toxicology.[363,364] Endocrine-disrupting phytoestrogen-rich ingredients, especially soy and alfalfa, as primary protein sources are expected in natural ingredient (aka grain-based or cereal-based) diets. Phytoestrogens are recognized to have influences on rodent reproduction, immunity, cardiovascular, neoplastic, and other conditions.[365,366] Animal byproducts, bone meal, and fish meal are used in many natural ingredient diets and are a source of nitrites and nitrosamines.[363,367]

Poor reporting of research-relevant diet factors such as differences between purified and natural ingredient diets have attracted attention and concern recently.[10,358,368] Research diets are frequently provided ad libitum to rodents on shorter term studies. Diet restriction in long-term studies usually improves survival and reduces neoplastic, kidney, inflammatory, and other lesions.[369–371]

Water

Contemporary water sources and delivery methods frequently include reverse osmosis, filtration, hyperchlorination, acidification, or some combination of these, delivered by water bottles, glass, or various plastics, tinted or untinted, and/or automated watering systems.

Acidification became a common practice for research rodents to control opportunistic bacteria (especially *P. aeuroginosa*) causing morbidity mortality in immune-deficient rodents that were further immunosuppressed by irradiation that further compromised or eliminated their innate immunity. Water treatments including administered drugs can affect water consumption and have immune or other effects that warrant reporting in publications.[343,372–374]

Husbandry and Biosecurity

Special husbandry needs of immunodeficient mice are largely related to protection from agents that may cause morbidity and mortality. Such agents may be harbored by clinically "healthy" immune sufficient mice, or possibly by human handlers, and may be transferred by common equipment and other fomites. Proximity to immune sufficient mice or to any mouse cohort with different microbial status warrants special procedures and policies for sanitation and sterilization of caging, feed, water and other materials, sequence of animal handling, and microbial surveillance. GEM models may also manifest unexpected immunodeficiencies.[375] Immunomodulatory effects by common agents (Table 5) demand that immune relevant research must pay greater attention to microbial exclusion lists and definition of the specific pathogen free (SPF) status in the vivarium as well as in reporting. Use of the term SPF requires specification of the excluded agents.[316,376–378]

Some of the most concerning opportunistic agents in contemporary immunodeficient mice, such as *Staphylococcus xylosus*, *Corynebacterium bovis*, and *Pneumocystis murina*, are fairly common and usually subclinical in immune sufficient mice.[379–384] (see also Table 5)

Microbiota and Microbiome

Autochthonous (commensal and symbiotic) microbiota

Systemic and mucosal immunity in mice are influenced by the intestinal flora (microbiota).[27,375,385] The intestinal microbiota are important to effective mucosal immunity and to immune responses beyond the gut. As an example, segmented filamentous bacteria (SFB) have been identified as an important antigenic stimulus in inducing Th17 responses, and murine Th17 responses are blunted in mice that lack SFB.[395] Also SFB are shown to influence neuroinflammation in EAE models, diabetes

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susceptibility in NOD mice, and development of autoimmune arthritis in some models.[387–390] SFB normally colonize the distal small intestine of infant mice and decline with the maturation of the mucosal barrier and local IgA levels.[391] In mice with deficient adaptive immunity or Ig production, or mice specifically deficient in IgA, SFB persist with expanded distribution throughout the small intestine.[392,393] SFB are difficult to propagate *in vitro* and have not been included in the standardized communities of intestinal microbiota (e.g., Altered Schaedler flora) specifically maintained in some sources of laboratory mice to uniform the influence of microbiota on the experimental conditions. In this context, SFB are not expected in immune deficient mice from certain commercial vendors that maintain the mice in isolators with defined or highly restricted flora.[394,395]

Strain-associated and vendor-dependent differences in the gut microflora of laboratory mice have been identified and are implicated in variability in research results (see Table 5).[378,385,396–400] Flora with more *Bacteroides* spp. and *Parabacteroides* spp. such as *Parabacteroides distasonis* may mitigate DSS-induced colitis.[401] Mice of similar strains but from sources with more simplified or restricted microbiota, lacking SFB, have quite different dendritic cell profiles and Th17 responses.[402] In several immune relevant GEM including IL10, T cell receptor alpha, and IL2 knockout mice, intestinal inflammation also is substantially influenced by intestinal microbiome.[403,404] Enteric protists are common in mice (but usually excluded from commercial sources) and also have been shown to influence Th17 and Th1 responses as well.[405,406] The microbiota or autochthonous microflora of research animals are increasingly recognized as highly research relevant. The restricted microflora of naïve mice from reputable commercial sources have been presented as a research concern, but their well-characterized microbiota also represent an opportunity for this area of immune relevant research.[407–408]

Allochthonous (noncommensal) agents

Morbidity, mortality, and other adverse or confounding effects of infectious agents on research have led to great effort and expense toward microbial definition and exclusion by commercial sources of mice and for quarantine and surveillance by research programs to protect animals and research from infections.[410] Immune deficient mice are notoriously susceptible to disease and death from pathogens and opportunists. The same agents in immune sufficient mice may result in subclinical infections or a spectrum of disease phenotypes that are influenced by genetic background, age, sex, and other factors. But any agent detected by an immune system can be expected to elicit an immune response, or "immunomodulate." Table 5 summarizes examples of microbial effects on immunity and particular concerns for morbidity and mortality in immune deficient mice.[411,412]

Viruses with selective tropisms for immune cells include some of the murine parvoviruses, herpesviruses, and retroviruses. Many of the parvoviruses infecting mice are lymphocytotropic, altering both CD4+ and CD8+ T cell-mediated responses during acute infection.[378,413,414] Although long-term immune effects may not be identified with natural infections by some parvoviruses, significant immunomodulation is well documented with infection by others (Table 5).[378] Parvoviruses replicate in actively dividing cells and are studied as oncolytic agents in combined anti-cancer therapies.[415] Several mouse parvoviruses were identified originally as contaminants in biological materials such as tumor cell lines. They remain among the most common agents identified in research mice, pet store and feral mice, and biological materials. Despite the usual absence of clinical signs in parovirus-infected mice, these agents should be especially concerning in immune relevant and cancer studies.[409,416–420]

Although mouse herpesviruses are not expected in contemporary research colonies, mice are host to several lymphocytotropic herpesviruses that are reported in pet store and feral mice.[421,422] Mouse thymic virus infection in newborn mice causes thymic necrosis, with selective targeting of T cells, and transient immunosuppression.[413] This agent or a close relative was recently classified under the genus *Roseolovirus* similar to human roseoloviruses.[423,424] Murine cytomegalovirus is used to model human cytomegalovirus infection and targets hematolymphoid tissues and salivary glands. Disease manifestations vary with the genetic background.[425] Occult (seronegative) murine cytomegalovirus infection has been shown to affect responses to allografts.[426]

Murine gammaherpesvirus 68, a natural pathogen of bank voles, is related to human gamma herpesviruses Epstein-Barr virus (EBV) and Kaposi sarcoma-associated herpesvirus and is used to study the pathogenesis of gammaherpesviruses in experimentally infected mice. However, *Mus musculus* ssp. are not the natural host, and horizontal transmission between laboratory mice is not expected.[422,426–430] EBV is a human B-lymphotropic gamma herpesvirus that infects more than 90% of the human population. Human infections are subclinical (latent) when effectively controlled or can result in infectious mononucleosis or malignancies such as Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's lymphoma, and post-transplant lymphoproliferative disorders. Immunodeficient and humanized mice have been informative preclinical tools for studying the pathogenesis of some of the conditions associated with EBV.[431–433] EBV-induced post-transplant lymphoproliferative disorders are also increasingly recognized to complicate research with human patient derived xenografts in severely immunodeficient mice[434–436] and may be amenable to suppression of human lymphocyte proliferation in the donor tissue.[437,438]

Exogenous retroviruses and active endogenous retroviruses have lymphocyte tropisms and roles in immune modulation and lymphoproliferative conditions as well as in mammary carcinogenesis, sarcoma development, and lymphomagenesis. Exogenous horizontally transmitted retroviruses have been eliminated from commercially available mice but are identified in wild mice. Insertional mutagenesis with reintegration of endogenous retroviruses or transposition of retroelements has resulted in spontaneous mutations including some immune relevant ones such as *Foxn1*^{nu}, *Lep*^{ob}, and *Fas*^{lpr}.[439–441] Mice infected with LP-BM5 (defective) murine leukemia virus develop murine acquired immunodeficiency syndrome and have been widely used as a preclinical model to study the pathogenesis of human retroviral infections (Table 5).[442,443] Lifelong expression of viral proteins encoded by endogenous retroviruses/retroelements may be responsible for most of the spontaneous immune-mediated conditions observed in some inbred strains during aging, including glomerulonephritis and polyarteritis.[440,444] Strain-specific variations in the composition and activity of endogenous retroviruses/retroelements and immune response against retroviral antigens also play a role in the susceptibility of specific mouse backgrounds to experimental autoimmune conditions including SLE and T1D.[445–448]

While the parvoviruses, herpesviruses, and exogenous retroviruses have been eliminated from commercial sources of contemporary laboratory mice because of disease or other confounding effects on research, recent interest in the "normal" immunity of wild or pet store mice may render these agents, as

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well as historically important mouse disease problems and zoonotic concerns, more relevant.[449,450]

Biological Materials

Biological materials, including transplantable tumors, cell lines, serum, embryos, and gametes, can harbor a diversity of mouse viruses (parvoviruses, ectromelia virus, MHV, lactose dehydrogenase elevating virus, and retroviruses), human viruses, and bacteria, notoriously the Mycoplasmas.[420,451,452] They therefore represent a substantial concern as a source of pathogens and microbial confounders, especially in studies that involve immunodeficient rodents. Reporting recommendations plead for QA of cell lines: genetic QA (authentication to confirm the identity of the cell lines), and microbial QA (to assure freedom from pathogens).[453–456]

Unintended Consequences of Genetic Engineering Strategies

Genetic engineering strategies have immune effects that may have unintended or unexpected consequences for diverse research areas.

Cre/loxP-based DNA recombination technology is used for conditional (tissue-specific) gene targeting. The endonuclease activity of Cre recombinase, including the "illegitimate" targeting of the numerous pseudo-loxP sites across the mouse genome, results in the strong induction of an antiviral response. This is due to the recruitment of the specific cytosolic DNA sensor stimulator of interferon genes (STING), concurrent with Cre-dependent DNA damage and the accumulation of cytoplasmic DNA fragments. Given the primary role of STING in the activation of antiviral immune pathways (including type-I IFN), Cre expression can impact multiple immune parameters in Cre/loxP-based mouse models. Appropriate Cre-only controls may help in distinguishing signal from noise.[457]

The tamoxifen-inducible Cre/loxP system (Cre-ERT2) allows site- and time-specific gene targeting in the mouse. Tamoxifen has immune relevant effects, as well as toxic and genotoxic effects. The estrogen-dependent and -independent effects of tamoxifen have been demonstrated to promote a shift from a Th1- to a Th2-mediated immune responses. Such effects can especially impact allergy and autoimmune models involving activation of Th1-mediated immunity (e.g., EAE and some SLE models).[458,459] Recently, oral ivermectin treatment has been specifically linked to the unintended activation of Cre-ERT2 system in T cells.[460]

Tetracycline-controlled transcriptional activation (Tet-Off/Tet-On) systems allow site-specific, reversible, and dose-dependent control of gene expression in mice. Doxycycline (a tetracycline derivative) is administered or withdrawn to regulate target gene expression. Doxycycline in mice interferes with and modulates immune and inflammatory responses relevant to allotransplant rejection, response to LPS, and neutrophil chemotaxis, among others.[461–463] Recent works have also unveiled the effect of doxycycline on murine gut microbiota and how the resulting dysbiosis might affect the immune response in diverse experimental settings.[461–463] DNA binding by tetracycline/doxycycline-controlled Tet-transactivator (tTA) and its reverse is apparently sufficient to induce apoptosis in activated lymphocytes. These findings indicate that a major experimental bias exists in the use of the Tet-On/Off system for lymphocyte targeting as the approach may (1) limit the extent of the adaptive immune reaction and (2) favor the outgrowth of apoptosis-resistant subpopulations of lymphoid cells.[464]

Expression of fluorescent or enzymatic reporters driven by gene-specific regulatory elements is used to study *in vivo* or ex vivo activity and distribution of specific molecular targets or mutant alleles in GEM models. However, an increasing number of studies show that reporters can be highly immunogenic. Indeed, response of the mouse immune system against classical reporter molecules (including enhanced green fluorescent protein, luciferase, and β-galactosidase) has been demonstrated. The inherent immunogenicity of reporter gene's products depends on different factors including the mouse's background strain as well as level of expression and tissue distribution/accumulation. It is therefore extremely important to consider carefully any potential variable associated with the use of genetic reporter systems for immunological studies in mice.[465–470]

Even the most recent and sophisticated strategies for genome editing, including the revolutionary CRISPR-Cas9 system, have demonstrated experimental caveats influencing the immune system. In addition to the potential immunogenicity of viral vectors in viral delivery systems, human and mice have demonstrated preexisting adaptive immunity to Cas9 homologues expressed by common bacteria such as *Staphylococcus aureus* and *Streptococcus pyogenes*. The inherent immunogenicity of Cas9 is a concern not only for the preclinical application of the CRISPR-Cas9 system, but also for its potential clinical use as gene therapy strategy.[471,472]

Future Directions in Mouse Immunology

Human Surrogate/"Avatar" Approaches

Options to take advantage of humanized mice and other animals to study human derived immune elements in nonhuman surrogates are reviewed elsewhere. These present a diversity of opportunities for better understanding of human disease conditions as well as a number challenges that also may be informative if approached critically and scientifically.[431,566,567] For a comprehensive overview on this topic, readers are encouraged to consult the contribution from Simons et al. in the present issue of the *ILAR Journal*.

Genetic Approaches

Options to take advantage of the spectrum of mouse genetic and immune diversity include factorial study design and Collaborative Cross (CC)-derived RI strains and Diversity Outbred (DO) mice. In a factorial study design, significance can be achieved with relatively small "n" from several strains selected for informative differences in immune relevant genotypes and phenotypes.[568,569] Recognizing that an inbred strain represents an intentionally limited fraction of the spectrum of genetic variability of laboratory mice not designed or suited to model immunological endpoints at a population scale,[570,571] the CC-derived RI strains represent the genetic variability across the 7 major families of mice and offer fairly new options for dissecting genetic and molecular mechanisms of immunity and disease.[572,573] The CC is a mouse reference population with high allelic diversity constructed by a breeding strategy that systematically outcrosses 8 founder strains, followed by inbreeding to obtain new RI strains. Five of the 8 founder strains are "classical" laboratory strains including 129S1/SvImJ, A/J, C57BL/6J, NOD/ShiLtJ, and NZO/HlLtJ. Three founder strains are "wild-derived": CAST/EiJ, PWK/PhJ, and WSB/EiJ. Currently available CC RI lines are distributed through consortia (e.g., http://csbio.unc.edu/CCstatus/index.py) and public repositories

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(e.g., https://www.jax.org/strain/027296). Since their inception, partially inbred CC mice have been characterized and compared for the identification of deviant immune traits or phenotypes. They have provided opportunities to study the evolution of complex genetic interactions.[573] The application of immunogenomics and immunonogenetics techniques on CC mice has identified QTLs, polymorphic regions, and candidate genes that control mouse immunodiversity[572] and have contributed to our understanding of susceptibilities to SARS coronavirus, West Nile virus, and *Aspergillus fumigatus*.[574–577] DO mice (https://www.jax.org/strain/009376) were developed by random outcross matings of 160 CC RI lines, and the breeding strategy of continued random matings is designed to maximize their genetic diversity.[578–581] The genetic heterogeneity of DO mice far exceeds that of genetically undefined mice, termed "outbred," that derive from the Swiss branch of the mouse family tree (e.g., CD-1, CFW, ICR, ND4, NMRI, SW) originating from Clara Lynch's original 9 albino mice brought to the United States from Switzerland in 1926. The genetic heterogeneity and heterozygosity among these mice is more limited and varies with their source.[582,583] While the literature is still fairly limited on CC RI strains and the derived DO mice, these represent translational research tools that take advantage of mouse genetic variability to identify disease mechanisms, select novel drug targets, and discover associated biomarkers.

Microbial Approaches

There is recent interest in the use of genetically and microbially "wild-like" mice as a more human like or human relevant strategy.[3,4,6,450,573,584] The studies make relevant and useful points about the naïve immune systems of "clean" C57BL/6 mice recently received from microbially restricted commercial sources. However, many mice bred in house in research institutions are not quite so naïve or microbially restricted.[378,585–587] Undefined or incompletely defined microbiota of pet store or feral mice raise concerns for infection related morbidity, mortality, and unpredictable experimental confounds as well as biosafety concerns related to zoonotic agents. Advances in gnotobiotics and microbiota characterization offer opportunities for defined and strategic approaches that will deliver important insights to immune modulation by autochthonous and allochthonous microflora.[385,409,449,450]

Conclusions

Mice have had important roles in advancing the field of immunology and fostering the development of new diagnostic and therapeutic avenues. Recognition of intrinsic and extrinsic contributors to immune phenotypes is crucial for the selection of more relevant and reproducible mouse models and generation of robust translational data. Known contributors can be intentionally used or intentionally avoided in the experimental system. Accurate reporting of animals and study conditions is mission critical to communicating biomedical research. Well-designed and reported research in mice has much to offer to our understanding of immunity and important diseases of humans and other species.

Supplementary Material

Supplementary material is available at *Institute for Laboratory Animal Research Journal* online.

References

1. Bryda EC. The mighty mouse: the impact of rodents on advances in biomedical research. *Mo Med*. 2013;110(3):207. http://www.ncbi.nlm.nih.gov/pubmed/23829104.
2. Justice MJ, Dhillon P. Using the mouse to model human disease: increasing validity and reproducibility. *Dis Model Mech*. 2016;9(2):101–103. doi:10.1242/dmm.024547.
3. Abolins S, King EC, Lazarou L, et al. The comparative immunology of wild and laboratory mice, *mus musculus domesticus*. *Nat Commun*. 2017;8:14811. doi:10.1038/ncomms14811. https://www.nature.com/articles/ncomms14811. Accessed Jan 5, 2018.
4. Cauwels A, Vandendriessche B, Brouckaert P. Of mice, men, and inflammation. *Proc Natl Acad Sci USA*. 2013;110(34):E3150. doi:10.1073/pnas.1308333110. http://www.pnas.org/content/110/34/E3150.extract.
5. Perlman RL. Mouse models of human disease: an evolutionary perspective. *Evol Med Public Health*. 2016;2016(1):170. http://www.ncbi.nlm.nih.gov/pubmed/27121451.
6. Mestas J, Hughes CCW. Of mice and not men: differences between mouse and human immunology. *J Immunol*. 2004;172(5):2731–2738.
7. Sellers RS. Translating mouse models. *Toxicol Pathol*. 2017;45(1):134–145. doi:10.1177/0192623316675767.
8. Sundberg JP, Schofield PN. Commentary: mouse genetic nomenclature. Standardization of strain, gene, and protein symbols. *Vet Pathol*. 2010;47(6):1100–1104. doi:10.1177/0300985810374837.
9. Fontaine DA, Davis DB. Attention to background strain is essential for metabolic research: C57BL/6 and the international knockout mouse consortium. *Diabetes*. 2016;65(1):25–33. doi:10.2337/db15-0982.
10. Omary MB, Cohen DE, El-Omar EM, et al. Not all mice are the same: standardization of animal research data presentation. *Gastroenterology*. 2016;150(7):1503–1504. doi:10.1053/j.gastro.2016.03.034.
11. Dunn LC, Grüneberg H, Snell GD. Report of the committee on mouse genetics nomenclature. *J Hered*. 1940;31(12):505–506. doi:10.1093/oxfordjournals.jhered.a104827. https://academic.oup.com/jhered/article/31/12/505/889124. Accessed May 8, 2018.
12. Carter TC, Dunn LC, Falconer DS, Grüneberg H, Heston WE, Snell GD. Standardized nomenclature for inbred strains of mice. *Cancer Res*. 1952;12(8):602–613.
13. Green MC, Grueneberg H, Hertwig P, et al. A revision of the standardized genetic nomenclature for mice. *J Hered*. 1963;54:159–162.
14. Staats J. Standardized nomenclature for inbred strains of mice: third listing. *Cancer Res*. 1964;24:147–168.
15. Staats J. Standardized nomenclature for inbred strains of mice: fourth listing. *Cancer Res*. 1968;28(3):391–420.
16. Staats J. Standardized nomenclature for inbred strains of mice: fifth listing. *Cancer Res*. 1972;32(8):1609–1646.
17. Staats J. Standardized nomenclature for inbred strains of mice: sixth listing. *Cancer Res*. 1976;36(12):4333–4377.
18. Staats J. Standardized nomenclature for inbred strains of mice: seventh listing for the international committee on standardized genetic nomenclature for mice. *Cancer Res*. 1980;40(7):2083–2128.
19. Staats J. Standardized nomenclature for inbred strains of mice: eighth listing. *Cancer Res*. 1985;45(3):945–977.
20. Festing M, Kondo K, Loosli R, Poiley SM, Spiegel A. International standardized nomenclature for outbred

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stocks of laboratory animals. *Z Versuchstierkd*. 1972;14(4): 215–224.
21. [NRC] National Research Council. *Guidance for the description of animal research in scientific publications*. Washington (DC): National Academies Press (US); 2011. http://www.ncbi.nlm.nih.gov/books/NBK84205/. Accessed May 10, 2018.
22. Everitt JI. The future of preclinical animal models in pharmaceutical discovery and development: a need to bring in cerebro to the in vivo discussions. *Toxicol Pathol*. 2015;43(1): 70–77. doi:10.1177/0192623314555162.
23. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol*. 2010;8(6): e1000412. doi:10.1371/journal.pbio.1000412.
24. Doran AG, Wong K, Flint J, Adams DJ, Hunter KW, Keane TM. Deep genome sequencing and variation analysis of 13 inbred mouse strains defines candidate phenotypic alleles, private variation and homozygous truncating mutations. *Genome Biol*. 2016;17(1):167. doi:10.1186/s13059-016-1024-y.
25. Adams DJ, Doran AG, Lilue J, Keane TM. The mouse genomes project: a repository of inbred laboratory mouse strain genomes. *Mamm Genome*. 2015;26(9–10):403–412. doi:10.1007/s00335-015-9579-6.
26. Rahim MMA, Tu MM, Mahmoud AB, et al. Ly49 receptors: innate and adaptive immune paradigms. *Front Immunol*. 2014;5:145. doi:10.3389/fimmu.2014.00145.
27. Sellers RS, Clifford CB, Treuting PM, Brayton C. Immunological variation between inbred laboratory mouse strains: points to consider in phenotyping genetically immunomodified mice. *Vet Pathol*. 2012;49(1):32–43. doi:10.1177/0300985811429314. https://www.ncbi.nlm.nih.gov/pubmed/22135019. Accessed May 3, 2018.
28. Liu J, Karypis G, Hippen KL, et al. Genomic view of systemic autoimmunity in MRLlpr mice. *Genes Immun*. 2006;7(2): 156–168. doi:10.1038/sj.gene.6364286. http://www.ncbi.nlm.nih.gov/pubmed/16508641.
29. Nose M, Nishihara M, Kamogawa J, Terada M, Nakatsuru S. Genetic basis of autoimmune disease in MRL/lpr mice: dissection of the complex pathological manifestations and their susceptibility loci. *Rev Immunogenet*. 2000;2(1):154–164.
30. Yamada A, Miyazaki T, Lu L, et al. Genetic basis of tissue specificity of vasculitis in MRL/lpr mice. *Arthritis Rheum*. 2003;48(5):1445–1451. doi:10.1002/art.10952.
31. Vidal S, Kono DH, Theofilopoulos AN. Loci predisposing to autoimmunity in MRL-fas lpr and C57BL/6-faslpr mice. *J Clin Invest*. 1998;101(3):696–702. doi:10.1172/JCI1817.
32. Takahashi S, Fossati L, Iwamoto M, et al. Imbalance towards Th1 predominance is associated with acceleration of lupus-like autoimmune syndrome in MRL mice. *J Clin Invest*. 1996;97(7):1597–1604. doi:10.1172/JCI118584.
33. Xu M, Hou R, Sato-Hayashizaki A, et al. Cd72(c) is a modifier gene that regulates fas(lpr)-induced autoimmune disease. *J Immunol*. 2013;190(11):5436–5445. doi:10.4049/jimmunol.1203576.
34. Hogarth MB, Slingsby JH, Allen PJ, et al. Multiple lupus susceptibility loci map to chromosome 1 in BXSB mice. *J Immunol*. 1998;161(6):2753–2761.
35. Kimura J, Ichii O, Nakamura T, Horino T, Otsuka S, Kon Y. BXSB-type genome causes murine autoimmune glomerulonephritis: pathological correlation between telomeric region of chromosome 1 and yaa. *Genes Immun*. 2014;15(3):182–189. doi:10.1038/gene.2014.4.
36. Choi Y, Simon-Stoos K, Puck JM. Hypo-active variant of IL-2 and associated decreased T cell activation contribute to impaired apoptosis in autoimmune prone MRL mice. *Eur J Immunol*. 2002;32(3):677–685. doi:AID-IMMU677>3.0.CO;2-I.
37. Poland A, Glover E. Characterization and strain distribution pattern of the murine ah receptor specified by the ahd and ahb-3 alleles. *Mol Pharmacol*. 1990;38(3):306–312.
38. De Souza VR, Cabrera WK, Galvan A, et al. Aryl hydrocarbon receptor polymorphism modulates DMBA-induced inflammation and carcinogenesis in phenotypically selected mice. *Int J Cancer*. 2009;124(6):1478–1482.
39. Esser C, Rannug A, Stockinger B. The aryl hydrocarbon receptor in immunity. *Trends Immunol*. 2009;30(9):447–454.
40. Esser C. The immune phenotype of AhR null mouse mutants: not a simple mirror of xenobiotic receptor overactivation. *Biochem Pharmacol*. 2009;77:597–607. http://www.sciencedirect.com/science/article/pii/S0006295208007193.
41. Tulone C, Tsang J, Prokopowicz Z, Grosvenor N, Chain B. Natural cathepsin E deficiency in the immune system of C57BL/6J mice. *Immunogenetics*. 2007;59(12):927–935.
42. Wetsel RA, Fleischer DT, Haviland DL. Deficiency of the murine fifth complement component (C5). A 2-base pair gene deletion in a 5'-exon. *J Biol Chem*. 1990;265(5):2435–2440.
43. Skerka C, Chen Q, Fremeaux-Bacchi V, Roumenina LT. Complement factor H related proteins (CFHRs). *Mol Immunol*. 2013;56:170–180. http://www.sciencedirect.com/science/article/pii/S0161589013004185.
44. Encinas JA, Wicker LS, Peterson LB, et al. QTL influencing autoimmune diabetes and encephalomyelitis map to a 0.15-cM region containing Il2. *Nat Genet*. 1999;21(2):158–160.
45. Javan MR, Shahraki S, Safa A, Zamani MR, Salmaninejad A, Aslani S. An interleukin 12 B single nucleotide polymorphism increases IL-12p40 production and is associated with increased disease susceptibility in patients with relapsing-remitting multiple sclerosis. *Neurol Res*. 2017;39(5):435–441.
46. Wen X, Chen S, Li P, et al. Single nucleotide polymorphisms of IL12B are associated with takayasu arteritis in Chinese Han population. *Rheumatol Int*. 2017;37(4):547–555.
47. Zwiers A, Fuss IJ, Seegers D, et al. A polymorphism in the coding region of Il12b promotes IL-12p70 and IL-23 heterodimer formation. *J Immunol*. 2011;186(6):3572–3580.
48. Staeheli P, Grob R, Meier E, Sutcliffe JG, Haller O. Influenza virus-susceptible mice carry mx genes with a large deletion or a nonsense mutation. *Mol Cell Biol*. 1988;8:4518–4523. http://mcb.asm.org/content/8/10/4518.abstract.
49. Verhelst J, Spitaels J, Nurnberger C, et al. Functional comparison of Mx1 from two different mouse species reveals the involvement of loop L4 in the antiviral activity against influenza A viruses. *J Virol*. 2015;89(21):10879–10890.
50. Lightfield KL, Persson J, Brubaker SW, et al. Critical function for Naip5 in inflammasome activation by a conserved carboxy-terminal domain of flagellin. *Nat Immunol*. 2008;9(10):1171–1178.
51. Tenthorey JL, Haloupek N, Lopez-Blanco JR, et al. The structural basis of flagellin detection by NAIP5: a strategy to limit pathogen immune evasion. *Science*. 2017;358(6365):888–893.
52. Fortier A, Min-Oo G, Forbes J, Lam-Yuk-Tseung S, Gros P. Single gene effects in mouse models of host: pathogen interactions. *J Leukoc Biol*. 2005;77(6):868–877.
53. Moayeri M, Crown D, Newman ZL, et al. Inflammasome sensor Nlrp1b-dependent resistance to anthrax is mediated by caspase-1, IL-1 signaling and neutrophil recruitment.

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PLoS Pathog. 2010;6(2):e1001222. doi:10.1371/journal.ppat.1001222. http://www.ncbi.nlm.nih.gov/pubmed/21170303.
54. Tschopp J, Martinon F, Burns K. NALPs: a novel protein family involved in inflammation. *Nat Rev Mol Cell Biol*. 2003;4(2):95–104.
55. Ulland TK, Jain N, Hornick EE, et al. Nlrp12 mutation causes C57BL/6J strain-specific defect in neutrophil recruitment. *Nat Commun*. 2016;7:13180. doi:10.1038/ncomms13180.
56. Chen L, Wilson JE, Koenigsknecht MJ, et al. NLRP12 attenuates colon inflammation by maintaining colonic microbial diversity and promoting protective commensal bacterial growth. *Nat Immunol*. 2017;18(5):541–551.
57. Courtney SC, Di H, Stockman BM, Liu H, Scherbik SV, Brinton MA. Identification of novel host cell binding partners of Oas1b, the protein conferring resistance to flavivirus-induced disease in mice. *J Virol*. 2012;86(15):7953–7963.
58. Kwong LS, Brown MH, Barclay AN, Hatherley D. Signal-regulatory protein alpha from the NOD mouse binds human CD47 with an exceptionally high affinity—implications for engraftment of human cells. *Immunology*. 2014;143(1):61–67.
59. Iwamoto C, Takenaka K, Urata S, et al. The BALB/c-specific polymorphic SIRPA enhances its affinity for human CD47, inhibiting phagocytosis against human cells to promote xenogeneic engraftment. *Exp Hematol*. 2014;42(3):171.e1. doi:10.1016/j.exphem.2013.11.005.
60. Detre C, Keszei M, Romero X, Tsokos GC, Terhorst C. SLAM family receptors and the SLAM-associated protein (SAP) modulate T cell functions. *Semin Immunopathol*. 2010;32:157–171. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2868096/.
61. Keszei M, Latchman YE, Vanguri VK, et al. Auto-antibody production and glomerulonephritis in congenic Slamf1-/- and Slamf2-/- B6.129] but not in Slamf1-/- and Slamf2-/- BALB/c.129] mice. *Int Immunol*. 2011;23(2):149–158.
62. Koh AE, Njoroge SW, Feliu M, et al. The SLAM family member CD48 (Slamf2) protects lupus-prone mice from autoimmune nephritis. *J Autoimmun*. 2011;37:48–57. http://www.ncbi.nlm.nih.gov/pubmed/21561736.
63. Crampton SP, Morawski PA, Bolland S. Linking susceptibility genes and pathogenesis mechanisms using mouse models of systemic lupus erythematosus. *Dis Model Mech*. 2014;7(9):1033–1046. doi:10.1242/dmm.016451. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4142724/.
64. Stober CB, Brode S, White JK, Popoff JF, Blackwell JM. Slc11a1, formerly Nramp1, is expressed in dendritic cells and influences major histocompatibility complex class II expression and antigen-presenting cell function. *Infect Immun*. 2007;75(10):5059–5067.
65. Osman GE, Hannibal MC, Anderson JP, Lasky SR, Ladiges WC, Hood L. FVB/N (H2(q)) mouse is resistant to arthritis induction and exhibits a genomic deletion of T-cell receptor V beta gene segments. *Immunogenetics*. 1999;49(10):851–859.
66. Osman GE, Hannibal MC, Anderson JP, et al. T-cell receptor vbeta deletion and valpha polymorphism are responsible for the resistance of SWR mouse to arthritis induction. *Immunogenetics*. 1999;49(9):764–772.
67. Behlke MA, Chou HS, Huppi K, Loh DY. Murine T-cell receptor mutants with deletions of beta-chain variable region genes. *Proc Natl Acad Sci USA*. 1986;83(3):767–771.
68. Poltorak A, He X, Smirnova I, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science*. 1998;282(5396):2085–2088.
69. Poltorak A, Smirnova I, He X, et al. Genetic and physical mapping of the lps locus: identification of the toll-4 receptor as a candidate gene in the critical region. *Blood Cells Mol Dis*. 1998;24(3):340–355.
70. Liaunardy-Jopeace A, Gay NJ. Molecular and cellular regulation of toll-like receptor-4 activity induced by lipopolysaccharide ligands. *Front Immunol*. 2014;5:473.
71. De Vooght V, Vanoirbeek JA, Luyts K, Haenen S, Nemery B, Hoet PH. Choice of mouse strain influences the outcome in a mouse model of chemical-induced asthma. *PLoS One*. 2010;5:e12581. http://www.ncbi.nlm.nih.gov/pubmed/20830207.
72. Watanabe H, Numata K, Ito T, Takagi K, Matsukawa A. Innate immune response in Th1- and Th2-dominant mouse strains. *Shock*. 2004;22(5):460–466.
73. Simon MM, Greenaway S, White JK, et al. A comparative phenotypic and genomic analysis of C57BL/6J and C57BL/6N mouse strains. *Genome Biol*. 2013;14(7):R82. doi:10.1186/gb-2013-14-7-r82.
74. Zurita E, Chagoyen M, Cantero M, et al. Genetic polymorphisms among C57BL/6 mouse inbred strains. *Transgenic Res*. 2011;20(3):481–489. doi:10.1007/s11248-010-9403-8.
75. Mekada K, Abe K, Murakami A, et al. Genetic differences among C57BL/6 substrains. *Exp Anim*. 2009;58(2):141–149.
76. Mekada K, Hirose M, Murakami A, Yoshiki A. Development of SNP markers for C57BL/6N-derived mouse inbred strains. *Exp Anim*. 2015;64(1):91–100. doi:10.1538/expanim.14-0061.
77. Mahajan VS, Demissie E, Mattoo H, et al. Striking immune phenotypes in gene-targeted mice are driven by a copy-number variant originating from a commercially available C57BL/6 strain. *Cell Rep*. 2016;15(9):1901–1909. doi:10.1016/j.celrep.2016.04.080.
78. Hedlund M, Tangvoranuntakul P, Takematsu H, et al. N-glycolylneuraminic acid deficiency in mice: implications for human biology and evolution. *Mol Cell Biol*. 2007;27(12):4340–4346. doi:10.1128/MCB.00379-07.
79. Purtha WE, Swiecki M, Colonna M, Diamond MS, Bhattacharya D. Spontaneous mutation of the Dock2 gene in Irf5-/- mice complicates interpretation of type I interferon production and antibody responses. *Proc Natl Acad Sci USA*. 2012;109(15):898. doi:10.1073/pnas.1118155109.
80. Freeman HC, Hugill A, Dear NT, Ashcroft FM, Cox RD. Deletion of nicotinamide nucleotide transhydrogenase: a new quantitive trait locus accounting for glucose intolerance in C57BL/6J mice. *Diabetes*. 2006;55(7):2153–2156. doi:10.2337/db06-0358.
81. Ronchi JA, Figueira TR, Ravagnani FG, Oliveira HCF, Vercesi AE, Castilho RF. A spontaneous mutation in the nicotinamide nucleotide transhydrogenase gene of C57BL/6J mice results in mitochondrial redox abnormalities. *Free Radic Biol Med*. 2013;63:446–456. doi:10.1016/j.freeradbiomed.2013.05.049.
82. Liron T, Raphael B, Hiram-Bab S, Bab IA, Gabet Y. Bone loss in C57BL/6J-OlaHsd mice, a substrain of C57BL/6J carrying mutated alpha-synuclein and multimerin-1 genes. *J Cell Physiol*. 2018;233(1):371–377. doi:10.1002/jcp.25895.
83. Specht CG, Schoepfer R. Deletion of the alpha-synuclein locus in a subpopulation of C57BL/6J inbred mice. *BMC Neurosci*. 2001;2(1):11. doi:10.1186/1471-2202-2-11. http://www.ncbi.nlm.nih.gov/pubmed/11591219.
84. López-Jiménez A, Walter NAR, Giné E, et al. A spontaneous deletion of α-Synuclein is associated with an increase in CB1 mRNA transcript and receptor expression in the hippocampus and amygdala: effects on alcohol consumption. *Synapse*. 2013;67(6):280–289. doi:10.1002/syn.21639. https://onlinelibrary.wiley.com/doi/abs/10.1002/syn.21639.

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86. Mattapallil MJ, Wawrousek EF, Chan C, et al. The Rd8 mutation of the Crb1 gene is present in vendor lines of C57BL/6N mice and embryonic stem cells, and confounds ocular induced mutant phenotypes. *Invest Ophthalmol Vis Sci*. 2012; 53(6):2921–2927. doi:10.1167/iovs.12-9662.
86. Mehalow AK, Kameya S, Smith RS, et al. CRB1 is essential for external limiting membrane integrity and photoreceptor morphogenesis in the mammalian retina. *Hum Mol Genet*. 2003;12(17):2179–2189. doi:10.1093/hmg/ddg232.
87. Davisson MT, Bergstrom DE, Reinholdt LG, Donahue LR. Discovery genetics—the history and future of spontaneous mutation research. *Curr Protoc Mouse Biol*. 2012;2:103–118. doi:10.1002/9780470942390.mo110200.
88. Davisson MT. Discovery genetics: serendipity in basic research. *ILAR J*. 2005;46(4):338–345.
89. [NRC] National Research Council. *Immunodeficient rodents: a guide to their immunobiology, husbandry, and use*. Washington (DC): National Academies Press (US); 1989. http://www.ncbi.nlm.nih.gov/books/NBK218200/. Accessed Nov 16, 2017.
90. Montagutelli X. Effect of the genetic background on the phenotype of mouse mutations. *J Am Soc Nephrol*. 2000;11 (Suppl 16):101.
91. Zeineldin M, Jensen D, Paranjape SR, et al. Human cancer xenografts in outbred nude mice can be confounded by polymorphisms in a modifier of tumorigenesis. *Genetics*. 2014;197(4):1365–1376. doi:10.1534/genetics.114.166587.
92. Nonoyama S, Ochs HD. Immune deficiency in SCID mice. *Int Rev Immunol*. 1996;13(4):289–300.
93. Nonoyama S, Smith FO, Bernstein ID, Ochs HD. Strain-dependent leakiness of mice with severe combined immune deficiency. *J Immunol*. 1993;150(9):3817–3824.
84. Hoshino K, Takeuchi O, Kawai T, et al. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the lps gene product. *J Immunol*. 1999;162(7):3749–3752.
95. Shultz LD, Schweitzer PA, Christianson SW, et al. Multiple defects in innate and adaptive immunologic function in NOD/LtSz-scid mice. *J Immunol*. 1995;154(1):180–191.
96. Caplazi P, Baca M, Barck K, et al. Mouse models of rheumatoid arthritis. *Vet Pathol*. 2015;52:819–826. http://www.ncbi.nlm.nih.gov/pubmed/26063174.
97. Pearson JA, Wong FS, Wen L. The importance of the non obese diabetic (NOD) mouse model in autoimmune diabetes. *J Autoimmun*. 2016;66:76–88. doi:10.1016/j.jaut.2015.08.019.
98. Constantinescu CS, Farooqi N, O'Brien K, Gran B. Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS). *Br J Pharmacol*. 2011;164:1079–1106. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3229753/.
99. Rottman JB, Willis CR. Mouse models of systemic lupus erythematosus reveal a complex pathogenesis. *Vet Pathol*. 2010;47(4):664–676. doi:10.1177/0300985810370005. http://journals.sagepub.com/doi/full/10.1177/0300985810370005.
100. Krishnamurthy B, Dudek NL, McKenzie MD, et al. Responses against islet antigens in NOD mice are prevented by tolerance to proinsulin but not IGRP. *J Clin Invest*. 2006;116(12):3258–3265. doi:10.1172/JCI29602.
101. Stadinski BD, Delong T, Reisdorph N, et al. Chromogranin A is an autoantigen in type 1 diabetes. *Nat Immunol*. 2010; 11(3):225–231. doi:10.1038/ni.1844.
102. Unanue ER, Ferris ST, Carrero JA. The role of islet antigen presenting cells and the presentation of insulin in the initiation of autoimmune diabetes in the NOD mouse. *Immunol Rev*. 2016;272(1):183–201. doi:10.1111/imr.12430.
103. Noble JA, Erlich HA. Genetics of type 1 diabetes. *Cold Spring Harb Perspect Med*. 2012;2:a007732. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3253030/.
104. Dwyer CJ, Bayer AL, Fotino C, et al. Altered homeostasis and development of regulatory T cell subsets represent an IL-2R-dependent risk for diabetes in NOD mice. *Sci Signal*. 2017;10(510). https://www.ncbi.nlm.nih.gov/pubmed/29259102.
105. Wong AS, Mortin-Toth S, Sung M, et al. Polymorphism in the innate immune receptor SIRPalpha controls CD47 binding and autoimmunity in the nonobese diabetic mouse. *J Immunol*. 2014;193(10):4833–4844.
106. Suri A, Walters JJ, Gross ML, Unanue ER. Natural peptides selected by diabetogenic DQ8 and murine I-A(g7) molecules show common sequence specificity. *J Clin Invest*. 2005;115:2268–2276. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1180544/.
107. Hattori M, Buse JB, Jackson RA, et al. The NOD mouse: recessive diabetogenic gene in the major histocompatibility complex. *Science*. 1986;231:733–735. http://science.sciencemag.org/content/sci/231/4739/733.full.pdf.
108. Dai H, Friday AJ, Abou-Daya KI, et al. Donor SIRPα polymorphism modulates the innate immune response to allogeneic grafts. *Sci Immunol*. 2017;2(12). doi:10.1126/sciimmunol.aam6202.
109. Mehta G, Ferreira VP, Pickering MC, Skerka C, Zipfel PF, Banda NK. New insights into disease-specific absence of complement factor H related protein C in mouse models of spontaneous autoimmune diseases. *Mol Immunol*. 2014;62:235–248. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4157073/.
110. Zekavat G, Mozaffari R, Arias VJ, et al. A novel CD93 polymorphism in non-obese diabetic (NOD) and NZB/W F1 mice is linked to a CD4+ iNKT cell deficient state. *Immunogenetics*. 2010;62:397–407. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2875467/.
111. Simecek P, Churchill GA, Yang H, et al. Genetic analysis of substrain divergence in non-obese diabetic (NOD) mice. *G3 (Bethesda)*. 2015;5:771–775. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4426365/.
112. Celhar T, Fairhurst A. Toll-like receptors in systemic lupus erythematosus: potential for personalized treatment. *Front Pharmacol*. 2014;5:265. doi:10.3389/fphar.2014.00265.
113. Perry D, Sang A, Yin Y, Zheng YY, Morel L. Murine models of systemic lupus erythematosus. *J Biomed Biotechnol*. 2011; 2011:271694.
114. Celhar T, Fairhurst A. Modelling clinical systemic lupus erythematosus: similarities, differences and success stories. *Rheumatology (Oxford)*. 2017;56(suppl_1):i88–i99. doi:10.1093/rheumatology/kew400.
115. Andrews BS, Eisenberg RA, Theofilopoulos AN, et al. Spontaneous murine lupus-like syndromes. clinical and immunopathological manifestations in several strains. *J Exp Med*. 1978;148(5):1198–1215.
116. Santiago-Raber M, Laporte C, Reininger L, Izui S. Genetic basis of murine lupus. *Autoimmun Rev*. 2004;3(1):33–39. doi:10.1016/S1568-9972(03)00062-4.
117. Górnikiewicz B, Ronowicz A, Madanecki P, Sachadyn P. Genome-wide DNA methylation profiling of the regenerative MRL/MpJ mouse and two normal strains. *Epigenomics*. 2017;9(8):1105–1122. doi:10.2217/epi-2017-0009.
118. Heber-Katz E. Oxygen, metabolism, and regeneration: lessons from mice. *Trends Mol Med*. 2017;23(11):1024–1036. doi:10.1016/j.molmed.2017.08.008.
119. McBrearty BA, Clark LD, Zhang XM, Blankenhorn EP, Heber-Katz E. Genetic analysis of a mammalian wound-

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healing trait. *Proc Natl Acad Sci USA*. 1998;95(20):11792–11797.
120. Nose M, Nishimura M, Ito MR, Toh J, Shibata T, Sugisaki T. Arteritis in a novel congenic strain of mice derived from MRL/lpr lupus mice: genetic dissociation from glomerulonephritis and limited autoantibody production. *Am J Pathol*. 1996;149(5):1763–1769.
121. Ghoreishi M, Dutz JP. Murine models of cutaneous involvement in lupus erythematosus. *Autoimmun Rev*. 2009;8(6):484–487. doi:10.1016/j.autrev.2009.02.028.
122. Nose M, Komori H, Miyazaki T, Mori S. Genomics of vasculitis: lessons from mouse models. *Ann Vasc Dis*. 2013;6(1):16–21. doi:10.3400/avd.oa.12.00096.
123. Nose M, Nishihara M, Fujii H. Genetic basis of the complex pathological manifestations of collagen disease: lessons from MRL/lpr and related mouse models. *Int Rev Immunol*. 2000;19(4–5):473–498.
124. Morse HC, Roths JB, Davidson WF, Langdon WY, Fredrickson TN, Hartley JW. Abnormalities induced by the mutant gene, lpr. patterns of disease and expression of murine leukemia viruses in SJL/J mice homozygous and heterozygous for lpr. *J Exp Med*. 1985;161:602–616. http://www.ncbi.nlm.nih.gov/pubmed/2982991.
125. Lane PW, Murphy ED. Susceptibility to spontaneous pneumonitis in an inbred strain of beige and satin mice. *Genetics*. 1972;72(3):451–460.
126. Murphy ED, Rohs JB. A Y chromosome associated factor in strain BXSB producing accelerated autoimmunity and lymphoproliferation. *Arthritis Rheum*. 1979;22(11):1188–1194.
127. Fairhurst AM, Hwang SH, Wang A, et al. Yaa autoimmune phenotypes are conferred by overexpression of TLR7. *Eur J Immunol*. 2008;38(7):1971–1978.
128. Santiago-Raber ML, Kikuchi S, Borel P, et al. Evidence for genes in addition to Tlr7 in the yaa translocation linked with acceleration of systemic lupus erythematosus. *J Immunol*. 2008;181(2):1556–1562.
129. Jain S, Ward JM, Shin D, et al. Associations of autoimmunity, immunodeficiency, lymphomagenesis, and gut microbiota in mice with knockins for a pathogenic autoantibody. *Am J Pathol*. 2017;187(9):2020–2033. doi:10.1016/j.ajpath.2017.05.017.
130. East J, de Sousa MA, Parrott DM. Immunopathology of New Zealand black (NZB) mice. *Transplantation*. 1965;3(6):711–729.
131. East J, Branca M. Autoimmune reactions and malignant changes in germ-free New Zealand black mice. *Clin Exp Immunol*. 1969;4(6):621–635.
132. Beck-Engeser GB, Ahrends T, Knittel G, et al. Infectivity and insertional mutagenesis of endogenous retrovirus in autoimmune NZB and B/W mice. *J Gen Virol*. 2015;96(11):3396–3410. doi:10.1099/jgv.0.000271.
133. Salerno E, Yuan Y, Scaglione BJ, et al. The New Zealand black mouse as a model for the development and progression of chronic lymphocytic leukemia. *Cytometry B Clin Cytom*. 2010;78(Suppl 1):98. doi:10.1002/cyto.b.20544.
134. Scatizzi JC, Haraldsson MK, Pollard KM, Theofilopoulos AN, Kono DH. The Lbw2 locus promotes autoimmune hemolytic anemia. *J Immunol*. 2012;188(7):3307–3314. doi:10.4049/jimmunol.1103561.
135. Kelley VE, Winkelstein A. Age- and sex-related glomerulonephritis in New Zealand white mice. *Clin Immunol Immunopathol*. 1980;16(2):142–150.
136. Mellors RC. Autoimmune and immunoproliferative diseases of NZB/bl mice and hybrids. *Int Rev Exp Pathol*. 1966;5:217–252.
137. Dubois EL, Horowitz RE, Demopoulos HB, Teplitz R. NZB/NZW mice as a model of systemic lupus erythematosus. *JAMA*. 1966;195(4):285–289.
138. Morel L, Wakeland EK. Susceptibility to lupus nephritis in the NZB/W model system. *Curr Opin Immunol*. 1998;10(6):718–725.
139. Rahman ZSM, Tin S, Buenaventura PL, et al. A novel susceptibility locus on chromosome 2 in the (New Zealand black x New Zealand white)F1 hybrid mouse model of systemic lupus erythematosus. *J Immunol*. 2002;168(6):3042–3049.
140. Rudofsky UH, Lawrence DA. New Zealand mixed mice: a genetic systemic lupus erythematosus model for assessing environmental effects. *Environ Health Perspect*. 1999;107(Suppl 5):713–721.
141. Morel L. Mapping lupus susceptibility genes in the NZM2410 mouse model. *Adv Immunol*. 2012;115:113–139.
142. Waters ST, Fu SM, Gaskin F, et al. NZM2328: a new mouse model of systemic lupus erythematosus with unique genetic susceptibility loci. *Clin Immunol*. 2001;100:372–383. http://www.sciencedirect.com/science/article/pii/S152166160195079X. Accessed Dec 1, 2018.
143. Moresco EMY, Li X, Beutler B. Going forward with genetics. *Am J Pathol*. 2013;182(5):1462–1473. doi:10.1016/j.ajpath.2013.02.002. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3644711/. Accessed Dec 1, 2018.
144. Coutinho A, Meo T. Genetic basis for unresponsiveness to lipopolysaccharide in C57BL/10Cr mice. *Immunogenetics*. 1978;7(1):17–24. doi:10.1007/BF01843983.
145. Sakaguchi S, Wing K, Miyara M. Regulatory T cells—a brief history and perspective. *Eur J Immunol*. 2007;37(Suppl 1):S116–S123. doi:10.1002/eji.200737593. http://www.ncbi.nlm.nih.gov/pubmed/17972355. Accessed Dec 1, 2018.
146. McClellan J, Macasocal R, Hare T, Horn M. Characterization of immunodeficient mouse models. *FASEB J*. 2017;31(1_suppl):807.14. doi:10.1096/fasebj.31.1_supplement.807.14. https://www.fasebj.org/doi/abs/10.1096/fasebj.31.1_supplement.807.14. Accessed Jun 7, 2018.
147. Joliat MJ, Shultz LD. The molecular bases of spontaneous immunological mutations in the mouse and their homologous human diseases. *Clin Immunol*. 2001;101(2):113–129. doi:10.1006/clim.2001.5120.
148. Izui S, Kelley VE, Masuda K, Yoshida H, Roths JB, Murphy ED. Induction of various autoantibodies by mutant gene lpr in several strains of mice. *J Immunol*. 1984;133(1):227–233.
149. Nose M, Terada M, Nishihara M, et al. Genome analysis of collagen disease in MRL/lpr mice: polygenic inheritance resulting in the complex pathological manifestations. *Int J Cardiol*. 2000;75(Suppl 1):S61.
150. Takahashi T, Tanaka M, Brannan CI, et al. Generalized lymphoproliferative disease in mice, caused by a point mutation in the fas ligand. *Cell*. 1994;76(6):969–976.
151. Roths JB, Murphy ED, Eicher EM. A new mutation, gld, that produces lymphoproliferation and autoimmunity in C3H/HeJ mice. *J Exp Med*. 1984;159(1):1–20.
152. Mosier DE, Stell KL, Gulizia RJ, Torbett BE, Gilmore GL. Homozygous scid/scid;beige/beige mice have low levels of spontaneous or neonatal T cell-induced B cell generation. *J Exp Med*. 1993;177(1):191–194.
153. Shibata S, Asano T, Ogura A, et al. SCID-bg mice as xenograft recipients. *Lab Anim*. 1997;31(2):163–168. doi:10.1258/002367797780600107.
154. Mosbach-Ozmen L, Fonteneau P, Loor F. The C57BL/6 nu/nu lpr/lpr mouse. I. Expression of the 'lpr phenotype' in the C57BL/6 genetic background. *Thymus*. 1985;7(4):221–232.

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155. Mosbach-Ozmen L, Fonteneau P, Loor F. The C57BL/6 nu/nu lpr/lpr mouse. II. Pedigree and preliminary characteristics. *Thymus*. 1985;7(4):233–245.
156. Trembleau S, Pflumio F, Kuntz L, Jachez B, Loor F. Immunoglobulin isotypes of C57BL/6 nu/nu, lpr/lpr mice. lack of direct intrinsic effect of the lpr gene on B cell hyperactivity. *Autoimmunity*. 1991;9(2):165–170.
157. Steinberg AD, Roths JB, Murphy ED, Steinberg RT, Raveche ES. Effects of thymectomy or androgen administration upon the autoimmune disease of MRL/mp-lpr/lpr mice. *J Immunol*. 1980;125(2):871–873.
158. Pisetsky DS, Caster SA, Piper M, Scott DW, Steinberg AD. Cloning of B cells from autoimmune MRL-lpr/lpr and MRL. xid mice. *Cell Immunol*. 1984;84(1):32–40.
159. Steinberg EB, Santoro TJ, Chused TM, Smathers PA, Steinberg AD. Studies of congenic MRL-ipr/ipr.xid mice. *J Immunol*. 1983;131(6):2789–2795.
160. Yasutomo K, Maeda K, Hisaeda H, Good RA, Kuroda Y, Himeno K. The fas-deficient SCID mouse exhibits the development of T cells in the thymus. *J Immunol*. 1997;158(10):4729–4733.
161. Pisetsky DS, Klatt C, Dawson D, Roths JB. The influence of yaa on anti-DNA responses of B6-lpr mice. *Clin Immunol Immunopathol*. 1985;37(3):369–376.
162. Suzuka H, Yoshifusa H, Nakamura Y, Miyawaki S, Shibata Y. Morphological analysis of autoimmune disease in MRL-lpr,yaa male mice with rapidly progressive systemic lupus erythematosus. *Autoimmunity*. 1993;14(4):275–282.
163. Pflumio F, Fonteneau P, Gavériaux C, Cammisuli S, Loor F. The C57BL/6 nude, beige mouse: a model of combined T cell and NK effector cell immunodeficiency. *Cell Immunol*. 1989;120(1):218–229.
164. Sadoff DA, Giddens WE, DiGiacomo RF, Vogel AM. Neoplasms in NIH type II athymic (nude) mice. *Lab Anim Sci*. 1988;38(4):407–412.
165. Azar HA, Hansen CT, Costa J. N:NIH(S)-nu/nu mice with combined immunodeficiency: a new model for human tumor heterotransplantation. *J Natl Cancer Inst*. 1980;65(2):421–430.
166. Sainte-Marie G, Peng FS. Lymph nodes of the N:NIH(S)II-nu/nu mouse. *Lab Invest*. 1985;52(6):631–637.
167. Waggie KS, Wu-Owens J, Hollifield V, Hansen CT. Lymphoblastic lymphoma in a colony of N:NIH(S)-bg-nu-xid mice. *Lab Anim Sci*. 1992;42(4):375–377.
168. Ishigaki Y, Yasuda K, Hashimoto N, Nikaido H, Nikaido O, Hayakawa JI. Enhanced human tumor cell transplantability in a new congenic immunodeficient mouse; KSN-BNX. *Folia Microbiol (Praha)*. 1998;43(5):493–494.
169. Lozzio BB, Lopez DM, Coulson P, Lair SV. High incidence of mammary tumors in mice with inherited asplenia carriers for the nude gene. *Cancer Res*. 1979;39(5):1529–1533.
170. Bender AT, Pereira A, Fu K, et al. Btk inhibition treats TLR7/IFN driven murine lupus. *Clin Immunol*. 2016;164:65–77. doi:10.1016/j.clim.2016.01.012. http://www.sciencedirect.com/science/article/pii/S1521661616300134. Accessed Jan 23, 2018.
171. Smee DF, Dagley A, Downs B, Hagloch J, Tarbet EB. Enhanced efficacy of cidofovir combined with vaccinia immune globulin in treating progressive cutaneous vaccinia virus infections in immunosuppressed hairless mice. *Antimicrob Agents Chemother*. 2015;59(1):520–526. https://www.ncbi.nlm.nih.gov/pubmed/25385098. Accessed Dec 1, 2018.
172. Godfrey VL, Wilkinson JE, Rinchik EM, Russell LB. Fatal lymphoreticular disease in the scurfy (sf) mouse requires T cells that mature in a sf thymic environment: potential model for thymic education. *Proc Natl Acad Sci USA*. 1991;88:5528–5532. doi:10.1073/pnas.88.13.5528. http://www.pnas.org/content/88/13/5528. Accessed Jan 24, 2018.
173. Sundberg JP. *Handbook of mouse mutations with skin and hair abnormalities: animal models and biomedical tools*. Boca Raton, FL: CRC Press; 1994.
174. Nonaka S, Naito T, Chen H, et al. Intestinal gamma delta T cells develop in mice lacking thymus, all lymph nodes, Peyer's patches, and isolated lymphoid follicles. *J Immunol*. 2005;174(4):1906–1912.
175. Griesemer A, Yamada K, Sykes M. Xenotransplantation: immunological hurdles and progress toward tolerance. *Immunol Rev*. 2014;258(1):241–258. doi:10.1111/imr.12152.
176. Pearson T, Greiner DL, Shultz LD. Creation of "humanized" mice to study human immunity. *Curr Protoc Immunol*. 2008; Chapter 15:Unit 15.21. doi:10.1002/0471142735.im1521s81.
177. Iwakawa M, Noda S, Ohta T, et al. Different radiation susceptibility among five strains of mice detected by a skin reaction. *J Radiat Res*. 2003;44(1):7–13.
178. Mori N, Matsumoto Y, Okumoto M, Suzuki N, Yamate J. Variations in prkdc encoding the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) and susceptibility to radiation-induced apoptosis and lymphomagenesis. *Oncogene*. 2001;20(28):3609–3619. doi:10.1038/sj.onc.1204497.
179. Mukherjee S, Sainis KB, Deobagkar DD. F1 hybrids of BALB/c and C57BL/6 mouse strains respond differently to low-dose ionizing radiation exposure. *J Genet*. 2014;93(3):667–682.
180. Degg NL, Weil MM, Edwards A, et al. Adenoma multiplicity in irradiated apc(min) mice is modified by chromosome 16 segments from BALB/c. *Cancer Res*. 2003;63(10):2361–2363.
181. Yu Y, Okayasu R, Weil MM, et al. Elevated breast cancer risk in irradiated BALB/c mice associates with unique functional polymorphism of the prkdc (DNA-dependent protein kinase catalytic subunit) gene. *Cancer Res*. 2001;61(5):1820–1824.
182. Lombardini ED, Pacheco-Thompson ME, Melanson MA. Chapter 44 radiation and other physical agents—dimensions. In: Haschek WM, Rousseaux CG, Wallig MA, Bolon B, Ochoa R, eds. *Haschek and Rousseaux's handbook of toxicologic pathology third edition*. London: Academic Press; 2013: 1421–1503. https://app.dimensions.ai/details/publication/pub.1010167368. Accessed May 14, 2018.
183. Hernández-Godoy J, Silvestre DP, Hernández BB. Immediate and short-, mid- and long-term effects of in vivo ionizing radiation exposure in BALB/c mice: i. activation of lymphocytes and subpopulations. *In Vivo*. 2010; 24(5):719–726.
184. Manda K, Glasow A, Paape D, Hildebrandt G. Effects of ionizing radiation on the immune system with special emphasis on the interaction of dendritic and T cells. *Front Oncol*. 2012;2:102. doi:10.3389/fonc.2012.00102.
185. Wensinck F, Van Bekkum DW, Renaud H. The prevention of *Pseudomonas aeruginosa* infections in irradiated mice and rats. *Radiat Res*. 1957;7(5):491–499.
186. Chiang CS, McBride WH, Withers HR. Myelin-associated changes in mouse brain following irradiation. *Radiother Oncol*. 1993;27(3):229–236.
187. Larsen SR, Kingham JA, Hayward MD, Rasko JEJ. Damage to incisors after nonmyeloablative total body irradiation may complicate NOD/SCID models of hemopoietic stem cell transplantation. *Comp Med*. 2006;56(3):209–214.
188. Schwarte S, Bremer M, Fruehauf J, Sorge Y, Skubich S, Hoffmann MW. Radiation protocols determine acute graft-versus-host disease incidence after allogeneic bone marrow

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transplantation in murine models. *Int J Radiat Biol*. 2007;83 (9):625–636. doi:10.1080/09553000701534572. https://www.ncbi.nlm.nih.gov/pubmed/17654104. Accessed Dec 1, 2018.
189. Borges HL, Chao C, Xu Y, Linden R, Wang JYJ. Radiation-induced apoptosis in developing mouse retina exhibits dose-dependent requirement for ATM phosphorylation ofp53. *Cell Death Differ*. 2004;11(5):494–502. doi:10.1038/sj.cdd.4401366.
190. Down JD, Tarbell NJ, Warhol M, Mauch P. Dose-limiting complications from upper half body irradiation in C3H mice. *Int J Radiat Oncol Biol Phys*. 1988;14(3):483–489.
191. Gartner LP, Hiatt JL, Provenza DV. Effects of ionizing radiation on incisor development of the prenatal mouse. *Acta Anat (Basel)*. 1977;98(4):367–375.
192. Jaggi JS, Seshan SV, McDevitt MR, LaPerle K, Sgouros G, Scheinberg DA. Renal tubulointerstitial changes after internal irradiation with alpha-particle-emitting actinium daughters. *J Am Soc Nephrol*. 2005;16(9):2677–2689. doi:10.1681/ASN.2004110945.
193. Medak H, Weinreb M, Sicher H, Weinmann JP, Schour I. The effect of single doses of irradiation upon the tissues of the upper rat incisor. *J Dent Res*. 1952;31(4):559–574. doi:10.1177/00220345520310040601.
194. Pearson AE, Phelps TA. Radiation effects on mouse incisor teeth following whole-body doses of up to 16 gray. *Int J Radiat Biol Relat Stud Phys Chem Med*. 1981;39(4):409–417.
195. Schmidt SL, Vitral RW, Linden R. Effects of prenatal ionizing irradiation on the development of the ganglion cell layer of the mouse retina. *Int J Dev Neurosci*. 2001;19(4):469–473.
196. Byrne SN, Spinks N, Halliday GM. Ultraviolet a irradiation of C57BL/6 mice suppresses systemic contact hypersensitivity or enhances secondary immunity depending on dose. *J Invest Dermatol*. 2002;119(4):858–864. doi:10.1046/j.1523-1747.2002.00261.x.
197. Schwarz T. The dark and the sunny sides of UVR-induced immunosuppression: photoimmunology revisited. *J Invest Dermatol*. 2010;130(1):49–54. doi:10.1038/jid.2009.217.
198. González Maglio DH, Paz ML, Leoni J. Sunlight effects on immune system: is there something else in addition to UV-induced immunosuppression? *Biomed Res Int*. 2016;2016:1–10. doi:10.1155/2016/1934518. https://www.ncbi.nlm.nih.gov/pubmed/28070504. Accessed Dec 1, 2018.
199. Zuluaga AF, Salazar BE, Rodriguez CA, Zapata AX, Agudelo M, Vesga O. Neutropenia induced in outbred mice by a simplified low-dose cyclophosphamide regimen: characterization and applicability to diverse experimental models of infectious diseases. *BMC Infect Dis*. 2006;6:55. doi:10.1186/1471-2334-6-55.
200. Brownstein DG. Pathogenesis of bacteremia due to pseudomonas aeruginosa in cyclophosphamide-treated mice and potentiation of virulence of endogenous streptococci. *J Infect Dis*. 1978;137(6):795–801.
201. De Bernardis F, Palliola E, Lorenzini R, Antonucci G. Evaluation of the experimental pathogenicity of some cryptococcus species in normal and cyclophosphamide-immunodepressed mice. *Microbiol Immunol*. 1987;31(5):449–460.
202. Sistigu A, Viaud S, Chaput N, Bracci L, Proietti E, Zitvogel L. Immunomodulatory effects of cyclophosphamide and implementations for vaccine design. *Semin Immunopathol*. 2011;33(4):369–383. doi:10.1007/s00281-011-0245-0.
203. Peng KW, Myers R, Greenslade A, et al. Using clinically approved cyclophosphamide regimens to control the humoral immune response to oncolytic viruses. *Gene Ther*. 2013;20(3):255–261. doi:10.1038/gt.2012.31.
204. Heylmann D, Bauer M, Becker H, et al. Human CD4+CD25+ regulatory T cells are sensitive to low dose cyclophosphamide: implications for the immune response. *PLoS One*. 2013;8(12):e83384. doi:10.1371/journal.pone.0083384.
205. Wilkinson FL, Sergijenko A, Langford-Smith KJ, Malinowska M, Wynn RF, Bigger BW. Busulfan conditioning enhances engraftment of hematopoietic donor-derived cells in the brain compared with irradiation. *Mol Ther*. 2013;21(4):868–876. doi:10.1038/mt.2013.29.
206. Robert-Richard E, Ged C, Ortet J, et al. Human cell engraftment after busulfan or irradiation conditioning of NOD/SCID mice. *Haematologica*. 2006;91(10):1384.
207. Ghiringhelli F, Bruchard M, Apetoh L. Immune effects of 5-fluorouracil: ambivalence matters. *Oncoimmunology*. 2013;2 (3):e23139. doi:10.4161/onci.23139.
208. Vincent J, Mignot G, Chalmin F, et al. 5-Fluorouracil selectively kills tumor-associated myeloid-derived suppressor cells resulting in enhanced T cell-dependent antitumor immunity. *Cancer Res*. 2010;70(8):3052–3061. doi:10.1158/0008-5472.CAN-09-3690.
209. Halloran PF, Helms LM, Kung L, Noujaim J. The temporal profile of calcineurin inhibition by cyclosporine in vivo. *Transplantation*. 1999;68(9):1356–1361.
210. Matsue H, Yang C, Matsue K, Edelbaum D, Mummert M, Takashima A. Contrasting impacts of immunosuppressive agents (rapamycin, FK506, cyclosporin A, and dexamethasone) on bidirectional dendritic cell-T cell interaction during antigen presentation. *J Immunol*. 2002;169(7):3555–3564.
211. Barequet IS, Platner E, Sade K, et al. Topical tacrolimus for the management of acute allergic conjunctivitis in a mouse model. *Graefes Arch Clin Exp Ophthalmol*. 2013;251(7):1717–1721. doi:10.1007/s00417-013-2333-4.
212. Niwa Y, Terashima T, Sumi H. Topical application of the immunosuppressant tacrolimus accelerates carcinogenesis in mouse skin. *Br J Dermatol*. 2003;149(5):960–967.
213. Obayashi K, Tomonari M, Yoshimatsu H, et al. Dosing time-dependency of the arthritis-inhibiting effect of tacrolimus in mice. *J Pharmacol Sci*. 2011;116(3):264–273.
214. Shin J, Park HR, Kim SW, et al. The effect of topical FK506 (tacrolimus) in a mouse model of allergic rhinitis. *Am J Rhinol Allergy*. 2012;26(2):71. doi:10.2500/ajra.2012.26.3743.
215. Vandenbussche C, Van der Hauwaert C, Dewaeles E, et al. Tacrolimus-induced nephrotoxicity in mice is associated with microRNA deregulation. *Arch Toxicol*. 2018;92(4):1539–1550. doi:10.1007/s00204-018-2158-3.
216. Furukawa A, Wisel SA, Tang Q. Impact of immune-modulatory drugs on regulatory T cell. *Transplantation*. 2016;100(11):2288–2300. doi:10.1097/TP.0000000000001379.
217. Bonkhoff H. Estrogen receptor signaling in prostate cancer: implications for carcinogenesis and tumor progression. *Prostate*. 2018;78(1):2–10. doi:10.1002/pros.23446.
218. Nelles JL, Hu W, Prins GS. Estrogen action and prostate cancer. *Expert Rev Endocrinol Metab*. 2011;6(3):437–451. doi:10.1586/eem.11.20.
219. Song W, Soni V, Soni S, Khera M. Testosterone inhibits the growth of prostate cancer xenografts in nude mice. *BMC Cancer*. 2017;17(1):635. doi:10.1186/s12885-017-3569-x.
220. Turo R, Smolski M, Esler R, et al. Diethylstilboestrol for the treatment of prostate cancer: past, present and future. *Scand J Urol*. 2014;48(1):4–14. doi:10.3109/21681805.2013.861508.
221. Ngobili TA, Daniele MA. Nanoparticles and direct immunosuppression. *Exp Biol Med*. 2016;241(10):1064–1073. doi:10.

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1177/1535370216650053. http://journals.sagepub.com/doi/full/10.1177/1535370216650053. Accessed Dec 1, 2018.
222. Luo Y, Chang LW, Lin P. Metal-based nanoparticles and the immune system: activation, inflammation, and potential applications. *Biomed Res Int*. 2015;2015:143720. http://www.ncbi.nlm.nih.gov/pubmed/26125021. Accessed Dec 1, 2018.
223. Zolnik BS, González-Fernández A, Sadrieh N, Dobrovolskaia MA. Nanoparticles and the immune system. *Endocrinology*. 2010;151(2):458–465. doi:10.1210/en.2009-1082.
224. Daley JM, Thomay AA, Connolly MD, Reichner JS, Albina JE. Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice. *J Leukoc Biol*. 2008;83(1):64–70. doi:10.1189/jlb.0407247.
225. Kruisbeek AM. In vivo depletion of CD4- and CD8-specific T cells. *Curr Protoc Immunol*. 2001;Chapter 4: Unit4.1. doi:10.1002/0471142735.im0401s01.
226. Montalvao F, Garcia Z, Celli S, et al. The mechanism of anti-CD20-mediated B cell depletion revealed by intravital imaging. *J Clin Invest*. 2013;123(12):5098–5103. doi:10.1172/JCI70972.
227. Vergani A, D'Addio F, Jurewicz M, et al. A novel clinically relevant strategy to abrogate autoimmunity and regulate alloimmunity in NOD mice. *Diabetes*. 2010;59(9):2253–2264. doi:10.2337/db09-1264.
228. Xia C, Chernatynskaya AV, Wasserfall CH, et al. Anti-thymocyte globulin (ATG) differentially depletes naïve and memory T cells and permits memory-type regulatory T cells in nonobese diabetic mice. *BMC Immunol*. 2012;13:70. doi:10.1186/1471-2172-13-70.
229. Jahrsdörfer B, Weiner GJ. CpG oligodeoxynucleotides as immunotherapy in cancer. *Update Cancer Ther*. 2008;3(1):27–32. doi:10.1016/j.uct.2007.11.003.
230. Liu W, Yang X, Wang N, et al. Multiple immunosuppressive effects of CpG-c41 on intracellular TLR-mediated inflammation. *Mediators Inflamm*. 2017;2017:6541729. doi:10.1155/2017/6541729.
231. Asano M, Toda M, Sakaguchi N, Sakaguchi S. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *J Exp Med*. 1996;184(2):387–396.
232. Dooley J, Erickson M, Gillard GO, Farr AG. Cervical thymus in the mouse. *J Immunol*. 2006;176(11):6484–6490.
233. Many MC, Drexhage HA, Denef JF. High frequency of thymic ectopy in thyroids from autoimmune prone nonobese diabetic female mice. *Lab Invest*. 1993;69(3):364–367.
234. Bronte V, Pittet MJ. The spleen in local and systemic regulation of immunity. *Immunity*. 2013;39(5):806–818. doi:10.1016/j.immuni.2013.10.010.
235. Lin H, Faunce DE, Stacey M, et al. The macrophage F4/80 receptor is required for the induction of antigen-specific efferent regulatory T cells in peripheral tolerance. *J Exp Med*. 2005;201(10):1615–1625. doi:10.1084/jem.20042307.
236. Higashijima J, Shimada M, Chikakiyo M, et al. Effect of splenectomy on antitumor immune system in mice. *Anticancer Res*. 2009;29(1):385–393.
237. Datta K, Hyduke DR, Suman S, Moon B, Johnson MD, Fornace AJ. Exposure to ionizing radiation induced persistent gene expression changes in mouse mammary gland. *Radiat Oncol*. 2012;7:205. doi:10.1186/1748-717x-7-205. https://www.ncbi.nlm.nih.gov/pubmed/23216862. Accessed May 28, 2018.
238. Nakatsukasa H, Tsukimoto M, Tokunaga A, Kojima S. Repeated gamma irradiation attenuates collagen-induced arthritis via up-regulation of regulatory T cells but not by damaging lymphocytes directly. *Radiat Res*. 2010;174(3):313–324. doi:10.1667/RR2121.1.
239. Pecaut MJ, Nelson GA, Gridley DS. Dose and dose rate effects of whole-body gamma-irradiation: i. lymphocytes and lymphoid organs. *In Vivo*. 2001;15(3):195–208.
240. Rivina L, Davoren MJ, Schiestl RH. Mouse models for radiation-induced cancers. *Mutagenesis*. 2016;31(5):491–509. doi:10.1093/mutage/gew019.
241. Tsukimoto M, Nakatsukasa H, Sugawara K, Yamashita K, Kojima S. Repeated 0.5-gy gamma irradiation attenuates experimental autoimmune encephalomyelitis with up-regulation of regulatory T cells and suppression of IL17 production. *Radiat Res*. 2008;170(4):429–436.
242. Willey JS, Lloyd SAJ, Nelson GA, Bateman TA. Ionizing radiation and bone loss: space exploration and clinical therapy applications. *Clin Rev Bone Miner Metab*. 2011;9(1):54–62. doi:10.1007/s12018-011-9092-8.
243. Worgul BV, Smilenov L, Brenner DJ, Junk A, Zhou W, Hall EJ. Atm heterozygous mice are more sensitive to radiation-induced cataracts than are their wild-type counterparts. *Proc Natl Acad Sci USA*. 2002;99(15):9836–9839. doi:10.1073/pnas.162349699.
244. Bellés M, Gonzalo S, Serra N, et al. Environmental exposure to low-doses of ionizing radiation. Effects on early nephrotoxicity in mice. *Environ Res*. 2017;156:291–296. doi:10.1016/j.envres.2017.03.034. https://www.ncbi.nlm.nih.gov/pubmed/28371757. Accessed May 28, 2018.
245. Bryan RA, Jiang Z, Howell RC, et al. Radioimmunotherapy is more effective than antifungal treatment in experimental cryptococcal infection. *J Infect Dis*. 2010;202(4):633. doi:10.1086/654813. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2904428/. Accessed Jun 1, 2018.
246. Gorin J, Guilloux Y, Morgenstern A, Chérel M, Davodeau F, Gaschet J. Using α radiation to boost cancer immunity? *Oncoimmunology*. 2014;3(9):e954925. doi:10.4161/21624011.2014.954925. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4292714/. Accessed Jun 1, 2018.
247. Jaggi JS, Seshan SV, McDevitt MR, Sgouros G, Hyjek E, Scheinberg DA. Mitigation of radiation nephropathy after internal α-particle irradiation of kidneys. *Int J Radiat Oncol Biol Phys*. 2006;64(5):1503–1512. doi:10.1016/j.ijrobp.2005.11.036. https://www.redjournal.org/article/S0360-3016(05)02985-8/fulltext. Accessed Jun 1, 2018.
248. Denkins Y, Fidler IJ, Kripke ML. Exposure of mice to UV-B radiation suppresses delayed hypersensitivity to candida albicans. *Photochem Photobiol*. 1989;49(5):615–619.
249. Jeevan A, Kripke ML. Effect of a single exposure to ultraviolet radiation on mycobacterium bovis bacillus calmette-guerin infection in mice. *J Immunol*. 1989;143(9):2837–2843.
250. Kaplan DH, Jenison MC, Saeland S, Shlomchik WD, Shlomchik MJ. Epidermal langerhans cell-deficient mice develop enhanced contact hypersensitivity. *Immunity*. 2005;23(6):611–620. doi:10.1016/j.immuni.2005.10.008.
251. Miyauchi-Hashimoto H, Tanaka K, Horio T. Enhanced inflammation and immunosuppression by ultraviolet radiation in xeroderma pigmentosum group A (XPA) model mice. *J Invest Dermatol*. 1996;107(3):343–348.
252. Miyauchi-Hashimoto H, Sugihara A, Tanaka K, Horio T. Ultraviolet radiation-induced impairment of tumor rejection is enhanced in xeroderma pigmentosum a gene-deficient mice. *J Invest Dermatol*. 2005;124(6):1313–1317. doi:10.1111/j.0022-202x.2005.23717.x.
253. Obhrai JS, Oberbarnscheidt M, Zhang N, et al. Langerhans cells are not required for efficient skin graft rejection.

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J Invest Dermatol. 2008;128(8):1950–1955. doi:10.1038/jid.2008.52.
254. Pronk C, Oldenziel H, Lequin HC. A method for determination of serum iron, total iron binding capacity and iron in urine by atomic absorption spectrophotometry with manganese as internal standard. *Clin Chim Acta*. 1974;50(1):35–41.
255. Rana S, Rogers LJ, Halliday GM. Immunosuppressive ultraviolet-A radiation inhibits the development of skin memory CD8 T cells. *Photochem Photobiol Sci*. 2010;9(1):25–30. doi:10.1039/b9pp00051h.
256. Reeve VE, Tyrrell RM. Heme oxygenase induction mediates the photoimmunoprotective activity of UVA radiation in the mouse. *Proc Natl Acad Sci USA*. 1999;96(16):9317–9321.
257. Shen J, Bao S, Reeve VE. Modulation of IL-10, IL-12, and IFN-gamma in the epidermis of hairless mice by UVA (320-400 nm) and UVB (280–320 nm) radiation. *J Invest Dermatol*. 1999;113(6):1059–1064. doi:10.1046/j.1523-1747.1999.00782.x.
258. Yuen KS, Nearn MR, Halliday GM. Nitric oxide-mediated depletion of langerhans cells from the epidermis may be involved in UVA radiation-induced immunosuppression. *Nitric Oxide*. 2002;6(3):313–318. doi:10.1006/niox.2001.0414.
259. Altman A, Hochberg Z, Silbermann M. Interactions between growth hormone and dexamethasone in skeletal growth and bone structure of the young mouse. *Calcif Tissue Int*. 1992;51(4):298–304.
260. Cain DW, Cidlowski JA. Immune regulation by glucocorticoids. *Nat Rev Immunol*. 2017;17(4):233–247. doi:10.1038/nri.2017.1.
261. Ersek A, Santo AIE, Vattakuzhi Y, George S, Clark AR, Horwood NJ. Strain dependent differences in glucocorticoid-induced bone loss between C57BL/6J and CD-1 mice. *Sci Rep*. 2016;6:36513. doi:10.1038/srep36513.
262. McLaughlin F, Mackintosh J, Hayes BP, et al. Glucocorticoid-induced osteopenia in the mouse as assessed by histomorphometry, microcomputed tomography, and biochemical markers. *Bone*. 2002;30(6):924–930.
263. Purton JF, Monk JA, Liddicoat DR, et al. Expression of the glucocorticoid receptor from the 1A promoter correlates with T lymphocyte sensitivity to glucocorticoid-induced cell death. *J Immunol*. 2004;173(6):3816–3824.
264. Rousseaux CG, Bolon B. Chapter 62—embryo and fetus. In: Haschek W, Rousseaux C, Wallig M, eds. *Haschek and Rousseaux's handbook of toxicologic pathology*. 3rd ed. Boston: Academic Press; 2013:2695–2759. https://www.sciencedirect.com/science/article/pii/B9780124157590000625. Accessed Jun 1, 2018.
265. Silverstone AE, Frazier DE, Fiore NC, Soults JA, Gasiewicz TA. Dexamethasone, beta-estradiol, and 2,3,7,8-tetrachlorodibenzo-p-dioxin elicit thymic atrophy through different cellular targets. *Toxicol Appl Pharmacol*. 1994;126(2):248–259. doi:10.1006/taap.1994.1114.
266. Anton E. Delayed toxicity of cyclophosphamide on the bladder of DBA/2 and C57BL/6 female mouse. *Int J Exp Pathol*. 2002;83(1):47–53.
267. Bracci L, Moschella F, Sestili P, et al. Cyclophosphamide enhances the antitumor efficacy of adoptively transferred immune cells through the induction of cytokine expression, B-cell and T-cell homeostatic proliferation, and specific tumor infiltration. *Clin Cancer Res*. 2007;13(2 Pt 1):644–653. doi:10.1158/1078-0432.CCR-06-1209.
268. Golubeva AV, Zhdanov AV, Mallel G, Dinan TG, Cryan JF. The mouse cyclophosphamide model of bladder pain syndrome: tissue characterization, immune profiling, and relationship to metabotropic glutamate receptors. *Physiol Rep*. 2014;2(3):e00260. doi:10.1002/phy2.260.
269. Kawakami T, Nakamura Y, Karibe H. Cyclophosphamide inhibits root development of molar teeth in growing mice. *Odontology*. 2015;103(2):143–151. doi:10.1007/s10266-014-0158-1.
270. Nakahara T, Uchi H, Lesokhin AM, et al. Cyclophosphamide enhances immunity by modulating the balance of dendritic cell subsets in lymphoid organs. *Blood*. 2010;115(22):4384–4392. doi:10.1182/blood-2009-11-251231.
271. Xu X, Zhang X. Effects of cyclophosphamide on immune system and gut microbiota in mice. *Microbiol Res*. 2015;171:97–106. doi:10.1016/j.micres.2014.11.002.
272. Yasunami R, Bach JF. Anti-suppressor effect of cyclophosphamide on the development of spontaneous diabetes in NOD mice. *Eur J Immunol*. 1988;18(3):481–484. doi:10.1002/eji.1830180325.
273. Zhao D, Wang C, Zhao Y, et al. Cyclophosphamide causes osteoporosis in C57BL/6 male mice: suppressive effects of cyclophosphamide on osteoblastogenesis and osteoclastogenesis. *Oncotarget*. 2017;8(58):98163–98183. doi:10.18632/oncotarget.21000.
274. Swindell WR. Meta-analysis of 29 experiments evaluating the effects of rapamycin on life span in the laboratory mouse. *J Gerontol A Biol Sci Med Sci*. 2017;72(8):1024–1032. doi:10.1093/gerona/glw153.
275. Thomson AW, Turnquist HR, Raimondi G. Immunoregulatory functions of mTOR inhibition. *Nat Rev Immunol*. 2009;9(5):324–337. doi:10.1038/nri2546.
276. Bhoopalam N, Price KS, Norgello H, Fried W. Busulfan-induced suppression of natural killer cell activity. *Exp Hematol*. 1985;13(11):1127–1132.
277. Gibson FM, Andrews CM, Diamanti P, et al. A new model of busulphan-induced chronic bone marrow aplasia in the female BALB/c mouse. *Int J Exp Pathol*. 2003;84(1):31–48.
278. Hassan M. The role of busulfan in bone marrow transplantation. *Med Oncol*. 1999;16(3):166–176.
279. Molyneux G, Andrews M, Sones W, et al. Haemotoxicity of busulphan, doxorubicin, cisplatin and cyclophosphamide in the female BALB/c mouse using a brief regimen of drug administration. *Cell Biol Toxicol*. 2011;27(1):13–40. doi:10.1007/s10565-010-9167-1.
280. Sjöö F, Hassan Z, Abedi-Valugerdi M, et al. Myeloablative and immunosuppressive properties of treosulfan in mice. *Exp Hematol*. 2006;34(1):115–121. doi:10.1016/j.exphem.2005.09.015.
281. Buettner M, Bornemann M, Bode U. Skin tolerance is supported by the spleen. *Scand J Immunol*. 2013;77(4):238–245. doi:10.1111/sji.12034.
282. Fotiadis C, Zografos G, Aronis K, et al. The effect of various types of splenectomy on the development of B-16 melanoma in mice. *Anticancer Res*. 1999;19(5B):4235–4239.
283. Chung DT, Korn T, Richard J, et al. Anti-thymocyte globulin (ATG) prevents autoimmune encephalomyelitis by expanding myelin antigen-specific Foxp3+ regulatory T cells. *Int Immunol*. 2007;19(8):1003–1010. doi:10.1093/intimm/dxm078.
284. Bakheet SA, Attia SM, Alwetaid MY, et al. B-1,3-glucan reverses aflatoxin B1-mediated suppression of immune responses in mice. *Life Sci*. 2016;152:1–13. doi:10.1016/j.lfs.2016.03.030.
285. Norton EB, Clements JD, Voss TG, Cárdenas-Freytag L. Prophylactic administration of bacterially derived immunomodulators improves the outcome of influenza virus infection in a murine model. *J Virol*. 2010;84(6):2983–2995. doi:10.1128/JVI.01805-09.

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286. Maki T, Ichikawa T, Blanco R, Porter J. Long-term abrogation of autoimmune diabetes in nonobese diabetic mice by immunotherapy with anti-lymphocyte serum. *Proc Natl Acad Sci USA*. 1992;89(8):3434–3438.
287. Bouaziz J, Yanaba K, Venturi GM, et al. Therapeutic B cell depletion impairs adaptive and autoreactive CD4+ T cell activation in mice. *Proc Natl Acad Sci USA*. 2007;104(52): 20878–20883. doi:10.1073/pnas.0709205105.
288. Chen L, Mehta ND, Zhao Y, DiPietro LA. Absence of CD4 or CD8 lymphocytes changes infiltration of inflammatory cells and profiles of cytokine expression in skin wounds, but does not impair healing. *Exp Dermatol*. 2014;23(3): 189–194. doi:10.1111/exd.12346.
289. Jing W, Gershan JA, Johnson BD. Depletion of CD4 T cells enhances immunotherapy for neuroblastoma after syngeneic HSCT but compromises development of antitumor immune memory. *Blood*. 2009;113(18):4449–4457. doi:10.1182/blood-2008-11-190827.
290. Rosenblum MD, Remedios KA, Abbas AK. Mechanisms of human autoimmunity. *J Clin Invest*. 2015;125:2228–2233. http://www.ncbi.nlm.nih.gov/pubmed/25893595. Accessed Dec 1, 2018.
291. Melli K, Friedman RS, Martin AE, et al. Amplification of autoimmune response through induction of dendritic cell maturation in inflamed tissues. *J Immunol*. 2009;182:2590–2600. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3057894/. Accessed Dec 1, 2018.
292. Schmidt RE, Grimbacher B, Witte T. Autoimmunity and primary immunodeficiency: two sides of the same coin? *Nat Rev Rheumatol*. 2017;14(1):7–18.
293. Petkova SB, Yuan R, Tsaih SW, Schott W, Roopenian DC, Paigen B. Genetic influence on immune phenotype revealed strain-specific variations in peripheral blood lineages. *Physiol Genomics*. 2008;34(3):304–314.
294. deLuca LES, Pikor NB, O'Leary J, et al. Substrain differences reveal novel disease-modifying gene candidates that alter the clinical course of a rodent model of multiple sclerosis. *J Immunol*. 2010;184:3174–3185. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3160975/. Accessed Dec 1, 2018.
295. Begley CG, Ellis LM. Drug development: raise standards for preclinical cancer research. *Nature*. 2012;483(7391): 531–533.
296. Rice AS, Cimino-Brown D, Eisenach JC, et al. Animal models and the prediction of efficacy in clinical trials of analgesic drugs: a critical appraisal and call for uniform reporting standards. *Pain*. 2008;139(2):243–247.
297. Bevaart L, Vervoordeldonk MJ, Tak PP. Evaluation of therapeutic targets in animal models of arthritis: how does it relate to rheumatoid arthritis? *Arthritis Rheum*. 2010;62: 2192–2205. http://www.ncbi.nlm.nih.gov/pubmed/20506322.
298. Farkas B, Boldizsar F, Tarjanyi O, et al. BALB/c mice genetically susceptible to proteoglycan-induced arthritis and spondylitis show colony-dependent differences in disease penetrance. *Arthritis Res Ther*. 2009;11:R21. http://www.ncbi.nlm.nih.gov/pubmed/19220900.
299. Glant TT, Radacs M, Nagyeri G, et al. Proteoglycan (PG)-induced arthritis (PGIA) and recombinant human PG-G1 domain-induced arthritis (GIA) in BALB/c mice resembling two subtypes of rheumatoid arthritis. *Arthritis Rheum*. 2011;63:1312–1321. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3086933/.
300. Boldizsar F, Tarjanyi O, Nemeth P, Mikecz K, Glant TT. T(h)1/T(h)17 polarization and acquisition of an arthritogenic phenotype in arthritis-susceptible BALB/c, but not in MHC-matched, arthritis-resistant DBA/2 mice. *Int Immunol*. 2009;21:511–522. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2675029/.
301. Voskuhl RR, Gold SM. Sex-related factors in multiple sclerosis: genetic, hormonal and environmental contributions. *Nat Rev Neurol*. 2012;8:255–263. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4435786/.
302. Bernard CC, Mackay IR. Experimental autoimmune encephalomyelitis in mice: lyt and ia phenotypes of the effector and suppressor cells. *Prog Clin Biol Res*. 1984;146: 277–284. http://www.ncbi.nlm.nih.gov/pubmed/6232623.
303. Bernard CC. Suppressor T cells prevent experimental autoimmune encephalomyelitis in mice. *Clin Exp Immunol*. 1977; 29:100–109. http://www.ncbi.nlm.nih.gov/pubmed/302168.
304. Bernard CC, Leydon J, Mackay IR. T cell necessity in the pathogenesis of experimental autoimmune encephalomyelitis in mice. *Eur J Immunol*. 1976;6:655–660. http://www.ncbi.nlm.nih.gov/pubmed/1087243.
305. Croxford AL, Kurschus FC, Waisman A. Mouse models for multiple sclerosis: historical facts and future implications. *Biochim Biophys Acta*. 2011;1812(2):177–183. doi:10.1016/j.bbadis.2010.06.010.
306. Bernard CC, Carnegie PR. Experimental autoimmune encephalomyelitis in mice: immunologic response to mouse spinal cord and myelin basic proteins. *J Immunol*. 1975;114:1537–1540. http://www.ncbi.nlm.nih.gov/pubmed/47366.
307. Behlke MA, Loh DY. Alternative splicing of murine T-cell receptor β-chain transcripts. *Nature*. 1986;322(6077):379. doi:10.1038/322379a0. https://proxy.library.upenn.edu:2611/articles/322379a0. Accessed May 3, 2018.
308. Bernard CC. Experimental autoimmune encephalomyelitis in mice: genetic control of susceptibility. *J Immunogenet*. 1976;3:263–274. http://www.ncbi.nlm.nih.gov/pubmed/1109134.
309. Kawamura K, Yao K, Shukaliak-Quandt JA, et al. Different development of myelin basic protein agonist- and antagonist-specific human TCR transgenic T cells in the thymus and periphery. *J Immunol*. 2008;181(8):5462–5472.
310. Sato F, Tanaka H, Hasanovic F, Tsunoda I. Theiler's virus infection: pathophysiology of demyelination and neurodegeneration. *Pathophysiology*. 2011;18(1):31–41. doi:10.1016/j.pathophys.2010.04.011.
311. Turrin NP. Central nervous system toll-like receptor expression in response to Theiler's murine encephalomyelitis virus-induced demyelination disease in resistant and susceptible mouse strains. *Virol J*. 2008;5:154. doi:10.1186/1743-422x-5-154.
312. McCarthy DP, Richards MH, Miller SD. Mouse models of multiple sclerosis: experimental autoimmune encephalomyelitis and Theiler's virus-induced demyelinating disease. *Methods Mol Biol*. 2012;900:381–401. doi:10.1007/978-1-60761-720-4_19.
313. Cowley TJ, Weiss SR. Murine coronavirus neuropathogenesis: determinants of virulence. *J Neurovirol*. 2010;16(6): 427–434. doi:10.3109/13550284.2010.529238.
314. Kishore A, Kanaujia A, Nag S, et al. Different mechanisms of inflammation induced in virus and autoimmune-mediated models of multiple sclerosis in C57BL6 mice. *Biomed Res Int*. 2013;2013:589048. doi:10.1155/2013/589048.
315. Knobler RL, Tunison LA, Lampert PW, Oldstone MB. Selected mutants of mouse hepatitis virus type 4 (JHM strain) induce different CNS diseases. pathobiology of disease induced by wild type and mutants ts8 and ts15 in BALB/c and SJL/J mice. *Am J Pathol*. 1982;109(2):157–168.

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316. [NRC] National Research Council. *Guide for the care and use of laboratory animals: eighth edition*. Washington, DC: The National Academies Press; 2011. doi:10.17226/12910. https://www.nap.edu/catalog/12910/guide-for-the-care-and-use-of-laboratory-animals-eighth.
317. Clough G. Suggested guidelines for the housing and husbandry of rodents for aging studies. *Neurobiol Aging*. 1991;12(6):653–658.
318. Everds NE, Snyder PW, Bailey KL, et al. Interpreting stress responses during routine toxicity studies: a review of the biology, impact, and assessment. *Toxicol Pathol*. 2013;41(4):560–614. doi:10.1177/0192623312466452.
319. Rowan AN. Refinement of animal research technique and validity of research data. *Fundam Appl Toxicol*. 1990;15(1):25–32.
320. Laber K, Veatch LM, Lopez MF, Mulligan JK, Lathers DMR. Effects of housing density on weight gain, immune function, behavior, and plasma corticosterone concentrations in BALB/c and C57BL/6 mice. *J Am Assoc Lab Anim Sci*. 2008;47(2):16–23.
321. Anisman H, Prakash P, Merali Z, Poulter MO. Corticotropin releasing hormone receptor alterations elicited by acute and chronic unpredictable stressor challenges in stressor-susceptible and resilient strains of mice. *Behav Brain Res*. 2007;181(2):180–190. doi:10.1016/j.bbr.2007.04.002.
322. David JM, Knowles S, Lamkin DM, Stout DB. Individually ventilated cages impose cold stress on laboratory mice: a source of systemic experimental variability. *J Am Assoc Lab Anim Sci*. 2013;52(6):738–744.
323. Ferrecchia CE, Jensen K, Van Andel R. Intracage ammonia levels in static and individually ventilated cages housing C57BL/6 mice on 4 bedding substrates. *J Am Assoc Lab Anim Sci*. 2014;53(2):146–151.
324. Mexas AM, Brice AK, Caro AC, Hillanbrand TS, Gaertner DJ. Nasal histopathology and intracage ammonia levels in female groups and breeding mice housed in static isolation cages. *J Am Assoc Lab Anim Sci*. 2015;54(5):478–486.
325. Rosenbaum MD, VandeWoude S, Volckens J, Johnson T. Disparities in ammonia, temperature, humidity, and airborne particulate matter between the micro-and macroenvironments of mice in individually ventilated caging. *J Am Assoc Lab Anim Sci*. 2010;49(2):177–183.
326. Bartolomucci A. Social stress, immune functions and disease in rodents. *Front Neuroendocrinol*. 2007;28(1):28–49. doi:10.1016/j.yfrne.2007.02.001.
327. Gordon CJ, Becker P, Ali JS. Behavioral thermoregulatory responses of single- and group-housed mice. *Physiol Behav*. 1998;65(2):255–262.
328. Maher RL, Barbash SM, Lynch DV, Swoap SJ. Group housing and nest building only slightly ameliorate the cold stress of typical housing in female C57BL/6J mice. *Am J Physiol Regul Integr Comp Physiol*. 2015;308(12):1070. doi:10.1152/ajpregu.00407.2014.
329. Nicholson A, Malcolm RD, Russ PL, et al. The response of C57BL/6J and BALB/cJ mice to increased housing density. *J Am Assoc Lab Anim Sci*. 2009;48(6):740–753.
330. Weber EM, Dallaire JA, Gaskill BN, Pritchett-Corning KR, Garner JP. Aggression in group-housed laboratory mice: why can't we solve the problem? *Lab Anim (NY)*. 2017;46(4):157–161. doi:10.1038/laban.1219.
331. Gaskill BN, Gordon CJ, Pajor EA, Lucas JR, Davis JK, Garner JP. Heat or insulation: behavioral titration of mouse preference for warmth or access to a nest. *PLoS One*. 2012;7(3):e32799. doi:10.1371/journal.pone.0032799.
332. Johnson JS, Taylor DJ, Green AR, Gaskill BN. Effects of nesting material on energy homeostasis in BALB/cAnNCrl, C57BL/6NCrl, and crl:CD1(ICR) mice housed at 20 °C. *J Am Assoc Lab Anim Sci*. 2017;56(3):254–259.
333. Song Y, Gan Y, Wang Q, et al. Enriching the housing environment for mice enhances their NK cell antitumor immunity via sympathetic nerve-dependent regulation of NKG2D and CCR5. *Cancer Res*. 2017;77(7):1611–1622. doi:10.1158/0008-5472.CAN-16-2143.
334. Van Loo PLP, Van der Meer E, Kruitwagen CLJJ, Koolhaas JM, Van Zutphen LFM, Baumans V. Long-term effects of husbandry procedures on stress-related parameters in male mice of two strains. *Lab Anim*. 2004;38(2):169–177. doi:10.1258/002367704322968858.
335. Arranz L, De Castro NM, Baeza I et al. Environmental enrichment improves age-related immune system impairment: long-term exposure since adulthood increases life span in mice. *Rejuvenation Res*. 2010;13(4):415–428. doi:10.1089/rej.2009.0989.
336. Brod S, Gobbetti T, Gittens B, Ono M, Perretti M, D'Acquisto F. Findings from Queen Mary University of London in granulocytes provides new insights (the impact of environmental enrichment on the murine inflammatory immune response). *Obes Fit Wellness Week*. 2017;2(7):817. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5374068/.
337. Messmer MN, Kokolus KM, Eng JW, Abrams SI, Repasky EA. Mild cold-stress depresses immune responses: implications for cancer models involving laboratory mice. *Bioessays*. 2014;36(9):884–891. doi:10.1002/bies.201400066.
338. Bailoo JD, Murphy E, Varholick JA, Novak J, Palme R, Würbel H. Evaluation of the effects of space allowance on measures of animal welfare in laboratory mice. *Sci Rep*. 2018;8(1):713. doi:10.1038/s41598-017-18493-6.
339. Hylander BL, Repasky EA. Thermoneutrality, mice, and cancer: a heated opinion. *Trends Cancer*. 2016;2(4):166–175. doi:10.1016/j.trecan.2016.03.005.
340. Hylander BL, Eng JW, Repasky EA. The impact of housing temperature-induced chronic stress on preclinical mouse tumor models and therapeutic responses: an important role for the nervous system. *Adv Exp Med Biol*. 2017;1036:173–189. doi:10.1007/978-3-319-67577-0_12.
341. Toth LA, Trammell RA, Ilsley-Woods M. Interactions between housing density and ambient temperature in the cage environment: effects on mouse physiology and behavior. *J Am Assoc Lab Anim Sci*. 2015;54(6):708. http://www.ncbi.nlm.nih.gov/pubmed/26632780.
342. Eichner M, Purcell JE, Fortman JD. Effects of intracage ammonia on markers of pulmonary endothelial integrity in mice housed in static microisolation cages. *J Am Assoc Lab Anim Sci*. 2018;57(1):18. https://www.ncbi.nlm.nih.gov/pubmed/29402347.
343. Langgartner D, Foertsch S, Füchsl AM, Reber SO. Light and water are not simple conditions: fine tuning of animal housing in male C57BL/6 mice. *Stress*. 2017;20(1):10–18. doi:10.1080/10253890.2016.1254186.
344. Peirson SN, Brown LA, Pothecary CA, Benson LA, Fisk AS. Light and the laboratory mouse. *J Neurosci Methods*. 2018;300:26–36. doi:10.1016/j.jneumeth.2017.04.007.
345. Scheiermann C, Kunisaki Y, Frenette PS. Circadian control of the immune system. *Nat Rev Immunol*. 2013;13(3):190–198. doi:10.1038/nri3386.
346. Kim S, Neuendorff N, Alaniz RC, Sun Y, Chapkin RS, Earnest DJ. Shift work cycle-induced alterations of

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circadian rhythms potentiate the effects of high-fat diet on inflammation and metabolism. *FASEB J*. 2018;32(6): 3085–3095. doi:10.1096/fj.201700784R.
347. Maestroni GJ. The photoperiod transducer melatonin and the immune-hematopoietic system. *J Photochem Photobiol B*. 1998;43(3):186–192.
348. Turner JG, Parrish JL, Hughes LF, Toth LA, Caspary DM. Hearing in laboratory animals: strain differences and nonauditory effects of noise. *Comp Med*. 2005;55(1):12–23.
349. Norton JN, Kinard WL, Reynolds RP. Comparative vibration levels perceived among species in a laboratory animal facility. *J Am Assoc Lab Anim Sci*. 2011;50(5):653–659.
350. Münzel T, Daiber A, Steven S, et al. Effects of noise on vascular function, oxidative stress, and inflammation: mechanistic insight from studies in mice. *Eur Heart J*. 2017;38(37): 2838–2849. doi:10.1093/eurheartj/ehx081.
351. Atanasov NA, Sargent JL, Parmigiani JP, Palme R, Diggs HE. Characterization of train-induced vibration and its effect on fecal corticosterone metabolites in mice. *J Am Assoc Lab Anim Sci*. 2015;54(6):737–744.
352. Ambery AG, Tackett L, Penque BA, Hickman DL, Elmendorf JS. Effect of corncob bedding on feed conversion efficiency in a high-fat diet-induced prediabetic model in C57Bl/6J mice. *J Am Assoc Lab Anim Sci*. 2014;53(5):449–451.
353. Jacobs BB, Dieter DK. Spontaneous hepatomas in mice inbred from ha:ICR Swiss stock: effects of sex, cedar shavings in bedding, and immunization with fetal liver or hepatoma cells. *J Natl Cancer Inst*. 1978;61(6):1531–1534.
354. Potgieter FJ, Törrönen R, Wilke PI. The in vitro enzyme-inducing and cytotoxic properties of South African laboratory animal contact bedding and nesting materials. *Lab Anim*. 1995;29(2):163–171. doi:10.1258/002367795780740212.
355. Sabine JR. Exposure to an environment containing the aromatic red cedar, Juniperus virginiana: procarcinogenic, enzyme-inducing and insecticidal effects. *Toxicology*. 1975;5(2):221–235.
356. Villalon Landeros R, Morisseau C, Yoo HJ, Fu SH, Hammock BD, Trainor BC. Corncob bedding alters the effects of estrogens on aggressive behavior and reduces estrogen receptor-α expression in the brain. *Endocrinology*. 2012;153(2):949–953. doi:10.1210/en.2011-1745.
357. Whiteside TE, Thigpen JE, Kissling GE, Grant MG, Forsythe D. Endotoxin, coliform, and dust levels in various types of rodent bedding. *J Am Assoc Lab Anim Sci*. 2010;49(2): 184–189.
358. [NRC] National Research Council. *Nutrient requirements of laboratory animals*. 4th revised ed. Washington, DC: The National Academies Press; 1995. doi:10.17226/4758. https://www.nap.edu/catalog/4758/nutrient-requirements-of-laboratory-animals-fourth-revised-edition-1995.
359. [NRC] National Research Council. *Mineral tolerance of domestic animals*. Washington, DC: The National Academies Press; 1980. doi:10.17226/25. https://www.nap.edu/catalog/25/mineral-tolerance-of-domestic-animals.
360. [NRC] National Research Council. *Vitamin tolerance of animals*. Washington, DC: The National Academies Press; 1987. doi:10.17226/949. https://www.nap.edu/catalog/949/vitamin-tolerance-of-animals.
361. Clarke HE, Coates ME, Eva JK, et al. Dietary standards for laboratory animals: report of the laboratory animals centre diets advisory committee. *Lab Anim*. 1977;11(1):1–28. doi:10.1258/002367777780959175.
362. Newberne PM, Fox JG. Nutritional adequacy and quality control of rodent diets. *Lab Anim Sci*. 1980;30(2 Pt 2):352–365.
363. Rao GN, Knapka JJ. Contaminant and nutrient concentrations of natural ingredient rat and mouse diet used in chemical toxicology studies. *Fundam Appl Toxicol*. 1987;9(2):329–338.
364. Mesnage R, Defarge N, Rocque L, Spiroux de Vendômois J, Séralini G. Laboratory rodent diets contain toxic levels of environmental contaminants: implications for regulatory tests. *PLoS One*. 2015;10(7):e0128429. doi:10.1371/journal.pone.0128429.
365. Jensen MN, Ritskes-Hoitinga M. How isoflavone levels in common rodent diets can interfere with the value of animal models and with experimental results. *Lab Anim*. 2007; 41(1):1–18. doi:10.1258/002367707779399428.
366. Thigpen JE, Setchell KDR, Kissling GE, et al. The estrogenic content of rodent diets, bedding, cages, and water bottles and its effect on bisphenol A studies. *J Am Assoc Lab Anim Sci*. 2013;52(2):130–141.
367. Silverman J, Adams JD. N-nitrosamines in laboratory animal feed and bedding. *Lab Anim Sci*. 1983;33(2):161–164.
368. Pellizzon MA, Ricci MR. The common use of improper control diets in diet-induced metabolic disease research confounds data interpretation: the fiber factor. *Nutr Metab (Lond)*. 2018;15:3. doi:10.1186/s12986-018-0243-5.
369. NTP. Effect of dietary restriction on toxicology and carcinogenesis studies in F344/N rats and B6C3F1 mice. *Natl Toxicol Program Tech Rep Ser*. 1997;460:1–414. https://www.ncbi.nlm.nih.gov/pubmed/12587016.
370. Moraal M, Leenaars PP, Arnts H, et al. The influence of food restriction versus ad libitum feeding of chow and purified diets on variation in body weight, growth and physiology of female Wistar rats. *Lab Anim*. 2012;46(2): 101–107. doi:10.1258/la.2011.011011.
371. Spindler SR. Review of the literature and suggestions for the design of rodent survival studies for the identification of compounds that increase health and life span. *Age (Dordr)*. 2012;34(1):111–120. doi:10.1007/s11357-011-9224-6.
372. Hermann LM, White WJ, Lang CM. Prolonged exposure to acid, chlorine, or tetracycline in the drinking water: effects on delayed-type hypersensitivity, hemagglutination titers, and reticuloendothelial clearance rates in mice. *Lab Anim Sci*. 1982;32(6):603–608.
373. McIntyre AR, Lipman NS. Amoxicillin-clavulanic acid and trimethoprim- sulfamethoxazole in rodent feed and water: effects of compounding on antibiotic stability. *J Am Assoc Lab Anim Sci*. 2007;46(5):26–32.
374. Redelsperger IM, Taldone T, Riedel ER, Lepherd ML, Lipman NS, Wolf FR. Stability of doxycycline in feed and water and minimal effective doses in tetracycline-inducible systems. *J Am Assoc Lab Anim Sci*. 2016;55(4):467–474.
375. Franklin CL. Microbial considerations in genetically engineered mouse research. *ILAR J*. 2006;47(2):141–155. doi:10.1093/ilar.47.2.141. https://academic.oup.com/ilarjournal/article/47/2/141/671013. Accessed May 13, 2018.
376. Nicklas W, Keubler L, Bleich A. Maintaining and monitoring the defined microbiota status of gnotobiotic rodents. *ILAR J*. 2015;56(2):241–249. https://academic.oup.com/ilarjournal/article/56/2/241/651412. doi:10.1093/ilar/ilv029. Accessed May 13, 2018.
377. Hedrich HJ, Nicklas W. Chapter 4. 1—housing and maintenance. In: Hedrich H, ed. *The laboratory mouse*. 2nd ed. Boston: Academic Press; 2012:521–545. https://www.sciencedirect.com/science/article/pii/B9780123820082000222. Accessed May 13, 2018.

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378. Treuting PM, Clifford CB, Sellers RS, Brayton CF. Of mice and microflora: considerations for genetically engineered mice. *Vet Pathol*. 2012;49(1):44–63.
379. Dagnæs-Hansen F, Poulsen K. Pneumocystis murina infection in immunodeficient mice in a closed barrier unit: a case report. *Scand J Lab Anim Sci*. 2011;38(2):91–96.
380. Burr HN, Lipman NS, White JR, Zheng J, Wolf FR. Strategies to prevent, treat, and provoke corynebacterium-associated hyperkeratosis in athymic nude mice. *J Am Assoc Lab Anim Sci*. 2011;50(3):378. https://www.ncbi.nlm.nih.gov/pubmed/21640035. Accessed May 13, 2018.
381. Santagostino SF, Arbona RJR, Nashat MA, White JR, Monette S. Pathology of aging in NOD scid gamma female mice. *Vet Pathol*. 2017;54(5):855–869. doi:10.1177/0300985817698210.
382. Scanziani E, Gobbi A, Crippa L, et al. Hyperkeratosis-associated coryneform infection in severe combined immunodeficient mice. *Lab Anim*. 1998;32(3):330–336. doi:10.1258/002367798780559239.
383. Acuff NV, LaGatta M, Nagy T, Watford WT. Severe dermatitis associated with spontaneous staphylococcus xylosus infection in rag-/-Tpl2-/- mice. *Comp Med*. 2017;67(4):344–349.
384. Russo M, Invernizzi A, Gobbi A, Radaelli E. Diffuse scaling dermatitis in an athymic nude mouse. *Vet Pathol*. 2013;50(4):722–726. doi:10.1177/0300985812463408.
385. Tao L, Reese TA. Making mouse models that reflect human immune responses. *Trends Immunol*. 2017;38(3):181–193. doi:10.1016/j.it.2016.12.007.
386. Ivanov II, Littman DR. Segmented filamentous bacteria take the stage. *Mucosal Immunol*. 2010;3(3):209–212. doi:10.1038/mi.2010.3.
387. Ivanov II, Atarashi K, Manel N, et al. Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell*. 2009;139(3):485–498. doi:10.1016/j.cell.2009.09.033.
388. Kriegel MA, Sefik E, Hill JA, Wu H, Benoist C, Mathis D. Naturally transmitted segmented filamentous bacteria segregate with diabetes protection in nonobese diabetic mice. *Proc Natl Acad Sci USA*. 2011;108(28):11548–11553.
389. Lee YK, Menezes JS, Umesaki Y, Mazmanian SK. Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci USA*. 2011;108(Suppl 1):4615–4622. doi:10.1073/pnas.1000082107.
390. Wu H, Ivanov II, Darce J, et al. Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. *Immunity*. 2010;32(6):815–827. doi:10.1016/j.immuni.2010.06.001.
391. Ericsson AC, Hagan CE, Davis DJ, Franklin CL. Segmented filamentous bacteria: commensal microbes with potential effects on research. *Comp Med*. 2014;64(2):90–98.
392. Jiang HQ, Bos NA, Cebra JJ. Timing, localization, and persistence of colonization by segmented filamentous bacteria in the neonatal mouse gut depend on immune status of mothers and pups. *Infect Immun*. 2001;69(6):3611–3617. doi:10.1128/IAI.69.6.3611-3617.2001.
393. Suzuki K, Meek B, Doi Y, et al. Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut. *Proc Natl Acad Sci USA*. 2004;101(7):1981–1986. doi:10.1073/pnas.0307317101.
394. Schnupf P, Gaboriau-Routhiau V, Gros M, et al. Growth and host interaction of mouse segmented filamentous bacteria in vitro. *Nature*. 2015;520(7545):99–103. doi:10.1038/nature14027.
395. Wymore Brand M, Wannemuehler MJ, Phillips GJ, et al. The altered schaedler flora: continued applications of a defined murine microbial community. *ILAR J*. 2015;56(2):169–178. doi:10.1093/ilar/ilv012.
396. Ericsson AC, Franklin CL. Manipulating the gut microbiota: methods and challenges. *ILAR J*. 2015;56(2):205–217. doi:10.1093/ilar/ilv021.
397. Friswell MK, Gika H, Stratford IJ, et al. Site and strain-specific variation in gut microbiota profiles and metabolism in experimental mice. *PLoS One*. 2010;5(1):e8584. doi:10.1371/journal.pone.0008584.
398. Hufeldt MR, Nielsen DS, Vogensen FK, Midtvedt T, Hansen AK. Variation in the gut microbiota of laboratory mice is related to both genetic and environmental factors. *Comp Med*. 2010;60(5):336–347.
399. Cadwell K, Liu JY, Brown SL, et al. A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal paneth cells. *Nature*. 2008;456(7219):259–263. doi:10.1038/nature07416.
400. Cadwell K, Patel KK, Maloney NS, et al. Virus-plus-susceptibility gene interaction determines Crohn's disease gene Atg16L1 phenotypes in intestine. *Cell*. 2010;141(7):1135–1145. doi:10.1016/j.cell.2010.05.009.
401. Kverka M, Zakostelska Z, Klimesova K, et al. Oral administration of parabacteroides distasonis antigens attenuates experimental murine colitis through modulation of immunity and microbiota composition. *Clin Exp Immunol*. 2011;163(2):250–259. doi:10.1111/j.1365-2249.2010.04286.x.
402. Denning TL, Norris BA, Medina-Contreras O, et al. Functional specializations of intestinal dendritic cell and macrophage subsets that control Th17 and regulatory T cell responses are dependent on the T cell/APC ratio, source of mouse strain, and regional localization. *J Immunol*. 2011;187(2):733–747. doi:10.4049/jimmunol.1002701.
403. Hart ML, Ericsson AC, Franklin CL. Differing complex microbiota alter disease severity of the IL-10-/- mouse model of inflammatory bowel disease. *Front Microbiol*. 2017;8:792. doi:10.3389/fmicb.2017.00792.
404. Peloquin JM, Nguyen DD. The microbiota and inflammatory bowel disease: insights from animal models. *Anaerobe*. 2013;24:102–106. doi:10.1016/j.anaerobe.2013.04.006.
405. Chudnovskiy A, Mortha A, Kana V, et al. Host-protozoan interactions protect from mucosal infections through activation of the inflammasome. *Cell*. 2016;167(2):456.e14. doi:10.1016/j.cell.2016.08.076.
406. Escalante NK, Lemire P, Cruz Tleugabulova M, et al. The common mouse protozoa tritrichomonas muris alters mucosal T cell homeostasis and colitis susceptibility. *J Exp Med*. 2016;213(13):2841–2850. doi:10.1084/jem.20161776.
407. Ericsson AC, Davis JW, Spollen W, et al. Effects of vendor and genetic background on the composition of the fecal microbiota of inbred mice. *PLoS One*. 2015;10(2):e0116704. doi:10.1371/journal.pone.0116704.
408. Franklin CL, Ericsson AC. Microbiota and reproducibility of rodent models. *Lab Anim (NY)*. 2017;46(4):114–122. doi:10.1038/laban.1222.
409. Rosshart SP, Vassallo BG, Angeletti D, et al. Wild mouse gut microbiota promotes host fitness and improves disease resistance. *Cell*. 2017;171(5):1028.e13. doi:10.1016/j.cell.2017.09.016.
410. Barthold SW. Microbes and the evolution of scientific fancy mice. *ILAR J*. 2008;49(3):265–271.
411. Arapović J, Arapović M, Golemac M, et al. The specific NK cell response in concert with perforin prevents CD8(+) T cell-mediated immunopathology after mouse cytomegalovirus infection. *Med Microbiol Immunol*. 2015;204(3):335–344. doi:10.1007/s00430-015-0409-y.

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412. Wetzel JL, Fensterl V, Sen GC. Sendai virus pathogenesis in mice is prevented by Ifit2 and exacerbated by interferon. *J Virol*. 2014;88(23):13593–13601. doi:10.1128/JVI.02201-14.
413. Morse SS, Sakaguchi N, Sakaguchi S. Virus and autoimmunity: induction of autoimmune disease in mice by mouse T lymphotropic virus (MTLV) destroying CD4+ T cells. *J Immunol*. 1999;162(9):5309–5316.
414. Jacoby Ro, Ball-Goodrich L. Chapter 4—parvoviruses. In: Fox JG, Davisson MT, Quimby FW, Barthold SW, Newcomer CE, Smith AL, eds. *The mouse in biomedical research*. (2nd ed). Burlington: Academic Press; 2007:93–103. https://www.sciencedirect.com/science/article/pii/B9780123694546500327. Accessed May 14, 2018.
415. Riolobos L, Valle N, Hernando E, Maroto B, Kann M, Almendral JM. Viral oncolysis that targets raf-1 signaling control of nuclear transport. *J Virol*. 2010;84(4):2090–2099. doi:10.1128/JVI.01550-09.
416. Henderson KS, Pritchett-Corning KR, Perkins CL, et al. A comparison of mouse parvovirus 1 infection in BALB/c and C57BL/6 mice: susceptibility, replication, shedding, and seroconversion. *Comp Med*. 2015;65(1):5–14.
417. Watson J. Unsterilized feed as the apparent cause of a mouse parvovirus outbreak. *J Am Assoc Lab Anim Sci*. 2013;52(1):83–88.
418. Beura LK, Hamilton SE, Bi K, et al. Normalizing the environment recapitulates adult human immune traits in laboratory mice. *Nature*. 2016;532(7600):512. doi:10.1038/nature17655. https://www.nature.com/articles/nature17655. Accessed Mar 4, 2018.
419. Roble GS, Gillespie V, Lipman NS. Infectious disease survey of mus musculus from pet stores in New York City. *J Am Assoc Lab Anim Sci*. 2012;51(1):37–41.
420. Mahabir E, Bauer B, Schmidt J. Rodent and germplasm trafficking: risks of microbial contamination in a high-tech biomedical world. *ILAR J*. 2008;49(3):347–355.
421. Parker SE, Malone S, Bunte RM, Smith AL. Infectious diseases in wild mice (mus musculus) collected on and around the University of Pennsylvania (Philadelphia) campus. *Comp Med*. 2009;59(5):424. https://www.ncbi.nlm.nih.gov/pubmed/19887025. Accessed Jun 10, 2018.
422. Ehlers B, Küchler J, Yasmum N, et al. Identification of novel rodent herpesviruses, including the first gammaherpesvirus of mus musculus. *J Virol*. 2007;81(15):8091–8100. doi:10.1128/JVI.00255-07.
423. Patel SJ, Zhao G, Penna VR, et al. A murine herpesvirus closely related to ubiquitous human herpesviruses causes T-cell depletion. *J Virol*. 2017;91(9). doi:10.1128/JVI.02463-16.
424. Patel SJ, Yokoyama WM. Reply to "murine roseolovirus, historically known as murine thymic lymphotropic virus". *J Virol*. 2017;91(18). doi:10.1128/JVI.00956-17.
425. Krmpotic A, Bubic I, Polic B, Lucin P, Jonjic S. Pathogenesis of murine cytomegalovirus infection. *Microbes Infect*. 2003;5(13):1263–1277.
426. Thomas AC, Forster MR, Bickerstaff AA, et al. Occult cytomegalovirus in vivarium-housed mice may influence transplant allograft acceptance. *Transpl Immunol*. 2010;23(-1–2):86–91. doi:10.1016/j.trim.2010.03.005.
427. Hughes DJ, Kipar A, Sample JT, Stewart JP. Pathogenesis of a model gammaherpesvirus in a natural host. *J Virol*. 2010;84(8):3949–3961. doi:10.1128/JVI.02085-09.
428. Olivadoti M, Toth LA, Weinberg J, Opp MR. Murine gammaherpesvirus 68: a model for the study of epstein-barr virus infections and related diseases. *Comp Med*. 2007;57(1):44–50.
429. Aligo J, Brosnan K, Walker M, et al. Murine gammaherpesvirus-68 (MHV-68) is not horizontally transmitted amongst laboratory mice by cage contact. *J Immunotoxicol*. 2015;12(4):330–341. doi:10.3109/1547691x.2014.980020.
430. Dong S, Forrest JC, Liang X. Murine gammaherpesvirus 68: a small animal model for gammaherpesvirus-associated diseases. *Adv Exp Med Biol*. 2017;1018:225–236. doi:10.1007/978-981-10-5765-6_14.
431. Walsh NC, Kenney LL, Jangalwe S, et al. Humanized mouse models of clinical disease. *Annu Rev Pathol*. 2017;12:187–215. doi:10.1146/annurev-pathol-052016-100332.
432. Ahmed EH, Baiocchi RA. Murine models of Epstein-Barr virus-associated lymphomagenesis. *ILAR J*. 2016;57(1):55–62. doi:10.1093/ilar/ilv074.
433. Wagar EJ, Cromwell MA, Shultz LD, et al. Regulation of human cell engraftment and development of EBV-related lymphoproliferative disorders in hu-PBL-scid mice. *J Immunol*. 2000;165(1):518–527.
434. Radaelli E, Hermans E, Omodho L, et al. Spontaneous post-transplant disorders in NOD.cg- prkdcscid Il2rgtm1Sug/JicTac (NOG) mice engrafted with patient-derived metastatic melanomas. *PLoS One*. 2015;10(5):e0124974. doi:10.1371/journal.pone.0124974.
435. Taurozzi AJ, Beekharry R, Wantoch M, et al. Spontaneous development of Epstein-Barr virus associated human lymphomas in a prostate cancer xenograft program. *PLoS One*. 2017;12(11):e0188228. doi:10.1371/journal.pone.0188228.
436. Facompre ND, Sahu V, Montone KT, et al. Barriers to generating PDX models of HPV-related head and neck cancer. *Laryngoscope*. 2017;127(12):2777–2783. doi:10.1002/lary.26679.
437. Butler KA, Hou X, Becker MA, et al. Prevention of human lymphoproliferative tumor formation in ovarian cancer patient-derived xenografts. *Neoplasia*. 2017;19(8):628–636. doi:10.1016/j.neo.2017.04.007.
438. Corso S, Cargnelutti M, Durando S, et al. Rituximab treatment prevents lymphoma onset in gastric cancer patient-derived xenografts. *Neoplasia*. 2018;20(5):443–455. doi:10.1016/j.neo.2018.02.003.
439. Morse HC. Chapter 10—retroelements in the mouse. In: Fox JG, Davisson MT, Quimby FW, Barthold SW, Newcomer CE, Smith AL, eds. *The mouse in biomedical research*. 2nd ed. Burlington: Academic Press; 2007:269–279. https://www.sciencedirect.com/science/article/pii/B9780123694546500388. Accessed May 14, 2018.
440. Barthold SW, Griffey SM, Percy DH. Mouse. In: Barthold SW, Griffey SM, Percy DH, eds. *Pathology of laboratory rodents and rabbits*. John Wiley & Sons; 2016:1–118.
441. Nagata S, Suda T. Fas and fas ligand: lpr and gld mutations. *Immunol Today*. 1995;16(1):39–43.
442. Hiromatsu K, Usami J, Aoki Y, Makino M, Yoshikai Y. Accelerated progression of a murine retrovirus-induced immunodeficiency syndrome in fas mutant C57BL/6 lpr/lpr mice. *Microbiol Immunol*. 1997;41(3):221–227. https://www.ncbi.nlm.nih.gov/pubmed/9130234. Accessed May 14, 2018.
443. Rastad JL, Green WR. Myeloid-derived suppressor cells in murine AIDS inhibit B-cell responses in part via soluble mediators including reactive oxygen and nitrogen species, and TGF-β. *Virology*. 2016;499:9–22. doi:10.1016/j.virol.2016.08.031.
444. Radaelli E, Castiglioni V, Recordati C, et al. The pathology of aging 129S6/SvEvTac mice. *Vet Pathol*. 2016;53(2):477–492. doi:10.1177/0300985815608673.
445. Baudino L, Yoshinobu K, Morito N, Santiago-Raber ML, Izui S. Role of endogenous retroviruses in murine SLE. *Autoimmun*

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Rev. 2010;10(1):27–34. doi:10.1016/j.autrev.2010.07.012. https://www.ncbi.nlm.nih.gov/pubmed/20659589. Accessed May 14, 2018.
446. Hanafusa T, Miyagawa J, Nakajima H, et al. The NOD mouse. *Diabetes Res Clin Pract*. 1994;24(Suppl):S307–S311. https://www.ncbi.nlm.nih.gov/pubmed/7859625. Accessed May 14, 2018.
447. Ito K, Baudino L, Kihara M, et al. Three sgp loci act independently as well as synergistically to elevate the expression of specific endogenous retroviruses implicated in murine lupus. *J Autoimmun*. 2013;43:10. doi:10.1016/j.jaut.2013.01.014. https://www.ncbi.nlm.nih.gov/pubmed/23465716. Accessed May 14, 2018.
448. Bashratyan R, Regn D, Rahman MJ, et al. Type 1 diabetes pathogenesis is modulated by spontaneous autoimmune responses to endogenous retrovirus antigens in NOD mice. *Eur J Immunol*. 2017;47(3):575–584. doi:10.1002/eji.201646755.
449. Graham DM. A walk on the wild side. *Lab Anim (NY)*. 2017;46:423–427. doi:10.1038/laban.1372.
450. Zeiss CJ, Brayton CF. Immune responses to the real world. *Lab Anim (NY)*. 2017;47(1):13–14. doi:10.1038/laban.1384.
451. Peterson NC. From bench to cageside: risk assessment for rodent pathogen contamination of cells and biologics. *ILAR J*. 2008;49(3):310–315.
452. Nicklas W, Kraft V, Meyer B. Contamination of transplantable tumors, cell lines, and monoclonal antibodies with rodent viruses. *Lab Anim Sci*. 1993;43(4):296–300.
453. Almeida JL, Cole KD, Plant AL. Standards for cell line authentication and beyond. *PLoS Biol*. 2016;14(6):e1002476. doi:10.1371/journal.pbio.1002476.
454. Geraghty RJ, Capes-Davis A, Davis JM, et al. Guidelines for the use of cell lines in biomedical research. *Br J Cancer*. 2014;111(6):1021–1046. doi:10.1038/bjc.2014.166.
455. Meehan TF, Conte N, Goldstein T, et al. PDX-MI: minimal information for patient-derived tumor xenograft models. *Cancer Res*. 2017;77(21):e66. doi:10.1158/0008-5472.CAN-17-0582.
456. Yu M, Selvaraj SK, Liang-Chu MMY, et al. A resource for cell line authentication, annotation and quality control. *Nature*. 2015;520(7547):307–311. doi:10.1038/nature14397.
457. Pepin G, Ferrand J, Honing K, et al. Cre-dependent DNA recombination activates a STING-dependent innate immune response. *Nucleic Acids Res*. 2016;44(11):5356–5364. http://www.ncbi.nlm.nih.gov/pubmed/27166376. doi:10.1093/nar/gkw405.
458. Behjati S, Frank MH. The effects of tamoxifen on immunity. *Curr Med Chem*. 2009;16(24):3076–3080. https://www.ncbi.nlm.nih.gov/pubmed/19689284. doi:10.2174/092986709788803042.
459. Sthoeger ZM, Zinger H, Mozes E. Beneficial effects of the anti-oestrogen tamoxifen on systemic lupus erythematosus of (NZBxNZW)F1 female mice are associated with specific reduction of IgG3 autoantibodies. *Ann Rheum Dis*. 2003;62(4):341–346. http://www.ncbi.nlm.nih.gov/pubmed/12634234. doi:10.1136/ard.62.4.341.
460. Corbo-Rodgers E, Staub ES, Zou T, Smith A, Kambayashi T, Maltzman JS. Oral ivermectin as an unexpected initiator of CreT2-mediated deletion in T cells. *Nat Immunol*. 2012;13(3):197–198. doi:10.1038/ni.2232.
461. Bellahsene A, Forsgren A. Effect of doxycycline on immune response in mice. *Infect Immun*. 1985;48(2):556–559. http://iai.asm.org/content/48/2/556.abstract.
462. Milano S, Arcoleo F, D'Agostino P, Cillari E. Intraperitoneal injection of tetracyclines protects mice from lethal endotoxemia downregulating inducible nitric oxide synthase in various organs and cytokine and nitrate secretion in blood. *Antimicrob Agents Chemother*. 1997;41(1):117–121.
463. Boynton FDD, Ericsson AC, Uchihashi M, Dunbar ML, Wilkinson JE. Doxycycline induces dysbiosis in female C57BL/6NCrl mice. *BMC Res Notes*. 2017;10(1):644. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5708113/. doi:10.1186/s13104-017-2960-7. Accessed Feb 7, 2018.
464. Ottina E, Peperzak V, Schoeler K, et al. DNA-binding of the tet-transactivator curtails antigen-induced lymphocyte activation in mice. *Nat Commun*. 2017;8:1. doi:10.1038/s41467-017-01022-4. https://www.nature.com/articles/s41467-017-01022-4.
465. Ansari A, Ahmed A, Matsangos A, et al. Cellular GFP toxicity and immunogenicity: potential confounders in in vivo cell tracking experiments. *Stem Cell Rev*. 2016;12(5):553–559. doi:10.1007/s12015-016-9670-8. https://www.ncbi.nlm.nih.gov/pubmed/27435468.
466. Jeon YH, Choi Y, Kang JH, et al. Immune response to firefly luciferase as a naked DNA. *Cancer Biol Ther*. 2007;6(5):781–786.
467. McPherson SW, Yang J, Chan C, Dou C, Gregerson DS. Resting CD8 T cells recognize beta-galactosidase expressed in the immune-privileged retina and mediate autoimmune disease when activated. *Immunology*. 2003;110(3):386–396.
468. Podetz-Pedersen KM, Vezys V, Somia NV, Russell SJ, McIvor RS. Cellular immune response against firefly luciferase after sleeping beauty-mediated gene transfer in vivo. *Hum Gene Ther*. 2014;25(11):955–965. doi:10.1089/hum.2014.048.
469. Stripecke R, Carmen Villacres M, Skelton D, Satake N, Halene S, Kohn D. Immune response to green fluorescent protein: implications for gene therapy. *Gene Ther*. 1999;6(7):1305–1312.
470. Gossa S, Nayak D, Zinselmeyer BH, McGavern DB. Development of an immunologically tolerated combination of fluorescent proteins for in vivo two-photon imaging. *Sci Rep*. 2014;4:6664. doi:10.1038/srep06664.
471. Ledford H. How the immune system could stymie some CRISPR gene therapies. http://www.nature.com/articles/d41586-018-00335-8. Updated 2018. Accessed Feb 17, 2018.
472. Chew WL, Tabebordbar M, Cheng JKW, et al. A multifunctional AAV-CRISPR-Cas9 and its host response. *Nat Methods*. 2016;13(10):868–874. doi:10.1038/nmeth.3993.
473. Kalenova LF, Sukhovei YG, Fisher TA. Specific and nonspecific reactions of mouse immune system under the effect of short-term exposure in warm and/or cold water. *Bull Exp Biol Med*. 2005;140(6):720–722. doi:10.1007/s10517-006-0065-8. https://link.springer.com/article/10.1007/s10517-006-0065-8. Accessed Jun 17, 2018.
474. Karp CL. Unstressing intemperate models: how cold stress undermines mouse modeling. *J Exp Med*. 2012;209(6):1069. doi:10.1084/jem.20120988. https://www.ncbi.nlm.nih.gov/pubmed/22665703. Accessed Jun 17, 2018.
475. Kokolus KM, Capitano ML, Lee C, et al. Baseline tumor growth and immune control in laboratory mice are significantly influenced by subthermoneutral housing temperature. *Proc Natl Acad Sci USA*. 2013;110(50):20176. doi:10.1073/pnas.1304291110. https://www.pnas.org/content/110/50/20176. Accessed Jun 17, 2018.
476. Nguyen KD, Qiu Y, Cui X, et al. Alternatively activated macrophages produce catecholamines to sustain adaptive

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thermogenesis. *Nature*. 2011;480(7375):104. doi:10.1038/nature10653. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3371761/. Accessed Jun 17, 2018.
477. Baldwin AL. Effects of noise on rodent physiology. *East Eur J Enterp Technol*. 2014;2(12(68)):72. https://escholarship.org/uc/item/04m5m3h1. doi:10.15587/1729-4061.2014.23865.
478. Fonken LK, Lieberman RA, Weil ZM, Nelson RJ. Dim light at night exaggerates weight gain and inflammation associated with a high-fat diet in male mice. *Endocrinology*. 2013;154(10):3817–3825. doi:10.1210/en.2013-1121. https://www.ncbi.nlm.nih.gov/pubmed/23861373. Accessed Jun 17, 2018.
479. Gardner EM. Caloric restriction decreases survival of aged mice in response to primary influenza infection. *J Gerontol A Biol Sci Med Sci*. 2005;60(6):688–694. doi:10.1093/gerona/60.6.688. https://academic.oup.com/biomedgerontology/article/60/6/688/590315. Accessed Jun 17, 2018.
480. Suárez-Souto MA, Lara-Padilla E, Reyna-Garfias H, et al. Caloric restriction modifies both innate and adaptive immunity in the mouse small intestine. *J Physiol Biochem*. 2012;68(2):163–173. doi:10.1007/s13105-011-0128-9.
481. Rogers NH, Walsh H, Alvarez-Garcia O, et al. Metabolic benefit of chronic caloric restriction and activation of hypothalamic AGRP/NPY neurons in male mice is independent of ghrelin. *Endocrinology*. 2016;157(4):1430–1442. doi:10.1210/en.2015-1745.
482. Spaulding CC, Walford RL, Effros RB. Calorie restriction inhibits the age-related dysregulation of the cytokines TNF-alpha and IL-6 in C3B10RF1 mice. *Mech Ageing Dev*. 1997;93(1–3):87–94.
483. Vega VL, De Cabo R, De Maio A. Age and caloric restriction diets are confounding factors that modify the response to lipopolysaccharide by peritoneal macrophages in C57BL/6 mice. *Shock*. 2004;22(3):248–253.
484. Brown EM, Wlodarska M, Willing BP, et al. Diet and specific microbial exposure trigger features of environmental enteropathy in a novel murine model. *Nat Commun*. 2015;6:7806. doi:10.1038/ncomms8806.
485. Iyer SS, Chatraw JH, Tan WG, et al. Protein energy malnutrition impairs homeostatic proliferation of memory CD8 T cells. *J Immunol*. 2012;188(1):77–84. doi:10.4049/jimmunol.1004027.
486. Maier EA, Weage KJ, Guedes MM, et al. Protein-energy malnutrition alters IgA responses to rotavirus vaccination and infection but does not impair vaccine efficacy in mice. *Vaccine*. 2013;32(1):48–53. doi:10.1016/j.vaccine.2013.10.072.
487. Gruver AL, Sempowski GD. Cytokines, leptin, and stress-induced thymic atrophy. *J Leukoc Biol*. 2008;84(4):915–923. doi:10.1189/jlb.0108025.
488. Barouei J, Bendiks Z, Martinic A, et al. Microbiota, metabolome, and immune alterations in obese mice fed a high-fat diet containing type 2 resistant starch. *Mol Nutr Food Res*. 2017;61(11). doi:10.1002/mnfr.201700184.
489. Crevel RW, Friend JV, Goodwin BF, Parish WE. High-fat diets and the immune response of C57Bl mice. *Br J Nutr*. 1992;67(1):17–26.
490. Olefsky JM, Glass CK. Macrophages, inflammation, and insulin resistance. *Annu Rev Physiol*. 2010;72:219–246. doi:10.1146/annurev-physiol-021909-135846.
491. Cheng D, Wan Z, Zhang X, Li J, Li H, Wang C. Dietary chlorella vulgaris ameliorates altered immunomodulatory functions in cyclophosphamide-induced immunosuppressive mice. *Nutrients*. 2017;9(7). doi:10.3390/nu9070708.
492. Weise C, Heunemann C, Loddenkemper C et al. Dietary docosahexaenoic acid in combination with arachidonic acid ameliorates allergen-induced dermatitis in mice. *Pediatr Allergy Immunol*. 2011;22(5):497–504. doi:10.1111/j.1399-3038.2010.01133.x.
493. Blewett HJ, Taylor CG. Dietary zinc deficiency in rodents: effects on T-cell development, maturation and phenotypes. *Nutrients*. 2012;4(6):449–466. doi:10.3390/nu4060449.
494. Fraker PJ, King LE, Laakko T, Vollmer TL. The dynamic link between the integrity of the immune system and zinc status. *J Nutr*. 2000;130(5S Suppl):406S. doi:10.1093/jn/130.5.1399S.
495. Fraker PJ, King LE. A distinct role for apoptosis in the changes in lymphopoiesis and myelopoiesis created by deficiencies in zinc. *FASEB J*. 2001;15(14):2572–2578. doi:10.1096/fj.01-0430rev.
496. King LE, Osati-Ashtiani F, Fraker PJ. Apoptosis plays a distinct role in the loss of precursor lymphocytes during zinc deficiency in mice. *J Nutr*. 2002;132(5):974–979. doi:10.1093/jn/132.5.974.
497. Ross AC. Vitamin A and retinoic acid in T cell-related immunity. *Am J Clin Nutr*. 2012;96(5):72S. doi:10.3945/ajcn.112.034637.
498. Spencer SP, Wilhelm C, Yang Q, et al. Adaptation of innate lymphoid cells to a micronutrient deficiency promotes type 2 barrier immunity. *Science*. 2014;343(6169):432–437. doi:10.1126/science.1247606.
499. Stephensen CB, Moldoveanu Z, Gangopadhyay NN. Vitamin A deficiency diminishes the salivary immunoglobulin A response and enhances the serum immunoglobulin G response to influenza A virus infection in BALB/c mice. *J Nutr*. 1996;126(1):94–102. doi:10.1093/jn/126.1.94.
500. Mora JR, Iwata M, von Andrian UH. Vitamin effects on the immune system: vitamins A and D take centre stage. *Nat Rev Immunol*. 2008;8(9):685–698. doi:10.1038/nri2378.
501. Cooke PS, Simon L, Denslow ND. Chapter 37—endocrine disruptors. In: Haschek WM, Rousseaux CG, Wallig MA, eds. *Haschek and Rousseaux's handbook of toxicologic pathology*. 3rd ed. Boston: Academic Press; 2013:1123–1154. https://www.sciencedirect.com/science/article/pii/B9780124157590000376. Accessed Jun 18, 2018.
502. Pestka JJ. Deoxynivalenol-induced IgA production and IgA nephropathy-aberrant mucosal immune response with systemic repercussions. *Toxicol Lett*. 2003;140–141:287–295.
503. Xu J, Huang G, Guo TL. Developmental bisphenol A exposure modulates immune-related diseases. *Toxics*. 2016;4(4). doi:10.3390/toxics4040023.
504. Kuper CF, Ruehl-Fehlert C, Elmore SA, Parker GA. Chapter 49—immune system. In: Haschek WM, Rousseaux CG, Wallig MA, eds. *Haschek and Rousseaux's handbook of toxicologic pathology*. 3rd ed. Boston: Academic Press; 2013:1795–1862. https://www.sciencedirect.com/science/article/pii/B9780124157590000492. Accessed Jun 18, 2018.
505. Schecter AJ, Olson J, Papke O. Exposure of laboratory animals to polychlorinated dibenzodioxins and polychlorinated dibenzofurans from commerical rodent chow. *Chemosphere*. 1996;32(3):501–508.
506. Kozul CD, Ely KH, Enelow RI, Hamilton JW. Low-dose arsenic compromises the immune response to influenza A infection in vivo. *Environ Health Perspect*. 2009;117(9):1441–1447. doi:10.1289/ehp.0900911.
507. de Souza MS, Smith AL. Characterization of accessory cell function during acute infection of BALB/cByJ mice with mouse hepatitis virus (MHV), strain JHM. *Lab Anim Sci*. 1991;41(2):112–118.
508. Godfraind C, Holmes KV, Coutelier JP. Thymus involution induced by mouse hepatitis virus A59 in BALB/c mice. *J Virol*. 1995;69(10):6541–6547.

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509. Jolicoeur P, Lamontagne L. Impairment of bone marrow pre-B and B cells in MHV3 chronically-infected mice. *Adv Exp Med Biol*. 1995;380:193–195.
510. Brownstein DG, Weir EC. Immunostimulation in mice infected with sendai virus. *Am J Vet Res*. 1987;48(12):1692–1696.
511. Clark EA, Russell PH, Egghart M, Horton MA. Characteristics and genetic control of NK-cell-mediated cytotoxicity activated by naturally acquired infection in the mouse. *Int J Cancer*. 1979;24(5):688–699.
512. Streilein JW, Shadduck JA, Pakes SP. Effects of splenectomy and sendai virus infection on rejection of male skin isografts by pathogen-free C57BL/6 female mice. *Transplantation*. 1981;32(1):34–37.
513. Kay MM. Long term subclinical effects of parainfluenza (SENDAI) infection on immune cells of aging mice. *Proc Soc Exp Biol Med*. 1978;158(3):326–331.
514. Hsu CC, Paik J, Treuting PM, et al. Infection with murine norovirus 4 does not alter helicobacter-induced inflammatory bowel disease in Il10(-/-) mice. *Comp Med*. 2014;64(4):256–263.
515. Mumphrey SM, Changotra H, Moore TN, et al. Murine norovirus 1 infection is associated with histopathological changes in immunocompetent hosts, but clinical disease is prevented by STAT1-dependent interferon responses. *J Virol*. 2007;81(7):3251–3263. doi:10.1128/JVI.02096-06.
516. Hsu CC, Piotrowski SL, Meeker SM, Smith KD, Maggio-Price L, Treuting PM. Histologic lesions induced by murine norovirus infection in laboratory mice. *Vet Pathol*. 2016;53(4):754–763. doi:10.1177/0300985815618439.
517. Andrews DM, Andoniou CE, Granucci F, Ricciardi-Castagnoli P, Degli-Esposti MA. Infection of dendritic cells by murine cytomegalovirus induces functional paralysis. *Nat Immunol*. 2001;2(11):1077–1084. doi:10.1038/ni724.
518. O'Donoghue HL, Lawson CM, Reed WD. Autoantibodies to cardiac myosin in mouse cytomegalovirus myocarditis. *Immunology*. 1990;71(1):20–28.
519. Popkin DL, Watson MA, Karaskov E, Dunn GP, Bremner R, Virgin HW. Murine cytomegalovirus paralyzes macrophages by blocking IFN gamma-induced promoter assembly. *Proc Natl Acad Sci USA*. 2003;100(24):14309–14314. doi:10.1073/pnas.1835673100.
520. Trgovcich J, Stimac D, Polić B, et al. Immune responses and cytokine induction in the development of severe hepatitis during acute infections with murine cytomegalovirus. *Arch Virol*. 2000;145(12):2601–2618.
521. Wood BA, Dutz W, Cross SS. Neonatal infection with mouse thymic virus: spleen and lymph node necrosis. *J Gen Virol*. 1981;57(Pt 1):139–147. doi:10.1099/0022-1317-57-1-139.
522. McKisic MD, Macy JD, Delano ML, Jacoby RO, Paturzo FX, Smith AL. Mouse parvovirus infection potentiates allogeneic skin graft rejection and induces syngeneic graft rejection. *Transplantation*. 1998;65(11):1436–1446.
523. McKisic MD, Paturzo FX, Smith AL. Mouse parvovirus infection potentiates rejection of tumor allografts and modulates T cell effector functions. *Transplantation*. 1996;61(2):292–299.
524. Segovia JC, Bueren JA, Almendral JM. Myeloid depression follows infection of susceptible newborn mice with the parvovirus minute virus of mice (strain i). *J Virol*. 1995;69(5):3229–3232. http://jvi.asm.org/content/69/5/3229.abstract.
525. Gaskins HR, Prochazka M, Hamaguchi K, Serreze DV, Leiter EH. Beta cell expression of endogenous xenotropic retrovirus distinguishes diabetes-susceptible NOD/lt from resistant NON/lt mice. *J Clin Invest*. 1992;90(6):2220–2227. doi:10.1172/JCI116107.
526. Morse HC, Yetter RA, Via CS, et al. Functional and phenotypic alterations in T cell subsets during the course of MAIDS, a murine retrovirus-induced immunodeficiency syndrome. *J Immunol*. 1989;143(3):844–850.
527. Morse HC, Chattopadhyay SK, Makino M, Fredrickson TN, Hügin AW, Hartley JW. Retrovirus-induced immunodeficiency in the mouse: MAIDS as a model for AIDS. *AIDS*. 1992;6(7):607–621.
528. Mosier DE, Yetter RA, Morse HC. Retroviral induction of acute lymphoproliferative disease and profound immunosuppression in adult C57BL/6 mice. *J Exp Med*. 1985;161(4):766–784.
529. Tsumura H, Miyazawa M, Ogawa S, Wang JZ, Ito Y, Shimura K. Detection of endogenous retrovirus antigens in NOD mouse pancreatic beta-cells. *Lab Anim*. 1998;32(1):86–94. doi:10.1258/002367798780559464.
530. Li W, Hofer MJ, Jung SR, Lim S, Campbell IL. IRF7-dependent type I interferon production induces lethal immune-mediated disease in STAT1 knockout mice infected with lymphocytic choriomeningitis virus. *J Virol*. 2014;88(13):7578–7588. doi:10.1128/JVI.03117-13.
531. Oldstone MBA. Biology and pathogenesis of lymphocytic choriomeningitis virus infection. *Curr Top Microbiol Immunol*. 2002;263:83–117.
532. Zhou X, Ramachandran S, Mann M, Popkin DL. Role of lymphocytic choriomeningitis virus (LCMV) in understanding viral immunology: past, present and future. *Viruses*. 2012;4(11):2650–2669. doi:10.3390/v4112650.
533. Foreman O, Kavirayani AM, Griffey SM, Reader R, Shultz LD. Opportunistic bacterial infections in breeding colonies of the NSG mouse strain. *Vet Pathol*. 2011;48(2):495–499. doi:10.1177/0300985810378282.
534. Godfrey VL. Chapter 20—fungal diseases in laboratory mice. In: Fox JG, Davisson MT, Quimby FW, Barthold SW, Newcomer CE, Smith AL, eds. *The mouse in biomedical research*. 2nd ed. Burlington: Academic Press; 2007:507–515. https://www.sciencedirect.com/science/article/pii/B9780123694546500480. Accessed Jun 22, 2018.
535. Kobayashi-Sakamoto M, Tamai R, Isogai E, Kiyoura Y. Gastrointestinal colonisation and systemic spread of Candida albicans in mice treated with antibiotics and prednisolone. *Microb Pathog*. 2018;117:191–199. doi:10.1016/j.micpath.2018.02.043.
536. Odds FC. *Candida and candidosis: F. C. Odds*. Baltimore: University Park Press; 1978. https://trove.nla.gov.au/version/22995412. Accessed Jun 22, 2018.
537. Furuta T, Nagata T, Kikuchi T, Kikutani H. Fatal spontaneous pneumocystosis in CD40 knockout mice. *J Eukaryot Microbiol*. 2001;Suppl:129S–130S. https://www.ncbi.nlm.nih.gov/pubmed/11906023.
538. Sakala IG, Chaudhri G, Scalzo AA, et al. Evidence for persistence of ectromelia virus in inbred mice, recrudescence following immunosuppression and transmission to naïve mice. *PLoS Pathog*. 2015;11(12):e1005342. doi:10.1371/journal.ppat.1005342.
539. Ingle A, Ghim S, Joh J, Chepkoech I, Bennett Jenson A, Sundberg JP. Novel laboratory mouse papillomavirus (MusPV) infection. *Vet Pathol*. 2011;48(2):500–505. doi:10.1177/0300985810377186.
540. MacDuff DA, Reese TA, Kimmey JM, et al. Phenotypic complementation of genetic immunodeficiency by chronic herpesvirus infection. *Elife*. 2015;4. doi:10.7554/eLife.04494.
541. Labelle P, Hahn NE, Fraser JK, et al. Mousepox detected in a research facility: case report and failure of mouse antibody production testing to identify ectromelia virus in contaminated mouse serum. *Comp Med*. 2009;59(2):180–186.

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542. Fenske TS, McMahon C, Edwin D, et al. Identification of candidate alkylator-induced cancer susceptibility genes by whole genome scanning in mice. *Cancer Res*. 2006;66(10):5029–5038. doi:10.1158/0008-5472.CAN-05-3404.
543. Haggerty HG, Holsapple MP. Role of metabolism in dimethylnitrosamine-induced immunosuppression: a review. *Toxicology*. 1990;63(1):1–23.
544. Shimada Y, Nishimura M, Kakinuma S, et al. Genetic susceptibility to thymic lymphomas and K-ras gene mutation in mice after exposure to X-rays and N-ethyl-N-nitrosourea. *Int J Radiat Biol*. 2003;79(6):423–430.
545. Freund YR, Dousman L, MacGregor JT, Mohagheghpour N. Oral treatment with trimethoprim-sulfamethoxazole and zidovudine suppresses murine accessory cell-dependent immune responses. *Toxicol Sci*. 2000;55(2):335–342.
546. Blakley BR, Rousseaux CG. Effect of ivermectin on the immune response in mice. *Am J Vet Res*. 1991;52(4):593–595.
547. Yan S, Ci X, Chen N, et al. Anti-inflammatory effects of ivermectin in mouse model of allergic asthma. *Inflamm Res*. 2011;60(6):589–596. doi:10.1007/s00011-011-0307-8.
548. Berndt A, Ackert-Bicknell C, Silva KA, et al. Genetic determinants of fibro-osseous lesions in aged inbred mice. *Exp Mol Pathol*. 2016;100(1):92–100. doi:10.1016/j.yexmp.2015.11.018.
549. Brown N, Nagarkatti M, Nagarkatti PS. Diethylstilbestrol alters positive and negative selection of T cells in the thymus and modulates T-cell repertoire in the periphery. *Toxicol Appl Pharmacol*. 2006;212(2):119–126. doi:10.1016/j.taap.2005.07.012.
550. Kalland T, Forsberg JG. Natural killer cell activity and tumor susceptibility in female mice treated neonatally with diethylstilbestrol. *Cancer Res*. 1981;41(12 Pt 1):5134–5140.
551. Khan D, Ansar Ahmed S. The immune system is a natural target for estrogen action: opposing effects of estrogen in two prototypical autoimmune diseases. *Front Immunol*. 2015;6:635. doi:10.3389/fimmu.2015.00635.
552. Lindberg MK, Weihua Z, Andersson N, et al. Estrogen receptor specificity for the effects of estrogen in ovariectomized mice. *J Endocrinol*. 2002;174(2):167–178.
553. Arredouani MS. New insights into androgenic immune regulation. *Oncoimmunology*. 2014;3(9):e954968. doi:10.4161/21624011.2014.954968.
554. Kissick HT, Sanda MG, Dunn LK, et al. Androgens alter T-cell immunity by inhibiting T-helper 1 differentiation. *Proc Natl Acad Sci USA*. 2014;111(27):9887–9892. doi:10.1073/pnas.1402468111.
555. Ramaiah L, Bounous DI, Elmore SA. Chapter 50—hematopoietic system. In: Haschek WM, Rousseaux CG, Wallig MA, eds. *Haschek and Rousseaux's handbook of toxicologic pathology*. 3rd ed. Boston: Academic Press; 2013:1863–1933. https://www.sciencedirect.com/science/article/pii/B9780124157590000509. Accessed Jun 23, 2018.
556. Roden AC, Moser MT, Tri SD, et al. Augmentation of T cell levels and responses induced by androgen deprivation. *J Immunol*. 2004;173(10):6098–6108.
557. Muller YD, Golshayan D, Ehirchiou D, et al. Immunosuppressive effects of streptozotocin-induced diabetes result in absolute lymphopenia and a relative increase of T regulatory cells. *Diabetes*. 2011;60(9):2331–2340. doi:10.2337/db11-0159.
558. Chen H, Dorrigan A, Saad S, Hare DJ, Cortie MB, Valenzuela SM. In vivo study of spherical gold nanoparticles: inflammatory effects and distribution in mice. *PLoS One*. 2013;8(2):e58208. doi:10.1371/journal.pone.0058208.
559. Ilinskaya AN, Dobrovolskaia MA. Immunosuppressive and anti-inflammatory properties of engineered nanomaterials. *Br J Pharmacol*. 2014;171(17):3988–4000. doi:10.1111/bph.12722.
560. Mitchell LA, Lauer FT, Burchiel SW, McDonald JD. Mechanisms for how inhaled multiwalled carbon nanotubes suppress systemic immune function in mice. *Nat Nanotechnol*. 2009;4(7):451–456. doi:10.1038/nnano.2009.151.
561. Shen C, Liang H, Wang C, Liao M, Jan T. Iron oxide nanoparticles suppressed T helper 1 cell-mediated immunity in a murine model of delayed-type hypersensitivity. *Int J Nanomedicine*. 2012;7:2729–2737. doi:10.2147/IJN.S31054.
562. Su C, Chen T, Chang C, et al. Comparative proteomics of inhaled silver nanoparticles in healthy and allergen provoked mice. *Int J Nanomedicine*. 2013;8:2783–2799. doi:10.2147/IJN.S46997.
563. Sumbayev VV, Yasinska IM, Garcia CP, et al. Gold nanoparticles downregulate interleukin-1β-induced pro-inflammatory responses. *Small*. 2013;9(3):472–477. doi:10.1002/smll.201201528.
564. Baklaushev VP, Kilpeläinen A, Petkov S, et al. Luciferase expression allows bioluminescence imaging but imposes limitations on the orthotopic mouse (4T1) model of breast cancer. *Sci Rep*. 2017;7(1):7715. doi:10.1038/s41598-017-07851-z.
565. Yang Y, Haecker SE, Su Q, Wilson JM. Immunology of gene therapy with adenoviral vectors in mouse skeletal muscle. *Hum Mol Genet*. 1996;5(11):1703–1712.
566. Ernst W. Humanized mice in infectious diseases. *Comp Immunol Microbiol Infect Dis*. 2016;49:29–38. doi:10.1016/j.cimid.2016.08.006.
567. Skelton JK, Ortega-Prieto AM, Dorner M. A hitchhiker's guide to humanized mice: new pathways to studying viral infections. *Immunology*. 2018;154(1):50–61. doi:10.1111/imm.12906.
568. Festing MFW. Genetically defined strains in drug development and toxicity testing. *Methods Mol Biol*. 2016;1438:1–17. doi:10.1007/978-1-4939-3661-8_1.
569. Festing MFW, Nevalainen T. The design and statistical analysis of animal experiments: introduction to this issue. *ILAR J*. 2014;55(3):379–382. doi:10.1093/ilar/ilu046.
570. Brodin P, Davis MM. Human immune system variation. *Nat Rev Immunol*. 2017;17(1):21. doi:10.1038/nri.2016.125. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5328245/. Accessed Mar 3, 2018.
571. Davis MM, Tato CT, Furman D. Systems immunology: just getting started. *Nat Immunol* 2017;18(7):725. doi:10.1038/ni.3768. https://search.proquest.com/docview/1917969297.
572. Graham JB, Swarts JL, Mooney M, et al. Extensive homeostatic T cell phenotypic variation within the collaborative cross. *Cell Rep*. 2017;21(8):2313–2325. doi:10.1016/j.celrep.2017.10.093.
573. Phillippi J, Xie Y, Miller DR, et al. Using the emerging collaborative cross to probe the immune system. *Genes Immun*. 2014;15(1):38–46. doi:10.1038/gene.2013.59. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4004367/. Accessed Dec 15, 2017.
574. Durrant C, Tayem H, Yalcin B, et al. Collaborative cross mice and their power to map host susceptibility to aspergillus fumigatus infection. *Genome Res*. 2011;21(8):1239–1248. doi:10.1101/gr.118786.110.
575. Graham JB, Thomas S, Swarts J, et al. Genetic diversity in the collaborative cross model recapitulates human west nile virus disease outcomes. *mBio*. 2015;6(3):493. doi:10.1128/mBio.00493-15. http://mbio.asm.org/content/6/3/e00493-15.
576. Gralinski LE, Ferris MT, Aylor DL, et al. Genome wide identification of SARS-CoV susceptibility loci using the collaborative

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cross. *PLoS Genet*. 2015;11(10):e1005504. doi:10.1371/journal.pgen.1005504. https://www.ncbi.nlm.nih.gov/pubmed/26452100. Accessed Dec 15, 2017.
577. Green R, Wilkins C, Thomas S, et al. Transcriptional profiles of WNV neurovirulence in a genetically diverse collaborative cross population. *Genom Data*. 2016;10:137–140. doi:10.1016/j.gdata.2016.10.005. http://europepmc.org/abstract/MED/27872814, http://europepmc.org/articles/PMC5107684/?report=abstract. Accessed Dec 15, 2017.
578. Churchill GA, Gatti DM, Munger SC, Svenson KL. The diversity outbred mouse population. *Mamm Genome*. 2012;23(9–10):713–718. doi:10.1007/s00335-012-9414-2.
579. Gatti DM, Svenson KL, Shabalin A, et al. Quantitative trait locus mapping methods for diversity outbred mice. *G3 (Bethesda)*. 2014;4(9):1623–1633. doi:10.1534/g3.114.013748.
580. Logan RW, Robledo RF, Recla JM, et al. High-precision genetic mapping of behavioral traits in the diversity outbred mouse population. *Genes Brain Behav*. 2013;12(4):424–437. doi:10.1111/gbb.12029.
581. Svenson KL, Gatti DM, Valdar W, et al. High-resolution genetic mapping using the mouse diversity outbred population. *Genetics*. 2012;190(2):437–447. doi:10.1534/genetics.111.132597.
582. Chia R, Achilli F, Festing MFW, Fisher EMC. The origins and uses of mouse outbred stocks. *Nat Genet*. 2005;37(11):1181–1186. doi:10.1038/ng1665.
583. Yalcin B, Flint J. Association studies in outbred mice in a new era of full-genome sequencing. *Mamm Genome*. 2012;23(9–10):719–726. doi:10.1007/s00335-012-9409-z.
584. Rivera J, Tessarollo L. Genetic background and the dilemma of translating mouse studies to humans. *Immunity*. 2008;28(1):1–4. doi:10.1016/j.immuni.2007.12.008. http://www.sciencedirect.com/science/article/pii/S1074761307005912. Accessed Feb 26, 2018.
585. Marx JO, Gaertner DJ, Smith AL. Results of survey regarding prevalence of adventitial infections in mice and rats at biomedical research facilities. *J Am Assoc Lab Anim Sci*. 2017;56(5):527–533.
586. Mähler Convenor M, Berard M, Feinstein R, et al. FELASA recommendations for the health monitoring of mouse, rat, hamster, guinea pig and rabbit colonies in breeding and experimental units. *Lab Anim*. 2014;48(3):178–192. doi:10.1177/0023677213516312.
587. Pritchett-Corning KR, Cosentino J, Clifford CB. Contemporary prevalence of infectious agents in laboratory mice and rats. *Lab Anim*. 2009;43(2):165–173. doi:10.1258/la.2008.008009.

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Review Article

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Of Mice, Dogs, Pigs, and Men: Choosing the Appropriate Model for Immuno-Oncology Research

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Abstract

The immune system plays dual roles in response to cancer. The host immune system protects against tumor formation via immunosurveillance; however, recognition of the tumor by immune cells also induces sculpting mechanisms leading to a Darwinian selection of tumor cell variants with reduced immunogenicity. Cancer immunoediting is the concept used to describe the complex interplay between tumor cells and the immune system. This concept, commonly referred to as the three E's, is encompassed by 3 distinct phases of elimination, equilibrium, and escape. Despite impressive results in the clinic, cancer immunotherapy still has room for improvement as many patients remain unresponsive to therapy. Moreover, many of the preclinical results obtained in the widely used mouse models of cancer are lost in translation to human patients.

To improve the success rate of immuno-oncology research and preclinical testing of immune-based anticancer therapies, using alternative animal models more closely related to humans is a promising approach. Here, we describe 2 of the major alternative model systems: canine (spontaneous) and porcine (experimental) cancer models. Although dogs display a high rate of spontaneous tumor formation, an increased number of genetically modified porcine models exist. We suggest that the optimal immuno-oncology model may depend on the stage of cancer immunoediting in question. In particular, the spontaneous canine tumor models provide a unique platform for evaluating therapies aimed at the escape phase of cancer, while genetically engineered swine allow for elucidation of tumor-immune cell interactions especially during the phases of elimination and equilibrium.

Key words: cancer immunoediting; canine cancer models; comparative oncology; immunotherapy; porcine cancer models; translational immunology

Introduction

Cancer has recently surpassed cardiovascular diseases as the leading cause of death worldwide.[1] The increasing cancer incidence combined with the emergence of improved therapeutic strategies has driven research into fields such as how the immune system influences cancer development and progression. The term immunosurveillance has traditionally been used to describe how the immune system can protect the host from tumor development.[2] However, because immunocompetent individuals still develop tumors, the hypothesis of immunosurveillance being a

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fully protective mechanism is challenged.[3] It has become well-recognized that the interplay between tumor cells and the immune system is extremely complex, and the ability of tumor cells to avoid immune destruction has been included as an official hallmark of cancer.[4] Cancer immunoediting describes a complex interplay in which the immune system not only protects against cancer but also induces tumor-sculpting mechanisms leading to reduced immunogenicity of tumor cell variants.[5,6] The concept of cancer immunoediting is composed of 3 phases: elimination, equilibrium, and escape[7,8] (Table 1). The kinetics by which each of the 3 cancer immunoediting steps occurs is speculated to differ between tumors, with aggressive tumors accelerating faster through these phases.[8,9]

The elimination phase encompasses the original concept of immunosurveillance, where the innate and adaptive immune systems collaborate to destroy the developing tumor.[6,10] Although more work is needed to fully elucidate the mechanisms behind this antitumor immunity, it is known to be partly mediated by release of cytotoxic granules from $CD8^+$ T cells and Natural Killer (NK) cells in addition to cytokine release from $CD4^+$ T cells and Natural Killer T (NKT) cells[11] (Table 1). A more detailed mechanism behind the elimination phase has been proposed by Dunn et al (2002).[6] In brief, the tumor becomes invasive when reaching a size that requires a distinct blood supply controlled in part by the production of angiogenic proteins.[12] Such invasive growth results in small disruptions in the adjacent tissue, thereby inducing inflammation, which leads to intratumoral infiltration of innate immune cells like dendritic cells (DCs), NK cells, NKT cells, γδ T cells, and macrophages. Upon recognition of tumor cells, these innate immune subsets produce interferon (IFN)-γ, which can induce tumor cell death by antiproliferative and apoptotic mechanisms. Moreover, these innate immune cells produce chemokines with the capacity to limit blood vessel formation. Tumor cell debris is then taken up by DCs, which migrate to the draining lymph node and induce tumor-specific $CD4^+$ T helper cells and tumor-specific $CD8^+$ T cells. Finally, these activated T cells home to the tumor, where the $CD8^+$ T cells in particular mediate antitumor activities.[6] If the immune system succeeds in completing this phase, the host is cleared of cancer with no clinical symptoms or progression to the additional editing stages[6,10] (Table 1).

However, as well as protecting the host, antitumor immunity can also induce tumor-sculpting mechanisms resulting in tumor editing.[5,8,13,14] Consequently, tumor cell variants with increased capacity to avoid immune recognition can develop, thereby entering the equilibrium phase (Table 1). This is a dynamic equilibrium that can last for several years and is believed to be the longest of the 3 phases.[6,8,15] Several underlying molecular mechanisms at the genetic and epigenetic level have been suggested to contribute to reduced immunogenicity of cancer cells during the equilibrium phase. In particular, increased genetic instability, reduced Major Histocompatibility Complex (MHC) class I expression, and defective antigen processing have been implicated in reducing tumor immunogenicity and facilitating tumor escape.[8,10,16–23] Enhanced secretion of immunosuppressive cytokines by tumor cells, increased induction of regulatory T cells, and tumor insensitivity towards IFN-γ have also been reported as important factors[24–27] (Table 1).

After a prolonged suboptimal immune response, selected tumor cell variants with reduced immunogenicity can become insensitive to immune recognition resulting in uncontrolled tumor growth. This is referred to as the escape phase,[6–8,28] and the tumor is now capable of proliferating in a fully immunocompetent host environment (Table 1), although the degree of immune cell infiltration still affects the prognosis of the patient.[29–31] Additional work is required to fully understand the complex interplay between cancer and the immune system, highlighting the need for animal models appropriately mimicking the human situation. Different animal models can provide unique insights into the distinct immunoediting stages (elimination, equilibrium, and escape) of cancer progression and empower cancer researchers to rationally combine various modeling systems necessary to generate high-value and translationally relevant immunobiologic data from future research investigations.

Table 1 Common Immunological, Tumoral, and Clinical Characteristics of Cancer Immunoediting

Phase	Immunological Characteristics	Tumor Characteristics	Clinical Characteristics
Elimination	Active immunosurveillance. Initial infiltration of tumors with DCs, NK cells, NKT cells, γδ T cells, and macrophages. Production of IFN-γ and chemokines. Recruitment of adaptive immune cells followed by antitumor reactivity mediated by $CD8^+$ T cells, NK cells, $CD4^+$ T cells, and NKT cells.	High expression level of MHC class I, efficient antigen processing, and presentation of tumor antigens to T cells. Production of angiogenic proteins, tissue disruption, and induction of inflammation.	No clinical symptoms. Potentially full regression of the developing tumor.
Equilibrium	Dynamic equilibrium between the tumor and the immune system. Anti-tumor immunity remains present.	Expansion of tumor cell variants with reduced immunogenicity. Lowered MHC class I expression and increased genetic instability and avoidance of immune recognition. Enhanced secretion of immunosuppressive cytokines. Increased induction of Tregs and insensitivity towards IFN-γ.	The longest of the three phases, which may last for several years.
Escape	Suppression of antitumor immunity and/or lack of recognition. T cells impaired by inhibitory cytokines and checkpoint molecules, limitations in nutrient availability, metabolic competition, reduction of oxygen levels, and increase in lactate production by the tumor cells.	Defective antigen processing and reduced antigen presentation to T cells. Insensitivity to immune recognition. Immunosuppressive tumor microenvironment.	Uncontrolled tumor growth in an immunocompetent host.

References.[6–11,13–20,22,24–28]

Abbreviations: DC, dendritic cell; NK cell, natural killer cell; NKT cell, natural killer T cell; MHC, Major Histocompatibility Complex; Treg, regulatory T cell.

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Mouse Models of Immuno-Oncology

Syngeneic Mouse Models

For many years, mice have been the most commonly used animal model for immunological research and have provided a crucial elucidation of complex immunological pathways.[32–35] This in part reflects mice displaying reduced genetic variability, short generation intervals, easy maintenance, and the large number of commercially available reagents.[32,36] In cancer immunology, the most widely used mouse models involve inoculation of histocompatible (syngeneic) tumor cell lines into recipient mice, often of C57/BL6 or BALB/c background.[34,37,38] These syngeneic tumor models offer several advantages including reproducible tumor growth and simplicity in measuring tumor development over time, especially if the tumor cells are inoculated subcutaneously.[33,34,39] However, the off-site (heterotopic) injection of tumor cells in the subcutaneous tissues largely fails to recapitulate the normal microenvironment in which most tumor cells develop, and hence the operative mechanisms of immunosurveillance are likewise artificial. Additionally, the tumor cell lines tend to grow aggressively post injection, which causes studies to be terminated within a relatively short time due to ethical considerations and temporally constrains the time allowed for trafficking of immune cells and the natural development of antitumor immunity. Furthermore, the tumor cell lines differ in their intrinsic immunogenicity; therefore, the resulting tumor microenvironment often does not represent what is seen in human patients.[40,41]

Orthotopic implantation is administration of a given tumor cell line into the relevant tissue for that specific tumor. In contrast to subcutaneous injection, orthotopic implantation has been shown to better recapitulate the tumor biology, tumor environment, and disease progression.[42] In particular, the early steps of metastasis and angiogenesis have been modelled more appropriately using orthotopically implanted tumors.[42–45] Moreover, orthotopically implanted tumors have provided a valuable system for evaluation and understanding of checkpoint inhibition in various preclinical cancer models.[46–48] To date, several types of orthotopically implanted tumor models have been established amongst others, including transplantation in the brain (GL261 cells),[49] the mammary fat pad (4T1 and EMT6 cells),[50,51] intrasplenic (Panc02 cells),[52,53] and in the bladder (MBT-2 cells).[54] Overall, these models may serve as more clinically relevant systems, although the technicality of transplanting the tumor cells is more complex and labor-intensive compared to subcutaneous administration.[42]

Genetically Engineered Mouse Models

Although syngeneic mouse models are immunocompetent, they do not offer the opportunity for directly testing human targets. For this reason, syngeneic models are increasingly replaced by genetically engineered mouse (GEM) models, human xenograft, and patient-derived xenograft models.[39] An almost unlimited number of GEM models exist, with those for cancer research purposes typically produced through deletion, mutation, or overexpression of genes known to be crucial for cellular transformation and malignancy.[55] GEM models are very useful for studying the effect of specific mutations on tumor progression in an immunocompetent host.[55–58] By changing the genetic profile of these mice, it is possible to introduce mutations resulting in conditional expression/overexpression or loss/gain of function of genes known to be involved in transformation and tumorigenesis.[55,58] Moreover, tissue-/organ-specific targeting of the mutation or targeting to specific developmental stages during disease progression are valuable research tools for understanding the complex mechanisms underlying transformation and malignancy.[55,59]

Despite this, GEM models often fail in mimicking the complexity of human tumors that are often driven by stochastic genomic instability.[55] Some mouse models of cancer appear to be driven by homozygous mutations, whereas human cancers are most likely heterozygous with a functional wild-type allele. As such, the knockout of specific genes or pathways in GEM models may fail to recapitulate the chaotic manner in which malignant transformation occurs during spontaneous tumor development in human cancer patients. Although no ideal animal model can fully recapitulate the stochastic nature of human tumorigenesis, certain strategies have been developed to generate GEM models with more heterogeneous tumors of clinical relevance. Such approaches include, for instance, single-cell knockouts to achieve sporadic loss of gene expression and subsequently in vivo mosaics[59,60] as well as chemical- or UV-induced models, which can result in heterogeneous tumors arising from a multistep process.[61,62]

Xenograft Models and Humanized Mice

Xenograft models, which involve the transplantation of human cancer cell lines, or patient-derived tumor cells in the case of patient-derived xenograft models, into immunodeficient mice represent another commonly used mouse model for cancer research.[63–65] These models offer a unique tool for testing anticancer drugs targeting human proteins in mutated cancer as well as individualized and patient-specific treatments.[55] Moreover, engraftment of surgically resected tumor biopsies into these immunodeficient mice allows for an in vivo system, where interactions between, for instance, tumor cells and stromal cells can be evaluated.[65] Xenograft models undeniably add valuable knowledge to the research field; however, they are fairly expensive and labor intensive.[66,67] Also, the arising tumor is not exposed to any immune-mediated pressure due to the lack of an endogenous immune system.

To address the limitations associated with using an immunodeficient host, humanized mice have been developed. These mice are either genetically engineered to carry human genes[57] or developed through engraftment of human immune cells into an immunodeficient host.[68–71] Notably, humanized mice have provided an important tool for obtaining knowledge within the field of checkpoint inhibitors targeting, for instance, cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4), programmed cell death-1 (PD-1), and programmed death ligand-1 (PD-L1).[72] Moreover, therapies combining chimeric antigen receptor (CAR) T-cell therapies with checkpoint inhibition have been tested in humanized mice.[73,74] Despite this, humanized mice are often on the *Il2rg*$^{-/-}$ background; they lack both lymph nodes and Peyer's patches,[75–77] which are major secondary lymphoid organs necessary for mature DCs to interact and potentially activate naïve T- and B-lymphocytes. As such, humanized mice are devoid of key organized immune microenvironments critical to initiating robust immune responses. Furthermore, humanized mice are challenged in their capacity to restore MHC class I and II-selecting elements, which are crucial for shaping the T-cell repertoire.[78]

It is becoming increasingly recognized that mice often poorly mimic human diseases, even when sophisticatedly manipulated with genetic techniques.[79,80] An ideal animal model for cancer research should preferably be fully immunocompetent to properly mimic human immune responses.[39,81] Although some mouse

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models are immunocompetent, they often still display a very narrow MHC class I representation due to inbreeding. Consequently, this might result in unrepresentative results when compared to outbred animals and humans.[32] Overall, no perfect animal model capable of fully recapitulating the complexity of human disease exists. Mouse models have indeed provided the field of immuno-oncology with invaluable insight, but there remains a need for large animal models encompassing a fully competent immune system, which may function as a link between murine studies and the clinic. Given their comparable body size and metabolic physiology to human beings, as well as their well-annotated genomes, canine and porcine models of human cancer are uniquely situated to serve as excellent comparative tumor models.

Canine Models Of Immuno-Oncology

Cancer in pet dogs is common and has been reported as a leading cause of death in aging dogs, accounting for greater than 1 in 4 deaths.[82,83] As cancer in dogs occurs spontaneously and displays similar characteristics to many specific human tumor histologies, canine models are becoming more widely used in preclinical cancer research.[84–86] Representative of this research opportunity, in 2003 the National Cancer Institute's (NCI) Center for Cancer Research established the Comparative Oncology Program to facilitate and support the design, sponsorship, and execution of translational trials in pet dogs to test novel anti-cancer drugs prior to human clinical trials.[87] There are several advantages unique to canine models that were recognized and leveraged to expedite novel drug development ultimately slated for human usage. Because dogs are companion animals, they often live together with humans; therefore, they are exposed to the same environmental risk factors and might to a certain extent have a diet similar to humans.[88,89] As with humans, a correlation between spontaneous tumor incidence and age is found in dogs.[90] Additionally, from an evolutionary point of view, dogs are more closely related to humans than mice[91,92] and share more similar physiologic and immunobiologic traits. Lastly, the high degree of homology in the human and canine genome makes analysis of DNA damage as well as epigenetic changes during tumor development and progression more facilely traceable and possible in outbred dogs.[91,93,94]

Recently, several canine tumor histologies have been intensely studied using molecular cytogenetic techniques such as comparative genomic hybridization, oligonucleotide arrays, fluorescence in situ hybridization, and gene expression profiling. Based upon these genomic investigations, several conserved genetic similarities have been identified between canine and human tumors, including DNA copy number variations, structural chromosome aberrations, and differential gene expression patterns.[95–107] These findings of shared genetic perturbations associated with distinct tumor histologies in both dogs and human beings further support the potential value of pet dogs with certain types of naturally occuring tumors as a unique model system for human-relevant cancer research. Importantly, canine tumors believed to be immunogenic including osteosarcoma, lymphoma, urothelial carcinoma, mammary gland carcinoma, melanoma, and brain cancers have been the primary focus of most genomic-based investigations.[95–97,99,100,102,104–107]

The Canine Immune System

The canine immune system demonstrates a close homology to the human counterpart,[108–110] and many of the same immune markers have been validated in the canine species. Because tumors in pet dogs arise in an immunocompetent host, canine models enable the design of experiments that elucidate the complex interplay between cancer cells and the immune system as well as the natural progression of malignant transformation under the evolutionary pressures exerted by host immunosurveillance. Using human antibodies toward T-cell markers, it is now possible to distinguish canine activated T cells and central memory T cells by flow cytometry,[110] thus providing an important tool for vaccine research purposes. Adding to the strength of dogs to cancer vaccine research is their recognized breed-specific restriction in MHC expressions,[111–113] thereby allowing cancer researchers to focus efforts on "high-value" neoantigen discovery most likely to elicit potent cytotoxic T-cell responses. Despite being limited in scope to date, some studies have evaluated tumor immune cell infiltrates in canine cancer models. Flow cytometric analysis has shown the presence of both $CD4^+$ and $CD8^+$ tumor infiltrating lymphocytes within canine mammary tumors.[114] Another study using dogs with metastatic lesions showed an increased CD4/CD8 T-cell ratio, which also correlated with decreased survival rate.[114] In studies of canine B cell lymphoma, a worse prognosis was found in dogs with increased representation of tumor-associated macrophages, myeloid-derived suppressor cells, and regulatory T cells,[115–117] and cytotoxic T-cell-mediated killing of autologous lymphoma cells has been demonstrated in vitro.[116] Collectively, these preclinical and clinical findings provide strong support for including the canine species as an immune competent model system for immuno-oncology research.

Immunotherapy Research Using Canine Models

Leveraging the immune system to fight cancer can take many different, yet synergistic, strategies that engage the cellular players comprising the innate and/or adaptive immune systems. Classically, innate immune cells including neutrophils, marcrophages, and NK cells can be activated through engagement of diverse cellular receptors with cognate ligands of exogenous (pathogen associated molecular patterns) or endogenous (alarmins) nature, while cells of the adaptive immune system including B and T lymphocytes can be activated by primed antigen presenting cells. In addition, eliciting adaptive antitumor immunity can be mediated by both active and passive immunotherapeutic interventions such as vaccines and monoclonal antibodies, respectively. As immunobiologic reagents and therapeutics have become more readily available, many of these different approaches for stimulating both innate and adaptive systems, either passively or actively, have been investigated in pet dogs with cancer and a nonexhaustive list of example strategies are summarized in Table 2, with some of the most recent strategies further described below.

For immunotherapy purposes, canine tumor models offer a very powerful research tool. As monoclonal antibodies blocking CTLA-4, PD-1, and PD-L1 have provided impressive results in the clinic, it is desirable to have a preclinical animal model expressing these molecules. CTLA-4, PD-1, and PD-L1 expression have all been shown in a variety of canine solid and hematopoietic tumors.[118–123] In fact, the PD-1/PD-L1 pathway in dogs is associated with T-cell exhaustion, as often reported for humans.[119] Due to limitations in commercially available canine reagents, detailed studies with checkpoint inhibitors in dogs remain preliminary in scope and nature; however, early evidence demonstrates that blockade of PD-1/PD-L1 can lead to enhanced T-cell proliferation and cytokine release.[120,122,123] Whether these

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Table 2 Strategies for Stimulating the Innate and Adaptive Immune System in Pet Dog Cancer Models

Immune Arm	Immunotherapeutic Strategy	Specific Methodology	Tumor Type	Reference
Innate				
	Innate immune cell activation	Localized radiation and autologous NK cell intratumoral transfer	Osteosarcoma	229
	Modulation of immune signaling	Localized radiation, TLR activation, and indolamine-2,3-Dioxygenase inhibition	Melanoma, STS	230
	Macrophage activation	Liposome MTP-PE infusion	Osteosarcoma	200
Adaptive (passive)				
	Exogenous cytokine therapy	Intravenous liposome-DNA complexes with interleukin-2 gene	Osteosarcoma	231
		Inhalation therapy with liposome interleukin-2	Osteosarcoma	232
		Intralesional interleukin-2	Urothelial carcinoma	233
		Intratumoral interleukin-2	Transmissible venereal tumor	234
	Monoclonal antibody therapy	*Ex vivo* PD-L1 blockade to mitigate T cell exhaustion	Various solid tumors	119
		In vitro PD-1 blockade to induce TIL activation	STS, adenocarcinoma	122
		In vivo PD-L1 blockade in cancer-bearing dogs	Melanoma, STS	120
Adaptive (active)				
	Adoptive transfer of T cells	Autologous T cell transfer following cytokine activation	B cell lymphoma	235
		Autologous lymphokine-activated T cell transfer	Melanoma, others	236
	Genetically-modified T cells (CAR-T)	Generation of CAR-expressing T cells specific to HER2 epitope-in vitro	Osteosarcoma	237
		Generation of CAR-expressing T cells specific to CD20	B-cell lymphoma	127
	Vaccination	HER2-targeting Listeria monocytogenes vaccination	Osteosarcoma	139
		Adenovirus DNA-electro-gene-transfer targeting dog telomerase reverse transcriptase	B-cell lymphoma	238,239
		Lipoplexes with HSV-TK and canine INFβ; tumor extract vaccine + cytokines	Melanoma	240
		Xenogeneic human tyrosinase DNA vaccine	Melanoma	241

Abbreviations: CAR, chimeric antigen receptor; NK, natural killer; PD-1, Programmed cell death-1; PD-L1, Programmed death-ligand 1; STS, soft tissue sarcoma; TIL, tumor-infiltrating lymphocyte; TLR, Toll-like Receptor.

observed immunobiologic activities will be adequate to produce robust clinical benefit in a substantial fraction of treated pet dogs remains to be determined, yet early results indicate some measurable immunobiologic activity against specific solid tumors including oral melanoma and soft tissue sarcoma.[120]

Most recently, genetically engineering of CAR T cells has been heralded as an immunologic breakthrough for the management of pediatric acute lymphoblastic leukemia in human beings.[124,125] Although this genetic manipulation technology remains in its infancy for veterinary medicine, CAR T cells have shown promising results in dogs as a proof-of-concept for the management of both hematopoietic (B-cell lymphoma) and solid (osteosarcoma) tumors.[126,127] Therefore, pet dogs might in the future serve as an important model in elucidating the design of treatment regimens that maximize therapeutic benefit yet minimize adverse events often observed upon CAR T-cell therapy.[128]

The establishment of active adaptive immunotherapy through tumor vaccination strategies remains a priority in human cancer patients. Although preventative vaccines against hepatitis B virus and human papillomavirus have dramatically decreased the incidence of hepatocellular and cervical cancers, respectively,[129,130] the utility of therapeutic cancer vaccines remains limited. In 2010, the FDA approved sipuleucel-T (Provenge), a vaccine that utilizes tumor lysate-loaded dendritic cells to activate the immune system against castration-resistant prostate cancer,[131,132] and to date this remains the only approved therapeutic cancer vaccine in people. In terms of cancer vaccine trials in dogs, whole tumor cell lysate vaccines have been tested either as combination therapy or stand-alone treatment.[133–135] Most notably, in 2007, a xenogeneic DNA vaccine (Oncept) targeting the human tyrosinase protein was the first therapeutic vaccine to be approved for treatment of canine oral melanoma.[136,137] Although considered the first of its kind, the definitive immunostimulatory potential and clinically benefit derived from this xenogeneic DNA vaccine strategy would be substantially bolstered through the conductance of a large, prospective, randomized phase III clinical trial in pet dogs. In addition, canine vaccine trials targeting telomerase reverse transcriptase, heat-shock proteins, and the human vascular endothelial growth factor protein have been performed.[92,136,138] Notably, these trials all share the aim of treating cancer in dogs rather than using the canine tumor models as a link between rodent studies and human clinical trials. However, at least 2 examples exist that seek to leverage the pet dog as a comparative tumor model for the development of immunotherapeutic strategies to be employed in human cancer patients. First, a *Listeria monocytogenes* vaccine strategy has been evaluated in pet dogs with osteosarcoma, and initial results support the generation of a potent adaptive immune response translating into substantive improvements in overall survival time.[139] Second, a DC-based vaccine in combination with IFN-γ administration has been demonstrated to improve the clinical outcome in tumor-bearing dogs,

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thereby supporting the use of canine models for preclinical testing of human anti-cancer therapies.[140]

Despite the many benefits of canine cancer models, their use for therapeutic cancer vaccine development has a number of important drawbacks. The low number of known canine tumor antigens,[138] the increasing ethical regulation of experiments on companion animals,[89] and the limited number of commercially available reagents undeniably make canine translational research more difficult.[90] Although dogs are more outbred than mice, modern dog breeds are the results of line inbreeding, thus questioning whether canine models can properly mimic human heterogeneity.[36] Therefore, although canine models provide some important advantages over murine models, there is still a need for alternative large animal cancer models, and the most robust investigations will likely be derived from the utilization of a panel of animal models.

Porcine Models of Immuno-Oncology

Pigs are valuable models for studying immune responses toward infections.[141–143] Moreover, porcine models are becoming increasingly used for human biomedical research and as unique research tools for surgical procedural training.[144–146] The advancement in using porcine models is due to the high degree of homology in anatomy, physiology, size, cell biology, key metabolizing enzymes, genetics, and epigenetics between pigs and humans.[147–157] In addition, the life-span of the pig also offers an opportunity to monitor and characterize disease development and progression over a human-relevant amount of time.[36,149,158] Importantly for cancer research, porcine somatic cells, consistent with human cells, suppress telomerase activity in most tissues, which is then reactivated during tumorigenesis.[159,160]

Although mice are closer to humans phylogenetically, pigs and humans share a higher similarity in protein structure.[161] A detailed comparison of immune-related genes across several species revealed that pigs are more closely related to humans at the immunome level than mice.[141] In addition, the number of species-unique immune-related genes is considerably lower in pigs than in mice.[141] Using orthology preservation analysis of the immunome, the authors found 188 genes shared across humans, mice, and pigs. When evaluating species-unique immune-related genes, humans and pigs showed 37 and 16 genes, respectively. In contrast, 174 genes relating to various immunological pathways were found to be present only in the mouse,[141] clearly indicating crucial differences in the immune system between rodents and larger animals, including pigs and humans. Recently, the same authors compared the inflammasome across humans, pigs, and mice. Here, they clearly showed a murine expansion in the number of 7 different pattern recognition receptors compared to the 2 other species analyzed.[161] For instance, mice displayed 57 different receptors belonging to the NK cell receptor subfamily of the C-type lectin superfamily, whereas only 24 and 23 were found in the human and porcine system, respectively.[161] As NK cells are crucial players of mediating antitumor immunity and limiting tumor metastasis,[162,163] such differences need to be taken into account when interpreting immuno-oncology research. Combined, these data support the notion that preclinical results obtained in porcine models have several advantages compared to rodent models.

The Porcine Immune System

Overall, the porcine immune system comprises the same immune cell populations as demonstrated in humans.[143,164] For instance, the porcine Treg population expresses markers similar to the human population, namely CD4, CD25, and FoxP3.[165,166] However, some important differences do exist between the porcine and the human immune system. Porcine peripheral blood comprises a large number of γδ T cells, representing up to 50% of the total blood lymphocyte population in young animals.[167] In contrast, the representation of γδ T cells in human peripheral blood sampled across the world is less than 10%.[168] Although the functional properties of γδ T cells are not fully understood, it is suggested that these cells display both cytolytic activity and capacity to perform antigen presentation.[165]

Another notable difference is that the porcine T-cell pool comprises a large proportion of $CD4^+$ T cells coexpressing the CD8α homodimer in peripheral tissues.[169,170] In pigs, these $CD4^+CD8\alpha^+$ T cells are defined as an activated/memory $CD4^+$ T-cell population recognizing antigens in the context of MHC class II.[165,171] As this $CD4^+$ T-cell population expresses the $CD8\alpha^+$ homodimer, expression of the CD8β molecule is commonly used to define porcine cytotoxic T cells.[164,165] In addition, the lymphocyte migration pattern differs slightly between pigs and humans due to the porcine lymph nodes being structurally inverted.[172] Consequently, porcine lymphocytes, similar to humans, enter the lymph node via L-selectin$^+$ high endothelial venules. However, porcine T and B cells leave the lymph node by directly entering the blood stream via high endothelial venules rather than migrating out via the efferent lymph as in humans.[172,173] Despite the increased representation of $CD4^+CD8\alpha^+$ T cells in porcine peripheral blood and the inverted lymph node morphology, there are currently no indications of these differences resulting in any significant functional differences between the human and porcine immune system.[173]

The porcine MHC molecule is commonly referred to as the swine leukocyte antigen (SLA). As pigs are largely outbred compared to rodents, fully immunocompetent porcine models display a high MHC class I allelic diversity with the number of known alleles continuously expanding with improved typing methods and growing interest in swine for biomedical research.[174,175] In particular, the development of a Next Generation Sequencing-based SLA-typing approach has allowed a fast identification of expressed SLA class I molecules,[174] thereby allowing selection of MHC-matched animals to be used for instance in a vaccine protocol or other immunological assays.

Immunotherapy Research Using Porcine Models

Although pigs have provided valuable findings for infectious diseases, porcine models have had limited use thus far in experimental oncology. The 2 most common cancer types found in pigs are lymphosarcoma and melanoma.[176] Porcine skin is very similar to human skin both in terms of morphology and functional characteristics,[177] providing a unique model for studying skin cancers like melanoma. For many years, the Sinclair minipig and the melanoblastoma-bearing Libechov minipig (MeLiM) model have been the 2 most commonly used porcine spontaneous melanoma models, although the underlying genetic changes resulting in the melanoma development are not well understood.[176,178] Despite this, a study in the MeLiM model has contributed to a better understanding of melanoma progression and identified RACK1 as a potential marker of malignancy in human melanoma.[179] In recent years, porcine severe combined immunodeficiency models have also been developed.[180–185] As in the rodent equivalents, porcine porcine severe combined immunodeficiency animals lack T and B cells, allowing them to be used for xenotransplantation studies including engraftment of human tumor and immune cells.

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Genetically Engineered Porcine Models

To expand the use of pigs in experimental oncology, several genetically modified porcine models of human cancer have been developed. By overexpressing the human *GLI2* gene, it was possible to develop a model with basal cell carcinoma-like lesions.[186] In addition, colorectal cancer[187,188] and breast cancer[189,190] models have been developed, although these animals either lacked in vivo tumor development or displayed lethality issues. Modification of either the tumor suppressor gene *TP53* or the oncogene *KRAS* has enabled the development of porcine models giving rise to various cancer types. Mutational silencing of the *TP53* tumor suppressive pathway is observed in approximately 33% of human cancers.[191] Such mutations in the *TP53* gene are often associated with increased cell proliferation, survival, invasiveness, and metastasis.[192] The porcine models express the $TP53^{R167H}$ dominant negative mutation, which is equivalent to the frequently observed $TP53^{R175H}$ mutation in humans.[191,193] Upon expression of $TP53^{R167H}$, the pigs develop both lymphoma and osteogenic tumors.[194]

Furthermore, the *RAS* gene is mutated in approximately 25% of all human cancers, with *KRAS* being the most commonly mutated isoform.[191] The RAS protein is a GTPase driving cellular proliferation, and oncogenic RAS especially promotes progrowth, proangiogenic, and antiapoptotic signals.[195] Specifically for $KRAS^{G12D}$, this oncogenic activating mutation promotes metastasis in human pancreatic cancer in part by downregulating E-cadherin.[196] Although histopathology is yet to be determined, a porcine model with inducible $KRAS^{G12}$ has been developed.[194] Upon xenotransplantation, in vitro- transformed porcine mesenchymal stem cells expressing both the $TP53^{R167H}$ mutation and the $KRAS^{G12D}$ mutation have successfully established tumors in immunodeficient mice.[197] However, the only transgenic pig combining both the $TP53^{R167H}$ dominant negative mutation and the $KRAS^{G12D}$ oncogenic activating mutation is a model known as the Oncopig.[191] The expression of the 2 mutations is under control of a CAG promoter. Due to the internal ribosome entry site element, bicistronic expression of the mutated transgenes, $KRAS^{G12D}$ and $TP53^{R167H}$, is possible. Because every cell in the Oncopig has this expression construct, the model enables induction of a broad range of cancer types upon exposure to Cre recombinase.[191]

In vivo induction of sarcomas with regional leiomyosarcomas has been shown upon intramuscular, testicular, and subcutaneous injection of adenoviral vectors encoding Cre recombinase into Oncopigs.[191] Successful in vitro transformation of 11 different Oncopig cell lines has been established, as described in detail elsewhere.[36] Although limited in scope, some immunological characterization of the Oncopig intratumoral landscape has been performed. Using immunohistochemistry, infiltration of $CD3^+$ cells was shown in Oncopig hepatocellular carcinoma.[198] A more detailed and T-cell-focused evaluation of the immunological landscape in Oncopig sarcomas was recently performed, where pronounced T-cell infiltration to the tumor site was demonstrated (Overgaard et al, 2018, submitted). The tumor microenvironment was especially enriched with cytotoxic and activated immune cells. This, in conjunction with RNA-seq analysis revealing elevated gene expression of the immunosuppressive molecules *CTLA4*, *PDL1*, and *indoleamine 2,3-dioxygenase 1* in tumor tissue, supports the use of this transgenic porcine model for evaluation of the complex interplay between the tumor and the immune system of the host.

Ongoing and Future Translational Opportunities

Efforts are made to promote a One Health approach to evaluate new treatment options for cancer in canine animal models through the Comparative Oncology Trials Consortium at NCI as a major clinical trial hub across Northern America (United States and Canada). Further, a group of Academic Veterinary Teaching Hospitals in the United States/Canada recently established the Comparative Brain Tumor Consortium to improve the knowledge, development of, and access to naturally occurring canine brain cancers, specifically glioma, as a model for human disease.[199] Supporting the merits for the NCI's (Comparative Oncology Trials Consortium and Comparative Brain Tumor Consortium) translational efforts, existing evidence for the value of pet dogs with cancer in expediting anticancer drug development are multiple. Perhaps the best example for pet dogs to be included in the new drug or biological agent development path is mifamurtide, which is liposome encapsulated MTP-PE.[200] Although the data packet for mifamurtide was deemed insufficient for FDA approval, the European Medicines Agency was convinced of mifamurtide's activity and in 2004 approved its use for the treatment of high-grade, nonmetastatic, resectable osteosarcoma in human beings. In addition to mifamurtide, other investigational agents that included pet dogs with cancer in the pathway towards investigational new drug designation and human Phase I clinical trials include GS-9219, KPT-335, and PAC-1.[107,201–205]

Given the immune competency of pet dogs with cancer, and underscoring the unique and valuable potential of large animal models in cancer research, the NCI recently launched a request for proposals to support canine clinical studies evaluating the feasibility and activity of immunotherapeutic agents and novel drug combinations such as immune modulators, molecular targeted agents, chemotherapy, and/or radiation.[206] Clinical studies will be accompanied by laboratory correlative studies that seek to describe, characterize, and understand the cellular and molecular mechanisms that determine the antitumor response (or lack of response) in dogs with spontaneous tumors. Specifically, the spontaneous tumor types that have been deliberately targeted as comparative for immunotherapeutic development include lymphoma,[92,98,207,208] osteosarcoma,[95,97,209–212] mammary gland cancer,[106,107,213,214] brain cancer,[199,215–217] melanoma,[218–220] and transitional cell carcinoma[221,222] (Table 3).

Complementing spontaneous tumor models in pet dogs, the development of genetically modified pigs has allowed for several tumor types to be studied in these large experimental animal models. In particular, basal cell carcinoma,[186] colorectal cancer,[187] breast cancer,[189,190] soft-tissue sarcoma,[191,223] hepatocellular carcinoma,[198] pancreatic ductal adenocarcinoma (Principt et al., 2018, submitted), lymphoma,[193] and osteosarcoma[193,197] (Table 3) are among the tumor types that are currently in focus. However and as previously mentioned, both the colorectal cancer[187,188] and breast cancer[189,190] models currently either lack in vivo tumor development or display issues with lethality. Although there are obvious ethical problems in development of genetically modified pet animals for cancer studies, several genetically modified swine have already been developed to study cancer development as outlined above. With the emergence of precision gene editing tools, such as CRISPR/Cas9 or TALEN technologies, the potential for development of point-mutation models as well as single and multiplexed recombinants using homology-directed repair is a real and accessible option for development of new complex cancer models as well as complex comorbidity models.[149]

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Table 3 Cancer Types Mimicked Either by Spontaneous Canine Models or Genetically Engineered Porcine Models

Spontaneous Canine Tumor Models (NCI Recognized)[167]	References	Genetically Engineered Porcine Tumor Models	References
Lymphoma	72,168–170	Lymphoma	193
Osteosarcoma	171–176	Osteosarcoma	193,197
Mammary gland cancer	177–180	Breast cancer	189,190
Brain cancer	166,181–183	Soft-tissue sarcoma	191,223
Melanoma	184–186	Hepatocellular carcinoma	198
Transitional cell carcinoma	187,188	Pancreatic ductal adenocarcinoma	Principe et al., 2018, submitted
		Basal cell carcinoma	186
		Colorectal cancer	187

Abbreviations: NCI, National Cancer Institute.

Because cancer is not one disease and different tumor types require specific treatment strategies,[224] a "one size fits all" universal animal model for preclinical testing or studying the complex pathways of tumor/immune cell interactions does not seem realistic. With the concept of cancer immunoediting in mind, it could be suggested that different large animal models should be used for evaluating the different phases of cancer immunoediting. For instance, and although complete histological regression of human melanoma lesions is a rare occurrence limited to relatively few case studies,[225] melanoma remains one of the human tumor types most commonly displaying spontaneous regression.[226] Interestingly, lesions of porcine melanoma models display a high tendency of spontaneous regression, with the MeLiM model showing complete clearance in up to 96% of the cases.[227,228] From this, it could be speculated that porcine models with their apparant efficient antitumor immunity provide a unique model for studying both the elimination and equilibrium phases of cancer. In contrast, the spontaneous canine tumor models with well-established, long-term tumors provide a platform for studying and testing immunotherapeutic agents aimed at the escape phase of cancer. By those means, pigs and dogs have the potential to contribute very differently to some of the unmet clinical needs within immuno-oncology.

Despite the growing interest in large animal models for biomedical research, a major limitation to distributing the use of both canine and porcine models for immuno-oncology lies within the reduction in funding provided for veterinary immunological research. Although the large animal models presented here offer promising in vivo systems for testing human anticancer therapies, they are labor-intensive, time-consuming, and expensive compared to rodents. Moreover, large animal models encompass additional challenges relating to housing, ethical regulation, and breeding difficulties as well as a limited number of commercially available reagents. For this reason, there is a need for specific calls addressing the continued development of immune relevant large animal cancer models, which will also secure a continued expansion of both the canine and porcine immunological toolboxes in addition to training of translational onco-immunologists. In conclusion, porcine and canine cancer models may complement unmet aspects of oncology research, but these large animal models should not replace the broad selection of mouse models, which continuously provide valuable knowledge to the research field. Instead, canine and porcine models offer a crucial link between mice and men; thus, choosing the appropriate combination of animal models for immuno-oncology research might increase the success rate when translating preclinical findings to the clinic.

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References

1. WHO | Estimates for 2000–2015. *WHO* 2017. Available at: http://www.who.int/healthinfo/global_burden_disease/estimates/en/index1.html. Accessed July 11, 2017.
2. Burnet FM. The concept of immunological surveillance. *Prog Exp Tumor Res*. 1970;13:1–27.
3. de Visser KE, Eichten A, Coussens LM. Paradoxical roles of the immune system during cancer development. *Nat Rev Cancer*. 2006;6(1):24–37. doi:10.1038/nrc1782.
4. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144(5):646–674. doi:10.1016/j.cell.2011.02.013.
5. Shankaran V, Ikeda H, Bruce AT, et al. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature*. 2001;410(6832):1107–1111. doi:10.1038/35074122.
6. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol*. 2002;3(11):991–998. doi:10.1038/ni1102-991.
7. Dunn GP, Old LJ, Schreiber RD. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity*. 2004;21(2):137–148. doi:10.1016/j.immuni.2004.07.017.
8. Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. *Annu Rev Immunol*. 2004;22(1):329–360. doi:10.1146/annurev.immunol.22.012703.104803.
9. Hadrup S, Donia M, Thor Straten P. Effector CD4 and CD8 T cells and their role in the tumor microenvironment. *Cancer Microenviron*. 2013;6(2):123–133. doi:10.1007/s12307-012-0127-6.
10. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science*. 2011;331(6024):1565–1570. doi:10.1126/science.1203486.
11. van der Burg SH, Arens R, Ossendorp F, van Hall T, Melief CJM. Vaccines for established cancer: overcoming the challenges posed by immune evasion. *Nat Rev Cancer*. 2016;16(4):219–233. doi:10.1038/nrc.2016.16.

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12. Kerbel RS. Tumor angiogenesis: past, present and the near future. *Carcinogenesis*. 2000;21(3):505–515. doi:10.1093/carcin/21.3.505.
13. Dighe AS, Richards E, Old LJ, Schreiber RD. Enhanced in vivo growth and resistance to rejection of tumor cells expressing dominant negative IFN gamma receptors. *Immunity*. 1994;1(6):447–456. doi:10.1016/1074-7613(94)90087-6.
14. Smyth MJ, Dunn GP, Schreiber RD. Cancer immunosurveillance and immunoediting: the roles of immunity in suppressing tumor development and shaping tumor immunogenicity. *Adv Immunol*. 2006;90:1–50. doi:10.1016/S0065-2776(06)90001-7.
15. Loeb LA, Loeb KR, Anderson JP. Multiple mutations and cancer. *Proc Natl Acad Sci USA*. 2003;100(3):776–781. doi:10.1073/pnas.0334858100.
16. Takeda K, Nakayama M, Hayakawa Y, et al. IFN-γ is required for cytotoxic T cell-dependent cancer genome immunoediting. *Nat Commun*. 2017;8:14607. doi:10.1038/ncomms14607.
17. Garrido F, Aptsiauri N, Doorduijn EM, Garcia Lora AM, van Hall T. The urgent need to recover MHC class I in cancers for effective immunotherapy. *Curr Opin Immunol*. 2016;39:44–51. doi:10.1016/j.coi.2015.12.007.
18. Reiman JM, Kmieciak M, Manjili MH, Knutson KL. Tumor immunoediting and immunosculpting pathways to cancer progression. *Semin Cancer Biol*. 2007;17(4):275–287. doi:10.1016/j.semcancer.2007.06.009.
19. Kloor M, Becker C, Benner A, et al. Immunoselective pressure and human leukocyte antigen class I antigen machinery defects in microsatellite unstable colorectal cancers. *Cancer Res*. 2005;65(14):6418–6424. doi:10.1158/0008-5472.CAN-05-0044.
20. Rabinovich GA, Gabrilovich D, Sotomayor EM. Immunosuppressive strategies that are mediated by tumor cells. *Annu Rev Immunol*. 2007;25(1):267–296. doi:10.1146/annurev.immunol.25.022106.141609.
21. Lengauer C, Kinzler KW, Vogelstein B. Genetic instabilities in human cancers. *Nature*. 1998;396(6712):643–649. doi:10.1038/25292.
22. Garrido F, Ruiz-Cabello F, Cabrera T, et al. Implications for immunosurveillance of altered HLA class I phenotypes in human tumours. *Immunol Today*. 1997;18(2):89–95. doi:10.1016/S0167-5699(96)10075-X.
23. Algarra I, Cabrera T, Garrido F. The HLA crossroad in tumor immunology. *Hum Immunol*. 2000;61(1):65–73. doi:10.1016/S0198-8859(99)00156-1.
24. Sakaguchi S, Sakaguchi N, Shimizu J, et al. Immunologic tolerance maintained by CD25+ CD4+ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunol Rev*. 2001;182:18–32. doi:10.1034/j.1600-065×.2001.1820102.x.
25. Khong HT, Restifo NP. Natural selection of tumor variants in the generation of "tumor escape" phenotypes. *Nat Immunol*. 2002;3(11):999–1005. doi:10.1038/ni1102-999.
26. Kaplan DH, Shankaran V, Dighe AS, et al. Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. *Proc Natl Acad Sci USA*. 1998;95(13):7556–7561.
27. Zaidi MR, Merlino G. The two faces of interferon-γ in cancer. *Clin Cancer Res*. 2011;17(19):6118–6124. doi:10.1158/1078-0432.CCR-11-0482.
28. Dunn GP, Koebel CM, Schreiber RD. Interferons, immunity and cancer immunoediting. *Nat Rev Immunol*. 2006;6(11):836–848. doi:10.1038/nri1961.
29. Pagès F, Galon J, Dieu-Nosjean M-C, Tartour E, Sautès-Fridman C, Fridman W-H. Immune infiltration in human tumors: a prognostic factor that should not be ignored. *Oncogene*. 2010;29(8):1093–1102. doi:10.1038/onc.2009.416.
30. Pagès F, Kirilovsky A, Mlecnik B, et al. In situ cytotoxic and memory T cells predict outcome in patients with early-stage colorectal cancer. *J Clin Oncol*. 2009;27(35):5944–5951. doi:10.1200/JCO.2008.19.6147.
31. Fridman WH, Pagès F, Sautès-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer*. 2012;12(4):298–306. doi:10.1038/nrc3245.
32. Griffin J. A strategic approach to vaccine development: animal models, monitoring vaccine efficacy, formulation and delivery. *Adv Drug Deliv Rev*. 2002;54(6):851–861. doi:10.1016/S0169-409×(02)00072-8.
33. Becher OJ, Holland EC. Genetically engineered models have advantages over xenografts for preclinical studies. *Cancer Res*. 2006;66(7):3355–3359. doi:10.1158/0008-5472.CAN-05-3827.
34. Zitvogel L, Pitt JM, Daillère R, Smyth MJ, Kroemer G. Mouse models in oncoimmunology. *Nat Rev Cancer*. 2016;16(12):759–773. doi:10.1038/nrc.2016.91.
35. Gillet J-P, Calcagno AM, Varma S, et al. Redefining the relevance of established cancer cell lines to the study of mechanisms of clinical anti-cancer drug resistance. *Proc Natl Acad Sci USA*. 2011;108(46):18708–18713. doi:10.1073/pnas.1111840108.
36. Schachtschneider KM, Schwind RM, Newson J, et al. The oncopig cancer model: an innovative large animal translational oncology platform. *Front Oncol*. 2017;7:190. doi:10.3389/FONC.2017.00190.
37. Suggitt M, Bibby MC. 50 years of preclinical anticancer drug screening: empirical to target-driven approaches. *Clin Cancer Res*. 2005;11(3):971–981.
38. Ostrand-Rosenberg S. Animal models of tumor immunity, immunotherapy and cancer vaccines. *Curr Opin Immunol*. 2004;16(2):143–150. doi:10.1016/j.coi.2004.01.003.
39. Sanmamed MF, Chester C, Melero I, Kohrt H. Defining the optimal murine models to investigate immune checkpoint blockers and their combination with other immunotherapies. *Ann Oncol*. 2016;27(7):1190–1198. doi:10.1093/annonc/mdw041.
40. Borrell B. How accurate are cancer cell lines? *Nature*. 2010;463(7283):858–858. doi:10.1038/463858a.
41. Dranoff G. Experimental mouse tumour models: what can be learnt about human cancer immunology? *Nat Rev Immunol*. 2011;12(1):61–66. doi:10.1038/nri3129.
42. Sharpless NE, DePinho RA. The mighty mouse: genetically engineered mouse models in cancer drug development. *Nat Rev Drug Discov*. 2006;5(9):741–754. doi:10.1038/nrd2110.
43. Yang J, Mani SA, Donaher JL, et al. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell*. 2004;117(7):927–939. doi:10.1016/j.cell.2004.06.006.
44. Hoffman RM. Orthotopic metastatic mouse models for anticancer drug discovery and evaluation: a bridge to the clinic. *Invest New Drugs*. 1999;17(4):343–359.
45. Bibby MC. Orthotopic models of cancer for preclinical drug evaluation. *Eur J Cancer*. 2004;40(6):852–857. doi:10.1016/j.ejca.2003.11.021.
46. Zhao X, Li L, Starr TK, Subramanian S. Tumor location impacts immune response in mouse models of colon cancer. *Oncotarget*. 2017;8(33):54775–54787. doi:10.18632/oncotarget.18423.

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47. Friedman D, Baird JR, Young KH, et al. Programmed cell death-1 blockade enhances response to stereotactic radiation in an orthotopic murine model of hepatocellular carcinoma. *Hepatol Res*. 2017;47(7):702–714. doi:10.1111/hepr.12789.
48. Zeng J, See AP, Phallen J, et al. Anti-PD-1 blockade and stereotactic radiation produce long-term survival in mice with intracranial gliomas. *Int J Radiat Oncol*.2013;86(2):343–349. doi:10.1016/j.ijrobp.2012.12.025.
49. Oh T, Fakurnejad S, Sayegh ET, et al. Immunocompetent murine models for the study of glioblastoma immunotherapy. *J Transl Med*. 2014;12(1):107. doi:10.1186/1479-5876-12-107.
50. Connolly EM, Harmey JH, O'Grady T, et al. Cyclo-oxygenase inhibition reduces tumour growth and metastasis in an orthotopic model of breast cancer. *Br J Cancer*. 2002;87(2): 231–237. doi:10.1038/sj.bjc.6600462.
51. Oh K, Lee O-Y, Shon SY, et al. A mutual activation loop between breast cancer cells and myeloid-derived suppressor cells facilitates spontaneous metastasis through IL-6 trans-signaling in a murine model. *Breast Cancer Res*. 2013; 15(5):R79. doi:10.1186/bcr3473.
52. Little EC, Wang C, Watson PM, Watson DK, Cole DJ, Camp ER. Novel immunocompetent murine models representing advanced local and metastatic pancreatic cancer. *J Surg Res*. 2012;176(2):359–366. doi:10.1016/j.jss.2011.10.025.
53. Soares KC, Foley K, Olino K, et al. A preclinical murine model of hepatic metastases. *J Vis Exp*. 2014;(91):51677. doi:10.3791/51677.
54. Tamura K, Kikuchi E, Konno T, et al. Therapeutic effect of intravesical administration of paclitaxel solubilized with poly(2-methacryloyloxyethyl phosphorylcholine-co-n-butyl methacrylate) in an orthotopic bladder cancer model. *BMC Cancer*. 2015;15(1):317. doi:10.1186/s12885-015-1338-2.
55. Richmond A, Su Y. Mouse xenograft models vs GEM models for human cancer therapeutics. *Dis Model Mech*. 2008;1(2–3): 78–82. doi:10.1242/dmm.000976.
56. Merlino G, Flaherty K, Acquavella N, et al. Meeting report: the future of preclinical mouse models in melanoma treatment is now. *Pigment Cell Melanoma Res*. 2013;26(4):E8–E14. doi:10.1111/pcmr.12099.
57. Frese KK, Tuveson DA. Maximizing mouse cancer models. *Nat Rev Cancer*. 2007;7(9):654–658. doi:10.1038/nrc2192.
58. Gopinathan A, Tuveson DA. The use of GEM models for experimental cancer therapeutics. *Dis Model Mech*. 2008;1 (2–3):83–86. doi:10.1242/dmm.000570.
59. Walrath JC, Hawes JJ, Van Dyke T, Reilly KM. Genetically engineered mouse models in cancer research. *Adv Cancer Res*. 2010;106:113–164. doi:10.1016/S0065-230×(10)06004-5.
60. Lozano G, Behringer RR. New mouse models of cancer: single-cell knockouts. *Proc Natl Acad Sci USA*. 2007;104(11): 4245–4246. doi:10.1073/pnas.0700173104.
61. Wu X, Pandolfi PP. Mouse models for multistep tumorigenesis. *Trends Cell Biol*. 2001;11(11):S2–S9. doi:10.1016/S0962-8924(01)02127-4.
62. Day C-P, Merlino G, Van Dyke T. Preclinical mouse cancer models: a maze of opportunities and challenges. *Cell*. 2015; 163(1):39–53. doi:10.1016/j.cell.2015.08.068.
63. Morton CL, Houghton PJ. Establishment of human tumor xenografts in immunodeficient mice. *Nat Protoc*. 2007;2(2): 247–250. doi:10.1038/nprot.2007.25.
64. Freeman A, Bridge JA, Maruthayanar P, et al. Comparative immune phenotypic analysis of cutaneous squamous cell carcinoma and intraepidermal carcinoma in immunecompetent individuals: proportional representation of CD8+ T-cells but not FoxP3+ regulatory T-cells is associated with disease stage. *PLoS One*. 2014;9(10):e110928. doi:10.1371/journal.pone.0110928.
65. Tentler JJ, Tan AC, Weekes CD, et al. Patient-derived tumour xenografts as models for oncology drug development. *Nat Rev Clin Oncol*. 2012;9(6):338–350. doi:10.1038/nrclinonc.2012.61.
66. Jung J. Human tumor xenograft models for preclinical assessment of anticancer drug development. *Toxicol Res*. 2014;30(1):1–5. doi:10.5487/TR.2014.30.1.001.
67. Kelland LR. Of mice and men: values and liabilities of the athymic nude mouse model in anticancer drug development. *Eur J Cancer*. 2004;40(6):827–836. doi:10.1016/j.ejca.2003.11.028.
68. Shultz LD, Ishikawa F, Greiner DL. Humanized mice in translational biomedical research. *Nat Rev Immunol*. 2007;7 (2):118–130. doi:10.1038/nri2017.
69. Traggiai E, Chicha L, Mazzucchelli L, et al. Development of a human adaptive immune system in cord blood cell-transplanted mice. *Science*. 2004;304(5667):104–107. doi:10.1126/science.1093933.
70. Ishikawa F, Yasukawa M, Lyons B, et al. Development of functional human blood and immune systems in NOD/SCID/IL2 receptor chainnull mice. *Blood*. 2005;106(5): 1565–1573. doi:10.1182/blood-2005-02-0516.
71. Shultz LD, Lyons BL, Burzenski LM, et al. Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. *J Immunol*. 2005;174(10):6477–6489. doi:10.4049/jimmunol.174.10.6477.
72. Carrillo MA, Zhen A, Kitchen SG. The use of the humanized mouse model in gene therapy and immunotherapy for HIV and cancer. *Front Immunol*. 2018;9:746. doi:10.3389/fimmu.2018.00746.
73. Suarez ER, Chang D-K, Sun J, et al. Chimeric antigen receptor T cells secreting anti-PD-L1 antibodies more effectively regress renal cell carcinoma in a humanized mouse model. *Oncotarget*. 2016;7(23):34341–34355. doi:10.18632/oncotarget.9114.
74. Gay F, D'Agostino M, Giaccone L, et al. Immuno-oncologic approaches: CAR-T cells and checkpoint inhibitors. *Clin Lymphoma Myeloma Leuk*. 2017;17(8):471–478. doi:10.1016/j.clml.2017.06.014.
75. Cao X, Shores EW, Hu-Li J, et al. Defective lymphoid development in mice lacking expression of the common cytokine receptor gamma chain. *Immunity*. 1995;2(3):223–238.
76. DiSanto JP, Müller W, Guy-Grand D, Fischer A, Rajewsky K. Lymphoid development in mice with a targeted deletion of the interleukin 2 receptor gamma chain. *Proc Natl Acad Sci USA*. 1995;92(2):377–381.
77. Ohbo K, Suda T, Hashiyama M, et al. Modulation of hematopoiesis in mice with a truncated mutant of the interleukin-2 receptor gamma chain. *Blood*. 1996;87(3):956–967.
78. Bernard D, Peakman M, Hayday AC. Establishing humanized mice using stem cells: maximizing the potential. *Clin Exp Immunol*. 2008;152(3):406–414. doi:10.1111/j.1365-2249.2008.03659.x.
79. Seok J, Warren HS, Cuenca AG, et al. Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proc Natl Acad Sci USA*. 2013;110(9):3507–3512. doi:10.1073/pnas.1222878110.
80. Mak IWY, Evaniew N, Ghert M. Lost in translation: animal models and clinical trials in cancer treatment. *Am J Transl Res*. 2014;6(2):114–118. doi:AJTR1312010.

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81. Wei W-Z, Jones RF, Juhasz C, Gibson H, Veenstra J. Evolution of animal models in cancer vaccine development. *Vaccine*. 2015;33(51):1–7. doi:10.1016/j.vaccine.2015.07.075.
82. Fleming JM, Creevy KE, Promislow DEL. Mortality in North American dogs from 1984 to 2004: an investigation into age-, size-, and breed-related causes of death. *J Vet Intern Med*. 2011;25(2):187–198. doi:10.1111/j.1939-1676.2011.0695.x.
83. Adams VJ, Evans KM, Sampson J, Wood JLN. Methods and mortality results of a health survey of purebred dogs in the UK. *J Small Anim Pract*. 2010;51(10):512–524. doi:10.1111/j.1748-5827.2010.00974.x.
84. Rowell JL, McCarthy DO, Alvarez CE. Dog models of naturally occurring cancer. *Trends Mol Med*. 2011;17(7):380–388. doi:10.1016/j.molmed.2011.02.004.
85. Pinho SS, Carvalho S, Cabral J, Reis CA, Gärtner F. Canine tumors: a spontaneous animal model of human carcinogenesis. *Transl Res*. 2012;159(3):165–172. doi:10.1016/j.trsl.2011.11.005.
86. Gardner HL, Fenger JM, London CA. Dogs as a model for cancer. *Annu Rev Anim Biosci*. 2016;4(1):199–222. doi:10.1146/annurev-animal-022114-110911.
87. Gordon I, Paoloni M, Mazcko C, Khanna C. The Comparative Oncology Trials Consortium: using spontaneously occurring cancers in dogs to inform the cancer drug development pathway. *PLoS Med*. 2009;6(10):e1000161. doi:10.1371/journal.pmed.1000161.
88. Król M, Motyl T. Exploiting cancer genomics in pet animals to gain advantage for personalized medicine decisions. *J Appl Genet*. 2014;55(3):337–341. doi:10.1007/s13353-014-0206-0.
89. Vail DM, MacEwen EG. Spontaneously occurring tumors of companion animals as models for human cancer. *Cancer Invest*. 2000;18(8):781–792.
90. Park JS, Withers SS, Modiano JF, et al. Canine cancer immunotherapy studies: linking mouse and human. *J Immunother cancer*. 2016;4(1):97. doi:10.1186/s40425-016-0200-7.
91. Lindblad-Toh K, Wade CM, Mikkelsen TS, et al. Genome sequence, comparative analysis and haplotype structure of the domestic dog. *Nature*. 2005;438(7069):803–819. doi:10.1038/nature04338.
92. Richards KL, Suter SE. Man's best friend: what can pet dogs teach us about non-Hodgkin's lymphoma? *Immunol Rev*. 2015;263(1):173–191. doi:10.1111/imr.12238.
93. Kirkness EF, Bafna V, Halpern AL, et al. The dog genome: survey sequencing and comparative analysis. *Science*. 2003;301(5641):1898–1903. doi:10.1126/science.1086432.
94. Cadieu E, Ostrander EA. Canine genetics offers new mechanisms for the study of human cancer. *Cancer Epidemiol Biomarkers Prev*. 2007;16(11):2181–2183. doi:10.1158/1055-9965.EPI-07-2667.
95. Angstadt AY, Thayanithy V, Subramanian S, Modiano JF, Breen M. A genome-wide approach to comparative oncology: high-resolution oligonucleotide aCGH of canine and human osteosarcoma pinpoints shared microaberrations. *Cancer Genet*. 2012;205(11):572–587. doi:10.1016/J.CANCERGEN.2012.09.005.
96. Angstadt AY, Motsinger-Reif A, Thomas R, et al. Characterization of canine osteosarcoma by array comparative genomic hybridization and RT-qPCR: signatures of genomic imbalance in canine osteosarcoma parallel the human counterpart. *Genes Chromosomes Cancer*. 2011;50(11):859–874. doi:10.1002/gcc.20908.
97. Paoloni M, Davis S, Lana S, et al. Canine tumor cross-species genomics uncovers targets linked to osteosarcoma progression. *BMC Genomics*. 2009;10(1):625. doi:10.1186/1471-2164-10-625.
98. Breen M, Modiano JF. Evolutionarily conserved cytogenetic changes in hematological malignancies of dogs and humans-man and his best friend share more than companionship. *Chromosom Res*. 2008;16(1):145–154. doi:10.1007/s10577-007-1212-4.
99. Richards KL, Motsinger-Reif AA, Chen H-W, et al. Gene profiling of canine B-cell lymphoma reveals germinal center and postgerminal center subtypes with different survival times, modeling human DLBCL. *Cancer Res*. 2013;73(16):5029–5039. doi:10.1158/0008-5472.CAN-12-3546.
100. Thomas R, Seiser EL, Motsinger-Reif A, et al. Refining tumor-associated aneuploidy through "genomic recoding" of recurrent DNA copy number aberrations in 150 canine non-Hodgkin lymphomas. *Leuk Lymphoma*. 2011;52(7):1321–1335. doi:10.3109/10428194.2011.559802.
101. Roode SC, Rotroff D, Avery AC, et al. Genome-wide assessment of recurrent genomic imbalances in canine leukemia identifies evolutionarily conserved regions for subtype differentiation. *Chromosome Res*. 2015;23(4):681–708. doi:10.1007/s10577-015-9475-7.
102. Shapiro SG, Raghunath S, Williams C, et al. Canine urothelial carcinoma: genomically aberrant and comparatively relevant. *Chromosome Res*. 2015;23(2):311–331. doi:10.1007/s10577-015-9471-y.
103. Hedan B, Thomas R, Motsinger-Reif A, et al. Molecular cytogenetic characterization of canine histiocytic sarcoma: a spontaneous model for human histiocytic cancer identifies deletion of tumor suppressor genes and highlights influence of genetic background on tumor behavior. *BMC Cancer*. 2011;11(1):201. doi:10.1186/1471-2407-11-201.
104. Thomas R, Duke SE, Wang HJ, et al. "Putting our heads together": insights into genomic conservation between human and canine intracranial tumors. *J Neurooncol*. 2009;94(3):333–349. doi:10.1007/s11060-009-9877-5.
105. Poorman K, Borst L, Moroff S, et al. Comparative cytogenetic characterization of primary canine melanocytic lesions using array CGH and fluorescence in situ hybridization. *Chromosome Res*. 2015;23(2):171–186. doi:10.1007/s10577-014-9444-6.
106. Uva P, Aurisicchio L, Watters J, et al. Comparative expression pathway analysis of human and canine mammary tumors. *BMC Genomics*. 2009;10(1):135. doi:10.1186/1471-2164-10-135.
107. Klopfleisch R, Lenze D, Hummel M, Gruber AD. Metastatic canine mammary carcinomas can be identified by a gene expression profile that partly overlaps with human breast cancer profiles. *BMC Cancer*. 2010;10(1):618. doi:10.1186/1471-2407-10-618.
108. Cobbold S, Metcalfe S. Monoclonal antibodies that define canine homologues of human CD antigens: summary of the First International Canine Leukocyte Antigen Workshop (CLAW). *Tissue Antigens*. 1994;43(3):137–154. doi:10.1111/j.1399-0039.1994.tb02315.x.
109. Isotani M, Katsuma K, Tamura K, et al. Efficient generation of canine bone marrow-derived dendritic cells. *J Vet Med Sci*. 2006;68(8):809–814. doi:10.1292/jvms.68.809.
110. Hartley AN, Tarleton RL. Chemokine receptor 7 (CCR7)-expression and IFNγ production define vaccine-specific canine T-cell subsets. *Vet Immunol Immunopathol*. 2015;164(3–4):127–136. doi:10.1016/j.vetimm.2015.02.001.
111. Ross P, Buntzman AS, Vincent BG, et al. Allelic diversity at the DLA-88 locus in Golden Retriever and Boxer breeds is limited. *Tissue Antigens*. 2012;80(2):175–183. doi:10.1111/j.1399-0039.2012.01889.x.

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112. Angles JM, Kennedy LJ, Pedersen NC. Frequency and distribution of alleles of canine MHC-II DLA-DQB1, DLA-DQA1 and DLA-DRB1 in 25 representative American Kennel Club breeds. *Tissue Antigens*. 2005;66(3):173–184. doi:10.1111/j.1399-0039.2005.00461.x.
113. Ross P, Holmes JC, Gojanovich GS, Hess PR. A cell-based MHC stabilization assay for the detection of peptide binding to the canine classical class I molecule, DLA-88. *Vet Immunol Immunopathol*. 2012;150(3–4):206–212. doi:10.1016/j.vetimm.2012.08.012.
114. Estrela-Lima A, Araújo MS, Costa-Neto JM, et al. Immunophenotypic features of tumor infiltrating lymphocytes from mammary carcinomas in female dogs associated with prognostic factors and survival rates. *BMC Cancer*. 2010;10(1):256. doi:10.1186/1471-2407-10-256.
115. Raposo T, Gregório H, Pires I, Prada J, Queiroga FL. Prognostic value of tumour-associated macrophages in canine mammary tumours. *Vet Comp Oncol*. 2014;12(1):10–19. doi:10.1111/j.1476-5829.2012.00326.x.
116. Mitchell L, Dow SW, Slansky JE, Biller BJ. Induction of remission results in spontaneous enhancement of antitumor cytotoxic T-lymphocyte activity in dogs with B cell lymphoma. *Vet Immunol Immunopathol*. 2012;145(3–4):597–603. doi:10.1016/j.vetimm.2012.01.006.
117. Mucha J, Majchrzak K, Taciak B, Hellmén E, Król M. MDSCs mediate angiogenesis and predispose canine mammary tumor cells for metastasis via IL-28/IL-28RA (IFN-λ) signaling. *PLoS One*. 2014;9(7):e103249. doi:10.1371/journal.pone.0103249.
118. Tagawa M, Maekawa N, Konnai S, Takagi S. Evaluation of costimulatory molecules in peripheral blood lymphocytes of canine patients with histiocytic sarcoma. *PLoS One*. 2016;11(2):e0150030. doi:10.1371/journal.pone.0150030.
119. Maekawa N, Konnai S, Ikebuchi R, et al. Expression of PD-L1 on canine tumor cells and enhancement of IFN-γ production from tumor-infiltrating cells by PD-L1 blockade. *PLoS One*. 2014;9(6):e98415. doi:10.1371/journal.pone.0098415.
120. Maekawa N, Konnai S, Takagi S, et al. A canine chimeric monoclonal antibody targeting PD-L1 and its clinical efficacy in canine oral malignant melanoma or undifferentiated sarcoma. *Sci Rep*. 2017;7(1):8951. doi:10.1038/s41598-017-09444-2.
121. Maekawa N, Konnai S, Okagawa T, et al. Immunohistochemical analysis of PD-L1 expression in canine malignant cancers and PD-1 expression on lymphocytes in canine oral melanoma. *PLoS One*. 2016;11(6):e0157176. doi:10.1371/journal.pone.0157176.
122. Coy J, Caldwell A, Chow L, Guth A, Dow S. PD-1 expression by canine T cells and functional effects of PD-1 blockade. *Vet Comp Oncol*. 2017;15(4):1487–1502. doi:10.1111/vco.12294.
123. Hartley G, Faulhaber E, Caldwell A, et al. Immune regulation of canine tumour and macrophage PD-L1 expression. *Vet Comp Oncol*. 2017;15(2):534–549. doi:10.1111/vco.12197.
124. Kalos M, Levine BL, Porter DL, et al. T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. *Sci Transl Med*. 2011;3(95):95ra73. doi:10.1126/scitranslmed.3002842.
125. Porter DL, Levine BL, Kalos M, Bagg A, June CH. Chimeric antigen receptor–modified T cells in chronic lymphoid leukemia. *N Engl J Med*. 2011;365(8):725–733. doi:10.1056/NEJMoa1103849.
126. Mata M, Vera JF, Gerken C, et al. Toward immunotherapy with redirected T cells in a large animal model. *J Immunother*. 2014;37(8):407–415. doi:10.1097/CJI.0000000000000052.
127. Panjwani MK, Smith JB, Schutsky K, et al. Feasibility and safety of RNA-transfected CD20-specific chimeric antigen receptor T cells in dogs with spontaneous B cell lymphoma. *Mol Ther*. 2016;24(9):1602–1614. doi:10.1038/mt.2016.146.
128. Morgan RA, Yang JC, Kitano M, Dudley ME, Laurencot CM, Rosenberg SA. Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. *Mol Ther*. 2010;18(4):843–851. doi:10.1038/mt.2010.24.
129. Frazer IH. Prevention of cervical cancer through papillomavirus vaccination. *Nat Rev Immunol*. 2004;4(1):46–55. doi:10.1038/nri1260.
130. Qu C, Chen T, Fan C, et al. Efficacy of neonatal HBV vaccination on liver cancer and other liver diseases over 30-year follow-up of the Qidong Hepatitis B Intervention Study: a cluster randomized controlled trial. *PLoS Med*. 2014;11(12):e1001774. doi:10.1371/journal.pmed.1001774.
131. Kantoff PW, Higano CS, Shore ND, et al. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *N Engl J Med*. 2010;363(5):411–422. doi:10.1056/NEJMoa1001294.
132. Higano CS, Schellhammer PF, Small EJ, et al. Integrated data from 2 randomized, double-blind, placebo-controlled, phase 3 trials of active cellular immunotherapy with sipuleucel-T in advanced prostate cancer. *Cancer*. 2009;115(16):3670–3679. doi:10.1002/cncr.24429.
133. U'Ren LW, Biller BJ, Elmslie RE, Thamm DH, Dow SW. Evaluation of a novel tumor vaccine in dogs with hemangiosarcoma. *J Vet Intern Med*. 2007;21(1):113–120. doi:10.1111/j.1939-1676.2007.tb02936.x.
134. Andersen BM, Pluhar GE, Seiler CE, et al. Vaccination for invasive canine meningioma induces in situ production of antibodies capable of antibody-dependent cell-mediated cytotoxicity. *Cancer Res*. 2013;73(10):2987–2997. doi:10.1158/0008-5472.CAN-12-3366.
135. Pluhar GE, Grogan PT, Seiler C, et al. Anti-tumor immune response correlates with neurological symptoms in a dog with spontaneous astrocytoma treated by gene and vaccine therapy. *Vaccine*. 2010;28(19):3371–3378. doi:10.1016/j.vaccine.2010.02.082.
136. Anderson K, Modiano J. Progress in adaptive immunotherapy for cancer in companion animals: success on the path to a cure. *Vet Sci*. 2015;2(4):363–387. doi:10.3390/vetsci2040363.
137. Atherton MJ, Morris JS, McDermott MR, Lichty BD. Cancer immunology and canine malignant melanoma: a comparative review. *Vet Immunol Immunopathol*. 2016;169:15–26. doi:10.1016/j.vetimm.2015.11.003.
138. Regan D, Guth A, Coy J, Dow S. Cancer immunotherapy in veterinary medicine: current options and new developments. *Vet J*. 2016;207:20–28. doi:10.1016/j.tvjl.2015.10.008.
139. Mason NJ, Gnanandarajah JS, Engiles JB, et al. Immunotherapy with a HER2-targeting listeria induces HER2-specific immunity and demonstrates potential therapeutic effects in a phase I trial in canine osteosarcoma. *Clin Cancer Res*. 2016;22(17):4380–4390. doi:10.1158/1078-0432.CCR-16-0088.
140. Mito K, Sugiura K, Ueda K, et al. IFN markedly cooperates with intratumoral dendritic cell vaccine in dog tumor models. *Cancer Res*. 2010;70(18):7093–7101. doi:10.1158/0008-5472.CAN-10-0600.
141. Dawson HD, Loveland JE, Pascal G, et al. Structural and functional annotation of the porcine immunome. *BMC Genomics*. 2013;14:332. doi:10.1186/1471-2164-14-332.
142. Groenen MAM, Archibald AL, Uenishi H, et al. Analyses of pig genomes provide insight into porcine demography and

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evolution. *Nature*. 2012;491(7424):393–398. doi:10.1038/nature11622.
143. Meurens F, Summerfield A, Nauwynck H, Saif L, Gerdts V. The pig: a model for human infectious diseases. *Trends Microbiol*. 2012;20(1):50–57. doi:10.1016/j.tim.2011.11.002.
144. Swindle MM, Makin A, Herron AJ, Clubb FJ, Frazier KS. Swine as models in biomedical research and toxicology testing. *Vet Pathol*. 2012;49(2):344–356. doi:10.1177/0300985811402846.
145. Helke KL, Nelson KN, Sargeant AM, et al. Pigs in toxicology. *Toxicol Pathol*. 2016;44(4):575–590. doi:10.1177/0192623316639389.
146. Helke KL, Swindle MM. Animal models of toxicology testing: the role of pigs. *Expert Opin Drug Metab Toxicol*. 2013;9(2):127–139. doi:10.1517/17425255.2013.739607.
147. Ho C-S, Lunney JK, Ando A, et al. Nomenclature for factors of the SLA system, update 2008. *Tissue Antigens*. 2009;73(4):307–315. doi:10.1111/j.1399-0039.2009.01213.x.
148. Flisikowska T, Kind A, Schnieke A. The new pig on the block: modelling cancer in pigs. *Transgenic Res*. 2013;22(4):673–680. doi:10.1007/s11248-013-9720-9.
149. Watson AL, Carlson DF, Largaespada DA, Hackett PB, Fahrenkrug SC. Engineered swine models of cancer. *Front Genet*. 2016;7:78. doi:10.3389/fgene.2016.00078.
150. Schachtschneider KM, Madsen O, Park C, Rund LA, Groenen MAM, Schook LB. Adult porcine genome-wide DNA methylation patterns support pigs as a biomedical model. *BMC Genomics*. 2015;16(1):743. doi:10.1186/s12864-015-1938-x.
151. Choi M, Lee J, Le MT, et al. Genome-wide analysis of DNA methylation in pigs using reduced representation bisulfite sequencing. *DNA Res*. 2015;22(5):343–355. doi:10.1093/dnares/dsv017.
152. Schook LB, Beever JE, Rogers J, et al. Swine Genome Sequencing Consortium (SGSC): a strategic roadmap for sequencing the pig genome. *Comp Funct Genomics*. 2005;6(4):251–255. doi:10.1002/cfg.479.
153. Paoloni MC, Khanna C. Comparative oncology today. *Vet Clin North Am Small Anim Pract*. 2007;37(6):1023–1032. doi:10.1016/j.cvsm.2007.08.003.
154. Breen M. Update on genomics in veterinary oncology. *Top Companion Anim Med*. 2009;24(3):113–121. doi:10.1053/j.tcam.2009.03.002.
155. Schook L, Beattie C, Beever J, et al. Swine in biomedical research: creating the building blocks of animal models. *Anim Biotechnol*. 2005;16(2):183–190. doi:10.1080/10495390500265034.
156. Gray MA, Pollock CB, Schook LB, Squires EJ. Characterization of porcine pregnane X receptor, farnesoid X receptor and their splice variants. *Exp Biol Med*. 2010;235(6):718–736. doi:10.1258/ebm.2010.009339.
157. Pollock CB, Rogatcheva MB, Schook LB. Comparative genomics of xenobiotic metabolism: a porcine-human PXR gene comparison. *Mamm Genome*. 2007;18(3):210–219. doi:10.1007/s00335-007-9007-7.
158. Gün G, Kues WA. Current progress of genetically engineered pig models for biomedical research. *Biores Open Access*. 2014;3(6):255–264. doi:10.1089/biores.2014.0039.
159. Pathak S, Multani AS, McConkey DJ, Imam AS, Amoss MS. Spontaneous regression of cutaneous melanoma in sinclair swine is associated with defective telomerase activity and extensive telomere erosion. *Int J Oncol*. 2000;17(6):1219–1224. doi:10.3892/ijo.17.6.1219.
160. Stewart SA, Weinberg RA. Telomerase and human tumorigenesis. *Semin Cancer Biol*. 2000;10(6):399–406. doi:10.1006/scbi.2000.0339.
161. Dawson HD, Smith AD, Chen C, Urban JF. An in-depth comparison of the porcine, murine and human inflammasomes; lessons from the porcine genome and transcriptome. *Vet Microbiol*. 2017;202:2–15. doi:10.1016/j.vetmic.2016.05.013.
162. Guillerey C, Huntington ND, Smyth MJ. Targeting natural killer cells in cancer immunotherapy. *Nat Immunol*. 2016;17(9):1025–1036. doi:10.1038/ni.3518.
163. Glasner A, Ghadially H, Gur C, et al. Recognition and prevention of tumor metastasis by the NK receptor NKp46/NCR1. *J Immunol*. 2012;188(6):2509–2515. doi:10.4049/jimmunol.1102461.
164. Piriou-guzylack L, Salmon H. Review article membrane markers of the immune cells in swine: an update. *Vet Res*. 2008;39(54). doi:10.1051/vetres:2008030.
165. Charerntantanakul W, Roth JA. Biology of porcine T lymphocytes. *Anim Health Res Rev*. 2006;7(1–2):81–96. doi:10.1017/S1466252307001235.
166. Käser T, Gerner W, Hammer SE, Patzl M, Saalmüller A. Phenotypic and functional characterisation of porcine CD4+CD25high regulatory T cells. *Vet Immunol Immunopathol*. 2008;122(1–2):153–158. doi:10.1016/j.vetimm.2007.08.002.
167. Gerner W, Käser T, Saalmüller A. Porcine T lymphocytes and NK cells-an update. *Dev Comp Immunol*. 2009;33(3):310–320. doi:10.1016/j.dci.2008.06.003.
168. Esin S, Shigematsu M, Nagai S, Eklund A, Wigzell H, Grunewald J. Different percentages of peripheral blood gamma delta + T cells in healthy individuals from different areas of the world. *Scand J Immunol*. 1996;43(5):593–596.
169. Saalmüller A, Reddehase MJ, Bühring HJ, Jonjić S, Koszinowski UH. Simultaneous expression of CD4 and CD8 antigens by a substantial proportion of resting porcine T lymphocytes. *Eur J Immunol*. 1987;17(9):1297–1301. doi:10.1002/eji.1830170912.
170. Overgaard NH, Jung J-W, Steptoe RJ, Wells JW. CD4+/CD8+ double-positive T cells: more than just a developmental stage? *J Leukoc Biol*. 2015;97(1):31–38. doi:10.1189/jlb.1RU0814-382.
171. Zuckermann FA, Husmann RJ. Functional and phenotypic analysis of porcine peripheral blood CD4/CD8 double-positive T cells. *Immunology*. 1996;87(3):500–512.
172. Binns RM, Pabst R. Lymphoid tissue structure and lymphocyte trafficking in the pig. *Vet Immunol Immunopathol*. 1994;43(1–3):79–87. doi:10.1016/0165-2427(94)90123-6.
173. Bode G, Clausing P, Gervais F, et al. The utility of the minipig as an animal model in regulatory toxicology. *J Pharmacol Toxicol Methods*. 2010;62(3):196–220. doi:10.1016/j.vascn.2010.05.009.
174. Sørensen MR, Ilsøe M, Strube ML, et al. Sequence-based genotyping of expressed swine leukocyte antigen class I alleles by next-generation sequencing reveal novel swine leukocyte antigen class I haplotypes and alleles in Belgian, Danish, and Kenyan fattening pigs and Göttingen minipigs. *Front Immunol*. 2017;8:701. doi:10.3389/fimmu.2017.00701.
175. Gao C, Quan J, Jiang X, Li C, Lu X, Chen H. Swine leukocyte antigen diversity in Canadian specific pathogen-free Yorkshire and Landrace Pigs. *Front Immunol*. 2017;8:282. doi:10.3389/fimmu.2017.00282.
176. Flisikowska T, Kind A, Schnieke A. Pigs as models of human cancers. *Theriogenology*. 2016;86(1):433–437. doi:10.1016/j.theriogenology.2016.04.058.
177. Boisgard R, Vincent-Naulleau S, Leplat J-J, et al. A new animal model for the imaging of melanoma: correlation of FDG PET with clinical outcome, macroscopic aspect and

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histological classification in Melanoblastoma-bearing Libechov Minipigs. *Eur J Nucl Med Mol Imaging*. 2003;30(6):826–834. doi:10.1007/s00259-003-1152-y.
178. Grossi AB, Hyttel P, Jensen HE, Leifsson PS. Porcine melanotic cutaneous lesions and lymph nodes. *Vet Pathol*. 2015;52(1):83–91. doi:10.1177/0300985814521637.
179. Egidy G, Julé S, Bossé P, et al. Transcription analysis in the MeLiM swine model identifies RACK1 as a potential marker of malignancy for human melanocytic proliferation. *Mol Cancer*. 2008;7:34. doi:10.1186/1476-4598-7-34.
180. Suzuki S, Iwamoto M, Saito Y, et al. Il2rg gene-targeted severe combined immunodeficiency pigs. *Cell Stem Cell*. 2012;10(6):753–758. doi:10.1016/j.stem.2012.04.021.
181. Watanabe M, Nakano K, Matsunari H, et al. Generation of interleukin-2 receptor gamma gene knockout pigs from somatic cells genetically modified by zinc finger nuclease-encoding mRNA. *PLoS One*. 2013;8(10):e76478. doi:10.1371/journal.pone.0076478.
182. Huang J, Guo X, Fan N, et al. RAG1/2 knockout pigs with severe combined immunodeficiency. *J Immunol*. 2014;193(3):1496–1503. doi:10.4049/jimmunol.1400915.
183. Ito T, Sendai Y, Yamazaki S, et al. Generation of recombination activating gene-1-deficient neonatal piglets: a model of T and B cell deficient severe combined immune deficiency. *PLoS One*. 2014;9(12):e113833. doi:10.1371/journal.pone.0113833.
184. Powell EJ, Graham J, Ellinwood NM, et al. T cell lymphoma and leukemia in severe combined immunodeficiency pigs following bone marrow transplantation: a case report. *Front Immunol*. 2017;8:813. doi:10.3389/fimmu.2017.00813.
185. Lee K, Kwon D-N, Ezashi T, et al. Engraftment of human iPS cells and allogeneic porcine cells into pigs with inactivated RAG2 and accompanying severe combined immunodeficiency. *Proc Natl Acad Sci USA*. 2014;111(20):7260–7265. doi:10.1073/pnas.1406376111.
186. McCalla-Martin AC, Chen X, Linder KE, Estrada JL, Piedrahita JA. Varying phenotypes in swine versus murine transgenic models constitutively expressing the same human Sonic hedgehog transcriptional activator, K5-HGLI2ΔN. *Transgenic Res*. 2010;19(5):869–887. doi:10.1007/s11248-010-9362-0.
187. Flisikowska T, Merkl C, Landmann M, et al. A porcine model of familial adenomatous polyposis. *Gastroenterology*. 2012;143(5):1173–1175.e7. doi:10.1053/j.gastro.2012.07.110.
188. Tan W, Carlson DF, Lancto CA, et al. Efficient nonmeiotic allele introgression in livestock using custom endonucleases. *Proc Natl Acad Sci USA*. 2013;110(41):16526–16531. doi:10.1073/pnas.1310478110.
189. Yamakawa H, Nagai T, Harasawa R, et al. Production of transgenic pig carrying MMTV/v-Ha-ras. *J Reprod Dev*. 1999;45(2):111–118. doi:10.1262/jrd.45.111.
190. Luo Y, Li J, Liu Y, et al. High efficiency of BRCA1 knockout using rAAV-mediated gene targeting: developing a pig model for breast cancer. *Transgenic Res*. 2011;20(5):975–988. doi:10.1007/s11248-010-9472-8.
191. Schook LB, Collares TV, Hu W, et al. A genetic porcine model of cancer. *PLoS One*. 2015;10(7):e0128864. doi:10.1371/journal.pone.0128864.
192. Muller PAJ, Vousden KH. p53 mutations in cancer. *Nat Cell Biol*. 2013;15(1):2–8. doi:10.1038/ncb2641.
193. Sieren JC, Meyerholz DK, Wang X-J, et al. Development and translational imaging of a TP53 porcine tumorigenesis model. *J Clin Invest*. 2014;124(9):4052–4066. doi:10.1172/JCI75447.
194. Li S, Edlinger M, Saalfrank A, et al. Viable pigs with a conditionally-activated oncogenic KRAS mutation. *Transgenic Res*. 2015;24(3):509–517. doi:10.1007/s11248-015-9866-8.
195. Pylayeva-Gupta Y, Grabocka E, Bar-Sagi D. RAS oncogenes: weaving a tumorigenic web. *Nat Rev Cancer*. 2011;11(11):761–774. doi:10.1038/nrc3106.
196. Rachagani S, Senapati S, Chakraborty S, et al. Activated $KrasG^{12}D$ is associated with invasion and metastasis of pancreatic cancer cells through inhibition of E-cadherin. *Br J Cancer*. 2011;104(6):1038–1048. doi:10.1038/bjc.2011.31.
197. Saalfrank A, Janssen K-P, Ravon M, et al. A porcine model of osteosarcoma. *Oncogenesis*. 2016;5(3):e210. doi:10.1038/oncsis.2016.19.
198. Schachtschneider KM, Schwind RM, Darfour-Oduro KA, et al. A validated, transitional and translational porcine model of hepatocellular carcinoma. *Oncotarget*. 2017;8(38):63620–63634. doi:10.18632/oncotarget.18872.
199. LeBlanc AK, Mazcko C, Brown DE, et al. Creation of an NCI comparative brain tumor consortium: informing the translation of new knowledge from canine to human brain tumor patients. *Neuro Oncol*. 2016;18(9):1209–1218. doi:10.1093/neuonc/now051.
200. MacEwen EG, Kurzman ID, Rosenthal RC, et al. Therapy for osteosarcoma in dogs with intravenous injection of liposome-encapsulated muramyl tripeptide. *J Natl Cancer Inst*. 1989;81(12):935–938. doi:10.1093/jnci/81.12.935.
201. Vail DM, Thamm DH, Reiser H, et al. Assessment of GS-9219 in a pet dog model of non-Hodgkin's lymphoma. *Clin Cancer Res*. 2009;15(10):3503–3510. doi:10.1158/1078-0432.CCR-08-3113.
202. London CA, Bernabe LF, Barnard S, et al. Preclinical evaluation of the novel, orally bioavailable Selective Inhibitor of Nuclear Export (SINE) KPT-335 in spontaneous canine cancer: results of a phase I study. *PLoS One*. 2014;9(2):e87585. doi:10.1371/journal.pone.0087585.
203. Joshi AD, Botham RC, Schlein LJ, et al. Synergistic and targeted therapy with a procaspase-3 activator and temozolomide extends survival in glioma rodent models and is feasible for the treatment of canine malignant glioma patients. *Oncotarget*. 2017;8(46):80124–80138. doi:10.18632/oncotarget.19085.
204. Botham RC, Roth HS, Book AP, Roady PJ, Fan TM, Hergenrother PJ. Small-molecule procaspase-3 activation sensitizes cancer to treatment with diverse chemotherapeutics. *ACS Cent Sci*. 2016;2(8):545–559. doi:10.1021/acscentsci.6b00165.
205. Peterson QP, Hsu DC, Novotny CJ, et al. Discovery and canine preclinical assessment of a nontoxic procaspase-3-activating compound. *Cancer Res*. 2010;70(18):7232–7241. doi:10.1158/0008-5472.CAN-10-0766.
206. Institute NC. RFA-CA-17-001: canine immunotherapy trials and correlative studies (U01). Available at: https://grants.nih.gov/grants/guide/rfa-files/RFA-CA-17-001.html. Accessed February 12, 2018.
207. Ito D, Frantz AM, Modiano JF. Canine lymphoma as a comparative model for human non-Hodgkin lymphoma: recent progress and applications. *Vet Immunol Immunopathol*. 2014;159(3–4):192–201. doi:10.1016/j.vetimm.2014.02.016.
208. Seelig D, Avery A, Ehrhart E, Linden M. The comparative diagnostic features of canine and human lymphoma. *Vet Sci*. 2016;3(4):11. doi:10.3390/vetsci3020011.
209. Fan T, Khanna C. Comparative aspects of osteosarcoma pathogenesis in humans and dogs. *Vet Sci*. 2015;2(4):210–230. doi:10.3390/vetsci2030210.

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210. Simpson S, Dunning MD, de Brot S, Grau-Roma L, Mongan NP, Rutland CS. Comparative review of human and canine osteosarcoma: morphology, epidemiology, prognosis, treatment and genetics. *Acta Vet Scand*. 2017;59(1):71. doi:10.1186/s13028-017-0341-9.
211. Fenger JM, London CA, Kisseberth WC. Canine osteosarcoma: a naturally occurring disease to inform pediatric oncology. *ILAR J*. 2014;55(1):69–85. doi:10.1093/ilar/ilu009.
212. Morello E, Martano M, Buracco P. Biology, diagnosis and treatment of canine appendicular osteosarcoma: similarities and differences with human osteosarcoma. *Vet J*. 2011; 189(3):268–277. doi:10.1016/J.TVJL.2010.08.014.
213. Lutful Kabir FM, Alvarez CE, Bird RC. Canine mammary carcinomas: a comparative analysis of altered gene expression. *Vet Sci*. 2016;3(1):1. doi:10.3390/vetsci3010001.
214. Klopfleisch R, Klose P, Weise C, et al. Proteome of metastatic canine mammary carcinomas: similarities to and differences from human breast cancer †. *J Proteome Res*. 2010;9(12):6380–6391. doi:10.1021/pr100671c.
215. Herranz C, Fernández F, Martín-Ibáñez R, et al. Spontaneously arising canine glioma as a potential model for human glioma. *J Comp Pathol*. 2016;154(2–3):169–179. doi:10.1016/J.JCPA.2015.12.001.
216. Hicks J, Platt S, Kent M, Haley A. Canine brain tumours: a model for the human disease? *Vet Comp Oncol*. 2017;15(1): 252–272. doi:10.1111/vco.12152.
217. Bentley RT, Ahmed AU, Yanke AB, Cohen-Gadol AA, Dey M. Dogs are man's best friend: in sickness and in health. *Neuro Oncol*. 2016;19(3):312–322. doi:10.1093/neuonc/now109.
218. Nishiya A, Massoco C, Felizzola C, et al. Comparative aspects of canine melanoma. *Vet Sci*. 2016;3(4):7. doi:10.3390/vetsci3010007.
219. Gillard M, Cadieu E, De Brito C, et al. Naturally occurring melanomas in dogs as models for non-UV pathways of human melanomas. *Pigment Cell Melanoma Res*. 2014;27(1): 90–102. doi:10.1111/pcmr.12170.
220. Simpson RM, Bastian BC, Michael HT, et al. Sporadic naturally occurring melanoma in dogs as a preclinical model for human melanoma. *Pigment Cell Melanoma Res*. 2014;27(1): 37–47. doi:10.1111/pcmr.12185.
221. Dhawan D, Paoloni M, Shukradas S, et al. Comparative gene expression analyses identify luminal and basal subtypes of canine invasive urothelial carcinoma that mimic patterns in human invasive bladder cancer. *PLoS One*. 2015; 10(9):e0136688. doi:10.1371/journal.pone.0136688.
222. Knapp DW, Ramos-Vara JA, Moore GE, Dhawan D, Bonney PL, Young KE. Urinary bladder cancer in dogs, a naturally occurring model for cancer biology and drug development. *ILAR J*. 2014;55(1):100–118. doi:10.1093/ilar/ilu018.
223. Schachtschneider KM, Liu Y, Mäkeläinen S, et al. Oncopig soft-tissue sarcomas recapitulate key transcriptional features of human sarcomas. *Sci Rep*. 2017;7(1):2624. doi:10.1038/s41598-017-02912-9.
224. Teng MWL, Galon J, Fridman W-H, Smyth MJ. From mice to humans: developments in cancer immunoediting. *J Clin Invest*. 2015;125(9):3338–3346. doi:10.1172/JCI80004.
225. High WA, Stewart D, Wilbers CRH, Cockerell CJ, Hoang MP, Fitzpatrick JE. Completely regressed primary cutaneous malignant melanoma with nodal and/or visceral metastases: a report of 5 cases and assessment of the literature and diagnostic criteria. *J Am Acad Dermatol*. 2005;53(1): 89–100. doi:10.1016/j.jaad.2005.03.006.
226. Papac RJ. Spontaneous regression of cancer: possible mechanisms. *In Vivo*. 1996;12(6):571–578. doi:10.1.1.326.4921.
227. Flisikowski K, Flisikowska T, Sikorska A, et al. Germline gene polymorphisms predisposing domestic mammals to carcinogenesis. *Vet Comp Oncol*. 2017;15(2):289–298. doi:10.1111/vco.12186.
228. Vincent-Naulleau S, Le Chalony C, Leplat J-J, et al. Clinical and histopathological characterization of cutaneous melanomas in the melanoblastoma-bearing Libechov minipig model. *Pigment Cell Res*. 2004;17(1):24–35. doi:10.1046/j.1600-0749.2003.00101.x.
229. Canter RJ, Grossenbacher SK, Foltz JA, et al. Radiotherapy enhances natural killer cell cytotoxicity and localization in pre-clinical canine sarcomas and first-in-dog clinical trial. *J Immunother cancer*. 2017;5(1):98. doi:10.1186/s40425-017-0305-7.
230. Monjazeb AM, Kent MS, Grossenbacher SK, et al. Blocking indolamine-2,3-dioxygenase rebound immune suppression boosts antitumor effects of radio-immunotherapy in murine models and spontaneous canine malignancies. *Clin Cancer Res*. 2016;22(17):4328–4340. doi:10.1158/1078-0432.CCR-15-3026.
231. Dow S, Elmslie R, Kurzman I, MacEwen G, Pericle F, Liggitt D. Phase I study of liposome-DNA complexes encoding the interleukin-2 gene in dogs with osteosarcoma lung metastases. *Hum Gene Ther*. 2005;16(8):937–946. doi:10.1089/hum.2005.16.937.
232. Khanna C, Anderson PM, Hasz DE, Katsanis E, Neville M, Klausner JS. Interleukin-2 liposome inhalation therapy is safe and effective for dogs with spontaneous pulmonary metastases. *Cancer*. 1997;79(7):1409–1421.
233. Konietschke U, Teske E, Jurina K, Stockhaus C. Palliative intralesional interleukin-2 treatment in dogs with urinary bladder and urethral carcinomas. *In Vivo*. 2012; 26(6): 931–935.
234. Den Otter W, Hack M, Jacobs JJL, Tan JFV, Rozendaal L, Van Moorselaar RJA. Treatment of transmissible venereal tumors in dogs with intratumoral interleukin-2 (IL-2). A pilot study. *Anticancer Res*. 2015;35(2):713–717.
235. O'Connor CM, Sheppard S, Hartline CA, et al. Adoptive T-cell therapy improves treatment of canine non-Hodgkin lymphoma post chemotherapy. *Sci Rep*. 2012;2(1):249. doi:10.1038/srep00249.
236. Mie K, Shimada T, Akiyoshi H, Hayashi A, Ohashi F. Change in peripheral blood lymphocyte count in dogs following adoptive immunotherapy using lymphokine-activated T killer cells combined with palliative tumor resection. *Vet Immunol Immunopathol*. 2016;177:58–63. doi:10.1016/j.vetimm.2016.06.007.
237. Mata M, Vera JF, Gerken C, et al. Toward immunotherapy with redirected T cells in a large animal model: ex vivo activation, expansion, and genetic modification of canine T cells. *J Immunother*. 2014;37(8):407–415. doi:10.1097/CJI.0000000000000052.
238. Gavazza A, Lubas G, Fridman A, et al. Safety and efficacy of a genetic vaccine targeting telomerase plus chemotherapy for the therapy of canine B-cell lymphoma. *Hum Gene Ther*. 2013;24(8):728–738. doi:10.1089/hum.2013.112.
239. Peruzzi D, Gavazza A, Mesiti G, et al. A vaccine targeting telomerase enhances survival of dogs affected by B-cell lymphoma. *Mol Ther*. 2010;18(8):1559–1567. doi:10.1038/mt.2010.104.

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240. Finocchiaro LME, Fondello C, Gil-Cardeza ML, et al. Cytokine-enhanced vaccine and interferon-β plus suicide gene therapy as surgery adjuvant treatments for spontaneous canine melanoma. *Hum Gene Ther*. 2015;26(6): 367–376. doi:10.1089/hum.2014.130.

241. Bergman PJ, McKnight J, Novosad A, et al. Long-term survival of dogs with advanced malignant melanoma after DNA vaccination with xenogeneic human tyrosinase: a phase I trial. *Clin Cancer Res*. 2003;9(4): 1284–1290.

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Review Article

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Animal Models for Preclinical Development of Allogeneic Hematopoietic Cell Transplantation

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Abstract

Since its inception in the 1950s, hematopoietic cell transplantation (HCT) has become a highly effective clinical treatment for malignant and nonmalignant hematological disorders. This milestone in cancer therapy was only possible through decades of intensive research using murine and canine animal models that overcame what appeared in the early days to be insurmountable obstacles. Conditioning protocols for tumor ablation and immunosuppression of the recipient using irradiation and chemotherapeutic drugs were developed in mouse and dog models as well as postgrafting immunosuppression methods essential for dependable donor cell engraftment. The random-bred canine was particularly important in defining the role of histocompatibility barriers and the development of the nonmyeloablative transplantation procedure, making HCT available to elderly patients with comorbidities. Two complications limit the success of HCT: disease relapse and graft versus host disease. Studies in both mice and dogs have made significant progress toward reducing and to some degree eliminating patient morbidity and mortality associated with both disease relapse and graft versus host disease. However, more investigation is needed to make HCT more effective, safer, and available as a treatment modality for other non-life-threatening diseases such as autoimmune disorders. Here, we focus our review on the contributions made by both the murine and canine models for the successful past and future development of HCT.

Key words: canine; cell; hematopoietic; model; murine; preclinical; transplantation

Introduction

Hematopoietic cell transplantation (HCT) is a widely used therapy for malignant and nonmalignant hematological disorders.[1,2] Hematopoietic stem cells correct nonmalignant hematopoietic disorders such as immunodeficiency diseases and anemias as well as allow clinicians to use more aggressive marrow-toxic irradiation protocols for the treatment of hematological malignancies. More than 70 years of studies in animal models have been essential for achieving success in human patients and will be necessary to refine the procedure to minimize toxicity and improve outcomes.

Researchers concluded from early experiments that protection from lethal irradiation was due to humoral factors rather than engraftment of donor cells.[3–6] In 1956, three independent groups (Rijswijk Radiobiology Lab, the Netherlands; Harwell

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Radiochemistry Labs, UK; and Oakridge National Labs, USA) provided clear evidence that a cellular mechanism was responsible for the rescue of mice from the lethal effects of irradiation of irradiation.[7] The cellular hypothesis gained indisputable acceptance following a series of critical studies showing that recipient mice given stem allogeneic marrow were protected from lethal irradiation and were tolerant to donor skin grafts.[8,9] These studies clearly indicated that living cells and not humoral factors are responsible for recovery following lethal irradiation. These early experiments laid the groundwork for therapeutic HCT.

Further experiments using mice with established leukemia showed that mice survived longer after irradiation when given an injection of homologous (allogenic) versus isologous bone marrow.[10,11] This was the first demonstration of an immune-mediated control of hematopoietic malignancy known as the graft-versus-leukemia (GVL) or graft-versus-tumor (GVT) effect. However, animals receiving allogenic HCT (allo-HCT) eventually succumbed to a wasting disease, presumed at the time to be malnourishment from radiation-damaged intestinal tissue.[11,12]

Translation of initial HCT studies in mice to human patients was disappointingly unsuccessful. Although an initial human study of six cases demonstrated the safety of marrow transplantation, only transient engraftment was observed and only in one patient.[13] HCT in patients with refractory leukemia, in which the donor was an identical twin, resulted in successful donor hematopoietic cell engraftment; however, the patients ultimately relapsed, indicating a lack of GVL response.[14] These studies were a clear indication that matching of donor and recipients was necessary for engraftment, yet some genetic difference was required for donor targeting of tumor cells. The first persistent allograft was described in 1965 in a patient with leukemia; however, the patient eventually died from secondary syndrome, which was most likely chronic graft-versus-host disease (GVHD).[15]

Clinical trials for patients with hematological malignancies were failing due to premature application of results from murine studies to human clinical trials. In 1970, this opinion was further substantiated in a report covering 200 patients treated with HCT for hematological diseases in which all recipients had died of either graft failure, GVHD, infections, or disease relapse.[16] More knowledge surrounding what was required for successful engraftment and tumor targeting was necessary.

In subsequent years, researchers directed their attention toward identifying alternative animal models that would better predict results in the clinic. Research using large animal models, primarily dogs, supplemented research using inbred mice. The focus was on conditioning regimens, matching of donor and recipient pairs, and GVHD prophylaxis. Studies in these large animal preclinical models were successfully translated to the clinic as effective treatment protocols for malignant and nonmalignant hematopoietic diseases.

Future studies are especially required to address two major problems: disease relapse and nonrelapse mortality. Once these issues are firmly under control, allo-HCT may result in better survival of patients with hematological disorders and be of medical benefit for less life-threatening diseases such as autoimmune disorders and solid organ/tissue transplantation.[17] The purpose of the current report is to describe the role of animal models, primarily the mouse and dog, in the successful development of allo-HCT protocols and their potential in future research efforts towards unresolved issues.

Current Clinical Need

Traditional myeloablative pretransplant conditioning involves high-dose total body irradiation (TBI) and/or chemotherapy to reduce the patient's tumor burden before HCT and suppress the host immune system to prevent rejection of the donor graft. These myeloablative protocols are generally only acceptable in younger patients or older patients with limited comorbidities. This is particularly confounding, since hematological malignancies often occur in older patients; the median age for acute myelogenous leukemia and non-Hodgkin lymphoma range from 65 to 75 years. Studies in the dog model established a regimen of reduced-intensity, non-myeloablative conditioning and postgrafting immunosuppression that reduces allo-transplant-related toxicities and maintains donor cell engraftment.[18]

A recent review of 1092 patients with advanced hematologic malignancies given non-myeloablative conditioning with fludarabine and 2 cGy TBI followed by a human leukocyte antigen (HLA)-matched related or unrelated HCT showed an overall 5-year relapse mortality rate of 18% to 60%, depending on risk of relapse. Most relapse occurs within the first 2 years and depends on the disease and disease burden.[19,20] A combined analysis of other reduced intensity conditioning (RIC) and non-myeloablative regimens yielded similar results, with a 43% average rate of relapse (range, 22–65%). A phase III trial by the Blood and Marrow Transplant Clinical Trials Network comparing myeloablative and RIC regimens showed that progression-free survival was significantly lower in the RIC arm of the study (47.3% vs. 67.8% in myeloablative arm).[21] These data suggest that reduced intensity regimens may not be sufficient in all patients for achieving disease remission. Increasing the intensity of the conditioning regimen to reduce the incidence of relapse would be too toxic for patients with high comorbidities and unnecessary for a majority of the patients. Therefore, less toxic means for reducing relapse need to be identified.

In the Blood and Marrow Transplant Clinical Trials Network study described above, the primary cause of death in the myeloablative arm was GVHD (52%). In the analysis described by Storb et al.,[19,20] 43% of related recipients and 59% of unrelated recipients developed grade II-IV acute GVHD, and the cumulative incidence of both acute and chronic GVHD was 75%. Overall, the 5-year nonrelapse mortality was 24%, and the majority (20.2%) were associated with GVHD. Specifically, 16% of patients died from complications related to either acute GVHD alone or from acute GVHD progressing to chronic GVHD, and an additional 4.2% died with de novo chronic GVHD. In this analysis, acute GVHD conveyed no additional beneficial GVL/GVT effect but significantly increased the hazard ratios for both nonrelapse mortality and the incidence of chronic GVHD, which increased from 25% to close to 50%.[20]

A number of reports showed significantly higher rates of chronic GVHD with peripheral blood HCT compared to marrow transplant, most likely due to higher numbers of lymphocytes contained in the peripheral blood graft.[22–29] Conversely, when the number of transplanted lymphocytes was reduced by in vitro or in vivo T-cell depletion (anti-thymocyte gamma globulin, alemtuzumab, post-HCT cyclophosphamide),[30,31] the rates of chronic GVHD declined significantly. However, the benefit from decreasing the incidence of chronic GVHD is offset by significantly increased relapse rates in patients with hematological malignancies.

Reduced-intensity regimens have the benefit of lower toxicity and are appropriate for a greater number of patients. However, the risk of relapse is significant, and therefore,

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reduced-intensity regimens need to be improved to effectively treat the malignancy and prevent relapse without increasing the risk of acute or chronic GVHD. However, myeloablative approaches remain effective in a number of situations, thereby necessitating new approaches to prevent and treat GVHD.

Animal Models

Once HCT was successful in the first human patients, a long history ensued in which studies in preclinical animal models played a key role in reducing toxicity and improving patient outcomes. Studies in mice and canines focused on MHC typing[32–37] and pretransplant conditioning regimens such as TBI,[38–45] chemical immunosuppression,[46–48] and radioimmunological ablation.[49,50] Other critical studies evaluated hematopoietic stem cell dosage,[51–54] post-transplant immunosuppression,[18,55–61] prevention of graft failure and disease relapse,[62–65] and nonrelapse mortality, primarily acute and chronic GVHD.[66,67]

Mice are unquestionably the most cost-effective research animal model. Mice require little laboratory space, are easily maintained and handled, and antibodies specific to a wide variety of cellular antigens are abundantly available. Mice are small and, therefore, efficiently dosed on a mg/kg basis with costly early phase development drugs and biologicals. Mice are genetically well defined and are ideal research animals for genetic manipulation to study pathways and mechanisms. Mice also facilitate study of a wide variety of hematopoietic disease models, including murine hematopoietic tumors and human tumor xenografts in immune-deficient models.

Weaknesses of the murine HCT model are well documented.[68] Early HCT studies in mice did not translate well to the clinic. Mice have a short life span, making long-term engraftment studies impossible. Although specific strains of mice can mimic various individual aspects of the GVHD syndromes seen in humans, GVHD studies in mice fail to reproduce the full spectrum of the disease (see below). A single stem cell can repopulate a mouse immune system,[69] but repopulation of human and dog hematopoietic systems has always been polyclonal.[70] Mice are generally maintained under gnotobiotic conditions, and thus, the impact of complex microbiota on the murine immune system is largely absent.

Large animals, primarily dogs, and to some lesser extent nonhuman primates, have been used to supplement studies using inbred strains of mice. Dogs are superior to mouse models for HCT for several reasons. Dogs are particularly well-suited due to their mixed genetic background as a result of long-term random breeding in the laboratory setting. Dogs display phenotypic diversity and longer life spans compared to mice.[71] Dogs used in experimental research are not raised under gnotobiotic conditions, and therefore they are subject to similar immune conditions imposed by intestinal microbiota following HCT as are humans.[72,73] Dogs also possess a relatively short gestation period (average 63 days) and have large-sized litters, allowing for successful studies evaluating matching for MHC antigens, known as dog leukocyte antigens (DLA). Importantly, DLA class I and II genes simulate HLA matching for donor and recipient pairs.[74,75]

Canine and human CD34+ marrow cells possess similar in vitro and in vivo characteristics.[76] Although nonhuman primates are also similar to human in many respects, dogs are easier to handle, less expensive to purchase and maintain, and lack virulent primate pathogens transmissible to humans. Importantly, successful canine HCT studies in DLA-matched donor and recipient pairs with postgrafting immunosuppression reestablished a sense of confidence that HCT for aplastic anemia and hematological malignancies could be successful in patients.[34,77]

However, the dog model does have limitations. Antibodies directed against dog hematopoietic cell antigens are not as common as those for mice and nonhuman primates. Dogs are more expensive to purchase and house compared to mice but are less expensive than nonhuman primates. Currently, dogs are not as amenable to knock in/knock out studies as are mice. However, both dogs and primates have been used effectively in stem cell gene therapy.[70] Additional comparisons between the three species are described by Stolfi et al.[78] Table 1 lists the suitability of mouse, dog, and nonhuman primate for a variety of HCT characteristics described above.

Disease Relapse

Disease relapse, or progression of the underlying malignancy, remains a critical cause for failure of allogeneic HCT in the clinical setting.[19] GVL and GVT effects are the result of an active immune process involving donor T cells and, likely, donor NK cells. Postgrafting immune suppression for GVHD prevention and slow development of the donor immune system contribute to limited donor GVL/GVT activity early after transplant.[20] Increasing the intensity of the conditioning regimen to reduce tumor burden would increase regimen-related toxicity in medically infirm patients. New approaches to reduce tumor burden and boost donor immune cell function are needed to overcome the problem of relapse.

Animal Models

Mouse models for disease relapse after HCT have the advantage that a large number of transplantable tumors exist that are specific to various strains of mice. These tumors often show genetic instability and can be injected into mice with intact immune systems before HCT. However, mouse tumor models often do not share characteristics with human tumors such as latency period, biology of metastasis, or clinical outcome to new therapies.[79] Many of the murine hematological tumor cell lines have been extensively cultured in vitro and have become highly sensitive to chemotherapy and alloreactive T cell targeting.[78] In contrast, most human tumors have adopted means of avoiding immune recognition and resisting chemotherapy. Spontaneous murine hematological tumors have been developed, such as murine chronic myelogenous leukemia after transfecting marrow cells with BCR/ABL,[80] and can be viewed as a more appropriate model for development of therapeutic interventions for disease relapse.

Dogs develop lymphomas, sarcomas, and melanomas.[78] Cancers in companion dogs share histological features with human tumors such as tumor growth over time, tumor heterogeneity, disease relapse, and metastatic microenvironment characteristics.[81] The obvious deficiency in using animals with spontaneous tumors is the difficulty in conducting controlled randomized studies in a timely manner. However, several reports have been published describing HCT treatment of lymphoma in companion dogs.[82–84]

Genetic modification of canine hematopoietic stem cells prior to transplant opens up the possibility of generating leukemia in vivo. Two dogs transplanted with autologous genetically modified donor CD34+ hematopoietic stem cells overexpressing HOXB4 developed myeloid leukemia within approximately 2 years.[85] Moreover, accidental transfusion of trace numbers of cells from one of these dogs into two immune-suppressed dogs

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Table 1 Comparison of suitable features for animal model HCT development

	Mouse	Canine	Nonhuman primate
Early contributions to HCT	3	3	1
Comparable cell surface receptors (mAb)	1	1	2
Reduced-intensity conditioning	1	3	1
Multilineage chimerism	1	3	3
Clinical translation	1	3	3
Genetic relevance	1	2	3
Metabolic similarity	1	2	3
Genetic manipulation studies[a]	3	2	2
Acute GVHD modeling	3	3	1
Chronic GVHD modeling	1	3	1
Cost	3	1	1
Accessibility	3	2	1

Suitability is based on the overall contribution of the species to each criterion and is on a scale of 1 to 3, with 3 being the greatest.
[a]Includes knock in-out studies and gene transfer experiments.

resulted in development of myeloid leukemia in both animals.[86] These studies indicate that generating canine models of leukemia are possible and may provide a model for investigating GVL and relapse.

Cell-Based Targeting of Tumors

One approach to treating disease relapse with low risk of toxicity is with post-transplant donor lymphocyte infusion (DLI) and/or cytokine therapy to boost the anti-tumor effect of donor immune cells.[87] Weiss and colleagues used co-infusion of BCL1 cells, a B-cell leukemia cell line, with T-cell-depleted donor bone marrow after lethal irradiation to establish a model of minimal residual disease for evaluating the effect of DLI and cytokine therapy.[88] A low level of disease (10^4 BCL1 cells) was eliminated with IL-2 alone or cells alone, yet a higher level of disease (10^5 BCL1 cells) required DLI and IL-2 to eliminate the leukemia.

In a mouse model of acute myeloid leukemia, mice were infused with leukemia cells prior to irradiation and transplant with bone marrow and spleen cells from syngeneic, congenic, and allogeneic donors.[89] At a tumor burden of 10^5 mouse model of acute myeloid leukemia cells, transplant with allogeneic cells was curative, yet congenic transplant was unsuccessful. Mice receiving post-transplant IL-2 in addition to congenic transplant were able to eliminate the tumor, suggesting cytokine therapy after reduced intensity or non-myeloablative transplant could stimulate the GVL/GVT effect without increasing GVHD.

In dogs, non-myeloablative preconditioning followed by a DLA-identical marrow transplant and postgrafting immune suppression results in stable mixed hematopoietic chimerism.[18] Targeting host hematopoietic cells with the aim to increase donor chimerism can act as a surrogate for studying GVL activity and prevention of disease relapse. Infusion of donor lymphocytes after establishing stable mixed chimerism in dogs failed to increase donor chimerism, even with the addition of a 2-week course of IL-2 to stimulate proliferation.[90,91] If the donors were first sensitized to minor histocompatibility antigens by a recipient-to-donor skin graft, DLI resulted in a rapid shift to full donor chimerism, clearly demonstrating a graft-versus-host effect.[90,92] Recently,[93] sensitized female dogs to male antigens using adenovirus constructs encoding sections of SMCY and the entire SRY genes. After female-to-male non-myeloablative transplants, male antigen-sensitized DLI from the female donor caused a shift in donor chimerism in two of three male recipients.

Adoptive immunotherapy with natural killer (NK) cells has the potential to reduce tumor burden without increasing the risk of GVHD. Transplant of expanded donor NK cells in addition to marrow and spleen cells in a mouse model of leukemia improved survival compared to controls and resulted in less severe GVHD, suggesting NK cells may be able to reduce tumor burden.[94] A trial in human patients in which a single dose of NK cells is added to non-myeloablative haploidentical transplant with post-transplant cyclophosphamide showed potential for improved progression-free survival at 2 years, indicating further study and optimization is merited.

Chimeric antigen receptor (CAR) engineered T cells have yielded impressive results in the treatment of B cell malignancies and afford a novel approach towards preventing disease relapse. These results have been achieved using CD19-specific,[95–97] CD20-specific,[98] or CD30-specific[99] CAR-T cells for treating B cell malignancies. In a study of 30 children and adults diagnosed with ALL, 90% achieved complete remission of their disease.[96] Development of this technology depended on validation in primarily murine tumor models. Long-term survival was established in nude mice bearing NIH3T3 cells expressing human ERBB2 antigen.[100] Second- and third-generation CAR constructs containing costimulatory molecule signaling domains were also validated in murine models.[101,102]

Validation of CAR-T cell efficacy is also possible in dog spontaneous tumor models. Canine T cells can be expanded ex vivo, transfected with a CD20-ζ targeting domain, and used to treat dogs with relapsed B cell lymphoma. In a study by,[103] three injections of CAR-T cells into a dog with relapsed B cell lymphoma were safely tolerated and led to transient anti-tumor effects. The advantage of using the dog as a model for CAR-T cell development/validation is that a spontaneous tumor replicates the complexities of the tumor microenvironment of human B-cell neoplasia and is considered a relevant and predictive model for the development of therapies for the treatment of non-Hodgkin lymphoma.[104–106]

Antibody-Based Targeting of Tumors

Antibody-radionuclide conjugates can specifically target toxic radiation to the tumor and reduce off-target effects of TBI. Historically, iodine-131 and yttrium-90, which are both β-particle-emitting isotopes, have been used in the majority of

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radioimmunotherapy preclinical and clinical studies.[107] Radioimmunotherapy using yttrium-90-anti-CD22 in conjunction with unconjugated anti-CD20 IgG successfully cured 80% of nude mice grafted with the human B-cell lymphoma, Ramos.[108] Use of both isotopes has been described in hundreds of clinical trials that attest to their efficacy for the treatment of hematological and solid malignancies. One potential advantage of using beta- and gamma-emitting radionuclides is that the path length of radiation is long (0.8–11.3 mm, respectively), resulting in a "cross-fire" effect against non-antigen-bearing tumor cells. However, the long path length may also result in targeting of nonmalignant "bystander cells." Moreover, iodine-131 and yttrium-90 both have a long half-life of 2.5 and 8 days, respectively, and low energy emissions of 0.7 and 2.3 MeV, respectively.

Alternatively, radioimmunoconjugates using alpha emitters such as astatine-211 and bismuth-213 possess a short half-life (7.2 hours and 1 hour, respectively) and a limited path length (0.04–0.06 mm), thereby limiting off-target effects. Moreover, they have a high energy transfer (5.9 MeV and 8 MeV, respectively) and are expected to more effectively eliminate target cells. A bismuth-213-labeled antibody specific to Thy-1.2 specifically eliminated a Thy-1.2 + EL-4 murine tumor cell line in vivo.[109] A bismuth-213-labeled anti-CD45 antibody administered at 3.3 mCi/kg provided sufficient preconditioning for stable donor hematopoietic engraftment in a DLA-identical canine model of allo-HCT.[110] However, the widespread use of bismuth-213 is precluded by high cost and limited availability.

Orozco and colleagues[111] showed that astatine-211 was able to substitute for bismuth, showing that astatine-labeled anti-CD45 antibody improved the median survival time of mice bearing leukemic cells in a dose-dependent manner, indicating the potential in reducing tumor burden prior to transplant. Similar to the results seen with bismuth-213, astatine-211-labeled anti-CD45 was able to substitute for TBI conditioning in a DLA-identical HCT model.[112] Seven of eight dogs conditioned for transplantation with 155 to 165 μCi/kg of the astatine-211 immunoconjugate developed long-term donor chimerism. Collectively, these studies suggest that administering radioimmunoconjugates may be appropriate for reducing tumor burden and improving relapse rates without adding toxicity or impairing donor immune recovery.

GVHD

In human patients GVHD is broadly defined as either acute, occurring within 100 days, or chronic, which develops 100 days and beyond after transplantation, with the disease lasting up to several years. The two syndromes differ in their clinical presentation. Acute GVHD typically manifests with a systemic syndrome of weight loss, diarrhea, skin rash, and high mortality. Up to 80% of patients given HLA-identical allo-HCT develop acute GVHD.[66] Chronic GVHD presents as an autoimmune condition with systemic fibrosis and the production of auto-antibodies.[113] Both diseases are induced by donor T cells, and the targeted tissues are primarily the skin, intestinal tract, lung (chronic GVHD), and liver.

GVHD was first described as "secondary disease," a wasting syndrome that occurred following transplant. Decades of preclinical work in both mice and canine models clearly demonstrated that this syndrome is the result of donor T cells attacking host tissues.[114–124] The ability to match donors and recipients based on HLA typing reduces the risk of GVHD, yet fatal GVHD can develop still develop, presumably as a result of minor histocompatibility antigen mismatches.[34,125–131]

Animal Models of Acute GVHD

Mouse models of acute GVHD generally involve myeloablative conditioning using a lethal dose of irradiation (600–1300 cGy, depending on the strain), followed by transplant of H-2 incompatible bone marrow supplemented with donor lymphocytes, either splenocytes or lymph node T cells. The result is a systemic disease that normally affects the GI tract, liver, and skin and is lethal between 10 and 30 days after transplant. Sensitivity to radiation dose is dependent on the strain such that B6 mice are more resistant to radiation than BALB/c, and F1 progeny are more resistant than parental strains.[132]

Murine models of acute GVHD include MHC-mismatched models, minor histocompatibility antigen (miHA)-mismatched models, and xenogenic models. The most common MHC-mismatched model of acute GVHD is a transplant from C57/Bl6 (H^{2b}) donors to Balb/c (H^{2d}) recipients. Parent-to-F1 transplants using C57Bl/6 parental donors also generate acute GVHD; however, not all parental strains are able to induce acute GVHD, and disease development depends on irradiation. Interestingly, transplant from C57/Bl6 ($H2^{b}$) parental donors to recipients with mutations in MHC I (B6.C-$H2^{bm1}$) and/or MHC II (B6.C-$H2^{bm12}$) demonstrated that a mismatch in both MHC class I and class II is required for development of acute GVHD, suggesting that both CD4+ and CD8+ T cells are involved in disease induction.[133]

The miHA-mismatched models also rely on pretransplant irradiation, typically ranging from 600 to 1000 cGy. Either CD4+ or CD8+ T cells can contribute to disease pathology in the miHA-mismatch setting. Many of the models display systemic disease, but there is variation. For example, a transplant from a B10 ($H2^{b}$) donor to a BALB.b ($H2^{b}$) recipient generates acute GVHD without any skin involvement. Xenotransplant of human peripheral blood mononuclear cells into immune-deficient mice results in systemic disease. Immune-deficient mice require a lower dose of irradiation, typically 200 to 300 cGy depending on the strain. It is a CD4+ T cell-dependent model, as human APCs are required to process mouse antigens and T cell recognition of MHC molecules is restricted by species.

Mouse models of acute GVHD represent a controlled experimental system that allows analysis of single variables. However, humans exhibit genetic and phenotypic diversity, varied exposure to microorganisms, and variation in health status that all can affect outcomes. Mice are generally housed in specific-pathogen-free conditions; however, the microbiome has the potential to contribute to the generation of intestinal GVHD and may determine severity.

Exposure to a radioactive source, such as ^{137}Cs, is typically used as conditioning in mouse models of HCT. For human patients, a linear accelerator is typically used to generate and emit high-energy x-rays, which penetrate deeper into tissue for TBI. Moreover, unlike in mouse models, conditioning in human patients varies in intensity from non-myeloablative to myeloablative, depending on age, disease status, and comorbidities, and may involve chemotherapy and/or TBI. Moreover, postgrafting immune suppression as GVHD prophylaxis is rarely used in mouse models yet is standard of care in human patients. Postgrafting immune suppression will impact onset of GVHD and tumor progression.

The dog model uses more clinically relevant conditioning regimens[46–48,134] and postgrafting immune suppression with such

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individual agents as methotrexate (MTX), cyclosporine (CSP) azathioprine, succinyl acetone, and tacrolimus alone[56,58–60,135] or in combination.[56,60,136,137] Studies in the nonhuman primate HCT model generally rely on conditioning and postgrafting immunosuppression.[138–140] Early studies in dogs demonstrated that successful marrow grafts in lethally irradiated dogs results in graft versus host reactions.[141] Once serotyping was established to determine histocompatibility, consistent GVHD is induced in dogs using myeloablative TBI followed by transplant of marrow from mismatched and unrelated dogs.[142] The tissues targeted and the resulting pathology closely resemble human acute GVHD.[143,144]

Preventing and Treating Acute GVHD

Dogs given CSP after a DLA-mismatched and unrelated transplant had a lower incidence of GVHD, yet a significant number failed to engraft.[145] The combination of MTX and CSP was most effective for delaying onset of acute GVHD and permitted stable engraftment.[145,146] A similar synergism was observed when MTX was combined with another calcineurin inhibitor, tacrolimus.[60] The effectiveness of the combination of CSP and MTX was confirmed in randomized, phase III studies in humans.[147,148]

Mycophenolate mofetil (MMF), an inhibitor of DNA synthesis, appeared no better than MTX, CSP, or tacrolimus for preventing GVHD in dogs.[137] However, MMF showed synergism when combined with CSP and proved very effective both in enhancing engraftment after non-myeloablative conditioning and in controlling acute GVHD.[18,137] A further canine study showed that rapamycin (sirolimus) could be substituted for MMF in combination with CSP.[149] MMF/CSP or MMF/tacrolimus is now widely used for human allogeneic HCT after reduced-intensity conditioning regimens and represents the current state-of-the-art in that setting.[150]

If acute GVHD develops, corticosteroids are the first and most effective treatment option.[151] However, steroids have a number of undesirable side effects, and GVHD can become steroid-refractory, prompting a number of studies to find new means of treating the disease. Studies in mouse models showed that IL-11, IL-1β antagonists, TNFα antagonists, and IL-6 antagonists successfully treat acute GVHD.[152–158] However, clinical trials in humans were unsuccessful and, in the case of IL-11 therapy, generated adverse side effects that resulted in unexpectedly high mortality.[159–164] In these cases, the mouse models of acute GVHD were unable to predict outcomes in human patients. More recent studies in mouse models have identified HDAC inhibitors and JAK1/2 inhibitors as potential treatment options.[165–167] Studies in human patients are promising. Vorinostat, an HDAC inhibitor, reduces the severity of acute GVHD,[168,169] and ruxolitinib was able to achieve a complete response rate of 46.3% in patients with steroid-refractory acute GVHD.[170]

Costimulatory Blockade

Blocking costimulatory molecules required for activation and expansion of T cells is predicted to be effective in abrogating pathogenic T cell responses after allo-HCT. Costimulatory molecule blockade has been investigated as a means to prevent or treat GVHD in mouse models.[171–175] Following myeloablative allo-HCT, selectively blocking CD28 and ICOS, but not CTLA4, prevented acute GVHD in mice more effectively than blocking either CD28 or ICOS alone.

Blocking the CD28 costimulatory signal in the dog model with human CTLA4-Ig, in combination with MTX/CSP immunosuppression, increased survival and resulted in a lower incidence of GVHD.[176] Currently, abatacept (human CTLA4-Ig) is in clinical trials as GVHD prophylaxis. Directly targeting CD28 with an anti-CD28 mAb while leaving the coinhibitory pathway of CTLA-4 intact has been proposed.[177] Our lab produced an anti-CD28 mAb with in vitro antagonistic activity.[178] However, injection of the anti-CD28 mAb into normal dogs produced a "cytokine storm" analogous to that seen in human volunteers, suggesting that anti-CD28 Fab or Fab constructs without cross-linking ability may be superior for human applications.[179]

Animal Models of Chronic GVHD

The murine models used to study chronic GVHD have shorter disease onset periods of 14 to 49 days, and disease manifestations are restricted in organ involvement. It is rare to recapitulate all chronic GVHD disease manifestations in a single mouse model. Transplant from C57/Bl6 (H^{2b}) donors to recipients with mutations in MHC I (B6.C-H2bm1) resulted in mild chronic GVHD, whereas transplant into mice with mutations in MHC II (B6.C-H2bm12) resulted in severe systemic chronic GVHD,[133,180] suggesting that CD4+ T cells are the main contributors to disease pathology.

Mouse models of chronic GVHD are varied. The scleroderma-tous models typically involve lethal irradiation followed by an miHA-mismatched transplant, and the resulting disease is primarily T_H2-dependent fibrosis of the skin.[181] The autoantibody/lupus-like models are generally parent-to-F1 MHC-mismatched transplants and may or may not involve TBI. The most common model is a DBA/2 ($H2^d$) to B6D2F1 ($H2^{b/d}$) transplant, which results in lymphadenopathy, splenomegaly, and autoantibody production.

Some models claim multi-organ involvement; however, the disease is normally restricted to a small number of tissues. A DBA/2 (H^{2d}) to BALB/c (H^{2d}) transplant results in production of autoantibodies and scleroderma.[182] A C57/B6 ($H2^b$) to B10.Br ($H2^k$) transplant results mainly in bronchiolitis obliterans, but mild pathology was detected in oral mucosa and autoantibodies detected in the liver.[183]

In contrast, fibrosis in human chronic GVHD can be systemic or pleiotropic, the repertoire of autoantibodies is more diverse, and lymphadenopathy and splenomegaly do not occur. Nephritis in human GVHD is rare but common in mouse model. Therefore, murine models of chronic GVHD do not adequately recapitulate the human disease.

Dogs conditioned with 8.5 to 9.2 Gy of total body irradiation followed by infusion of marrow from DLA-nonidentical littermates and postgrafting immunosuppression with MTX developed two distinct clinical forms of GVHD.[143] The median onset for acute GVHD was 13 days after transplant, while the chronic form developed at a median of 124 days after transplant. This temporal relationship recapitulates the human clinical condition better than does the mouse model.

The canine chronic GVHD model was not pursued at this time because investigators believed that solutions to treating the disease would be identified clinically. This assumption did not materialize, and chronic GVHD has remained a major problem in humans that is difficult to treat. Recently, we described a protocol in which dogs, conditioned with 9.2 Gy TBI and transplanted with DLA-mismatched unrelated marrow and buffy coat cells followed by postgrafting immunosuppression with MTX and CSP, developed de novo chronic GVHD, the clinical course of which resembled chronic GVHD seen in human.[184] Moreover, the target organs in the canine model (skin, liver,

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gastrointestinal tract, and lungs) exhibited the same pathology as that observed in the human condition.[185]

Treating Chronic GVHD

Compared to acute GVHD, the mouse model of chronic GVHD has provided few insights into treatment options.[67] The recent characterization of the canine model of chronic GVHD opens up the possibility of testing new drugs and biomolecules and investigating the role of the costimulatory pathways. Specifically, ICOS was upregulated on CD3+ cells within the blood, lymph nodes, and spleen in dogs affected by chronic GVHD, providing a potential target for therapy.[186] Administration of an antibody specific to canine ICOS resulted in a temporary remission of chronic GVHD symptoms and a significant prolongation in survival from the onset of the disease compared to control dogs.[119]

Accessibility and Acceptability

Mice are more accessible for study than dogs. Small animal vivaria are common at most major research institutions and large academic institutions. Canine and nonhuman primate vivaria have limited access. Special needs must be met for large animals such as treatment rooms, surgical suite(s), and kennels designed to humanely serve the dog's or the primate's needs. Sterile technique, surgical methods, and anesthesia are on par with that used in hospitals. For total body irradiation, a costly linear accelerator or linear accelerator is commonly used for an external beam radiation source for large animals, while for mouse irradiation, a far less expensive cesium irradiator in a shielded container can easily suffice.

A veterinarian is required for both small and large animal care, as are highly trained technical staff for caring, handling, and treating large animals. Protocols for experimental testing for large animals are exceedingly detailed and require investigators comply with strict regulations that ensure humane care and treatment of the animals. A great deal of the investigators' time is required to comply with the institutional animal care and use committee forms and validation processes. These issues have been recently examined and a need for consolidation and revamping of current practices suggested.[187] Overall, the cost and space required have a great impact on the accessibility of conducting large animal studies versus small animal studies.

Acceptability is an issue for any animal experimentation required in the testing of new therapeutic approaches for HCT. There are levels of pain that must be addressed and mitigated with the proper anesthesia and analgesia so that suffering is eliminated. The dog models have special concerns to researchers and the public, as dogs are companion animals while mice are generally not. It is important to note that what has been learned in the laboratory for HCT using the canine model has been returned to general dog healthcare by making available to dog owners the procedures for treating canine hematological and solid malignancies.[82–84,188,189] Further education of the public with regards to the benefit of the appropriate use of animal models is essential for acceptability.

Overview and Next Steps

There are limitations to any animal model used for evaluating new drugs and protocols for clinical translation. The complexity of the MHC of different species and how it relates to environmental conditions, tumor heterogeneity, drug pharmacokinetics, and intensity of conditioning regiments all play a part in translation of therapies to the clinic. Nevertheless, animal models provide a critical role in drug and protocol development between in vitro testing and clinical application from perspectives of toxicity, pharmacology, and efficacy. As in all studies, investigators should keep in mind that selection of the appropriate animal model for studies in HCT should be based on past performance of the model and not on availability/accessibility or lowest cost.

Both the murine and canine models have been invaluable in making HCT a highly successful therapy for the treatment of malignant and nonmalignant hematopoietic disorders. Despite tremendous success, two important areas of concern remain: disease relapse and GVHD. Undoubtedly, both mouse and dog will continue to contribute toward elucidation of these two problems.

In regards to disease relapse, the murine model will have significant impact in the development of next-generation CAR-T cells using human tumor xenografts in NOD/SCID or humanized mice. Dog HCT models with spontaneous or HOXB4-transformed hematopoietic cells[85] can better replicate the human condition for testing new CAR-T cell safety and efficacy. Natural killer cells, used to supplement/replace T cells in HCT or in adoptive immunotherapy following relapse, require further vetting in mouse and dog HCT models for their attractive GVT effects without inducing GVHD.[190,191] Again, the canine model can be used provided the appropriate mAbs are developed for NK cell selection and expansion ex vivo.

GVHD also remains a significant complication to successful HCT. Only recently has there been reported a protocol for reliably inducing chronic GVHD in dogs that recapitulates all the manifestations of the disease seen in the human setting. Continued investigation into the application of costimulatory molecule blockade to chronic GVHD and especially steroid refractory GVHD needs to be tested in the canine HCT model.

Another important factor in looking forward is to extend to the animal models proteomics and genomic approaches that are widely applied to human systems. Identification and function of candidate molecules that are under investigation should bear close similarity between the two species so that mechanistic analyses and translations to the clinic can be properly made.[192–194]

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References

1. Appelbaum FR. Hematopoietic-cell transplantation at 50. *N Engl J Med*. 2007;357(15):1472–1475.
2. Thomas ED. Bone marrow transplantation—past, present and future: Nobel lecture, December 8, 1990. In: Frängsmyr T, ed. *Les Prix Nobel: The Nobel Prizes 1990*. Stockholm: Nobel Foundation; 1990:222–230.
3. Congdon CC, Lorenz E. Humoral factor in irradiation protection: Modification of lethal irradiation injury in mice by injection of rat bone marrow. *Am J Physiol*. 1954;176(2): 297–300.
4. Jacobson LO, Marks EK, Robson MJ, Gaston EO, Zirkle RE. Effect of spleen protection on mortality following x-irradiation. *J Lab Clin Med*. 1949;34:1538–1543.
5. Lorenz E, Uphoff D, Reid TR, Shelton E. Modification of irradiation injury in mice and guinea pigs by bone marrow injections. *J Natl Cancer Inst*. 1951;12:197–201.
6. Lorenz E, Congdon CC, Uphoff D. Prevention of irradiation-induced lymphoid tumors in C57BL mice by spleen protection. *J Natl Cancer Inst*. 1953;14(2):291–301.
7. van Bekkum DW, de Vries MJ. *Radiation chimaeras*. London: Logos Press Limited; 1967.
8. Main JM, Prehn RT. Successful skin homografts after the administration of high dosage X radiation and homologous bone marrow. *J Natl Cancer Inst*. 1955;15:1023–1029.
9. Trentin JJ. Mortality and skin transplantability in x-irradiated mice receiving isologous or heterologous bone marrow. *Proc Soc Exp Biol Med*. 1956;92:688–693.
10. Barnes DWH, Corp MJ, Loutit JF, Neal FE. Treatment of murine leukaemia with x-rays and homologous bone marrow. Preliminary communication. *Br Med J*. 1956;2:626–627.
11. Mathe G, Bernard J. [Trial therapy, by x-irradiation followed by the administration of homologous bone marrow cells, of highly-advanced spontaneous leukemia in AK mice]. *Bull Assoc Fr Etud Cancer*. 1958;45(3):289–300.
12. Barnes DWH, Loutit JF. Treatment of murine leukaemia with x-rays and homologous bone marrow: II. *Br J Haematol*. 1957;3:241–252.
13. Thomas ED, Lochte HL Jr, Lu WC, Ferrebee JW. Intravenous infusion of bone marrow in patients receiving radiation and chemotherapy. *N Engl J Med*. 1957;257:491–496.
14. Thomas ED, Lochte HL Jr, Cannon JH, Sahler OD, Ferrebee JW. Supralethal whole body irradiation and isologous marrow transplantation in man. *J Clin Invest*. 1959;38:1709–1716.
15. Mathe G, Amiel JL, Schwarzenberg L, Cattan A, Schneider M. Adoptive immunotherapy of acute leukemia: experimental and clinical results. *Cancer Res*. 1965;25(9):1525–1531.
16. Bortin MM. A compendium of reported human bone marrow transplants. *Transplantation*. 1970;9:571–587.
17. Sachs DH, Sykes M, Kawai T, Cosimi AB. Immunointervention for the induction of transplantation tolerance through mixed chimerism. *Semin Immunol*. 2011;23(3): 165–173.
18. Storb R, Yu C, Wagner JL, et al. Stable mixed hematopoietic chimerism in DLA-identical littermate dogs given sublethal total body irradiation before and pharmacological immunosuppression after marrow transplantation. *Blood*. 1997;89(8):3048–3054.
19. Storb R, Sandmaier BM. Nonmyeloablative allogeneic hematopoietic cell transplantation. *Haematologica*. 2016;101 (5):521–530.
20. Storb R, Gyurkocza B, Storer BE, et al. Graft-versus-host disease and graft-versus-tumor effects after allogeneic hematopoietic cell transplantation. *J Clin Oncol*. 2013;31(12): 1530–1538.
21. Scott BL, Pasquini MC, Logan BR, et al. Myeloablative versus reduced-intensity hematopoietic cell transplantation for acute myeloid leukemia and myelodysplastic syndromes. *J Clin Oncol*. 2017;35(11):1154–1161.
22. Bensinger WI, Martin PJ, Storer B, et al. Transplantation of bone marrow as compared with peripheral-blood cells from HLA-identical relatives in patients with hematologic cancers. *N Engl J Med*. 2001;344(3):175–181.
23. Cutler C, Giri S, Jeyapalan S, Paniagua D, Viswanathan A, Antin JH. Acute and chronic graft-versus-host disease after allogeneic peripheral-blood stem-cell and bone marrow transplantation: A meta-analysis. *J Clin Oncol*. 2001;19(16): 3685–3691.
24. Flowers MED, Parker PM, Johnston LJ, et al. Comparison of chronic graft-versus-host disease after transplantation of peripheral blood stem cells versus bone marrow in allogeneic recipients: Long-term follow-up of a randomized trial. *Blood*. 2002;100(2):415–419.
25. Friedrichs B, Tichelli A, Bacigalupo A, et al. Long-term outcome and late effects in patients transplanted with mobilised blood or bone marrow: A randomised trial. *Lancet Oncol*. 2010;11(4):331–338.
26. Mohty M, Kuentz M, Michallet M, et al., Societe Francaise de Greffe de Moelle et de Therapie Cellulaire (SFGM-TC). Chronic graft-versus-host disease after allogeneic blood stem cell transplantation: Long-term results of a randomized study. *Blood*. 2002;100(9):3128–3134.
27. Schmitz N, Beksac M, Bacigalupo A, et al. Filgrastim-mobilized peripheral blood progenitor cells versus bone marrow transplantation for treating leukemia: 3-year results from the EBMT randomized trial. *Haematologica*. 2005; 90(5):643–648.
28. Schmitz N, Eapen M, Horowitz MM, et al. Long-term outcome of patients given transplants of mobilized blood or bone marrow: A report from the International Bone Marrow Transplant Registry and the European Group for Blood and Marrow Transplantation. *Blood*. 2006;108(13):4288–4290.
29. Stem Cell Trialists' Collaborative Group. Allogeneic peripheral blood stem-cell compared with bone marrow transplantation in the management of hematologic malignancies: An individual patient data meta-analysis of nine randomized trials. *J Clin Oncol*. 2005;23(22):5074–5087.
30. Marmont AM, Horowitz MM, Gale RP, et al. T-cell depletion of HLA-identical transplants in leukemia. *Blood*. 1991;78: 2120–2130.
31. Wagner JE, Thompson JS, Carter SL, Kernan NA. Effect of graft-versus-host disease prophylaxis on 3-year disease-free survival in recipients of unrelated donor bone marrow (T-cell Depletion Trial): A multi-centre, randomised phase II-III trial. *Lancet*. 2005;366(9487):733–741.
32. Mollen N, Cannon FD, Ferrebee JW, St John D. Lymphocyte typing in allografted beagles. *Transplantation*. 1968;6(8):939–940.
33. Snell GD, Stimpfling JH. Genetics of tissue transplantation. In: Green EL, Jackson Laboratory (Bar Harbor M), eds. *Biology of the Laboratory Mouse*. 2nd ed. New York: Dover Publications, Inc.; 1966. http://www.informatics.jax.org/greenbook/
34. Storb R, Epstein RB, Bryant J, Ragde H, Thomas ED. Marrow grafts by combined marrow and leukocyte infusions in unrelated dogs selected by histocompatibility typing. *Transplantation*. 1968;6(4):587–593.

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35. Wagner JL, Burnett RC, DeRose SA, Storb R. Molecular analysis and polymorphism of the DLA-DQA gene. *Tissue Antigens* 1996a;48:199–204.
36. Wagner JL, Burnett RC, Storb R. Molecular analysis of the DLA DR region. *Tissue Antigens* 1996b;48(5):549–553.
37. Wagner JL, Hayes-Lattin B, Works JD, Storb R. Molecular analysis and polymorphism of the DLA-DQB genes. *Tissue Antigens*. 1998;52:242–250.
38. Bull MI, Herzig GP, Graw RG Jr, Krueger GR, Bowles CA. Canine allogeneic bone marrow transplantation. Technique and variables influencing engraftment. *Transplantation*. 1976;22(2):150–159.
39. Deeg HJ, Storb R, Weiden PL, Schumacher D, Shulman H, Graham T, Thomas ED. High-dose total-body irradiation and autologous marrow reconstitution in dogs: Dose-rate-related acute toxicity and fractionation-dependent long-term survival. *Radiat Res* 1981b;88(2):385–391.
40. Deeg HJ, Storb R, Longton G, et al. Single dose or fractionated total body irradiation and autologous marrow transplantation in dogs: Effects of exposure rate, fraction size and fractionation interval on acute and delayed toxicity. *Int J Radiat Oncol Biol Phys*. 1988;15(3):647–653.
41. Graves SS, Storer BE, Butts TM, Storb R. Comparing high and low total body irradiation dose rates for minimum-intensity conditioning of dogs for dog leukocyte antigen-identical bone marrow grafts. *Biol Blood Marrow Transplant*. 2013;19(11):1650–1654.
42. Kolb HJ, Rieder I, Bodenberger U, et al. Dose rate and dose fractionation studies in total body irradiation of dogs. *Pathologie Biologie*. 1979;27:370–372.
43. Storb R, Deeg HJ. Failure of allogeneic canine marrow grafts after total body irradiation: Allogeneic resistance vs transfusion induced sensitization. *Transplantation*. 1986;42(6): 571–580.
44. Storb R, Raff RF, Appelbaum FR, et al. What radiation dose for DLA-identical canine marrow grafts? *Blood*. 1988;72(4): 1300–1304.
45. Thomas ED, LeBlond R, Graham T, Storb R. Marrow infusions in dogs given midlethal or lethal irradiation. *Radiat Res*. 1970;41(1):113–124.
46. Epstein RB, Storb R, Clift RA, Thomas ED. Autologous bone marrow grafts in dogs treated with lethal doses of cyclophosphamide. *Cancer Res* 1969a;29(5):1072–1075.
47. Kolb HJ, Storb R, Weiden PL, et al. Immunologic, toxicologic and marrow transplantation studies in dogs given dimethyl myleran. *Biomedicine*. 1974;20(5):341–351.
48. Storb R, Epstein RB, Rudolph RH, Thomas ED. Allogeneic canine bone marrow transplantation following cyclophosphamide. *Transplantation*. 1969;7(3):378–386.
49. Appelbaum FR, Brown P, Sandmaier B, et al. Antibody-radionuclide conjugates as part of a myeloblative preparative regimen for marrow transplantation. *Blood*. 1989;73(8): 2202–2208.
50. Bethge WA, Wilbur DS, Storb R, et al. Selective T-cell ablation with bismuth-213-labeled anti-TCRalphabeta as non-myeloablative conditioning for allogeneic canine marrow transplantation. *Blood*. 2003;101(12):5068–5075.
51. Appelbaum FR. Hemopoietic reconstitution following autologous bone marrow and peripheral blood mononuclear cell infusions. *Exp Hematol*. 1979;7(Suppl 5):7–11.
52. Appelbaum FR, Herzig GP, Graw RG, et al. Study of cell dose and storage time on engraftment of cryopreserved autologous bone marrow in a canine model. *Transplantation*. 1978; 26:245–248.
53. Bodenberger U, Kolb HJ, Rieder I, et al. Fractionated total body irradiation and autologous bone marrow transplantation in dogs: Hemopoietic recovery after various marrow cell doses. *Exp Hematol*. 1980;8(4):384–394.
54. Sullivan RD, Stecher G, Sternberg SS. Value of bone marrow and spleen cell suspensions for survival of lethally irradiated dogs. *J Natl Cancer Inst*. 1959;23:367–383.
55. Deeg HJ, Storb R, Weiden PL, Graham T, Atkinson K, Thomas ED. Cyclosporin-A: Effect on marrow engraftment and graft-versus-host disease in dogs. *Transplant Proc* 1981a; 13(1):402–409.
56. Deeg HJ, Storb R, Weiden PL, et al. Cyclosporin A and methotrexate in canine marrow transplantation: Engraftment, graft-versus-host disease, and induction of tolerance. *Transplantation* 1982b;34(1):30–35.
57. Kolb HJ, Storb R, Graham TC, Kolb H, Thomas ED. Antithymocyte serum and methotrexate for control of graft-versus-host disease in dogs. *Transplantation*. 1973;16 (1):17–23.
58. Storb R, Epstein RB, Graham TC, Thomas ED. Methotrexate regimens for control of graft-versus-host disease in dogs with allogeneic marrow grafts. *Transplantation*. 1970;9(3): 240–246.
59. Storb R, Kolb HJ, Deeg HJ, et al. Prevention of graft-versus-host disease by immunosuppressive agents after transplantation of DLA-nonidentical canine marrow. *Bone Marrow Transplant* 1986c;1(2):167–177.
60. Storb R, Raff RF, Appelbaum FR, et al. FK-506 and methotrexate prevent graft-versus-host disease in dogs given 9.2 Gy total body irradiation and marrow grafts from unrelated dog leukocyte antigen-nonidentical donors. *Transplantation*. 1993;56(4):800–807.
61. Storb R, Yu C, Barnett T, et al. Stable mixed hematopoietic chimerism in dog leukocyte antigen-identical littermate dogs given lymph node irradiation before and pharmacologic immunosuppression after marrow transplantation. *Blood*. 1999;94(3):1131–1136.
62. Carlens S, Remberger M, Aschan J, Ringdén O. The role of disease stage in the response to donor lymphocyte infusions as treatment for leukemic relapse. *Biol Blood Marrow Transplant*. 2001;7:31–38.
63. Mattsson J, Ringdén O, Storb R. Graft failure after allogeneic hematopoietic cell transplantation. *Biol Blood Marrow Transplant*. 2008;14 (Suppl. 1):165–170.
64. Slavin S, Weiss L, Ackerstein A, et al. Prevention and treatment of relapse by bone marrow transplantation. *Bone Marrow Transplant*. 1993;12(Suppl 3):S54–S56.
65. Zetterquist H, Mattsson J, Uzunel M, et al. Mixed chimerism in the B cell lineage is a rapid and sensitive indicator of minimal residual disease in bone marrow transplant recipients with pre-B cell acute lymphoblastic leukemia. *Bone Marrow Transplant*. 2000;25(8):843–851.
66. Ram R, Storb R. Pharmacologic prophylaxis regimens for acute graft-versus-host disease: Past, present and future. *Leuk Lymphoma*. 2013;54(8):1591–1601.
67. Zeiser R, Blazar BR. Preclinical models of acute and chronic graft-versus-host disease: How predictive are they for a successful clinical translation? *Blood*. 2016;127(25): 3117–3126.
68. Chu YW, Gress RE. Murine models of chronic graft-versus-host disease: Insights and unresolved issues (Review). *Biol Blood Marrow Transplant*. 2008;14(4):365–378.
69. Osawa M, Hanada K, Hamada H, Nakauchi H. Long-term lymphohematopoietic reconstitution by a single CD34-low/

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negative hematopoietic stem cell. *Science*. 1996;273(5272): 242–245.
70. Trobridge GD, Kiem HP. Large animal models of hematopoietic stem cell gene therapy (Review). *Gene Ther*. 2010;17(8): 939–948.
71. Ostrander EA, Wayne RK. The canine genome (Review). *Genome Res*. 2005;15(12):1706–1716.
72. Ghimire S, Weber D, Mavin E, Wang XN, Dickinson AM, Holler E. Pathophysiology of GvHD and other HSCT-related major complications. *Front Immunol*. 2017;8:79.
73. Jenq RR, Ubeda C, Taur Y, et al. Regulation of intestinal inflammation by microbiota following allogeneic bone marrow transplantation. *J Expl Med*. 2012;209(5):903–911.
74. Venkataraman GM, Stroup P, Graves SS, Storb R. An improved method for dog leukocyte antigen 88 typing and two new major histocompatibility complex class I alleles, DLA-88*01101 and DLA-88*01201. *Tissue Antigens*. 2007;70(1): 53–57.
75. Wagner JL, Burnett RC, Storb R. Organization of the canine major histocompatibility complex: Current perspectives. *J Hered*. 1999;90(1):35–38.
76. Suter SE, Gouthro TA, McSweeney PA, et al. Isolation and characterization of pediatric canine bone marrow CD34 + cells. *Veterinary Immunology & Immunopathology*. 2004;101:31–47.
77. Epstein RB, Storb R, Clift RA, Thomas ED. Transplantation of stored allogeneic bone marrow in dogs selected by histocompatibility typing. *Transplantation* 1969b;8(4):496–501.
78. Stolfi JL, Pai CC, Murphy WJ. Preclinical modeling of hematopoietic stem cell transplantation—advantages and limitations. *FEBS J*. 2016;283(9):1595–1606.
79. Ranieri G, Gadaleta CD, Patruno R, et al. A model of study for human cancer: Spontaneous occurring tumors in dogs. Biological features and translation for new anticancer therapies. *Crit Rev Oncol Hematol*. 2013;88(1):187–197.
80. Zhang X, Ren R. Bcr/Abl efficiently induces a myeloproliferative disease and production of excess interleukin-3 and granulocyte macrophage colony-stimulating factor in mice: a novel model for chronic myelogenous leukemia. *Blood* 1998;92:3829–3840.
81. Vail DM, MacEwen EG. Spontaneously occurring tumors of companion animals as models for human cancer (Review). *Cancer Invest*. 2000;18(8):781–792.
82. Deeg HJ, Appelbaum FR, Weiden PL, Hackman RC, Graham TC, Storb R. Autologous marrow transplantation as consolidation therapy for canine lymphoma: Efficacy and toxicity of various regimens of total body irradiation. *Am J Vet Res*. 1985;46(9):2016–2018.
83. Weiden PL, Storb R, Deeg HJ, Graham TC. Total body irradiation and autologous marrow transplantation as consolidation therapy for spontaneous canine lymphoma in remission. *Exp Hematol* (Suppl.5) 1979a;7:160–163.
84. Weiden PL, Storb R, Deeg HJ, Graham TC, Thomas ED. Prolonged disease-free survival in dogs with lymphoma after total-body irradiation and autologous marrow transplantation consolidation of combination-chemotherapy-induced remissions. *Blood* 1979b;54(5):1039–1049.
85. Zhang XB, Schwartz JL, Humphries RK, Kiem HP. Effects of HOXB4 overexpression on ex vivo expansion and immortalization of hematopoietic cells from different species. *Stem Cells*. 2007;25:2074–2081.
86. Thakar MS, Zhang XB, Beard BC, et al. Transmission and expansion of HOXB4-induced leukemia in two immunosuppressed dogs: Implications for a new canine leukemia model. *Exp Hematol*. 2009;37(10):1157–1166.
87. Introna M, Lussana F, Algarotti A, et al. Phase II study of sequential infusion of donor lymphocyte infusion and cytokine-induced killer cells for patients relapsed after allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. 2017;23(12):2070–2078.
88. Weiss L, Reich S, Slavin S. Use of recombinant human interleukin-2 in conjunction with bone marrow transplantation as a model for control of minimal residual disease in malignant hematological disorders: I. Treatment of murine leukemia in conjunction with allogeneic bone marrow transplantation and IL-2-activated cell-mediated immunotherapy. *Cancer Invest*. 1992;10(1):19–26.
89. Vourka-Karussis U, Karussis D, Ackerstein A, Slavin S. Enhancement of GVL effect with rhIL-2 following BMT in a murine model for acute myeloid leukemia in SJL/J mice. *Exp Hematol*. 1995;23(3):196–201.
90. Georges GE, Storb R, Thompson JD, et al. Adoptive immunotherapy in canine mixed chimeras after nonmyeloablative hematopoietic cell transplantation. *Blood*. 2000;95(10): 3262–3269.
91. Georges GE, Storb R, Zaucha JM, Taranova AG, Gooley T, Nash RA. IL-2 does not enhance the conversion to complete donor chimerism following nonmyeloablative hematopoietic cell transplantation in dogs. *Bone Marrow Transplant*. 2003;31: 1027–1031.
92. Graves SS, Hogan WJ, Kuhr C, et al. Adoptive immunotherapy against allogeneic kidney grafts in dogs with stable hematopoietic trichimerism. *Biol Blood Marrow Transplant*. 2008;14:1201–1208.
93. Rosinski SL, Stone B, Graves SS, et al. Development of a minor histocompatibility antigen vaccine regimen in the canine model of hematopoietic cell transplantation. *Transplantation* 2015a;99(10):2083–2094.
94. Chen G, Wu D, Wang Y, et al. Expanded donor natural killer cell and IL-2, IL-15 treatment efficacy in allogeneic hematopoietic stem cell transplantation. *Eur J Haematol*. 2008;81(3): 226–235.
95. Lee DW, Kochenderfer JN, Stetler-Stevenson M, et al. T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: A phase 1 dose-escalation trial. *Lancet*. 2015;385(9967):517–528.
96. Maude SL, Frey N, Shaw PA, et al. Chimeric antigen receptor T cells for sustained remissions in leukemia. *N Engl J Med*. 2014;371(16):1507–1517.
97. Turtle CJ, Hanafi LA, Berger C, et al. CD19 CAR-T cells of defined CD4+:CD8+ composition in adult B cell ALL patients. *J Clin Invest*. 2016;126(6):2123–2138.
98. Zhang WY, Wang Y, Guo YL, et al. Treatment of CD20-directed Chimeric Antigen Receptor-modified T cells in patients with relapsed or refractory B-cell non-Hodgkin lymphoma: an early phase IIa trial report. *Signal Transduct Target Ther*. 2016;1:16002.
99. Wang CM, Wu ZQ, Wang Y, et al. Autologous T cells expressing CD30 chimeric antigen receptors for relapsed or refractory hodgkin lymphoma: An open-label phase I trial. *Clin Cancer Res*. 2017;23(5):1156–1166.
100. Moritz D, Wels W, Mattern J, Groner B. Cytotoxic T lymphocytes with a grafted recognition specificity for ERBB2-expressing tumor cells. *Proc Natl Acad Sci USA*. 1994;91(10): 4318–4322.
101. Kowolik CM, Topp MS, Gonzalez S, et al. CD28 costimulation provided through a CD19-specific chimeric antigen receptor enhances in vivo persistence and antitumor efficacy of adoptively transferred T cells. *Cancer Res*. 2006;66 (22):10995–11004.

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102. Milone MC, Fish JD, Carpenito C, et al. Chimeric receptors containing CD137 signal transduction domains mediate enhanced survival of T cells and increased antileukemic efficacy in vivo. *Molecular Therapy*. 2009;17(8):1453–1464.
103. Panjwani MK, Smith JB, Schutsky K, et al. Feasibility and safety of RNA-transfected CD20-specific chimeric antigen receptor T cells in dogs with spontaneous B cell lymphoma. *Mol Ther*. 2016;24(9):1602–1614.
104. Jubala CM, Wojcieszyn JW, Valli VE, et al. CD20 expression in normal canine B cells and in canine non-Hodgkin lymphoma. *Vet Pathol*. 2005;42(4):468–476.
105. Modiano JF, Breen M, Valli VE, Wojcieszyn JW, Cutter GR. Predictive value of p16 or Rb inactivation in a model of naturally occurring canine non-Hodgkin's lymphoma. *Leukemia*. 2007;21(1):184–187.
106. O'Connor CM, Sheppard S, Hartline CA, et al. Adoptive T-cell therapy improves treatment of canine non-Hodgkin lymphoma post chemotherapy. *Sci Rep*. 2012;2:249.
107. Larson SM, Carrasquillo JA, Cheung NK, Press OW. Radioimmunotherapy of human tumours. *Nat Rev Cancer*. 2015;15(6):347–360.
108. Mattes MJ, Sharkey RM, Karacay H, Czuczman MS, Goldenberg DM. Therapy of advanced B-lymphoma xenografts with a combination of 90Y-anti-CD22 IgG (epratuzumab) and unlabeled anti-CD20 IgG (veltuzumab). *Clin Cancer Res*. 2008;14(19):6154–6160.
109. Macklis RM, Kinsey BM, Kassis AI, et al. Radioimmunotherapy with alpha-particle-emitting immunoconjugates. *Science*. 1988;240(4855):1024–1026.
110. Nakamae H, Kerbauy FR, Wilbur DS, et al. Pilot study of a 213bismuth-labeled anti-CD45 mAb as a novel nonmyeloablative conditioning for DLA-haploidentical littermate hematopoietic transplantation. *Transplantation*. 2010;89(11):1336–1340.
111. Orozco JJ, Kenoyer A, Balkin ER, et al. Anti-CD45 radioimmunotherapy without TBI before transplantation facilitates persistent hapoidentical donor engraftment. *Blood*. 2016;127(3):352–359.
112. Chen Y, Kornblit B, Hamlin DK, et al. Durable donor engraftment after radioimmunotherapy using alpha-emitter astatine-211-labeled anti-CD45 antibody for conditioning in allogeneic hematopoietic cell transplantation. *Blood*. 2012;119(5):1130–1138.
113. Schroeder MA, DiPersio JF. Mouse models of graft-versus-host disease: Advances and limitations (Review). *Dis Model Mech*. 2011;4(3):318–333.
114. Anderson BE, McNiff JM, Matte C, Athanasiadis I, Shlomchik WD, Shlomchik MJ. Recipient CD4+ T cells that survive irradiation regulate chronic graft-versus-host disease. *Blood*. 2004;104(5):1565–1573.
115. Busca A, Aversa F. In-vivo or ex-vivo T cell depletion or both to prevent graft-versus-host disease after hematopoietic stem cell transplantation. *Expert Opin Biol Ther*. 2017;17(11):1401–1415.
116. Doney KC, Weiden PL, Buckner CD, Storb R, Thomas ED. Treatment of severe aplastic anemia using antithymocyte globulin with or without an infusion of HLA haploidentical marrow. *Exp Hematol*. 1981;9(8):829–834.
117. Glucksberg H, Storb R, Fefer A, et al. Clinical manifestations of graft-versus-host disease in human recipients of marrow from HL-A-matched sibling donors. *Transplantation*. 1974;18(4):295–304.
118. Gorer PA, Boyse EA. Pathological changes in F1 hybrid mice following transplantation of spleen cells from donors of the parental strains. *Immunology*. 1959;2(2):182–193.
119. Graves SS, Parker MH, Stone D, et al. Anti-inducible costimulator monoclonal antibody treatment of canine chronic graft-versus-host disease. *Biol Blood Marrow Transplant* 2017a;24(1):50–54.
120. Miller J, Pierce JC, Martinez C, Good RA. An assay of graft-host interactions across strong and weak histocompatibility barriers in mice. *J Exp Med*. 1963;117(5):863–878.
121. Remlinger K, Martin PJ, Hansen JA, Doney KC, Smith A, Deeg HJ, Sullivan K, Storb R, Thomas ED. Murine monoclonal anti-T cell antibodies for treatment of steroid-resistant acute graft-versus-host disease. *Hum Immunol*. 1984;9(1):21–35.
122. Storb R. Graft-versus-host disease after marrow transplantation. In: Meryman HT, ed. *Transplantation: Approaches to Graft Rejection*. New York: Alan R. Liss, Inc.; 1986:139–157.
123. Storb R, Thomas ED. Graft-versus-host disease in dog and man: The Seattle experience (Review). *Immunol Rev*. 1985;88:215–238.
124. Thiele DL, Eigenbrodt ML, Bryde SE, Eigenbrodt EH, Lipsky PE. Intestinal graft-versus-host disease is initiated by donor T cells distinct from classic cytotoxic T lymphocytes. *J Clin Invest*. 1989;84(6):1947–1956.
125. Epstein RB, Storb R, Ragde H, Thomas ED. Cytotoxic typing antisera for marrow grafting in littermate dogs. *Transplantation*. 1968;6(1):45–58.
126. Goulmy E, Gratama JW, Blokland E, Zwaan FE, van Rood JJ. A minor transplantation antigen detected by MHC-restricted cytotoxic T lymphocytes during graft-versus-host disease. *Nature*. 1983;302(5904):159–161.
127. Kaminski ER. How important is histocompatibility in bone marrow transplantation? *Bone Marrow Transplant*. 1989;4(4):439–444.
128. Milosevic S, Bachnick B, Karim K, et al. Identification of MHC II-restricted minor histocompatibility antigens after HLA-identical stem-cell transplantation. *Transplantation*. 2010;90(9):1030–1035.
129. Mori S, El-Baki H, Mullen CA. Analysis of immunodominance among minor histocompatibility antigens in allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplant*. 2003;31(10):865–875.
130. Murphy GF, Whitaker D, Sprent J, Korngold R. Characterization of target injury of murine acute graft-versus-host disease directed to multiple minor histocompatibility antigens elicited by either CD4+ or CD8+ effector cells. *Am J Pathol*. 1991;138(4):983–990.
131. Takayanagi T, Nishihori H, Matsuki N, Iwabuchi K, Ogasawara K, Onoe K. Effects of non-major histocompatibility antigens on acute graft-versus-host reaction after allogeneic bone marrow transplantation. *Bone Marrow Transplant*. 1997;20(4):297–304.
132. Reddy P, Negrin R, Hill GR. Mouse models of bone marrow transplantation (Review) [Erratum appears in Biol Blood Marrow Transplant. 2008 Nov;14(11):1317–8]. *Biol Blood Marrow Transplant*. 2008a;14(1 Suppl 1):129–135.
133. Rolink AG, Pals ST, Gleichmann E. Allosuppressor and allohelper T cells in acute and chronic graft-vs.-host disease. II. F1 recipients carrying mutations at H-2K and/or I-A. *J Exp Med*. 1983;157(2):755–771.
134. Storb R, Weiden PL, Graham TC, Lerner KG, Nelson N, Thomas ED. Hemopoietic grafts between DLA-identical canine littermates following dimethyl myleran. Evidence for resistance to grafts not associated with DLA and abrogated by antithymocyte serum. *Transplantation*. 1977;24(5):349–357.

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135. Raff RF, Storb R, Graham T, et al. Pharmacologic, toxicologic and marrow transplantation studies in dogs given succinyl acetone. *Transplantation*. 1992;54:813–820.
136. Yu C, Storb R, Deeg HJ, et al. Tacrolimus (FK506) and methotrexate regimens to prevent graft-versus-host disease after unrelated dog leukocyte antigen (DLA) nonidentical marrow transplantation. *Bone Marrow Transplant*. 1996;17:649–653.
137. Yu C, Seidel K, Nash RA, et al. Synergism between mycophenolate mofetil and cyclosporine in preventing graft-versus-host disease among lethally irradiated dogs given DLA-nonidentical unrelated marrow grafts. *Blood*. 1998;91(7):2581–2587.
138. Kean LS, Gangappa S, Pearson TC, Larsen CP. Transplant tolerance in non-human primates: Progress, current challenges and unmet needs. *Am J Transplant*. 2006;6(5 Pt 1):884–893.
139. Larsen CP, Page A, Linzie KH, et al. An MHC-defined primate model reveals significant rejection of bone marrow after mixed chimerism induction despite full MHC matching. *Am J Transplant*. 2010;10(11):2396–2409.
140. Zheng HB, Watkins B, Tkachev V, et al. The knife's edge of tolerance: Inducing stable multilineage Mixed chimerism but with a significant risk of CMV reactivation and disease in rhesus macaques. *Am J Transplant*. 2017;17(3):657–670.
141. Thomas ED, Collins JA, Herman EC Jr, Ferrebee JW. Marrow transplants in lethally irradiated dogs given methotrexate. *Blood*. 1962;19(2):217–228.
142. Storb R, Kolb HJ, Graham TC, et al. Marrow grafts between histoincompatible canine family members. (Short communication). *Eur J Clin Biol Res*. 1972;17(7):680–685.
143. Atkinson K, Shulman HM, Deeg HJ, et al. Acute and chronic graft-versus-host disease in dogs given hemopoietic grafts from DLA-nonidentical littermates: Two distinct syndromes. *Am J Pathol*. 1982;108(2):196–205.
144. Sung AD, Chao NJ. Concise review: Acute graft-versus-host disease: immunobiology, prevention, and treatment. *Stem Cells Transl Med*. 2013;2(1):25–32.
145. Deeg HJ, Storb R, Raff RF, Weiden PL, Atkinson K, Thomas ED. Long-term survival of dogs given marrow grafts from unrelated DLA-nonidentical donors and treated with Cyclosporin A and Methotrexate—Is tolerance mediated by suppressor cells? In: Baum SJ, Ledney GD, Thierfelder S, eds. *Experimental Hematology Today*. Basel: S. Karger; 1982a:127–133.
146. Deeg HJ, Storb R, Appelbaum FR, Kennedy MS, Graham TC, Thomas ED. Combined immunosuppression with cyclosporine and methotrexate in dogs given bone marrow grafts from DLA-haploidentical littermates. *Transplantation*. 1984;37(1):62–65.
147. Storb R, Deeg HJ, Farewell V, et al. Marrow transplantation for severe aplastic anemia: Methotrexate alone compared with a combination of methotrexate and cyclosporine for prevention of acute graft-versus-host disease. *Blood* 1986a;68(1):119–125.
148. Storb R, Deeg HJ, Whitehead J, et al. Methotrexate and cyclosporine compared with cyclosporine alone for prophylaxis of acute graft versus host disease after marrow transplantation for leukemia. *N Engl J Med* 1986b;314(12):729–735.
149. Hogan WJ, Little MT, Zellmer E, et al. Postgrafting immunosuppression with sirolimus and cyclosporine facilitates stable mixed hematopoietic chimerism in dogs given sublethal total body irradiation before marrow transplantation from DLA-identical littermates. *Biol Blood Marrow Transplant*. 2003;9:489–495.
150. Sandmaier BM, Storb R. Reduced-intensity conditioning followed by hematopoietic cell transplantation for hematologic malignancies. In: Appelbaum FR, Forman SJ, Negrin RS, Blume KG, eds. *Thomas' Hematopoietic Cell Transplantation*. Oxford: Wiley-Blackwell; 2009:1043–1058.
151. Mielcarek M, Storer BE, Boeckh M, et al. Initial therapy of acute graft-versus-host disease with low-dose prednisone does not compromise patient outcomes. *Blood*. 2009;113(13):2888–2894.
152. Chen X, Das R, Komorowski R, et al. Blockade of interleukin-6 signaling augments regulatory T-cell reconstitution and attenuates the severity of graft-versus-host disease. *Blood*. 2009;114(4):891–900.
153. Hill GR, Cooke KR, Teshima T, et al. Interleukin-11 promotes T cell polarization and prevents acute graft-versus-host disease after allogeneic bone marrow transplantation. *J Clin Invest*. 1998;102(1):115–123.
154. Hill GR, Teshima T, Gerbitz A, et al. Differential roles of IL-1 and TNF-alpha on graft-versus-host disease and graft versus leukemia. *J Clin Invest*. 1999;104(4):459–467.
155. McCarthy PL Jr, Abhyankar S, Neben S, et al. Inhibition of interleukin-1 by an interleukin-1 receptor antagonist prevents graft-versus-host disease. *Blood*. 1991;78:1915–1918.
156. Schmaltz C, Alpdogan O, Muriglan SJ, et al. Donor T cell-derived TNF is required for graft-versus-host disease and graft-versus-tumor activity after bone marrow transplantation. *Blood*. 2003;101(6):2440–2445.
157. Tawara I, Koyama M, Liu C, et al. Interleukin-6 modulates graft-versus-host responses after experimental allogeneic bone marrow transplantation. *Clin Cancer Res*. 2011;17(1):77–88.
158. Teshima T, Hill GR, Pan L, et al. IL-11 separates graft-versus-leukemia effects from graft-versus-host disease after bone marrow transplantation. *J Clin Invest*. 1999;104(3):317–325.
159. Antin JH, Lee SJ, Neuberg D, et al. A phase I/II double-blind, placebo-controlled study of recombinant human interleukin-11 for mucositis and acute GVHD prevention in allogeneic stem cell transplantation. *Bone Marrow Transplant*. 2002;29:373–377.
160. Couriel DR, Saliba R, de Lima M, et al. A phase III study of infliximab and corticosteroids for the initial treatment of acute graft-versus-host disease. *Biol Blood Marrow Transplant*. 2009;15(12):1555–1562.
161. Kennedy GA, Varelias A, Vuckovic S, et al. Addition of interleukin-6 inhibition with tocilizumab to standard graft-versus-host disease prophylaxis after allogeneic stem-cell transplantation: A phase 1/2 trial. *Lancet Oncol*. 2014;15(13):1451–1459.
162. McCarthy PL Jr, Williams L, Harris-Bacile M, et al. A clinical phase I/II study of recombinant human interleukin-1 receptor in glucocorticoid-resistant graft-versus-host disease. *Transplantation*. 1996;62(5):626–631.
163. van Groningen LF, Liefferink AM, de Haan AF, et al. Combination therapy with inolimomab and etanercept for severe steroid-refractory acute graft-versus-host disease. *Biol Blood Marrow Transplant*. 2016;22(1):179–182.
164. Veeraputhiran M, Mangan K. Sudden loss of the GVL effect following use of the TNF inhibitor infliximab in a chronic myelogenous leukemia patient with chronic GVHD. *Bone Marrow Transplant*. 2010;45(6):1113–1114.
165. Reddy P, Maeda Y, Hotary K, et al. Histone deacetylase inhibitor suberoylanilide hydroxamic acid reduces acute

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graft-versus-host disease and preserves graft-versus-leukemia effect. *Proc Natl Acad Sci USA*. 2004;101(11):3921–3926.
166. Reddy P, Sun Y, Toubai T, Duran-Struuck R, Clouthier SG, Weisiger E, Maeda Y, Tawara I, Krijanovski O, Gatza E, Liu C, Malter C, Mascagni P, Dinarello CA, Ferrara JL. Histone deacetylase inhibition modulates indoleamine 2,3-dioxygenase-dependent DC functions and regulates experimental graft-versus-host disease in mice. *J Clin Invest* 2008b;118(7):2562–2573.
167. Spoerl S, Mathew NR, Bscheider M, et al. Activity of therapeutic JAK 1/2 blockade in graft-versus-host disease. *Blood*. 2014;123(24):3832–3842.
168. Choi SW, Braun T, Chang L, et al. Vorinostat plus tacrolimus and mycophenolate to prevent graft-versus-host disease after related-donor reduced-intensity conditioning allogeneic haemopoietic stem-cell transplantation: A phase 1/2 trial. *Lancet Oncol*. 2014;15(1):87–95.
169. Choi SW, Braun T, Henig I, et al. Vorinostat plus tacrolimus/methotrexate to prevent GVHD after myeloablative conditioning, unrelated donor HCT. *Blood*. 2017;130(15):1760–1767.
170. Zeiser R, Burchert A, Lengerke C, et al. Ruxolitinib in corticosteroid-refractory graft-versus-host disease after allogeneic stem cell transplantation: a multicenter survey. *Leukemia*. 2015;29(10):2062–2068.
171. Li J, Semple K, Suh WK, et al. Roles of CD28, CTLA4, and inducible costimulator in acute graft-versus-host disease in mice. *Biol Blood Marrow Transplant*. 2011;17(7):962–969.
172. Ogawa S, Nagamatsu G, Watanabe M, et al. Opposing effects of anti-activation-inducible lymphocyte-immunomodulatory molecule/inducible costimulator antibody on the development of acute versus chronic graft-versus-host disease. *J Immunol*. 2001;167(10):5741–5748.
173. Tajima N, Tezuka K, Tanimoto A, et al. JTA-009, a fully human antibody against human AILIM/ICOS, ameliorates graft-vs-host reaction in SCID mice grafted with human PBMCs. *Exp Hematol*. 2008;36(11):1514–1523.
174. Taylor PA, Panoskaltsis-Mortari A, Freeman GJ, et al. Targeting of inducible costimulator (ICOS) expressed on alloreactive T cells down-regulates graft-versus-host disease (GVHD) and facilitates engraftment of allogeneic bone marrow (BM). *Blood*. 2005;105(8):3372–3380.
175. Yu XZ, Liang Y, Nurieva RI, Guo F, Anasetti C, Dong C. Opposing effects of ICOS on graft-versus-host disease mediated by CD4 and CD8 T cells. *J Immunol*. 2006;176(12):7394–7401.
176. Yu C, Linsley P, Seidel K, et al. Cytotoxic T lymphocyte antigen 4-immunoglobulin fusion protein combined with methotrexate/cyclosporine as graft-versus-host disease prevention in a canine dog leukocyte antigen-nonidentical marrow transplant model. *Transplantation*. 2000;69(3):450–454.
177. Daikh D, Wofsy D, Imboden JB. The CD28-B7 costimulatory pathway and its role in autoimmune disease (Review). *J Leukoc Biol*. 1997;62(2):156–162.
178. Graves SS, Stone DM, Loretz C, et al. Antagonistic and agonistic anti-canine CD28 monoclonal antibodies: Tools for allogeneic transplantation. *Transplantation*. 2011;91(8):833–840.
179. Rosinski SL, Storb R, Strong RK, et al. Anti-CD28 antibody-initiated cytokine storm in canines. *Transplant Direct* 2015b;1(2):e7. doi:10.1097/TXD.0000000000000516.
180. Brown GR, Lee E, Thiele DL. TNF-TNFR2 interactions are critical for the development of intestinal graft-versus-host disease in MHC class II-disparate (C57BL/6J—>C57BL/6J x bm12)F1 mice. *J Immunol*. 2002;168(6):3065–3071.
181. Levy S, Nagler A, Okon S, Marmary Y. Parotid salivary gland dysfunction in chronic graft-versus-host disease (cGVHD): A longitudinal study in a mouse model. *Bone Marrow Transplant*. 2000;25(10):1073–1078.
182. Zhang C, Todorov I, Zhang Z, et al. Donor CD4+ T and B cells in transplants induce chronic graft-versus-host disease with autoimmune manifestations. *Blood*. 2006;107(7):2993–3001.
183. Srinivasan M, Flynn R, Price A, et al. Donor B-cell alloantibody deposition and germinal center formation are required for the development of murine chronic GVHD and bronchiolitis obliterans. *Blood*. 2012;119(6):1570–1580.
184. Graves SS, Rezvani A, Sale G, Stone D, Parker M, Rosinski S, Spector M, Swearingen B, Kean L, Storb R. A canine model of chronic graft-vs.-host disease. *Biol Blood Marrow Transplant* 2017b;23(3):420–427.
185. Shulman HM, Sullivan KM, Weiden PL, et al. Chronic graft-versus-host syndrome in man. A long-term clinicopathologic study of 20 Seattle patients. *Am J Med*. 1980;69:204–217.
186. Sato M, Storb R, Loretz C, et al. Inducible costimulator (ICOS) up-regulation on activated T cells in graft-versus-host disease after dog leukocyte antigen-nonidentical hematopoietic cell transplantation: A potential therapeutic target. *Transplantation*. 2013;96(1):34–41.
187. Cornwall W. Revamp animal research rules, report urges. *Science*. 2017;358(6362):434.
188. Gerdes AJ, Storb R. The repopulation of irradiated bone marrow by the infusion of stored autologous marrow. *Radiology*. 1970;94(2):441–445.
189. Lupu M, Sullivan EW, Westfall TE, et al. Use of multigeneration-family molecular dog leukocyte antigen typing to select a hematopoietic cell transplant donor for a dog with T-cell lymphoma. *J Am Vet Med Assoc*. 2006;228(5):728–732.
190. Miller JS, Soignier Y, Panoskaltsis-Mortari A, et al. Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. *Blood*. 2005;105(8):3051–3057.
191. Olson JA, Leveson-Gower DB, Gill S, Baker J, Beilhack A, Negrin RS. NK cells mediate reduction of GVHD by inhibiting activated, alloreactive T cells while retaining GVT effects. *Blood*. 2010;115(21):4293–4301.
192. Deckert M, Montesinos-Rongen M, Brunn A, Siebert R. Systems biology of primary CNS lymphoma: From genetic aberrations to modeling in mice. *Acta Neuropathol*. 2014;127(2):175–188.
193. Guryanova OA, Levine RL. Advances in the development of animal models of myeloid leukemias. *Semin Hematol*. 2013;50(2):145–155.
194. Schoenebeck JJ, Ostrander EA. Insights into morphology and disease from the dog genome project. *Annu Rev Cell Dev Biol*. 2014;30:535–560.

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Review Article

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Keeping the Engine Running: The Relevance and Predictive Value of Preclinical Models for CAR-T Cell Development

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Abstract

The cellular immunotherapy field has achieved important milestones in the last 30 years towards the treatment of a variety of cancers due to improvements in ex-vivo T cell manufacturing processes, the invention of synthetic T cell receptors, and advances in cellular engineering. Here, we discuss major preclinical models that have been useful for the validation of chimeric antigen receptor (CAR)-T cell therapies and also promising new models that will fuel future investigations towards success. However, multiple unanswered questions in the CAR-T cell field remain to be addressed that will require innovative preclinical models. Key challenges facing the field include premature immune rejection of universal CAR-T cells and the immune suppressive tumor microenvironment. Immune competent models that accurately recapitulate tumor heterogeneity, the hostile tumor microenvironment, and barriers to CAR-T cell homing, toxicity, and persistence are needed for further advancement of the field.

Key words: CAR-T cells; preclinical models; solid tumors; translational research

Introduction

Chimeric antigen receptors (CARs) are synthetic T cell receptors composed of an extracellular single-chain variable fragment (scFv) derived from the variable heavy and variable light domains of a given antibody, fused to transmembrane and intracellular signaling domains, which are derived from components sufficient for activation of the native T cell receptor. Clinical manufacturing of CAR-T cells involves the collection of autologous lymphocytes (which are usually obtained from the peripheral blood by leukapheresis), activation, and expansion with agonistic antibody-coated beads and cytokines, cell transduction with retroviral virions carrying the CAR, an expansion phase, and finally reinfusion into the patient. The advantages of CAR-T cells to other cellular approaches are (1) MHC-independence, (2) in cis costimulatory and activation activity, and (3) expanded targets for tumor recognition that go beyond peptide detection to include recognition of post-translational modifications of surface proteins and lipids.

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Human CAR Clinical Successes in Leukemia/Lymphoma

Since the first concept of engineering CAR-T cells,[1,2] continuous improvements, optimizations, and joint partnerships between the pharmaceutical industry and academia have led to successful multicentric phase II trials, where heavily pretreated patients with various hematological tumors experienced benefits of tumors response, improvement to quality of life, and overall survival. Pediatric B cell malignancies (young adult/pediatric leukemia (B-ALL)) were first targeted with CAR-T cells specific for a B cell lineage marker CD19.[3–5] Thereafter, anti-CD19 CAR-T cells were evaluated in adult patients with high-grade diffuse large B cell lymphomas previously refractory to standard therapies.[6] As examples of the therapeutic potency, adult patients with diffuse large B cell lymphomas and follicular lymphoma experienced over 80% response rates and long-lasting remissions, even in patient populations with predicted overall survival not longer than a few weeks with existing standard management. These compelling findings led the U.S. Food and Drug Administration to grant the approval for Kymriah–tisagenlecleucel and Yescarta-axicabtagene in August and October of 2017, respectively.

Cellular Therapies for the Treatment of Infectious Diseases

The therapeutic potential of cytotoxic T cells is not limited to only malignancies, but significant impacts are also possible for the treatment of viral infections. Immunocompromised patients post allogeneic stem cell or cord transplant develop potential life-threatening viral infections, most notably due to cytomegalovirus (CMV). Catherine Bollard and colleagues provided proof of safety and efficacy of adoptively transferred donor-derived, virus-specific T cells in preventing viral infections post transplant.[7,8] In fact, this research allowed the isolation of MHC class I restricted CMV-specific T cell clones from related donors that recognize structural proteins of the virus, such as CMVpp65. As a result, naïve T cell transfers from seronegative healthy donors were able to provide long-term CMV/EBV-free protection.[9,10]

The development of gene-edited autologous CD4 T cells for adoptive transfer showed promise as well for the treatment of aviremic HIV patients undergoing active antiretroviral therapies.[11] In first efforts to reduce the expression of CCR5, the major co-receptor responsible for HIV cellular entry, researchers provided ex-vivo CD28 costimulation of patient T cells before adoptive transfer, which showed improved CD4 T cell functionality and persistence in HIV-infected patients.[12] The advent of gene editing technology, such as zinc finger nucleases (ZFNs) and now CRISPR/Cas9, has provided cellular engineers the ability to permanently disrupt genes of therapeutic interest. Tebas et al treated 12 aviremic patients with ZFN-modified autologous CCR5-negative CD4 T cells. One single infusion of 10 billion cells, in which 10% to 28% were ZFN-modified, allowed safe interruption of antiretroviral therapies with a significant decrease in HIV viral load in the peripheral blood and sustained CD4 T cell counts.[13] This technology could also be applied to other cellular receptors that are necessary for viral entry, such as CXCR4.

Both virus-specific and gene-edited T cell therapies have been explored in different clinical settings, and demonstrable responses provide much promise for their future development and use in the coming years.

Human CAR Clinical Challenges

The aforementioned trials uncovered information about the toxicity profile of this new class of therapy. For anti-CD19 CAR-T cell therapy, an expected side effect is B cell aplasia, which is due to the elimination of all cells expressing CD19, a pan-B cell marker that is not tumor specific. This side effect was observed in preclinical, syngeneic mouse models evaluating the efficacy of anti-CD19 CAR-T cells. However, other side effects observed clinically were unexpected, including cytokine release syndrome (CRS), macrophage activation syndrome, and transient and fatal neurotoxicities.

Cytokine Release Syndrome

CRS is a complex, potentially life-threatening phenomenon that involves hypotension, acute vascular leak, high fever, consumptive coagulopathy, and sometimes organ failure with hepatic, renal, cardiac, dermatologic, and GI dysfunctions or acute respiratory distress. These clinical manifestations require intensive care management in the acute phase (generally within the first week after CAR-T cell infusion), pressor support for hypotension, intubation and ventilation support for respiratory distress, anti-IL-6 agents, and high-dose steroids.[3,14] The imputability of IL-6 as the hallmark cytokine in CRS was described after comparative cytokine analysis with clinical biomarkers established its predictive value for CRS in a cohort of 51 patients treated with Kymriah.[15] Unmodified, endogenous macrophages, more than CAR-T cells, secrete high concentrations of IL-1 and IL-6 and are considered responsible for the occurrence of CRS. Preclinical data suggest IL-1Rα blockade may be a potential therapy to protect anti-CD19 CAR-T patients from CRS.[16,17]

Tocilizumab, the first humanized monoclonal antibody counteracting IL-6, received FDA approval in 2017 for the treatment of CRS. It was initially used as an immunosuppressing agent in various autoimmune diseases, such as rheumatoid arthritis,[18] systemic juvenile arthritis, or giant cell temporal arteritis. By blocking the IL-6 receptor, tocilizumab abrogates CRS but may also increase the level of circulating IL-6. For that reason, siltuximab, which binds soluble IL-6, has been used in CAR-T trials for brain tumors and has the advantage of not relying on brain penetration for effectiveness.[19]

Macrophage Activation Syndrome

Macrophage activation syndrome (MAS) derives from the uncontrolled proliferation of lymphocytes and mature macrophages typically seen in autoimmune disorders. Accompanying markers are pancytopenia, high levels of bilirubin and creatinine, ferritinemia, low fibrinogen, and bone marrow hemaphagocytosis.[20] Management of MAS symptoms in CAR-T cell patients is the same as CRS.[21] Interestingly, recent preclinical evidence implicated the proinflammatory cytokine IL-18 in the pathogenesis of MAS using a genetically engineered mouse model, which opens new opportunities for biomarker prediction and therapeutic targeting.[20,22] Better CAR-T cell expansion and persistence correlates with durable antitumor activity, and this desirable feature can be facilitated by engineering CAR-T cells to constitutively secrete IL-18.[23] However, thorough preclinical evaluation of this next-generation CAR-T approach and its potential to induce MAS is needed before translation into clinical investigation.

Neurotoxicities

Neurotoxicities observed in the anti-CD19 CAR-T cell trials varied from clinical manifestations of confusion, loss of consciousness,

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ataxia, aphasia, and epilepsy to critical clinical encephalopathy (CAR-T cell related encephalopathy syndrome) and acute brain edema. Initial pathophysiological interpretation of these neurological symptoms implicated heavy trafficking of T cells into the brain[14,24] and/or passage of inflammatory cytokines (mainly IL-6) through the blood brain barrier as contributing factors.[25,26] Recent evidence identified CNS endothelial activation through secretion of integrin and angiopoetin-2, which triggers enhanced pericyte activation and endothelial permeabilization, as major mechanisms for initiation of the fatal neurotoxicities after CD19 CAR-T cell therapies.[27] This event mediates local destruction of the blood brain barrier, which may explain the bleedings and edema observed in the clinic.

The causative role that a variety of factors related to CAR-T cell treatment play in the induction of neurotoxicity still remains to be elucidated. These factors include CAR-T cell interaction with bystander cells, such as microglia or endothelial cells, the recruitment of endogenous T cells, the cytotoxic cytokines responsible for neuronal disruption, and the contributions of the CAR costimulatory domain or the targeted antigen.

Antigen Escape.

The eradication of target-expressing tumor cells is the ultimate objective of CAR-T cell therapy. This event is usually achieved in the context of the B-cell malignancies by anti-CD19 CAR-T cells.[3,14,28,29] However, antigen escape following CAR-T cell therapy has been a frequent occurrence in patients and has been demonstrated by 2 different mechanisms. First, heterogeneous populations of tumor cells may exist in which the targeted antigen is not ubiquitously expressed, and targeted depletion of antigen-positive cells allows enrichment of antigen-negative cells after CAR-T cell therapy. This type of relapse may also include tumor cells expressing alternative splice variants of the targeted antigen, resulting in the expression of truncated protein.[30] The second mechanism of antigen escape are epigenetic or posttranslational modifications that result in phenotype switching, as reported after CD19 CAR-T cells[31] or antibody-drug conjugate therapy.[32] To overcome antigen escape, it will be necessary to develop bi- or multidirectional CAR platforms.

On-Target and Off-Target Toxicities.

On-target toxicity refers to toxicity against healthy tissues bearing the targeted antigen. Besides the expected B-cell aplasia that occurs after CD19-directed CAR-T cell therapy, the most cited T cell immunotherapy-related examples of on-target toxicity are fatalities that occurred after treatment of patients with melanoma and esophageal cancer with a transgenic TCR T cell therapy targeting MAGE-A3,[33] which triggered a fatal necrotizing leukoencephalopathy, or a fatality that occurred after the treatment of a colon cancer patient with a third-generation CAR targeting ERBB2 (Her2/neu), which recognized low-level Her2 expression in the lungs and caused pulmonary distress, alveolar damage, and multiple organ failure.[34] In both cases, high-affinity binding by the TCR or the CAR may have allowed recognition of very low-level target expression in the brain and lung to trigger these toxicities. A critical feature to consider in the design of variable domains of TCRs and the scFv domain for CARs is the target affinity. When scFv affinity is increased in certain CAR molecules, very low levels of antigen expression in normal tissues can trigger CAR-T cell stimulation and may potentiate life-threatening inflammation and damage, such as encephalitis caused by a high-affinity anti-GD2 CAR.[35]

Off-target toxicity is not due to the expected cytotoxic effect of CAR-T cells upon target recognition but on cross-reactive binding to a mimotope,[36] which is an epitope that mimics the binding site of the targeted epitope.[37,38] To date, these have only been demonstrated for TCR-engineered T cells and not for CAR-T cells, but it may also be possible for CAR scFvs to recognize unintended targets. For more details of the reported on-target and off-target toxicities by engineered T cell therapies, the reader can refer to the following references.[39,40] The field is rapidly evolving with multiple novel safety switch constructs to mitigate these toxicities in the future.[41]

Preclinical Investigation Of CAR-T Cells In Mouse Models

The Declaration of Helsinki, which outlines the ethics for medical research in humans, states that investigation of new therapeutics must be based on "adequate laboratory and, as appropriate, animal experimentation." To this end, animal models (primarily mouse models) have been instrumental in the preclinical investigation and development of CAR-T cell therapies. However, there are both advantages and disadvantages with each model, and these have determined what we have been able to learn about CAR-T cell therapies throughout preclinical development. As will be addressed later, the field is now generating new models for investigating some of the toxicities associated with CAR-T cells that occurred unexpectedly in human patients and were not predicted from preclinical models. Here, we will review the animal models that have been used for CAR-T cell development and the benefits and deficits of those models.

Immunocompetent (Syngeneic/Transgenic) Mice

Inbred, immunocompetent mice have been useful for investigating the efficacy of CAR-T cells against a specific antigen. However, low protein homology between murine and human tumor-associated antigens is a limitation for translation of murine-specific CAR-T cells into human clinical trials. Similarly, murine anti-CD19 CAR-T cells demonstrated aplasia of both normal splenic B cells and κ-light chain positive 38c13 murine lymphoma cells in C57BL/6 mice when mice were sacrificed and observed 63 days post T cell infusion.[42] Unfortunately, engineered murine T cells do not demonstrate substantial persistence in immune competent mice; this persistence can be enhanced through lymphodepletion or whole body irradiation of the mice prior to T cell infusion. One explanation for the difference in the in vivo persistence of human and mouse CAR-T cells may be a polymorphism in the cytoplasmic tail of CD28, which is required for CD28-induced NFκB activation in human T cells and is deficient in mouse T cells.[43] This polymorphism is often also included in the cytoplasmic tail of murine CAR-T cells and may influence the lack of T cell persistence observed in immunocompetent mouse models. Importantly, immunocompetent mouse models have also provided evidence of antigen spread, where EGFRviii-targeting CAR-T cells eliminated EGFRviii-positive glioma cells, and cured mice were protected from tumor growth after rechallenge with EGFRviii-negative glioma cells.[44] These data demonstrate that CAR-T cells, at least in mice, can enhance the host immune response against additional tumor antigens after an initial cytolysis by engineered T cells.

A strategy to evaluate targeting of human tumor-associated antigens in syngeneic models is the generation of transgenic tumor cell lines or transgenic mice. For example, in the first animal model investigating CAR-T cells against a tumor-

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associated antigen, Hwu and colleagues inoculated C57BL/6 mice with 1 million cells of the mouse sarcoma line 24JK that has been transduced to express human alpha-folate receptor through intravenous injection.[45] The mice were treated 3 days later with a single dose of 27 million murine lymphocytes that were either nontransduced or retrovirally transduced with a CAR transgene targeting human alpha-folate receptor and 9 doses of IL-2. Eight days after T cell infusion, the average number of lung tumors in the mice was significantly reduced if the mice were treated with alpha-folate receptor targeting CAR-T cells compared with mice treated with nontransduced T cells (13 tumors per mouse vs 195 tumors per mouse). In a more recent model, Pennell and colleagues utilized a transgenic mouse model expressing human CD19 in a lineage-restricted manner and demonstrated that murine CAR-T cells targeting human CD19 cause B cell aplasia and acute toxicities.[46] The benefit of this model, although not utilizing human T cells that may outperform the function of murine T cells in vivo, is the evaluation of targeting a human antigen in the context of normal organismal expression; this allows researchers to investigate both the efficacy of the CAR-T cells and any potential toxicology. As a reminder to readers, acute toxicities of anti-CD19 CAR-T cells were first observed clinically and not in preclinical models.

An under-utilized preclinical model that falls in the category of immunocompetent mice are genetically engineered mice that develop spontaneous, autochthonous tumors. In this regard, induction of tumorigenic transgenes, such as $KRAS^{G12D}$, or loss of tumor suppressors, such as TRP53, with either the injection of a virus producing Cre recombinase[47] or under the control of a tissue-specific Cre, such as Pdx1-Cre,[48] can give rise to large lung or pancreatic ductal adenocarcinomas in immunocompetent mice. The speed of tumor formation in these models may not accurately reflect tumorigenesis in humans because recombinase activity induces multiple tumor-initiating cells at the same time, but the autochthonous growth of the tumor(s) allows investigators to study extratumoral factors that inhibit immunotherapy, such as neovascularization and stromal involvement. These models have been used to evaluate treatment with transgenic mesothelin-specific TCR-expressing murine T cells[49] but have yet to be utilized to study CAR-T cell efficacy and toxicities. A beautiful synergy may come later in the generation of spontaneous, autochthonous models with transgenic expression of human tumor-associated antigens.

Immunocompetent mice can also be useful to model the synergistic effect of therapeutic combinations, such as immune checkpoint blockers, conventional targeted therapies, or classic anti-mitotic drugs, which may enhance the efficacy of CAR-T cells[50] and can only be modeled in hosts with intact immune systems.

For detailed listing of advantages and drawbacks of this model in a broader context of cancer immunology, the reader can refer to the following reference.[51]

Immunodeficient (Xenograft/PDX) Mice

Immunodeficient mice provided the majority of preclinical data of CAR-T cells prior to translation into human clinical trials.[52,53] Immunodeficiency allows sufficient engraftment of xenotransplanted tumor cells and lymphocytes without great potential for rejection by the host immune system, given that these mice lack either some or almost all adaptive immune cell populations. These models have allowed for comparison of human CAR-T cell persistence in mice, and differences observed between CAR signaling variants have been upheld in human clinical evaluations.[54] The most utilized mouse model in this regard is the genotype NOD, $Prkdc^{scid}$ (loss of function mutation of the *prkdc* gene responsible for the defective recombination of the heavy/light chain of the B cell receptor and alpha/beta chain of the TCR), and $Il2rg^{null}$ (knockout of IL2 receptor common gamma chain) (NSG). NSG mice lack T cells, B cells, and NK cell function but also bear defective dendritic cells, macrophages, and multiple cytokine/chemokine signaling pathways.[55] An alternative model of immunodeficiency is SCID beige ($Prkdc^{scid}$, *Lystbg-J*). SCID beige mice have severe B and T cell lymphopenia, inferior NK cell activity compared with other SCID mice, but functional monocytes and macrophages. This last feature has proved useful to study CRS.[16,56] In these models, CAR-T cell activity induces endogenous macrophages to produce IL-6; prior macrophage depletion ameliorates acute toxicity and IL-6 production. Previous studies of CRS were conducted using NSG mice,[57] which have maturation and functional defects in the myeloid lineage and an impaired response of recipient's monocytes and macrophages to IL-1 and IFNγ stimulation. Moreover, Giavridis et al suggested that CAR-T cells activate the monocyte lineage compartment through a variety of cell surface receptors, such as the CD40 antigen, and cytokines, such as IFN-γ, macrophage inflammatory protein 1α, and granulocyte macrophage colony stimulating factor.[16] This cell-cell interaction in turn triggers inducible nitric oxide synthase in macrophages responsible for IL6 and IL1 secretion.

Xenografts allow researchers to evaluate the efficacy of CAR-T cells targeting human tumor-associated antigens utilizing the engraftment of either human tumor cell lines or freshly resected tumor samples, known as patient-derived xenografts (PDX). PDX models are thought to increase the xenografted tumor complexity, because the tumors may also include cellular and physical components of the TME.[58] However, progressive replacement of the TME by murine inflammatory and stromal cells, murine cytokines, chemokines, and angiogenic structures may significantly alter the original tumors in these models.[59]

Humanized Mice

Immunodeficient animal models are limited in their ability to interrogate interactions between the innate and adaptive arms of the immune system. In this regard, humanized mouse models, reconstituted with human CD34+ cells in their simplest format, can overcome these obstacles. More complex humanized models involve genetic modification of the mouse strains to allow secretion of human cytokines, such as the MISTR and MISTRG mice, that facilitate better engraftment of innate immune cells from normal hematopoeisis.[60] In recent work, CAR-T cells produced from peripheral blood lymphocytes of humanized mice generated acute toxicities that phenocopy the CRS seen in clinical trials leading to identification of the best model of CRS to date.[17] Anti-CD19 CAR-T cells, developed from humanized mice, were infused into either nonhumanized (no myeloid cells) or humanized (high number of myeloid cells) mice. Only the humanized mice developed severe CRS, and ablation of monocytes in these mice through treatment with anti-CD44v6 CAR-T cells prevented the development of CRS, confirming the critical role of monocytes and macrophages as IL-6/IL-1 secreting cells of origin.

An important concern for clinical translation of significant preclinical findings is the inability of traditional mouse models to properly determine the safety of anti-human cytotoxic T cell therapies. Infamous cases of unpredicted toxicities include, but

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are not limited to, a fatal lung toxicity and multiple organ failure reportedly due to a Her2-28BBz CAR,[34,61] previously investigated in SCID mice, and a case of neurotoxicity with the MAGE-A3 TCR-T cell therapy,[33] which was not predicted during the preclinical development of the TCR in HLA-A*0201 transgenic mice. These unfortunate and unpredicted events demonstrate the potential irrelevance and unreliability of safety data stemming from mouse studies. Similarly, mouse models can overestimate the efficaciousness of potential therapeutics, which may be due to the use of common, homogenous human cell lines that do not recapitulate the heterogeneity of spontaneous tumors, as already extensively described.[62] Also, the immune environment of the immunocompromised or humanized mice may lack significant value due to missing adaptive and innate interactions, as mentioned previously in the discussion of CRS modeling in immunodeficient mice. Additionally, the scale of work in mice is daunted by the inability to acquire and manufacture large quantities of cells from leukapheresis, which can be performed in large animal models such as dogs and nonhuman primates (NHPs).

The value of a model is limited by what the model can deliver. To infer important information about CAR-T cell biology and to improve the efficacy and safety of these therapies, we must ask better questions and develop better models. Simply put, the road to future CAR therapies cannot be solely paved by additional mouse models.

Preclinical Advancements Of CAR-T Cells from Dog Models

Until recently, mouse models have not provided researchers with the ability to demonstrate and interrogate CRS or neurotoxicities from CAR-T cells. Additionally, it is still difficult to model on-target, off -tumor toxicities due to a lack of significant tumor-associated antigen protein homology between mice and humans and the absence of relevant transgenic mouse models. Lastly, the sterile and controlled environment of experimentation in mice provides data for CAR-T cell efficacy only in the context of specific effector-to-target ratios and on tumor burdens with limited variance; this limited scope of disease does not fully represent the variation of disease presentation in humans. These limitations require the identification of more relevant preclinical models to investigate cancer immunology. The potential benefits of developing better models are reduction in clinical research costs and increased correlation between positive results in preclinical experimentation and success in human clinical trials. For this reason, we and others believe that immunocompetent pet dogs with spontaneous tumors provide a parallel patient population of high relevance to study cancer immunology and cellular immunotherapy. This concept has been supported by field leaders[63,64] and was reinforced at the U.S. National Academy of Medicine's National Cancer Policy Forum held in 2015[65] and in 2017 by the inclusion of canine cancer immunotherapy clinical trials and correlative studies in the Cancer Moonshot Initiative.

Dogs frequently develop spontaneous cancers, with an incidence rate of 5300 per 100 000 canines, a rate approximately 10 times higher than humans.[66] Unlike mice, dogs represent an outbred population, although pronounced breed dispositions to certain cancer types exist, most likely due to selective inbreeding for desired phenotypic traits. Malignancies that occur spontaneously in canines include lymphoma; hemangiosarcoma; osteosarcoma; soft tissue sarcomas; melanoma; mammary, prostate, and squamous cell carcinomas; urothelial carcinoma; and glioma. Many of these tumors share the same histologies, oncogenic driver mutations, or chromosomal translocations as their human counterparts.[67–70] They also exhibit similar clinical and biological behavior to human patients, including chemoresistance, recurrence, and metastases, providing a parallel patient population in which to evaluate the ability of genetically engineered cells to safely provide durable remissions or cures. Furthermore, canine tumors frequently exhibit an immune suppressive microenvironment with regulatory T cells, myeloid-derived suppressor cells, and tumor-associated macrophages serving to inhibit natural and induced antitumor immunity[71–76] Importantly, environmental cues and the microbiome are also shared between humans and their pet dogs,[77] and these factors may influence tumor initiation, progression, and response to immune therapy.[78] Finally, current treatments for canine cancers include chemotherapy, radiation therapy, and surgery, providing a parallel system for evaluating combination therapies that accurately reflect those that could be employed in the human clinic. Together, these findings indicate that the pet dog is relevant for the study of tumorigenesis, the safety and efficacy of immune-directed therapies, and the identification of correlative biomarkers that together may serve to inform human clinical trial design. Historically, canines were important in the validation of haploidentical hematopoietic stem cell transplantation, which is extremely relevant to the progression of allogenic and "universal" CAR-T cell approaches.[79–82]

Pioneering marrow-grafting studies in canines led by E. D. Thomas and colleagues were instrumental in the progress achieved for the treatment of leukemias in the clinic. This model provided invaluable insights on the metrics of success for allogeneic marrow transplant engraftment. In fact, histocompatibility typing in the dog with the detection of dog leukocyte antigen and successful marrow engraftment between dog littermates led the way for bone marrow transplantation in humans with donor-recipient–matched HLA.[83–86]

Moreover, the practical details of marrow pheresis, isolation, cryopreservation protocols,[87] conditioning regimes,[88] and graft versus host reactivity prophylaxis (methotrexate, anti-thymocyte serum)[89,90] used in the clinic have been possible thanks to research in dogs.

In summary, research on large animals, and importantly canines, has explained how the immune system can accept or reject grafts and also demonstrated the curative potential against malignancies, which has paved the way for modern era cancer immunotherapy.

Advantages of Dogs Over Mice

Canine cancer patients provide the opportunity to study acute toxicities of CAR-T cells in the spontaneous tumor setting and in the presence of an intact immune system. Functional interactions between the adaptive and innate immune compartments have been necessary to demonstrate CRS in mice; however, these were models that were developed in retrospect. Canines provide the opportunity to study these toxicities in relevant clinical settings where immune and nonimmune system networks are intact. Furthermore, specialists in clinical veterinary medicine and veterinary pathology provide expert clinical and pathological evaluations of canine patients and patient biopsies providing better recognition of adverse effects. For example, evaluation of canines using clinical motor and sensory tests together with advanced MRI imaging and CSF/brain biopsy can be performed to identify CAR-T–related neurotoxicity. Lastly, as mentioned above, the protein homology of tumor-associated

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antigens between canines and humans in some cases allows the utilization of cross-reactive scFvs to determine efficacy and safety in a clinically relevant large animal model.[91–94]

Anti-Tumor Activity and Rejection of RNA CAR-T Cells

In recent proof-of-concept studies, canine T cells were expanded ex vivo with manufacturing platforms that mirror clinical expansion of human T cells and electroporated with mRNA encoding for a canine CAR targeting CD20.[91] The resulting anti-CD20 canine CAR-T cells demonstrated selective target recognition and specific lysis of tumor cells. Infusion of autologous CD20 targeted mRNA CAR-T cells in a dog with spontaneous lymphoma temporarily stunted tumor growth. However, the development of Canine Anti-Mouse Antibodies occurred, presumably directed against the murine anti-canine CD20 scFv, recapitulating a key mechanism of CAR-T cell rejection that has also been reported in humans following repeated injections of mesothelin-specific CAR-T cells.[95] These findings have fueled efforts to humanize CAR scFv components to overcome this obstacle. Similar strategies to develop canine scFvs to enhance durable persistence of CAR-T cells in canines are also underway.

Challenges to Advance the Model

Despite these first encouraging results, practical hurdles still exist for canine CAR-T cell production that must be overcome. First, genetic engineering of canine T cells is less developed than for human T cells, and transduction with lentiviral vectors in canine cells must be optimized. Canines are not known to be susceptible to lentiviral infection, unlike humans, NHPs, and felines, and it may be important to investigate whether resistance mechanisms exist in canine T cells. Similarly, there is a requirement to develop new reagents for both correlative studies and translational studies in canines; for instance, anti-human and anti-mouse CD19 antibodies do not cross-react with canine CD19 and canine-specific CD19 monoclonal antibodies are not commercially or publically available.

Preclinical Advancements Of CAR-T Cells From NHP Models

The NHP is a unique model that provides high translational relevance to humans for safety evaluations because of significant protein homology, but primates are most commonly used as a late-stage validation step of investigation. For example, a cross-reactive CD171-specific CAR-T cell therapy was tested in a rhesus macaque trial to evaluate possible toxicity after infusion of high-dose CAR-T cells (which would not be administered in humans). The limitation of this advanced model for CAR-T cell evaluation is the inability to assess antitumor efficacy, because most NHP models are deficient of tumor.

Model of Neurotoxicity and Cytokine Release Syndrome

Another example of the modeling CAR-T cell-induced neurotoxicity and CRS was recently performed by Taraseviciute and colleagues in NHP.[26] In these experiments, rhesus anti-CD20–specific CAR-T cells were infused into rhesus macaques (n = 3) and the macaques were observed for B cell aplasia and symptoms of acute toxicities. Post infusion, the macaques presented neurological symptoms similar to those observed in humans and exhibited CRS characterized by elevated serum levels of IL-6, IL-8, IL1RA, MIG, and CXCR11. This study provided new information that CAR-T–induced neurotoxicity is associated with high concentrations of IL-6, IL-2, and granulocyte macrophage colony stimulating factor in the cerebral spinal fluid and an increased T cell migration in the brain parenchyma.

Other Tissue-Specific Toxicities

An advantage in using NHP as a preclinical CAR-T cell toxicology model is the conservation of homology with many cell surface markers. For example, Berger et al investigated the orphan tyrosine kinase receptor (ROR1) as a potential target for ROR+ malignancies and treated 2 healthy rhesus macaques with second-generation CAR-T cells with an equal ratio of CD4 and CD8 cells. The infusion of this autologous T cell product demonstrated a lack of toxicity, notably in the pancreas and adipocytes where low levels of ROR1 expression is observed, even at supra-therapeutic doses.[96]

Persistence

In a SHIV model of viral infection, pigtail macaques were treated with hematopoietic stem cells transduced with CAR targeting the viral envelope protein gp120.[97] The engineered T cells exhibited long-term persistence, accumulation in lymphoid tissues, decreased viral load, and increased CD4/CD8 ratios in the gut. These effects were attributed to increased engraftment of the engineered progenitor cells. It is assumed that persistence in these NHP models will be predictive of responses in human trials given the evolutionary conservation. Respective to CAR-T cells derived from peripheral blood T cells, there are several factors that are thought to affect the in vivo persistence, including exhaustion, memory phenotypes, and lymphodepletion strategies. Many of these factors have been investigated in mouse models and will likely be evaluated in dogs in the future. Berger and colleagues have developed several NHP models to also address questions pertaining to exhaustion and function of adoptively transferred T cells, the potential benefit of T cell memory enrichment, as well as the impact different preconditioning regimens have on T cell persistence, albeit in the absence of spontaneously arising tumors.[98]

T Cell Expansion

Ex vivo T cell expansion in primates has been studied mainly for its application for HIV treatment. Various protocols allow the expansion of large numbers of autologous T cells,[99,100] and the development of such methods is crucial for implementation of further trials in primates. Using the rhesus macaque model, researchers recently reported testing the function, expansion, and persistence of gene-edited CD33-knockout CD34+ cells.[101] The authors treated 2 healthy macaques and demonstrated feasibility of HSPC mobilization, apheresis, autologous gene-edited T cell transplantation, and total body irradiation with rigorous clinical and biological follow-up.

Despite the informative resource of the NHP research, there are several obstacles to consider that make broad application of this model difficult. First, accessibility to NHP is restricted to a few centers and their maintenance and housing are expensive. Also, given the potential for high grade toxicities, such as CRS and neurotoxicity, post adoptive transfer of genetically modified T cells, the need for specialized veterinary intensive care is crucial. Finally, the lack of spontaneous tumors in primates is a major obstacle to the study of therapeutic efficacy. The latter point is a major difference with the dog model, which has a high incidence of spontaneous liquid and solid tumors, as mentioned previously. As a result, the NHP model remains highly demanding in terms of resources, expertise, and availability

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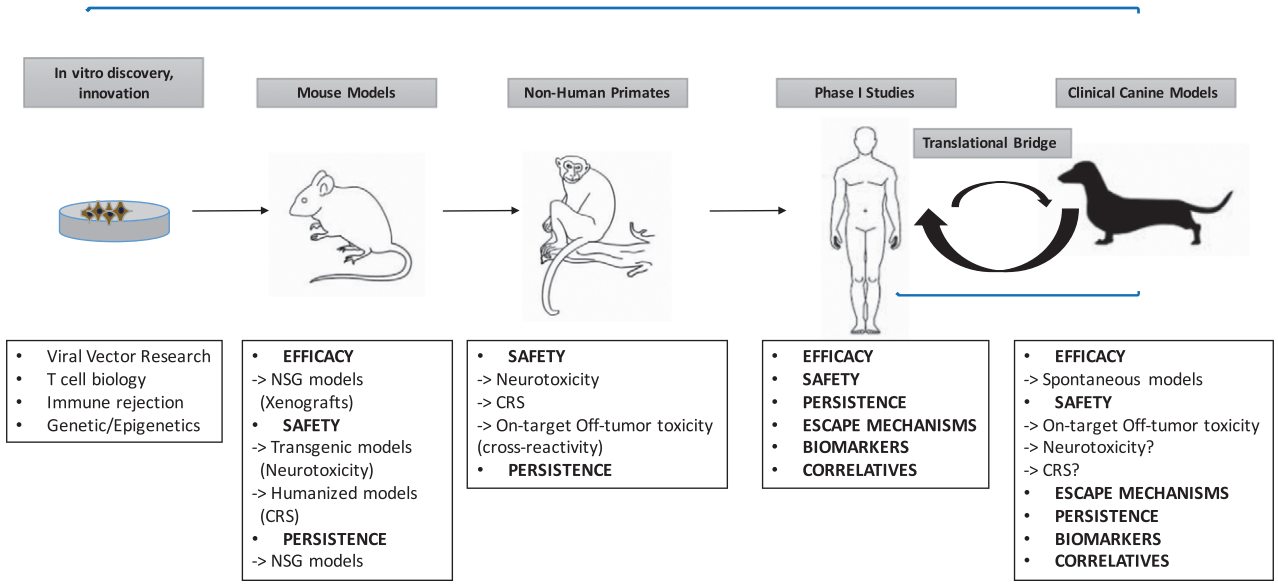

Figure 1. Representative steps of preclinical therapeutic validation and translation into human studies, including the advantages of each model system, as well as potential feedback through clinical canine models.

and provides little prediction of translational expectations outside of high-level safety data.

Conclusions

A variety of preclinical models exist for the investigation of CAR-T cell efficacy and safety profiles, including mouse models, spontaneous dog models, and NHP models. Unfortunately, the lack of appropriate models to study CAR-T cell side effects was not fully recognized prior to the first-in-human studies, and improved preclinical models were developed in retrospect. There remain many challenges in this rapidly growing field, which include the absence of models to interrogate the mechanisms of persistence and rejection of gene-edited "universal" CAR-T cells. Development and validation of models that enable relevant investigation of CAR-T cell homing and penetration into solid tumors, the inhibitory effects of the TME on CAR-T cell function and survival, and in vivo CAR-T cell metabolism will go a long way to accelerating the translation of new and improved next-generation CAR-T cell therapies into the human clinic. In the personal opinion of the authors, clinical studies in canines with spontaneous tumors add an additional immune competent preclinical model for CAR-T cells and present the best opportunities to gain relevant efficacy, toxicity, and correlative data for rapid translation into human trials (Figure 1).

References

1. Gross G, Waks T, Eshhar Z. Expression of immunoglobulin-T-cell receptor chimeric molecules as functional receptors with antibody-type specificity. *Proc Natl Acad Sci USA*. 1989; 86:10024–10028.
2. Eshhar Z, Waks T, Gross G, et al. Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the gamma or zeta subunits of the immunoglobulin and T-cell receptors. *Proc Natl Acad Sci USA*. 1993;90:720–724.
3. Maude SL, Frey N, Shaw PA, et al. Chimeric antigen receptor T cells for sustained remissions in leukemia. *N Engl J Med*. 2014;371:1507–1517.
4. Maude SL, Laetsch TW, Buechner J, et al. Tisagenlecleucel in Children and Young Adults with B-Cell Lymphoblastic Leukemia. *N Engl J Med*. 2018;378:439–448.
5. Park JH, Riviere I, Gonen M, et al. Long-Term Follow-up of CD19 CAR Therapy in Acute Lymphoblastic Leukemia. *N Engl J Med*. 2018;378:449–459.
6. Neelapu SS, Locke FL, Bartlett NL, et al. Axicabtagene ciloleucel CAR T-cell therapy in refractory large B-cell lymphoma. *N Engl J Med*. 2017;377:2531–2544.
7. Cruz CR, Micklethwaite KP, Savoldo B, et al. Infusion of donor-derived CD19-redirected virus-specific T cells for B-cell malignancies relapsed after allogeneic stem cell transplant: a phase 1 study. *Blood*. 2013;122:2965–2973.
8. Hanley PJ, Cruz CR, Savoldo B, et al. Functionally active virus-specific T cells that target CMV, adenovirus, and EBV can be expanded from naive T-cell populations in cord blood and will target a range of viral epitopes. *Blood*. 2009;114:1958–1967.
9. Hanley PJ, Melenhorst JJ, Nikiforow S, et al. CMV-specific T cells generated from naive T cells recognize atypical epitopes and may be protective in vivo. *Sci Transl Med*. 2015;7: 285ra63.
10. Bollard CM, Gottschalk S, Torrano V, et al. Sustained complete responses in patients with lymphoma receiving autologous cytotoxic T lymphocytes targeting Epstein-Barr virus latent membrane proteins. *J Clin Oncol*. 2014;32: 798–808.
11. Perez EE, Wang J, Miller JC, et al. Establishment of HIV-1 resistance in CD4+ T cells by genome editing using zinc-finger nucleases. *Nat Biotechnol*. 2008;26:808–816.
12. Levine BL, Bernstein WB, Aronson NE, et al. Adoptive transfer of costimulated CD4+ T cells induces expansion of peripheral T cells and decreased CCR5 expression in HIV infection. *Nat Med*. 2002;8:47–53.
13. Tebas P, Stein D, Tang WW, et al. Gene editing of CCR5 in autologous CD4 T cells of persons infected with HIV. *N Engl J Med*. 2014;370:901–910.
14. Grupp SA, Kalos M, Barrett D, et al. Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. *N Engl J Med*. 2013;368:1509–1518.

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15. Teachey DT, Lacey SF, Shaw PA, et al. Identification of predictive biomarkers for cytokine release syndrome after chimeric antigen receptor T-cell therapy for acute lymphoblastic leukemia. *Cancer Discov*. 2016;6:664–679.
16. Giavridis T, van der Stegen SJC, Eyquem J, et al. CAR T cell-induced cytokine release syndrome is mediated by macrophages and abated by IL-1 blockade. *Nat Med*. 2018;24(6):731–738.
17. Norelli M, Camisa B, Barbiera G, et al. Monocyte-derived IL-1 and IL-6 are differentially required for cytokine-release syndrome and neurotoxicity due to CAR T cells. *Nat Med*. 2018;24(6):739–748.
18. Gabay C, Emery P, van Vollenhoven R, et al. Tocilizumab monotherapy versus adalimumab monotherapy for treatment of rheumatoid arthritis (ADACTA): a randomised, double-blind, controlled phase 4 trial. *Lancet*. 2013;381:1541–1550.
19. O'Rourke DM, Nasrallah MP, Desai A, et al. A single dose of peripherally infused EGFRvIII-directed CAR T cells mediates antigen loss and induces adaptive resistance in patients with recurrent glioblastoma. *Sci Transl Med*. 2017;9(399):eaaa0984. doi: 10.1126/scitranslmed.aaa0984.
20. Daver N, Kantarjian H. Malignancy-associated haemophagocytic lymphohistiocytosis in adults. *Lancet Oncol*. 2017;18:169–171.
21. Neelapu SS, Tummala S, Kebriaei P, et al. Chimeric antigen receptor T-cell therapy—assessment and management of toxicities. *Nat Rev Clin Oncol*. 2018;15:47–62.
22. Kaplanski G. Interleukin-18: biological properties and role in disease pathogenesis. *Immunol Rev*. 2018;281:138–153.
23. Hu B, Ren J, Luo Y, et al. Augmentation of antitumor immunity by human and mouse CAR T cells secreting IL-18. *Cell Rep*. 2017;20:3025–3033.
24. Kochenderfer JN, Dudley ME, Carpenter RO, et al. Donor-derived CD19-targeted T cells cause regression of malignancy persisting after allogeneic hematopoietic stem cell transplantation. *Blood*. 2013;122:4129–4139.
25. Hu Y, Sun J, Wu Z, et al. Predominant cerebral cytokine release syndrome in CD19-directed chimeric antigen receptor-modified T cell therapy. *J Hematol Oncol*. 2016;9:70.
26. Taraseviciute A, Tkachev V, Ponce R, et al. Chimeric antigen receptor T cell-mediated neurotoxicity in nonhuman primates. *Cancer Discov*. 2018;8:750–763.
27. Gust J, Hay KA, Hanafi LA, et al. Endothelial activation and blood-brain barrier disruption in neurotoxicity after adoptive immunotherapy with CD19 CAR-T cells. *Cancer Discov*. 2017;7:1404–1419.
28. Porter DL, Hwang WT, Frey NV, et al. Chimeric antigen receptor T cells persist and induce sustained remissions in relapsed refractory chronic lymphocytic leukemia. *Sci Transl Med*. 2015;7:303ra139.
29. Porter DL, Levine BL, Kalos M, et al. Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. *N Engl J Med*. 2011;365:725–733.
30. Sotillo E, Barrett DM, Black KL, et al. Convergence of acquired mutations and alternative splicing of CD19 enables resistance to CART-19 immunotherapy. *Cancer Discov*. 2015;5:1282–1295.
31. Gardner R, Wu D, Cherian S, et al. Acquisition of a CD19-negative myeloid phenotype allows immune escape of MLL-rearranged B-ALL from CD19 CAR-T-cell therapy. *Blood*. 2016;127:2406–2410.
32. Fry TJ, Shah NN, Orentas RJ, et al. CD22-targeted CAR T cells induce remission in B-ALL that is naive or resistant to CD19-targeted CAR immunotherapy. *Nat Med*. 2018;24:20–28.
33. Morgan RA, Chinnasamy N, Abate–Daga D, et al. Cancer regression and neurological toxicity following anti-MAGE-A3 TCR gene therapy. *J Immunother*. 2013;36:133–151.
34. Morgan RA, Yang JC, Kitano M, et al. Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. *Mol Ther*. 2010;18:843–851.
35. Richman SA, Nunez–Cruz S, Moghimi B, et al. High-affinity GD2-specific CAR T cells induce fatal encephalitis in a preclinical neuroblastoma model. *Cancer Immunol Res*. 2018;6:36–46.
36. Geysen HM, Rodda SJ, Mason TJ. A priori delineation of a peptide which mimics a discontinuous antigenic determinant. *Mol Immunol*. 1986;23:709–715.
37. Cameron BJ, Gerry AB, Dukes J, et al. Identification of a Titin-derived HLA-A1-presented peptide as a cross-reactive target for engineered MAGE A3-directed T cells. *Sci Transl Med*. 2013;5:197ra103.
38. Linette GP, Stadtmauer EA, Maus MV, et al. Cardiovascular toxicity and titin cross-reactivity of affinity-enhanced T cells in myeloma and melanoma. *Blood*. 2013;122:863–871.
39. Bedoya F, Frigault MJ, Maus MV. The flipside of the power of engineered T cells: observed and potential toxicities of genetically modified T cells as therapy. *Mol Ther*. 2017;25:314–320.
40. June CH, Warshauer JT, Bluestone JA. Is autoimmunity the Achilles' heel of cancer immunotherapy? *Nat Med*. 2017;23:540–547.
41. Ren J, Zhao Y. Advancing chimeric antigen receptor T cell therapy with CRISPR/Cas9. *Protein Cell*. 2017;8:634–643.
42. Kochenderfer JN, Yu Z, Frasheri D, et al. Adoptive transfer of syngeneic T cells transduced with a chimeric antigen receptor that recognizes murine CD19 can eradicate lymphoma and normal B cells. *Blood*. 2010;116:3875–3886.
43. Porciello N, Grazioli P, Campese AF, et al. A non-conserved amino acid variant regulates differential signalling between human and mouse CD28. *Nat Commun*. 2018;9:1080.
44. Sampson JH, Choi BD, Sanchez–Perez L, et al. EGFRvIII mCAR-modified T-cell therapy cures mice with established intracerebral glioma and generates host immunity against tumor-antigen loss. *Clin Cancer Res*. 2014;20:972–984.
45. Hwu P, Yang JC, Cowherd R, et al. In vivo antitumor activity of T cells redirected with chimeric antibody/T-cell receptor genes. *Cancer Res*. 1995;55:3369–3373.
46. Pennell CA, Barnum JL, McDonald–Hyman CS, et al. Human CD19-targeted mouse T cells induce B cell aplasia and toxicity in human CD19 transgenic mice. *Mol Ther*. 2018;26:1423–1434.
47. Jackson EL, Willis N, Mercer K, et al. Analysis of lung tumor initiation and progression using conditional expression of oncogenic K-ras. *Genes Dev*. 2001;15:3243–3248.
48. Hingorani SR, Wang L, Multani AS, et al. Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. *Cancer Cell*. 2005;7:469–483.
49. Stromnes IM, Schmitt TM, Hulbert A, et al. T cells engineered against a native antigen can surmount immunologic and physical barriers to treat pancreatic ductal adenocarcinoma. *Cancer Cell*. 2015;28:638–652.
50. Smith TT, Moffett HF, Stephan SB, et al. Biopolymers codelivering engineered T cells and STING agonists can eliminate heterogeneous tumors. *J Clin Invest*. 2017;127:2176–2191.
51. Zitvogel L, Pitt JM, Daillere R, et al. Mouse models in oncoimmunology. *Nat Rev Cancer*. 2016;16:759–773.

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52. Johnson LA, Scholler J, Ohkuri T, et al. Rational development and characterization of humanized anti-EGFR variant III chimeric antigen receptor T cells for glioblastoma. *Sci Transl Med*. 2015;7:275ra22.
53. Brown CE, Starr R, Aguilar B, et al. Stem-like tumor-initiating cells isolated from IL13Ralpha2 expressing gliomas are targeted and killed by IL13-zetakine-redirected T Cells. *Clin Cancer Res*. 2012;18:2199–2209.
54. Milone MC, Fish JD, Carpenito C, et al. Chimeric receptors containing CD137 signal transduction domains mediate enhanced survival of T cells and increased antileukemic efficacy in vivo. *Mol Ther*. 2009;17:1453–1464.
55. Shultz LD, Lyons BL, Burzenski LM, et al. Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. *J Immunol*. 2005;174:6477–6489.
56. van der Stegen SJ, Davies DM, Wilkie S, et al. Preclinical in vivo modeling of cytokine release syndrome induced by ErbB-retargeted human T cells: identifying a window of therapeutic opportunity? *J Immunol*. 2013;191:4589–4598.
57. Singh N, Hofmann TJ, Gershenson Z, et al. Monocyte lineage-derived IL-6 does not affect chimeric antigen receptor T-cell function. *Cytotherapy*. 2017;19:867–880.
58. Byrne AT, Alferez DG, Amant F, et al. Interrogating open issues in cancer medicine with patient-derived xenografts. *Nat Rev Cancer*. 2017;17:632.
59. Delitto D, Pham K, Vlada AC, et al. Patient-derived xenograft models for pancreatic adenocarcinoma demonstrate retention of tumor morphology through incorporation of murine stromal elements. *Am J Pathol*. 2015;185:1297–1303.
60. Rongvaux A, Willinger T, Martinek J, et al. Development and function of human innate immune cells in a humanized mouse model. *Nat Biotechnol*. 2014;32:364–372.
61. Zhao Y, Wang QJ, Yang S, et al. A herceptin-based chimeric antigen receptor with modified signaling domains leads to enhanced survival of transduced T lymphocytes and antitumor activity. *J Immunol*. 2009;183:5563–5574.
62. Jamal–Hanjani M, Quezada SA, Larkin J, et al. Translational implications of tumor heterogeneity. *Clin Cancer Res*. 2015; 21:1258–1266.
63. Kalos M, June CH. Adoptive T cell transfer for cancer immunotherapy in the era of synthetic biology. *Immunity*. 2013;39: 49–60.
64. Mata M, Vera JF, Gerken C, et al. Toward immunotherapy with redirected T cells in a large animal model: ex vivo activation, expansion, and genetic modification of canine T cells. *J Immunother*. 2014;37:407–415.
65. LeBlanc AK, Breen M, Choyke P, et al. Perspectives from man's best friend: National Academy of Medicine's Workshop on Comparative Oncology. *Sci Transl Med*. 2016;8:324ps5.
66. Schiffman JD, Breen M. Comparative oncology: what dogs and other species can teach us about humans with cancer. *Philos Trans R Soc Lond B Biol Sci*. 2015;370.
67. Kirkness EF, Bafna V, Halpern AL, et al. The dog genome: survey sequencing and comparative analysis. *Science*. 2003; 301:1898–1903.
68. Breen M, Modiano JF. Evolutionarily conserved cytogenetic changes in hematological malignancies of dogs and humans—man and his best friend share more than companionship. *Chromosome Res*. 2008;16:145–154.
69. Richards KL, Motsinger–Reif AA, Chen HW, et al. Gene profiling of canine B-cell lymphoma reveals germinal center and postgerminal center subtypes with different survival times, modeling human DLBCL. *Cancer Res*. 2013;73:5029–5039.
70. Wang G, Wu M, Maloneyhuss MA, et al. Actionable mutations in canine hemangiosarcoma. *PLoS One*. 2017;12:e0188667.
71. Goulart MR, Pluhar GE, Ohlfest JR. Identification of myeloid derived suppressor cells in dogs with naturally occurring cancer. *PLoS One*. 2012;7:e33274.
72. Pinheiro D, Chang YM, Bryant H, et al. Dissecting the regulatory microenvironment of a large animal model of non-Hodgkin lymphoma: evidence of a negative prognostic impact of FOXP3+ T cells in canine B cell lymphoma. *PLoS One*. 2014;9:e105027.
73. Pinheiro D, Singh Y, Grant CR, et al. Phenotypic and functional characterization of a CD4(+) CD25(high) FOXP3(high) regulatory T-cell population in the dog. *Immunology*. 2011; 132:111–122.
74. Biller BJ, Guth A, Burton JH, et al. Decreased ratio of CD8+ T cells to regulatory T cells associated with decreased survival in dogs with osteosarcoma. *J Vet Intern Med*. 2010;24: 1118–1123.
75. Muir CF, Priestnall SL, Hibbert A, et al. Prevalence of FoxP3 (+) cells does not correlate with Ki67 expression in canine diffuse large B-cell lymphoma. *J Comp Pathol*. 2017;157: 15–22.
76. Sakai K, Maeda S, Yamada Y, et al. Association of tumour-infiltrating regulatory T cells with adverse outcomes in dogs with malignant tumours. *Vet Comp Oncol*. 2018;16: 330–336.
77. Coelho LP, Kultima JR, Costea PI, et al. Similarity of the dog and human gut microbiomes in gene content and response to diet. *Microbiome*. 2018;6:72.
78. Gopalakrishnan V, Spencer CN, Nezi L, et al. Gut microbiome modulates response to anti-PD-1 immunotherapy in melanoma patients. *Science*. 2018;359:97–103.
79. Panse JP, Bastianelli C, Santos EB, et al. Dog leukocyte antigen nonidentical unrelated canine marrow grafts: enhancement of engraftment by CD4 and CD8 T cells. *Transplantation*. 2003; 76:474–480.
80. Ladiges WC, Storb R, Raff RF, et al. Failure of anti-MHC antibodies to prevent GVHD in DLA mismatched unrelated canine marrow recipients. *Bone Marrow Transplant*. 1990;5:43–46.
81. Graves SS, Hogan W, Kuhr CS, et al. Stable trichimerism after marrow grafting from 2 DLA-identical canine donors and nonmyeloablative conditioning. *Blood*. 2007; 110:418–423.
82. Jochum C, Beste M, Zellmer E, et al. CD154 blockade and donor-specific transfusions in DLA-identical marrow transplantation in dogs conditioned with 1-Gy total body irradiation. *Biol Blood Marrow Transplant*. 2007;13:164–171.
83. Epstein RB, Storb R, Ragde H, et al. Cytotoxic typing antisera for marrow grafting in littermate dogs. *Transplantation*. 1968; 6:45–58.
84. Fefer A, Buckner CD, Thomas ED, et al. Cure of hematologic neoplasia with transplantation of marrow from identical twins. *N Engl J Med*. 1977;297:146–148.
85. Storb R, Rudolph RH, Thomas ED. Marrow grafts between canine siblings matched by serotyping and mixed leukocyte culture. *J Clin Invest*. 1971;50:1272–1275.
86. Thomas E, Storb R, Clift RA, et al. Bone-marrow transplantation (first of two parts). *N Engl J Med*. 1975;292:832–843.
87. Storb R, Epstein RB, Le Blond RF, et al. Transplantation of allogeneic canine bone marrow stored at –80 degrees C in dimethyl sulfoxide. *Blood*. 1969;33:918–923.
88. Storb R, Epstein RB, Rudolph RH, et al. Allogeneic canine bone marrow transplantation following cyclophosphamide. *Transplantation*. 1969;7:378–386.

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89. Storb R, Epstein RB, Graham TC, et al. Methotrexate regimens for control of graft-versus-host disease in dogs with allogeneic marrow grafts. *Transplantation*. 1970;9:240–246.
90. Storb R, Kolb HJ, Graham TC, et al. Treatment of established graft-versus-host disease in dogs by antithymocyte serum or prednisone. *Blood*. 1973;42:601–609.
91. Panjwani MK, Smith JB, Schutsky K, et al. Feasibility and safety of RNA-transfected CD20-specific chimeric antigen receptor T cells in dogs with spontaneous B cell lymphoma. *Mol Ther*. 2016;24:1602–1614.
92. Yin Y, Boesteanu AC, Binder ZA, et al. Checkpoint blockade reverses anergy in IL-13Ralpha2 humanized scFv-based CAR T cells to treat murine and canine gliomas. *Mol Ther Oncolytics*. 2018;11:20–38.
93. Gordon I, Paoloni M, Mazcko C, et al. The Comparative Oncology Trials Consortium: using spontaneously occurring cancers in dogs to inform the cancer drug development pathway. *PLoS Med*. 2009;6:e1000161.
94. Singer J, Weichselbaumer M, Stockner T, et al. Comparative oncology: ErbB-1 and ErbB-2 homologues in canine cancer are susceptible to cetuximab and trastuzumab targeting. *Mol Immunol*. 2012;50:200–209.
95. Beatty GL, Haas AR, Maus MV, et al. Mesothelin-specific chimeric antigen receptor mRNA-engineered T cells induce anti-tumor activity in solid malignancies. *Cancer Immunol Res*. 2014;2:112–120.
96. Berger C, Sommermeyer D, Hudecek M, et al. Safety of targeting ROR1 in primates with chimeric antigen receptor-modified T cells. *Cancer Immunol Res*. 2015;3:206–216.
97. Zhen A, Peterson CW, Carrillo MA, et al. Correction: long-term persistence and function of hematopoietic stem cell-derived chimeric antigen receptor T cells in a nonhuman primate model of HIV/AIDS. *PLoS Pathog*. 2018;14:e1006891.
98. Berger C, Jensen MC, Lansdorp PM, et al. Adoptive transfer of effector CD8+ T cells derived from central memory cells establishes persistent T cell memory in primates. *J Clin Invest*. 2008;118:294–305.
99. Aoyama A, Klarin D, Yamada Y, et al. Low-dose IL-2 for In vivo expansion of CD4+ and CD8+ regulatory T cells in non-human primates. *Am J Transplant*. 2012;12:2532–2537.
100. Dons EM, Raimondi G, Zhang H, et al. Ex vivo-expanded cynomolgus macaque regulatory T cells are resistant to alemtuzumab-mediated cytotoxicity. *Am J Transplant*. 2013;13:2169–2178.
101. Kim MY, Yu KR, Kenderian SS, et al. Genetic inactivation of CD33 in hematopoietic stem cells to enable CAR T cell immunotherapy for acute myeloid leukemia. *Cell*. 2018; 173:1439–1453.e19.

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Review Article

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Xenotransplantation: Progress Along Paths Uncertain from Models to Application

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Abstract

For more than a century, transplantation of tissues and organs from animals into man, xenotransplantation, has been viewed as a potential way to treat disease. Ironically, interest in xenotransplantation was fueled especially by successful application of allotransplantation, that is, transplantation of human tissue and organs, as a treatment for a variety of diseases, especially organ failure because scarcity of human tissues limited allotransplantation to a fraction of those who could benefit. In principle, use of animals such as pigs as a source of transplants would allow transplantation to exert a vastly greater impact than allotransplantation on medicine and public health. However, biological barriers to xenotransplantation, including immunity of the recipient, incompatibility of biological systems, and transmission of novel infectious agents, are believed to exceed the barriers to allotransplantation and presently to hinder clinical applications. One way potentially to address the barriers to xenotransplantation is by genetic engineering animal sources. The last 2 decades have brought progressive advances in approaches that can be applied to genetic modification of large animals. Application of these approaches to genetic engineering of pigs has contributed to dramatic improvement in the outcome of experimental xenografts in nonhuman primates and have encouraged the development of a new type of xenograft, a reverse xenograft, in which human stem cells are introduced into pigs under conditions that support differentiation and expansion into functional tissues and potentially organs. These advances make it appropriate to consider the potential limitation of genetic engineering and of current models for advancing the clinical applications of xenotransplantation and reverse xenotransplantation.

Key words: adaptive immunity; clinical xenotransplantation; gene editing; innate immunity; molecular incompatibility; nonhuman primate; reverse xenograft; transgenic pig; xenotransplantation; zoonosis

Introduction

Few, if any, subjects of research and fields of medical practice provoke as much excitement and as much controversy as xenotransplantation. The excitement stems from the prospect that lethal and debilitating diseases might be conquered by replacing sick or damaged organs with healthy organs using an inexhaustible supply provided by animals and from progressively improving results of experimental xenotransplants, some surviving for a year or more, suggesting that xenotransplantation could soon emerge from the laboratory and enter (actually reenter) the clinic.[1–3] The controversies stem from the limited supply of human organs that impels consideration of xenotransplantation, from the apparent need to introduce human genes into the germline of animals to facilitate acceptance of foreign tissue grafts, and from fears about yet-unknown organisms that might originate from the clinical application of xenotransplantation.

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Below we summarize the rationale for pursuing xenotransplantation, the obstacles to success, and recent advances in overcoming those obstacles. We deliberately avoid consideration of fundamental advances that are not pertinent to practical applications. These advances may prove the most enduring legacy of research in xenotransplantation, but they distract from addressing such questions as what advances are needed for application xenotransplantation in preference to alternative therapies. We emphasize the evolution of technologies of genetic engineering, as they might be applied to the animals that likely would serve as sources of xenografts. We do so not to focus on or endorse particular genetic manipulations but rather to provide a sense about how problems yet undetected will be approached, that is, by modifying source of a graft in preference to treating a patient. We also focus on the ways animal models provide sound inference about what would occur if the tissue or organ from an animal were transplanted into a patient and on some of the ways animal models might misinform about the likely outcome of xenografts.

Definitions

Xenotransplantation refers to the deliberate transfer of living cells, tissues, or organs from individuals of one species to individuals of another. Cells, tissues, or organs so transferred are called xenografts.[4] Allotransplantation/allograft refers to transfers between individuals of the same species. An isograft refers to a transplant between genetically identical individuals, an autograft to a transplant from an individual to itself. The term "heterograft" was previously used to refer to xenografts and the term homograft to allograft, but in some instances heterograft was applied to both xenografts and allografts. Today, xenotransplantation is usually taken to refer more narrowly to the transplantation of cells tissues or organs from animals into humans or to animal models that represent such clinical transplants. There is also interest in what we shall call "reverse xenotransplantation" (Figure 1) or the transplantation of human cells and tissues into animal hosts for the purpose of either expanding the human cells or developing humanized tissues and organs. The term xenotransplantation is not usually applied to accidental exchanges of living cells between species or to infestations by parasites. Xenogeneic cells introduced into animals to investigate properties of the transferred cells (eg, cancer cells implanted in immunodeficient mice) and devitalized structures such as xenogeneic heart valves are sometimes called xenografts, but these applications are not commonly taken to represent xenotransplantation. Here we shall summarize the current understanding of the biological barriers to xenotransplantation of cells, tissues, and organs of animals into humans and the extent to which these barriers are represented in models commonly used today. We will also briefly consider reverse xenotransplantation as it applies to large animals models.

Insights from Early Experiences in Xenotransplantation

Clinical xenotransplantation (and allotransplantation) of skin has been performed throughout history. The skin grafts were usually to provide covering of burn or traumatic wounds (to prevent excess loss of water, scar formation, etc.).[5,6] Skin allografts taken from amputated limbs or cadavers were found to remain in place for days to weeks but most often failed with time. Skin xenografts, sometimes used when human skin was not available, were usually reported to behave like allografts,

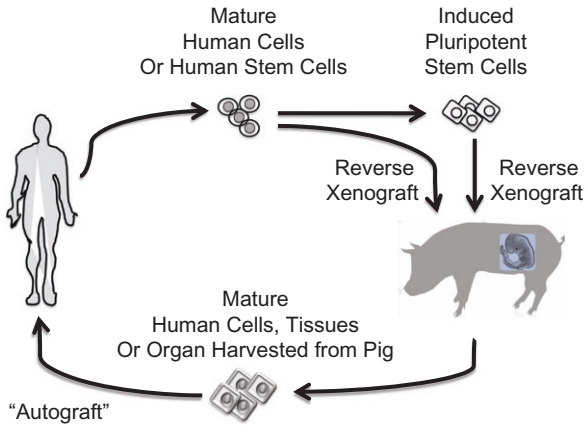

Figure 1 Reverse xenotransplantation. The term "reverse xenotransplantation" is used to refer to the transplantation of human cells into animals. Reverse xenotransplantation has been explored as an approach that might be used to expand a population of mature human cells or to coax the differentiation of human stem cells to generate mature human cells, or a tissue or organ for transplantation as an autograft into the individual who provide the original human cells. The figure illustrates several examples of reverse xenografts. As one example, mature cells such as fibroblasts might be harvested from an individual with organ failure. The fibroblasts would be converted to induced pluripotent stem cells. These stem cells would be treated to begin tissue or organ-specific differentiation and then transplanted into a mature pig or the undifferentiated stem cells might be transplanted into a fetal pig. In the mature or fetal pig, the stem cells would undergo further differentiation and begin organogenesis. Depending on the organ or tissue needed, the maturing human cells, tissue, or primordial organ would be harvested from the pig and then implanted into the individual with organ failure. Reverse xenotransplantation might offer biologically more efficiently and less costly ways to use stem cells for replacement of tissues and organs. Not illustrated in the figure but discussed in the text are various genetic changes that might be introduced in pigs to facilitate engraftment and differentiation of human cells.

effectively covering wounds for days and sometimes weeks but also ultimately failing.[5,6] Although some claimed that xenografts were comparable to allografts, the impression emerged that xenografts were less enduring than allografts and allografts between unrelated individuals were less enduring than allografts between closely related individuals. In contrast to allografts and xenografts, autografts usually survived permanently. Microscopic examination of skin xenografts, allografts, and autografts also suggested relatedness of the transplant and the recipient determined histologic integrity.[7,8] These experiences, however, did not deter use of xenogeneic skin as temporary covering for wounds in the past and proposals for such use today.[9,10]

The experience in transplanting organs within and between species was quite different. Development of techniques to allow the surgical joining of the cut ends of blood vessels (the vascular anastomosis) sparked attempts to transplant intact organs.[11,12] The first "successful" vascularized kidney allografts were performed in dogs in 1905.[13,14] These successes led almost immediately to several attempts to use the technique to treat patients with kidney failure. Because it was not then clear that human organs could be obtained even from deceased individuals (because some reasoned that the presence of living cells during the hours after death precluded ethical harvesting of human organs), the first clinical kidney transplants were performed using kidneys harvested from pigs and sheep.[13,15] One

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of these first clinical xenografts did not function, the other issued a few drops of urine and then it too ceased to function. These results and presumed failure of a clinical kidney allograft widely reported to have been performed[16] discouraged all but a few experimental attempts at clinical kidney transplantation. In the 1960s, when immunosuppressive agents had been developed, transplantation resurfaced as a potential approach to treatment of failure of the kidneys, liver, and heart.[17,18] In that era, as before and since, availability of human organs was considered the preeminent limitation to the application of transplantation for treatment of disease. On a few particularly urgent settings, animals—monkeys or chimpanzees—in lieu of humans were used as the source of organs for transplantation.[19,20] With the recipients receiving immunosuppressive agents then available, most clinical kidney xenografts from chimpanzees functioned for ~2 months and one functioned 9 months; clinical kidney xenografts from baboons functioned days to weeks (Table 1).

The early experiences in experimental and clinical xenotransplantation within and between species fueled some controversies that are still unsettled and pertinent for models and potential clinical applications of xenotransplantation today. One controversy concerned the cause of graft failure. Some believed allografts and xenografts, particularly cancers but also normal tissues, evoke immune responses that destroy the transplants.[21,22] Others believed that biochemical incompatibilities between individuals within a species and between different species, but not immunity per se, cause the failure and destruction of grafts.[8] Today we understand that immunity was then and is still the main obstacle to successful transplantation between different individuals, and the clinical practice of transplantation today is predicated on the continuous provision of immunosuppressive agents (and on availability of antimicrobial agents to address toxicities imposed by immunosuppression).

Although immunity is the most important barrier to successful transplantation, it is not the only barrier. Despite the availability of powerful and highly effective regimens of immunosuppression, up to one-half of all allografts ultimately fail over time. Which grafts are likely to fail and why some fail and some persist are subjects of intense research. One possibility is that the diversity of individuals within species and between species creates incompatibilities that are not amenable to immunosuppression, and it is these that determine the fate of grafts. Below we shall discuss emerging evidence that when immunosuppression is optimized, properties of organ transplants other than antigens and properties of recipients other than the capacity to respond to antigens may determine whether and how well an organ transplant functions. Modeling these determinants especially in large animals poses a considerable challenge but also an opportunity because it presently represents the main cause of failure of grafts.

Rational and Applications for Xenotransplantation

Organ Failure

Transplantation is the preferred treatment for severe failure of the heart, kidneys, liver, and lungs. Although organ transplantation can dramatically reverse the pathophysiology of organ failure, the impact of organ transplantation on public health is limited by a severe shortage in the supply of human organs available for transplantation (Figure 2). The limited supply of human organs and tissues for transplantation remains the preeminent rationale for developing xenotransplantation as an alternative to allotransplantation. Advances in therapeutics and preventative medicine might decrease the incidence of organ failure and lessen the demand for organ transplantation for a period of time after advances are introduced. However, advances in medicine that affect longevity are likely to eventually increase the prevalence of organ failure owing to increased prevalence of diseases of aging. For example, increased attention to blood pressure, cholesterol, and lifestyle and the advent of statins undoubtedly helped to limit the prevalence of cardiac disease and accentuated the relative contribution of cancer among causes of death.[23] But, as advances in cancer treatment further increase longevity, heart and kidney failure will take on renewed significance. Accordingly, we speculate that advances in medicine and public health ultimately increase the prevalence of diseases of aging, including failure of the heart and kidneys, and hence the potential impact of xenotransplantation.[24,25] Xenotransplantation might also find favor in cultures that eschew organ donation and in areas that lack the infrastructure needed to support use of artificial organs. We can also imagine that lower costs we expect to be associated with xenotransplantation could fuel some demand.

Models for Evaluating Impact of Transplantation for Organ Failure

Organ failure significantly affects the outcome of clinical transplantation, increasing the risk of infection, early graft failure, and other complications. Unfortunately, few if any of the preclinical (ie, large animal) models used to investigate transplantation faithfully represent this impact. Generally, healthy animals with or without acute organ failure are used to represent conditions that over periods of months or years eventuate in organ failure. These relatively healthy recipients of organ transplants bypass comorbidities, such as atherosclerosis, chronic changes in vascular resistance, kidney disease, autoimmunity, cancer, etc. that limit the success of organ allotransplantation. However, the limitations of animal models used to

Table 1 Experience in Clinical Xenotransplantation of the Kidney[a]

Source (Author)	Number	Outcome	Reference
Chimpanzee (Reemtsma)	12	1 immediate failure 11 function 2–9 months Infection, not rejection, caused most deaths	294
Baboon (Starzl)	6	Function 10 days–2 months 2 functioning but failing xenografts removed when allografts available; 2 fully ceased function; 2 rejected; 2 regrafted, the regrafts failing at death from sepsis. 1 death from pneumonia, 1 from multiple pulmonary emboli	20

[a]Adapted from[127] and references listed.

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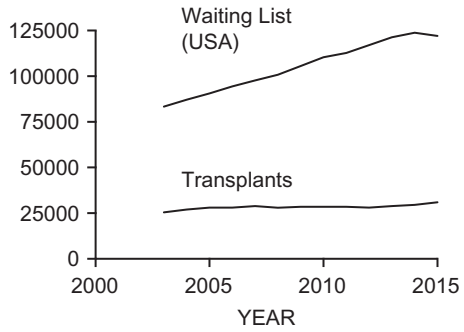

Figure 2 The shortage of human organs for transplantation. Displayed are the number of persons on the waiting lists for transplantation and the number of transplants performed in the United States during the time periods shown. The data are drawn from 2016 Annual Data Report of the Scientific Registry of Transplant Recipients http://srtr.transplant.hrsa.gov/annual_reports/Default.aspx.

study application of allotransplantation for treatment of organ failure have not hindered preclinical testing of novel drugs and regimens for clinical allotransplantation, and there is no reason to think the experience in xenotransplantation will differ. However, the absence of models representing acute and chronic failure of the liver and insulin dependent (type 1) diabetes have slowed development of therapies in general and could particularly limit testing the efficacy of xenotransplantation as a treatment for these conditions. For example, after experimental xenotransplantation of the liver, incompatibilities between the complement and coagulation systems of the liver of the foreign species appear to amplify (rather than resolve) insufficiencies in these systems caused by liver failure. As a result, xenotransplantation of the liver in animal model systems is quite difficult physiologically. However, patients with liver failure often have baseline insufficiencies of complement and coagulation systems, and treatments used to secure survival of experimental xenografts could be more toxic than experimental work would suggest. In treatment of autoimmune diabetes, xenogeneic islets conceivably could pose a lower or higher hurdle to success; if residual "autoimmunity" did not target xenogeneic islets the hurdle would be less, if heighted inflammation associated with xenotransplantation amplifies autoimmunity the impact could be greater if epitopes were similar between species. In the absence of suitable models, the impact of xenotransplantation on liver failure and diabetes might thus be difficult or impossible to predict.

Preemptive Transplantaion

Rapid advances in diagnostics, including molecular profiling, genomics, and molecular imaging, expand the opportunities to detect disease before clinical manifestations appear and to identify individuals at high risk for development disabling or lethal disease. These diseases include cardiac malformations and arrhythmias, inherited defects and deficiencies of metabolic pathways of liver and other organs, and cancer of various types. Identification of individuals with incipient or early-stage disease encourages consideration of preemptive therapies, including transplantation. The benefits versus risks of early diagnosis and the weighing of preemptive therapy versus "watchful waiting" are topics of great interest in medicine. Although preemptive transplantation is practiced,[26,27] practice and investigation of benefits versus risks is limited for the most part to kidney transplantation for which living donors can provide organs.[28,29] Obviously, xenotransplantation would make it possible to introduce preemptive transplantation of other organs and other settings. For investigation of xenotransplantation as a preemptive therapy, physiologically normal recipients, such as those used today, likely provide a reasonable model.

Metabolic Disease

Transplantation of the liver, hepatocytes, pancreas, or islets is performed to correct metabolic diseases. Investigation of xenotransplantation for these conditions has focused mainly on immunological hurdles, and for that purpose physiologically normal recipients provide a reasonable model. Some metabolic diseases have been modeled in mutant mice; however, weighing the potential efficacy versus risks of allo- or xenotransplantation versus other therapies requires development large animal models.

Genetic Engineering for Xenotransplantation

Rationale

One important and sensational rationale for xenotransplantation and reverse xenotransplantation (Figure 1) is the opportunity to engineer the genome of the animal used as the source of the transplant or the host for human cells. Genetic engineering of pigs was first proposed for suppression of complement-mediated injury[30,31] and later for eradication of antigen.[32] The first transgenic pigs generated for this purpose expressed human complement regulatory proteins at low levels but still evaded the immediate complement-mediated injury thought to preclude clinical xenotransplantation.[33] During the 20 years since then, genetic engineering of pigs has been appreciated as a key strategy for advancing xenotransplantation toward clinical practice (see Tables 2 and 3 and[34] and[1] for examples). Genetic engineering of the sources of xenografts potentially decreases the need to administer toxic agents to recipients and, if modifications are stably represented in the germline, allows the extension of favorable characteristics by breeding rather than by manipulation of individual animals. Before the rationale for specific manipulations of the genome is discussed, it is helpful to consider some merits and limitations of approaches used to modify the genome of large animals that could be used as sources of xenografts or as hosts for human cells (Table 3).

Approaches to Genetic Engineering of Large Animals

Genetic engineering of pigs for xenotransplantation initially relied on pronuclear injection of DNA constructs in early zygotes and was restricted to gain-of-function modifications (see[35] for review). These approaches were costly and inefficient and could not be used for targeted inactivation of genes. Thus, although complement might be suppressed by expressing heterologous complement regulatory proteins, suppression of antigen production depended on expression of proteins that could hinder (via competition for substrate) synthesis of the carbohydrate of interest.[36]

Still, the possibility of directly targeting the synthesis of antigenic targets was enabled when the seminal work of Smithies and Cappechi[37,38] proved homologous recombination could introduce mutations in precise regions of the genome and set the stage for gene targeting. This advance and successes in generating gene "knock out mice" sparked the first proposals to target the enzyme responsible for the synthesis of the carbohydrate antigen that had been identified as the initial target of immunity in xenotransplantation.[32,39] However, the

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Table 2 Some Outcomes of Experimental Pig Organ Xenografts in Nonhuman Primates[a]

	Target of Genetic Modification			Outcome (Survival)	Reference
	Ag	C Reg	Coag & Hemost Reg		
Heart Xenograft (n)					
6	α1,3GT KO	Hu CD46	Hu TM	159–945 days	295
5	α1,3GT KO	Hu CD46		42–236 days	295
8	α1,3GT KO			23–179 days	296
Kidney xenograft (n)					
1	α1,3GT KO	Hu CD46 Hu CD55	Hu TM EPCR CD39	136 days	297
5	α1,3GT KO	Hu CD55		6–133 days	298
5				24–229 days	299
7	α1,3GT KO			18–83 days	300

Abbreviations: Ag, antigen; C, complement; Coag & Hemost Reg, coagulation and hemostasis regulation; α1,3GT KO, α1,3-galactosyltransferase knockout; Hu, human; TM, thrombomodulin transgenic; EPCR, endothelial protein c receptor transgenic.
[a]The table shows results from the references cited. Most recipients were baboons. Recipients received various regimens of immunosuppression, some designed to induce tolerance. Some recipients were treated with cobra venom factor to inhibit complement. The results should not be taken to indicate the genetic modifications were mainly responsible for the results but rather to indicate range of responses observed. The significance of this range is discussed in the text.

Table 3 Approaches to Genetic Modification of Animals for Xenotransplantation

Method	Target Cell	Selectable Marker	NHEJ	HDR	Reference
Pronuclear injection[a]	Zygote	No	0.9%	No	301
Random insertion and SCNT	Somatic cell	Yes	10^{-3}–10^{-4}	No	302,303
Conventional HR and SCNT	Somatic cell	Yes	10^{-3}–10^{-4}	10^{-5}–10^{-7}	46,73,304
Gene editing and SCNT	Somatic cell	No	1–50% MA 1–30% BA	2–5%	73,305 [b]
Gene editing and direct embryo injection	Zygote	No	10% MA 100% BA	3–80%	76,78,306

Abbreviations: BA, biallelic; HDR, homology directed repair; HR, homologous recombination; MA, mono-allelic; NHEJ, nonhomologous end-joining; SCNT, somatic cell nuclear transfer.
[a]Efficiency per embryo injected and transferred (combination of 20 projects).
[b]Of the many manuscripts in this area, those selected report results in multiple loci using multiple targets/loci and as such represent what can be expected.

low efficiency of homologous recombination precluded targeting of genes in mature animals or embryos. One potential avenue to targeting of genes in animals was to perform gene targeting and selection in embryonic stem (ES) cells in culture and then introduce the manipulated ES cells into primitive embryos, that is, generating germline chimeras, some of the offspring of which transmit the trait to subsequent generations.[40,41]

Availability of ES cells of mice enabled the generation of lines of gene-targeted mice that have played an essential role in biomedical research. The advances in mice spurred efforts to generate ES cells that could be used for gene targeting in large animals, especially pigs.[42,43] However, despite over 20 years of research in many laboratories worldwide, no ES cell line that could be used for generating gene-targeted pigs was found. As a result, generation of complex transgenic pigs for xenotransplantation was slow and limited to a few research groups.

In 1997, however, Wilmut and Campbell[44] reported that nuclei of somatic cells from sheep removed and inserted into an enucleated egg underwent full reprogramming and could generate a living animal (Dolly), the cells of which, including the germ cells, had the chromosomal DNA of the somatic cell. Thus, somatic cell nuclear transfer (SCNT) could generate animals, cloned from a mature cell, and genetic modification of animals might be undertaken without ES cells or the inefficiencies of microinjection of DNA.

This approach was soon applied to other mammalian species, including swine.[45] The ability to generate offspring from somatic cells meant that ES cells could be bypassed and living animals generated after genetic modification of the somatic cells in vitro. SCNT thus had a major impact in pig transgenesis and xenotransplantation because it enabled the generation of the first α1,3-galactosyltransferase knockout pigs.[46] The combination of conventional homologous recombination and SCNT allowed the generation of multiple transgenic pig lines (reviewed in[47]); however, the low rate of recombination in somatic cells[48,49] limited the progress that could be made in developing complex transgenic animals.

The application of zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9 to gene editing in cultured cells provided the efficiency and specificity needed to generate complex genetic changes. The 3 systems increased rates of targeted modification several orders of magnitude beyond conventional homologous recombination. Even biallelic inactivation and targeted insertions/gene replacements were now achievable at high efficacy (reviewed by[50,51]). The frequency in both cases can range between 10% and 80%, making identification of the correct event a simple task. With these tools, multiple groups have now reported the ability to simultaneously generate mutations in more than one locus.[52–55] These technologies also allow gene replacement and knock-in (placing

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a gene into a preselected genomic region).[56,57] CRISPR-Cas9, in particular, has shown wide applicability and ease of use.[50] Initial concerns regarding high frequency of off target effects (OTE) persist but may be addressed in part by generation of Cas9 enzymes with greater fidelity[58] and in part by improvement in approaches to detecting OTE.[59] Still, the impact OTE on the functioning of organ xenografts could be subtle, and the possibility should be considered when genetic manipulations fail to achieve expected improvements in outcome, as later discussed.

Gene Editing Applied to Pigs

The gene editing technologies have been applied to pigs. The initial gene-edited pigs were generated using ZFN,[60,61] including development of IL2RG KO pigs,[62] but high costs and complex rules of assembly and target selection limited its wide applicability. The advent of TALENs[51,63] and novel assembly methods[64] enabled rapid application of the technology to pigs.[65–69] However, CRISPR-Cas9, with its simplicity of use and lower costs, rapidly eclipsed ZFN and TALENs as the method of choice for generating transgenic pigs. Since then, multiple gene-edited pigs have been generated using CRISPR-cas9 combined with SCNT.[70–74] A recent search of Pub Med yielded >60 reports, including several for xenotransplantation.[75]

The CRISPR-Cas9 system is now being used to rapidly modify pigs by direct injection in zygotes. For reasons still unclear, the efficiency of gene editing in zygotes is even higher than the efficiency in somatic cells, sometimes yielding frequencies of 100% bialleic modification and even multi locus modification.[76–78] Although zygotic injection results in very effective gene editing, the use of SCNT makes it possible to carry out multiple rounds of mutations in pigs without the need for breeding and increases the ability to generate multi-transgenic animals carrying both gene inactivation and gene replacements. Use of SCNT drastically reduces generational intervals and costs associated with breeding multi-transgenic animals where independent segregation can lead to complex litters. In pigs, genetically modified fetuses obtained at day 32–42 of gestation can be used for the next round of modification. We have successfully performed 3 sequential SCNT rounds yielding viable offspring (J. Piedrahita, unpublished observations), and other groups have performed 6 rounds in cattle and 25 rounds in mice.[79,80] Thus, the combination of CRISPR-Cas9 gene editing of somatic cells, direct injection into zygotes and SCNT allows the rapid and efficient generation of essentially any genetic modifications needed in pigs.

Reverse Xenotransplantation

Rationale

Xenotransplantation of human cells to animals (Figure 1), "reverse xenotransplantation," has been envisioned as a way to generate and expand human cells, tissues, and organs for transplantation.[81–83] Reverse xenotransplantation offers certain obvious advantages, histocompatibility, and physiologic compatibility of the human cells upon return to the stem cell source. As we envisioned it, stem cells from an individual needing transplantation (or stem cells generated from differentiated cells or nuclei that after transfer to an egg would be reprogrammed to yield stem cells) might be introduced into fetal animals, the microenvironment of which would coax differentiation or development into mature immunodeficient animals and in these environments the human stem cells might differentiate and grow to human primordia. The primordial could be harvested and implanted in the individual from whom the stem cells were generated and undergo organogenesis. For some purposes, for example, to generate hepatocytes, islets, or hematopoietic cells, the human stem cells would be allowed to fully differentiate in the animal host whereupon the mature cells or tissues could be harvested and transferred to the patient. Generation of human stem cells, transfer to fetal animals, and differentiation and development in the xenogeneic hosts has been accomplished and extensively studied in mice (humanized mice).[84–87] Although essential to progress in many fields, humanized mice will not be considered here. We shall consider the status of transplanting human cells into large animals (humanized pigs) for generation of sizable masses of human cells, tissues, and organs that for clinical purposes might be transplanted into humans.

Seminal work in sheep demonstrated that human hematopoietic stem cells administered to the fetus establish multi-lineage hematopoietic chimerism.[88–90] However, reverse xenotransplantation in pigs would offer certain advantages, including cost, multi-parity, and the large body of knowledge regarding biological barriers to engraftment. However, experience in human-pig reverse xenotransplantation is quite limited. Human stem cells transferred to fetal pigs have been shown to contribute to formation of some nephrons in kidney, segments of skin, and to the thymus[91] and hematopoietic system.[91–93] Mature pigs generated from these fetuses have some human T cells selected and matured in the chimeric thymus that can generate human restricted responses to antigen.[93] The potential of reverse xenotransplantation to address clinical problems, however, remains to be determined. Application could well depend on optimizing the sources and types of human cells, the approach to delivery (eg, devising approaches to deliver cells to mature rather than to fetal pigs), and on minimizing hurdles posed by immunity and biological incompatibility. Below we discuss some of the model systems in which progress is being made.

Intra-Uterine Stem Cell Transplantation (IUSCT) to Achieve Xenogeneic Engraftment

The first approach used for delivery of human cells to animals involved IUSCT. Introduction in utero averts rejection and provides a more nurturing microenvironment. Sheep were initially preferred as hosts for IUSCT because the fetus would tolerate manipulation and the size made surgical intervention easier. Transplantation of allogeneic hematopoietic stem cells early in gestation of wild-type sheep fetuses yielded sustained multilineage hematopoietic chimerism.[90,94] The approach was used for other types of stem cells.[95–97] However, engraftment was quite low (<1%), making this model impractical.[88,89]

As mentioned, size, anatomic, genetic, and physiological similarity to humans and extensive information already assembled about zoonosis and compatibilities make the pig a more attractive host for IUSCT intended to have clinical applications. Thus, others[92] and we[93,98] successfully performed IUSCT, introducing human hematopoietic stem cells in porcine fetuses and detecting mature progeny years after birth. The introduction of human stem cells in the porcine fetus facilitated development of tolerance by the host.[93] Still application of IUSCT in pigs was limited by the low level of human cell engraftment.

Although the IUSCT host animals exhibited immune tolerance, engraftment is potentially limited by innate immunity (NK cells), a niche that is less than optimally supportive of xenogeneic cells and incompatibility of growth factors between species. Some of these hurdles can be overcome by delivering

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more human cells to the fetus, by administering human growth factors with the transplant, and/or by depleting some of the porcine cells that compete with the transplant for growth factors. In mice, success has been most readily achieved by genetic engineering (see[99–101] for review), and that is the approach others and we have pursued in pigs.

Genetic Engineering to Achieve Xenogeneic Engraftment

An absolute requirement for engraftment of human cells in other individuals of the same or disparate species is suppression or elimination of adaptive immunity. This barrier was minimized by using the fetus at a gestation that precedes development of mature lymphocytes, particularly T lymphocytes, as a recipient of foreign cells, introduced by IUSCT, as described above. However, to avoid IUSCT, foreign cells could be introduced into animals that were immunodeficient. For decades mice with naturally arising immunodeficiency, such as nude mice, have been used to harbor and study malignant human cells.[102] However, full and enduring engraftment of normal cells was never achieved. Hence, with the advent of genetic engineering, efforts were made to more completely remove immune barriers to engraftment posed by NK cells and adaptive immunity. The greatest success achieved by targeted disruption of IL2RG and either RAG-1 or RAG-2 (see[103] for review). In addition to averting innate and adaptive immunity, optimal and enduring engraftment of human cells in mice was achieved by providing human growth factors (eg, IL-3, hM-CSF, GM-CSF, thrombopoietin) and a phagocytosis suppressor SIRP-α (see[104] for review) and by limiting the competition of murine stem cells (see[105] for review) or mature cells.[106]

For reasons discussed above, we and a few others have begun to use genetic engineering to generate immunodeficient pigs as potential hosts for reverse xenografts. Transgenic *IL2RG*$^{-/y}$ pigs exhibit some features of X-linked severe combined immunodeficiency syndrome, including marked decreases but not complete absence of T cells and NK cells in peripheral blood and spleen (~2.3% of normal) but normal B cell numbers.[62,107] The pigs accept grafts of semiallogeneic but not human hematopoietic stem grafts and therefore are not likely to prove useful for reverse xenotransplants. *RAG-1*$^{-/-}$ and *RAG-2*$^{-/-}$ transgenic pigs have a hypoplastic thymus and significantly decreased numbers of T cells and B cells in the circulation and in spleen, although some CD3 + cells, likely NK cells, are detected in spleen.[68] Biallelic RAG-2$^{-/-}$ pigs have been reported to have a phenotype similar to that of pigs deficient in both RAG-1 and RAG-2 and to accept transplants of human induced pluripotent stem cells, developing teratomas, and transplanted allogeneic trophoblast cells.[108] Whether the pigs would accept normal cells remains unknown. Pigs with targeted biallelic disruption of genes encoding RAG-2 and IL2RG have been reported.[78] As might be expected, the pigs have a ~100-fold decrease in circulating T cells and B cells but a small decrease in NK cells, reflecting some residual IL2RG function and inability to clear norovirus. Whether the pigs accept foreign grafts is unknown.

We have generated pigs with targeted disruption of RAG2, RAG1, and IL2RG (J. Piedrahita, unpublished observation). The pigs accept allogeneic stem cells and in so doing reconstitute the immune system. The pigs also accept xenogeneic cells; however, our experience indicates, perhaps not surprisingly, that hurdles beyond innate and adaptive immunity limit xenogeneic engraftment. We expect advances in gene editing discussed above will allow us to overcome this limitation in the near future.

Animal Species as Sources of Xenografts

Nonhuman Primates

When transplantation was introduced into clinical practice at a few academic centers and donated organs were scarce, xenotransplantation was seen as a reasonable alternative "in certain rare circumstances"[17] and nonhuman primates, because of taxonomic and physiologic proximity to humans, were used as the source of most organs used for clinical xenografts.[19] Nearly all of the xenografts functioned at least briefly, but none provided enduring support and all patients died either because of infection or rejection of the transplant. The results of some renal xenografts from nonhuman primates to human patients are summarized in Table 2.

Certainly better results and perhaps enduring function could be achieved today. Yet, nonhuman primates have been excluded as potential sources of organs in part for reasons of ethics, but especially because nonhuman primates are too scarce to have any meaningful impact on the shortage of human organs. There is also concern that transplantation might convey lethal infection. Furthermore, although tissue physiology of nonhuman primates may resemble that of humans, the smaller size of chimpanzees and monkeys limit the physiologic impact the organs would have as xenografts in mature humans. On the other hand, nonhuman primates are commonly used to model human xenograft recipients, as discussed below.

Pigs

During recent decades the pig has received universal acclaim as the preferred source of xenografts.[30,109,110] Pigs are plentiful enough to fulfill any conceivable need. Early in life the size of pigs overlaps with human. Pigs can be genetically engineered and owing to sizable litters, readily bred, as described below. Because pigs have long existed in proximity to humans, the susceptibility of infectious diseases and potential for transmission to humans is understood well enough to formulate detailed approaches to screening and prevention.[111,112] As discussed below, experience and investigation have also tempered some concerns that use of pigs in xenotransplantation might generate exotic microorganisms.[3]

Because present interest focuses almost exclusively on pigs as sources of tissues and organs for clinical xenotransplantation, modeling of clinical xenotransplantation today also generally uses pigs as a source and primates as recipients. Therefore we shall focus mainly on xenografts in which pigs are used as a source. Still, experimental xenografts between various combinations of species (eg, guinea pig-to-rat, rat-to-mouse, pig-to-dog) have contributed to the body of knowledge about xenotransplantation. Where appropriate, we shall refer to these models without offering detailed review.

Biological Barriers to Xenotransplantation

Introduction

The biological barriers to xenotransplantation include the immune response of the recipient against the graft, physiological and biochemical incompatibility between the graft and the recipient, and the potential for transmission of infection between the graft and the recipient and the consequences thereof including potential generation of novel microorganisms.[113–116] Although typically these barriers are investigated independently, sometimes

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using divergent models, the elements of the barriers intersect in origin, pathogenesis, and manifestations (Figure 3). For example, ischemia-reperfusion injury associated with transplantation of a pig organ into a nonhuman primate incites activation of complement, the control of which is thwarted by incompatibility of complement regulatory proteins.[117] At the same time natural antibodies of nonhuman primates directed against Galα1-3Gal[39] and antibodies others directed against neoantigen on ischemic cells[118] increase the extent of complement activation,[119] which in turn amplifies B cell[120] and T cell[121,122] responses to foreign antigens. The inflammatory and immune environment associated with ischemia-reperfusion injury and innate immunity modifies the physiology of parenchymal cells and endothelium, potentially effacing control of viral latency[123,124] but also potentially circumscribing infectious agents in the recipient or carried with graft.[125,126] The intersection of immunity, physiologic incompatibility, and infection underscore the importance of taking account of the limits of simplified experimental systems, such as cell cultures and small animals, in predicting the impact of the various barriers as they would be manifest in swine-to-human transplants.

Immunity as a Barrier

The immune response of the recipient to a xenograft has been considered the most daunting barrier to xenotransplantation. The importance of the immune barrier emerged from repeated failures to achieve permanent engraftment organs from between disparate species, such as pig organs in nonhuman primates.[127] Recent reports of long-term (>1 year) survival of some heterotopic cardiac xenografts and kidney xenografts might suggest, however, that the hurdle posed by immunity can be overcome and clinical trials might soon commence.[1,3,128] These promising results were achieved, however, using immunosuppressive agents and regimens more severe than those typically used for clinical transplantation. We briefly discuss some aspects of the immunology of xenotransplantation pertinent to animal models currently used and some of the limitations inherent in those models. More detailed reviews of the immune response to xenotransplantation can be found in other publications.[129–131]

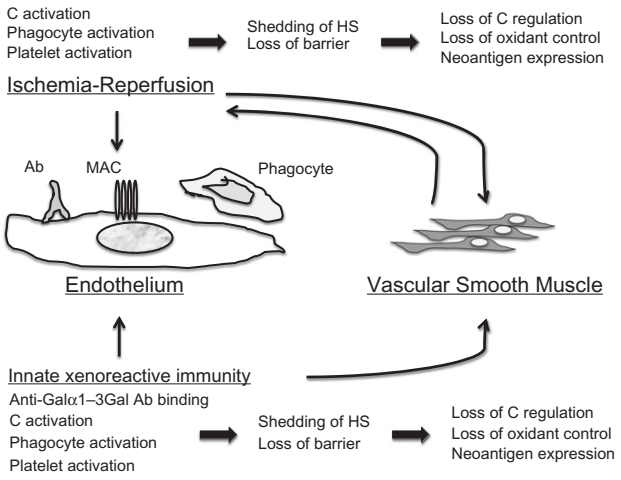

Figure 3 Integration of pathogenesis of ischemia-reperfusion injury with pathogenesis injury caused by xenoreactive antibodies and xenoreactive phagocytes. Ischemia reperfusion injury and innate immunity directed at xenografts converge to amplify complement-mediated early graft injury. Activation of complement (C) by ischemia (through several pathways) and/or by xenoreactive antibodies increases the amount and kinetics of membrane attack complex (MAC) assembly, which increases membrane injury. C activation generates C3a and C5a, which activate leukocytes and C3bi, thereby tethering phagocytes to endothelium. C activation also causes smooth muscle contraction, decreasing blood flow and increasing the extent and duration of ischemia. C activation causes shedding of heparan sulfate (HS) from cell surfaces, compromising barrier functions; impairing regulation of complement, coagulation, and platelet activation; and hindering control of oxidants. Leukocytes, platelets, and endothelial cells release proteases that expose neoantigen on endothelium, heightening the reaction. Ischemia and innate immunity also amplify adaptive immunity (not shown).

Innate Immunity

Xenotransplantation potentially recruits every facet of innate immunity through the response to ischemia-reperfusion injury and through recognition of the graft as "foreign by natural antibodies, complement, and phagocytes (Figure 3). The 2 processes are truly synergistic because each compromises resistance to the other and each promotes smooth muscle contraction, amplifying ischemia (Figure 3). Both are further amplified by relative ineffectiveness of controls of complement, coagulation, and platelet activation. Hence the barrier posed by innate immunity is particularly significant in xenotransplantation.

Complement

The most dramatic example of dysregulation of innate immunity is the early pathogenic impact of activation of the complement system. Complement can be activated by one or more of the several distinct initiating mechanisms: (1) the classical pathway, typically initiated by binding of complement-fixing antibodies; (2) the alternative pathway, typically initiated generation of C3b and association with factor B exceeds the control exerted by circulating (factor H) and membrane associated (CD46) complement regulators; (3) the lectin pathway, initiated by the binding of mannose binding lectin or ficolin with mannose-binding lectin associated serine proteases 1, 2, and/or 3; and (4) the properdin pathway, initiated by the binding of properdin directly to a target. These canonical pathways belie a much larger number of mechanisms that can initiate the complement cascade (eg, antibodies bound to a surface can activate the alternative and/or properdin pathway, and C1q can attach directly to injured cells).

Upon reperfusion of organ xenografts (or introduction of xenogeneic tissue into blood[132–134]) ischemia-reperfusion injury and binding of xenoreactive antibodies activates the complement system. The extent and the kinetics of complement activation are governed at key steps by regulatory proteins in blood, such as factor H; on cell membranes, such as CD46, CD55, and CD59;[135,136] and by the condition of cell surfaces.[137] Some complement regulatory proteins may function more effectively in homologous than in heterologous systems,[30,117,138,139] although some have challenged this concept based on work using isolated cells.[140] Although challenges to the concept of homologous restriction of complement are welcome, heterologous complement has always appeared more active than homologous complement cell lysis assays.[141] More to the point, observation obtained over decades in transplanting organs between various combinations of disparate species (and hence the principle of in vivo veritas) provides compelling support for the importance of species specificity of complement regulation. For example, heart and kidney xenografts from pigs into unmanipulated nonhuman primates invariably undergo hyperacute rejection triggered by anti-Galα1-3Gal antibodies, the concentrations and functions of which resemble isohemagglutinins,[142] leading to activation of complement. In contrast, ABO-incompatible

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allografts rarely undergo hyperacute rejection.[143] Consistent with this concept, expression of small amounts of human complement regulators in transgenic pigs can prevent this type of rejection,[33] and pigs developed as sources of organs for xenotransplantation often incorporate one or more transgenes for expression of such proteins.[34]

Perhaps more important than the cell-associated complement regulators is factor H, a plasma protein that regulates the alternative pathway of complement (by facilitating dissociation C3bBb complexes and by acting as a co-factor for factor I-mediated cleavage of C3b). To exert its function, factor H attaches to acidic moieties on cell surfaces (eg, heparan sulfate and sialic acid), and it is the interaction with cells surfaces that may limit the activity of the protein on foreign surfaces.[144] If factor H fails to control complement on surfaces (eg, rat factor H fails on guinea pig cell surfaces), immediate and severe complement-mediated injury, that is, hyperacute rejection, ensues.[145] Fortunately, human (and nonhuman primate) factor H regulates human complement on porcine cells and hence this limitation is not often discussed. However, factor H might sterically compete with properdin (a protein that promotes the alternative complement pathway)[146,147] and other proteins for binding to cell surfaces, and it is conceivable that novel mixtures of proteins in xenograft recipients could hinder the regulation of complement.

Natural Antibodies

The natural antibodies of greatest interest in xenotransplantation are natural antibodies specific for Galα1-3Gal.[148,149] Galα1-3Gal is the product of a galactosyltransferase (α1,3-galactosyltransferase) that is produced by New World monkeys and lower mammals, including the pig, but not by humans, apes, and Old World monkeys.[150] Mammals lacking Galα1-3Gal produce natural antibodies specific for that saccharide, much as humans lacking blood group substances A or B produce isohemagglutinins directed at the corresponding substances.[142] When a porcine organ is transplanted into a nonhuman primate with natural antibodies specific for Galα1-3Gal, the binding of those antibodies triggers immediate, complement-mediated rejection of the organ[119] and (if immediate rejection is avoided) antibody-mediated rejection (also called acute vascular rejection).[151] The importance of antibodies specific for Galα1-3Gal in xenotransplantation led to the generation of pigs with targeted (α1,3-galactosyltransferase) (Gal KO pigs).[152,153] It should be noted, however, that the ability of anti-Galα1-3Gal to trigger immediate rejection and even antibody-mediated rejection depends very much on failure of complement regulation, as the presence of even low level of human complement regulatory proteins in a xenograft thwarts immediate rejection[33] and temporary removal of natural antibodies against blood groups prevents hyperacute and antibody-mediated rejection.[154]

Human natural antibodies against structures other than Galα1-3Gal might initiate rejection of xenografts.[155] The significance of these natural antibodies in xenotransplantation remains uncertain because the antibodies have been studied in systems in which antibody interaction with Galα1-3Gal cannot occur (eg, when Gal KO organs are transplanted into nonhuman primates). Some of the antigens might be "neoantigen" produced in the absence of α1, 3-galactosyltransferase and some antigens might be recognized by natural antibodies of only a fraction of nonhuman primates and humans. Therefore, it is difficult to know a priori whether targeting of the corresponding glycosyltransferases will confer more benefit than harm.

Still another type of natural antibody, the polyreactive antibody, could have an effect on xenotransplants. Polyreactive antibodies recognize multiple antigens, as the name indicates, including autoantigens and are produced by a distinct subset of B cells.[30,156] Polyreactive antibodies have been implicated in the pathogenesis of ischemia-reperfusion injury.[118,157] In this setting, the antibodies can attach to neoantigen formed by degradation or oxidation of normal molecules or to antigens exposed by injury of cell membranes. Polyreactive antibodies also can initiate the repair of injured cells and tissues[158–160] and thus potentially benefit a transplant.[154] Polyreactive antibodies bind xenogeneic to endothelial cells in culture and can be found in xenografts.[161] What impact polyreactive antibodies have on the fate of xenografts is unknown, but it is not unreasonable to think that impact is exaggerated over the effect exerted in allotransplants[161] and the "autoreactivity" of the antibodies should be considered when in evaluation of the specificities in serum.[162]

Cellular Innate Immunity

Leukocytes (natural killer cells, macrophages, neutrophils, and T cells), platelets, fibrocytes, and other cells are found in inflammatory reactions of every type, including those observed in xenografts. These cells are thought to participate in the innate immune reactions that accompany ischemia and surgical disruption of tissues and acute and chronic rejection of transplants,[163–166] modifying the functions of endothelial cells, especially in transplants.[167–169] Cellular elements can recognize foreign or injured-autologous cell surfaces and products released from cells (eg, agonists of inflammatory receptors) and initiate the ensuing reactions, or cellular elements can be recruited to inflammatory reactions begun by other recognitive systems (eg, responses to binding of Ab or activation of complement). Cellular elements can also play a tangential role in pathogenesis (eg, severe complement-mediated injury can destroy an organ whether or not inflammatory cells are present). Although effector and regulatory pathways and specific cellular interactions are often identified in cell culture systems, the contribution of cells to pathologic processes must be ascertained in vivo, typically by use of animal models. For example, foreign NK cells rapidly bind to and kill or activate foreign endothelial cells, including xenogeneic cells[167,170–172]; yet NK cells do not evidently cause early injury of porcine organs transplanted into nonhuman primates but rather might contribute to chronic vascular injury.[173]

Inflammatory reactions associated with xenotransplantation appear to be greater than those typically seen in ischemia-reperfusion injury or in allogeneic transplant reactions. Whether interactions between macrophages, NK cells, and other cells and xenografts initiate or cause that increase or whether these interactions merely reflect greater tissue damage caused by antibodies, complement, etc. is not entirely clear. However, some of the pathways that constrain cellular interactions in autologous systems fail in allogeneic and especially in xenogeneic systems. For example, NK activity is suppressed by interaction of inhibitory receptors with classical or nonclassical MHC class I, but porcine MHC class I (and some allogeneic MHC class I) fail to interact with these inhibitory receptors.[174,175] Macrophage activity is suppressed in autologous systems by interaction of signal regulatory protein (SIRP)-alpha, with CD47, expressed by nearly all cells.[176] Failure of these interactions increases interaction and effector activity of NK cells and macrophages with xenografts.[130] As potential solutions to the incompatibilities, pigs have been

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genetically engineered to express transgenes for human HLA-E and human CD47.[177–179]

Adaptive Immunity

More than 100 years have elapsed since Ehrlich and Morgenroth showed that foreign cells elicit abundant antibody responses,[180] Nuttall[181] showed that those responses recognize multiple determinants, and Fleisher[182] observed a more intense "leukocytic reaction" in xenografts than in allografts. Still, the rapidly destructive impact of innate immunity and biological incompatibilities generated by admixing of cells of disparate species left some uncertainty about the dimensions of the barrier to xenotransplantation posed by adaptive immunity, particularly cell-mediated immunity.[127] Long-term survival of porcine organs in nonhuman primates is presently pursued by genetic engineering to minimize the impact of innate immunity (ie, use of pigs deficient α1,3-galactosyltransferase and expressing human complement regulatory proteins) and by administration of regimens of immunosuppressive agents more severe than those typically used in clinical transplantation. Because innate and adaptive immunity intersect (eg, complement activation promotes B cell responses) and because both innate and adaptive immunity induce thrombosis, coagulation, and inflammation, advances in survival of experimental xenografts have been realized through use of combinations of intense regimens of immunosuppression (to suppress adaptive immunity) and genetic engineering to efface innate immunity, thrombosis, coagulation, etc. However, as clinical application of xenotransplantation approaches, it will be important to determine whether less intrusive regimens of immunosuppression can be employed. Identifying the model(s) that can reliably and efficiently evaluate immunosuppression for clinical xenotransplantation thus should be a key objective in the field.

Cell-Mediated Immunity

Isolated xenogeneic proteins and intact cells elicit cell-mediated immunity in humans and animals. Because the number of foreign peptides in xenogeneic antigens exceeds the number in allogeneic antigens, one might expect cellular immune reaction to the former would be especially robust. Therefore, it was striking, to say the least, when in vitro analyses of T cell responses to xenogeneic cells revealed profound limitations of cell-mediated responses.[183,184] These limitations mainly resulted from incompatibility of cytokines and co-recognition receptors between species under conditions that minimized the impact of recognition of foreign peptides presented with self-MHC (ie, mixed leukocyte cultures, which preferentially detect responses of naïve T cells to intact foreign MHC but not de novo responses to foreign peptides).[185] The observations raised the possibility that cell-mediated immunity to xenografts might be vulnerable in ways that allogeneic responses are not. Consistent with that possibility, work in mice revealed that antibodies against CD4 suppress T cell responses to and cellular rejection of xenografts.[186] Subsequent investigation revealed that anti-CD4 antibodies, engineered to block co-recognition but not to deplete CD4+ T cells, induce tolerance to xenogeneic protein.[187] Today, most work in xenotransplantation employs agents such as anti-CD154 and anti-CD40 that disrupt co-stimulation more broadly.[188] The toxicity of these broadly active agents will probably encourage a revisiting of less severe approaches or the possibility of inducing tolerance.

For reasons described above, immunosuppressive regimens for xenotransplantation are commonly investigated using nonhuman primates as recipients of porcine xenografts. Although nonhuman primates probably offer a more stringent model of the cellular immune barrier to xenotransplantation than small animals, including "humanized"-mice, nonhuman primates also potentially can mislead. One problem is that nonhuman primates might develop immunity to "humanized" antibodies or to human proteins expressed as transgenes in pig organs. If such immunity blocks the action of immunosuppressive agents, rejection could ensue or higher doses or more severe regimens might be needed to sustain the graft. For this reason, selection of the optimal and least intrusive immunosuppressive regimens for swine-to-human xenotransplantation might prove futile in nonhuman primates and might rather depend on analysis in early clinical trials.

One potential avenue that might be optimally tested first in nonhuman primates is the induction of tolerance. Some have argued that successful application of xenotransplantation might depend on devising approaches for the induction of immune tolerance.[188–191] Depending on the approach and regimen used, tolerance might well be extended to human proteins expressed in a xenograft and to "humanized" agents used for tolerance induction or maintenance.

As xenotransplantation approaches clinical application, the emphasis of research will almost certainly shift from preventing and treating acute rejection to the promoting of long-term function and avoidance of chronic disease of the graft and such comorbidities as cancer and cardiovascular disease. Toward that objective, it will be important to consider whether manipulation of the recipient and/or genetic engineering of swine modify cell-mediated immunity in ways that promote or hinder long-term function and well-being. As only one example, consider the impact of transgenic expression of human CD46 in a swine organ to control activation of complement in the graft.[192,193] CD46 facilitates the proteolytic action of factor I on C3b, generating C3d, among other fragments, which can amplify the effector activity of cell-mediated immunity.[122] Therefore, if efforts to decrease the intensity of immunosuppression and/or to induce tolerance fail, there might be reason to revisit the approaches used to control complement. For reasons given above, pig-to-nonhuman primate models are best suited if not essential for testing the impact of genetic engineering in xenotransplantation.

Humoral Immunity

There is general appreciation that elicited antibody responses might limit the success of xenotransplantation. In the absence of immunosuppression, all xenografts and nearly all heterologous proteins elicit T cell-dependent B cell responses. The specificity, concentration, and avidity of antibody responses to xenotransplantation in immunosuppressed nonhuman primates have been the subject of a few reports.[194–197] However, the full range of antibody responses in xenotransplant recipients is incompletely understood at best. Given the successes achieved by targeting the α1,3-galactosyltransferase gene, there might be some temptation to catalogue the specificities of elicited antibody responses to xenotransplantation. Hopefully, consideration of the potential diversity of these responses and the variation in specificities likely to be found between recipients will build resistance to such temptations.

Several obstacles hinder investigation of elicited antibody responses to xenotransplantation. Natural antibody responses to Galα1-3Gal and other antigens can increase after transplantation, possibly owing to inflammation, making these responses more

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difficult to distinguish from de novo responses. Another obstacle, and one that also impairs full understanding of donor-specific antibody responses in allotransplantation, is that a functioning graft, especially an intact organ graft, can absorb enormous amounts of antibody and the antibodies so absorbed will be of the highest affinity and specific for the antigens of greatest density.[198–200] Hence, the antibodies remaining in the serum do not necessarily represent the antibodies of greatest importance.[201] Another obstacle is that antibodies and complement bound to healthy cells can be taken up and processed, impairing the ability of immunopathology to detect early stages of injury. These problems are potentially overcome by investigation of B cell responses.[201]

Some antibody responses to xenotransplantation may induce antigen-specific pathophysiology. Human kidney allotransplant recipients with the X-linked Alport syndrome (mutant gene encoding collagen type IV alpha 5 chain) sometimes produce antibodies against the wild-type collagen in the transplant, leading to antiglomerular basement membrane nephritis.[202] Similarly, human kidney transplant recipients sometimes produce antibodies against angiotensin receptor allotypic variants, evoking malignant hypertension.[203] In principle, all kidney xenograft recipients are at risk for producing antibodies directed against the swine homologues of these or other pathophysiologically significant targets. Thus, antibody-inhibitors of heterologous factor VIII have been described in xenograft recipients.[197] Because every protein in a xenograft is a potential immunogen, identifying the most common pathophysiologically vulnerable targets could prove challenging but clinically significant. Although this problem is likely to be idiosyncratic, it is best pursued in animal models in which the importance of antibody binding to a specific antigen can be distinguished from pathology caused by antibody binding to any target and solutions, such as antigen specific tolerance, can be explored.

Pig-to-nonhuman primate xenotransplantation models offer 2 important advantages for investigation of elicited humoral immune responses to transplantation. One advantage is the high frequency of antibody-mediated rejection (compared to clinical allografts) makes it easier to link B cell and antibody responses to the development of graft pathology. The other advantage is the ready access to blood and tissue samples. The main disadvantage is the relative paucity of information concerning non-uman primate variable region genes and the mixture of species (baboon and monkey) used as recipients.

The Impact of Immunity on Xenografts

Three distinct factors determine the impact of innate and adaptive immunity on xenografts. These factors are: (1) the source(s) of the blood vessels in the graft; (2) the intrinsic and induced resistance of the graft to immune and inflammatory injury (a condition we named accommodation); and (3) the nature and kinetics of immunity directed at the graft. The nature and kinetics of immunity to xenografts were discussed above. But as important as the intensity of immunity may be, the impact of immunity on a graft is to a large extent determined by the origin of blood vessels—whether derived from the recipient by ingrowth or originating with the source (as in organ grafts)—which determines the pathogenic processes invoked by immunity (Figure 4). Intrinsic and induced resistance to injury determines whether the immune-induced pathogenic pathways destroy the graft or allow repair and recovery from immune assault.

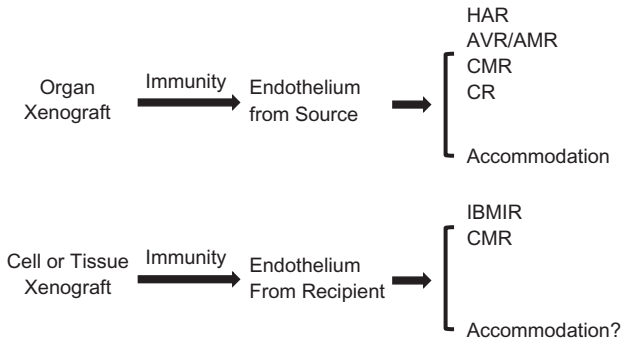

Figure 4 The type of xenograft determines the source of endothelium, which in turn determines the impact of immunity on graft pathology. Organ xenografts (top) contain blood vessels originating from the source of the xenograft. Antibodies and complement directly attack the endothelial lining of organ xenografts, causing hyperacute rejection (HAR) in minutes to a few hours. If HAR is averted, acute vascular rejection (AVR) also called antibody-mediated rejection (AMR) and also caused by antibodies and complement, can ensue over the next days, weeks, or months. Organ grafts are also susceptible to chronic rejection (CR), sometimes caused by antibodies and complement, developing over months or years. Immunity also can induce changes in endothelium that render blood vessels and other cells resistant to injury (accommodation). These resistive changes counter pathogenesis and allow function to persist in the face of immunity to the transplant. Cell or tissue xenografts (bottom) contain blood vessels and endothelium of the recipient. Recipient blood vessels are not directly targeted by xenoreactive antibodies and hence are not susceptible to HAR, AVR, and CR (usually). However, T cells and phagocytes actively penetrate blood vessels, and hence cell and tissue grafts are susceptible to CMR. When cell and tissue xenografts are introduced into blood vessels of the recipient (eg, into the portal vein), antibodies and complement can attack the grafts, causing "instant blood mediated inflammatory reaction" (IBMIR). Injury begun by IBMR can persist after the grafts pass out of the circulation, but susceptibility to de novo IBMR ceases once engraftment outside of blood vessels occurs. Studies in cell culture systems suggest cell and tissue grafts, like organ grafts, may be protected by accommodation.

Source of Blood Vessels in a Graft

We have long emphasized that the origin of blood vessels in a xenograft determines the conditions potentially induced by the immune response of the recipient.[114,130] Organ xenografts, in which blood vessels are mainly of donor origin, are susceptible to conditions generated by the direct action of antibodies, complement, and inflammatory cells on graft endothelium (Figure 4). Cell and tissue xenografts (eg, pancreatic islet and hepatocyte, respectively) in extravascular sites are not susceptible to these conditions (hyperacute and acute antibody mediated rejection) because all IgM and most IgG and complement are retained within blood vessels. Cell and tissue xenografts introduced via the blood (rather than injection into extravascular spaces) are susceptible to injury by antibodies and complement during the period of passage through the blood of the recipient, but once engrafted, this susceptibility wanes.[204,205] All xenografts are susceptible to cellular rejection because stimulated lymphocytes and phagocytes migrate actively through blood vessel walls.[206,207]

The pathology of ischemia and rejection of xenografts reflects the distinct assaults by immunity on blood vessels.[30,126] Rapid activation of abundant amounts of complement and assembly of terminal complement complexes cause endothelium to lose functions (especially barrier and vasoregulatory functions), eventuating immediately in the pathology of hyperacute rejection. Activation of smaller amounts of complement or interaction of leukocytes (macrophages, NK cells, T cells) changes the transcriptional program and physiology of

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endothelial cells, causing the cells to promote coagulation, leukocyte activation, and migration, etc. and eventuating in coagulation and intra- and perivascular inflammation. In allografts, this condition is often called antibody-mediated rejection and that term could be applied to xenografts. However, antibodies do not necessarily mediate this condition, and therefore we have preferred to use the term acute vascular rejection because blood vessels are the target and the instrument of pathological processes.[30,126,208] Migration of activated T cells and macrophages through otherwise undamaged endothelium causes the cellular infiltrates typical of cellular rejection and, perhaps more importantly ,increases interstitial pressure and exposes regional cells to cytokines, proteases etc., the clearance of which is impaired.

Baseline and Induced Resistance to Injury (Accommodation)

Allografts and xenografts exhibit a wide range of responses to assault by ischemia and immunity. In part these differences reflect the intensity of the ischemic insult and of the immune response of the recipient directed at the transplant. Indeed, the intensity of injury to the graft is often used as an index of ischemia or immunity. Yet decades of investigation have established that properties of target can govern the outcome of inflammation and immunity.[209,210] Thus, among pairs of recipients from the same renal transplant donors, up to 60% of early and late outcome can be ascribed to the donor organ.[211–213] Some graft-associated determinants of the outcome of transplants (besides MHC) are inherited. In clinical transplantation, the race of the donor influences early and possibly long-term outcome. Among inherited factors in donors are polymorphisms encoding variants of APOL1, caveolin-1, ABCB1, and eNOS, and donor-recipient pairing of certain alleles beyond MHC[214] have a discernable impact as well.[215,216]

One property of grafts, especially xenografts, that determines outcome is the ability to resist and repair injury. We refer to this ability as "accommodation."[30] Accommodation was first observed in ABO-incompatible kidney transplants and in heterotopic cardiac xenografts that continued to function despite the presence of antibodies against the grafts in the blood of the recipients.[30] Accommodation in ABO-incompatible transplants explained why in some circumstances antibodies that can initiate devastating injury sometimes fail to do so and why surveys of anti-graft antibodies in the blood of recipients often revealed little or no relationship to the presence or severity of graft injury in ABO-incompatible transplants.[217,218] Accommodation is also observed in conventional (ABO-compatible) organ allografts, but the frequency is unclear because alloantibodies specific for HLA can be absorbed in and taken up by organ transplants.[199,219]

Accommodation develops over a period of days, and establishment of that condition obviously requires sufficient baseline resistance to injury. As a working hypothesis, we suggest that baseline resistance to injury reflects some constitutive properties of cells,[210,220] and heightened expression of the products of "cytoprotective genes"[221] allow cells, tissues, and organs to repair initial damage and dispose of waste. More enduring changes increase the efficiency of these processes, restoring function and shifting the level of resistance to cytotoxicity.[137,222,223]

Accommodation, sometimes referred to by other terms, has been implicated in the cancer phenotype, responses to infection and physical injury, and autoimmunity.[200,222,224,225] Efforts to identify genes involved in accommodation in clinical settings have met with limited success,[226] in part because surveys focused on "cytoprotective genes," which are also expressed in rejection. Genome-wide association studies (GWAS) reveal regions of the genome and heritable traits that confer resistance to injury from infection[227,228] and improved quality of meat.[229] GWAS in models of tissue injury in pigs and transcriptional profiling of human subjects with rejection reveal potential involvement of tissue repair.[230,231] Still, identifying the optimal genetic background and understanding how expression of sets of genes over time drives (or allows) accommodation to occur will require a more incisive analysis, probably both GWAS and dynamic gene expression.[232,233]

The more proximal consideration, however, is that pigs, like humans, undoubtedly vary greatly in the levels of baseline and induced resistance to immune and inflammatory injury. Differences in genetic background thus may confound efforts to compare efficacy of therapeutic regimens or genetic manipulations of xenografts from distinct sources. Table 2 shows outcomes of heart and kidney transplants with various genetic manipulations performed in nonhuman primates. Some of the variation in outcome reflects the efficacy of genetic manipulations but some reflects differences in the background of the transplants and some differences in immunity between recipients. There is a tendency to think that introduction of more human genes that counter pathologic changes will improve results,[34] but comparison of the outcomes of kidney xenografts listed in Table 2 might suggest otherwise. This problem can be addressed in part by cloning to make the genetic background of the source homogeneous. However, cloning (or inbreeding) potentially fixes in the background gene variants that undermine resistance to injury and restoration of function. A recent report on the outcome of kidney transplants from "multi-transgenic" inbred mini-pigs (a1,3 GT KO, and combinations of human CD55, Hu CD46, Hu CD59, and Hu CD39) in baboons revealed little or no advantage of the transgenes and survival at 3–14 days.[234] These results, disappointing compared to the results shown in Table 2, could reflect various aspects of the regimens or transgenes used, but they could also reflect properties of the genetic background of the source. Nor can one be certain whether off-target effects of genetic manipulation have affected physiology (the heart xenografts are mainly nonfunctional heterotopic grafts, the kidney xenografts are functional). Because the more dramatic barriers to xenotransplantation are overcome and clinical application and long-term function are prized, there will be much potentially to be gained by focusing on function rather than survival and pathology and potentially from optimizing the background of the sources through breeding or engineering or both.

Physiological and Biochemical Barriers to Xenotransplantation (Incompatibilities)

During the first half of the twentieth century, the forebears of transplantation biology and immunology struggled to understand why grafts of foreign tissue fail. One theory, put forward by Leo Loeb, held that proteins produced by genetically-different individuals and especially by individuals of disparate species fail to support healthy and functional interactions between cells from those different individuals.[235] The differences between proteins of different individuals might be assayed by serologic methods, but the failure of grafts and attendant pathology reflected the extent of incompatibility. Although Loeb was recognized widely for his contributions,[236] his theory

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of individuality obscured recognition of immunity as a cause of allograft failure. However, the development of Loeb's theory presaged the challenges one inevitably faces in attempting to understand why xenografts fail.

Because human cells can survive enduringly in immunodeficient mice[99] and immune-competent pigs,[93] whatever incompatibilities may exist between disparate species need not preclude survival of xenografts. However, comparison of protein structure and physiology between species could suggest that xenografts would likely fail to meet the physiologic needs of recipients and incompatibilities of biochemical systems could engender distinct toxicities,[237,238] as first suggested by Loeb. Obviously, the preeminent question for clinical application of xenotransplantation is whether and to what extent biochemical and physiologic incompatibility between species diminish the value of xenotransplantation and whether the defects can be overcome without converting the swine genome to human. These questions have been addressed at least in part by demonstration that xenografts of swine lungs, kidneys, hearts, and pancreatic islets can temporarily support the life of nonhuman primates.[239–245] In contrast, orthotopic porcine liver xenografts can engender life-threatening complications (eg, thrombocytopenia), and some believe incompatibility of the swine liver precludes successful xenotransplantation. However, porcine liver xenografts do exhibit measurable function for a period of days,[246] and whole porcine livers[247] and isolated porcine hepatocytes in liver assist devices[248] can augment functions in patients with acute liver failure, suggesting physiology is not limiting. The observations on transplantation of porcine tissues and organs other than liver into nonhuman primates thus argue against Loeb's idea that xenografts inevitably fail because of incompatibilities generate lethal toxicity.[235] The observations also argue against the proposition that incompatibilities decrease the level or modify the nature of physiologic support to the point where xenografts might not offer an acceptable replacement for a failing tissue or organ.

The acceptable function of porcine xenografts in nonhuman primates for periods of months or even years cannot be taken as evidence of absence of significant incompatibilities between species that would impair clinical utility. Regardless of the extent of species-specific regulation of complement[30,117,138,139] (or lack thereof[140]), experience during the past 20 years provides numerous examples of the benefit for xenograft function and survival conferred by expression of human complement regulators in swine tissues. This benefit indicates that, for whatever reason, xenotransplantation effectuates a functional deficiency of complement regulation. However, functional deficiency or incompatibility are not necessarily apparent immediately but rather might appear months or years after birth (in the case of inherited deficiencies) or transplantation. Some individuals with inherited deficiency (or nonfunction) of complement factor H, factor I, and CD46 (regulators of the alternative complement pathway) develop thrombotic microangiopathy of the kidney (atypical hemolytic uremic syndrome) but do so years after birth or in adulthood.[249,250] Some never develop this condition. A xenotransplantation model functioning for months or even years might very well fail to reveal clinical evidence of some incompatibilities of complement regulation.

Xenotransplantation of swine organs in humans or nonhuman primates would generate incompatibility between the coagulation system of the recipient and coagulation regulators, particularly thrombomodulin, expressed in blood vessels of the transplant.[251,252] Thrombomodulin expressed in porcine blood vessels is appreciably incompatible with human protein C[113,253,254] and that should eventuate in excess generation of thrombin and coagulation and/or inflammation.[255] This incompatibility sparked the development of transgenic pigs expressing human thrombomodulin, among other modifications, and testing with favorable results in pig-to-nonhuman primate organ xenograft models.[256,257] These and more recent results encourage the view that generation of pigs expressing multiple transgenes has advanced xenotransplantation toward clinical application.[3] Whether correct or not, the successes achieved by expression of multiple transgenes, such as those listed in Table 2, should not be taken as critical proof that the transgenes address key molecular incompatibilities. Using the expression of human thrombomodulin as only one example, the incompatibility of the human protein for swine has been proved but the importance of the incompatibility has not. Thrombotic microangiopathy, as observed in organ xenografts, is characteristic of inherited defects in regulation of the alternative pathway of complement (eg, atypical hemolytic uremic syndrome). In contrast, inherited deficiency of thrombomodulin activity typically causes late-onset large vessel or coronary thrombosis,[258] if it causes any disease at all.[259] That is not to question the benefit of expressing human thrombomodulin in xenografts. Transgenic expression of human thrombomodulin is certainly more convenient and less toxic than administration of anticoagulant agents (or correction of what we think might be the more fundamental problem with complement regulation). Rather, it argues that molecular incompatibilities may have less impact than vitro experiments suggest. Inherited defects in regulation of complement or coagulation (among other pathways) are not immediately pathogenic because the system adjusts to increased pathway activity. Such adjustment is likely to be found for countless biochemical or structural "incompatibilities."

Infection Between Species as a Barrier to Xenotransplantation

The possibility that xenotransplantation would convey or heighten the risk of infection has been viewed as a significant barrier to xenotransplantation.[260–262] Accordingly, approaches to prevention and surveillance and standards for microbiological safety have been extensively discussed and recently reviewed.[263–268] Although these approaches will continue to be applied, dimensions of this barrier are now generally viewed as "small"[269] and "manageable."[3] Here we shall consider the general nature of the biological barrier infection poses and the extent to which current models can provide useful insights. For reasons we shall mention, this consideration must remain a matter of speculation until xenotransplantation enters clinical practice.

Transplants of every type potentially convey infectious agents, particularly viruses, to the recipient. That risk is greatest when transplants originate from deceased donors because only limited time can be devoted to screening and because screening might fail to detect a recently acquired transmissible infectious agent. When transplants originate from living human donors, more time and resources can be devoted to screening and clinicians can weigh the risks against potential benefits if the donor has a transmissible virus. In xenotransplantation, the potential for screening is greater because multiple generations of source animals can be evaluated and risks can be further decreased by isolation of source animals, breeding, treatment, vaccination, or genetic engineering to eliminate existing agents.[270] Therefore, in principle, the risk transmitting an infectious agent from a graft to a recipient should be lower

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in clinical xenotransplantation than in allotransplantation. This risk is further decreased because some agents capable of infecting pigs are not infectious for humans.

However, xenotransplantation does potentially engender several risks distinct from those experienced in allotransplantation. One such risk is that infectious agents harbored by the graft, whether or not transferred to human cells, might be less effectively controlled by human immunity, particularly T cells and cytokines, and in this setting cause tissue or organ damage. Porcine CMV has been found to be activated in xenotransplants, capable of activating endothelial cells and associated with thrombotic microangiopathy.[271,272] It is possible that immunity to the graft and rejection causes activation of the virus as virus activation was seen in early rejection.[273] But it is also possible that this or some other virus underlies damage or dysfunction observed over time in xenotransplants. On the other hand, if the swine agent is controlled by the immune system of the recipient, it is possible the agent could serve as a source of peptide targeted by cell-mediated rejection.

Another risk of infection pertinent to xenotransplantation is the possibility that innocuous retroelements or an endogenous retrovirus of the pig could undergo activation and/or recombination to generate a novel virus transferable to the human recipient and potentially more broadly in society. The porcine endogenous retrovirus (PERV) has been thought potentially to be such an agent.[260] A gammaretrovirus, PERV can be activated and transferred to human cells in culture. Concern about PERV has fueled efforts to eliminate elements from the porcine genome by selection and gene targeting.[274] However, humans subjects exposed to pigs in the workplace and subjects whose blood was perfused through porcine livers for treatment of liver failure or through porcine kidneys for kidney failure or recipients of porcine xenografts of skin or other tissues reveal no evidence of PERV transmission to humans.[275–278] Consequently, concern about potential risk of PERV transmission has decreased substantially.[3]

Yet another "infectious" risk unique to xenotransplantation involves the potential consequences of genetic recombination caused by spontaneous fusion of swine and human cells.[91,279] Although fusion of heterologous cells is probably rare, when it occurs, the potential for recombination is increased by aberrant hybridization and DNA breaks, among other events, and recombination potentially generates novel genes.[280–282] Cell fusion has been considered mainly from the perspective of risks of oncogenesis and tumor progression,[282,283] but the same mechanism potentially can underlie emergence or evolution of viruses, for example, acquisition of a ligand for an existing cell surface receptor.[279] Although this mechanism might explain rare emergence of new viruses by evolutionary leaps, we think the likelihood that swine to human xenotransplantation would cause emergence of new viruses by this mechanism is exceedingly low because pigs and humans have lived in proximity for thousands of years and blood is continuously exchanged on farms and other settings (some of those engaged in agriculture could have immunodeficiency or receive immunosuppressive agents). The introduction of human genes in the swine genome at increasing numbers of loci, however, does potentially increase the potential for recombination in hybrids and that might warrant consideration in the future.

On the other hand, because pigs and nonhuman primates do not naturally share habitats and exchange flora, the use of nonhuman primates as recipients of experimental xenografts does potentially generate conditions that could increase the rate of viral evolution. Although accelerated viral evolution is probably not a unique risk of xenotransplantation, for reasons mentioned above, these models potentially offer an opportunity to investigate processes important for public health. As a related consideration, however, experimental transplants of tissues or organs from pigs into nonhuman primates probably offer a poor (and exaggerated) model of the infectious risks of clinical xenotransplantation. Not only are humans better adapted than nonhuman primates to pig flora, but the sophisticated diagnostic tools, range of therapeutic agents, and established regimens and doses of antimicrobials in the clinical setting, among many other factors, probably decrease the risk and improve the outcome of infection in the clinical setting.

Concluding Remarks on Potential Limitations of Current Models of Xenotransplantation for Clinical Application

Advances in experimental xenotransplantation have generated much excitement and the perception that xenotransplantation is rapidly advancing toward clinical application.[1,2,284] To a large extent, this excitement and the perception of progress spring from improvements in the survival, now sometimes exceeding 1 year, of porcine islets, hearts, and kidneys transplanted into nonhuman primates. At this juncture, then, it would seem appropriate to consider how well the preclinical models of xenotransplantation are likely to predict the outcome of clinical xenografts performed for treatment of disease. The questions we think most timely are two. The first question is whether the results in experimental models suggest that in a given condition and circumstance (eg, unavailability of an allogeneic organ), a xenograft could provide a better option than alternative therapies. This question is frequently addressed by practitioners and regulators and hence needs little comment here. The second question, not adequately addressed in the literature, is whether and how pig-to-nonhuman primate models depart systematically from what might be expected of pig-to-human xenografts performed in the clinical setting.

We believe clinical xenografts might well perform better than experimental xenografts discussed above. One reason for this view is that the resources, expertise, fund of knowledge, diagnostics, therapeutics, etc. that can be directed at the recipient of a clinical xenograft vastly exceed what can be directed at recipients in animal models. Another reason for this view is that much of the genetic modification of pigs for xenotransplantation has gained expression of human genes, the products of which (eg, CD46 and thrombomodulin) better regulate complement and coagulation of humans than of nonhuman primates. Even if these proteins have normal function in isolation, the proteins may interact aberrantly in complex networks, the impact of which extends beyond complement and coagulation, potentially influencing expression and function of a broad set of genes,[232,285,286] cellular functions, and signaling pathways,[287] components of which can be physiologically discontinuous between species.[288] One extended network potentially pertinent here concerns coagulation and complement. Nearly all components of the coagulation system vary greatly in the population, reflecting tuning by regulation,[289] and modifying one protein at one anatomic location changes the system in others.[290] Although nonhuman primates are used to model humans, individual proteins and complex systems of nonhuman primates likely have some incompatibility with human proteins and systems. Such incompatibilities might explain some of the abnormal function of organs from nonhuman primates transplanted into patients (Table 1). As a related concern, when nonhuman primates are used as recipients of porcine organ

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xenografts, the nonhuman primates might develop immunity to human proteins expressed as the products of transgenes in pigs. Immune responses of nonhuman primates to human proteins might thus limit the duration or level of action of the human proteins in pig-to-nonhuman primate xenograft models. Immunity to the human proteins would be far less likely to compromise the function porcine xenografts in human recipients.

The potential usefulness of xenotransplantation in clinical settings remains a matter of speculation. Because nonhuman primates do not model the diseases and pathophysiologies that would be addressed by transplantation, it is impossible to accurately compare the potential efficacy of xenotransplantation against the efficacy of other therapies for most conditions. Only clinical trials can be expected to test the efficacy for some potential applications of xenotransplantation.

Two exceptions might be transplantation of islets for treatment of diabetes and transplantation of hepatocytes for treatment of acute liver failure. Both applications are limited at least in part by availability of human tissues and in both settings retransplantation could be performed if the initial transplant failed. Xenotransplantation of hepatocytes for treatment of severe acute liver failure might be especially compelling. Orthotopic liver transplantation is the only life-saving treatment currently available for most severely afflicted individuals. Xenotransplantation might be considered if a human organ (or an effective liver assist device) was not available. Orthotopic liver xenotransplantation seems unlikely to provide a permanent solution, although it might serve as a surgically intrusive bridge to allotransplantation. Hepatocyte xenotransplantation, on the other hand, might avoid removal of the native liver and potentially allow the diseased liver to regenerate.[291] Therefore, the development of a model for acute liver failure in nonhuman primates[292] is a timely advance.

Pig-to-nonhuman primate models for xenotransplantation have proven essential for advancing xenotransplantation. The models established the significance of immune and biochemical barriers to xenotransplantation, especially the significance of Galα1-3Gal as a target natural antibodies and defective control of complement as a mechanism responsible heightened susceptibility of xenografts to complement-mediated injury. Pig-to-nonhuman primate models have been essential to testing physiologic incompatibility of xenogeneic blood vessels with primate coagulation and thromboregulation. Finally, pig-to-nonhuman primate models have proven essential to the testing of genetic engineering as a central approach to addressing those barriers. However, we also believe it is important now to consider the limitations of pig-to-nonhuman primate models, especially as the models are used to test genetic engineering. Little attention has been devoted to incompatibilities between nonhuman primates and humans that might confound efforts to test more subtle genetic manipulations. One consequence could be the introduction of genes to solve problems that would not exist in pig-to-human xenografts. Another might be that the models underestimate the survival and function would be exhibited by pig xenografts in humans. On the other hand, we also suspect that once clinical trials are begun, barriers unappreciated in pig-to-nonhuman primate models will be found and these might well be addressed by introduction of further genetic modifications in pigs and tested in nonhuman primates or perhaps sometimes preferably in "humanized" mice[101,293] to avert limitations of nonhuman primate models.

Having commented extensively on the models used to advance xenotransplantation toward clinical application, we would be remiss not to add that a byproduct of the preclinical investigation of xenotransplantation includes fundamental discoveries. Fundamental discoveries will have value and affect whether xenotransplantation becomes part of clinical practice. These discoveries include the importance of endothelial cells as the engine of changes in tissues targeted by immune responses, accommodation as a response by cellular targets of immunity that subverts injury, rekindled interest in humoral immunity as a determinant of the outcome of organ transplants, including allotransplants, and an impetus for discoveries and applications at the nexus of developmental biology and genetics.

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References

1. Puga Yung GL, Rieben R, Buhler L, Schuurman HJ, Seebach J. Xenotransplantation: where do we stand in 2016? *Swiss Med Wkly*. 2017;147:w14403.
2. McGregor CGA, Byrne GW. Porcine to human heart transplantation: is clinical application now appropriate? *J Immunol Res*. 2017;2017:2534653.
3. Cooper DKC, Pierson RN, 3rd, Hering BJ, et al. Regulation of clinical xenotransplantation-time for a reappraisal. *Transplantation*. 2017;101(8):1766–1769.
4. Snell GD. The terminology of tissue transplantation. *Transplantation*. 1964;2:655–657.
5. Ehrenfried A. Reverdin and other methods of skin-grafting. *Boston Med Surg J*. 1909;CLXI(26):911–927.
6. Davis JS. II. Skin grafting at the Johns Hopkins Hospital. *Ann Surg*. 1909;50(3):542–549.
7. Loeb L. Heterotransplantation of the thyroid gland. *J Exp Med*. 1920;31(6):765–784.
8. Loeb L. Transplantation and individuality. *Biol Bull*. 1921;40:143–180.
9. Hermans MH. Porcine xenografts vs. (cryopreserved) allografts in the management of partial thickness burns: is there a clinical difference? *Burns*. 2014;40(3):408–415.
10. Leonard DA, Mallard C, Albritton A, et al. Skin grafts from genetically modified alpha-1,3-galactosyltransferase knockout miniature swine: a functional equivalent to allografts. *Burns*. 2017;43(8):1717–1724.
11. Guthrie CC. *Blood-vessel surgery and its applications*. New York: Longmans, Green & Co; 1912.
12. Lexer E. Free transplantation. *Ann Surg*. 1914;60(2):166–194.
13. Ullman E. Tissue and organ transplantation. *Ann Surg*. 1914;60:195–219.
14. Carrel A, Guthrie CC. Functions of a transplanted kidney. *Science*. 1905;22(563):473.
15. Jaboulay M. De reins au pli du coude par soutures arterielles et veineuses. *Lyon Med*. 1906;107:575–577.
16. Anonymous. Dr. Hammond gives patient new kidney. *The New York Times*. 11-14-11, 1911.
17. Moore FD, Burch GE, Harken DE, Swan HJ, Murray JE, Lillihei CW. Cardiac and other organ transplantation. In the setting of transplant science as a national effort. *JAMA*. 1968;206(11):2489–2500.
18. Russell PS, Monaco AP. The biology of tissue transplantation. *N Engl J Med*. 1964;271:553–562 CONTD.

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19. Reemtsma K, McCracken BH, Schlegel JU, et al. Renal heterotransplantation in man. *Ann Surg*. 1964;160:384–410.
20. Starzl TE, Marchioro TL, Peters GN, et al. Renal heterotransplantation from baboon to man: experience with 6 cases. *Transplantation*. 1964;2:752–776.
21. Bashford EF. The immunity reaction to cancer. *Proc R Soc Med*. 1910;3(Ther Pharmacol Sect):69–81.
22. Murphy JB. Factors of resistance to heteroplastic tissue-grafting. *J Exp Med*. 1914;19:513–522.
23. United States Department of Health and Human Services, Center for Disease Control, National Center for Health Statistics (U.S.). *Health, United States, 2015: With Special Feature on Racial and Ethnic Health Disparities*. Hyattsville, MD: National Center for Health Statistics; 2016.
24. Platt JL, Cascalho M. New and old technologies for organ replacement. *Curr Opin Organ Transplant*. 2013;18(2):179–185.
25. Platt JL, Cascalho M. Cardiac xenotransplantation, in Congestive Heart Failure and Cardiac Transplantation: clinical, pathology, imaging and molecular profiles. In: Garry DJ, Wilson RF, Vlodaver Z, SpringerLink (Online service), eds. 1st ed. Cham (Switzerland): Springer International Publishing; 2017. doi:10.1007/978-3-319-44577-9.
26. Roake JA, Cahill AP, Gray CM, Gray DW, Morris PJ. Preemptive cadaveric renal transplantation—clinical outcome. *Transplantation*. 1996;62(10):1411–1416.
27. Vats AN, Donaldson L, Fine RN, Chavers BM. Pretransplant dialysis status and outcome of renal transplantation in North American children: a NAPRTCS Study. North American Pediatric Renal Transplant Cooperative Study. *Transplantation*. 2000;69(7):1414–1419.
28. Huang Y, Samaniego M. Preemptive kidney transplantation: has it come of age? *Nephrol Ther*. 2012;8(6):428–432.
29. Jay CL, Dean PG, Helmick RA, Stegall MD. Reassessing preemptive kidney transplantation in the United States: are we making progress? *Transplantation*. 2016;100(5):1120–1127.
30. Platt JL, Vercellotti GM, Dalmasso AP, et al. Transplantation of discordant xenografts: a review of progress. *Immunol Today*. 1990;11:450–456.
31. Platt JL, Bach FH. The barrier to xenotransplantation. *Transplantation*. 1991;52:937–947.
32. Cooper DK, Koren E, Oriol R. Genetically engineered pigs. *Lancet*. 1993;342(8872):682–683.
33. McCurry KR, Kooyman DL, Alvarado CG, et al. Human complement regulatory proteins protect swine-to-primate cardiac xenografts from humoral injury. *Nat Med*. 1995;1:423–427.
34. Chan JL, Singh AK, Corcoran PC, et al. Encouraging experience using multi-transgenic xenografts in a pig-to-baboon cardiac xenotransplantation model. *Xenotransplantation*. 2017;24(6):e12330.
35. Wall RJ. Transgenic livestock: progress and prospects for the future *Theriogenology*. 1996;45(1):57–68.
36. Sharma A, Okabe JF, Birch P, Platt JL, Logan JS. Reduction in the level of Gal (a1,3) Gal in transgenic mice and pigs by the expression of an a(1,2) fucosyltransferase. *Proc Natl Acad Sci USA*. 1996;93:7190–7195.
37. Smithies O, Koralewski MA, Song KY, Kucherlapati RS. Homologous recombination with DNA introduced into mammalian cells. *Cold Spring Harb Symp Quant Biol*. 1984;49:161–170.
38. Folger K, Thomas K, Capecchi MR. Analysis of homologous recombination in cultured mammalian cells. *Cold Spring Harb Symp Quant Biol*. 1984;49:123–138.
39. Cooper DKC, Good AH, Koren E, et al. Identification of a-galactosyl and other carbohydrate epitopes that are bound by human anti-pig antibodies: relevance to discordant xenografting in man. *Transpl Immunol*. 1993;1:198–205.
40. Thomas KR, Capecchi MR. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell*. 1987;51(3):503–512.
41. Koller BH, Hagemann LJ, Doetschman T, et al. Germ-line transmission of a planned alteration made in a hypoxanthine phosphoribosyltransferase gene by homologous recombination in embryonic stem cells. *Proc Natl Acad Sci USA*. 1989;86:8927–8931.
42. Brevini TA, Pennarossa G, Gandolfi F. No shortcuts to pig embryonic stem cells. *Theriogenology*. 2010;74(4):544–550.
43. Koh S, Piedrahita JA. From "ES-like" cells to induced pluripotent stem cells: a historical perspective in domestic animals. *Theriogenology*. 2014;81(1):103–111.
44. Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH. Viable offspring derived from fetal and adult mammalian cells. *Nature*. 1997;385(6619):810–813.
45. Polejaeva IA, Chen S, Vaught TD, et al. Cloned pigs produced by nuclear transfer from adult somatic cells. *Nature*. 2000;407:86–90.
46. Dai Y, Vaught TD, Boone J, et al. Targeted disruption of thealpha1,3-galactosyltransferase gene in cloned pigs. *Nat Biotechnol*. 2002;20(3):251–255.
47. Piedrahita JA, Olby N. Perspectives on transgenic livestock in agriculture and biomedicine: an update. *Reprod Fertil Dev*. 2011;23(1):56–63.
48. Mir B, Piedrahita JA. Nuclear localization signal and cell synchrony enhance gene targeting efficiency in primary fetal fibroblasts. *Nucleic Acids Res*. 2004;32(3):e25.
49. Arbones ML, Austin HA, Capon DJ, Greenburg G. Gene targeting in normal somatic cells: inactivation of the interferon-gamma receptor in myoblasts. *Nat Genet*. 1994;6(1):90–97.
50. Mojica FJ, Montoliu L. On the origin of CRISPR-Cas technology: from prokaryotes to mammals. *Trends Microbiol*. 2016;24(10):811–820.
51. Joung JK, Sander JD. TALENs: a widely applicable technology for targeted genome editing. *Nat Rev Mol Cell Biol*. 2013;14(1):49–55.
52. Cong L, Ran FA, Cox D, et al. Multiplex genome engineering using CRISPR/Cas systems. *Science*. 2013;339(6121):819–823.
53. Cheng AW, Wang H, Yang H, et al. Multiplexed activation of endogenous genes by CRISPR-on, an RNA-guided transcriptional activator system. *Cell Res*. 2013;23(10):1163–1171.
54. Seruggia D, Montoliu L. The new CRISPR-Cas system: RNA-guided genome engineering to efficiently produce any desired genetic alteration in animals. *Transgenic Res*. 2014;23(5):707–716.
55. Zhu Z, Verma N, Gonzalez F, Shi ZD, Huangfu D. A CRISPR/Cas-mediated selection-free knockin strategy in human embryonic stem cells. *Stem Cell Reports*. 2015;4(6):1103–1111.
56. Suzuki K, Tsunekawa Y, Hernandez-Benitez R, et al. In vivo genome editing via CRISPR/Cas9 mediated homology-independent targeted integration. *Nature*. 2016;540(7631):144–149.
57. He X, Tan C, Wang F, et al. Knock-in of large reporter genes in human cells via CRISPR/Cas9-induced homology-dependent and independent DNA repair. *Nucleic Acids Res*. 2016;44(9):e85.
58. Tsai SQ, Joung JK. Defining and improving the genome-wide specificities of CRISPR-Cas9 nucleases. *Nat Rev Genet*. 2016;17(5):300–312.

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59. Tsai SQ, Zheng Z, Nguyen NT, et al. GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. *Nat Biotechnol*. 2015;33(2):187–197.
60. Watanabe M, Umeyama K, Matsunari H, et al. Knockout of exogenous EGFP gene in porcine somatic cells using zinc-finger nucleases. *Biochem Biophys Res Commun*. 2010;402(1): 14–18.
61. Hauschild J, Petersen B, Santiago Y, et al. Efficient generation of a biallelic knockout in pigs using zinc-finger nucleases. *Proc Natl Acad Sci USA*. 2011;108(29):12013–12017.
62. Watanabe M, Nakano K, Matsunari H, et al. Generation of interleukin-2 receptor gamma gene knockout pigs from somatic cells genetically modified by zinc finger nuclease-encoding mRNA. *PLoS One*. 2013;8(10):e76478.
63. Hockemeyer D, Wang H, Kiani S, et al. Genetic engineering of human pluripotent cells using TALE nucleases. *Nat Biotechnol*. 2011;29(8):731–734.
64. Cermak T, Doyle EL, Christian M, et al. Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. *Nucleic Acids Res*. 2011;39(12):e82.
65. Carlson DF, Tan W, Lillico SG, et al. Efficient TALEN-mediated gene knockout in livestock. *Proc Natl Acad Sci USA*. 2012;109(43):17382–17387.
66. Xin J, Yang H, Fan N, et al. Highly efficient generation of GGTA1 biallelic knockout inbred mini-pigs with TALENs. *PLoS One*. 2013;8(12):e84250.
67. Yao J, Huang J, Hai T, et al. Efficient bi-allelic gene knockout and site-specific knock-in mediated by TALENs in pigs. *Sci Rep*. 2014;4:6926.
68. Huang J, Guo X, Fan N, et al. RAG1/2 knockout pigs with severe combined immunodeficiency. *J Immunol*. 2014;193(3): 1496–1503.
69. Gao H, Zhao C, Xiang X, et al. Production ofalpha1,3-galactosyltransferase and cytidine monophosphate-N-acetylneuraminic acid hydroxylase gene double-deficient pigs by CRISPR/Cas9 and handmade cloning. *J Reprod Dev*. 2017;63(1):17–26.
70. Zhou X, Xin J, Fan N, et al. Generation of CRISPR/Cas9-mediated gene-targeted pigs via somatic cell nuclear transfer. *Cell Mol Life Sci*. 2015;72(6):1175–1184.
71. Chen F, Wang Y, Yuan Y, et al. Generation of B cell-deficient pigs by highly efficient CRISPR/Cas9-mediated gene targeting. *J Genet Genomics*. 2015;42(8):437–444.
72. Ruan J, Li H, Xu K, et al. Highly efficient CRISPR/Cas9-mediated transgene knockin at the H11 locus in pigs. *Sci Rep*. 2015;5:14253.
73. Sper RB, Koh S, Zhang X, et al. Generation of a stable transgenic swine model expressing a porcine histone 2B-eGFP fusion protein for cell tracking and chromosome dynamics studies. *PLoS One*. 2017;12(1):e0169242.
74. Zhang W, Wang G, Wang Y, et al. Generation of complement protein C3 deficient pigs by CRISPR/Cas9-mediated gene targeting. *Sci Rep*. 2017;7(1):5009.
75. Salomon DR. A CRISPR way to block PERVs—engineering organs for transplantation. *N Engl J Med*. 2016;374(11): 1089–1091.
76. Whitworth KM, Lee K, Benne JA, et al. Use of the CRISPR/Cas9 system to produce genetically engineered pigs from in vitro-derived oocytes and embryos. *Biol Reprod*. 2014;91(3):78.
77. Wang X, Cao C, Huang J, et al. One-step generation of triple gene-targeted pigs using CRISPR/Cas9 system. *Sci Rep*. 2016; 6:20620.
78. Lei S, Ryu J, Wen K, et al. Increased and prolonged human norovirus infection in RAG2/IL2RG deficient gnotobiotic pigs with severe combined immunodeficiency. *Sci Rep*. 2016;6:25222.
79. Kuroiwa Y, Kasinathan P, Matsushita H, et al. Sequential targeting of the genes encoding immunoglobulin-mu and prion protein in cattle. *Nat Genet*. 2004;36(7):775–780.
80. Wakayama S, Kohda T, Obokata H, et al. Successful serial recloning in the mouse over multiple generations. *Cell Stem Cell*. 2013;12(3):293–297.
81. Cascalho M, Platt JL. Xenotransplantation and other means of organ replacement. *Nat Rev Immunol*. 2001;1(2):154–160.
82. Ogle BM, Cascalho M, Platt JL. Fusion of approaches to the treatment of organ failure. *Am J Transplant*. 2004;4 (Suppl 6): 74–77.
83. Cascalho M, Platt J. New technologies for organ replacement and augmentation. *Mayo Clin Proc*. 2005;80(3):370–378.
84. Liu H, Kim Y, Sharkis S, Marchionni L, Jang YY. In vivo liver regeneration potential of human induced pluripotent stem cells from diverse origins. *Sci Transl Med*. 2011;3(82):82ra39.
85. Nagamoto Y, Takayama K, Ohashi K, et al. Transplantation of a human iPSC-derived hepatocyte sheet increases survival in mice with acute liver failure. *J Hepatol*. 2016;64(5): 1068–1075.
86. Irudayaswamy A, Muthiah M, Zhou L, et al. Long-term fate of human fetal liver progenitor cells transplanted in injured mouse livers. *Stem Cells*. 2018;36(1):103–113.
87. Bantounas I, Ranjzad P, Tengku F, et al. Generation of functioning nephrons by implanting human pluripotent stem cell-derived kidney progenitors. *Stem Cell Reports*. 2018;10(3): 766–779.
88. Zanjani ED, Pallavicini MG, Ascensao JL, et al. Engraftment and long-term expression of human fetal hemopoietic stem cells in sheep following transplantation in utero. *J Clin Invest*. 1992;89(4):1178–1188.
89. Zanjani ED, Flake AW, Rice H, Hedrick M, Tavassoli M. Long-term repopulating ability of xenogeneic transplanted human fetal liver hematopoietic stem cells in sheep. *J Clin Invest*. 1994;93(3):1051–1055.
90. Zanjani ED, Almeida-Porada G, Ascensao JL, MacKintosh FR, Flake AW. Transplantation of hematopoietic stem cells in utero. *Stem Cells*. 1997;15(Suppl 1):79–92; discussion 93.
91. Ogle BM, Butters KB, Plummer TB, et al. Spontaneous fusion of cells between species yields transdifferentiation and retroviral in vivo. *FASEB J*. 2004;18:548–550.
92. Fujiki Y, Fukawa K, Kameyama K, et al. Successful multilineage engraftment of human cord blood cells in pigs after in utero transplantation. *Transplantation*. 2003;75(7):916–922.
93. Ogle BM, Knudsen BE, Nishitai R, Ogata K, Platt JL. Toward the development of human T cells in swine for potential use in adoptive T cell immunotherapy. *Tiss Eng*. 2009;15(5): 1031–1040.
94. Zanjani ED. The human/sheep xenograft model for assay of human HSC (discussion). *Stem Cells*. 1997;15(Suppl 1):209.
95. Almeida-Porada G, Crapnell K, Porada C, et al. In vivo haematopoietic potential of human neural stem cells. *Br J Haematol*. 2005;130(2):276–283.
96. Chamberlain J, Yamagami T, Colletti E, et al. Efficient generation of human hepatocytes by the intrahepatic delivery of clonal human mesenchymal stem cells in fetal sheep. *Hepatology*. 2007;46(6):1935–1945.
97. Narayan AD, Chase JL, Lewis RL, et al. Human embryonic stem cell-derived hematopoietic cells are capable of engrafting primary as well as secondary fetal sheep recipients. *Blood*. 2006;107(5):2180–2183.
98. Ogle BM, Butters KA, Plummer TB, et al. Spontaneous fusion of cells between species yields transdifferentiation and retroviral transfer in vivo. *FASEB J*. 2004;18(3):548–550.

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99. Kalscheuer H, Onoe T, Dahmani A, et al. Xenograft tolerance and immune function of human T cells developing in pig thymus xenografts. *J Immunol*. 2014;192(7):3442–3450.
100. Theocharides AP, Rongvaux A, Fritsch K, Flavell RA, Manz MG. Humanized hemato-lymphoid system mice. *Haematologica*. 2016;101(1):5–19.
101. Li HW, Vishwasrao P, Holzl MA, et al. Impact of mixed xenogeneic porcine hematopoietic chimerism on human NK cell recognition in a humanized mouse model. *Am J Transplant*. 2017;17(2):353–364.
102. Rygaard J, Povlsen CO. Heterotransplantation of a human malignant tumour to "Nude" mice. *Acta Pathol Microbiol Scand*. 1969;77(4):758–760.
103. Fujiwara S. Humanized mice: a brief overview on their diverse applications in biomedical research. *J Cell Physiol*. 2018;233(4):2889–2901.
104. Rongvaux A, Willinger T, Martinek J, et al. Development and function of human innate immune cells in a humanized mouse model. *Nat Biotechnol*. 2014;32(4):364–372.
105. Schoner A, Adham I, Mauceri G, et al. Partial rescue of the KIT-deficient testicular phenotype in KitW-v/KitW-v Tg (TSPY) mice. *Biol Reprod*. 2010;83(1):20–26.
106. Azuma H, Paulk N, Ranade A, et al. Robust expansion of human hepatocytes in $Fah^{-/-}/Rag2^{-/-}/Il2rg^{-/-}$ mice. *Nat Biotechnol*. 2007;25(8):903–910.
107. Suzuki S, Iwamoto M, Saito Y, et al. Il2rg gene-targeted severe combined immunodeficiency pigs. *Cell Stem Cell*. 2012;10(6):753–758.
108. Lee K, Kwon DN, Ezashi T, et al. Engraftment of human iPS cells and allogeneic porcine cells into pigs with inactivated RAG2 and accompanying severe combined immunodeficiency. *Proc Natl Acad Sci USA*. 2014;111(20):7260–7265.
109. Cooper DKC, Human PA, Lexer G, et al. Effects of cyclosporine and antibody adsorption on pig cardiac xenograft survival in the baboon. *J Heart Transplant*. 1988;7:238–246.
110. White D, Wallwork J. Xenografting: probability, possibility, or pipe dream? *Lancet*. 1993;342:879–880.
111. Schuurman HJ. Microbiological safety of clinical xenotransplantation products: monitoring strategies and regulatory aspects. A commentary. *Xenotransplantation*. 2016;23(6):440–443.
112. Crossan C, O'Hara Z, Mourad N, Gianello P, Scobie L. Examining the potential for porcine-derived islet cells to harbour viral pathogens. *Xenotransplantation*. 2018;25(2):e12375.
113. Lawson JH, Platt JL. Molecular barriers to xenotransplantation. *Transplantation*. 1996;62:303–310.
114. Platt JL. New directions for organ transplantation. *Nature*. 1998;392(Suppl 6679):11–17.
115. Michaels MG, Simmons RL. Xenotransplant-associated zoonoses. *Transplantation*. 1994;57:1–7.
116. Allan JS. Xenograft transplantation of the infectious disease conundrum. *ILAR J*. 1995;37(1):37–48.
117. Dalmasso AP, Vercellotti GM, Platt JL, Bach FH. Inhibition of complement-mediated endothelial cell cytotoxicity by decay accelerating factor: potential for prevention of xenograft hyperacute rejection. *Transplantation*. 1991;52:530–533.
118. Weiser MR, Williams JP, Moore FDJ, et al. Reperfusion injury of ischemic skeletal muscle is mediated by natural antibody and complement. *J Exp Med*. 1996;183:2343–2348.
119. Lin SS, Kooyman DL, Daniels LJ, et al. The role of natural anti-Gala1-3Gal antibodies in hyperacute rejection of pig-to-baboon cardiac xenotransplants. *Transpl Immunol*. 1997;5:212–218.
120. Fearon DT, Locksley RM. The instructive role of innate immunity in the acquired immune response. *Science*. 1996;272:50–53.
121. Freeley S, Kemper C, Le Friec G. The "ins and outs" of complement-driven immune responses. *Immunol Rev*. 2016;274(1):16–32.
122. Platt JL, Silva I, Balin SJ, et al. C3d regulates immune checkpoint blockade and enhances antitumor immunity. *JCI Insight*. 2017;2(9):pii: 90201.
123. Hummel M, Abecassis MM. A model for reactivation of CMV from latency. *J Clin Virol*. 2002;25(Suppl 2):S123–S136.
124. Cook CH, Trgovcich J. Cytomegalovirus reactivation in critically ill immunocompetent hosts: a decade of progress and remaining challenges. *Antiviral Res*. 2011;90(3):151–159.
125. Kapadia SB, Levine B, Speck SH, Virgin HW, 4th. Critical role of complement and viral evasion of complement in acute, persistent, and latent gamma-herpesvirus infection. *Immunity*. 2002;17(2):143–155.
126. Saadi S, Wrenshall LE, Platt JL. Regional manifestations and control of the immune system. *FASEB J*. 2002;16(8):849–856.
127. Auchincloss H, Jr. Xenogeneic transplantation. *Transplantation*. 1988;46:1–20.
128. Meier RPH, Muller YD, Balaphas A, et al. Xenotransplantation: back to the future? *Transplant Int*. 2018;31(5):465–477.
129. Auchincloss H, Jr., Sachs DH. Xenogeneic transplantation. *Annu Rev Immunol*. 1998;16:433–470.
130. Cascalho M, Platt JL. The immunological barrier to xenotransplantation. *Immunity*. 2001;14:437–446.
131. Vadori M, Cozzi E. Immunological challenges and therapies in xenotransplantation. *Cold Spring Harb Perspect Med*. 2014;4(4):a015578.
132. Bennet W, Sundberg B, Groth CG, et al. Incompatibility between human blood and isolated islets of Langerhans: a finding with implications for clinical intraportal islet transplantation? *Diabetes*. 1999;48(10):1907–1914.
133. Bennet W, Sundberg B, Lundgren T, et al. Damage to porcine islets of langerhans after exposure to human blood in vitro, or after intraportal transplantation to cynomologus monkeys. *Transplantation*. 2000;69(5):711–719.
134. Samy KP, Davis RP, Gao Q, et al. Early barriers to neonatal porcine islet engraftment in a dual transplant model. *Am J Transplant*. 2018;18(4):998–1006.
135. Liszewski MK, Atkinson JP. Complement regulators in human disease: lessons from modern genetics. *J Intern Med*. 2015;277(3):294–305.
136. Bajic G, Degn SE, Thiel S, Andersen GR. Complement activation, regulation, and molecular basis for complement-related diseases. *EMBO J*. 2015;34(22):2735–2757.
137. Platt JL, Wrenshall LE, Johnson GB, Cascalho M. Heparan sulfate proteoglycan metabolism and the fate of grafted tissues. *Adv Exp Med Biol*. 2015;865:123–140.
138. Lachmann PJ. The control of homologous lysis. *Immunol Today*. 1991;12:312–315.
139. Atkinson JP, Oglesby TJ, White D, Adams EA, Liszewski MK. Separation of self from non-self in the complement system: a role for membrane cofactor protein and decay accelerating factor. *Clin Exp Immunol*. 1991;86(Suppl 1):27–30.
140. Morgan BP, Berg CW, Harris CL. "Homologous restriction" in complement lysis: roles of membrane complement regulators. *Xenotransplantation*. 2005;12(4):258–265.

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141. Todd C, White RG. On the haemolytic immune isolysins of the ox and their relation to the question of individuality and blood-relationship. *J Hyg (Lond)*. 1910;10(2):185–195.
142. Parker W, Lundberg-Swanson K, Holzknecht ZE, et al. Isohemagglutinins and xenoreactive antibodies are members of a distinct family of natural antibodies. *Hum Immunol*. 1996;45:94–104.
143. Garcia de Mattos Barbosa M, Cascalho M, Platt JL. Accommodation in ABO-incompatible organ transplants. *Xenotransplantation*. 2018;25(3):e12418.
144. Pangburn MK, Ferreira VP, Cortes C. Discrimination between host and pathogens by the complement system. *Vaccine*. 2008;26(Suppl 8):I15–I21.
145. Miyagawa S, Hirose H, Shirakura R, et al. The mechanism of discordant xenograft rejection. *Transplantation*. 1988;46: 825–830.
146. Hourcade DE. Properdin and complement activation: a fresh perspective. *Curr Drug Targets*. 2008;9(2):158–164.
147. Kemper C, Atkinson JP, Hourcade DE. Properdin: emerging roles of a pattern-recognition molecule. *Annu Rev Immunol*. 2010;28:131–155.
148. Galili U, Macher BA, Buehler J, Shohet SB. Human natural anti-a-galactosyl IgG. II. the specific recognition of a (1–3)-linked glactose residues. *J Exp Med*. 1985;162:573–582.
149. Oriol R, Koren E, Cooper DKC. Carbohydrate antigens of pig tissues reacting with human natural antibodies as potential targets for hyperacute vascular rejection in pig-to-man organ xenotransplantation. *Transplantation*. 1993;56:1433–1442.
150. Galili U, Clark MR, Shohet SB, Buehler J, Macher BA. Evolutionary relationship between the natural anti-Gal antibody and the Gal a1-3Gal epitope in primates. *Proc Natl Acad Sci USA*. 1987;84:1369–1373.
151. Lin SS, Weidner BC, Byrne GW, et al. The role of antibodies in acute vascular rejection of pig-to-baboon cardiac transplants. *J Clin Invest*. 1998;101:1745–1756.
152. Lai L, Kolber-Simonds D, Park KW, et al. Production of a-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. *Science*. 2002;295(5557):1089–1092.
153. Phelps CJ, Koike C, Vaught TD, et al. Production of alpha 1,3-galactosyltransferase-deficient pigs. *Science*. 2003;299 (5605):411–414.
154. Cascalho MI, Chen BJ, Kain M, Platt JL. The paradoxical functions of B cells and antibodies in transplantation. *J Immunol*. 2013;190(3):875–879.
155. Byrne GW, McGregor CGA, Breimer ME. Recent investigations into pig antigen and anti-pig antibody expression. *Int J Surg*. 2015;23(Pt B):223–228.
156. Casali P, Notkins AL. Probing the human B-cell repertoire with EBV: polyreactive antibodies and CD5+ B lymphocytes. *Annu Rev Immunol*. 1989;7:513–535.
157. Kulik L, Fleming SD, Moratz C, et al. Pathogenic natural antibodies recognizing annexin IV are required to develop intestinal ischemia-reperfusion injury. *J Immunol*. 2009;182 (9):5363–5373.
158. Avrameas S. Natural autoantibodies: from 'horror autotoxicus' to 'gnothi seauton'. *Immunol Today*. 1991;12:154–159.
159. Silverman GJ, Vas J, Gronwall C. Protective autoantibodies in the rheumatic diseases: lessons for therapy. *Nat Rev Rheumatol*. 2013;9(5):291–300.
160. Platt JL, Cascalho M. IgM in the kidney: a multiple personality disorder. *Kidney Int*. 2015;88(3):439–441.
161. Geller RL, Bach FH, Turman MA, Casali P, Platt JL. Evidence that polyreactive antibodies are deposited in rejected discordant xenografts. *Transplantation*. 1993;55:168–172.
162. Hoffman W, Lakkis FG, Chalasani G. B cells, antibodies, and more. *Clin J Am Soc Nephrol*. 2016;11(1):137–154.
163. Rabb H, Daniels F, O'Donnell M, et al. Pathophysiological role of T lymphocytes in renal ischemia-reperfusion injury in mice. *Am J Physiol Renal Physiol*. 2000;279(3):F525–F531.
164. Rajalingam R. The impact of HLA class I-specific killer cell immunoglobulin-like receptors on antibody-dependent natural killer cell-mediated cytotoxicity and organ allograft rejection. *Front Immunol*. 2016;7:585.
165. Halloran PF, Venner JM, Madill-Thomsen KS, et al. Review: the transcripts associated with organ allograft rejection. *Am J Transplant*. 2018;18(4):785–795.
166. Paul P, Picard C, Sampol E, et al. Genetic and functional profiling of CD16-dependent natural killer activation identifies patients at higher risk of cardiac allograft vasculopathy. *Circulation*. 2018;137(10):1049–1059.
167. Malyguine AM, Saadi S, Holzknecht RA, et al. Induction of procoagulant function in porcine endothelial cells by human NK cells. *J Immunol*. 1997;159:4659–4664.
168. Geller RL, Ihrcke NS, Platt JL. Release of endothelial cell-associated heparan sulfate proteoglycan by activated T cells. *Transplantation*. 1994;57:770–774.
169. Bustos M, Saadi S, Platt JL. Platelet-mediated activation of endothelial cells: implications for the pathogenesis of transplant rejection. *Transplantation*. 2001;72:509–515.
170. Bender JR, Pardi R, Karasek MA, Engleman EG. Phenotypic and functional characterization of lymphocytes that bind human microvascular endothelial cells in vitro. Evidence for preferential binding of natural killer cells. *J Clin Invest*. 1987;79(6):1679–1688.
171. Inverardi L, Samaja M, Motterlini R, Mangili F, Bender JR, Pardi R. Early recognition of a discordant xenogeneic organ by human circulating lymphocytes. *J Immunol*. 1992;149: 1416–1423.
172. Sommaggio R, Cohnen A, Watzl C, Costa C. Multiple receptors trigger human NK cell-mediated cytotoxicity against porcine chondrocytes. *J Immunol*. 2012;188(5):2075–2083.
173. Hisashi Y, Yamada K, Kuwaki K, et al. Rejection of cardiac xenografts transplanted fromalpha1,3-galactosyltransferase gene-knockout (GalT-KO) pigs to baboons. *Am J Transplant*. 2008;8(12):2516–2526.
174. Inverardi L, Clissi B, Stolzer AL, Bender JR, Sandrin MS, Pardi R. Human natural killer lymphocytes directly recognize evolutionarily conserved oligosaccharide ligands expressed by xenogeneic tissues. *Transplantation*. 1997;63: 1318–1330.
175. Itescu S, Kwiatkowski P, Artrip JH, et al. Role of natural killer cells, macrophages, and accessory molecule interactions in the rejection of pig-to-primate xenografts beyond the hyperacute period. *Hum Immunol*. 1998;59(5):275–286.
176. Oldenborg PA, Gresham HD, Lindberg FP. CD47-signal regulatory protein alpha (SIRPalpha) regulates Fcgamma and complement receptor-mediated phagocytosis. *J Exp Med*. 2001;193(7):855–862.
177. Weiss EH, Lilienfeld BG, Muller S, et al. HLA-E/human beta2-microglobulin transgenic pigs: protection against xenogeneic human anti-pig natural killer cell cytotoxicity. *Transplantation*. 2009;87(1):35–43.
178. Tena AA, Sachs DH, Mallard C, et al. Prolonged survival of pig skin on baboons after administration of pig cells expressing human CD47. *Transplantation*. 2017;101(2):316–321.
179. Laird CT, Burdorf L, French BM, et al. Transgenic expression of human leukocyte antigen-E attenuates GalKO.hCD46 porcine lung xenograft injury. *Xenotransplantation*. 2017;24(2):e12294.

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180. Ehrlich P, Morgenroth J. On hæmolysins. Third communication. In: Himmelweit F, ed. *The Collected Papers of Paul Ehrlich*. 2nd vol. London: Pergamon; 1957:205–212.
181. Nuttall GHF, Graham-Smith GS, Pigg-Strangeways TS. *Blood Immunity and Blood Relationship*. Cambridge: University Press; 1904.
182. Fleisher MS. Immunity and tissue transplantation: III. A comparison of heterotransplantation and homoiotransplantation. *J Med Res*. 1918;38(3):353–369.
183. Lindahl KF, Bach FH. Genetic and cellular aspects of xenogeneic mixed leukocyte culture reaction. *J Exp Med*. 1976; 144:305–318.
184. Alter BJ, Bach FH. Cellular basis of the proliferative response of human T cells to mouse xenoantigens. *J Exp Med*. 1990;191:333–338.
185. Moses RD, Pierson RN, 3rd, Winn HJ, Auchincloss HJ. Xenogeneic proliferation and lymphokine production are dependent on CD4+ helper T cells and self antigen-presenting cells in the mouse. *J Exp Med*. 1990;172:567–575.
186. Pierson RN, Winn HJ, Russell PS, Auchincloss H. Xenogeneic skin graft rejection is especially dependent on CD4+ T cells. *J Exp Med*. 1989;170:991–996.
187. Winsor-Hines D, Merrill C, O'Mahony M, et al. Induction of immunological tolerance/hyporesponsiveness in baboons with a nondepleting CD4 antibody. *J Immunol*. 2004;173(7): 4715–4723.
188. Higginbotham L, Ford ML, Newell KA, Adams AB. Preventing T cell rejection of pig xenografts. *Int J Surg*. 2015;23(Pt B):285–290.
189. Sykes M. Mixed chimerism and transplant tolerance. *Immunity*. 2001;14(4):417–424.
190. Vagefi PA, Shah JA, Sachs DH. Progress towards inducing tolerance of pig-to-primate xenografts. *Int J Surg*. 2015;23 (Pt B):291–295.
191. Yamada K, Sykes M, Sachs DH. Tolerance in xenotransplantation. *Curr Opin Organ Transplant*. 2017;22(6):522–528.
192. Diamond LE, Quinn CM, Martin MJ, Lawson J, Platt JL, Logan JS. A human CD46 transgenic pig model system for the study of discordant xenotransplantation. *Transplantation*. 2001;71: 132–142.
193. Mohiuddin MM, Singh AK, Corcoran PC, et al. Chimeric 2C10R4 anti-CD40 antibody therapy is critical for long-term survival of GTKO.hCD46.hTBM pig-to-primate cardiac xenograft. *Nat Commun*. 2016;7:11138.
194. McCurry KR, Parker W, Cotterell AH, et al. Humoral responses in pig-to-baboon cardiac transplantation: implications for the pathogenesis and treatment of acute vascular rejection and for accommodation. *Hum Immunol*. 1997;58: 91–105.
195. Richards AC, Davies HF, McLaughlin ML, et al. Serum anti-pig antibodies as potential indicators of acute humoral xenograft rejection in pig-to-cynomolgus monkey kidney transplantation. *Transplantation*. 2002;73(6):881–889.
196. Thompson P, Badell IR, Lowe M, et al. Islet xenotransplantation using gal-deficient neonatal donors improves engraftment and function. *Am J Transplant*. 2011;11: 2593–2602.
197. Stewart JM, Tarantal AF, Hawthorne WJ, et al. Rhesus monkeys and baboons develop clotting factor VIII inhibitors in response to porcine endothelial cells or islets. *Xenotransplantation*. 2014;21(4):341–352.
198. Parker W, Lin SS, Platt JL. Antigen expression in xenotransplantation: how low must it go? *Transplantation*. 2001;71: 313–319.
199. Platt JL, Cascalho M. Donor specific antibodies after transplantation. *Pediatr Transplant*. 2011;15(7):686–690.
200. Platt JL. Accommodation: how you see it, how you don't. *Am J Transplant*. 2011;11(10):2007–2008.
201. Lynch RJ, Silva IA, Chen BJ, Punch JD, Cascalho M, Platt JL. Cryptic B cell response to renal transplantation. *Am J Transplant*. 2013;13(7):1713–1723.
202. Ding J, Zhou J, Tryggvason K, Kashtan CE. COL4A5 deletions in three patients with Alport syndrome and post-transplant antiglomerular basement membrane nephritis. *J Am Soc Nephrol*. 1994;5(2):161–168.
203. Zhang G, Wang H, Wang F, et al. Gene polymorphisms of the renin-angiotensin-aldosterone system and angiotensin II type 1-receptor activating antibodies in renal rejection. *Tohoku J Exp Med*. 2007;213(3):203–214.
204. Nagata H, Nishitai R, Shirota C, et al. Prolonged survival of porcine hepatocytes in cynomolgus monkeys. *Gastroenterology*. 2007;132:321–329.
205. Soltys KA, Setoyama K, Tafaleng EN, et al. Host conditioning and rejection monitoring in hepatocyte transplantation in humans. *J Hepatol*. 2017;66(5):987–1000.
206. Walch JM, Zeng Q, Li Q, et al. Cognate antigen directs CD8+ T cell migration to vascularized transplants. *J Clin Invest*. 2013;123(6):2663–2671.
207. Pober JS, Merola J, Liu R, Manes TD. Antigen presentation by vascular cells. *Front Immunol*. 2017;8:1907.
208. Saadi S, Takahashi T, Holzknecht RA, Platt JL. Pathways to acute humoral rejection. *Am J Pathol*. 2004;164(3):1073–1080.
209. Pellegrino MA, Ferrone S, Cooper NR, Dierich MP, Reisfeld RA. Variation in susceptibility of a human lymphoid cell line to immune lysis during the cell cycle. Lack of correlation with antigen density and complement binding. *J Exp Med*. 1974;140(2):578–590.
210. Ohanian SH, Borsos T. Lysis of tumor cells by antibody and complement. II. Lack of correlation between amount of C4 and C3 fixed and cell lysis. *J Immunol*. 1975;114(4):1292–1295.
211. Cosio FG, Qiu W, Henry ML, et al. Factors related to the donor organ are major determinants of renal allograft function and survival. *Transplantation*. 1996;62(11):1571–1576.
212. Louvar DW, Li N, Snyder J, Peng Y, Kasiske BL, Israni AK. "Nature versus nurture" study of deceased-donor pairs in kidney transplantation. *J Am Soc Nephrol*. 2009;20(6):1351–1358.
213. Gautreaux MD, Freedman BI. Genotypic variation and outcomes in kidney transplantation: donor and recipient effects. *Kidney Int*. 2013;84(3):431–433.
214. Mesnard L, Muthukumar T, Burbach M, et al. Exome sequencing and prediction of long-term kidney allograft function. *PLoS Comput Biol*. 2016;12(9):e1005088.
215. Phelan PJ, Conlon PJ, Sparks MA. Genetic determinants of renal transplant outcome: where do we stand? *J Nephrol*. 2014;27(3):247–256.
216. Chand S, McKnight AJ, Borrows R. Genetic polymorphisms and kidney transplant outcomes. *Curr Opin Nephrol Hypertens*. 2014;23(6):605–610.
217. Chopek MW, Simmons RL, Platt JL. ABO-incompatible renal transplantation: initial immunopathologic evaluation. *Transplant Proc*. 1987;19:4553–4557.
218. Ishida H, Kondo T, Shimizu T, Nozaki T, Tanabe K. Postoperative rebound of antiblood type antibodies and antibody-mediated rejection after ABO-incompatible living-related kidney transplantation. *Transpl Int*. 2015;28(3): 286–296.
219. Tait BD, Susal C, Gebel HM, et al. Consensus guidelines on the testing and clinical management issues associated

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with HLA and non-HLA antibodies in transplantation. *Transplantation*. 2013;95(1):19–47.
220. Koch CA, Kanazawa A, Nishitai R, et al. Intrinsic resistance of hepatocytes to complement-mediated injury. *J Immunol*. 2005;174:7302–7309.
221. Bach FH, Ferran C, Hechenleitner P, et al. Accommodation of vascularized xenografts: expression of "protective genes" by donor endothelial cells in a host Th2 cytokine environment. *Nat Med*. 1997;3:196–204.
222. Cascalho M, Platt JL. Harnessing B cells in immunotherapy. *Immunotherapy*. 2016;8(3):237–239.
223. Platt JL, Kaufman CL, Garcia de Mattos Barbosa M, Cascalho M. Accommodation and related conditions in vascularized composite allografts. *Curr Opin Organ Transplant*. 2017;22(5):470–476.
224. Koch CA, Khalpey ZI, Platt JL. Accommodation: preventing injury in transplantation and disease. *J Immunol*. 2004;172(9):5143–5148.
225. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144(5):646–674.
226. Dean PG, Park WD, Cornell LD, Gloor JM, Stegall MD. Intragraft gene expression in positive crossmatch kidney allografts: ongoing inflammation mediates chronic antibody-mediated injury. *Am J Transplant*. 2012;12(6):1551–1563.
227. Yang T, Wilkinson J, Wang Z, Ladinig A, Harding J, Plastow G. A genome-wide association study of fetal response to type 2 porcine reproductive and respiratory syndrome virus challenge. *Sci Rep*. 2016;6:20305.
228. Bertolini F, Harding JC, Mote B, Ladinig A, Plastow GS, Rothschild MF. Genomic investigation of piglet resilience following porcine epidemic diarrhea outbreaks. *Anim Genet*. 2017;48(2):228–232.
229. Sato S, Uemoto Y, Kikuchi T, et al. SNP- and haplotype-based genome-wide association studies for growth, carcass, and meat quality traits in a Duroc multigenerational population. *BMC Genet*. 2016;17:60.
230. Adapala NS, Kim HK. Comprehensive genome-wide transcriptomic analysis of immature articular cartilage following ischemic osteonecrosis of the femoral head in piglets. *PLoS One*. 2016;11(4):e0153174.
231. Vitalone MJ, Sigdel TK, Salomonis N, Sarwal RD, Hsieh SC, Sarwal MM. Transcriptional perturbations in graft rejection. *Transplantation*. 2015;99(9):1882–1893.
232. Nicod J, Davies RW, Cai N, et al. Genome-wide association of multiple complex traits in outbred mice by ultra-low-coverage sequencing. *Nat Genet*. 2016;48(8):912–918.
233. da Silva SR, Perrone GC, Dinis JM, de Almeida RM. Reproducibility enhancement and differential expression of non predefined functional gene sets in human genome. *BMC Genomics*. 2014;15:1181.
234. Le Bas-Bernardet S, Tillou X, Branchereau J, et al. Bortezomib, C1-inhibitor and plasma exchange do not prolong the survival of multi-transgenic GalT-KO pig kidney xenografts in baboons. *Am J Transplant*. 2015;15(2):358–370.
235. Loeb L. *The Biological Basis of Individuality*. 1st ed. Springfield: Charles C. Thomas; 1945.
236. Blumenthal HT. Leo Loeb, experimental pathologist and humanitarian. *Science*. 1960;131(3404):907–908.
237. Hammer C. Physiological obstacles after xenotransplantation. *Ann N Y Acad Sci*. 1998;862:19–27.
238. Hammer C, Thein E. Physiological aspects of xenotransplantation, 2001. *Xenotransplantation*. 2002;9(5):303–305.
239. Xu H, Gundry SR, Hancock WW, et al. Prolonged discordant xenograft survival and delayed xenograft rejection in a pig-to-baboon orthotopic cardiac xenograft model. *J Thorac Cardiovasc Surg*. 1998;115(6):1342–1349.
240. Vial CM, Ostlie DJ, Bhatti FN, et al. Life supporting function for over one month of a transgenic porcine heart in a baboon. *J Heart Lung Transplant*. 2000;19(2):224–229.
241. Daggett CW, Yeatman M, Lodge AJ, et al. Total respiratory support from swine lungs in primate recipients. *J Thorac Cardiovasc Surg*. 1998;115:19–27.
242. Nguyen BN, Azimzadeh AM, Zhang T, et al. Life-supporting function of genetically modified swine lungs in baboons. *J Thorac Cardiovasc Surg*. 2007;133(5):1354–1363.
243. Hering BJ, Wijkstrom M, Graham ML, et al. Prolonged diabetes reversal after intraportal xenotransplantation of wild-type porcine islets in immunosuppressed nonhuman primates. *Nat Med*. 2006;12(3):301–303.
244. Cardona K, Korbutt GS, Milas Z, et al. Long-term survival of neonatal porcine islets in nonhuman primates by targeting costimulation pathways. *Nat Med*. 2006;12(3):304–306.
245. van der Windt DJ, Bottino R, Casu A, et al. Long-term controlled normoglycemia in diabetic nonhuman primates after transplantation with hCD46 transgenic porcine islets. *Am J Transplant*. 2009;9(12):2716–2726.
246. Ramirez P, Chavez R, Majado M, et al. Life-supporting human complement regulator decay accelerating factor transgenic pig liver xenograft maintains the metabolic function and coagulation in the nonhuman primate for up to 8 days. *Transplantation*. 2000;70:989–998.
247. Chari RS, Collins BH, Magee JC, et al. Treatment of hepatic failure with ex vivo pig-liver perfusion followed by liver transplantation. *N Engl J Med*. 1994;331(4):234–237.
248. Demetriou AA, Brown RS Jr, Busuttil RW, et al. Prospective, randomized, multicenter, controlled trial of a bioartificial liver in treating acute liver failure. *Ann Surg*. 2004;239(5):660–667; discussion 667–670.
249. Meri S. Complement activation in diseases presenting with thrombotic microangiopathy. *Eur J Intern Med*. 2013;24(6):496–502.
250. Vieira-Martins P, El Sissy C, Bordereau P, Gruber A, Rosain J, Fremeaux-Bacchi V. Defining the genetics of thrombotic microangiopathies. *Transfus Apher Sci*. 2016;54(2):212–219.
251. Schmelzle M, Schulte Esch J, 2nd, Robson SC. Coagulation, platelet activation and thrombosis in xenotransplantation. *Curr Opin Organ Transplant*. 2010;15(2):212–218.
252. Cowan PJ, Robson SC. Progress towards overcoming coagulopathy and hemostatic dysfunction associated with xenotransplantation. *Int J Surg*. 2015;23(Pt B):296–300.
253. Lawson JH, Daniels L, Platt JL. The evaluation of thrombomodulin activity in porcine to human xenotransplantation. *Transplant Proc*. 1997;29:884–885.
254. Siegel JB, Grey ST, Lesnikoski BA, et al. Xenogeneic endothelial cells activate human prothrombin. *Transplantation*. 1997;64(6):888–896.
255. Bongoni AK, Klymiuk N, Wolf E, Ayares D, Rieben R, Cowan PJ. Transgenic expression of human thrombomodulin inhibits HMGB1-induced porcine aortic endothelial cell activation. *Transplantation*. 2016;100(9):1871–1879.
256. Mohiuddin MM, Singh AK, Corcoran PC, et al. One-year heterotopic cardiac xenograft survival in a pig to baboon model. *Am J Transplant*. 2014;14(2):488–489.
257. Iwase H, Ekser B, Satyananda V, et al. Pig-to-baboon heterotopic heart transplantation—exploratory preliminary experience with pigs transgenic for human thrombomodulin and comparison of three costimulation blockade-based regimens. *Xenotransplantation*. 2015;22(3):211–220.

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258. Lobato RL, White WD, Mathew JP, et al. Thrombomodulin gene variants are associated with increased mortality after coronary artery bypass surgery in replicated analyses. *Circulation*. 2011;124(11 Suppl):S143–S148.
259. Ahmad A, Sundquist K, Zoller B, Svensson PJ, Sundquist J, Memon AA. Identification of genetic aberrations in thrombomodulin gene in patients with recurrent venous thromboembolism. *Clin Appl Thromb Hemost*. 2017;23(4):319–328.
260. Patience C, Takeuchi Y, Weiss RA. Infection of human cells by an endogenous retrovirus of pigs. *Nat Med*. 1997;3: 282–286.
261. Chapman LE, Fishman JA. Xenotransplantation and infectious diseases. In: Cooper DKC, Kemp E, Platt JL, eds. *Xenotransplantation: The Transplantation of Organs and Tissues between Species*. 2nd ed. Berlin: Springer; 1997: 736–748.
262. Bach F, Fishman J, Daniels N, et al. Uncertainty in xenotransplantation: individual benefit versus colletctive risk. *Nat Med*. 1998;4:141–144.
263. Fishman JA, Patience C. Xenotransplantation: infectious risk revisited. *Am J Transplant*. 2004;4(9):1383–1390.
264. Takeuchi Y, Magre S, Patience C. The potential hazards of xenotransplantation: an overview. *Rev Sci Tech*. 2005;24(1): 323–334.
265. Denner J. Infectious risk in xenotransplantation—what post-transplant screening for the human recipient? *Xenotransplantation*. 2011;18(3):151–157.
266. Fishman JA, Scobie L, Takeuchi Y. Xenotransplantation-associated infectious risk: a WHO consultation. *Xenotransplantation*. 2012;19(2):72–81.
267. Spizzo T, Denner J, Gazda L, et al. First update of the International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes—Chapter 2a: source pigs—preventing xenozoonoses. *Xenotransplantation*. 2016;23 (1):25–31.
268. Denner J, Tonjes RR, Takeuchi Y, Fishman J, Scobie L. First update of the International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes—Chapter 5: recipient monitoring and response plan for preventing disease transmission. *Xenotransplantation*. 2016;23(1):53–59.
269. Fishman JA. Assessment of infectious risk in clinical xenotransplantation: the lessons for clinical allotransplantation. *Xenotransplantation*. 2014;21(4):307–308.
270. Denner J, Mueller NJ. Preventing transfer of infectious agents. *Int J Surg*. 2015;23(Pt B):306–311.
271. Mueller NJ, Barth RN, Yamamoto S, et al. Activation of cytomegalovirus in pig-to-primate organ xenotransplantation. *J Virol*. 2002;76(10):4734–4740.
272. Gollackner B, Mueller NJ, Houser S, et al. Porcine cytomegalovirus and coagulopathy in pig-to-primate xenotransplantation. *Transplantation*. 2003;75(11):1841–1847.
273. Yamada K, Tasaki M, Sekijima M, et al. Porcine cytomegalovirus infection is associated with early rejection of kidney grafts in a pig to baboon xenotransplantation model. *Transplantation*. 2014;98(4):411–418.
274. Niu D, Wei HJ, Lin L, et al. Inactivation of porcine endogenous retrovirus in pigs using CRISPR-Cas9. *Science*. 2017; 357(6357):1303–1307.
275. Hermida-Prieto M, Domenech N, Moscoso I, et al. Lack of cross-species transmission of porcine endogenous retrovirus (PERV) to transplant recipients and abattoir workers in contact with pigs. *Transplantation*. 2007;84(4):548–550.
276. Patience C, Patton GS, Takeuchi Y, et al. No evidence of pig DNA or retroviral infection in patients with short-term extracorporeal connection to pig kidneys. *Lancet*. 1998;352 (9129):699–701.
277. Paradis K, Langford G, Long Z, et al. Search for cross-species transmission of porcine endogenous retrovirus in patients treated with living pig tissue. *Science*. 1999;285: 1236–1241.
278. Morozov VA, Wynyard S, Matsumoto S, Abalovich A, Denner J, Elliott R. No PERV transmission during a clinical trial of pig islet cell transplantation. *Virus Res*. 2017;227:34–40.
279. Ogle BM, Cascalho M, Platt JL. Biological implications of cell fusion. *Nat Rev Mol Cell Biol*. 2005;6:567–575.
280. Zhou X, Merchak K, Lee W, Grande JP, Cascalho M, Platt JL. Cell Fusion connects oncogenesis with tumor evolution. *Am J Pathol*. 2015;185(7):2049–2060.
281. Platt JL, Zhou X, Lefferts AR, Cascalho M. Cell fusion in the war on cancer: a perspective on the inception of malignancy. *Int J Mol Sci*. 2016;17(7):e1118.
282. Lazebnik Y. The shock of being united: Another lesson from plants? *Cell Cycle*. 2014;13(15):2323–2329.
283. Duelli DM, Padilla-Nash HM, Berman D, Murphy KM, Ried T, Lazebnik Y. A virus causes cancer by inducing massive chromosomal instability through cell fusion. *Curr Biol*. 2007;17(5):431–437.
284. Cowan PJ, Tector AJ. The resurgence of xenotransplantation. *Am J Transplant*. 2017;17(10):2531–2536.
285. Kristjansson RP, Oddsson A, Helgason H, et al. Common and rare variants associating with serum levels of creatine kinase and lactate dehydrogenase. *Nat Commun*. 2016;7: 10572.
286. Visscher PM, Wray NR, Zhang Q, et al. 10 years of GWAS discovery: biology, function, and translation. *Am J Hum Genet*. 2017;101(1):5–22.
287. Kumar S. Cellular and molecular pathways of renal repair after acute kidney injury. *Kidney Int*. 2018;93(1):27–40.
288. Jablonski D. Approaches to macroevolution: 1. General concepts and origin of variation. *Evol Biol*. 2017;44(4): 427–450.
289. Brummel-Ziedins KE, Orfeo T, Rosendaal FR, et al. Empirical and theoretical phenotypic discrimination. *J Thromb Haemost*. 2009;7(Suppl 1):181–186.
290. Brummel-Ziedins KE, Lam PH, Gissel M, Gauthier E, Schneider DJ. Depletion of systemic concentrations of coagulation factors in blood from patients with atherosclerotic vascular disease. *Coron Artery Dis*. 2013;24(6):468–474.
291. Soltys KA, Soto-Gutiérrez A, Nagaya M, et al. Barriers to the successful treatment of liver disease by hepatocyte transplantation. *J Hepatol*. 2010;53(4):769–774.
292. Yannam GR, Han B, Setoyama K, et al. A nonhuman primate model of human radiation-induced venocclusive liver disease and hepatocyte injury. *Int J Radiat Oncol Biol Phys*. 2014;88(2):404–411.
293. Tan S, Li Y, Xia J, et al. Type 1 diabetes induction in humanized mice. *Proc Natl Acad Sci USA*. 2017;114(41): 10954–10959.
294. Reemtsma K. Renal heterotransplantation from nonhuman primates to man. *Ann N Y Acad Sci*. 1969;162:412–418.
295. Singh AK, Chan JL, Seavey CN, et al. CD4+CD25(Hi) FoxP3+ regulatory T cells in long-term cardiac xenotransplantation. *Xenotransplantation*. 2018;25(2):e12379.
296. Kuwaki K, Tseng YL, Dor FJ, et al. Heart transplantation in baboons usingalpha1,3-galactosyltransferase gene-knockout pigs as donors: initial experience. *Nat Med*. 2005;11(1):29–31.

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297. Iwase H, Liu H, Wijkstrom M, et al. Pig kidney graft survival in a baboon for 136 days: longest life-supporting organ graft survival to date. *Xenotransplantation*. 2015;22(4):302–309.
298. Higginbotham L, Mathews D, Breeden CA, et al. Pretransplant antibody screening and anti-CD154 costimulation blockade promote long-term xenograft survival in a pig-to-primate kidney transplant model. *Xenotransplantation*. 2015; 22(3):221–230.
299. Barth RN, Yamamoto S, LaMattina JC, et al. Xenogeneic thymokidney and thymic tissue transplantation in a pig-to-baboon model: I. Evidence for pig-specific T-cell unresponsiveness. *Transplantation*. 2003;75(10):1615–1624.
300. Griesemer AD, Hirakata A, Shimizu A, et al. Results of gal-knockout porcine thymokidney xenografts. *Am J Transplant*. 2009;9(12):2669–2678.
301. Pursel VG, Rexroad CE, Jr. Status of research with transgenic farm animals. *J Anim Sci*. 1993;71(Suppl 3):10–19.
302. Hao YH, Yong HY, Murphy CN, et al. Production of endothelial nitric oxide synthase (eNOS) over-expressing piglets. *Transgenic Res*. 2006;15(6):739–750.
303. Brunetti D, Perota A, Lagutina I, et al. Transgene expression of green fluorescent protein and germ line transmission in cloned pigs derived from in vitro transfected adult fibroblasts. *Cloning Stem Cells*. 2008;10(4):409–419.
304. Denning C, Dickinson P, Burl S, Wylie D, Fletcher J, Clark AJ. Gene targeting in primary fetal fibroblasts from sheep and pig. *Cloning Stem Cells*. 2001;3(4):221–231.
305. Tan W, Carlson DF, Lancto CA, et al. Efficient nonmeiotic allele introgression in livestock using custom endonucleases. *Proc Natl Acad Sci USA*. 2013;110(41):16526–16531.
306. Zhou X, Wang L, Du Y, et al. Efficient generation of gene-modified pigs harboring precise orthologous human mutation via CRISPR/Cas9-induced homology-directed repair in zygotes. *Hum Mutat*. 2016;37(1):110–118.

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Review Article

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Rational Design and In Vivo Characterization of Vaccine Adjuvants

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Abstract

Many different adjuvants are currently being developed for subunit vaccines against a number of pathogens and diseases. Rational design is increasingly used to develop novel vaccine adjuvants, which requires extensive knowledge of, for example, the desired immune responses, target antigen-presenting cell subsets, their localization, and expression of relevant pattern-recognition receptors. The adjuvant mechanism of action and efficacy are usually evaluated in animal models, where mice are by far the most used. In this review, we present methods for assessing adjuvant efficacy and function in animal models: (1) whole-body biodistribution evaluated by using fluorescently and radioactively labeled vaccine components; (2) association and activation of immune cell subsets at the injection site, in the draining lymph node, and the spleen; (4) adaptive immune responses, such as cytotoxic T-lymphocytes, various T-helper cell subsets, and antibody responses, which may be quantitatively evaluated using ELISA, ELISPOT, and immunoplex assays and qualitatively evaluated using flow cytometric and single cell sequencing assays; and (5) effector responses, for example, antigen-specific cytotoxic potential of $CD8^+$ T cells and antibody neutralization assays. While the vaccine-induced immune responses in mice often correlate with the responses induced in humans, there are instances where immune responses detected in mice are not translated to the human situation. We discuss some examples of correlation and discrepancy between mouse and human immune responses and how to understand them.

Key words: vaccine; adjuvant; immunogenicity assessment; biodistribution; antibody responses; cell mediated immune responses; cytotoxic T cell responses; in vivo tracking

Introduction: Adjuvants for Subunit Vaccines

Many vaccines currently licensed for human use are based on whole, inactivated, or attenuated pathogens, of which some have additionally been adjuvanted with aluminum salts. These vaccines are very effective for prevention of disease with a number of pathogens, for example, measles, mumps, and diphtheria.[1] Traditional vaccines mainly induce strong neutralizing antibody responses, and the target pathogens do not change their surface structure over time.[1,2] However, novel vaccine formulations are necessary to prevent or treat a number of difficult pathogen and disease targets, requiring complex immune responses. Possible targets include pandemic influenza, chlamydia, tuberculosis, HIV, and cancers.[1,2] For these, vaccines inducing concomitant humoral and cell-mediated immune responses or cytotoxic T-lymphocytes are necessary. Such vaccines can be prepared by including appropriate vaccine adjuvants designed to induce and control immune responses against co-administered antigens.

The term adjuvant covers delivery systems and immunostimulators, while some adjuvants possess both properties.[3–5] Adjuvants can be designed based on the characteristics and

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localization of the identified target cells and the immunostimulators required to induce the desired immune responses. Several types of delivery systems are applied in vaccines for humans or are being evaluated in preclinical and clinical studies, typically with the common feature of being particles, for example, aluminum salts, emulsions, liposomes, and virosomes.[6,7] The immunostimulators are introduced to induce the required immune responses by acting as ligands for pattern recognition receptors (PRRs), for example, Toll-like receptors (TLRs), C-type lectin receptors, retinoic acid-inducible gene-I-like receptors, and nucleotide-binding oligomerization domain (NOD)-like receptors.[8–11] Due to the diverse nature of the receptors, the ligands also arise from diverse classes of molecules, for example, lipids, proteins, peptides, sugars, DNA, and RNA, and different formulation approaches are required to incorporate them into the delivery systems.[12] The functionality and development of both delivery systems and immunostimulators as adjuvants are reviewed elsewhere.[6,13–19]

Aluminum-based adjuvants have been extensively used in human vaccines for almost a century, and for most of that period no other adjuvants were approved for human use.[20] The adjuvant effects of aluminum-based adjuvants were empirically discovered, while the exact mechanisms of action have remained relatively obscure until recently.[21,22] However, novel vaccine adjuvants are increasingly tailored to induce specific immune responses, which have been identified as critical for the prevention of target diseases. We have at our laboratory designed a palette of liposomal vaccine adjuvants capable of inducing various immune response profiles. The ones most advanced were made specifically to induce strong T-cell-mediated immune responses; Th1-skewed $CD4^+$ T-cell responses induced by CAF01 (dimethyldioctadecylammonium bromide [DDA] and trehalose-6,6´-dibehenate) and $CD8^+$ T-cell responses induced by CAF09 (DDA, synthetic monomycoloyl glycerol [MMG], and polyinosinic:polycytidylic acid [poly(I:C)]).[23,24] The adjuvants have been evaluated in vivo in different animal models using a variety of immunization routes.[24–26] Our experience with these vaccines, including the evaluation of their immunostimulatory and mechanistic profile, will be the basis for the present review.

Rational Design of Vaccine Adjuvants

Progress has been made in guiding immunity through a detailed mechanistic understanding of innate immune cell biology and the response of professional antigen-presenting cells (APCs) to various stimuli. Based on this, rational design can be applied, taking into account antigen type, target cell subsets and phenotype, and immunization routes, which guide the choice of delivery system and immunostimulators. Rational design basically means designing the vaccine to present sufficient amounts of the right antigen in the right conformation to the appropriate cell populations while supplying the right co-stimuli for a sufficient amount of time. Choice of conformation and dose of antigen are often handled by antigen discovery programs with focus on specific disease targets. In this review, we will focus on rational design of adjuvants, and thus immunogen design will not be further discussed here. Targeting of appropriate cell populations with the right co-stimuli and timing serve as guidance for rational design of novel adjuvants and require knowledge of numerous aspects: (1) what is the required immune response to prevent disease from a given pathogen, (2) which innate immune cells are relevant to induce said immune response, (3) where are these innate cell subsets located, and (4) which PRRs do the cells express (Figure 1). The questions can be answered by using animal models, as they enable mapping the whole-body effects of vaccination. Knowledge of the required immune responses, and the innate cells and cytokines involved, and the localization in the body can be acquired by evaluating stimulation and proliferation of innate and effector cells upon administration of the vaccine via a number of different assays as described below.

Evaluation of the Biodistribution and Cellular Association of Adjuvanted Vaccines

Many vaccines have been developed without detailed knowledge of the targeted cell populations. However, the biodistribution and cellular association patterns of adjuvanted vaccines are of utmost importance for the induction of specific immune responses. One such example is the CAF09 adjuvant that induces strong $CD8^+$ T-cell responses when given intraperitoneally (i.p.), but not upon subcutaneous (s.c.) or intramuscular (i.m.) immunization. This is presumably due to the formation of a persistent depot at the site of injection (SOI), which prevents targeting of the innate immune cell subsets specialized in $CD8^+$ T-cell induction.[24,27] The adjuvanted vaccines will predominantly be actively transported or drain via the lymphatics, and it is therefore of importance to know the draining lymph nodes (LNs) from a given injection site.

In vivo evaluation of the draining pattern from various injection sites can be performed by injection of fluorescently labelled particles followed by noninvasive imaging of the animal, allowing for assessment of the biodistribution pattern of the injected particles in the same animal over time (Figure 2a). For example, the draining LNs following i.p. administration were evaluated in rats by injection of near infra-red fluorescent quantum dots and human serum albumin conjugated with IR-Dye800.[28] The draining LNs and lymph flow was identified by imaging the rats in intervals up to 24 hours after i.p. administration, revealing primary and secondary draining LNs.[28] Alternatively, lymphatic mapping can be performed by using a visible dye such as Evans Blue dye, which was used to visualize the draining LNs following hind leg and lateral tail vein administration in mice.[29] Mapping the biodistribution pattern of a vaccine administered via a certain route provides important information about which compartments are affected by the vaccine. Thus, it may be possible to assess if the correct organs and cell types are targeted to induce the desired immune response.

Vaccine Interaction with Innate Immune Cells

Localization of vaccine components in the organs, particularly the LNs, has been illuminated by confocal microscopy (Figure 2a). The spatial localization of the vaccine components in the draining LNs was evaluated following s.c. immunization with the emulsion-based adjuvant MF59 fluorescently labeled with the lipid tracer dioctadecyl-tetramethylindodicarbocyanine perchlorate and intrinsically fluorescent PE-antigen.[30] The LNs were stained for relevant expression markers (eg, the macrophage marker F4/80 and the germinal center [GC] marker GL7), which made it possible to assess the co-localization of the vaccine components with specific LN compartments.[30] In a study evaluating the dependence of particle size on LN entry, red fluorescent 20-nm and green fluorescent 1000-nm particles were co-administered in the footpad of mice.[31] The results showed that the small particles likely entered the LNs freely, whereas the large particles required trafficking by dendritic cells (DCs).[31] In another study, fluorescently labelled chicken egg ovalbumin

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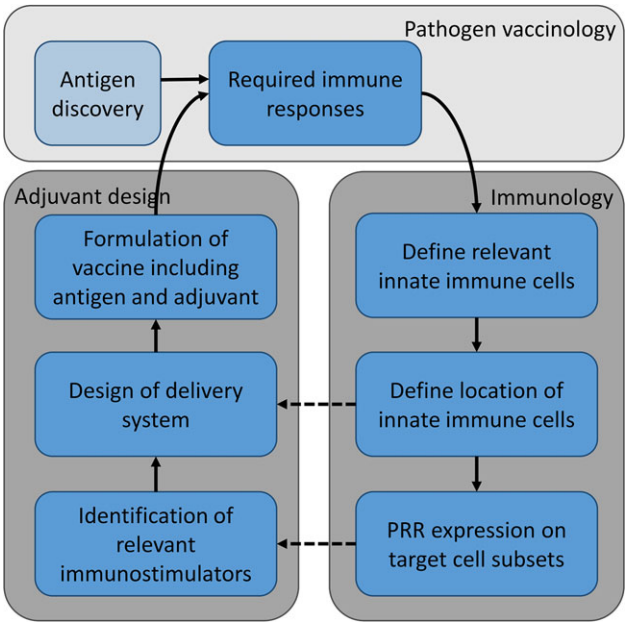

Figure 1. Rational design of vaccine adjuvants. The required immune responses are identified based on pathogen vaccinology and defined by the type of vaccine; different immune responses might be required for a prophylactic vaccine preventing disease, and a therapeutic vaccine treating disease or preventing clinical symptoms. Based on the required immune responses, the relevant innate immune cells must be identified along with evaluation of their localization within the body and PRR expression on target cells. Knowledge of these factors can be used to design the delivery system and identify relevant immunostimulatory molecules, respectively. The delivery systems are often nanoparticle-based structures of diverse origin, for example, liposomes, emulsion, virosomes, or aluminum salts. The design choices of delivery systems depend, amongst other things, on the location of the target innate cells, the route of administration, the chosen immunostimulators, and association mode of antigen. Relevant immunostimulators are often identified based on the PRR expression of target innate cells subsets. These may be antigen-presenting cells (eg, DCs and macrophages) or cells with bystander function. Antigen discovery programs can, independently of adjuvant design programs, identify immunogenic antigens for a given pathogen and be used to develop recombinant antigens that in combination with suitable adjuvants induce pathogen-specific immune responses. The vaccine formulation (adjuvant + antigen) is tested in vivo in relevant animal models to characterize the induced immune responses and, possibly, the response to a pathogen challenge.

(OVA) formulated in nanoparticles based on poly(lactide-co-hydroxymethylglycolic acid) was used to perform in vivo tracking over 13 days following s.c. administration with concomitant assessment of the levels of OVA at the SOI and in the draining LNs.[32]

Cellular association of fluorescently labeled vaccine components can be evaluated by flow cytometry, where cell subsets are detected by staining with appropriate fluorescently labeled antibodies.[27,33] This approach was used to identify targeting of DCs in the draining LNs by liposomal adjuvants (labeled with 7-nitro-2-1,3-benzoxadiazol-4-yl or 3,3′-dioctadecyloxacarbocyanine perchlorate) administered via different administration routes.[27,33] This allows for concomitant evaluation of the target lymphoid tissues the antigen drains to and the phenotype and activation of the targeted cell subsets (Figure 2b). Furthermore, the localization of fluorescently labeled antigen and adjuvant can be investigated by immunofluorescent staining and microscopy.

Using CAF09, we tracked fluorescently labeled antigen and noticed that i.p. administration targeted the $CD8^+$ T-cell priming $CD8\alpha^+$ DCs in LNs and spleen while s.c. and i.m. administration did not.[27] Thus, s.c./i.m. administration of CAF09-adjuvanted vaccines prevented efficient vaccine drainage to the intended target cells. Based on this information, as an example of rational vaccine design, we have recently demonstrated that reformulating the CAF09 adjuvant to limit the depot effect can be an effective means to obtain $CD8^+$ T-cell responses after s.c./i.m. immunization.[34]

Characterization of the association of the vaccine adjuvant and antigen with specific innate cell subsets at the injection site and in the lymphoid organs can be used to identify cell-mediated transport to the injection site and give an insight into the APC subsets priming the resulting immune response.[35–37] The cellular association of the emulsion-based adjuvant MF59 was evaluated in the injected muscle and the draining LNs using a flow cytometry panel of fluorescently labeled anti-Ly6C, -Ly6G, -CD11b, -CD11c, -F4/80, and -MHC-II antibodies, the expression patterns of which could be combined to identify neutrophils, eosinophils, inflammatory monocytes, macrophages, and different DC subsets.[35] MF59 was found to be mainly associated with neutrophils and inflammatory monocytes in the muscle tissue, which were thought to facilitate rapid cell-mediated transport to the draining LNs.[35] In another study, the liposome-based adjuvant AS01 was shown to induce a transient influx of neutrophils ($SSC^{high}CD11b^+Ly6G^{high}$) and monocytes ($Ly6C^{high}CD11b^+Ly6G^-$) into the muscle injection site.[36] Similar innate cell reactions were observed in rhesus macaques immunized with HIV-1 envelope protein adjuvanted with an aluminum-TLR7-ligand complex or MF59. In this study, fluorescently labelled antigen was found to associate with neutrophils, monocytes, and myeloid DCs at the muscle injection site. Furthermore, the study showed that priming of antigen-specific $CD4^+$ T cells happened exclusively in the draining LNs.[37] Flow cytometry coupled with high-throughput imaging of immunofluorescent staining, such as imagestream, represents a novel promising tool to investigate innate immune cell targeting, uptake, and subcellular location. For example, it was shown that the experimental adjuvant carbomer carbopol was located intracellularly in a number of different innate immune cells and that many cells had taken up multiple carbopol particles.[38]

Activation of DCs can be evaluated by assessing the increase in expression of activation markers such as CD40, CD80, CD86, and MHC-II using flow cytometry.[33,39,40] Thus, it is the change of surface marker expression on a single cell level that is evaluated, typically measured as the mean fluorescence intensity, rather than the number of cells expressing a certain cell marker. Mice immunized with the 2-component adjuvant IC31 showed significantly increased levels of CD40, CD80, and CD86 expression specifically on adjuvant-associated DCs in the LNs compared with control mice and mice immunized with the TLR-9-ligand CpG.[40] To investigate the heterogeneity of innate immune cells responding to vaccination, single cell sorting followed by RNA sequencing is a powerful technique. It enables genome-wide profiling of mRNA expression and has the potential to reveal the heterogeneity of APCs, otherwise masked at the bulk cell level.[41]

Quantitative Assessment of Vaccine Biodistribution

Injection of radiolabeled vaccine particles is also used for qualitative and quantitative assessment of the biodistribution on both organ and cellular levels (Figure 2a). Quantitative evaluation of the biodistribution of an adjuvanted vaccine can be performed by injection of radioactively labelled particles.[27,42–46] The method enables quantitative assessment of injected vaccine particles in separate excised organs, for example, the draining

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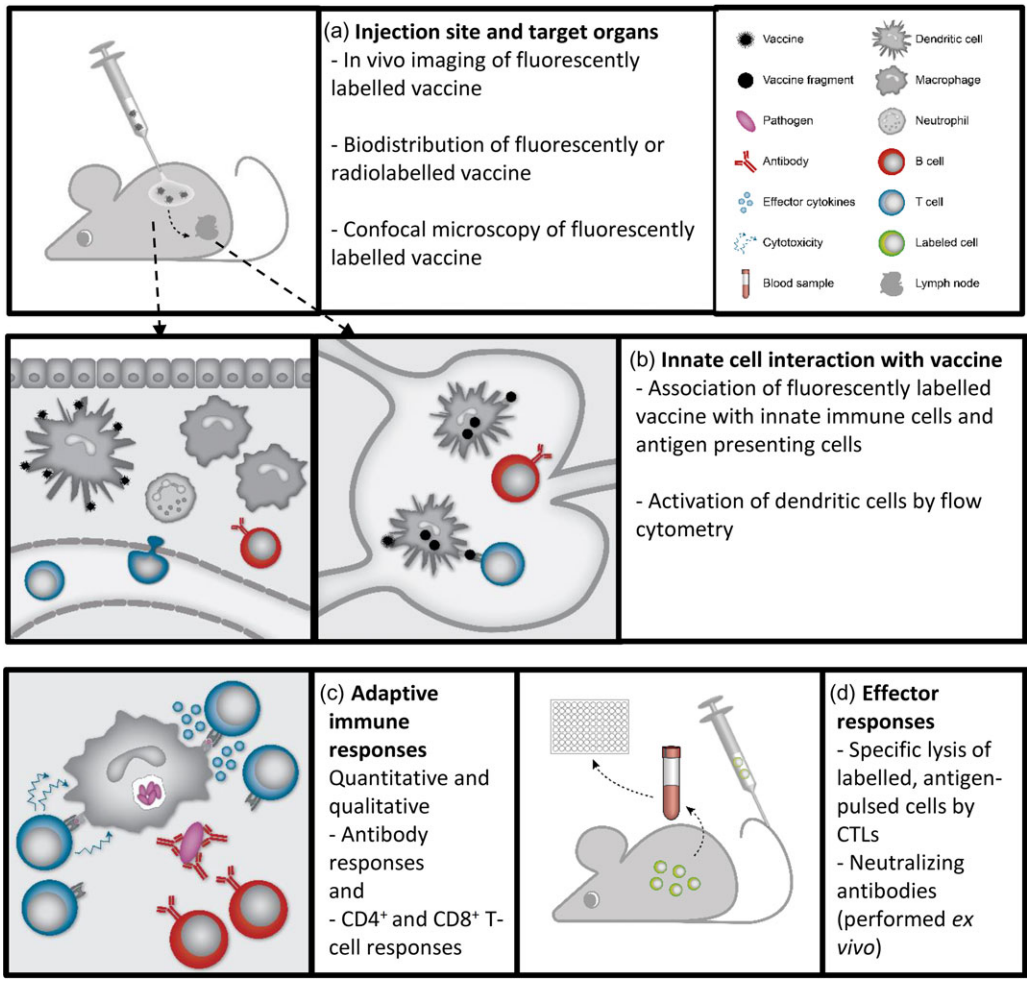

Figure 2. Evaluation of vaccines—from identification of target cells to desired immune response profile. A number of different assays can be utilized to assess adjuvanted subunit vaccine function and efficacy. (A) Assays evaluating biodistribution and organ localization of vaccine components on a whole-body level are often based on detection of fluorescently or radiolabeled adjuvants and antigens. (B) In target organs such as the injection site and draining lymph nodes, association of vaccine components with innate immune cells and antigen-presenting cells can be evaluated using flow cytometry-based assays. These assays can be used to elucidate the mechanism of action for vaccine adjuvants and which antigen presenting cells that are activated by the adjuvant. (C) Qualitative and quantitative evaluation of the adaptive immune responses to subunit vaccines is used to assess vaccine efficacy. Quantitative responses can be measured by ELISA, ELISPOT, and immunoplex assays, whereas flow cytometry-based assays and antibody avidity assays can be used to evaluate the qualitative immune responses. (D) Vaccine efficacy can be assessed using alternative assays to pathogen challenge models. The cytotoxic potential of $CD8^+$ T cells can be evaluated in antigen-specific lysis assays, while antibody functionality may be evaluated in neutralization assays or with methods to assess antibody Fc-dependent functionalities such as cytotoxicity, complement activation or phagocytosis.

LNs, the spleen, and the SOI, typically calculated as the ratio of the initial vaccine dose. One benefit of using radiolabeling of the vaccine is the possibility of performing concomitant evaluation of the levels of different components of the vaccine in various organs. Individual labeling of a liposomal adjuvant and a protein antigen with ^{1}H-cholesterol and covalent linkage with ^{125}I, respectively, enabled separate assessment of the relative antigen and adjuvant levels at the SOI and in the draining LNs.[43,46] This approach was used to evaluate the co-localization of antigen and adjuvant at the SOI and in the draining LNs as a consequence of protein and particle charge.[43] After i.m. administration, a negatively charged antigen adjuvanted with a cationic liposome remained at the SOI for a longer time than a positively charged antigen (lysozyme) adjuvanted with the same cationic liposome, while neutral liposomes also caused rapid drainage of the negatively charged antigen from the SOI.[43] In a study using similar techniques, the lipid bilayer fluidity of the cationic liposomal adjuvant was shown to be critical for the biodistribution pattern.[47] Thus, liposomes, which are in a rigid gel state at body temperature, form a depot at the SOI over the 15-day study period, whereas fluid liposomes do not form a depot at a SOI but enter the draining LNs in appreciable amounts already 1 day after administration.[47] Evaluation of how vaccine adjuvants associate with immune cells, locally and systemically, and how these cells are activated can provide valuable knowledge of the mechanism of action of the adjuvants. Furthermore, this knowledge can aid the design of novel vaccine adjuvants as the connection between activation of innate immune cells and the induced immune responses may be determined.

Characterization of Immunostimulators That Activate Target Cells

Identification of the optimal combination of immunostimulators to be used for activating specific subsets of immune cells requires knowledge of the expression of PRRs on the cells in

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question. As an example, TLR3 is expressed on both LN-resident CD8α$^+$ and migratory CD103$^+$ cross-presenting DCs, and activation of TLR3 is required to induce cross-priming of CD8$^+$ T cells.[48] Thus, TLR3 ligands are often used in adjuvant formulations intended for induction of CD8$^+$ T-cell responses.[49] Advances in sequencing as well as systems and computational immunology have provided the field with online databases such as the Immunological Genome Project (Immgen), where the PRR gene expression on a large number of immune cells can be found for mouse and human (www.immgen.org). Such databases can be used as a tool to identify possible immunostimulators in the design phase of novel adjuvant formulations. It should be noted that dependent on the activation signals, the PRR expression profile may change. Thus, whereas Immgen displays PRR expression in the unpertubed steady state, initial activation by adjuvants may change the PRR profile of target cells, possibly providing access to additional PRRs. Importantly, gene expression analyses do not necessarily reflect actual protein expression. Protein expression of target PRRs should therefore be confirmed by such means as flow cytometry or by proteomic approaches.

Due to the complexities of the immune system, which requires interactions of several different cell subsets to induce and maintain antigen-specific immune responses, functional evaluations of vaccine candidates are preferably performed in animal models. For example, antibody responses to T-cell-dependent antigens require that antigen is complexed, for example, via complement deposition, is transported/drained to lymphoid tissue, and then taken up by specialized macrophage and DC subsets and delivered to follicular B cells. At the same time, T cells must be activated by DCs and form contact with the B cells in the draining LN.

Immunostimulators are often evaluated in vitro to identify the activation pathways in the target cells. Furthermore, in vitro evaluation of the immunogenic effects of an immunostimulator on the chosen cell strain can indicate the effects achieved upon in vivo administration. Correlation between in vivo and in vitro studies has been shown for the TLR7/8-ligand R848, which produced cytokines corresponding to a Th1-skewed CD4$^+$ T-cell response both in human-derived leukocytes and following s.c. immunization in an o/w-emulsion.[50] A synergistic effect of co-administration of MMG and the TLR9-ligand CpG was observed in vitro in J774 macrophages measured as secretion of IL-6.[51] A similar synergistic effect was observed following immunization of mice with H56-adjuvanted CpG/DDA/MMG-liposomes with respect to IFN-γ and IL-17 secretion.[51] The TLR3-ligand poly(I:C) formulated in poly-(L-lysine)-microspheres stimulated in vitro CD8$^+$ T-cell proliferation by monocyte-derived DCs and secretion of IL-6, IL-12p70, and TNF-α.[52] In vivo administration to mice of poly(I:C) formulated with CAF01 similarly induced strong CD8$^+$ T-cell responses but low levels of IL-6 and TNF-α.[53] Thus, in vitro studies can provide insights into early stimulation patterns by immunostimulators but should not replace in vivo evaluation of the complete vaccine adjuvant.

The complex interplay between the different cells of the immune system may be the cause of the vastly different results obtained with the synthetic MMG analogue MMG-6 in both in vitro and in vivo studies, respectively.[54,55] In the in vitro studies, neat MMG-6 failed to stimulate monocyte-derived DCs, whereas MMG-6 incorporated into DDA-based liposomes were capable of inducing robust Th1-skewed CD4$^+$ T-cell and total IgG antibody responses in vivo.[54,55] This illustrates the importance of evaluating the immunostimulators as part of the final adjuvant, as the formulation might alter the configuration of the molecules and the mode of presentation to the receptors. Furthermore, in vitro studies often rely on the function of a single cell subset, whereas in vivo studies enable simultaneous activation of several types of cell subsets. Further, in vitro evaluation of the *Mycobacterium tuberculosis* (*M.tb*)-derived MMG showed stimulation of the human Mincle receptor, which was not induced in the murine Mincle receptor.[56] However, MMG induces strong immune responses when administered in combination with DDA-based liposomes in murine studies,[57] indicating that it either stimulates the immune response through unknown receptors or adopts a conformation within the liposome, which enables interaction with the Mincle receptor.

The signaling pathway of trehalose-6,6´-dibehenate has been Investigated via both in vitro stimulation of bone marrow macrophages and in vivo administration of CAF01.[58,59] In vitro stimulation of macrophages, measured as release of nitrites and G-CSF, is dependent on cellular expression of the Mincle receptor and independent on MyD88 expression.[58] In contrast, antigen-specific secretion of IFN-γ and IL-17 by draining LN-isolated cells required expression of both MyD88 and Mincle.[59] This illustrates that the signaling pathways in the in vivo situation may be different from those found in in vitro studies, possibly due to the interaction between several different immune cell subsets in vivo. Furthermore, the cell lines or peripheral blood mononuclear cells tested in in vitro studies may be vastly different in composition compared with the cellular populations at the injection site.

Evaluation of Vaccine-Induced Adaptive Immune Responses

Evaluation of Antibody Responses

The best-established correlate of protection against a number of diseases is antibody responses.[60] Antibody responses can be measured as antibody titer by standard enzyme-linked immunosorbent assay (ELISA)-based approaches or by more disease-specific approaches, such as virus or bacterial neutralization (Figure 2, c and d). Vaccination elicits B-cell activation in the draining LNs, followed by formation of GCs in which affinity maturation and antibody class-switching occurs.[61] Adjuvants may affect the magnitude of GC responses, which can be measured by flow cytometry or immunofluorescent staining and confocal microscopy. Some spontaneous GC formation may occur in naïve animals, and it may therefore be beneficial to include a fluorescently labeled antigenic probe in the analysis,[62] thus enabling detection of antigen-specific GC responses. It should be noted that the kinetics of GC responses might vary with properties of the antigen and adjuvant used, thus requiring kinetic studies to define the peak response for a given vaccine. The affinity maturation of the antibody response can also be followed, using ELISA-based methods, such as limiting antigen dilution or chaotrope methods. In the latter, the resistance of the antigen-antibody complex to urea or NaSCN is evaluated as a measure for antibody affinity. Antibody avidity may correlate with protection. For example, for a meningococcal vaccine, serum antibody avidity significantly correlated with bactericidal titres.[63] Other methods to measure the strength of antigen-antibody interactions include surface plasmon resonance.[64] Ultimately, the GC B cells may undergo 1 of 2 productive fates, which are desired for all infections requiring antibody-dependent protection: memory B cells or plasma cells. It has recently become appreciated that early GCs have a

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preponderance for generating memory B cells, while plasma cell formation requires more progressed GCs.[65] An interesting possibility would be to modify immunization protocols or adjuvants to change GC persistence and thereby possibly alter the plasma cell to memory B cell output ratio. Memory B cells are circulatory and can be followed using flow cytometry. The phenotype of memory B cells is quite diverse and both class-switched and IgM positive memory B cells exist.[66,67] The best way to quantify memory B cells is therefore to use a probe (fluorescently labeled antigen) in combination with standard memory B cell markers (eg, $B220^+$, IgD^+, $CD38^+$ in mice). Antigen-specific memory B cells can further be sorted by FACS and used to provide information on B-cell receptor heavy- and light-chain gene usage (variable, diversity, and joining genes) after vaccination, or single-cell sorted and used for production of antigen-specific monoclonal antibodies.[68] For example, in macaques immunized with HIV-1 Env, memory B cells were single-sorted for CD4 binding site-reactivity using 2 fluorescent probes and used to produce monoclonal CD4 binding site reactive antibodies. This allowed for the further studies of vaccine-induced antibody recombination events and CD4 binding site specificities.[69] B cell receptor sequencing may also be performed to investigate how immunoglobulin sequence repertoire changes following vaccination[70] or how the type of vaccine or adjuvants may potentially influence B cell receptor variable gene usage. For example, upon immunization with a Plasmodium vivax antigen, including a TLR agonist expanded the diversity of the variable region sequences in comparison with the use of an oil-in-water emulsion adjuvant alone.[71] It is also possible to stimulate plasma cell formation from memory B cells using B cell mitogens, such as the TLR7 agonist R848 or the TLR9 agonist CpG B.[72] The number of antibody secreting cells can then be evaluated by enzyme-linked immunospot (ELISPOT) or secreted antibodies can be measured by ELISA. Dependent on the vaccine, plasma cells can be maintained for lifetime. Long-lived plasma cells home to the bone marrow and can be phenotypically characterized by flow cytometry and their antibody secretion can be followed by ELISPOT.[73] Tetanus-specific plasma cells were evaluated 10 years post-vaccination by ELISPOT from bone marrow samples.[74]

Standard evaluation of vaccine-induced antibody responses include determination of antigen-specific serum IgG levels. Dependent on the properties of the vaccine antigen, or the type of adjuvant, different antibody isotypes may be elicited. For example, in response to protein antigens, the CAF01 adjuvant elicits a balanced Th1/Th2 profile, characterized by both IgG1 and IgG2a/c antibodies, while aluminum hydroxide induces mainly IgG1 antibody responses to the same antigens in mice.[75] These antibody subclasses may (due to the structural properties of the Fc region) differentially bind to Fc receptors (FcR), which in turn may affect FcR-mediated antibody functions such as antibody-dependent cellular cytotoxicity, complement activation, and phagocytosis.[76,77] In humans, an ENV GP120 vaccine (VAX003) elicited IgG4 antibodies that may have outcompeted more functional Ig subclasses (IgG1 and IgG3), and depletion of IgG4 gave higher antibody functional responses.[78] An intriguing possibility is also that vaccines may influence the antibody Fc region glycosylation patterns, which may also affect Fc receptor binding and thus antibody FcR-mediated functions.[79] For example, it was found that an aluminum-adjuvanted recombinant gp120 vaccine induced a different antibody FC region glycan profile compared with an adenovirus based HIV-1 envelope A vaccine.[79] Dependent on the disease target, it may therefore be important to broaden the evaluation of antibody responses to include antibody avidity as well as antibody isotypes and functional attributes. To probe correlates of vaccine-induced immunity in more detail, transcriptomics and metabolomics show great promise. For example, evaluation of innate and adaptive immunity to Herpes zoster vaccination in humans was supplemented with metabolomics to reveal an interconnected immune network of metabolic pathways that correlated with adaptive immune responses.[80]

Evaluation of $CD4^+$ and $CD8^+$ T-cell Responses

Antigen-specific $CD4^+$ and $CD8^+$ T cells are important to prevent or combat infectious diseases. Therefore, evaluation of antigen-specific $CD4^+$ and $CD8^+$ T cells induced by novel vaccine formulations is an important measure of vaccine efficacy (Figure 2c).

A well-established method for evaluating antigen-specific $CD4^+$ and $CD8^+$ T-cell responses is stimulation of single-cell suspensions from target organs with the subunit antigen and minimal CD8 epitope peptides, respectively. Intracellular flow cytometry can be applied to single cell suspensions stimulated for a short amount of time to assess the production of cytokines on a cellular level. Furthermore, harvested supernatants of single cell suspensions stimulated for a longer time (typically 3–5 days) may be used to quantify the cytokine production on a cell population level using ELISAs and multiplex assays such as Luminex and Meso Scale Discovery.[72,81,82]

The $CD8^+$ T-cell responses induced by immunization with CAF09-adjuvanted *M.tb.*-antigen TB10.3 (as the whole protein or the CD8 epitope-containing peptide, P1) were evaluated by stimulating single cell suspensions of splenocytes with the minimal CD8 epitope, IMYNYPAM.[24] Subsequent fluorescent antibody staining of the splenocytes permitted evaluation of IFN-γ, IL-2, and TNF-α expression by $CD4^+$ and $CD8^+$ T cells using flow cytometry.[24] A similar flow cytrometry panel was used to evaluate the $CD4^+$ and $CD8^+$ T-cell responses in splenocytes of rhesus macaques immunized with the influenza vaccine Fluzone adjuvanted with the cationic lipid/DNA complex-adjuvant JVRS-100. Immunization with the adjuvanted vaccine resulted in higher levels of multifunctional $CD4^+$ and $CD8^+$ T cells compared with macaques immunized with unadjuvanted Fluzone.[83] This assay evaluates the quantitative functionality of the $CD8^+$ T cells as the level of cytokine producing cells in response to the stimulus. Furthermore, the levels of polyfunctionality in the stimulated cells can be used to assess the potential of a vaccine to induce lasting immune responses. Thus, $IFN\text{-}\gamma^+$,$IL\text{-}2^+$,$TNF\text{-}\alpha^+$ $CD8^+$ T cells are considered memory T cells, which give rise to a long-lived immune response, whereas a short-lived effector response may be defined as $IFN\text{-}\gamma^+$,$TNF\text{-}\alpha^+$ and $IFN\text{-}\gamma^+$ $CD8^+$ T cells.[84]

The repertoire of induced $CD4^+$ T cells is critical for the induced functional immune responses; Th1 responses induce proinflammatory responses and help the induction and sustaining of $CD8^+$ T-cell responses, whereas Th2 responses help promote antibody class switching, and Th17 $CD4^+$ T cells are thought to be important for establishing mucosal immune responses.[85] Intracellular staining and flow cytometry on single cell suspensions stimulated with the antigen can also be used for evaluation of $CD4^+$ Th1-cell responses utilizing the $IFN\text{-}\gamma^+$, $IL\text{-}2^+$, and $TNF\text{-}\alpha^+$ intracellular staining assay,[83,84] which may also include anti-IL-17-antibodies to assess the Th17-skewed $CD4^+$ T-cell response.[85]

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Flow cytometry has limitations to the number of antibodies that can be analyzed at one time due to spectral overlap of the fluorophores conjugated to the antibodies. An alternative method for analysis of T-cell populations is cytometry by time-of-flight (CyTOF), where the antibodies are conjugated to heavy metal isotopes by metal chelating polymers rather than the fluorophores used for flow cytometry assays.[86] Staining of stimulated cells with heavy metal isotope-conjugated antibodies enables detection of the cells by using mass spectroscopy. Due to little overlap between the heavy metal isotopes, the number of antibodies used for each assay can be increased compared with flow cytometry. However, the data acquisition rate is low at 300 to 500 events/s compared with the acquisition rates of flow cytometry at orders of magnitude at 10^3 to 10^5 events/s.[86,87] Thus, 36 different antibodies were used to identify subpopulations of human $CD8^+$ memory and effector T cells. The functionality of subpopulations of $CD8^+$ T cells were identified by principal component analysis and combinatorial diversity achieved by Boolean gating, which were distinct for different virus-specific $CD8^+$ T cells.[88] These data analysis approaches are suitable for simultaneous assessment of different immune cell populations due the large amounts of data generated using CyTOF.[87] Thus, differences in cytokine and receptor expression patterns of immune cell subsets ($CD4^+$ and $CD8^+$ T cells, B cells and monocytes) were assessed for naïve and influenza-vaccinated mice after influenza challenge.[87] CyTOF has potential for assessing changes in type and functionality of immune cell subsets after immunization with different subunit vaccine adjuvants. It may be possible to identify correlates of protection in disease challenge models or show differences between different adjuvants. Furthermore, the method requires low sample volumes enabling longitudinal studies using blood samples.[87]

The cytokine levels in response to stimulation with antigen can also be assessed by using ELISPOT, where the released cytokines are captured by cytokine-specific antibodies adsorbed to the well.[89,90] This approach was used to quantify the expression of IFN-γ, IL-4, and IL-2 in mice immunized with virus-like particles, which showed that concomitant co-stimulation with poly(I:C) increased the cytokine responses.[89] ELISPOT is also very useful in other animal models where flow cytometry antibodies are scarce. For example, to assess the IFN-γ responses in splenocytes to different tetanus toxoid doses adjuvanted with CAF09 in a study in Göttingen minipigs. The study showed that low doses of tetanus toxoid (1 and 10 μg/dose) resulted in the induction of IFN-γ responses, which were diminished when the dose was increased to 100 μg.[90] In one study, Luminex, ELISPOT, and intracellular flow cytometry were used with antibody ELISA assays to compare different adjuvants in a DNA plasmid prime/adjuvanted protein boost regimen.[91] The combination of assays allowed the identification of adjuvants capable of robustly boosting the primed immune responses, while providing detailed information on the differences in induced cytokine levels and T-cell responses induced by the different adjuvants. Thus, the results showed that MPLA, ISCOMATRIX, and QS-21-based adjuvants were capable of inducing antibody responses towards the antigen, though the cytokine profiles differed.[91]

Identification of CD4 and CD8 epitopes in novel protein- or peptide-based antigens can be achieved by epitope-mapping, where splenocytes from immunized mice are stimulated with individual peptides spanning the entire protein. Assessment of cytokine-producing T cells by intracellular flow cytometry in response to the individual peptides serve to identify $CD4^+$ and $CD8^+$ T-cell epitopes. This approach was used to elucidate the induction of $CD4^+$ and $CD8^+$ T-cell responses following immunization with recombinant NS3 protein antigen or the corresponding peptide mix both adjuvanted with CAF09.[92] The epitope mapping revealed that immunization with the peptide mix resulted in recognition of more CD4 epitopes compared with the recombinant protein, whereas 2 CD8 epitopes were induced by the peptide mix, while none were observed with the NS3 protein.[92]

Pentamer/tetramer/dextramer-conjugated CD8 epitope-loaded MHC-I molecules are used to assess the level of antigen-specific $CD8^+$ T cells in the relevant organs using flow cytometry. In a DC-based vaccine pulsed with the antigen TRP2 and adjuvanted with soluble poly(I:C), the percentage of TRP2-specific $CD8^+$ T cells was assessed using a K^b/TRP2 tetramer and an anti-CD8 antibody.[93] The induction of $CD8^+$ T-cell responses against the antigens TB10.3-P1, OVA, Gag p24, and E7 adjuvanted with CAF09, compared with using CAF01 as adjuvant, were evaluated using the specific minimal CD8-epitopes loaded onto the appropriate pentamer/dextramer-conjugated MHC-I molecules. The cell subsets were identified by co-staining with anti-CD8, -CD4, -CD19, and -CD44 antibodies.[24] While assessment of the number of antigen-specific $CD8^+$ T cells give a good indication of the efficacy of the administered vaccine, the results should be evaluated in combination with functionality assays, for example, production of cytokines or antigen-specific cytotoxicity assays as described below.

The proliferation of antigen-specific $CD4^+$ and $CD8^+$ T cells upon therapeutic vaccination can be used as a measure of how well the cells respond to vaccination. In the bromodeoxyuridine (BrdU) assay, mice are fed BrdU in the drinking water, or by i.v. or i.p. administration, for a few days prior to euthanization. BrdU is incorporated into the DNA of proliferating cells and can be imaged by fluorescent anti-BrdU antibodies in flow cytometry assays.[94,95] In a study of therapeutic vaccination of mice infected with chronic lymphocytic choriomeningitis virus, it was shown that a low amount of antigen-specific $CD8^+$ T cells proliferated in presence of a chronic infection compared with non-infected, preimmunized control mice.[94] In another study, mice were vaccinated with recombinant *M.tb.* antigen adjuvanted with cationic liposomes for the prime and boosted as an adenovector. Following *M.tb.* pulmonary challenge, proliferative antigen-specific $CD4^+$ T cells were recruited to the lungs to a higher degree than antigen-specific $CD8^+$ T cells.[95]

It may be of interest to investigate where vaccine-induced, antigen-specific $CD4^+$ and $CD8^+$ T cells localize upon pathogen challenge. Evaluation of tissue and circulatory localization of immune cells can be performed by i.v. injection of fluorescently labelled anti-CD45 antibodies a few minutes before killing.[96] The antibodies bind to CD45-expressing lymphocytes in the blood, thus enabling sorting of circulatory immune cells ($CD45^+$) from tissue resident immune cells ($CD45^-$) in highly perfused organs, such as the lungs.[96,97] In a study of a *M.tb.* subunit vaccine, fluorescently labelled antigen-specific $CD4^+$ T cells were adoptively transferred from donor mice immunized with low (5 μg) and high (50 μg) doses of adjuvanted antigen into *M.tb*-infected mice. One day after adoptive transfer, the CD45-labelling assay was used to evaluate the level of transferred antigen-specific $CD4^+$ T cells that homed to the lung parenchyma. It was shown that $CD4^+$ T cells from mice immunized with a low dose of antigen homed most efficiently to the lung parenchyma.[97] In another study, the assay was used to evaluate how the immunization routes affected the levels of IgA^+ B cells levels in the lungs and vasculature.[82] It was shown that a s.c. priming followed by an intranasal booster vaccination with adjuvanted

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ScpA antigen induced higher levels of homing to the lung parenchyma of IgA$^+$ B cells compared with a subcutaneous booster vaccination.[82] There are several assays to assess the CD4$^+$ and CD8$^+$ T-cell responses in animal models. The choice of assay may depend on the animal model, as the use of flow cytometry requires the access to antibodies, which organs are being assayed, and whether information on an individual cellular, organ, or systemic level is required.

Evaluation of Antigen-Specific Cytotoxic Potential for CD8$^+$ T Cells

When assessing the efficacy of a subunit vaccine, it may be desirable to use alternatives to disease challenge models for evaluation of antigen-specific cytotoxicity of CD8$^+$ T cells. The study animals are spared from experiencing the target disease, which may cause discomfort and pain. Furthermore, it enables separation of adjuvant function and efficacy from immunity preventing disease. Specifically, the latter may not be completely elucidated, as is the case for *M.tb* infection, where for example a strong pathogen-derived antigen-specific CD8$^+$ T-cell response was not preventive of disease in a mouse model.[98]

Several different assays exist to measure cell-mediated cytotoxicity, where the ^{51}Cr release assay is regarded as the "golden standard."[99] Cell-mediated cytotoxicity is detected when radioactive ^{51}Cr is released from target cells, which were initially pulsed with sodium chromate.[99] The assay is performed ex vivo, which enables selection of specific target cell populations at different effector to target cell ratios.[100] In a mouse study of a cell-based vaccine against renal cell carcinoma, this approach was used to show that the vaccine induced tumor-specific cytotoxicity, with little lysis of tissue control cells.[100]

One assay is measuring the specific lysis of i.v.-injected, fluorescently labeled, minimal CD8 epitope-pulsed splenocytes into immunized animals. A weakness of the assay is that the transfer of epitope peptide-pulsed splenocytes to immunized mice limits the results to encompass only the chosen epitopes. Thus, synergistic (or opposing) immune responses involving simultaneous antibody, CD4$^+$, and CD8$^+$ T-cell responses cannot be evaluated using this method alone but must be done in combination with ex vivo stimulation of target cells.

In the specific lysis assay, single cell suspensions of splenocytes from naïve mice are pulsed with different concentrations of the cellular dye carboxyfluorescein succinimidyl ester (CFSE) resulting in distinct populations, which can be further pulsed with the minimal CD8$^+$ epitopes of interest, always leaving one population unpulsed. The pooled populations are injected i.v. into recipient mice, and the specific lysis of the pulsed splenocytes is determined typically after 24 hours by calculating the ratio of peptide-pulsed to unpulsed splenocytes in relevant organs in the recipient mice. In a study evaluating a CAF09-adjuvanted pepmix vaccine against hepatitis C virus, the level of specific lysis to 2 different peptides containing CD8 epitopes was compared by i.v. injection of splenocytes labeled with 3 different concentrations of CFSE and 10 μg/mL of each peptide.[92]

A complex protocol involving up to 216 separately fluorescently stained splenocyte populations was developed by Quah et al., intended for detailed in vivo assessment of CD8$^+$ T-cell avidity and concomitant evaluation of several CD8 epitopes.[101] Splenocyte populations derived from naïve mice were stained with 4-6 concentrations of the fluorescent dyes CFSE, celltrace violet, and cell proliferation dye, including a nonstained population, followed by pulsing with different concentrations of minimal CD8 epitopes prior to injection into immunized mice. Separation of donor and recipient cells was achieved by using B6.CD45.1 donor mice, thus allowing selective fluorescent antibody staining of CD45.1 in the B6.CD45.2 recipient mice. The avidity of induced antigen-specific CD8$^+$ T cells was shown to depend on the type of antigen, as SIINFEKL-specific CD8$^+$ T cells showed a high level of specific killing even at low peptide concentrations on donor cells. In contrast, the epitopes GP33 and NP68 resulted in lower avidities, with distinctly peptide-concentration dependent specific lysis levels by antigen-specific CD8$^+$ T cells.[101]

Consideration for Use of Animal Models to Predict Immunity in Humans

One big hurdle in vaccine development is to transfer novel vaccines and adjuvants from preclinical studies into clinical trials. An important aspect here is obviously the need for animal models that optimally reflect human (or target animal) vaccine-induced immunity, toxicology, and prevention of disease against the pathogens in question. The choice of animal model requires that the relevant parts of the immune system are comparable with the target species in receptor expression and cellular responses. By far, most in vivo vaccine efficacy studies are performed on inbred mice. The structure of the immune system in mice and humans is overall highly similar, but some characteristics are different and should be taken into consideration when using mouse models. Covering this issue in detail is not our scope with this review, although it deserves some attention. We have focused on a few important topics.

Innate Sensing

The innate immune system is conserved between all multicellular organisms in some form in contrast to the adaptive immune system, which is found in vertebrates only.[102] There are vast differences in the types, numbers, and functions of TLRs, C-type lectin receptors, retinoic acid-inducible gene-I-like receptors, and NOD-like receptors between species, which has been reviewed elsewhere.[102] Furthermore, there are differences in the immune cell compositions and functions (eg, the ratio of leukocytes and the responses to IFN-γ), and, importantly, resistance is favored in humans, whereas tolerance is favored in mice.[103]

TLR7 and TLR8 are often grouped as they are both activated by single-stranded RNA and imidazoquinolines, such as R848 and 3M-052. However, the 2 TLRs respond very differently to stimulation in mice and humans, with human TLR8 responding to stimulation with single-stranded RNA, while no response is raised by murine TLR8.[104,105] It has been suggested that murine TLR8 has no function, but it has been shown that TLR8-deficient mice have an increased expression of TLR7 and develop autoimmune diseases.[106] Thus, preclinical testing of R848 and 3M-052 as vaccine adjuvants in mice likely evaluates the activation of TLR7, whereas in humans, both TLR7 and TLR8 have a function in the induction of an immune response.[107,108]

Humoral Immune Responses

In all higher vertebrates, the initial antibody response to immunization is mainly produced by GC-independent plasmablasts or early plasma cells in the draining LN and constitutes primarily of IgM antibodies. This is followed by formation of GCs.[109,110] The kinetics of the GC responses, and the overall phenotype of

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the main cellular subsets involved (follicular B cells, DCs, and T follicular helper cells [T_{fh}]) are similar between species commonly used in vaccine research. However, there are well-known differences in the Ig isotypes between the species. Mice produce IgM, IgA, IgD, IgE, and 4 subtypes of IgG: IgG1, IgG2a/c, IgG2b, and IgG3.[111] The same applies to rat, but rat IgG2b corresponds to mouse IgG2a/c and rat IgG2c to mouse IgG3. Pigs have up to 6 different IgG subclasses and rabbits have only one. Humans also express IgM and have 2 subtypes of IgA, IgA1 and IgA2, in addition to IgD and IgE. In humans there are also 4 subtypes of IgG (IgG1, IgG2, IgG3, and IgG4), but these do not correspond directly to those found in the rodents.[111] A particularly important aspect related to vaccine research is production of IgG1. While this is related to an IL-4-driven Th2 response in mice and is often paralleled by concomitant IgE production, the same does not apply to humans, where IL-4 can instead drive IgG4 production. Vaccination with protein antigens generally induces IgG1, IgG2b, and IgG2a/c in mice, while IgG3 is mostly produced in response to T-independent antigens, such as TLR ligands or polysaccharides.[112,113] In humans, protein antigens stimulate mostly IgG1 and IgG3, and polysaccharides stimulate IgG2.[114] IgG4 is typically produced in chronic infections and may be stimulated with repeated immunizations using high antigen doses, which is utilized in allergen immunotherapy.[115] Notably, there is no mouse equivalent to human IgG4,[116] and the mice may therefore be suboptimal as a model for potential IgG4-mediated allergen immunotherapy.

Mucosal immune responses show some additional distinct features between mouse and man. Thus, while the primary mucosal antibody produced is IgA in both species, mice mainly produce dimeric IgA both in serum and in mucosal sites. In humans the secretory IgA is mainly dimeric or polymeric, whereas serum IgA is mainly monomeric, making it easy to distinguish between locally produced and serum IgA.[117,118] Secretory IgA is transported across epithelial cells via the polymeric Ig receptor (pIgR). In mice, large amounts of pIgA are cleared from plasma and transported to bile by pIgR-expressing hepatocytes. In contrast, in humans, biliary epithelial cells express pIgR and perform the pIgA secretion into bile. This means that in humans there is much less circulating pIgA transported into bile and that most IgA in bile is secretory IgA produced by local plasma cells.[117] Pigs may be a better model for elucidating mucosal IgA responses, as porcine IgA is more homologous to human IgA than mouse or rat IgA.[117] Mucosal immune responses can also be evaluated in pigs by measuring mucosal pIgR levels. Another limitation of mouse models in mucosal immune responses is the lack of FcαR, which is otherwise conserved in mammals.[119]

The functional attributes of antibodies are largely determined by their Fc properties and, similar to the differences in antibody classes and subclasses, FcR expression varies between species used for vaccine evaluation. Both mice and humans express the FcR for IgM (FcμR or TOSO).[120] However, in contrast to humans, mice lack FcαRI. Humans express the Fc receptors for IgG, FcγRI, FcγRIIA, FcγRIIC, and hFcγRIIIA, which are activating, and FcγRIIB, which is inhibitory.[111] Human IgGs can bind to all the FcγR receptors, except IgG2, which cannot bind FcγRI. FcRn, which is used for transport of Igs, can also bind to all IgG subclasses[111] and also exists in mice.[121] In addition, mice express FcγRI, FcγIIB, FcγRIII, and FcγRIV.[122] Similar to humans, FcγRIIB is inhibitory, whereas the rest of the FcγRs are activating.[122] Notably, mice, but not humans, express FcγRIV, which can only bind mouse IgG2a/b/c and not IgG1.[111,123] Since FcγRIV may function by mediating ADCC,[124] IgG2 antibodies may be more efficient to perform this function in mice. It should also be noted that great differences in expression pattern between mouse and human FcγR exist. For example, the expression of human FcγRIIIA is restricted to NK and monocytic cells, whereas this is not the case in mice.[111]

Cell-Mediated Immune Responses

The biggest challenges when it comes to correlating vaccine-induced, cell-mediated immune responses between species is that these responses are most often measured in cells derived from lymphoid organs or tissues, whereas the same analysis in humans is almost exclusively derived from blood samples. The two most well-described $CD4^+$ T-cell subsets, Th1 and Th2, are well characterized in humans, and a clinical trial with CAF01 showed good correlation between mouse and man regarding these subsets.[125] Correlates of induction of other subsets like Th17, T_{reg}, and T_{fh} cells on the other hand are still lacking behind.

Th17 $CD4^+$ T cells are thought to be critical for mucosal protection against pathogen entry and are identified by their ability to produce the cytokine IL-17. For example, the populations of Th17 $CD4^+$ T cells vary with *M.tb* infection status in humans, that is, recently infected, latently infected, and active disease.[126] It has also been shown that people with impaired IL-17 function often suffer from chronic mucocutaneous candidiasis, recurrent or persistent symptomatic infection of the nails, skin, and mucosae by *Candida albicans*.[127–129] Subunit vaccines adjuvanted with CAF01 have been shown to induce robust Th17 $CD4^+$ T-cell responses in both spleen and lungs in mice.[82,85] However, attempts to detect IL-17 induction in human blood samples after vaccinations using CAF01 have so far failed.[125] The reasons for this can be multiple. The mechanism of induction of Th17 $CD4^+$ T-cell responses have not been completely elucidated. Thus, though there are similarities between human and murine Th17 $CD4^+$ T cells, it is not certain that they are induced via similar pathways and that Th17 is in fact induced by CAF01 in humans. Maybe more likely, the amount of Th17 cells in blood samples is below detection level with the commonly used techniques like IL-17 cytokine ELISA/ELISPOT and flow cytometry. Th17 is induced and detectable but not with the biomarkers currently used for detection. IL-17-producing cells were detected in the blood in a recent study evaluating an oral enterotoxigenic *Eschericia coli* vaccine, ETVAX, with or without dmLT adjuvant.[130] This vaccine was found to induce the appearance of activated T cells with a Th17 and gut-homing phenotype in peripheral blood.[130] So far, similarly to Th17 cells, detection of T_{fh} responses in humans has been hampered by the difficulty to obtain the relevant tissue. However, a subset of circulating T_{fh} cells has been identified in mice and humans, which shares functional properties with GC T_{fh} cells.[130–132]

The primary function of T_{reg} is to maintain immunological homeostasis and prevent excessive inflammation. Consequently, T_{reg} might also interfere with vaccine-induced immunity. In a recent clinical study, the ability of 4 commonly used antiviral vaccines to induce human $CD4^+$ T_{reg} responses was investigated. Peripheral blood mononuclear cells obtained from healthy volunteers that had been vaccinated with either trivalent influenza vaccine with or without the addition of adjuvant MF59 (Fluad or Agrippal), a HBV subunit vaccine (Engerix-B) or a live attenuated yellow fever vaccine (Stamaril).[133] At several days post vaccination, the frequency and phenotype of $CD4^+$ T_{reg} subpopulations in peripheral blood was examined by flow cytometry. For comparison, mice were vaccinated with influenza and hepatitis B

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vaccines and the T_{reg} frequency was analyzed in draining LNs and spleen at several days post vaccination. Overall, the study showed that vaccination with vaccines with an already established safe profile have only minimal impact on frequencies and characteristics of T_{reg} over time. However, it also showed that the systemic changes in T_{reg} frequency found in mice were not identical to the human data. The authors suggest that this may be caused by the fact that the human systemic T_{reg} frequency was determined in blood and that of mice in the spleen, or that there are differences in T_{reg} definitions between species.[133]

The induction and evaluation of $CD8^+$ T-cell responses in humans is relatively well described. However, most successful CD8 T-cell-inducing vaccines are based on viral vectors, the reason most probably being that priming of antigen-specific $CD8^+$ T-cell responses by adjuvanted peptide-/protein-based vaccines requires presentation of a CD8 epitope on MHC-I on specialized cross-priming DCs.[134] In humans, the $CD141^+CLEC9A^+$ DCs have been identified as a superior cross-priming DC subset compared with other DC subsets,[135] which correspond to the cross-priming $CD8\alpha^+$ and $CD103^+$ DC subsets characterized in mice.[136,137] These DC subsets are genetically closely related between the species[138] and share expression of the receptors TLR3 and XCR1, which are important for the cross-priming functionality.[135,139] Thus, the mouse can generally be considered a suitable animal model for evaluating adjuvants for their ability to induce cross-priming and subsequent $CD8^+$ T-cell responses.

Conclusion

The use of mice to evaluate vaccine immunogenicity, and especially adjuvant mechanism, is highly relevant, albeit one has to take a few aspects into consideration when trying to predict the function in humans. Does the target receptor specificity, cell distribution, and functionality in mice reflect that in humans, and does the injection route commonly used in mice result in the same immune responses as the one intended to be used in humans? In addition, one has to reflect on whether the immune responses evaluated in mouse organs like LNs, spleen, lungs, intestines, skin, genital tract, etc. can also be detected in blood samples, which is often the only accessible sample material from humans. Therefore, when performing preclinical studies, it should be considered to do the same analysis in blood as done on other tissues to counteract setbacks in clinical development.

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References

1. Rappuoli R. Bridging the knowledge gaps in vaccine design. *Nat Biotechnol*. 2007;25(12):1361–1366.
2. Plotkin SA. Complex correlates of protection after vaccination. *Clin Infect Dis*. 2013;56(10):1458–1465.
3. Guy B. The perfect mix: recent progress in adjuvant research. *Nat Rev Microbiol*. 2007;5(7):505–517.
4. Pashine A, Valiante NM, Ulmer JB. Targeting the innate immune response with improved vaccine adjuvants. *Nat Med*. 2005;11(4 Suppl):S63–S68.
5. Lee S, Nguyen MT. Recent advances of vaccine adjuvants for infectious diseases. *Immune Netw*. 2015;15(2):51–57.
6. O'Hagan DT, Fox CB. New generation adjuvants—from empiricism to rational design. *Vaccine*. 2015;33(Supplement 2):B14–B20.
7. Chauhan N, Tiwari S, Iype T, Jain U. An overview of adjuvants utilized in prophylactic vaccine formulation as immunomodulators. *Expert Rev Vaccines*. 2017;16(5):491–502.
8. Newton K, Dixit VM. Signaling in innate immunity and inflammation. *Cold Spring Harb Perspect Biol*. 2012;4(3):pii: a006049.
9. Kaisho T, Akira S. Toll-like receptor function and signaling. *J Allergy Clin Immunol*. 2006;117(5):979–987.
10. Maisonneuve C, Bertholet S, Philpott DJ, De Gregorio E. Unleashing the potential of NOD- and Toll-like agonists as vaccine adjuvants. *Proc Natl Acad Sci USA*. 2014;111(34):12294–12299.
11. Geijtenbeek TBH, Gringhuis SI. Signalling through C-type lectin receptors: shaping immune responses. *Nat Rev Immunol*. 2009;9(7):465–479.
12. Tandrup Schmidt S, Foged C, Smith Korsholm K, Rades T, Christensen D. Liposome-based adjuvants for subunit vaccines: formulation strategies for subunit antigens and immunostimulators. *Pharmaceutics*. 2016;8(1):7.
13. Peek LJ, Middaugh CR, Berkland C. Nanotechnology in vaccine delivery. *Adv Drug Deliv Rev*. 2008;60(8):915–928.
14. Srivastava A, Gowda DV, Madhunapantula SV, Shinde CG, Iyer M. Mucosal vaccines: a paradigm shift in the development of mucosal adjuvants and delivery vehicles. *APMIS*. 2015;123(4):275–288.
15. Amorij J-P, Kersten GFA, Saluja V, et al. Towards tailored vaccine delivery: needs, challenges and perspectives. *J Control Release*. 2012;161(2):363–376.
16. Bonam SR, Partidos CD, Halmuthur SKM, Muller S. An overview of novel adjuvants designed for improving vaccine efficacy. *Trends Pharmacol Sci*. 2017;38(9):771–793.
17. O'Hagan DT, Friedland LR, Hanon E, Didierlaurent AM. Towards an evidence based approach for the development of adjuvanted vaccines. *Curr Opin Immunol*. 2017;47:93–102.
18. Schwendener RA. Liposomes as vaccine delivery systems: a review of the recent advances. *Ther Adv Vaccines*. 2014;2(6):159–182.
19. Leroux-Roels G. Unmet needs in modern vaccinology: adjuvants to improve the immune response. *Vaccine*. 2010;28:Suppl. 3:C25–C36.
20. De Gregorio E, Tritto E, Rappuoli R. Alum adjuvanticity: unraveling a century old mystery. *Eur J Immunol* 2008;38(8):2068–2071.
21. Kool M, Soullié T, van Nimwegen M, et al. Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. *J Exp Med*. 2008;205(4):869–882.
22. Seubert A, Monaci E, Pizza M, O'Hagan DT, Wack A. The adjuvants aluminum hydroxide and MF59 induce monocyte and granulocyte chemoattractants and enhance monocyte differentiation toward dendritic cells. *J Immunol*. 2008;180(8):5402–5412.
23. Davidsen J, Rosenkrands I, Christensen D, et al. Characterization of cationic liposomes based on dimethyldioctadecylammonium and synthetic cord factor from M. tuberculosis (trehalose 6,6′-dibehenate)—A novel adjuvant

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inducing both strong CMI and antibody responses. *Biochim Biophys Acta*. 2005;1718(1–2):22–31.
24. Korsholm KS, Hansen J, Karlsen K, et al. Induction of CD8$^+$ T-cell responses against subunit antigens by the novel cationic liposomal CAF09 adjuvant. *Vaccine*. 2014;32(31): 3927–3935.
25. Olsen AW, Follmann F, Erneholm K, Rosenkrands I, Andersen P. Protection against Chlamydia trachomatis infection and upper genital tract pathological changes by vaccine-promoted neutralizing antibodies directed to the VD4 of the major outer membrane protein. *J Infect Dis*. 2015; 212(6):978–989.
26. Lorenzen E, Follmann F, Bøje S, et al. Intramuscular priming and intranasal boosting induce strong genital immunity through secretory IgA in minipigs infected with Chlamydia trachomatis. *Front Immunol*. 2015;6:628.
27. Schmidt ST, Khadke S, Korsholm KS, et al. The administration route is decisive for the ability of the vaccine adjuvant CAF09 to induce antigen-specific CD8+ T-cell responses: the immunological consequences of the biodistribution profile. *J Control Release*. 2016;239:107–117.
28. Parungo C, Soybel D, Colson Y, et al. Lymphatic drainage of the peritoneal space: a pattern dependent on bowel lymphatics. *Ann Surg Oncol*. 2007;14(2):286–298.
29. Harrell MI, Iritani BM, Ruddell A. Lymph node mapping in the mouse. *J Immunol Methods*. 2008;332(1–2):170–174.
30. Cantisani R, Pezzicoli A, Cioncada R, et al. Vaccine adjuvant MF59 promotes retention of unprocessed antigen in lymph node macrophage compartments and follicular dendritic cells. *J Immunol*. 2015;194(4):1717–1725.
31. Manolova V, Flace A, Bauer M, Schwarz K, Saudan P, Bachmann MF. Nanoparticles target distinct dendritic cell populations according to their size. *Eur J Immunol*. 2008;38 (5):1404–1413.
32. Rahimian S, Kleinovink JW, Fransen MF, et al. Near-infrared labeled, ovalbumin loaded polymeric nanoparticles based on a hydrophilic polyester as model vaccine: in vivo tracking and evaluation of antigen-specific CD8+ T cell immune response. *Biomaterials*. 2015;37(0):469–477.
33. Kamath AT, Mastelic B, Christensen D, et al. Synchronization of dendritic cell activation and antigen exposure is required for the induction of Th1/Th17 responses. *J Immunol*. 2012;188(10):4828–4837.
34. Schmidt ST, Pedersen GK, Neustrup MA, et al. Induction of cytotoxic T-lymphocyte responses upon subcutaneous administration of a subunit vaccine adjuvanted with an emulsion containing the toll-like receptor 3 ligand poly(I:C). *Front Immunol*. 2018;9:898.
35. Calabro S, Tortoli M, Baudner BC, et al. Vaccine adjuvants alum and MF59 induce rapid recruitment of neutrophils and monocytes that participate in antigen transport to draining lymph nodes. *Vaccine*. 2011;29(9):1812–1823.
36. Didierlaurent AM, Collignon C, Bourguignon P, et al. Enhancement of adaptive immunity by the human vaccine adjuvant AS01 depends on activated dendritic cells. *J Immunol*. 2014;193(4):1920–1930.
37. Liang F, Lindgren G, Sandgren KJ, et al. Vaccine priming is restricted to draining lymph nodes and controlled by adjuvant-mediated antigen uptake. *Sci Transl Med*. 2017;9 (393):pii: eaal2094.
38. Gartlan KH, Krashias G, Wegmann F, et al. Sterile inflammation induced by Carbopol elicits robust adaptive immune responses in the absence of pathogen-associated molecular patterns. *Vaccine*. 2016;34(19):2188–2196.
39. Fan Y, Sahdev P, Ochyl LJ, Akerberg J, Moon JJ. Cationic liposome–hyaluronic acid hybrid nanoparticles for intranasal vaccination with subunit antigens. *J Control Release*. 2015; 208:121–129.
40. Kamath AT, Valenti MP, Rochat A-F, et al. Protective antimycobacterial T cell responses through exquisite in vivo activation of vaccine-targeted dendritic cells. *Eur J Immunol*. 2008;38(5):1247–1256.
41. Chattopadhyay PK, Gierahn TM, Roederer M, Love JC. Single-cell technologies for monitoring immune systems. *Nat Immunol*. 2014;15(2):128–135.
42. Oussoren C, Storm G. Liposomes to target the lymphatics by subcutaneous administration. *Adv Drug Deliv Rev*. 2001; 50(1–2):143–156.
43. Henriksen-Lacey M, Christensen D, Bramwell VW, et al. Liposomal cationic charge and antigen adsorption are important properties for the efficient deposition of antigen at the injection site and ability of the vaccine to induce a CMI response. *J Control Release*. 2010;145(2):102–108.
44. Moghimi SM, Moghimi M. Enhanced lymph node retention of subcutaneously injected IgG1-PEG2000-liposomes through pentameric IgM antibody-mediated vesicular aggregation. *Biochim Biophys Acta*. 2008;1778(1):51–55.
45. Allen TM, Hansen CB, Guo LSS. Subcutaneous administration of liposomes: a comparison with the intravenous and intraperitoneal routes of injection. *Biochim Biophys Acta*. 1993;1150(1):9–16.
46. Kaur R, Bramwell VW, Kirby DJ, Perrie Y. Pegylation of DDA: TDB liposomal adjuvants reduces the vaccine depot effect and alters the Th1/Th2 immune responses. *J Control Release*. 2012;158(1):72–77.
47. Christensen D, Henriksen-Lacey M, Kamath AT, et al. A cationic vaccine adjuvant based on a saturated quaternary ammonium lipid have different in vivo distribution kinetics and display a distinct CD4 T cell-inducing capacity compared to its unsaturated analog. *J Control Release*. 2012;160 (3):468–476.
48. Jelinek I, Leonard JN, Price GE, et al. TLR3-specific double-stranded RNA oligonucleotide adjuvants induce dendritic cell cross-presentation, CTL responses, and antiviral protection. *J Immunol*. 2011;186(4):2422–2429.
49. Hafner AM, Corthésy B, Merkle HP. Particulate formulations for the delivery of poly(I:C) as vaccine adjuvant. *Adv Drug Deliv Rev*. 2013;65(10):1386–1399.
50. Dowling DJ, van Haren SD, Scheid A, et al. TLR7/8 adjuvant overcomes newborn hyporesponsiveness to pneumococcal conjugate vaccine at birth. *JCI Insight*. 2017;2(6):e91020.
51. Karlsen K, Korsholm KS, Mortensen R, et al. A stable nanoparticulate DDA/MMG formulation acts synergistically with CpG ODN 1826 to enhance the CD4+ T-cell response. *Nanomedicine*. 2014;9(17):2625–2638.
52. Hafner AM, Corthésy B, Textor M, Merkle HP. Tuning the immune response of dendritic cells to surface-assembled poly(I:C) on microspheres through synergistic interactions between phagocytic and TLR3 signaling. *Biomaterials*. 2011; 32(10):2651–2661.
53. Nordly P, Rose F, Christensen D, et al. Immunity by formulation design: Induction of high CD8$^+$ T-cell responses by poly(I:C) incorporated into the CAF01 adjuvant via a double emulsion method. *J Control Release*. 2011;150(3):307–317.
54. Martin-Bertelsen B, Korsholm KS, Rose F, et al. The supramolecular structure is decisive for the immunostimulatory properties of synthetic analogues of a mycobacterial lipid in vitro. *RSC Adv*. 2013;3(43):20673–20683.

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55. Martin-Bertelsen B, Korsholm KS, Roces CB, et al. Nano-self-assemblies based on synthetic analogues of mycobacterial monomycoloyl glycerol and DDA: supramolecular structure and adjuvant efficacy. *Mol Pharm*. 2016;13(8):2771–2781.
56. Hattori Y, Morita D, Fujiwara N, et al. Glycerol monomycolate is a novel ligand for the human, but not mouse macrophage inducible C-type lectin, Mincle. *J Biol Chem*. 2014;289(22):15405–15412.
57. Nordly P, Korsholm KS, Pedersen EA, et al. Incorporation of a synthetic mycobacterial monomycoloyl glycerol analogue stabilizes dimethyldioctadecylammonium liposomes and potentiates their adjuvant effect in vivo. *Eur J Pharm Biopharm*. 2011;77(1):89–98.
58. Huber A, Kallerup RS, Korsholm KS, et al. Trehalose diester glycolipids are superior to the monoesters in binding to Mincle, activation of macrophages in vitro and adjuvant activity in vivo. *Innate Immun*. 2016;22(6):405–418.
59. Desel C, Werninghaus K, Ritter M, et al. The Mincle-activating adjuvant TDB induces MyD88-dependent Th1 and Th17 responses through IL-1R signaling. *PLoS One*. 2013;8(1):e53531.
60. Plotkin SA. Correlates of protection induced by vaccination. *Clin Vaccine Immunol*. 2010;17(7):1055–1065.
61. Mesin L, Ersching J, Victora GD. Germinal center B cell dynamics. *Immunity*. 2016;45(3):471–482.
62. Zuccarino-Catania GV, Sadanand S, Weisel FJ, et al. CD80 and PD-L2 define functionally distinct memory B cell subsets that are independent of antibody isotype. *Nat Immunol*. 2014;15(7):631–637.
63. Vermont CL, van Dijken HH, van Limpt CJ et al. Antibody avidity and immunoglobulin G isotype distribution following immunization with a monovalent meningococcal B outer membrane vesicle vaccine. *Infect Immun*. 2002;70(2):584–590.
64. Pedersen GK, Hoschler K, Oie Solbak SM, et al. Serum IgG titres, but not avidity, correlates with neutralizing antibody response after H5N1 vaccination. *Vaccine*. 2014;32(35):4550–4557.
65. Weisel FJ, Zuccarino-Catania GV, Chikina M, Shlomchik MJ. A temporal switch in the germinal center determines differential output of memory B and plasma cells. *Immunity*. 2016;44(1):116–130.
66. Sanz I, Wei C, Lee FE-H, Anolik J. Phenotypic and functional heterogeneity of human memory B cells. *Semin Immunol*. 2008;20(1):67–82.
67. Weisel F, Shlomchik M. Memory B cells of mice and humans. *Annu Rev Immunol*. 2017;35:255–284.
68. Tiller T, Meffre E, Yurasov S, Tsuiji M, Nussenzweig MC, Wardemann H. Efficient generation of monoclonal antibodies from single human B cells by single cell RT-PCR and expression vector cloning. *J Immunol Methods*. 2008;329(1–2):112–124.
69. Sundling C, Li Y, Huynh N, et al. High-resolution definition of vaccine-elicited B cell responses against the HIV primary receptor binding site. *Sci Transl Med*. 2012;4(142):142ra196.
70. Galson JD, Truck J, Fowler A, et al. Analysis of B cell repertoire dynamics following hepatitis B vaccination in humans, and enrichment of vaccine-specific antibody sequences. *EBioMedicine*. 2015;2(12):2070–2079.
71. Wiley SR, Raman VS, Desbien A, et al. Targeting TLRs expands the antibody repertoire in response to a malaria vaccine. *Sci Transl Med*. 2011;3(93):93ra69.
72. Pedersen GK, Ebensen T, Gjeraker IH, et al. Evaluation of the sublingual route for administration of influenza H5N1 virosomes in combination with the bacterial second messenger c-di-GMP. *PLoS One*. 2011;6(11):e26973.
73. Purtha WE, Tedder TF, Johnson S, Bhattacharya D, Diamond MS. Memory B cells, but not long-lived plasma cells, possess antigen specificities for viral escape mutants. *J Exp Med*. 2011;208(13):2599–2606.
74. Hammarlund E, Thomas A, Amanna IJ, et al. Plasma cell survival in the absence of B cell memory. *Nat Commun*. 2017;8(1):1781.
75. Knudsen NPH, Olsen A, Buonsanti C, et al. Different human vaccine adjuvants promote distinct antigen-independent immunological signatures tailored to different pathogens. *Sci Rep*. 2016;6:19570.
76. Bournazos S, Ravetch JV. Fcgamma receptor function and the design of vaccination strategies. *Immunity*. 2017;47(2):224–233.
77. Lu LL, Suscovich TJ, Fortune SM, Alter G. Beyond binding: antibody effector functions in infectious diseases. *Nat Rev Immunol*. 2018;18(1):46–61.
78. Chung AW, Ghebremichael M, Robinson H, et al. Polyfunctional Fc-effector profiles mediated by IgG subclass selection distinguish RV144 and VAX003 vaccines. *Sci Transl Med*. 2014;6(228):228ra238.
79. Mahan AE, Jennewein MF, Suscovich T, et al. Antigen-specific antibody glycosylation is regulated via vaccination. *PLoS Pathog*. 2016;12(3):e1005456.
80. Li S, Sullivan NL, Rouphael N, et al. Metabolic phenotypes of response to vaccination in humans. *Cell*. 2017;169(5):862–877.e817.
81. Schmidt ST, Neustrup MA, Harloff-Helleberg S, et al. Systematic investigation of the role of surfactant composition and choice of oil: design of a nanoemulsion-based adjuvant inducing concomitant humoral and CD4+ T-cell responses. *Pharm Res*. 2017;34(8):1716–1727.
82. Christensen D, Mortensen R, Rosenkrands I, Dietrich J, Andersen P. Vaccine-induced Th17 cells are established as resident memory cells in the lung and promote local IgA responses. *Mucosal Immunol*. 2016;10(1):260–270.
83. Lay M, Callejo B, Chang S, et al. Cationic lipid/DNA complexes (JVRS-100) combined with influenza vaccine (Fluzone®) increases antibody response, cellular immunity, and antigenically drifted protection. *Vaccine*. 2009;27(29):3811–3820.
84. Seder RA, Darrah PA, Roederer M. T-cell quality in memory and protection: implications for vaccine design. *Nat Rev Immunol*. 2008;8(4):247–258.
85. Lindenstrøm T, Woodworth J, Dietrich J, Aagaard C, Andersen P, Agger EM. Vaccine-induced Th17 cells are maintained long-term postvaccination as a distinct and phenotypically stable memory subset. *Infect Immun*. 2012;80(10):3533–3544.
86. Lin D, Maecker HT. Mass cytometry assays for antigen-specific T cells using CyTOF. *Methods Mol Biol*. 2018;1678:37–47.
87. Reeves PM, Sluder AE, Paul SR, Scholzen A, Kashiwagi S, Poznansky MC. Application and utility of mass cytometry in vaccine development. *FASEB J* 2018;32(1):5–15.
88. Newell EW, Sigal N, Bendall SC, Nolan GP, Davis MM. Cytometry by time-of-flight shows combinatorial cytokine expression and virus-specific cell niches within a continuum of CD8+ T cell phenotypes. *Immunity*. 2012;36(1):142–152.

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89. Wang C, Zheng X, Gai W, et al. Novel chimeric virus-like particles vaccine displaying MERS-CoV receptor-binding domain induce specific humoral and cellular immune response in mice. *Antiviral Res*. 2017;140:55–61.
90. Overgaard NH, Frøsig TM, Jakobsen JT, Buus S, Andersen MH, Jungersen G. Low antigen dose formulated in CAF09 adjuvant favours a cytotoxic T-cell response following intraperitoneal immunization in Göttingen minipigs. *Vaccine*. 2017;35(42):5629–5636.
91. Buglione-Corbett R, Pouliot K, Marty-Roix R, et al. Serum cytokine profiles associated with specific adjuvants used in a DNA prime-protein boost vaccination strategy. *PLoS One*. 2013;8(9):e74820.
92. Filskov J, Mikkelsen M, Hansen PR, et al. Broadening CD4+ and CD8+ T cell responses against hepatitis C virus by vaccination with NS3 overlapping peptide panels in cross-priming liposomes. *J Virol*. 2017;91(14):pii: e00130-17.
93. Salem ML, Kadima AN, Cole DJ, Gillanders WE. Defining the antigen-specific T-cell response to vaccination and poly(I:C)/TLR3 signaling: evidence of enhanced primary and memory CD8 T-cell responses and antitumor immunity. *J Immunother*. 2005;28(3):9.
94. Wherry EJ, Blattman JN, Ahmed R. Low CD8 T-cell proliferative potential and high viral load limit the effectiveness of therapeutic vaccination. *J Virol*. 2005;79(14):8960–8968.
95. Elvang T, Christensen JP, Billeskov R, et al. CD4 and CD8 T cell responses to the M. tuberculosis Ag85B-TB10.4 promoted by adjuvanted subunit, adenovector or heterologous prime boost vaccination. *PLoS One*. 2009;4(4):e5139.
96. Anderson KG, Mayer-Barber K, Sung H, et al. Intravascular staining for discrimination of vascular and tissue leukocytes. *Nat Protoc*. 2014;9(1):209–222.
97. Billeskov R, Lindenstrøm T, Woodworth J, et al. High antigen dose is detrimental to post-exposure vaccine protection against Tuberculosis. *Front Immunol*. 2018;8:1973.
98. Lindenstrøm T, Aagaard C, Christensen D, Agger EM, Andersen P. High-frequency vaccine-induced $CD8^+$ T cells specific for an epitope naturally processed during infection with M. tuberculosis do not confer protection. *Eur J Immunol*. 2014;44(6):1699–1709.
99. Zaritskaya L, Shurin MR, Sayers TJ, Malyguine AM. New flow cytometric assays for monitoring cell-mediated cytotoxicity. *Expert Rev Vaccines*. 2010;9(6):601–616.
100. Doehn C, Esser N, Pauels H-G, et al. Mode-of-action, efficacy, and safety of a homologous multi-epitope vaccine in a murine model for adjuvant treatment of renal cell carcinoma. *Eur Urol*. 2009;56(1):123–133.
101. Quah BJC, Wijesundara DK, Ranasinghe C, Parish CR. Fluorescent target array killing assay: a multiplex cytotoxic T-cell assay to measure detailed T-cell antigen specificity and avidity in vivo. *Cytometry A*. 2012;81A(8):679–690.
102. Bryant CE, Monie TP. Mice, men and the relatives: cross-species studies underpin innate immunity. *Open Biol*. 2012;2(4):120015.
103. Zschaler J, Schlorke D, Arnhold J. Differences in innate immune response between man and mouse. *Crit Rev Immunol*. 2014;34(5):433–454.
104. Kastenmüller K, Wille-Reece U, Lindsay RWB, et al. Protective T cell immunity in mice following protein-TLR7/8 agonist-conjugate immunization requires aggregation, type I IFN, and multiple DC subsets. *J Clin Invest* 2011;121(5):1782–1796.
105. Heil F, Hemmi H, Hochrein H, et al. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science*. 2004;303(5663):1526–1529.
106. Demaria O, Pagni PP, Traub S, et al. TLR8 deficiency leads to autoimmunity in mice. *J Clin Invest* 2010;120(10):3651–3662.
107. Van Hoeven N, Fox CB, Granger B, et al. A formulated TLR7/8 agonist is a flexible, highly potent and effective adjuvant for pandemic influenza vaccines. *Sci Rep*. 2017;7:46426.
108. Ilyinskii PO, Roy CJ, O'Neil CP, et al. Adjuvant-carrying synthetic vaccine particles augment the immune response to encapsulated antigen and exhibit strong local immune activation without inducing systemic cytokine release. *Vaccine*. 2014;32(24):2882–2895.
109. Ellebedy A, Jackson K, Boyd S, Ahmed R. Defining antigen specific plasmablast and memory B cell subsets in blood following viral infection and vaccination of humans. *Eur J Immunol*. 2016;46:300.
110. De Silva NS, Klein U. Dynamics of B cells in germinal centres. *Nat Rev Immunol*. 2015;15(3):137–148.
111. Bruhns P. Properties of mouse and human IgG receptors and their contribution to disease models. *Blood*. 2012;119(24):5640–5649.
112. Vinuesa CG, Sze DMY, Cook MC, et al. Recirculating and germinal center B cells differentiate into cells responsive to polysaccharide antigens. *Eur J Immunol*. 2003;33(2):297–305.
113. Pedersen GK, Adori M, Khoenkhoen S, Dosenovic P, Beutler B, Karlsson Hedestam GB. B-1a transitional cells are phenotypically distinct and are lacking in mice deficient in IkappaBNS. *Proc Natl Acad Sci USA*. 2014;111(39):E4119–E4126.
114. Hjelholt A, Christiansen G, Sorensen US, Birkelund S. IgG subclass profiles in normal human sera of antibodies specific to five kinds of microbial antigens. *Pathog Dis*. 2013;67(3):206–213.
115. van de Veen W, Akdis M. Role of IgG4 in IgE-mediated allergic responses. *J Allergy Clin Immunol*. 2016;138(5):1434–1435.
116. Vidarsson G, Dekkers G, Rispens T. IgG subclasses and allotypes: from structure to effector functions. *Front Immunol*. 2014;5:520.
117. Snoeck V, Peters IR, Cox E. The IgA system: a comparison of structure and function in different species. *Vet Res*. 2006;37(3):455–467.
118. Brandtzaeg P. Secretory IgA: designed for anti-microbial defense. *Front Immunol*. 2013;4:222.
119. Decot V, Woerly G, Loyens M, et al. Heterogeneity of expression of IgA receptors by human, mouse, and rat eosinophils. *J Immunol*. 2005;174(2):628–635.
120. Kubagawa H, Kubagawa Y, Jones D, Nasti TH, Walter MR, Honjo K. The old but new IgM Fc receptor (Fc mu R). *Curr Top Microbiol Immunol*. 2014;382:3–28.
121. Andersen JT, Daba MB, Berntzen G, Michaelsen TE, Sandlie I. Cross-species binding analyses of mouse and human neonatal Fc receptor show dramatic differences in immunoglobulin G and albumin binding. *J Biol Chem*. 2010;285(7):4826–4836.
122. Bruhns P, Jonsson F. Mouse and human FcR effector functions. *Immunol Rev*. 2015;268(1):25–51.
123. Nimmerjahn F, Bruhns P, Horiuchi K, Ravetch JV. FcgammaRIV: a novel FcR with distinct IgG subclass specificity. *Immunity*. 2005;23(1):41–51.

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124. Nimmerjahn F, Lux A, Albert H, et al. FcgammaRIV deletion reveals its central role for IgG2a and IgG2b activity in vivo. *Proc Natl Acad Sci USA*. 2010;107(45):19396–19401.
125. van Dissel JT, Joosten SA, Hoff ST, et al. A novel liposomal adjuvant system, CAF01, promotes long-lived Mycobacterium tuberculosis-specific T-cell responses in human. *Vaccine*. 2014;32(52):7098–7107.
126. Coulter F, Parrish A, Manning D, et al. IL-17 production from T helper 17, mucosal-associated invariant T, and γδ cells in tuberculosis infection and disease. *Front Immunol*. 2017;8:1252.
127. Puel A, Cypowyj S, Bustamante J, et al. Chronic mucocutaneous candidiasis in humans with inborn errors of interleukin-17 immunity. *Science*. 2011;332(6025):65–68.
128. Puel A, Cypowyj S, Maródi L, Abel L, Picard C, Casanova J-L. Inborn errors of human IL-17 immunity underlie chronic mucocutaneous candidiasis. *Curr Opin Allergy Clin Immunol*. 2012;12(6):616–622.
129. Tasaki S, Cho T, Nagao J-I, et al. Th17 cells differentiated with mycelial membranes of Candida albicans prevent oral candidiasis. *FEMS Yeast Res*. 2018:18(3). doi:10.1093/femsyr/foy018.
130. Cárdeno A, Magnusson MK, Quiding-Järbrink M, Lundgren A. Activated T follicular helper-like cells are released into blood after oral vaccination and correlate with vaccine specific mucosal B-cell memory. *Sci Rep*. 2018;8(1):2729.
131. Morita R, Schmitt N, Bentebibel S-E, et al. Human blood CXCR5+CD4+ T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity*. 2011;34(1):108–121.
132. Schmitt N, Bentebibel S-E, Ueno H. Phenotype and functions of memory Tfh cells in human blood. *Trends Immunol*. 2014;35(9):436–442.
133. de Wolf ACMT, van Aalst S, Ludwig IS, et al. Regulatory T cell frequencies and phenotypes following anti-viral vaccination. *PLoS One*. 2017;12(6):e0179942.
134. Kurts C, Robinson BWS, Knolle PA. Cross-priming in health and disease. *Nat Rev Immunol*. 2010;10(6):403–414.
135. Jongbloed SL, Kassianos AJ, McDonald KJ, et al. Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. *J Exp Med*. 2010;207(6):1247–1260.
136. van der Aa E, van Montfoort N, Woltman AM. BDCA3 +CLEC9A+ human dendritic cell function and development. *Semin Cell Dev Biol*. 2015;41:39–48.
137. Lauterbach H, Bathke B, Gilles S, et al. Mouse CD8α(+) DCs and human BDCA3(+) DCs are major producers of IFN-λ in response to poly IC. *J Exp Med*. 2010;207(12):2703–2717.
138. Robbins SH, Walzer T, Dembélé D, et al. Novel insights into the relationships between dendritic cell subsets in human and mouse revealed by genome-wide expression profiling. *Genome Biol*. 2008;9(1):R17.
139. Bachem A, Güttler S, Hartung E, et al. Superior antigen cross-presentation and XCR1 expression define human CD11c+CD141+ cells as homologues of mouse CD8+ dendritic cells. *J Exp Med* 2010;207(6):1273–1281.

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Review Article

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Animal Models for Influenza A Virus Infection Incorporating the Involvement of Innate Host Defenses: Enhanced Translational Value of the Porcine Model

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Abstract

Influenza is a viral respiratory disease having a major impact on public health. Influenza A virus (IAV) usually causes mild transitory disease in humans. However, in specific groups of individuals such as severely obese, the elderly, and individuals with underlying inflammatory conditions, IAV can cause severe illness or death. In this review, relevant small and large animal models for human IAV infection, including the pig, ferret, and mouse, are discussed. The focus is on the pig as a large animal model for human IAV infection as well as on the associated innate immune response. Pigs are natural hosts for the same IAV subtypes as humans, they develop clinical disease mirroring human symptoms, they have similar lung anatomy, and their respiratory physiology and immune responses to IAV infection are remarkably similar to what is observed in humans. The pig model shows high face and target validity for human IAV infection, making it suitable for modeling many aspects of influenza, including increased risk of severe disease and impaired vaccine response due to underlying pathologies such as low-grade inflammation. Comparative analysis of proteins involved in viral pattern recognition, interferon responses, and regulation of interferon-stimulated genes reveals a significantly higher degree of similarity between pig, ferret, and human compared with mice. It is concluded that the pig is a promising animal model displaying substantial human translational value with the ability to provide essential insights into IAV infection, pathogenesis, and immunity.

Key words: animal model; antiviral; inflammation; influenza A virus; innate immune response; microRNA; translational value; validity

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Introduction

Influenza A virus (IAV) infection is a leading cause of morbidity and mortality in the human population, with estimates of annual epidemics resulting in 3 to 5 million cases of severe disease and 290 000 to 650 000 deaths worldwide.[1] While substantial progress has been made toward understanding viral evolution, transmission, and pathogenicity, multiple aspects such as the involvement of host factors in pathogenesis, control, and clearance of the infection remain to be fully understood. Disease severity varies markedly among individuals and can be ascribed to differences in genetically determined susceptibility and in the level and type of immune responses between individual hosts. In otherwise healthy individuals, influenza is a transient disease and the patient will usually recover within 1 to 2 weeks. However, vulnerable population groups such as pregnant women, infants, the elderly, and severely obese individuals, as well as individuals with chronic inflammatory or autoimmune conditions including diabetes mellitus, are at higher risk of increased morbidity and mortality from IAV infections.[2–6] Furthermore, these high-risk groups tend to have more prolonged and invasive forms of IAV infections, and are likewise at higher risk of selection for drug-resistant IAV populations during treatment due to the prolonged infection and delayed viral clearance.[7–10] Also, vaccination, which remains the most effective method for prevention of IAV infection and reduction of IAV related disease, varies substantially in efficiency between individuals; a pronounced decrease in IAV vaccine responsiveness has been found in the high-risk groups including the elderly and severely obese individuals, often associated with immunosenescence or low-grade chronic inflammation.[11,12] Antiviral agents are available for treatment and prevention of IAV infections in immunocompromised patients, including matrix-2 (M2) protein inhibitors and neuraminidase (NA) inhibitors. However, resistance to both groups of antiviral drugs has been described and the number of resistant strains seems to be increasing.[13–15] Characterization of the impact of viral and host mechanisms respectively, on the course of disease is therefore paramount for the development of more broadly effective vaccines and antiviral therapies.

IAVs are enveloped, single-stranded, negative-sense RNA viruses of the family *Orthomyxoviridae*. This family comprises a number of genera, including *Influenza A*, *B*, and *C*. IAVs are further classified into subtypes based on the antigenic surface glycoproteins hemagglutinin (HA) and NA. Currently, 16 HA (H1–H16) and 9 NA (N1–N9) subtypes are found in the aquatic bird reservoir.[16] IAVs enter the respiratory system of the host through inhaled droplets or aerosols before they infect pulmonary epithelial cells through binding of viral HA surface glycoprotein to sialic acid (SA) containing receptors found on the host cell surface in the upper respiratory tract in humans. Even though the type of SA linkage is not the sole determinant of viral host and tissue tropism, the tissue distribution of α-2,3 and α-2,6-linked SA receptors is important for viral binding and infectivity.[17] Within the cell nucleus, genomic RNA of IAV is replicated and progeny IAVs are formed when the particles bud off from the plasma membrane. Newly synthesized IAVs are released from the cell by cleavage of the HA-SA binding by NA surface glycoproteins. Due to their segmented genomes, IAVs may undergo reassortment where new subtypes are generated by new combinations of gene segments (antigenic shift). Additionally, the mutation rate of the IAV RNA genome is high, leading to amino acid changes in important viral epitopes (antigenic drift). Combined, antigenic shift and drift causes the development of new virus variants that may escape previously acquired host immunity and give rise to new epidemics or even pandemics. Thus, the continuous evolution of new IAV subtypes evading antiviral drugs and previously acquired immunity by vaccines, together with the inter-individual variability in host responses towards IAV, stresses the need for reliable and valid animal models for development of novel anti-IAV therapeutics and prophylactic agents, thereby serving as a bridge for the translational gap to the clinic.[18]

In this review, we aim to summarize relevant characteristics of well-established animal models, including ferret and mouse models, in relation to IAV infection, all of which have provided extensive knowledge on the basic immunology, pathology, and transmission of IAV.[19] Furthermore, the validity of the models in relation to IAV infection will be evaluated; however, the main focus will be on the utility and relevance of the pig as a large animal model for human IAV infection. The pig is a well-established animal model in many areas of biomedical research and excels as a highly reliable translational model for human IAV infection and disease.

Animal Models for Study of IAV Infection

Although several animal species have been used for IAV research, including rodents, ferrets, pigs, and nonhuman primates,[20] the most frequently used animal model is the mouse. This is likely due to the low per animal costs, modest housing requirements, wide availability of immunological reagents, and ease of creating and obtaining specifically genetically modified mouse strains. Even though the mouse model has provided extensive knowledge regarding fundamental immunology of IAV infections, major differences in clinical manifestations and in the anatomy of the respiratory system compared with humans emphasize the urgent need for improved translational models. As summarized in Table 1, mice are not naturally infected with human IAV strains nor do they show clinical signs similar to humans such as fever, nasal secretion, and coughing after IAV infection.[21,25] The majority of IAV strains require adaptation to effectively replicate and cause disease in mice. These adaptations include mutations in the receptor-binding site of the viral HA and NA proteins, loss of glycosylation sites, and other changes that may affect viral host and tissue tropism.[50,51] Evidently, mutations introduced in the IAV genome during adaptation to a murine host could result in changes in phenotypic traits that are important for pathogenesis in humans. Other animal species are therefore being increasingly used for IAV studies, including pigs and ferrets. These species are susceptible to infection with human IAV, show clinical signs resembling those seen in humans, and generally reflect the human anatomy and pathogenesis more faithfully than mouse models (Table 1). Pig breeds of different sizes are available, and a study comparing the clinical signs upon experimental IAV infection in differently sized pigs showed no differences in clinical manifestation.[52] Additionally, infected pigs and ferrets can readily transmit the virus to naïve animals while transmission from infected to naïve mice is inefficient.[21,53] Table 1 summarizes some of the most relevant features of pig, ferret, and mouse models, highlighting their strengths and weaknesses in relation to their applicability for the study of human IAV infection. Seronegative pigs, ferrets, and mice are available. However, in contrast to the mouse, pig and ferret animal models are outbred, like humans, with an inherently larger inter-individual biological variation and therefore a higher number of animals are required to obtain adequate statistical power for these species compared with inbred mouse animal models. The influence of underlying inflammatory conditions

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Table 1 Comparison of clinical signs after IAV infection, morphology of the respiratory tract, and experimental requirements between mammalian models of human IAV infection

	Human	Pig	Ferret	Mouse	References
Naturally infected with human IAV	Yes	Yes	Yes	**No (Requires adaptation)**	21–24
Fever	Present	Present	Present	**Absent**	21,24–33
Nasal secretion	Present	Present	Present	**Absent**	21,24,26–28
Coughing	Present	Present	Present	**Absent**	21,26–28,30,32,34,35
Possesses tonsils	Yes	Yes	Yes	**No**	36–39
Nature of pulmonary pleura	Thick	Thick	**Thin**	**Thin**	40–43
Nature of pulmonary connective tissue	Extensive and interlobular	Extensive and interlobular	Little	**Little, if any**	41–43
Alveolar macrophages	Constitutive phagocytic cells	Constitutive phagocytic cells	Constitutive phagocytic cells	Constitutive phagocytic cells	44–46
Pulmonary intravascular macrophages	Induced phagocytic cells	**Constitutive phagocytic cells**	Induced phagocytic cells	Induced phagocytic cells	47,48
Availability of species-specific immunological reagents	High	**Moderate; increasing**	**Low**	High	–
Housing requirements	–	Large	Medium	Small	–
Experimental costs	–	Moderate/high	Moderate/high	Low	–

Modified from.[49] Characteristics highlighted in bold differ from those observed in humans.

on IAV infection can be studied by administration of bacterial lipopolysaccharide (LPS); however, the sensitivity to the toxic effects of LPS differs remarkably between animal species. While mice are highly insensitive to LPS compared with humans, pigs are more comparable to the LPS sensitivity of humans.[54,55] The number of studies on LPS administration in ferrets is limited, and in most of the studies LPS is administered through the respiratory tract[56–58] except from a study on 5-day-old ferrets, where LPS was i.p. injected.[59] The study designs as well as the limited number of studies makes it difficult to compare the sensitivity of LPS in ferrets with human.

Host Defense Against IAV Infection

The mammalian antiviral innate defense system is multifactorial, encompassing a variety of cell types and cellular and secreted proteins working in concert to prevent viral invasion and replication and to control and fine-tune inflammatory and immune responses. Every factor must play its role at the right time and place to ensure a balanced and efficient immune response resulting in the ultimate clearance of the virus with a minimal degree of collateral host tissue damage.

Upon IAV infection, as summarized in Figure 1, the pulmonary host immune response is initiated by a rapid and transient induction of pro- and anti-inflammatory cytokines, type I and type III interferons (IFNs), and IFN-Stimulated Genes (ISGs), establishing an antiviral state in the infected and neighboring host cells.[60,61,66–68] With a slightly later onset, the noncoding microRNA (miRNA) response sets in (Figure 1). These miRNAs are involved in posttranscriptional regulation of antiviral target genes, and a subpopulation of miRNAs persists throughout the infection and even after the virus has been cleared.[65,69] The number of natural killer (NK) cells increases from the beginning of infection and reaches its peak as the viral load is declining.[62,70] Within a week after influenza virus infection, IAV-specific antibodies emerge simultaneously with the clearance of the infection accomplished mainly by the innate immune response in cases of uncomplicated disease (Figure 1).

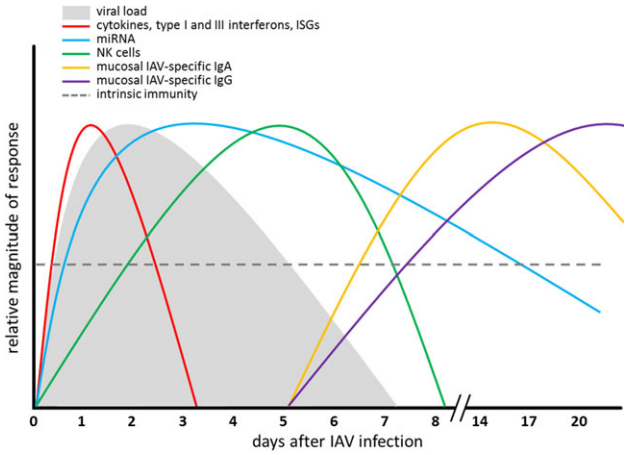

Figure 1 Schematic representation of different aspects of the antiviral host immune response against influenza. The x-axis shows the temporal progression of infection (days) and the y-axis denotes the relative magnitude of the different responses. The figure is based on data from several publications[60–64] and modified from[62] as well as our own work[65].

Intrinsic and Innate Barriers Against IAV Infection in Humans, Pigs, Ferrets, and Mice

To successfully infect and replicate within a host, IAV has to traverse several physiological and chemical barriers. The first challenge facing IAV upon host contact is the intrinsic respiratory mucus layer, a complex, viscous gel-like fluid secreted from goblet cells and submucosal glands of the conducting airways.[71] The distribution of goblet cells varies significantly between species. As shown in Figure 2, the distribution of goblet cells in the upper respiratory tract of humans, pigs, and ferrets is relatively similar, whereas mucus-secreting goblet cells are rare in the upper respiratory tract of mice.[40,72–76] The 2 secreted mucins (MUC) MUC5B and MUC5AC are major components of the human and pig upper respiratory mucus layer, and

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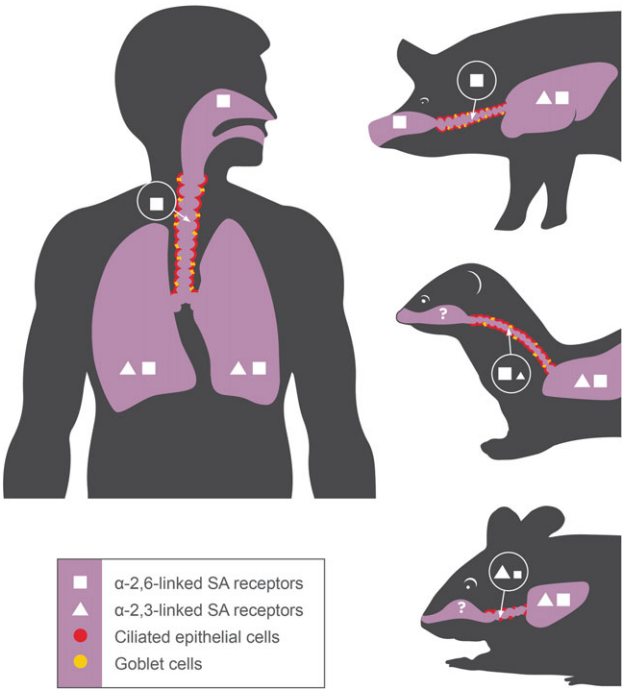

Figure 2 Schematic illustration of the distribution of α-2,3 and α-2,6-linked sialic acid (SA) containing receptors in the nasal cavity, trachea, and lung. Squares indicate α-2,6-linked SA containing receptors and triangles indicate α-2,3-linked SA containing receptors. The relative sizes of squares and triangles indicate the relative proportion of the SA receptors at each of the three locations in the respiratory tract. Question mark indicates that no literature reporting on examination of the specific tissues was found. Distribution and proportion of respiratory ciliated epithelial cells (red) and mucus producing goblet cells (yellow) in humans, pigs, ferrets, and mice are shown in the trachea.

other important components include membrane-associated MUC1, pulmonary surfactants such as surfactant protein type D (SP-D), lysozyme, and lactoferrin.[77–79] The respiratory human and pig mucus also contains SA that can function as "decoy receptors" binding to HA surface proteins of the invading virus and thus hindering interaction with the underlying epithelial cells.[80,81] Mucociliary clearance by ciliated epithelial cells ensures that inhaled and entrapped pathogens are continuously removed and swallowed. The percentage of ciliated respiratory epithelial cells varies among mice and other animal models (Figure 2). Similarly to humans, pigs and ferrets have a larger proportion of ciliated respiratory epithelial cells at the tracheal surface compared with mice.[74,75,82–84]

SP-D is a soluble, SA-containing constituent of the respiratory mucus layer and an important porcine defense lectin that with varying success has been demonstrated to reduce infection in MDCK cells by H1N1, H3N2, and H5N1 of human, swine, and avian origin.[85] Whereas SP-D has been found to be an important antiviral lectin in the mouse,[86] it has recently been shown not to play a major role in inhibition of different types of IAV in ferrets.[87,88] We have recently found the SP-D gene (*SFTPD*) to be constitutively expressed but not induced in the porcine lung during swIAV (H1N2) infection (Brogaard et al., unpublished work), suggesting that SP-D is an intrinsic pulmonary antiviral factor in pigs. After breaching the mucus layer, the viral HA binds to the terminal SA residues linked to host cell surface glycoproteins. Several factors, including local host proteases and the distribution of α-2,3 and α-2,6-linked SA receptors, are important for viral binding and infectivity. As indicated in Figure 2, the distribution of SAα-2,3 and SAα-2,6 receptors in the nasal cavity, trachea, and lung differs considerably between mice and humans, whereas less variation is seen between humans, pigs, and ferrets.[17,89–93] This might explain the difference in the location of respiratory infection caused by influenza virus, which in mice locates in the lower respiratory tract rather than in the upper respiratory tract as observed in humans, pigs, and ferrets during uncomplicated infections.[25] A study comparing the SA-containing receptor distribution in 2 differently sized pig breeds demonstrated the distribution to be similar.[52]

Comparison of Proteins Involved in Innate Antiviral Defense Between Animal Models

The human and mouse genomes were sequenced 15 years ago[94–96] and are now considered complete. Since the original sequencing was completed, each has undergone 38 major builds (assemblies of constructed sequences). In contrast, build 11.1 of the porcine genome was released in January 2017, and preliminary analysis indicates that it is almost complete (98%) but still contains a number of significant sequence and annotation errors (H. Dawson, personal communication). A draft version of build 1.0 of the ferret genome was published in 2014.[97] Although the size and number of genes are consistent with other mammalian species, the presence of sequence or annotation errors has not been reported.

A comprehensive literature search was conducted to identify human, pig, ferret, and mouse proteins involved in immune responses to IAV infections. Gene annotations for these proteins can be found in the porcine translational research database.[98] A comparative analysis of 91 antiviral proteins, where 1:1 orthology could be established between the 4 species,[98] is summarized in table 2. Orthologous sequences originate from a common ancestor and are inherited through speciation, and the functional specificity of such proteins is therefore assumed to be conserved. The proteins were divided into the categories Retinoic acid-Inducible Gene 1 (RIG-I)/Melanoma Differentiation-Associated protein 5 (MDA5), Toll-Like Receptor (TLR) and signaling, IFN and receptors, ISGs, DEAD/DEAH-box family, Inflammasome-associated proteins, and Miscellaneous antiviral proteins (Table 2). The amino acid sequences of all pig and ferret antiviral proteins included in the table were statistical significantly more similar (82.9 % and 81.9 %, respectively) to equivalent human proteins than were the mouse protein sequences (78.4 %). Pig and ferret proteins classified as or being involved in RIG-I/MDA5 signaling, IFN and Receptors, and ISGs (Table 2; Supplemental Table 1) exhibited statistical significantly greater human similarities than the corresponding mouse proteins. Furthermore, pig proteins classified as TLR or as being involved in TLR signaling (Table 2; Supplemental Table 1) had significantly greater similarity to orthologous human proteins than both ferret and mouse. No statistically significant differences in similarity to human orthologs were found between the 3 species with regards to the highly conserved DEAD (Asp-Glu-Ala-Asp) box polypeptide and DEAH (Asp-Glu-X-His) box polypeptide RNA helicases and proteins involved in inflammasome function (Table 2; Supplemental Table 1). Analysis of the 91 full-length ferret proteins (Table 2; Supplemental Table 1) required the use of 2 alternative gene sequences (IFNG, TLR8) and reassembly of one gene (KDM1A) due to the incomplete genome sequence.

For a number (24) of proteins associated with anti-IAV response, 1:1 orthology could not be established among all 4 species (Table 3). Mice have 12 and 3 times more nonorthologous genes than ferrets

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Table 2 Comparison of protein similarity of antiviral proteins between humans and pig, ferret, and mouse

		(mean % protein similarity ± SD)		
Classification	N	Pig	Ferret	Mouse
RIG-I/MDA5 and signaling	13	80.9[a] ± 11.0	81.8[a] ± 10.6	76.5[b] ± 11.8
TLR and signaling	9	90.4[a] ± 9.2	91.2[ab] ± 6.7	88.9[b] ± 10.3
IFN and receptors	9	65.2[a] ± 7.3	63.9[a] ± 6.3	53.1[b] ± 7.3
ISGs	25	73.7[a] ± 9.5	72.0[a] ± 11.6	67.5[b] ± 10.9
DEAD/DEAH-box family	8	81.0[a] ± 4.0	81.8[a] ± 8.6	76.5[a] ± 8.6
Inflammasome-associated proteins	3	78.3[a] ± 16.3	76.0[a] ± 18.7	74.0[a] ± 21.9
Miscellaneous antiviral proteins	24	88.7[a] ± 13.2	87.2[ab] ± 16.7	86.1[b] ± 15.6
Overall	91	82.9[a] ± 14.0	81.9[a] ± 15.1	78.4[b] ± 16.8

Analyses were performed using data obtained from the Porcine Translational Research Database, the NCBI reference protein database, and predicted sequences and NCBI blastp suite. 1:1 orthology of protein coding genes were determined by protein sequence similarity (best reciprocal BLAST hit to the human protein) and the presence of a corresponding gene in the syntenic region of the pig, ferret, and/or mouse genome. The values are calculated as the mean value of sequence identity shown in Supplemental Table 1. Means with different superscripts are significantly different at a level of $P < 0.05$ by matched paired ANOVA (JMP 12.2.0 SAS Institute Inc.). The type I interferon alpha superfamily was not compared because of previously noted difficulties establishing 1:1 orthology for these genes.[99]

Table 3 Nonorthologous genes associated with an anti-IAV response in humans, pigs, ferrets, and mice

Gene	Human	Pig	Ferret	Mouse
OAS3	X		X	X
Oas1b				X
Oas1c				X
Oas1d				X
Oas1e				X
Oas1f				X
Oas1g				X
Oas1h				X
Oasl2				X
IFIT5	X	X	X	
ITIT1B	X		X	X
Ifit1bl2				X
IFIT1L1		X		
Ifit3b				X
IFIT5L			X	
Ifitm6				X
Ifitm7				X
IFITM1L1		X		
IFITM1L2		X		
IFITM1L3		X		
IFITM2	X	X		X
NCR2	X	X	X	
NCR3	X	X	X	
HERC5	X	X	X	
Sum of nonorhologous genes	–	4	1	12

X indicates presence of the gene for one of the 24 proteins involved in antiviral immune response where 1:1 orthology could not be established. The total number of nonorthologous genes for each species is summed at the bottom, showing that mice have 12 times and 3 times more nonorthologous genes than ferrets and pigs, respectively. Analyses were performed using data obtained from the Porcine Translational Research Database and NCBI reference protein database and blastp suite.

and pigs, respectively. Notable differences between the 4 species were found in the following antiviral gene families: Oligoadenylate synthetase (OAS), IFN-induced protein with tetratricopeptide repeats (IFIT), and IFN-induced transmembrane protein (IFITM) (Table 3). OAS constitutes a family of anti-viral proteins that are important for controlling infections caused by IAV and other viruses. The enzyme initiates the degradation of viral RNA via synthesis of 2′,5′-oligoadenylates, which activates a latent ribonuclease (RNase L) leading to the degradation of viral RNA and inhibition of viral replication.[100,101] The human OAS family consists of 3 genes encoding active OAS enzymes (*OAS1-3*) and an OAS-Like (*OASL*) gene encoding an inactive protein. All 4 are induced by type I IFNs. Although all 3 OAS isoforms display 2-5As synthetic activity in various models, human OAS3 plays a dominant role in RNase L activation in response to poly (rI):poly (rC) as well as in response to infection of human cells with multiples viruses including IAV.[102] Ferrets and mice but not pigs have OAS3 orthologs.[103] We ecently found porcine *OAS1* and *OASL* to be highly upregulated in lung tissue 1 to 3 days after experimental infection with IAV H1N2.[65] Human and mouse IFIT1 proteins have no antiviral activity against IAV,[104] but porcine IFIT1, IFIT2, and IFIT3 reportedly inhibit the replication of swine influenza virus in vitro.[105] Humans have 5 IFITM family members (IFITM1, IFITM2, IFITM3, IFITM5, and IFITM10). Mice also carry these in addition to Ifitm6 and Ifitm7. All studied human and mouse IFITM proteins can restrict IAV with the exception of IFITM6/Ifitm6.[106] Ferret orthologs have been identified for all human IFITM family members except for IFITM2. Pigs have orthologs of all 5 human genes as well as 3 additional paralogs, *IFITM1L1*, *IFITM1L2*, and *IFITM1L3*. The functions of the 3 IFITM1 paralogs are unknown; however, they are variably induced during porcine viral infections.[107] Porcine *IFITM1* and *IFITM3* were recently found to be moderately upregulated 1 to 3 days after experimental infection with IAV H1N2.[65] Several genes with diverse antiviral functions are missing from one or more of the species reviewed here. The human natural cytotoxicity triggering receptors NKp44 (*NCR2*)[108] and NKp46 (*NCR1*)[109] bind to IAV HA. This binding is required for NK cell-mediated lysis of IAV-infected cells. Pigs and ferrets, but not mice, have *NCR2*[110] (Table 3), and T cells expressing *NCR1*, *NCR2* and *NCR3* have been found in the lungs of IAV infected pigs.[111]

In contrast to the mouse model, a higher degree of protein sequence similarity compared with human is observed in the pig and ferret models with regards to important antiviral protein families. Likewise, the mouse displays more nonorthologous antiviral genes compared with the other 3 species and has also previously been shown to carry greatly expanded PRR gene families relative to both pigs and humans.[112] This serves to emphasize the relevance of the pig as a model with high target validity and thereby higher translational value compared with the mouse model.

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Pig Models for the Study of IAV Infection

Pulmonary Host Response in Porcine Models of IAV Infection

After breaching the mucus layer and infecting the respiratory epithelial cells of the host, the receptor-mediated innate antiviral immune response takes over. For obvious reasons, respiratory epithelial tissue from patients with mild influenza virus infection is scarce, and characterization of the host response in IAV-infected human lung has only been reported from fatal cases after infection with, for example, the 2009 pandemic H1N1 or highly pathogenic avian H5N1 strains.[113–116] While these case studies are of great importance for elucidation of the mechanisms responsible for fatal outcomes of IAV infection, they may be less relevant for characterization of the host innate response to seasonal IAV infection. Instead, in vivo experimental IAV infection in pigs as well as in sophisticated porcine ex vivo cultured respiratory tissue using endemic strains of both human and swine origin have provided insight into the induction of the antiviral innate immune response at the site of viral infection and replication[61,65,117–123].

High-throughput methods like microarray technology, RNA sequencing, and microfluidic qPCR have been applied in several studies to obtain comprehensive transcriptional characterization of porcine respiratory tissue after IAV challenge. By identifying pathway enrichment for differentially expressed genes, these studies commonly report genes involved in viral recognition, pro-inflammatory responses by means of cytokine induction, chemotaxis and immune cell recruitment, apoptosis, and IFN and ISG responses to be centrally involved in the host response to IAV challenge.[117–119,123] Transcription of important viral pathogen recognition receptors such as *TLR3*, *TLR7*, RIG-I (*DDX58*), and MDA5 (*IFIH1*) is upregulated in pig lungs (in vivo, ex vivo) in response to swH1N1 and swH1N2 infections.[61,65,117,121] In vivo studies likewise report a strong chemokine response, dominated by CXCL10,[61,65,117] accompanied by a balanced pro- and anti-inflammatory cytokine response exemplified by the upregulation of *IL1B*, *IL6*, *IL1RN*, and *IL10*.[61,65] As described above, little is known about the local pulmonary response to low pathogenic IAV infection in humans. However, the abovementioned chemokines and inflammatory cytokines found to be regulated in porcine models have likewise been found to be highly expressed in human lung tissue samples of fatal cases, caused by the 2009 H1N1 or avian H5N1.[113,115]

The major hallmark of innate antiviral immunity is the IFN response. Accordingly, transcriptional studies of the porcine respiratory system after IAV infection consistently report the induction of IFNs and ISGs (Table 4) such as ISG15, PKR (*EIF2AK2*), MX1, OAS1, OASL, and IFITM1 and IFITM3.[61,65,117,120–122] A marked type I IFN response is commonly reported in porcine transcriptional studies of IAV-infected lung tissue with *IFNB1* gene expression being the primary constituent (Table 4)[61,65,118] while knowledge of type III IFN expression in the infected lung is sparse. Type III IFNs, or IFN-λ (Table 4), are the most recently identified family of IFNs and have been shown to induce a cellular antiviral state via ISGs that are remarkably similar to those activated by type I IFNs.[120,122,151] A study has reported on type III IFN gene expression in porcine lung tissue after IAV challenge, demonstrating a massive induction of *IL28B*, the porcine gene encoding IFN-λ3.[65] Another study has demonstrated ex vivo upregulation of *IL29* (encoding porcine IFN-λ1) in precision-cut porcine lung slices after swH3N2 infection.[120] Both studies showed that IFN-λ expression was accompanied by upregulation of *IFNB1* as well as a multitude of antiviral ISGs. In vitro investigation of the human type III IFN response to IAV has similarly demonstrated upregulation of both *IFNL1* and *IFNL2* in human alveolar type II epithelial cells after infection with human H1N1 or H3N2[135] (Table 4). Type III IFNs thus appear to be important contributors to the innate immune response in the IAV-infected lung, but additional studies are needed to elucidate the temporal dynamics of type I and III IFN expression and the interplay between these 2 important components of the antiviral immune response.

Systemic Host Response in Porcine Models of IAV Infection

In contrast to the local pulmonary response, the systemic transcriptional response to IAV infection in humans has been studied extensively, often with the aim to elucidate blood-based IAV-specific (or respiratory virus-specific) biomarker "signatures" or "classifiers."[67,152–156] Such a signature might prove a valuable diagnostic tool to determine disease etiology and intervention. Importantly, a substantial overlap of genes found to be upregulated in circulating leukocytes in pigs and humans, including the pathogen recognition receptors *DDX58* (RIG-I), *IFIH1* (MDA5), *TLR7*, *NOD1*, the ISGs *MX1*, *IFITM3*, and *OASL*, has recently been reported.[49] As such, the transcriptional systemic response to IAV infection is found to mirror pulmonary observations. IFN-α serum protein levels have been found to be elevated in pigs after swH1N1 infection,[138] mirroring our own observations of transcriptional upregulation of *IFNA1* in circulating leukocytes from swH1N2-infected pigs.[49] More comprehensive investigations of serum cytokine levels have been carried out in human patients with mild disease after pandemic H1N1 (2009) infection, with results likewise supporting our findings of transcriptional upregulation of *IFNA1*, *CXCL10*, *IL1RN*, *IL10*, and *CCL2* in the circulation of pigs infected with IAV.[49,157]

MicroRNA as a Possible Component of the Antiviral Response in Pigs and Humans

Host-encoded miRNAs are most commonly described as endogenous regulators of cellular protein translation, but several reports also describe the interaction between host-encoded miRNAs and viral RNA of the IAV.[158–161] Some DNA viruses and retroviruses encode their own miRNAs, as demonstrated by the annotated viral miRNAs included in the online miRNA repository, miRBase.[162] No influenza virus-encoded miRNAs can be found in miRbase, and the IAV genome has not been shown to contain any canonical miRNAs. miRNAs are evolutionarily highly conserved across species, making it likely that the study of host miRNA in the pig model will have great translational value for induction and function of miRNAs in settings of human disease.

Host-encoded miRNA regulation of the innate antiviral response after IAV infection has received some attention in recent years. We recently presented results suggesting that ssc-miR-15a, ssc-miR-18a, ssc-miR-21, ssc-miR-29b, and hsa-miR-590-3p are associated with the regulation of genes involved in viral pattern recognition, apoptosis, and inflammasome function in the lungs of pigs challenged with swH1N2.[65] Several of these, as well as other miRNAs found to be differentially expressed in pig lungs after IAV challenge,

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Table 4 Overview of key features of the host pulmonary intrinsic and innate antiviral response to IAV infection

Factor/characteristic	Human		Pig		Ferret		Mouse	
	Genes/ regulation	IAV strain, experimental model	Genes/regulation	IAV strain, experimental model	Genes/ regulation	IAV strain, experimental model	Genes/ regulation	IAV strain, experimental model
Pattern recognition receptors								
TLR genes	*TLR1-TLR10*		*TLR1-TLR10*		*TLR1-TLR8*, *TLR10*		*Tlr1-Tlr9*, *Tlr11-Tlr13*	
Expression of antiviral TLRs	↑ *TLR3* – *TLR7*	huH1N1, alveolar epithelial cells (in vitro)[124]	↑ *TLR3*, *TLR7*	swH1N2, lung (in vivo)[61,65]	– *TLR3*	huH1N1pdm09, lung (in vivo)[125,126]	↑ *TLR3*	huH3N2, pulmonary epithelial cells[127]
	↑ *TLR3*, *TLR7*	huH1N1, alveolar macrophages (in vitro)[128]	↑ *TLR3*, *TLR7*	swH3N2, alveolar macrophages (in vitro)[129]			↑ *TLR3*	huH1N1, nasal epithelium (in vivo)[130]
							↑ *TLR3*	huH1N1, alveolar epithelial cells, whole lung (in vivo)[131]
							↑ *TLR3*, *TLR7*	swH1N1pdm09, huH1N1pdm09, lung (in vivo)[132]
RLR genes	*DDX58*, *IFIH1*, *DHX58*		*DDX58*, *IFIH1*, *DHX58*		*DDX58*, *IFIH1*, *DHX58*		*Ddx58*, *Ifih1*, *Dhx58*	
Expression of antiviral RLRs	↑ *DDX58*	huH1N1, alveolar epithelial cells (in vitro)[124]	↑ *DDX58*, *IFIH1*	swH1N2, lung (in vivo)[61,65]	– *DDX58*	huH1N1pdm09, lung (in vivo)[126]	↑ *Dhx58*, *Ifih1*	huH1N1, lung (in vivo)[133]
	↑ *DDX58*, *IFIH1*	huH1N1, alveolar macrophages (in vitro)[128]	↑ *DDX58*, *IFIH1*	swH3N2, alveolar macrophages (in vitro)[129]			↑ *Ddx58*, *Ifih1*	huH1N1, nasal epithelium (in vivo)[130]
							↑ *Ddx58*, *Ifih1*	huH1N1, alveolar epithelial cells, whole lung (in vivo)[131]
							↑ *Ddx58*, *Ifih1*, *Dhx58*	swH1N1pdm09, huH1N1pdm09, lung (in vivo)[132]
Type I and III interferons								
Commonly investigated type I IFN genes	*IFNA*, *IFNB*		*IFNA*, *IFNB*		*IFNA*, *IFNB*		*Ifna*, *Ifnb*	
Type I IFN expression	↑ *IFNA1*, *IFNB1*	huH1N1, alveolar macrophages (in vitro)[128]	↑ *IFNB1* – *IFNA1*	swH1N2, lung (in vivo)[61,65]	↑ *IFNA1*, *IFNB1*	huH3N2, huH1N1pdm09, upper respiratory tract (in vivo)[134]	↑ *Ifnb1*	huH1N1, alveolar epithelial cells, whole lung (in vivo)[131]

Continued

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Table 4 Continued

Factor/characteristic	Human		Pig		Ferret		Mouse	
	Genes/ regulation	IAV strain, experimental model	Genes/regulation	IAV strain, experimental model	Genes/ regulation	IAV strain, experimental model	Genes/ regulation	IAV strain, experimental model
	↑ *IFNB1*	huH1N1, alveolar epithelial cells (in vitro)[135]	↑ *IFNA1*, *IFNB1*	swH3N2, alveolar macrophages (in vitro)[129]	– *IFNA1*, *IFNB1*	huH3N2, huH1N1pdm09, lung (in vivo)[134]	↑ IFN-β	huH1N1, bronchoalveolar lavage fluid[136]
	↑ *IFNB1*	huH1N1, huH3N2, airway epithelial cells (in vitro)[137]	↑ IFN-α	swH1N1, BALF and lung (in vivo)[138]			↑ IFN-α, IFN-β	huH1N1, bronchoalveolar lavage fluid[66]
			↑ IFN-α	swH1N1, lung (in vivo)[99]				
Type III IFN genes	*IFNL1*, *IFNL2*, *IFNL3*, *IFNL4*		*IL29* (IFN-λ1), *IL28B* (IFN-λ3)		*IFNL1*, *IFNL3*		*Ifnl2*, *Ifnl3*	
Type III IFN expression	↑ *IFNL1*, *IFNL2*	huH1N1, alveolar epithelial cells (in vitro)[135]	↑ *IL28B*	swH1N2, lung (in vivo)[65]	Uncharacterized		↑ IFN-λ2/3	huH1N1, bronchoalveolar lavage fluid[66]
	↑ *IFNL1*, *IFNL2*	huH1N1, alveolar macrophages (in vitro)[128]					↑ IFN-λ2/3	huH1N1, bronchoalveolar lavage fluid[139]
	↑ *IFNL2*	huH1N1, huH3N2, airway epithelial cells (in vitro)[137]						
Interferon-stimulated genes								
Commonly investigated antiviral ISGs	*EIF2AK2*, *MX1*, *ISG15*, *RNASEL*, *OAS1*, *OASL*, *IFITM1*, *IFITM3*		*EIF2AK2*, *MX1*, *ISG15*, *RNASEL*, *OAS1*, *OASL*, *IFITM1*, *IFITM3*		*EIF2AK2*, *MX1*, *ISG15*, *RNASEL*, *OAS1*, *OASL*		*Eif2ak2*, *Mx1*, *Isg15*, *Rnasel*, (OAS1 genes: *Oas1a*†, *Oas1b*, *Oas1c*, *Oas1d*, *Oas1e*, *Oas1f*, *Oas1g*†, *Oas1h*), (OASL genes: *Oasl1*, *Oasl2*), *Ifitm1*, *Ifitm3*	
ISG expression	↑ *MX1*, *ISG15*, *OAS1*, *OASL*, *IFITM1*	huH3N2, bronchial epithelial cells (in vitro)[140]	↑ *EIF2AK2*, *MX1*, *ISG15*, *RNASEL*, *OAS1*, *OASL*, *IFITM1*, *IFITM3*	swH1N2, lung (in vivo)[65]	↑ *EIF2AK2*, *ISG15*, *OAS1*, *OASL*	huH1N1pdm09, lung (in vivo)[141]	↑ *Mx1*, *Rnasel*, *Isg15*, *Oas1a*, *Oas1g*	swH1N1pdm09, huH1N1pdm09, lung (in vivo)[132]
	↑ *MX1*, *IFITM1*	huH1N1, alveolar macrophages (in vitro)[128]			↑ *EIF2AK2*, *ISG15*, *OAS1*	huH1N1, lung (in vivo)[141]	↑ *Mx1*, *Isg15*, *Oas1a*, *Oas1f*, *Oasl1*	huH1N1pdm09, lung (in vivo)[142]
Cytokines and chemokines								
Commonly investigated cytokines and chemokines	*IL1B*, *IL6*, *IL10*, *IL18*, *TNF* *CXCL8* (IL-8), *CXCL10*, *CCL2*		*IL1B*, *IL6*, *IL10*, *IL18*, *TNF* *CXCL8* (IL-8), *CXCL10*, *CCL2*		*IL1B*, *IL6*, *IL10*, *IL18*, *TNF* *CXCL8* (IL-8), *CXCL10*, *CCL2*		*Il1b*, *Il6*, *Il10*, *Il18*, *Tnf* *Cxcl10*, *Ccl2* (MCP-1)	
Cytokine and chemokine expression	↑ IL-6, IL-8, CCL2	huH1N1, alveolar epithelial cells (in vitro)[135]	↑ *IL1B*, *IL6*, *IL10*, *CCL2*	swH1N2, lung (in vivo)[61,65]	↑ *IL1B*, *TNF*, *CCL2*	huH1N1pdm09, lung (in vivo)[141]	↑ *Il1b*, *Il6*, *Tnf*	huH1N1pdm09, lung (in vivo)[142]
	↑ *IL6*, *TNF*	huH1N1, pharyngeal epithelial cells (in vitro)[143]	↓ *IL18*	swH1N2, lung (in vivo)[65]	↑ *IL6*, *TNF*, *CXCL8*, *CCL2*	huH1N1pdm09, lung (in vivo)[126]	↑ Il-1β, Il-6, Tnf	huH1N1, lung (in vivo)[144]

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	↑ *IL6*, *CXCL8*	huH1N1pdm09, bronchial epithelial cells (in vitro)[145]	– *CXCL8*	swH1N2, lung (in vivo)[61,65]	↑ *CXCL8*	huH1N1, huH3N2, nasal wash (in vivo)[146]	↑ Il-1β, Il-6, Il-18, TNF, MCP-1	huH1N1pdm09, lung (in vivo)[147]
	↑ IL-6, IL-8	huH1N1pdm09, bronchial epithelial cells (in vitro)[145]	↑ IL-1, IL-6, TNF	swH1N1, BALF and lung (in vivo)[138]				
CXCL10 as dominating pulmonary chemokine	↑ CXCL10	huH1N1pdm09, lung (in vivo, fatal cases)[113]	↑ *CXCL10*	swH1N2, lung (in vivo)[61,65]	↑ *CXCL10*	huH1N1pdm09, lung (in vivo)[148]	↑ *Cxcl10*	huH1N1, alveolar epithelial cells, whole lung (in vivo)[131]
	↑ CXCL10	huH1N1, alveolar macrophages (in vitro)[128]			↑ *CXCL10*	huH1N1pdm09, lung (in vivo)[149]		
miRNAs								
Number of annotated mature miRNA (miRBase v. 21)	2,588		411		None		1,915	

↑ indicates upregulation/high levels of gene expression under the examined condition; ↓ indicates downregulation/low levels of gene expression under the examined condition; —indicates unchanged levels of gene expression under the examined condition. Italics denotes studies of gene transcription; regular font denotes studies of protein levels. 'hu' and 'sw' prefixes indicate IAV strains of human and swine origin, respectively. † Oas1a and Oas1g are the only mouse OAS1 paralogs that possess enzymatic activity.[150]

have likewise been found to be regulated in human lung epithelial A549 cells after H1N1 infection.[163] However, other studies of IAV-infected A549 cells demonstrated little or no overlap of the differentially expressed miRNAs found in pig lungs after IAV challenge.[65,164,165] This calls for caution when interpreting miRNA expression results; their highly complex regulation likely causes their expression to be very sensitive to different experimental setups.

Recently, porcine ssc-miR-204 and ssc-miR-4331 were demonstrated to target HA and NS encoding gene segments of IAV (swH1N1) and inhibit viral replication in trachea cells isolated from newborn pigs.[161] ssc-miR-4331 has furthermore been shown to be upregulated in vivo in porcine pulmonary alveolar macrophages[166] as well as in total lung tissue of IAV-vaccinated pigs after swH1N2 challenge (Brogaard et al., unpublished work). However, host miRNA-IAV RNA interactions identified for one IAV strain are not necessarily applicable to other strains or subtypes due to the high mutation rate and risk of losing a miRNA binding site through antigenic drift. It would be of great interest to generate a "consensus genome" from a selection of IAVs of interest and identify host miRNA binding sites in the highly conserved portion of the IAV genome. Such an approach would help determine if host miRNA-IAV RNA interaction is a defining factor for virulence, transmissibility, or host range.

Systemic miRNAs are frequently ascribed great potential as biomarkers for various conditions due to their stability and availability in circulation.[167] As such, systemic miRNA expression after IAV infection has received some attention in pigs[49] as well as humans,[168–170] and importantly, around 70% of the miRNAs found to be regulated in porcine leukocytes after experimental IAV infection have likewise been reported to show altered expression in circulation of human patients after IAV infection.[49] So not only does the pig display a local antiviral immune response at the sites of IAV infection that parallels the corresponding responses in the human host, but there is also a substantial overlap in the systemic transcriptional response of protein coding and noncoding genes.

Validity of Porcine Models

The validity of animal models can be divided into face, target, and predictive validities.[171,172] In influenza research, face validity specifies how well the animal model mirrors human clinical condition and symptoms after IAV infection. Target validity concerns the similarity and homology of, for example, a specific signaling pathway or a protein critically important for the establishment and/or propagation of IAV infection in humans as compared with the animal model. Predictive validity refers to how accurately an animal model reflects the pharmacological effects of an antiviral drug or vaccine or other treatments in the human host.[173] Animal models with high face validity for studies of IAV pathogenesis and the involvement of host immunity, as well as models with high predictive validity for testing of vaccines and antiviral therapies, must be prioritized.[173,174] Mouse models for human IAV infection have low face validity due to the low similarity of clinical manifestations between human and mouse. Pig models, however, have high face validity due to the high similarity of clinical manifestations in humans and pigs such as fever, increased nasal secretion, and cough and similar clinical signs in pig breeds of different sizes have been observed.[52] However, a high face validity does not necessarily ensure high target validity, that is, that the underlying pathogenic mechanisms of the disease are

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similar. In the case of IAV infection, the innate immune mechanisms directed against the viral infection plays a central role in shaping the pathogenesis of the infection and need to be taken into account when assessing the optimal target validity compared to the human counterpart. This in turn ensures high predictive validity. As described above, genome and transcriptome comparison of immune- and inflammation-related gene families and protein sequences, including orthologous and non-orthologous proteins with antiviral properties, strongly supports the use of the pig as an animal model with optimal target validity for human IAV infection and disease.

Conclusion

Several factors warrant caution when applying the mouse as a model for human IAV infection, including its low face validity, the high number of unique (nonorthologous) murine genes compared with the human genome, the low murine antiviral protein sequence similarity to human proteins, and the need for adapted IAV strains. Pigs play an important role in cross-species transmission and epidemiology and are readily susceptible to infection with human IAV strains without the need for viral adaptation. Pigs and ferrets display higher sequence similarity to humans than mice with respect to a wide range of antiviral proteins. Upon infection with IAV, these 2 species present clinical signs and transcriptional immune responses, which closely mirror corresponding human responses. Using the pig as a model for IAV infections allows for the investigation of the local pulmonary immune response at the site of viral infection; this sample material is of great importance for the elucidation of pulmonary innate and adaptive antiviral immunity towards respiratory infections,[175,176] however not practically accessible in humans. Both ferret and pig models of IAV infection have extensive face and target validity; however, in-depth knowledge of the ferret innate and adaptive immune responses to IAV infections is currently limited weakening the proven target validity of ferret-based models. Last but not least, the pig is a suitable model for the study of IAV infection and impaired vaccine response in settings of underlying pathologies involving the innate immune system such as low-grade inflammation associated with obesity and aging, as this state is well characterized in the pig.[177–181] The pig is of great translational value in IAV research, and it will continue to provide essential insights into this important infection.

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Supplementary Material

Supplementary material is available at *Institute for Laboratory Animal Research Journal* online.

References

1. WHO | Influenza (Seasonal). WHO. 2018. http://www.who.int/mediacentre/factsheets/fs211/en/. Accessed February 14, 2018.
2. Thompson WW, Shay DK, Weintraub E, Brammer L, Cox N, Anderson LJ. Mortality associated with influenza and respiratory syncytial virus in the United States. *J Am Med Assoc*. 2003;289(2):179–186.
3. McElhaney JE. The unmet need in the elderly: designing new influenza vaccines for older adults. *Vaccine*. 2005;23(1):10–25. doi:10.1016/j.vaccine.2005.04.019.
4. Anar C, Bicmen C, Yapicioglu S, Unsal I, Halilcolar H, Yilmaz U. Evaluation of clinical data and antibody response following influenza vaccination in patients with chronic obstructive pulmonary disease. *New Microbiol*. 2010;33(2):117–127. http://www.ncbi.nlm.nih.gov/pubmed/20518273.
5. Glezen PW, Greenberg SB, Atmar RL, Pedro PA, Couch RB. Impact of respiratory virus infections on persons with chronic conditions. *JAMA*. 2000;283(4):499–505. doi:10.1001/jama.1996.03540180029029.
6. World Health Organization. (2015). A manual for estimating disease burden associated with seasonal influenza. World Health Organization. http://www.who.int/iris/handle/10665/178801.
7. To KKW, Hung IFN, Li IWS, et al. Delayed clearance of viral load and marked cytokine activation in severe cases of pandemic H1N1 2009 influenza virus infection. *Clin Infect Dis*. 2010;50(6):850–859. doi:10.1086/650581.
8. Meschi S, Selleri M, Lalle E, et al. Duration of viral shedding in hospitalized patients infected with pandemic H1N1. *BMC Infect Dis*. 2011;11(1):140. doi:10.1186/1471-2334-11-140.
9. Rajao DS, Loving CL, Waide EH, et al. Pigs with severe combined immunodeficiency are impaired in controlling influenza A virus infection. *J Innate Immun*. 2017;9(2):193–202. doi:10.1159/000451007.
10. van der Vries E, Stittelaar KJ, van Amerongen G, et al. Prolonged influenza virus shedding and emergence of antiviral resistance in immunocompromised patients and ferrets. *PLoS Pathog*. 2013;9(5). doi:10.1371/journal.ppat.1003343.
11. Lang P, Govind S, Bokum A, et al. Immune senescence and vaccination in the elderly. *Curr Top Med Chem*. 2013;13(20):2541–2550. doi:10.2174/15680266113136660181.
12. Tagliabue C, Principi N, Giavoli C, Esposito S. Obesity: impact of infections and response to vaccines. *Eur J Clin Microbiol Infect Dis*. 2016;35(3):325–331. doi:10.1007/s10096-015-2558-8.
13. Prachanronarong KL, Özen A, Thayer KM, et al. Molecular basis for differential patterns of drug resistance in influenza N1 and N2 neuraminidase. *J Chem Theory Comput*. 2016;12(12):6098–6108. doi:10.1021/acs.jctc.6b00703.
14. Jiang L, Liu P, Bank C, et al. A balance between inhibitor binding and substrate processing confers influenza drug resistance. *J Mol Biol*. 2016;428(3):538–553. doi:10.1016/j.jmb.2015.11.027.
15. Heider H, Adamczyk B, Presber HW, Schroeder C, Feldblum R, Indulen MK. Occurrence of amantadine- and rimantadine-resistant influenza. A virus strains during the 1980 epidemic. *Acta Virol*. 1981;25(6):395–400.
16. Shi Y, Wu Y, Zhang W, Qi J, Gao GF. Enabling the "host jump": structural determinants of receptor-binding specificity in influenza A viruses. *Nat Rev Microbiol*. 2014;12(12):822–831. doi:10.1038/nrmicro3362.
17. Shinya K, Ebina M, Yamada S, Ono M, Kasai N, Kawaoka Y. Avian flu: Influenza virus receptors in the human airway. *Nature*. 2006;440(7083):435–436. doi:10.1038/440435a.
18. Król E, Rychłowska M, Szewczyk B. Antivirals—current trends in fighting influenza. *Acta Biochim Pol*. 2014;61(3):495–504.
19. Thangavel RR, Bouvier NM. Animal models for influenza virus pathogenesis, transmission, and immunology. *J Immunol Methods*. 2014;410:60–79. doi:10.1016/j.jim.2014.03.023.
20. Margine I, Krammer F. Animal models for influenza viruses: implications for universal vaccine development. *Pathogens*. 2014;3(4):845–874. doi:10.3390/pathogens3040845.

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21. Rajao DS, Vincent AL. Swine as a model for influenza A virus infection and immunity. *ILAR J*. 2015;56(1):44–52. doi:10.1093/ilar/ilv002.
22. Bouvier NM, Lowen AC. Animal models for influenza virus pathogenesis and transmission. *Viruses*. 2010;2(8): 1530–1563. doi:10.3390/v20801530.
23. Kaplan MM, Payne AM-M. Serological survey in animals for type A influenza in relation to the 1957 pandemic. *Bull World Health Organ*. 1959;20(2–3):465–488.
24. Smith W, Andrewes CH, Laidlaw PP, Timbury MC. A virus obtained from influenza patients. *Lancet*. 1933;222:66–68. doi:10.1002/rmv.1980050402.
25. Radigan KA, Misharin AV, Chi M, Budinger GS. Modeling human influenza infection in the laboratory. *Infect Drug Resist*. 2015;8:311–320. doi:10.2147/IDR.S58551.
26. Monto AS, Gravenstein S, Elliott M, Colopy M, Schweinle J. Clinical signs and symptoms predicting influenza infection. *Arch Intern Med*. 2000;160:3243–3247.
27. Carrat F, Tachet A, Rouzioux C, Housset B, Valleron AJ. Evaluation of clinical case definitions of influenza: detailed investigation of patients during the 1995–1996 epidemic in France. *Clin Infect Dis*. 1999;28(2):283–290. doi:10.1086/515117.
28. Haesebrouck F, Pernsaert MB. Effect of intratracheal challenge of fattening pigs previously immunised with an activated influenza H1N1 vaccine. *Vet Microbiol*. 1986;11:239–249.
29. Dybing JK, Schultz-Cherry S, Swayne DE, Suarez DL, Perdue ML. Distinct pathogenesis of hong kong-origin H5N1 viruses in mice compared to that of other highly pathogenic H5 avian influenza viruses. *J Virol*. 2000;74(3):1443–1450. doi:10.1128/JVI.74.3.1443-1450.2000.
30. Zambon M, Potter CW. Influenza. In: Zuckerman AJ, Banatvala JE, Schoub BD, Griffiths PD, Mortimer P, eds. *Principles and Practice of Clinical Virology*. 6th ed. Chichester, UK:John Wiley & Sons, Ltd; 2009:373–408.
31. Majde JA, Bohnet SG, Ellis GA, et al. Detection of mouse-adapted human influenza virus in the olfactory bulbs of mice within hours after intranasal infection. *J Neurovirol*. 2007;13(5):399–409. doi:10.1080/13550280701427069.
32. Ohmit SE, Monto AS. Symptomatic predictors of influenza virus positivity in children during the influenza season. *Clin Infect Dis*. 2006;43(5):564–568. doi:10.1086/506352.
33. Yang YT, Evans CA. Hypothermia in mice due to influenza virus infection. *Proc Soc Exp Biol Med*. 1961;108(3):776–780.
34. Squires SL. The evaluation of compounds against influenza viruses. *Ann NY Acad Sci*. 1970;173:239–248.
35. Edenborough KM, Gilbertson BP, Brown LE. A mouse model for the study of contact-dependent transmission of influenza A virus and the factors that govern transmissibility. *J Virol*. 2012;86(23):12544–12551. doi:10.1128/JVI.00859-12.
36. Pabst R. Das lymphatische Gewebe der Nase (NALT) und des Kehlkopfes (LALT) im Speziesvergleich: Mensch, Ratte, Maus. *Pneumologie*. 2010;64(7):445–446. doi:10.1055/s-0030-1255509.
37. Evans H, An NQ. Anatomy of the ferret. In: Fox JG, Robert MP, eds. *Biology and Disease of the Ferret*. 3rd ed. Ames, Iowa, US: John Wiley & Sons, Inc.; 2014:23–67.
38. Williams DM, Rowland AC. The palatine tonsils of the pig—an afferent route to the lymphoid tissue. *J Anat*. 1972;113(1): 131–137.
39. Casteleyn C, Breugelmans S, Simoens P, Van Den Broeck W. The tonsils revisited: review of the anatomical localization and histological characteristics of the tonsils of domestic and laboratory animals. *Clin Dev Immunol*. 2011;2011:1–14. doi:10.1155/2011/472460.
40. Hyde DM, Samuelson DA, Blakeney WH, Kosch PC. A correlative light microscopy, transmission and scanning electron microscopy study of the ferret lung. *Scan Electron Microsc*. 1979;(3):891–898.
41. McLaughlin RF, Tyler WS, Canada RO. A study of the subgross pulmonary anatomy in various mammals. *Am J Anat*. 1961;108(2):149–165. doi:10.1002/aja.1001080203.
42. Tyler WS. Comparative subgross anatomy of lungs—pleuras, interlobular septa, and distal airways. *Am Rev Respir Dis*. 1983;128(2):S32–S36.
43. Peake JL, Pinkerton KE. Gross and subgross anatomy of lungs, pleura, connective tissue septa, distal airways, and structural units. In: *Parent RA, eds. Comparative Biology of the Normal Lung*. 2nd ed. Boston, MA: Elsevier Inc.; 2015:21–31. doi:10.1016/B978-0-12-404577-4.00003-5.
44. Holt PG. Alveolar macrophages. *Cell Immunol*. 1980;50:210–215.
45. Kim HM, Lee Y-W, Lee K-J, et al. Alveolar macrophages are indispensable for controlling influenza viruses in lungs of pigs. *J Virol*. 2008;82(9):4265–4274. doi:10.1128/JVI.02602-07.
46. Kim HM, Kang YM, Ku KB, et al. The severe pathogenicity of alveolar macrophage-depleted ferrets infected with 2009 pandemic H1N1 influenza virus. *Virology*. 2013;444(1–2): 394–403. doi:10.1016/j.virol.2013.07.006.
47. Schneberger D, Haronson-Raz K, Singh B. Pulmonary intravascular macrophages and lung health: what are we missing? *Am J Physiol Lung Cell Mol Physiol*. 2012;302(6):L498–L503.
48. Longworth KE. The comparative biology of pulmonary intravascular macrophages. *Front Biosci*. 1997;2:d232–d241.
49. Brogaard L, Heegaard PMH, Larsen LE, et al. Late regulation of immune genes and microRNAs in circulating leukocytes in a pig model of influenza A (H1N2) infection. *Sci Rep*. 2016; 6:21812. doi:10.1038/srep21812.
50. Kamal RP, Katz JM, York IA. Molecular determinants of influenza virus pathogenesis in mice. *Curr Top Microbiol Immunol*. 2014;385:243–274. doi:10.1007/82_2014_388.
51. Matsuoka Y, Swayne DE, Thomas C, et al. Neuraminidase stalk length and additional glycosylation of the hemagglutinin influence the virulence of influenza H5N1 viruses for mice. *J Virol*. 2009;83(9):4704–4708. doi:10.1128/JVI.01987-08.
52. Iwatsuki-horimoto K, Nakajima N, Shibata M, et al. The microminipig as an animal model for influenza A virus infection. *J Virol*. 2017;91(2):1–9.
53. Bouvier NM. Animal models for influenza virus transmission studies: a historical perspective. *Curr Opin Virol*. 2015; 13:101–108. doi:10.1016/j.coviro.2015.06.002.
54. Redl H, Bahrami S, Schlag G, Traber DL. Clinical detection of LPS and animal models of endotoxemia. *Immunobiology*. 1993;187(3–5):330–345. doi:10.1016/S0171-2985(11)80348-7.
55. Fink MP. Animal models of sepsis. *Virulence*. 2014;5(1): 143–153. doi:10.4161/viru.26083.
56. Kuss H, Hoefgen N, Johanssen S, Kronbach T, Rundfeldt C. In vivo efficacy in airway disease models of N- (3, 5-Dichloro-Acid Amide (AWD 12-281), a selective phosphodiesterase 4 inhibitor for inhaled administration. *Pharmacology*. 2003;307 (1):373–385. doi:10.1124/jpet.103.053942.Emesis.
57. Nials AT, Tralau-Stewart CJ, Gascoigne MH, Ball DI, Ranshaw LE, Knowles RG. In vivo characterization of GSK256066, a high-affinity inhaled phosphodiesterase 4 inhibitor. *J Pharmacol Exp Ther*. 2011;337(1):137–144. doi:10.1124/jpet.110.173641.
58. Kanoh S, Tanabe T, Rubin BK. Dapsone inhibits IL-8 secretion from human bronchial epithelial cells stimulated with lipopolysaccharide and resolves airway inflammation in the ferret. *Chest*. 2011;140(4):980–990. doi:10.1378/chest.10-2908.

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59. Jakeman KJ, Rushton DI, Smith H, Sweet C. Exacerbation of bacterial toxicity to infant ferrets by influenza virus: possible role in sudden infant death syndrome. *J Infect Dis*. 1991; 163(1):35–40.
60. Pomorska-Mól M, Markowska-Daniel I, Kwit K. Immune and acute phase response in pigs experimentally infected with H1N2 swine influenza virus. *FEMS Immunol Med Microbiol*. 2012;66(3):334–342. doi:10.1111/j.1574-695x.2012.01026.x.
61. Skovgaard K, Cirera S, Vasby D, et al. Expression of innate immune genes, proteins and microRNAs in lung tissue of pigs infected experimentally with influenza virus (H1N2). *Innate Immun*. 2013;19(5):531–544. doi:10.1177/1753425912473668.
62. Pommerenke C, Wilk E, Srivastava B, et al. Global transcriptome analysis in influenza-infected mouse lungs reveals the kinetics of innate and adaptive host immune responses. *PLoS One*. 2012;7(7). doi:10.1371/journal.pone.0041169.
63. Crisci E, Mussá T, Fraile L, Montoya M. Review: influenza virus in pigs. *Mol Immunol*. 2013;55(3–4):200–211. doi:10.1016/j.molimm.2013.02.008.
64. Larsen DL, Karasin A, Zuckermann F, Olsen CW. Systemic and mucosal immune responses to H1N1 influenza virus infection in pigs. *Vet Microbiol*. 2000;74(1–2):117–131. doi:10.1016/S0378-1135(00)00172-3.
65. Brogaard L, Larsen LE, Heegaard PM, et al. IFN-λ and microRNAs are important modulators of the pulmonary innate immune response against influenza A (H1N2) infection in pigs. *PLoS One*. 2018;13(4):e0194765. doi:10.1371/journal.pone.0194765.
66. Galani IE, Triantafyllia V, Eleminiadou E-E, et al. Interferon-λ mediates non-redundant front-line antiviral protection against influenza virus infection without compromising host fitness. *Immunity*. 2017;46(5):875–890. doi:10.1016/j.immuni.2017.04.025.
67. Zhai Y, Franco LM, Atmar RL, et al. Host transcriptional response to influenza and other acute respiratory viral infections—a prospective cohort study. *PLoS Pathog*. 2015;11(6):1–29. doi:10.1371/journal.ppat.1004869.
68. Carolan LA, Rockman S, Borg K, et al. Characterization of the localized immune response in the respiratory tract of ferrets following infection with influenza A and B viruses. *J Virol*. 2015;90(6):2838–2848. doi:10.1128/JVI.02797-15.
69. Pociask DA, Robinson KM, Chen K, et al. Epigenetic and transcriptomic regulation of lung repair during recovery from influenza infection. *Am J Pathol*. 2017;187(4):851–863. doi:10.1016/j.ajpath.2016.12.012.
70. Forberg H, Hauge AG, Valheim M, et al. Early responses of natural killer cells in pigs experimentally infected with 2009 pandemic H1N1 influenza a virus. *PLoS One*. 2014;9(6). doi:10.1371/journal.pone.0100619.
71. Jeffery PK. Morphologic features of airway surface epithelial cells and glands. *Am Rev Respir Dis*. 1983;128(60):S14–S20.
72. Spicer SS, Schulte BA, Thomopoulos GN. Histochemical properties of the respiratory tract epithelium in different species. *Am Rev Respir Dis*. 1983;128(2):S20–S26.
73. Pavelka M. Organ culture of adult rat and mouse tracheal epithelium: I. Ultrastructure following various culture periods. *Cell Tissue Res*. 1976;165(3). doi:10.1007/BF00222440.
74. Leigh MW, Cheng PW, Boat TF. Developmental changes of ferret tracheal mucin composition and biosynthesis. *Biochemistry*. 1989;28(24):9440–9446. doi:10.1021/bi00450a029.
75. Pack RJ, Al-Ugaily LH, Morris G, Widdicombe JG. The distribution and structure of cells in the tracheal epithelium of the mouse. *Cell Tissue Res*. 1980;208(1):65–84. doi:10.1007/BF00234174.
76. Korhonen LK, Holopainen E, Paavolainen M. Some histochemical characteristics of tracheobronchial tree and pulmonary neoplams. *Acta Histochem*. 1969;32(1):57–73.
77. Ganesan S, Comstock AT, Sajjan US. Barrier function of airway tract epithelium. *Tissue Barriers*. 2013;1(4):e24997. doi:10.4161/tisb.24997.
78. Dubin RF, Robinson SK, Widdicombe JH, et al. Secretion of lactoferrin and lysozyme by cultures of human airway epithelium secretion of lactoferrin and lysozyme by cultures of human airway epithelium. *Am J Physiol Lung Cell Mol Physiol*. 2004;8664(4):750–755. doi:10.1152/ajplung.00326.2003.
79. Ermund A, Meiss LN, Rodriguez-Pineiro AM, et al. The normal trachea is cleaned by MUC5B mucin bundles from the submucosal glands coated with the MUC5AC mucin. *Biochem Biophys Res Commun*. 2017;492(3):331–337. doi:10.1016/j.bbrc.2017.08.113.
80. Cohen M, Zhang X-Q, Senaati HP, et al. Influenza A penetrates host mucus by cleaving sialic acids with neuraminidase. *Virol J*. 2013;10(1):321. doi:10.1186/1743-422x-10-321.
81. Yang X, Steukers L, Forier K, et al. A beneficiary role for neuraminidase in influenza virus penetration through the respiratory mucus. *PLoS One*. 2014;9(10):1–11. doi:10.1371/journal.pone.0110026.
82. Zhang L, Button B, Gabriel SE, et al. CFTR delivery to 25% of surface epithelial cells restores normal rates of mucus transport to human cystic fibrosis airway epithelium. *PLoS Biol*. 2009;7(7). doi:10.1371/journal.pbio.1000155.
83. Wallace P, Kennedy JR, Mendicino J. Transdifferentiation of outgrowth cells and cultured epithelial cells from swine trachea. *In Vitr Cell Dev Biol Anim*. 1994;30A(3):168–180.
84. Leigh MW, Gambling TM, Carson JL, Collier AM, Wood RE, Boat TF. Postnatal development of tracheal surface epithelium and submucosal glands in the ferret. *Exp Lung Res*. 1986;10(2):153–169. doi:10.3109/01902148609061490.
85. Hillaire MLB, van Eijk M, van Trierum SE, et al. Assessment of the antiviral properties of recombinant porcine SP-D against various influenza A viruses in vitro. *PLoS One*. 2011;6(9). doi:10.1371/journal.pone.0025005.
86. Reading PC, Morey LS, Crouch EC. Collectin-mediated antiviral host defense of the lung: evidence from influenza virus infection of mice. *J Virol*. 1997;71(11):8204–8212.
87. Job ER, Deng Y-M, Tate MD, et al. Pandemic H1N1 influenza A viruses are resistant to the antiviral activities of innate immune proteins of the collectin and pentraxin superfamilies. *J Immunol*. 2010;185(7):4284–4291. doi:10.4049/jimmunol.1001613.
88. Job ER, Pizzolla A, Nebl T, et al. Neutralizing inhibitors in the airways of naïve ferrets do not play a major role in modulating the virulence of H3 subtype influenza A viruses. *Virology*. 2016;494:143–157. doi:10.1016/j.virol.2016.01.024.
89. Ning ZY, Luo MY, Qi WB, Yu B, Jiao PR, Liao M. Detection of expression of influenza virus receptors in tissues of BALB/c mice by histochemistry. *Vet Res Commun*. 2009;33:895–903. doi:10.1007/s11259-009-9307-3.
90. Kirkeby S, Martel CJM, Aasted B. Infection with human H1N1 influenza virus affects the expression of sialic acids of metaplastic mucous cells in the ferret airways. *Virus Res*. 2009;144(1–2):225–232. doi:10.1016/j.virusres.2009.05.004.
91. Xu Q, Wang W, Cheng X, Zengel J, Jin H. Influenza H1N1 A/Solomon Island/3/06 virus receptor binding specificity correlates with virus pathogenicity, antigenicity, and immunogenicity in ferrets. *J Virol*. 2010;84(10):4936–4945. doi:10.1128/JVI.02489-09.
92. Trebbien R, Larsen LE, Viuff BM. Distribution of sialic acid receptors and influenza A virus of avian and swine origin in

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experimentally infected pigs. *Virol J*. 2011;8(1):434. doi:10.1186/1743-422x-8-434.
93. Nelli RK, Kuchipudi SV, White GA, Perez BB, Dunham SP, Chang K-C. Comparative distribution of human and avian type sialic acid influenza receptors in the pig. *BMC Vet Res*. 2010;6:4. doi:10.1186/1746-6148-6-4.
94. Lander ES, Linton LM, Birren B, et al. Initial sequencing and analysis of the human genome. *Nature*. 2001;409(6822):860–921. doi:10.1038/35057062.
95. Venter JC, Adams MDD, Myers EWW, et al. The sequence of the human genome. *Science* 2001;291(5507):1304–1351. doi:10.1126/science.1058040.
96. Waterston RH, Lindblad-Toh K, Birney E, et al. Initial sequencing and comparative analysis of the mouse genome. *Nature*. 2002;420(6915):520–562. doi:10.1038/nature01262.
97. Peng X, Alföldi J, Gori K, et al. The draft genome sequence of the ferret (Mustela putorius furo) facilitates study of human respiratory disease. *Nat Biotechnol*. 2014;32(12):1250–1255. doi:10.1038/nbt.3079.
98. Dawson HD, Chen C, Gaynor B, Shao J, Urban JF. The porcine translational research database: a manually curated, genomics and proteomics-based research resource. *BMC Genomics*. 2017;18(1):643. doi:10.1186/s12864-017-4009-7.
99. Khatri M, Dwivedi V, Krakowka S, et al. Swine influenza H1N1 virus induces acute inflammatory immune responses in pig lungs: a potential animal model for human H1N1 influenza virus. *J Virol*. 2010;84(21):11210–11218. doi:10.1128/JVI.01211-10.
100. Zhou A, Hassel BA, Silverman RH. Expression cloning of 2-5A-dependent RNAase: a uniquely regulated mediator of interferon action. *Cell*. 1993;72(5):753–765. doi:10.1016/0092-8674(93)90403-D.
101. Silverman RH. Viral encounters with 2′,5′-oligoadenylate synthetase and RNase L during the interferon antiviral response. *J Virol*. 2007;81(23):12720–12729. doi:10.1128/JVI.01471-07.
102. Li Y, Banerjee S, Wang Y, et al. Activation of RNase L is dependent on OAS3 expression during infection with diverse human viruses. *Proc Natl Acad Sci USA*. 2016;113(8):2241–2246. doi:10.1073/pnas.1519657113.
103. Perelygin AA, Zharkikh AA, Scherbik SV, Brinton MA. The mammalian 2′-5′ oligoadenylate synthetase gene family: evidence for concerted evolution of paralogous Oas1 genes in Rodentia and Artiodactyla. *J Mol Evol*. 2006;63(4):562–576. doi:10.1007/s00239-006-0073-3.
104. Pinto AK, Williams GD, Szretter KJ, et al. Human and murine IFIT1 proteins do not restrict infection of negative-sense RNA viruses of the Orthomyxoviridae, Bunyaviridae, and Filoviridae families. *J Virol*. 2015;89(18):9465–9476. doi:10.1128/JVI.00996-15.
105. Li Y, Wen Z, Zhou H, et al. Porcine interferon-induced protein with tetratricopeptide repeats 3, poIFIT3, inhibits swine influenza virus replication and potentiates IFN-β production. *Dev Comp Immunol*. 2015;50(1):49–57. doi:10.1016/j.dci.2014.10.008.
106. Bailey CC, Zhong G, Huang I-C, Farzan M. IFITM-family proteins: the cell's first line of antiviral defense. *Annu Rev Virol*. 2014;1(1):261–283. doi:10.1146/annurev-virology-031413-085537.
107. Miller LC, Jiang Z, Sang Y, Harhay GP, Lager KM. Evolutionary characterization of pig interferon-inducible transmembrane gene family and member expression dynamics in tracheobronchial lymph nodes of pigs infected with swine respiratory disease viruses. *Vet Immunol Immunopathol*. 2014;159(3–4):180–191. doi:10.1016/j.vetimm.2014.02.015.
108. Arnon TI, Lev M, Katz G, Chernobrov Y, Porgador A, Mandelboim O. Recognition of viral hemagglutinins by NKp44 but not by NKp30. *Eur J Immunol*. 2001;31(9):2680–2689. doi:10.1002/1521-4141(200109)31:9<2680::AID-IMMU2680>3.0.CO;2-A.
109. Mandelboim O, Lieberman N, Lev M, et al. Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. *Nature*. 2001;409(6823):1055–1060. doi:10.1038/35059110.
110. Hollyoake M, Campbell RD, Aguado B. NKp30 (NCR3) is a pseudogene in 12 inbred and wild mouse strains, but an expressed gene in Mus caroli. *Mol Biol Evol*. 2005;22(8):1661–1672. doi:10.1093/molbev/msi162.
111. Mair KH, Stadler M, Talker SC, et al. Porcine CD3+NKp46+ lymphocytes have NK-cell characteristics and are present in increased frequencies in the lungs of influenza-infected animals. *Front Immunol*. 2016;7:1–17. doi:10.3389/fimmu.2016.00263.
112. Dawson HD, Smith AD, Chen C, Urban JF. An in-depth comparison of the porcine, murine and human inflammasomes; lessons from the porcine genome and transcriptome. *Vet Microbiol*. 2017;202:2–15. doi:10.1016/j.vetmic.2016.05.013.
113. Gao R, Bhatnagar J, Blau DM, et al. Cytokine and chemokine profiles in lung tissues from fatal cases of 2009 pandemic influenza A (H1N1): role of the host immune response in pathogenesis. *Am J Pathol*. 2013;183(4):1258–1268. doi:10.1016/j.ajpath.2013.06.023.
114. Mauad T, Hajjar LA, Callegari GD, et al. Lung pathology in fatal novel human influenza A (H1N1) infection. *Am J Respir Crit Care Med*. 2010;181(1):72–79. doi:10.1164/rccm.200909-1420OC.
115. Nakajima N, Van Tin N, Sato Y, et al. Pathological study of archival lung tissues from five fatal cases of avian H5N1 influenza in Vietnam. *Mod Pathol*. 2013;26(3):357–369. doi:10.1038/modpathol.2012.193.
116. Kongchanagul A, Suptawiwat O, Boonarkart C, et al. Decreased expression of surfactant protein D mRNA in human lungs in fatal cases of H5N1 avian influenza. *J Med Virol*. 2011;83(8):1410–1417. doi:10.1002/jmv.22105.
117. Li Y, Zhou H, Wen Z, et al. Transcription analysis on response of swine lung to H1N1 swine influenza virus. *BMC Genomics*. 2011;12(1):398. doi:10.1186/1471-2164-12-398.
118. Ma W, Belisle SE, Mosier D, et al. 2009 pandemic H1N1 influenza virus causes disease and upregulation of genes related to inflammatory and immune responses, cell death, and lipid metabolism in pigs. *J Virol*. 2011;85(22):11626–11637.
119. Go JT, Belisle SE, Tchitchek N, et al. 2009 pandemic H1N1 influenza virus elicits similar clinical course but differential host transcriptional response in mouse, macaque, and swine infection models. *BMC Genomics*. 2012;13(1):627. doi:10.1186/1471-2164-13-627.
120. Delgado-Ortega M, Melo S, Punyadarsaniya D, et al. Innate immune response to a H3N2 subtype swine influenza virus in newborn porcine trachea cells, alveolar macrophages, and precision-cut lung slices. *Vet Res*. 2014;45(1):1–18. doi:10.1186/1297-9716-45-42.
121. Dobrescu I, Levast B, Lai K, et al. In vitro and ex vivo analyses of co-infections with swine influenza and porcine reproductive and respiratory syndrome viruses. *Vet Microbiol*. 2014;169(1–2):18–32. doi:10.1016/j.vetmic.2013.11.037.

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122. Krishna VD, Roach E, Zaidman NA, et al. Differential induction of type I and type III interferons by swine and human origin H1N1 influenza a viruses in porcine airway epithelial cells. *PLoS One*. 2015;10(9). doi:10.1371/journal.pone.0138704.
123. Wilkinson JM, Gunvaldsen RE, Detmer SE, et al. Transcriptomic and epigenetic profiling of the lung of influenza-infected pigs: a comparison of different birth weight and susceptibility groups. *PLoS One*. 2015;10(9):1–24. doi:10.1371/journal.pone.0138653.
124. Wu W, Zhang W, Duggan ES, Booth JL, Zou MH, Metcalf JP. RIG-I and TLR3 are both required for maximum interferon induction by influenza virus in human lung alveolar epithelial cells. *Virology*. 2015;482:181–188. doi:10.1016/j.virol.2015.03.048.
125. Vidaña B, Martínez J, Martínez-Orellana P, et al. Heterogeneous pathological outcomes after experimental pH1N1 influenza infection in ferrets correlate with viral replication and host immune responses in the lung. *Vet Res*. 2014;45(1):1–14. doi:10.1186/s13567-014-0085-8.
126. Vidaña B, Martínez J, Martorell J, et al. Involvement of the different lung compartments in the pathogenesis of pH1N1 influenza virus infection in ferrets. *Vet Res*. 2016;47(1):1–11. doi:10.1186/s13567-016-0395-0.
127. Le Goffic R, Balloy V, Lagranderie M, et al. Detrimental contribution of the Toll-like receptor (TLR)3 to influenza A virus-induced acute pneumonia. *PLoS Pathog*. 2006;2(6):0526–0535. doi:10.1371/journal.ppat.0020053.
128. Wang J, Nikrad MP, Travanty EA, et al. Innate immune response of human alveolar macrophages during influenza a infection. *PLoS One*. 2012;7(3). doi:10.1371/journal.pone.0029879.
129. Zhang J, Miao J, Hou J, Lu C. The effects of H3N2 swine influenza virus infection on TLRs and RLRs signaling pathways in porcine alveolar macrophages. *Virol J*. 2015;12(1):1–8. doi:10.1186/s12985-015-0284-6.
130. Kim HJ, Kim CH, Kim MJ, et al. The induction of pattern-recognition receptor expression against influenza a virus through Duox2-derived reactive oxygen species in nasal mucosa. *Am J Respir Cell Mol Biol*. 2015;53(4):525–535. doi:10.1165/rcmb.2014-0334OC.
131. Stegemann-Koniszewski S, Jeron A, Gereke M, et al. Alveolar type II epithelial cells contribute to the anti-influenza A virus response in the lung by integrating pathogen- and microenvironmen-derived signals. *MBio*. 2016;7(3):1–11. doi:10.1128/mBio.00276-16.
132. Zou W, Chen D, Xiong M, et al. Insights into the increasing virulence of the swine-origin pandemic H1N1/2009 influenza virus. *Sci Rep*. 2013;3:1–9. doi:10.1038/srep01601.
133. Bao Y, Gao Y, Shi Y, Cui X. Dynamic gene expression analysis in a H1N1 influenza virus mouse pneumonia model. *Virus Genes*. 2017;53(3):357–366. doi:10.1007/s11262-017-1438-y.
134. Maines TR, Belser JA, Gustin KM, et al. Local innate immune responses and influenza virus transmission and virulence in ferrets. *J Infect Dis*. 2012;205(3):474–485. doi:10.1093/infdis/jir768.
135. Wang J, Oberley-Deegan R, Wang S, et al. Differentiated human alveolar type II cells secrete antiviral IL-29 (IFN-λ1) in response to influenza A infection. *J Immunol*. 2009;182(3):1296–1304. doi:10.4049/jimmunol.182.3.1296.
136. Jewell NA, Vaghefi N, Mertz SE, et al. Differential type I interferon induction by respiratory syncytial virus and influenza A virus in vivo. *J Virol*. 2007;81(18):9790–9800. doi:10.1128/JVI.00530-07.
137. Buggele WA, Johnson KE, Horvath CM. Influenza A virus infection of human respiratory cells induces primary microRNA expression. *J Biol Chem*. 2012;287(37):31027–31040. doi:10.1074/jbc.M112.387670.
138. Barbé F, Atanasova K, Van Reeth K. Cytokines and acute phase proteins associated with acute swine influenza infection in pigs. *Vet J*. 2011;187(1):48–53. doi:10.1016/j.tvjl.2009.12.012.
139. Jewell NA, Cline T, Mertz SE, et al. Lambda interferon is the predominant interferon induced by influenza A virus infection in vivo. *J Virol*. 2010;84(21):11515–11522. doi:10.1128/JVI.01703-09.
140. Hayashi S, Jibiki I, Asai Y, et al. Analysis of gene expression in human bronchial epithelial cells upon influenza virus infection and regulation by p38 mitogen-activated protein kinase and c-Jun-N-terminal kinase. *Respirology*. 2008;13(2):203–214. doi:10.1111/j.1440-1843.2007.01204.x.
141. Rowe T, León AJ, Crevar CJ, et al. Modeling host responses in ferrets during A/California/07/2009 influenza infection. *Virology*. 2010;401(2):257–265. doi:10.1016/j.virol.2010.02.020.
142. Almansa R, Martínez-Orellana P, Rico L, et al. Pulmonary transcriptomic responses indicate a dual role of inflammation in pneumonia development and viral clearance during 2009 pandemic influenza infection. *PeerJ*. 2017;5:e3915. doi:10.7717/peerj.3915.
143. Han Y, Bo ZJ, Xu MY, Sun N, Liu DH. The protective role of TLR3 and TLR9 ligands in human pharyngeal epithelial cells infected with influenza A virus. *Korean J Physiol Pharmacol*. 2014;18(3):225–231. doi:10.4196/kjpp.2014.18.3.225.
144. Indalao IL, Sawabuchi T, Takahashi E, Kido H. IL-1β is a key cytokine that induces trypsin upregulation in the influenza virus–cytokine–trypsin cycle. *Arch Virol*. 2017;162(1):201–211. doi:10.1007/s00705-016-3093-3.
145. Paquette SG, Banner D, Chi LTB, et al. Pandemic H1N1 influenza A directly induces a robust and acute inflammatory gene signature in primary human bronchial epithelial cells downstream of membrane fusion. *Virology*. 2014;448:91–103. doi:10.1016/j.virol.2013.09.022.
146. Svitek N, Rudd PA, Obojes K, Pillet S, von Messling V. Severe seasonal influenza in ferrets correlates with reduced interferon and increased IL-6 induction. *Virology*. 2008;376(1):53–59. doi:10.1016/j.virol.2008.02.035.
147. Han Y, Ling MT, Mao H, et al. Influenza virus-induced lung inflammation was modulated by cigarette smoke exposure in mice. *PLoS One*. 2014;9(1):e86166. doi:10.1371/journal.pone.0086166.
148. Huang SSH, Banner D, Degousee N, et al. Differential pathological and immune responses in newly weaned ferrets are associated with a mild clinical outcome of pandemic 2009 H1N1 infection. *J Virol*. 2012;86(24):13187–13201. doi:10.1128/JVI.01456-12.
149. León AJ, Banner D, Xu L, et al. Sequencing, annotation, and characterization of the influenza ferret infectome. *J Virol*. 2013;87(4):1957–1966. doi:10.1128/JVI.02476-12.
150. Elkhateeb E, Tag-El-Din-Hassan HT, Sasaki N, Torigoe D, Morimatsu M, Agui T. The role of mouse 2′,5′-oligoadenylate synthetase 1 paralogs. *Infect Genet Evol*. 2016;45:393–401. doi:10.1016/j.meegid.2016.09.018.
151. Jung K, Chae C. Expression of Mx protein and interferon-α in pigs experimentally infected with swine influenza virus. *Vet Pathol*. 2006;43(2):161–167. doi:10.1354/vp.43-2-161.
152. Ramilo O, Allman W, Chung W, et al. Gene expression patterns in blood leukocytes discriminate patients with acute infections. *Blood*. 2007;109(5):2066–2077.

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153. Zaas AK, Chen M, Varkey J, et al. Gene expression signatures diagnose influenza and other symptomatic respiratory viral infections in humans. *Cell Host Microbe*. 2009;6(3):207–217.
154. Huang Y, Zaas AK, Rao A, et al. Temporal dynamics of host molecular responses differentiate symptomatic and asymptomatic influenza a infection. *PLoS Genet*. 2011;7(8):e1002234. doi:10.1371/journal.pgen.1002234.
155. Andres-Terre M, McGuire HM, Pouliot Y, et al. Integrated, multi-cohort analysis identifies conserved transcriptional signatures across multiple respiratory viruses. *Immunity*. 2015;43(6):1199–1211. doi:10.1016/j.immuni.2015.11.003.
156. Sampson DL, Fox BA, Yager TD, et al. A four-biomarker blood signature discriminates systemic inflammation due to viral infection versus other etiologies. *Sci Rep*. 2017;7(1):2914. doi:10.1038/s41598-017-02325-8.
157. Bradley-Stewart A, Jolly L, Adamson W, et al. Cytokine responses in patients with mild or severe influenza A (H1N1)pdm09. *J Clin Virol*. 2013;58(1):100–107. doi:10.1016/j.jcv.2013.05.011.
158. Song L, Liu H, Gao S, Jiang W, Huang W. Cellular microRNAs inhibit replication of the H1N1 influenza A virus in infected cells. *J Virol*. 2010;84(17):8849–8860. doi:10.1128/JVI.00456-10.
159. Khongnomnan K, Makkoch J, Poomipak W, Poovorawan Y, Payungporn S. Human miR-3145 inhibits influenza A viruses replication by targeting and silencing viral PB1 gene. *Exp Biol Med*. 2015;240:1630–1639. doi:10.1177/1535370215589051.
160. Ma Y-J, Yang J, Fan X-L, et al. Cellular microRNA let-7c inhibits M1 protein expression of the H1N1 influenza A virus in infected human lung epithelial cells. *J Cell Mol Med*. 2012;16(10):2539–2546. doi:10.1111/j.1582-4934.2012.01572.x.
161. Zhang S, Wang R, Su H, et al. Sus scrofa miR-204 and miR-4331 negatively regulate swine H1N1/2009 influenza a virus replication by targeting viral HA and NS, respectively. *Int J Mol Sci*. 2017;18(4):1–18. doi:10.3390/ijms18040749.
162. Kozomara A, Griffiths-Jones S. MiRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Res*. 2014;42:D68–D73. doi:10.1093/nar/gkt1181.
163. Terrier O, Textoris J, Carron C, Marcel V, Bourdon J-C, Rosa-Calatrava M. Host microRNA molecular signatures associated with human H1N1 and H3N2 influenza A viruses reveal an unanticipated antiviral activity for miR-146a. *J Gen Virol*. 2013;94(Pt 5):985–995. doi:10.1099/vir.0.049528-0.
164. Makkoch J, Poomipak W, Saengchoowong S, et al. Human microRNAs profiling in response to influenza A viruses (subtypes pH1N1, H3N2, and H5N1). *Exp Biol Med*. 2016;241(4):409–420. doi:10.1177/1535370215611764.
165. Loveday EK, Svinti V, Diederich S, Pasick J, Jean F. Temporal- and strain-specific host microRNA molecular signatures associated with swine-origin H1N1 and avian-origin H7N7 influenza A virus infection. *J Virol*. 2012;86(11):6109–6122. doi:10.1128/JVI.06892-11.
166. Jiang P, Zhou N, Chen X, et al. Integrative analysis of differentially expressed microRNAs of pulmonary alveolar macrophages from piglets during H1N1 swine influenza A virus infection. *Sci Rep*. 2015;5:8167. doi:10.1038/srep08167.
167. Turchinovich A, Tonevitsky AG, Burwinkel B. Extracellular miRNA: a collision of two paradigms. *Trends Biochem Sci*. 2016;41(10):883–892. doi:10.1016/j.tibs.2016.08.004.
168. Tambyah PA, Sepramaniam S, Mohamed Ali J, et al. microRNAs in circulation are altered in response to influenza A Virus infection in humans. *PLoS One*. 2013;8(10):e76811. doi:10.1371/journal.pone.0076811.
169. Song H, Wang Q, Guo Y, et al. Microarray analysis of microRNA expression in peripheral blood mononuclear cells of critically ill patients with influenza A (H1N1). *BMC Infect Dis*. 2013;13(1):257. doi:10.1186/1471-2334-13-257.
170. Zhu Z, Qi Y, Ge A, et al. Comprehensive characterization of serum microRNA profile in response to the emerging avian influenza A (H7N9) virus infection in humans. *Viruses*. 2014;6:1525–1539. doi:10.3390/v6041525.
171. McKinney Jr WT, Bunney Jr WE. Animal model of depression: I. review of evidence: implications for research. *Arch Gen Psychiatry*. 1969;21(2):240.
172. Willner P. The validity of animal models of depression. *Psychopharmacology (Berl)*. 1984;83(1):1–16. doi:10.1007/BF00427414.
173. Denayer T, Stöhr T, Van Roy M. Animal models in translational medicine: validation and prediction. *New Horizons Transl Med*. 2014;2(1):5–11. doi:10.1016/j.nhtm.2014.08.001.
174. Mcgonigle P, Ruggeri B. Animal models of human disease: challenges in enabling translation (McGonigle and Ruggeri). *Biochem Pharmacol*. 2014;87(1):162–171. doi:10.1016/j.bcp.2013.08.006.
175. Pomorska-Mól M, Kwit K, Markowska-Daniel I, Kowalski C, Pejsak Z. Local and systemic immune response in pigs during subclinical and clinical swine influenza infection. *Res Vet Sci*. 2014;97(2):412–421. doi:10.1016/j.rvsc.2014.06.007.
176. Tchilian E, Holzer B. Harnessing local immunity for an effective universal swine influenza vaccine. *Viruses*. 2017;9(5):98. doi:10.3390/v9050098.
177. Ludvigsen TP, Kirk RK, Christoffersen BØ, et al. Göttingen minipig model of diet-induced atherosclerosis: influence of mild streptozotocin-induced diabetes on lesion severity and markers of inflammation evaluated in obese, obese and diabetic, and lean control animals. *J Transl Med*. 2015;13(312):1–12. doi:10.1186/s12967-015-0670-2.
178. Boonen HCM, Moesgaard SG, Birck MM, et al. Functional network analysis of obese and lean Göttingen minipigs elucidates changes in oxidative and inflammatory networks in obese pigs. *Pflugers Arch*. 2014;466(12):2167–2176. doi:10.1007/s00424-014-1486-2.
179. Toedebusch RG, Roberts MD, Wells KD, et al. Unique transcriptomic signature of omental adipose tissue in Ossabaw swine: a model of childhood obesity. *Physiol Genomics*. 2014;46(10):362–375. doi:10.1152/physiolgenomics.00172.2013.
180. Rødgaard T, Skovgaard K, Stagsted J, Heegaard PMH. Expression of innate immune response genes in liver and three types of adipose tissue in cloned pigs. *Cell Reprogram*. 2012;14(5):407–417. doi:10.1089/cell.2012.0026.
181. Mentzel CMJ, Alkan F, Keinicke H, et al. Joint profiling of miRNAs and mRNAs reveals miRNA mediated gene regulation in the Göttingen Minipig obesity model. *PLoS One*. 2016;11(11):1–16. doi:10.1371/journal.pone.0167285.

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Review

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Dietary Factors in Prevention of Pediatric *Escherichia coli* Infection: A Model Using Domestic Piglets

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Abstract

Enterotoxigenic *Escherichia coli* (ETEC) is the major etiological agent causing acute watery diarrhea that is most frequently seen in young children in lower-income countries. The duration of diarrheal symptom may be shortened by antibiotic treatment, but ETEC is relative refractory to common antibiotics. Burgeoning evidence suggests bioactive components that naturally occur in human milk (e.g., lysozyme and oligosaccharides) and plants (e.g., nondigestible carbohydrates and phytochemicals) contain antimicrobial functions are promising preventive measures to control ETEC infection. Although the exact protective mechanisms may vary for each compound and are still not completely understood, they generally act to (1) competitively inhibit the binding of pathogenic bacteria and toxins to gut epithelium; (2) directly kill pathogens; and (3) stimulate and/or enhance host mucosal and systemic immune defense against pathogenic microorganisms. An appropriate ETEC-challenge animal model is critical to evaluate the effect and unveil the mechanism of bioactive compounds in prevention of enteric infection. Despite wide application in biomedical research, rodents do not usually manifest typical clinical signs of enteric infections. The remarkable differences in digestive physiology, immune response, and gut microbiota between rodents and human beings necessitate the use of alternative animal models. Pigs are closely related to humans in terms of genomes, physiology, anatomy of gastrointestinal tracts, digestive enzymes, components of immune system, and gut microbiota. Like human infants and young children, nursing and nursery piglets are more susceptible to ETEC infection and reproduce the clinical signs as observed in humans. Hence, the ETEC-challenge piglet represents a valuable translational model to study pathogenesis and evaluate dietary factors (e.g., milk bioactive compounds, nondigestible carbohydrates, and phytochemicals) as preventive measures for ETEC infection in pediatrics.

Key words: bioactive compounds; diarrhea; enterotoxigenic *Escherichia coli*; pig

Introduction

Infectious diarrhea disease has long been one of the leading causes of morbidity and mortality in children living in developing countries.[1,2] Based on WHO UNICEF 2017 data, children in low-income countries experience on average 3 episodes of diarrhea each year. Even though the mortality has halved since 2000, diarrhea-caused under-5 deaths still account for 477 293 deaths in 2016.[3] One of the most common infectious agents causing diarrhea is *Escherichia coli* (*E. coli*), which includes 6 different categories: enteropathogenic *E. coli*, enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli*, enterohemorrhagic *E. coli*, diffusely adherent *E. coli*, and enteroaggregative *E. coli*.[4] In a systemic review of pathogens involved in diarrhea, enteropathogenic *E. coli*, ETEC, and enteroaggregative *E. coli* are responsible for 30% to 40% of all prolonged infections in children who live in low- and middle-income countries.[5] In another study conducted in Bangladesh, ETEC accounted for 19.5% cases of diarrhea in children under 2 years old, representing the most common pathogen followed by rotavirus.[6] Young children who live in areas with limited public healthcare and poor sanitation are the population most susceptible to ETEC infection.[7] ETEC strains colonize the small intestines and produce enterotoxins

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that produce diarrhea in both humans and animals.[8] ETEC is a noninvasive bacterium, and the pathogenesis mainly relies on 2 major virulent factors: colonization factors that are fimbrial structures on the bacterial surface, and the secretion of enterotoxins. Fimbriae help bacteria adhere to the small intestinal epithelial cells. Then the colonized ETEC produce one or more enterotoxins, such as heat-labile (LT), heat-stable (ST), or shiga toxins. These internalized enterotoxins in epithelial cells activate adenylate cyclase, which results in increased intracellular accumulation of cAMP that stimulates chloride secretion in the crypt cells, inhibits neutral sodium chloride in the villus, and consequently renders water loss, leading to dehydration and acidosis.[9–11] In this step, other components, including capsular polysaccharides, cell wall lipopolysaccharide (LPS), and iron-binding proteins, may also be involved in the pathogenicity of these bacteria in the host.[12] LPS and shiga toxin induce diseases through stimulating cytokine release or directly killing cells.[13,14] ETEC infection may activate host innate immune response and induce intestinal inflammation. This is supported by the detection of increased proinflammatory cytokines (e.g., IL-8, IL-1β, IL-6, IL-1RA, and IFN-γ) and leukocytes in fecal samples from children and adult travelers who were infected by ETEC.[15–17] The release of inflammatory mediators and recruitment of immune cells (e.g., neutrophil) are critical mechanisms for destroying and eliminating microorganisms; meanwhile, such inflammatory responses are deleterious to epithelium integrity and, in the case of chronic infection, impede repair of the epithelial barrier.[8] Neutrophil migration occurs through physical disruption of tight junctions between intestinal epithelial cells, which has been reported to increase paracellular permeability.[18] Despite broad understanding of the pathogenesis of ETEC infection, effective measures to enhance host resilience and prevent infection are still wanting.

Exclusive breastfeeding during infancy has been identified as the most effective intervention against infectious diarrheal disease in early childhood.[19–21] The protective effect of maternal milk is presumably attributed to an array of bioactive components such as secretory antibodies, bactericidal enzyme (e.g., lysozyme), oligosaccharides, and lactoferrin. Meanwhile, a growing number of phytochemicals derived from food plants were reported for their bactericidal and immune-stimulating functions and have been increasingly used as natural alternatives to antibiotics in animal feeds to prevent enteric infections.[22] Their application in pediatric nutrition is largely hampered by the concern or lack of understanding on the potential adverse effects. Nevertheless, due to the risk and ethical concern, it is extremely difficult or impossible to test the effectiveness of milk- or plant-derived bioactive compounds as prevention of infectious diarrhea in pathogen-challenge clinical trials with human subjects, especially young children.

A translatable pathogen-challenge animal model may serve as anvaluable approach to study the impact of pediatric nutrition on host resilience to enteric infections.[23,24] The animal model should not only resemble humans in terms of anatomy, gastrointestinal development, and digestive physiology, but also display similar symptoms and pathogenicity after infections and respond with comparable immune defense as young children. Rodent models are an important tool in biomedical research. Particularly, genetically modified mice are more readily available than any other model animals and are therefore extremely valuable and still going to be the predominant animal models in basic research.[25,26] Nevertheless, the remarkable differences between mice and human beings should also be mentioned, including their genomes, organ size, phases of development, lifestyle, behavior, and so forth.[27,28] The range of differences in the immune system between mice and humans is also very extensive and has been completely reviewed by other research groups.[29,30] A few researchers have applied small ruminants as comparative models for studies with mucosa immune function, but the model is highly restricted to research in the upper gastrointestinal tract.[31] Nonhuman primates are currently considered to be the most representative animal model for human research because of their irrefutable similarities to human; however, the ethical consideration, expensive raising and maintainance costs, and the high level of biosecurity requirement restricts the use of primates as research objects.[32,33] The comparisons of different animal models have been fully summarized by Jiminez et al.[28] The focus of this review is to highlight the suitability and significance of the pig model to explore the mechanisms of nutritional supplements on gut health with the emphasis on resistance to enteric ETEC infections.

Advantages and Limitations of using Domestic Piglet as a Model for Human Enteric Infectious Disease

Domestic pig (*Sus scrofa*) is a promising animal model that provides a number of translational advantages in the study of human nutrition and gastrointestinal pathophysiology.[24,34,35] They are readily available and very comparable to humans in genomics,[34] digestive physiology,[36,37] and immune system.[38] Use of pigs for biomedical research is also ethically more acceptable compared with nonhuman primates.[28,39] Pigs, likes humans, are omnivorous and have a glandular stomach lined with cardiac, gastric, and pyloric mucosa.[40] In comparison, the majority portion of stomach in rats is nonglandular.[41] The intestinal epithelial structure (the crypt-villus axis) and functions are very comparable between pigs and human beings, including absorption of nutrients and identification of self and non-self antigens.[36] As the major fermentation site, the colon of both pigs and humans are sacculated in comparison with a nonsacculated structure in rodents.[41] Our understanding about the modulatory role of gut microbiota on host metabolism has been significantly improved in the past several years. The intestinal microflora are broadly similar between pigs and human beings, whereas the majority (~85%) of gut microbiota of the mouse was not present in humans.[42] Furthermore, because pigs are omnivorous and precocious, they could be rapidly adapted to artificial feeding shortly after birth. It is feasible to manipulate the composition and feeding regimen of their liquid and solid diet at neonatal age for nutritional studies. Collectively, the domestic pig is an excellent model for translational research of pediatric nutrition.[36,43,44]

Pigs are also equipped with the whole innate and adaptive immune system, of which most effector clusters could match with their human counterparts. Additionally, the relevant features of the intestinal mucosal immunity between pigs and humans are very similar, including the distribution of Peyer's patches in the distal ileum and the pattern of intra-epithelial lymphocytes in the mucosal surfaces.[37] It has been implicated that the pig immune response resembles that of humans for 80% of analyzed parameters, whereas mice are similar in less than 10%.[45,46] There was remarkable improvement in genomic database and functional annotation of porcine immune-related homologenes compared with those identified in humans and

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rodents.[46,47] Hence, findings in pig immune response under various pathological states are translatable to humans and rodents. Pigs are naturally susceptible to many pathogens that are either identical or closely related to those infecting humans, such as *Rotavirus*, *influenza*, *E. coli*, and *Salmonella*. As we discussed above, ETEC-caused diarrhea is a huge disease burden in young children who live in areas with poor sanitary infrastructures. In swine production, ETEC also impose immense threat to nursing and nursery piglets.[48] However, rodents (mice and rats) are not normally susceptible to ETEC, and experimental challenges with the pathogen hardly reproduce clinical signs (e.g., diarrhea and dehydration) associated with ETEC, which is, at least partially, due to insufficient pathogen attachment to the mucosa of small intestine.[49] It was reported that strains of human ETEC and porcine K88 $(F4)^+$ strains cross-bound to isolated small intestinal cells from either humans or pigs, whereas the ETEC strain ($K99^+$) that prevalently infects calves and lambs failed to bind both human and pig small intestinal cells under the same conditions.[50] Consistently, gnotobiotic piglets challenged with the ETEC strain with the expression of porcine enterotoxins (pLT or pSTa) displayed equivalent colonization of pathogens and identical signs of disease as piglets inoculated with the isogenic ETEC strain constructed with human enterotoxins (hLT or hSTa).[51] Domestic piglets thus may be a superior model to study pediatric ETEC infection.

Despite the aforementioned advantages of pigs as a translational model, it is also important to recognize the differences between the 2 species in anatomy of gastrointestinal tracts, immunity, and gut microbiota. Compared with humans, pigs have bigger gastric capacity, longer absolute length of intestine, and a greater proportion of the large intestine.[41] Additionally, the cecum, the ascending and transverse colon, and the proximal section of descending colon are arranged in spiral coils, which structurally differs from that of humans.[52] As the pig placenta has 6 layers, the embryo is separated from the sow's blood supply during the intrauterine period. Piglets lack transplacental transfer of immunoglobins; therefore, colostral immunoglobulin intake is essential for their efficient immune function.[53] "Gut closure" for the macromolecule uptake occurs within 24 to 48 hours after birth.[54] During this period, colostral immunoglobulins are transported into piglets via enterocytes.[53,55] In addition to this major difference in passive immunity, other differences include the inversion of lymph nodes, types of Peyer's patches, and cluster of differentiation. Cell-surface proteins that allow the identification and characterization of various immune cells vary across species (human vs pig) have been thoroughly described by Brandtzaeg[56], Mair et al.[57], Rothkötter[53], and Summerfield and McCullough[58]. Although the majority of human intestinal microbiota are present in pigs, there were marked differences in relative abundance of gut bacteria at the phyla level between neonatal piglets and human infants.[35] In piglets, the predominant phyla are Bacteroidetes and Firmicutes (rather than Bifidobacteria), which represents more than 50% of 16S rRNA sequence in human neonates.[35] Given the important role of gut microbiota in modulation of host immune response, such innate differences between the neonates of the 2 species impose challenges using piglets for the study of pediatric enteric infections. Cautious explanation is warranted when translating results from piglets to human infants.

ETEC Challenge Piglet Model

Pigs are naturally susceptible to postweaning diarrhea, which is another reason that the pig is a very suitable model to study diarrhea or other environmentally acquired enteric infectious diseases. Postweaning *E. coli* diarrhea in pigs, characterized as anorexia, depression, rapid dehydration, decreased growth performance, and increased mortality, remains a major cause of loss for the pig industry.[59] *E. coli* that express F18 and F4 (K88) fimbria are the predominant strains that cause diarrhea in postweaning and preweaning pigs.[60] Several published studies thoroughly investigated the influences of F18 and F4 *E. coli* on gut morphology and immunity and systemic immunity of newly weaned pigs.[22,61–63] Other literature also reviewed the pathogenesis of K88 *E. coli* infection, which shares similarity and differences with the pathogenesis of F18 *E. coli*.[64–66] Both strains have been explored to use for *E. coli* challenge studies in pigs. As we mentioned above, 2 important virulence factors are involved in *E. coli* infection: fimbriae and toxins. The F4 and F18 receptors are located on different porcine chromosomes and are genetically determined by autosomal dominate genes.[67,68] It has been observed that the colonization of F4 *E. coli* in the small intestine may be quicker than that of F18 *E. coli*.[69] The peak excretion of F4 *E. coli* from feces was observed 2 days postinfection, whereas the peak excretion of F18 *E. coli* from feces was 1 to 3 days later than F4 *E. coli* infection.[69] Many factors could influence their adhesion, including the amount of fimbriae expressed by *E. coli*, the strength of their binding, and environmental factors in intestinal lumen that could impact the interaction of bacteria and their receptors.[69–71] F18 *E. coli* infection is more commonly observed in postweaning pigs (3 to 6 weeks of age) and may be the result of lack of expression of receptors in the small intestinal enterocytes of piglets for F18 fimbriae.[60] Different immune responses were also observed in F4+ and F18+ *E. coli*, depending on the toxins they expressed.[69,72,73]

In this review section, we will focus only on the description of F18 *E. coli* infection. In this disease challenge model, the F18 *E. coli* that was used for inoculation was derived from a field disease outbreak by the University of Illinois Veterinary Diagnostic Laboratory (isolate no.: U.IL-VDL #05-27242) and expressed LT, STb, and Shiga-like toxin 2. The inoculums were provided at 10^{10} cfu per dose per day in phosphate buffer saline for 3 consecutive days. It has been observed that F18 *E. coli* inoculation with the dose described above consistently induced moderate diarrhea (yellow to brown scours), as the peak of diarrhea was around 5 to 6 days postinoculation and most pigs recovered around 11 to 12 days after the first inoculation.[22,62,63] The fecal culture results were in agreement with the observations of diarrhea trend, as indicated that the majority of *E. coli* in feces were F18 *E. coli* on day 5 or 6 postinoculation, then the percentage of F18 *E. coli* in total coliforms gradually decreased after day 5 or 6.[22,63] Toxins (LT and STb) produced by *E. coli* are able to induce partial villus atrophy in young pigs.[74] In this disease challenge model, it was also observed that F18 *E. coli* infection reduced villus height in jejunum and ileum.[22] The villus volume is highly related to the nutrient absorptive capacity of the small intestine.[75] Therefore, the villus atrophy could induce decreased nutrient absorption, reduced feed intake, and finally reduced growth performance, which was also confirmed in this disease challenge model. The clinical signs observed in the *E. coli* challenge model with pigs are pretty similar to young children, including loss of appetite, watery diarrhea, severe dehydration, or potential growth retardation.[76]

In the F18 *E. coli* disease challenge model, it was also observed that F18 *E. coli* infection induced systemic inflammation, as indicated by the gradually increased total white blood cells, neutrophils, and lymphocytes postinoculation.[22,62,63] Consistent with this, several proinflammatory cytokines

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(i.e., TNF-α) and acute phase proteins (C-reactive protein and haptoglobin) in pig serum were also elevated by *E. coli* infection. Lymphocytes and neutrophils are the most abundant circulating immune cells in humans and play a fundamental role in the immune response. There is limited information about the nature of immune responses of ETEC in general, with the exception that the antibody responses against different virulence factors were determined in children or adult patients who were infected with ETEC.[77–79] Examination of white blood cells and neutrophils is particularly prevalent in patients with bacterial infection, and a similar trend is also observed in infected patients or clinical trials.[80] Therefore, data reflectinh systemic immunity confirmed that the *E. coli* disease challenge model with newly weaned pigs could provide a valuable tool to examine the immune responses of bacterial infection in young children.

As the first line of defense, the mucosal layer of the intestine is in direct contact with luminal contents; thus, mucosal immunity is very important for the immune defense against pathogens.[81] In the F18 *E. coli* challenge model, we also observed that *E. coli* infection enhanced specific local inflammation, as indicated by the increased neutrophil and macrophage recruitment in the distal ileum of weaned pigs.[22] During inflammatory responses, neutrophils are the first cells to migrate into infected tissues and then secret monocyte chemoattractants, which will contribute to the recruitment of other immune cells, such as macrophages.[82] The recruited neutrophils and macrophages in the infected sites will phagocytose bacteria and their particles, release large amounts of inflammatory mediators, and facilitate the resolution of inflammation. To characterize the effects of F18 *E. coli* infection on the expression of immune-related genes in ileal mucosa of weaned pigs, a porcine genome array was performed for the ileal mucosa samples collected from *E. coli*-infected pigs at day 5 postinoculation.[83] In summary, *E. coli* infection altered the expression of 418 of 5168 genes in the ileal mucosa. Within this, *E. coli* infection altered the expression level of genes related to LPS activation, cytokine and chemokine production, complement cascades, receptors and co-stimulators, heat stress, antigen presentation, cell apoptosis, and endoplasmic reticulum stress. Although the effects of *E. coli* infection on mucosa immunity have been well evaluated with mice or rat models, the results from this pig model provide more valuable information on the regulation of mucosal immune response against bacterial infection due to the intestinal similarities of pigs and humans.

Milk and Plant-derived Bioactive Compounds on Enteric ETEC Infection

Accumulating evidence has confirmed the importance of nutritional interventions, including modified feeding strategies and nutrient supplements, in the control of diarrheal diseases and prevention of enteric infection (Table 1).[84,85] For example, probiotics are probably the most popular supplements recommended to be used to treat or prevent infant diarrhea.[86–89] Supplementation with micronutrients, such as zinc, also showed a protective effect in both well-nourished and malnourished children with diarrhea.[90] To explore the novel nutritional strategies and decipher the underlying mechanisms, animal models are highly preferred prior to clinical trials with humans. In the F4 or F18 *E. coli* challenge pig model described above, the effects of different dietary factors or nutrient supplements on diarrhea, disease resistance, physiology, and immunity of newly weaned pigs could be assessed. Many nutritional strategies and/or feed additives have been applied to improve health and maximize the production of weaned pigs.[91–93] Those strategies target different aims: (1) improvement of nutrient digestion and absorption, (2) regulation of gut microbiota to more favorable bacterial species, and (3) immune modulation to enhance disease resistance of weaned pigs. In this review, we will focus only on a few milk- and plant-derived bioactive compounds, such as phytochemicals, oligosaccharides, and lysosomes, as examples to introduce novel interventions on enteric infection of young children using pigs as a model.

Phytochemicals

Phytochemicals are secondary plant metabolites and can be obtained naturally from plant materials. Phytochemicals can be used in solid powder form or as crude or concentrated extracts.

Table 1 Dietary Factors on Enteric Infection of Weaned Pigs

Pathogens[a]	Dietary Supplements	Outcome	Reference
ETEC, K88	Milk from human lysozyme transgenic goats	Reduced diarrhea, reduced bacterial translocation in mesenteric lymph nodes	172,182,183
ETEC, K88	Chito-oligosaccharide	Reduced diarrhea	184
ETEC, K88	Combination of raw potato starch and probiotic *E coli* strains	Reduced diarrhea, enhanced gut microbial diversity	185
ETEC, K88	Probiotics: *Pediococcus acidilactici*, *Sacharomyces cerevisiae boulardii*	Reduced ETEC attachment to ileal mucosa, upregulated inflammatory responses in gut	186
ETEC, K88	*Saccharomyces cerevisiae* fermented products	Enhanced appetite and ileal digesta bacteria richness, reduced ETEC adhering to the mucosa and colonic ammonia	187,188
ETEC, K88	Probiotics: *Lactobacillus plantarum* CJLP243	Enhanced growth performance, reduced diarrhea, reduced gut inflammation, enhanced gut barrier function	189,190
ETEC, K88	Phytogenics	Enhanced growth performance	191
ETEC, K88	Nucleotides	Enhanced growth performance and nutrient digestibility, reduced diarrhea	192
ETEC, F18	Clays (smectite, zeolite, kaolinite)	Reduced diarrhea, enhanced gut integrity	61,63
ETEC, F18	Phytochemicals (capsicum oleoresin, garlic botanical, turmeric, oleoresin)	Reduced diarrhea, enhanced gut morphology, decreased systemic and gut mucosal inflammation	22,83
ETEC, F18	β-Glucan	Enhanced gut barrier function, reduced systemic inflammation	62

[a]ETEC = enterotoxigenic *Escherichia coli*.

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Depending on the process used to derive the active ingredients, the extracts can be classified as essential oils that are volatile lipophilic substances obtained by cold extraction or distillation and oleoresins that are derived by nonaqueous solvents.[94] The major bioactive compounds in phytochemicals are polyphenols, terpenoids, alkaloids, and sulfur-containing compounds. The composition and concentration of bioactive compounds vary according to the plants, parts of the plant, geographical origins, harvesting season, environmental factors, storage conditions, and processing techniques.[95] Phytochemicals have been largely applied for human nutrition and improvement of human health due to their potential biological functions, such as, antiviral, antimicrobial, antioxidant, and antiinflammatory effects (Table 2).[96–99] It has been reported that various phytochemicals exhibit a wide spectrum of antibacterial activities against gram-negative and gram-positive bacteria[100–102] with several general modes of action. First, owing to the lipophilic nature, many essential oils exert their antibacterial effect through increasing permeability and fluidity of plasma membranes that cause leaking of intracellular materials (e.g., ions, proton).[103–105] Second, phytochemicals contain a high percentage of phenolic compounds, which possess strong antibacterial properties.[106,107] Third, the active components in phytochemicals could interfere with the enzyme system of bacteria, then block the microbe's virulence.[108] Fourth, certain bioactive components in phytochemicals may prevent the development of virulent structures in bacteria, such as flagella that is critical for bacterial adhesion.[109] Fifth, certain plant polyphenols could inhibit ETEC adhesion and toxin binding in vitro.[110] A low dose of phytochemicals has been recommended to serve as a potential natural antimicrobial in reconstituted infant rice cereal.[111]

The antiinflammatory effects of phytochemicals have been widely reported with in vitro cell culture models. Essential oils from clove, tea, garlic, cinnamon, and others have potential antiinflammatory activities because they are able to suppress the production of TNF-α, IL-1β, and nitric oxide from LPS-induced mouse and porcine macrophages.[99,112–114] In addition, Lang et al.[112] reported that garlic extract also can inhibit intestinal epithelial cell secretion of several chemokines, including IL-8, IP-10, and MIG, which mediate the inflammatory response by recruitment of various circulating leukocytes into the inflamed tissue. The modes of action for the antiinflammatory activities of phytochemicals are not clear, but evidence suggests that these effects are partially mediated by blocking the NF-κB activation pathway.[113,115,116] For example, curcumin can block cytokine-induced NF-κB DNA binding activity, RelA nuclear translocation, IκBα degradation, IκB serine 32 phosphorylation, and IκB kinase activity.[115]

In an ETEC challenge model with weaned pigs, it has been observed that dietary supplementation of 10 mg/kg of capsicum oleoresin, 20 mg/kg of garlic botanical, or 10 mg/kg of turmeric oleoresin alleviated signs of diarrhea in ETEC-infected pigs.[22] Capsicum and turmeric are extracted from oleoresins, which were standardized to 6% capsaicin and dihydrocapsaicin and 98% curcuminoides, respectively. Garlic botanical is standardized to 40% propyl thiosulfonates. Although the supplementation of those phytochemicals reduced diarrhea of ETEC-infected pigs, the proportions of β-hemolytic coliforms in feces were not affected, indicating that the dose of phytochemicals was probably too low to have a antimicrobial effect. Thus, the reduction of diarrhea may be due to other potential mechanisms instead of antimicrobial effects. The analysis of gene expression patterns by microarray showed

Table 2 Several Commonly Used Phytochemicals and Their Main Components Exhibiting Different Biological Activities

Common Name	Scientific Name	Main Components	General Modes of Action[a]
Cinnamon	*Cinnamomum verum* J. Presl *Cinnamomum osmophloeum*	Cinnamaldehyde	1. Antimicrobial effect[105,193–204] • Increase permeability and depolarize cytoplasmic membrane • Inhibit membrane-bound ATPase activity and impair ATP production • Inhibit bacterial thiol-containing enzymes and other enzymes involved in acetyl-CoA synthesis • Alter cellular metabolism • Compromise cellular antioxidant defense of bacterium • Downregulate transcription of virulence genes • Inhibit bacterial cytokinesis 2. Effect on host cells • Antioxidant • Antiinflammatory (suppress NF-κB expression/signaling pathway; inhibit TNF-α, IL-1β, IL-6, IP-10, MIG, and PGE2 production; suppress iNOS and COX-2 expression)
Clove	*Eugenia caryophyllus* Spreng. *Eugenia caryophylata* Thunb *Syzygium aromaticum* (L.)	Eugenol	
Fennel	*Eugenia caryophyllata* *Foeniculum vulgare* *Funicular vulgare*	Anethol Eugenol	
Garlic	*Allium saticum*	Allicin	
Ginger	*Zingiber officinale*	Curcumin Gingerol	
Oregano Thyme	*Origanum vulgare* spp. *Origanum onites* *Origanum minutiflorum*	Carvacrol	
Pepper	*Capsicum*	Capsaicin	
Pomegranate	*Punica granatum*	Ellagic acid	
Rutaceae	*Zanthoxylum schinifolium*	Citronellal *β*-Phellandrene	
Thyme	*Thymus vulgaris* L. *Thymbra spicata*	Thymol Carvacrol Terpinene	

Modified from Liu 2011.[66]

COX-2 = cyclooxygenase-2; IL = interleukin; iNOS = inducible nitric oxide synthase; IP-10 = interferon gamma-induce protein 10; NF- κB = nuclear factor kappa-light-chain-enhancer of activated B cells; MIG = monokine induced by gamma interferon; PGE2 = prostaglandin E2; TNF-α = tumor necrosis factor.

[a]The general modes of action were listed in the table, because many studies tested on essential oil containing a number of compounds rather than pure compound. The exact mode of action of each compound is not completely clear.

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that dietary phytochemicals affected the expression of genes related to mucin, membrane structure, and function in ileal mucosa of weaned pigs,[83] indicating consumption of phytochemicals may enhance gut mucosal health of *E. coli*-infected pigs.

Moreover, feeding those phytochemicals also reduced neutrophil and macrophage recruitment in the ileum of *E. coli*-infected pigs compared with pigs fed the control diet.[22] During the inflammatory response, neutrophils are the first cells to migrate into the infected gut as part of the host defense system.[82] The recruitment of other immune cells, such as macrophages, was activated by the secretion of monocyte chemoattractants from neutrophils in the infected tissues. Both neutrophils and macrophages can facilitate resolution of inflammation by phagocytizing bacteria and their particles and release large amounts of mediators. But excessive recruitment of those activated immune cells in the infected area will induce the excessive production of inflammatory mediators and then exacerbate gut inflammation. The reduced recruitment of immune cells suggests that weaned pigs supplemented with those phytochemicals actually had less gut inflammation compared with infected control. The microarray analysis also confirmed the reduced gut inflammation by feeding those phytochemicals to weaned pigs.[83] Compared with the ETEC-infected control pigs, feeding capsicum oleoresin, garlic botanical, or turmeric oleoresin altered the expression of 52 genes (18 up and 34 down), 117 genes (34 up and 83 down), or 84 genes (16 up and 68 down), respectively, often counteracting *E. coli* infection. The overall findings from this ETEC challenge study[22,83] indicate that supplementation of low-dose phytochemicals could enhance disease resistance and stimulate the recovery of young pigs from ETEC infection by modulating gut immunity and barrier functions.

Previous studies also demonstrated that perfusion of F4 *E. coli*-infected jejunal segments with black or green tea extract reduced net fluid and electrolyte losses, suggesting the antidiarrheal activity of those tea extracts.[117] Supplementation of 1 g/L of cranberry extract in drinking water remarkably reduced diarrhea of F18 *E. coli*-challenged piglets.[118] The use of herbal medicinal products and supplements has grown rapidly across the world over the past decades. There are more than 80% of people worldwide, representing the majority in the developing countries, relying on herbal medicines as primary healthcare.[119,120] A wide variety of herbal extracts are employed to treat diarrhea, especially in the developing world.[121–123] Although many promising potential benefits were observed in a good number of herbal products, many of them remain untested and their modes of action and potential side effects are not clear. A valuable animal model (i.e., pigs) will absolutely help us overcome the wide range of challenges of utilization of phytochemicals as medicine or nutritional therapy for fighting diarrhea in young children.

Prebiotics and Nondigestible Functional Carbohydrates

Prebiotics are a category of nutritional compounds that may not share similar structures but have the ability to improve the growth of beneficial microorganism in the gastrointestinal tract. It is important that prebiotics are resistant to hydrolysis by mammalian enzymes in humans and animals in the small intestine and preferentially utilized by *Lactobacilli* and *Bifidobacteria* in the large intestine, which confers benefit to gut health through competitive inhibition of pathogenic bacterial species.[124–126] Gibson et al.[126] offered a definition of prebiotics, which contains 3 key aspects: resistance to digestion, fermentation by the large intestinal microbiota, and a selective effect on the microbiota associated with health-promoting effects.

Many dietary fibers exhibit some prebiotic activity, but other nonfiber dietary components may be classified as prebiotics if they meet the requisite functional criteria. The number of potential prebiotic substances has grown beyond those that are naturally occurring, such as inulin found in chicory products, to include a large number of chemically/enzymatically manufactured prebiotics, the most notable of which is galacto-oligosaccharides (GOS), produced from lactose by β-galactosidase. The most well-characterized prebiotics are nondigestible oligosaccharides, such as inulin, fructo-oligosaccharides (FOS), GOS, lactulose, polydextrose, xylo-oligosaccharides, transgalactooligosaccharides, pyrodextrins, and isomalto-oligosaccharides.[127] Inulin, oligofructose, and FOS are considered inulin-type prebiotics, which have been commonly used in the pig industry and human foods.[128] GOS also have attracted interest, mainly because these are the compounds in human milk that have been associated with the improved colonic health of breast-fed infants.[129] Owing to the beneficial effect of human milk oligosaccharides, the use of prebiotics is encouraged in infant formula with the intention to simulate the effects of human milk oligosaccharides. A few other nondigestible carbohydrates not categorized as prebiotics, however, manifest health-promoting functions. For example, β-glucan is linear and branched polysaccharides that are produced by bacteria and are also found in cereals, algae, and fungi.[130] The use of β-glucan has drawn growing interest in the food industry due to its immunomodulatory effects as demonstrated in animal and humans.[131,132] In vitro study supports a prebiotic effect of nondairy bacterial origin β-glucan on 3 strains from *Lactobacillus* genus.[133] Oat β-glucan has been allowed to use to fortify cereals for young children (ages 1–3 years old) in the European Union. Clinical research on fermentation characters of β-glucan is still in scant.

The most notable effect of prebiotics is their modification of the balance of the microbiota, both in the lumen and at the mucosal surface. They can specifically stimulate growth of a limited number of beneficial microorganisms, generally *Bifidobacteria* and *Lactobacilli*, which suppress the growth of potentially pathogenic microorganisms such as *E. coli* by various means described below and therefore reduce the adverse effects caused by bacterial infection. For example, the desired bacteria produce short-chain fatty acids and lactic acid, which may indirectly and specifically kill or inhibit the growth of pathogens.[134] The reduction of the pH of the intestinal environment through production of acids creates an environment unsupportive of the growth of several pathogens.[135] The desired bacteria may produce antimicrobial compounds such as bacteriocins or antibiotics, although regulatory agencies try to avoid production of antibiotics.[136] The desired bacteria compete for the available nutrients against pathogens.[137]

More potential mechanisms are involved in the benefits of prebiotic supplements. For instance, the beneficial bacteria induced in the gastrointestinal tract by prebiotics could also inhibit the attachment of pathogens to the intestine by competing for binding sites on the intestinal wall,[138] by producing acids that may reduce pathogen binding,[135] by stimulating mucin production,[139] or by strengthening gut barrier functions.[140,141] Some prebiotics may contact with mucus to directly compete for intestinal binding sites.[142] In addition, some prebiotics and their subsequent increase in short-chain fatty acids appear to have direct immunomodulatory properties.[143–145] The most common studies in prebiotics and nondigestible carbohydrates and their potential modes of action are briefly summarized in Table 3.

Limited research has been published on the impacts of prebiotics on infectious diseases in young pigs, especially in GOS

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Table 3 Prebiotics and Nondigestible Carbohydrates and Their Potential Mechanisms of Action

Prebiotics/Nondigestible Carbohyrates	Major Sources	Mechanisms of Action[62,126,128,129,134–146,205]
Chito-oligosaccharides	Chitin	1. Inhibit pathogens by increasing population of desired bacteria
Cyclodextrins	Potato or maize starch	• Increase production of short chain fatty acids and lactic acid
Dextrins	Potato or maize starch	• Reduce pH of intestinal environment
Fructo-oligosaccharides (FOS)	Fruits and vegetables	• Stimulate production of antimicrobial compounds
Galacto-oligosaccharides (GOS)	Human milk	• Compete for available nutrients
Genti-oligosaccharides	Glucose	2. Inhibit pathogen attachment to intestine
Gluto-oligosaccharides	Saccarose, maltose	• Desired bacteria may compete for binding sites on intestinal wall
Inulin	Chicory root	• Prebiotics may contact mucus to compete for intestinal binding sites
Isomalto-oligosaccharides (IMO)	Maltose, sucrose	
Lactose	Milk, milk products	• Acid production may reduce pathogen binding
		• Stimulate mucin production, strengthen tight junctions, and enhance gut barrier function
Lactulose	Milk, milk products	3. Reduce expression of virulence gene
Levans	Fructans and soybean mucilage	4. Immunomodulatory properties
Maltodextrins	Potato or maize starch	• Modulate several types of immune cells
Oligofructose	Chicory root, wheat, onions, leeks	• Modulate levels of immunoglobulins
Pectic-oligosaccharides	Pectin	• Short chain fatty acids have immunomodulatory properties
Pyrodextrins	Potato or maize starch	
Resistant starch	Grains, cereals, legumes, seeds, nuts	5. Direct effects on host
Soybean oligosaccharides (SOS)	Soybean	• Short chain fatty acids as energy supply for enterocytes
Trans-galactooligosaccharides (TOS)	Human milk	• Decrease production of toxic amine
		• Increase mineral absorption because of lower pH and higher expression of mineral binding proteins or active carriers
Xylo-oligosaccharides (XOS)	Xylan	
β-Glucan	Algae, seaweed, yeast, grains	• Some desired bacteria may secrete digestive enzymes, enhancing nutrient digestion
		• Increase intestinal morphology

and transgalactooligosaccharides due to their relatively high cost. In a K88 ETEC challenge model with weaned pigs, it has been observed that supplementation of 8% inulin reduced the incidence and severity of postweaning diarrhea, probably by increasing short-chain fatty acid production in the cecum and proximal colon.[146] It has been also reported that the addition of FOS could prevent mortality and morbidity of weaned pigs infected with K88 ETEC.[147] Supplementation of β-glucan originated from different sources (yeast or algae) could enhance the resistance of pigs against ETEC infection.[62,148] The likely reasons may include enhanced gut integrity and health and reduced paracellular permeability,[149] reduced colonization of the small intestine with ETEC,[148] and boosted host immune response against ETEC infection.[62] Both dectin-1 and CR3 expressed on several immune cells (i.e., macrophages, neutrophils) are highly involved in the immuno-modulatory effects of β-glucan,[150] which need to be further elucidated.

Multiple studies have also evaluated the different combinations of oligosaccharides in pediatric research, suggesting that the preventive use of prebiotics could reduce the rate of acute infectious diseases requiring antibiotic therapy in infants and children younger than 2 years old.[151–153] Supplementation of prebiotic oligosaccharides to infant formula has also been shown to modify gut microflora of formula-fed infants closer to the flora in breast-fed infants.[154] Although a large amount of studies have been published to explore the potential benefits of prebiotics on human health and modes of action, the majority of research was done with in vitro cell culture models or laboratory animal models. The use of the pig model with ETEC challenge will provide more supportive data to validate the efficacy of different combinations of prebiotic carbohydrates against intestinal infection in young children and to help explore the potential mechanisms they may have.

Lysozyme

Lysozyme is an antimicrobial enzyme naturally present in body fluids (e.g., tears, saliva, and milk) of all mammalian species.[155–157] Its muramidase activity catalyzes the hydrolysis of the peptidoglycan layer of the bacterial cell wall that leads to cell lysis. Gram-positive bacteria is thus susceptible to the enzymatic degradation of lysozyme.[158] However, lysozyme also displayed bactericidal activity against a variety of Gram-positive and Gram-negative species through the mechanism that is independent of its enzymatic function.[158–160] Particularly, lysozyme has been found to act synergistically with lactoferrin in killing gram-negative bacteria.[155,157,161] It has also been reported for its antiinflammatory property that was mediated through inhibiting neutrophil migration.[162]

Early research reported that human milk contains an average of 390 mg/L lysozyme.[163] Based on data from 4 studies, Lönnerdal et al.[164] recently reported that the median concentration of lysozyme was 320 mg/L in human colostrum, peaked at 1100 mg/L in the second month of lactation, and decreased to 850 mg/L in the following month, whereas data of lysozyme concentration after 90 days in lactation were unavailable. The lysozyme concentration is remarkably low in milk of most livestock species (cow, sow, and goat). For instance, the lysozyme content of cow milk ranged from 0.18 to 0.45 mg/L across 5 dairy breeds,[165] whereas the lysozyme concentration is approximately 0.25 mg/L in goat milk and 0.065 mg/L in sow milk.[166,167] Deficiency in antimicrobial proteins in cow milk presumably contributes to the difference in gut microbiota profiles observed between breast-fed and formula-fed infants. For instance, breast-fed infants harbor fecal microbiota of more uniformity that is predominated by bifidobacteria[168]. In contrast, the microbiota of formula-fed newborns demonstrated greater diversity and higher prevalence of clostridia,

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streptococci, and *E. coli*.[169,170] In a clinical trial, children hospitalized with acute diarrhea had faster recovery and a lower relapse rate by receiving an oral rehydration solution supplemented with human lysozyme and human lactoferrin.[171] It has not been evaluated whether dietary supplementation of lysozyme per se could modulate microbiota and enhance host resistant to enteric infections in young children. The question has been addressed by a research group at the University of California, Davis (Drs. Elizabeth Maga and James Murray) and others through a translational model using milk from transgenic goats expressing human lysozyme at 68% of the level found in human milk, and young pigs as feeding subject.[172,173] Six-week-old crossbred domestic pigs were artificially reared and fed milk from either nontransgenic control goat or human lysozyme transgenic goat for 14 days. Consumption of lysozyme-rich milk significantly increased the proportion of Bacteroidetes and decreased the proportion of Firmicutes (Clostridia) in fecal microbial.[173] Within phyla, there was an enrichment in the abundance of Bifidobacteriaceae and Lactobacillaceae, families known for their health-promoting function in lower GI tract, whereas the abundance of bacteria (*Mycobacteriaceae*, *Streptococcaceae*, *Campylobacterales*) associated with diseases were underrepresented in response to consumption of lysozyme milk.[173] In another trial, after 14 days of feeding lysozyme milk, pigs were orally inoculated with porcine-specific ETEC (O149:F4 strain) at 2×10^7 total CFU for 4 times at 12-hour intervals.[172] Fecal score decreased from 24 to 96 hours post-ETEC inoculation, suggesting successful induction of diarrhea. The lowest score was observed at 24 to 48 hours postinoculation. In comparison with pigs that consumed control goat milk, feeding lysozyme-rich milk alleviated the severity of diarrhea and reduced total bacteria translocation into the mesenteric lymph nodes by 83%, which corresponded to a tendency of reduced fecal Enterobacteriaceae in pigs fed lysozyme-rich milk. Because many prevalent enteric pathogens such as *E. coli* and *Salmonella* belong to the family of Enterobacteriaceae, this possibly explained the dampened signs of diarrhea.[172]

A line of transgenic pigs that expresses high levels of recombinant human lysozyme (approximately 1300 mg/L) in their milk was also generated at China Agricultural University by Dr. Li's group.[174] Consumption of human lysozyme-rich milk reduced diarrhea, increased survival rate, and facilitated the recovery of neonatal pigs from F4 *E. coli* infection.[175] The observed benefits are likely due to the increase in the abundance of intestinal *Lactobacillus* as well as enhanced intestinal integrity and mucosa immunity of neonatal pigs consumed lysozyme.[175]

Further Applications and Conclusions

The pediatric population is especially vulnerable to ETEC infection. The tremendous infectious disease burden requires continued and extensive studies aimed at exploring more therapeutic/preventive interventions to improve young children's health/survival and to alleviate bacterial infection. Young pigs have demonstrated their potential as a new animal model for pediatric research. The translational features of the piglet model in terms of anatomy of the gastrointestinal tract, digestive physiology, components of the immune system, dynamics of neurodevelopment, and morphological structure of CNS foretells its broad applications for mechanistic research in human nutrition, immunity, and neurodevelopment in early life.[176–178] Considering that enteric infections are the leading causes of morbidity and mortality in early childhood, a well-characterized pig model incorporating enteric pathogen challenges presented by our group (bacterial infection) and others (viral infection)[179,180] is promising in preclinical studies to uncover the mechanism of pathogenesis and evaluate the effects of nutraceutical interventions in youth. The underlying mechanisms will be further explored by combining both functional measurements (i.e., gut permeability, feed efficiency, nutrient digestibility, etc.) and descriptive analysis (i.e., gut morphology and gut barrier function, etc.). The important roles of the gut microbiota in host resistance against invading pathogens in the small intestine should not be negligible.[181] The protective effects of gut microbiota against pathogenic bacterial infection could be deeply approached with this pig challenge model by investigating metagenomics and the changes of bacterial metabolites. Last but not least, this translational model could also be expanded to different pathogens, for instance, different strains of *E. coli*, *Salmonella*, other infectious agents, or combinations.

It is also important to keep in mind that each animal species shows some similarity to the physiology of humans and therefore provides valuable insights from different angles on the research of nutritional intervention in pediatric enteric infection. The purpose of this review is not to compare different animal models with their pros and cons. The overall objective is to highlight another potential model that could serve as a powerful tool for pediatric research.

References

1. Ramaswamy K, Jacobson K. Infectious diarrhea in children. *Gastroenterol Clin North Am*. 2001;30(3):611–624.
2. Cheng AC, McDonald JR, Thielman NM. Infectious diarrhea in developed and developing countries. *J Clin Gastroenterol*. 2005;39(9):757–773.
3. Estimates of child cause of dealth, diarrhoea. 2018. https://data.unicef.org/topic/child-health/diarrhoeal-disease. Accessed Feburary 2018.
4. Torres AG, Zhou X, Kaper JB. Adherence of diarrheagenic Escherichia coli strains to epithelial cells. *Infect Immun*. 2005;73(1):18–29.
5. Abba K, Sinfield R, Hart CA, Garner P. Pathogens associated with persistent diarrhoea in children in low and middle income countries: systematic review. *BMC Infect Dis*. 2009;9:88.
6. Qadri F, Saha A, Ahmed T, Al Tarique A, Begum YA, Svennerholm A-M. Disease burden due to enterotoxigenic Escherichia coli in the first 2 years of life in an urban community in Bangladesh. *Infect Immun*. 2007;75(8):3961–3968.
7. Qadri F, Svennerholm AM, Faruque AS, Sack RB. Enterotoxigenic Escherichia coli in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. *Clin Microbiol Rev*. 2005;18(3):465–483.
8. Sears CL, Kaper JB. Enteric bacterial toxins: mechanisms of action and linkage to intestinal secretion. *Microbiol Rev*. 1996;60(1):167–215.
9. Weikel CS, Guerrant RL. STb enterotoxin of Escherichia coli: cyclic nucleotide-independent secretion. *Ciba Found Symp*. 1985;112:94–115.
10. Forte LR, Thorne PK, Eber SL, et al. Stimulation of intestinal Cl- transport by heat-stable enterotoxin: activation of cAMP-dependent protein kinase by cGMP. *Am J Physiol*. 1992;263(3 Pt 1):C607–C615.
11. O'Brien AD, Holmes RK. Protein toxins of *Escherichia coli* and *Salmonella*. In: Neidhardt FC, ed. *Escherichia coli and*

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Samonella: Cellular and Molecular Biology. Washington, DC: ASM Press; 1996:2788–2802.
12. Gyles CL, Fairbrother JM. Escherichia coli. In: Gyles CL, Prescott JF, Songer JG, Thoen CO, eds. *Pathogenesis of Bacterial Infections in Animals*. Ames, IA: Blackwell Publishing; 2010:267–308.
13. Bergan J, Dyve Lingelem AB, Simm R, Skotland T, Sandvig K. Shiga toxins. *Toxicon*. 2012;60(6):1085–1107.
14. Bannerman DD, Goldblum SE. Mechanisms of bacterial lipopolysaccharide-induced endothelial apoptosis. *Am J Physiol Lung Cell Mol Physiol*. 2003;284(6):L899–L914.
15. Greenberg DE, Jiang ZD, Steffen R, Verenker MP, DuPont HL. Markers of inflammation in bacterial diarrhea among travelers, with a focus on enteroaggregative Escherichia coli pathogenicity. *J Infect Dis*. 2002;185(7):944–949.
16. Long KZ, Rosado JL, Santos JI, et al. Associations between mucosal innate and adaptive immune responses and resolution of diarrheal pathogen infections. *Infect Immun*. 2010; 78(3):1221–1228.
17. Mercado EH, Ochoa TJ, Ecker L, et al. Fecal leukocytes in children infected with diarrheagenic Escherichia coli. *J Clin Microbiol*. 2011;49(4):1376–1381.
18. Gayle J, Jones SL, Argenzio RA, Blikslager AT. Neutrophils increase paracellular permeability of restituted ischemic-injured porcine ileum. *Surgery*. 2002;132(3):461–470.
19. Arifeen S, Black RE, Antelman G, Baqui A, Caulfield L, Becker S. Exclusive breastfeeding reduces acute respiratory infection and diarrhea deaths among infants in Dhaka slums. *Pediatrics*. 2001;108(4):E67.
20. Brown KH, Black RE, Lopez de Romana G, Creed de Kanashiro H. Infant-feeding practices and their relationship with diarrheal and other diseases in Huascar (Lima), Peru. *Pediatrics*. 1989;83(1):31–40.
21. Team WCS. Effect of breastfeeding on infant and child mortality due to infectious diseases in less developed countries: a pooled analysis. WHO Collaborative Study Team on the Role of Breastfeeding on the Prevention of Infant Mortality. *Lancet*. 2000;355(9202):451–455.
22. Liu Y, Song M, Che TM, et al. Dietary plant extracts alleviate diarrhea and alter immune responses of weaned pigs experimentally infected with a pathogenic Escherichia coli. *J Anim Sci*. 2013;91(11):5294–5306.
23. Meurens F, Summerfield A, Nauwynck H, Saif L, Gerdts V. The pig: a model for human infectious diseases. *Trends Microbiol*. 2012;20(1):50–57.
24. Odle J, Lin X, Jacobi SK, Kim SW, Stahl CH. The suckling piglet as an agrimedical model for the study of pediatric nutrition and metabolism. *Annu Rev Anim Biosci*. 2014;2: 419–444.
25. Mizoguchi A. Animal models of inflammatory bowel disease. *Prog Mol Biol Transl Sci*. 2012;105:263–320.
26. Koleva PT, Valcheva RS, Sun X, Ganzle MG, Dieleman LA. Inulin and fructo-oligosaccharides have divergent effects on colitis and commensal microbiota in HLA-B27 transgenic rats. *Br J Nutr*. 2012;108(9):1633–1643.
27. Hedrich HJ, Bullock G. *The Laboratory Mouse*. Boston, MA: Academic Press; 2004.
28. Jiminez JA, Uwiera TC, Douglas Inglis G, Uwiera RR. Animal models to study acute and chronic intestinal inflammation in mammals. *Gut Pathog*. 2015;7:29.
29. Mestas J, Hughes CC. Of mice and not men: differences between mouse and human immunology. *J Immunol*. 2004; 172(5):2731–2738.
30. Gibbons DL, Spencer J. Mouse and human intestinal immunity: same ballpark, different players; different rules, same score. *Mucosal Immunol*. 2011;4(2):148–157.
31. Gerdts V, Uwiera RR, Mutwiri GK, et al. Multiple intestinal 'loops' provide an in vivo model to analyse multiple mucosal immune responses. *J Immunol Methods*. 2001;256(1–2):19–33.
32. Estes JD, Wong SW, Brenchley JM. Nonhuman primate models of human viral infections. *Nat Rev Immunol*. 2018;18(6): 390–404.
33. Friedman H, Ator N, Haigwood N, et al. The critical role of nonhuman primates in medical research. *Pathog Immun*. 2017;2(3):352–365.
34. Walters EM, Wolf E, Whyte JJ, et al. Completion of the swine genome will simplify the production of swine as a large animal biomedical model. *BMC Med Genomics*. 2012;5:55.
35. Wang M, Donovan SM. Human microbiota-associated swine: current progress and future opportunities. *ILAR J* 2015;56(1):63–73.
36. Patterson JK, Lei XG, Miller DD. The pig as an experimental model for elucidating the mechanisms governing dietary influence on mineral absorption. *Exp Biol Med (Maywood)*. 2008;233(6):651–664.
37. Zhang Q, Widmer G, Tzipori S. A pig model of the human gastrointestinal tract. *Gut Microbes*. 2013;4(3):193–200.
38. Bailey M, Christoforidou Z, Lewis MC. The evolutionary basis for differences between the immune systems of man, mouse, pig and ruminants. *Vet Immunol Immunopathol*. 2013; 152(1–2):13–19.
39. Guilloteau P, Zabielski R, Hammon HM, Metges CC. Nutritional programming of gastrointestinal tract development. Is the pig a good model for man? *Nutr Res Rev*. 2010; 23(1):4–22.
40. Yen JT. Anatomy of the digestive system and nutritional physiology. In: Lewis AJ, Southern LL, eds. *Swine Nutrition*. Washington, DC: CRC Press; 2001:31–64.
41. Kararli TT. Comparison of the gastrointestinal anatomy, physiology, and biochemistry of humans and commonly used laboratory animals. *Biopharm Drug Dispos*. 1995;16(5): 351–380.
42. Ley RE, Bäckhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI. Obesity alters gut microbial ecology. *Proc Natl Acad Sci USA*. 2005;102(31):11070–11075.
43. Joint FAO/WHO/UNU Expert Consultation on Protein and Amino Acid Requirements in Human Nutrition (2002: Geneva, Switzerland), Food and Agriculture Organization of the United Nations, World Health Organization & United Nations University. Protein and amino acid requirements in human nutrition: report of a joint FAO/WHO/UNU expert consultation. World Health Organization. 2007.
44. Jacobi SK, Odle J. Nutritional factors influencing intestinal health of the neonate. *Adv Nutr*. 2012;3(5):687–696.
45. Dawson HD. Comparative assessment of the pig, mouse, and human genomes: a structural and functional analysis of genes involved in immunity. In: McAnulty PA, Dayan A, Hastings KH, Ganderup N-C, eds. *The Minipig in Biomedical Research*. Boca Raton, FL: CRC Press; 2011:321–341.
46. Dawson HD, Loveland JE, Pascal G, et al. Structural and functional annotation of the porcine immunome. *BMC Genomics*. 2013;14:332–332.
47. Dawson HD, Chen C, Gaynor B, Shao J, Urban JF. The porcine translational research database: a manually curated, genomics and proteomics-based research resource. *BMC Genomics*. 2017;18:643.

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48. Sun Y, Kim SW. Intestinal challenge with enterotoxigenic Escherichia coli in pigs, and nutritional intervention to prevent postweaning diarrhea. *Anim Nutr*. 2017;3(4):322–330.
49. Wenzel H, Kaminski RW, Clarkson KA, et al. Improving chances for successful clinical outcomes with better preclinical models. *Vaccine*. 2017;35(49, Part A):6798–6802.
50. Deneke CF, McGowan K, Larson AD, Gorbach SL. Attachment of human and pig (K88) enterotoxigenic Escherichia coli strains to either human or porcine small intestinal cells. *Infect Immun*. 1984;45(2):522–524.
51. Zhang W, Robertson DC, Zhang C, Bai W, Zhao M, Francis DH. Escherichia coli constructs expressing human or porcine enterotoxins induce identical diarrheal diseases in a piglet infection model. *Appl Environ Microbiol*. 2008;74(18):5832–5837.
52. Swindle MM, Makin A, Herron AJ, Clubb FJ Jr, Frazier KS. Swine as models in biomedical research and toxicology testing. *Vet Pathol*. 2011;49(2):344–356.
53. Rothkotter HJ. Anatomical particularities of the porcine immune system—a physician's view. *Dev Comp Immunol*. 2009;33(3):267–272.
54. Leece JG. Effect of dietary regimen on cessation of uptake of macromolecules by piglet intestinal epithelium (closure) and transport to the blood. *J Nutr*. 1973;103(5):751–756.
55. Komuves LG, Heath JP. Uptake of maternal immunoglobulins in the enterocytes of suckling piglets: improved detection with a streptavidin-biotin bridge gold technique. *J Histochem Cytochem*. 1992;40(11):1637–1646.
56. Brandtzaeg P. The mucosal immune system and its integration with the mammary glands. *J Pediatr*. 2010;156(2 Suppl):S8–S15.
57. Mair KH, Sedlak C, Käser T, et al. The porcine innate immune system: an update. *Dev Comp Immunol*. 2014;45(2):321–343.
58. Summerfield A, McCullough KC. The porcine dendritic cell family. *Dev Comp Immunol*. 2009;33(3):299–309.
59. USDA. 2008. *Part IV: Changes in the U.S. Pork Industry, 1990–2006*. Fort Collins, CO: USDA-APHIS-VS, CEAH.
60. Nagy B, Whipp SC, Imberechts H, et al. Biological relationship between F18ab and F18ac fimbriae of enterotoxigenic and verotoxigenic Escherichia coli from weaned pigs with oedema disease or diarrhoea. *Microb Pathog*. 1997;22(1):1–11.
61. Almeida JA, Liu Y, Song M, et al. Escherichia coli challenge and one type of smectite alter intestinal barrier of pigs. *J Anim Sci Biotechnol*. 2013;4(1):52–59.
62. Kim K, Ehrlich A, Perng V, et al. Algae-derived β-glucan enhanced gut health and immune responses of weaned pigs experimentally infected with a pathogenic E. coli. *Anim Feed Sci Technol*. 2019;248:114–125.
63. Song M, Liu Y, Soares JA, et al. Dietary clays alleviate diarrhea of weaned pigs. *J Anim Sci*. 2012;90(1):345–360.
64. Nagy B, Fekete PZ. Enterotoxigenic Escherichia coli (ETEC) in farm animals. *Vet Res*. 1999;30(2–3):259–284.
65. Hampson DJ. Postweaning Escherichia coli diarrhea in pigs. In: Gyles CL, ed. *Escherichia coli in Domestic Animals and Humans*. Guildford, UK: CAB International; 1994:171–191.
66. Liu Y. *Effects of plant extracts on immune function and disease resistance in pigs*. Dissertation. Unviersity of Illinois, Urbana-Champaign; 2011.
67. Gibbons RA, Sellwood R, Burrows M, Hunter PA. Inheritance of resistance to neonatal E. coli diarrhoea in the pig: examination of the genetic system. *Theor Appl Genet*. 1977;51(2):65–70.
68. Vogeli P, Bertschinger HU, Stamm M, et al. Genes specifying receptors for F18 fimbriated Escherichia coli, causing oedema disease and postweaning diarrhoea in pigs, map to chromosome 6. *Anim Genet*. 1996;27(5):321–328.
69. Verdonck F, Cox E, van Gog K, et al. Different kinetic of antibody responses following infection of newly weaned pigs with an F4 enterotoxigenic Escherichia coli strain or an F18 verotoxigenic Escherichia coli strain. *Vaccine*. 2002;20(23–24):2995–3004.
70. Jones GW, Rutter JM. Role of the K88 antigen in the pathogenesis of neonatal diarrhea caused by Escherichia coli in piglets. *Infect Immun*. 1972;6(6):918–927.
71. Wittig W, Klie H, Gallien P, Lehmann S, Timm M, Tschape H. Prevalence of the fimbrial antigens F18 and K88 and of enterotoxins and verotoxins among Escherichia coli isolated from weaned pigs. *Zentralbl Bakteriol*. 1995;283(1):95–104.
72. Frydendahl K. Prevalence of serogroups and virulence genes in Escherichia coli associated with postweaning diarrhoea and edema disease in pigs and a comparison of diagnostic approaches. *Vet Microbiol*. 2002;85(2):169–182.
73. Loos M, Geens M, Schauvliege S, et al. Role of heat-stable enterotoxins in the induction of early immune responses in piglets after infection with enterotoxigenic Escherichia coli. *PLoS One*. 2012;7(7):e41041.
74. Rose R, Whipp SC, Moon HW. Effects of Escherichia coli heat-stable enterotoxin b on small intestinal villi in pigs, rabbits, and lambs. *Vet Pathol*. 1987;24(1):71–79.
75. Buddle JR, Bolton JR. The pathophysiology of diarrhoea in pigs. *Pig News Info*. 1992;13:41N–45N.
76. Makvana S, Krilov LR. Escherichia coli infections. *Pediatr Rev*. 2015;36(4):167–170; quiz 171.
77. Helander A, Wenneras C, Qadri F, Svennerholm AM. Antibody responses in humans against coli surface antigen 6 of enterotoxigenic Escherichia coli. *Infect Immun*. 1998;66(9):4507–4510.
78. Rao MR, Wierzba TF, Savarino SJ, et al. Serologic correlates of protection against enterotoxigenic Escherichia coli diarrhea. *J Infect Dis*. 2005;191(4):562–570.
79. Wenneras C, Qadri F, Bardhan PK, Sack RB, Svennerholm AM. Intestinal immune responses in patients infected with enterotoxigenic Escherichia coli and in vaccinees. *Infect Immun*. 1999;67(12):6234–6241.
80. Ishimine N, Honda T, Yoshizawa A, et al. Combination of white blood cell count and left shift level real-timely reflects a course of bacterial infection. *J Clin Lab Anal*. 2013;27(5):407–411.
81. Schenk M, Mueller C. The mucosal immune system at the gastrointestinal barrier. *Best Pract Res Clin Gastroenterol*. 2008;22(3):391–409.
82. Fournier BM, Parkos CA. The role of neutrophils during intestinal inflammation. *Mucosal Immunol*. 2012;5(4):354–366.
83. Liu Y, Song M, Che TM, et al. Dietary plant extracts modulate gene expression profiles in ileal mucosa of weaned pigs after an Escherichia coli infection. *J Anim Sci*. 2014;92(5):2050–2062.
84. Manary M, Iannotti L, Trehan I, Weisz A. Systematic review of the care of children with diarrhoea in the community-based management of severe acute malnutrition. 2012. http://www.who.int/nutrition/publications/guidelines/updates_management_SAM_infantandchildren_review4.pdf. Accessed April 12, 2019.
85. Gaffey MF, Wazny K, Bassani DG, Bhutta ZA. Dietary management of childhood diarrhea in low- and middle-income

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countries: a systematic review. *BMC Public Health*. 2013;13 (Suppl 3):S17.
86. Guandalini S, Pensabene L, Zikri MA, et al. Lactobacillus GG administered in oral rehydration solution to children with acute diarrhea: a multicenter European trial. *J Pediatr Gastroenterol Nutr*. 2000;30(1):54–60.
87. Kaila M, Isolauri E, Soppi E, Virtanen E, Laine S, Arvilommi H. Enhancement of the circulating antibody secreting cell response in human diarrhea by a human Lactobacillus strain. *Pediatr Res*. 1992;32(2):141–144.
88. Saavedra JM, Bauman NA, Oung I, Perman JA, Yolken RH. Feeding of Bifidobacterium bifidum and Streptococcus thermophilus to infants in hospital for prevention of diarrhoea and shedding of rotavirus. *Lancet*. 1994;344(8929): 1046–1049.
89. Lievin-Le Moal V. A gastrointestinal anti-infectious biotherapeutic agent: the heat-treated Lactobacillus LB. *Therap Adv Gastroenterol*. 2016;9(1):57–75.
90. Sazawal S, Black RE, Bhan MK, Bhandari N, Sinha A, Jalla S. Zinc supplementation in young children with acute diarrhea in India. *N Engl J Med*. 1995;333(13):839–844.
91. Pettigrew JE. Reduced use of antibiotic growth promoters in diets fed to weanling pigs: dietary tools, part 1. *Anim Biotechnol*. 2006;17(2):207–215.
92. Kil D, Stein H. Board invited review: management and feeding strategies to ameliorate the impact of removing antibiotic growth promoters from diets fed to weanling pigs. *Can J Anim Sci*. 2010;90(4):447–460.
93. Liu Y, Espinosa CD, Abelilla JJ, et al. Non-antibiotic feed additives in diets for pigs: a review. *Anim Nutr*. 2018;4(2): 113–125.
94. Gadde U, Kim WH, Oh ST, Lillehoj HS. Alternatives to antibiotics for maximizing growth performance and feed efficiency in poultry: a review. *Anim Health Res Rev*. 2017;18(1): 26–45.
95. Windisch W, Schedle K, Plitzner C, Kroismayr A. Use of phytogenic products as feed additives for swine and poultry. *J Anim Sci*. 2008;86(14 Suppl):E140–E148.
96. Baydar NG, Özkan G, Sağdiç O. Total phenolic contents and antibacterial activities of grape (Vitis vinifera L.) extracts. *Food Control*. 2004;15(5):335–339.
97. Sokmen M, Serkedjieva J, Daferera D, et al. In vitro antioxidant, antimicrobial, and antiviral activities of the essential oil and various extracts from herbal parts and callus cultures of Origanum acutidens. *J Agric Food Chem*. 2004;52(11): 3309–3312.
98. Dundar E, Olgun EG, Isiksoy S, Kurkcuoglu M, Baser KH, Bal C. The effects of intra-rectal and intra-peritoneal application of Origanum onites L. essential oil on 2,4,6-trinitrobenzenesulfonic acid-induced colitis in the rat. *Exp Toxicol Pathol*. 2008;59(6):399–408.
99. Liu Y, Song M, Che TM, Bravo D, Pettigrew JE. Antiinflammatory effects of several plant extracts on porcine alveolar macrophages in vitro. *J Anim Sci*. 2012;90(8):2774–2783.
100. Hammer KA, Carson CF, Riley TV. Antimicrobial activity of essential oils and other plant extracts. *J Appl Microbiol*. 1999;86(6):985–990.
101. Dorman HJ, Deans SG. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *J Appl Microbiol*. 2000;88(2):308–316.
102. Wong SY, Grant IR, Friedman M, Elliott CT, Situ C. Antibacterial activities of naturally occurring compounds against Mycobacterium avium subsp. paratuberculosis. *Appl Environ Microbiol*. 2008;74(19):5986–5990.
103. Carson CF, Mee BJ, Riley TV. Mechanism of action of Melaleuca alternifolia (tea tree) oil on Staphylococcus aureus determined by time-kill, lysis, leakage, and salt tolerance assays and electron microscopy. *Antimicrob Agents Chemother*. 2002;46(6):1914–1920.
104. Burt S. Essential oils: their antibacterial properties and potential applications in foods—a review. *Int J Food Microbiol*. 2004;94(3):223–253.
105. Xu J, Zhou F, Ji BP, Pei RS, Xu N. The antibacterial mechanism of carvacrol and thymol against Escherichia coli. *Lett Appl Microbiol*. 2008;47(3):174–179.
106. Farag RS, Daw ZY, Hewedi FM, El-Baroty GSA. Antimicrobial activity of some Egyptian spice essential oils. *J Food Prot*. 1989;52(9):665–667.
107. Lambert RJ, Skandamis PN, Coote PJ, Nychas GJ. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *J Appl Microbiol*. 2001;91(3):453–462.
108. Ankri S, Mirelman D. Antimicrobial properties of allicin from garlic. *Microbes Infect*. 1999;1(2):125–129.
109. Burt SA, van der Zee R, Koets AP, et al. Carvacrol induces heat shock protein 60 and inhibits synthesis of flagellin in Escherichia coli O157:H7. *Appl Environ Microbiol*. 2007;73 (14):4484–4490.
110. Verhelst R, Schroyen M, Buys N, Niewold T. The effects of plant polyphenols on enterotoxigenic Escherichia coli adhesion and toxin binding. *Livest Sci*. 2010;133(1):101–103.
111. Cetin-Karaca H, Newman MC. Antimicrobial efficacy of phytochemicals against Bacillus cereus in reconstituted infant rice cereal. *Food Microbiol*. 2018;69:189–195.
112. Lang A, Lahav M, Sakhnini E, et al. Allicin inhibits spontaneous and TNF-alpha induced secretion of proinflammatory cytokines and chemokines from intestinal epithelial cells. *Clin Nutr*. 2004;23(5):1199–1208.
113. Lee SH, Lee SY, Son DJ, et al. Inhibitory effect of 2'-hydroxycinnamaldehyde on nitric oxide production through inhibition of NF-kappa B activation in RAW 264.7 cells. *Biochem Pharmacol*. 2005;69(5):791–799.
114. Tung YT, Chua MT, Wang SY, Chang ST. Anti-inflammation activities of essential oil and its constituents from indigenous cinnamon (Cinnamomum osmophloeum) twigs. *Bioresour Technol*. 2008;99(9):3908–3913.
115. Jobin C, Bradham CA, Russo MP, et al. Curcumin blocks cytokine-mediated NF-kappa B activation and proinflammatory gene expression by inhibiting inhibitory factor I-kappa B kinase activity. *J Immunol*. 1999;163(6): 3474–3483.
116. Choi CY, Park KR, Lee JH, et al. Isoeugenol suppression of inducible nitric oxide synthase expression is mediated by down-regulation of NF-kappaB, ERK1/2, and p38 kinase. *Eur J Pharmacol*. 2007;576(1–3):151–159.
117. Bruins MJ, Cermak R, Kiers JL, van der Meulen J, van Amelsvoort JM, van Klinken BJ. In vivo and in vitro effects of tea extracts on enterotoxigenic Escherichia coli-induced intestinal fluid loss in animal models. *J Pediatr Gastroenterol Nutr*. 2006;43(4):459–469.
118. Coddens A, Loos M, Vanrompay D, Remon JP, Cox E. Cranberry extract inhibits in vitro adhesion of F4 and F18 (+)Escherichia coli to pig intestinal epithelium and reduces in vivo excretion of pigs orally challenged with F18(+) verotoxigenic E. coli. *Vet Microbiol*. 2017;202:64–71.
119. WHO. The global burden of disease. 2004. http://www.who.int/healthinfo/global_burden_disease/GBD_report_2004update_full.pdf. Accessed April 12, 2019.

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120. Ekor M. The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety. *Front Pharmacol*. 2014;4:177–186.
121. de Wet H, Nkwanyana MN, van Vuuren SF. Medicinal plants used for the treatment of diarrhoea in northern Maputaland, KwaZulu-Natal Province, South Africa. *J Ethnopharmacol*. 2010;130(2):284–289.
122. Njume C, Goduka NI. Treatment of diarrhoea in rural African communities: an overview of measures to maximise the medicinal potentials of indigenous plants. *Int J Environ Res Public Health*. 2012;9(11):3911–3933.
123. Palombo EA. Phytochemicals from traditional medicinal plants used in the treatment of diarrhoea: modes of action and effects on intestinal function. *Phytother Res*. 2006;20(9):717–724.
124. Macfarlane GT, Cummings JH. Probiotics and prebiotics: can regulating the activities of intestinal bacteria benefit health? *BMJ*. 1999;318(7189):999–1003.
125. Houdijk JGM, Hartemink R, Verstegen MWA, Bosch MW. Effects of dietary non-digestible oligosaccharides on microbial characteristics of ileal chime and faeces in weaned pigs. *Arch Anim Nutr*. 2002;56:297–307.
126. Gibson GR, Probert HM, Loo JV, Rastall RA, Roberfroid MB. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. *Nutr Res Rev*. 2004;17(2):259–275.
127. Macfarlane S, Macfarlane GT, Cummings JH. Review article: prebiotics in the gastrointestinal tract. *Aliment Pharmacol Ther*. 2006;24(5):701–714.
128. Kelly G. Inulin-type prebiotics—a review: part 1. *Altern Med Rev*. 2008;13(4):315–329.
129. Boehm G, Jelinek J, Stahl B, et al. Prebiotics in infant formulas. *J Clin Gastroenterol*. 2004;38(6 Suppl):S76–S79.
130. Zeković DB, Kwiatkowski S, Vrvić MM, Jakovljević D, Moran CA. Natural and modified (1→3)-β-D-glucans in health promotion and disease alleviation. *Crit Rev Biotechnol*. 2005;25(4):205–230.
131. Samuelsen AB, Schrezenmeir J, Knutsen SH. Effects of orally administered yeast-derived beta-glucans: a review. *Mol Nutr Food Res*. 2014;58(1):183–193.
132. Soltanian S, Stuyven E, Cox E, Sorgeloos P, Bossier P. Beta-glucans as immunostimulant in vertebrates and invertebrates. *Crit Rev Microbiol*. 2009;35(2):109–138.
133. Russo P, López P, Capozzi V, et al. Beta-glucans improve growth, viability and colonization of probiotic microorganisms. *Int J Mol Sci*. 2012;13(5):6026–6039.
134. Toure R, Kheadr E, Lacroix C, Moroni O, Fliss I. Production of antibacterial substances by bifidobacterial isolates from infant stool active against Listeria monocytogenes. *J Appl Microbiol*. 2003;95(5):1058–1069.
135. Gibson GR, Wang X. Regulatory effects of bifidobacteria on the growth of other colonic bacteria. *J Appl Bacteriol*. 1994;77(4):412–420.
136. Corr SC, Li Y, Riedel CU, O'Toole PW, Hill C, Gahan CG. Bacteriocin production as a mechanism for the antiinfective activity of Lactobacillus salivarius UCC118. *Proc Natl Acad Sci USA*. 2007;104(18):7617–7621.
137. Gibson GR, McCartney AL, Rastall RA. Prebiotics and resistance to gastrointestinal infections. *Br J Nutr*. 2005;93(Suppl 1):S31–S34.
138. Collado MC, Grzeskowiak L, Salminen S. Probiotic strains and their combination inhibit in vitro adhesion of pathogens to pig intestinal mucosa. *Curr Microbiol*. 2007;55(3):260–265.
139. Mack DR, Ahrne S, Hyde L, Wei S, Hollingsworth MA. Extracellular MUC3 mucin secretion follows adherence of Lactobacillus strains to intestinal epithelial cells in vitro. *Gut*. 2003;52(6):827–833.
140. Ten Bruggencate SJ, Bovee-Oudenhoven IM, Lettink-Wissink ML, Katan MB, van der Meer R. Dietary fructooligosaccharides affect intestinal barrier function in healthy men. *J Nutr*. 2006;136(1):70–74.
141. Zyrek AA, Cichon C, Helms S, Enders C, Sonnenborn U, Schmidt MA. Molecular mechanisms underlying the probiotic effects of Escherichia coli Nissle 1917 involve ZO-2 and PKCzeta redistribution resulting in tight junction and epithelial barrier repair. *Cell Microbiol*. 2007;9(3):804–816.
142. Schnaar RL. Glycosphingolipids in cell surface recognition. *Glycobiology*. 1991;1(5):477–485.
143. Pratt VC, Tappenden KA, McBurney MI, Field CJ. Short-chain fatty acid-supplemented total parenteral nutrition improves nonspecific immunity after intestinal resection in rats. *JPEN J Parenter Enteral Nutr*. 1996;20(4):264–271.
144. Bohmig GA, Krieger PM, Saemann MD, Wenhardt C, Pohanka E, Zlabinger GJ. n-butyrate downregulates the stimulatory function of peripheral blood-derived antigen-presenting cells: a potential mechanism for modulating T-cell responses by short-chain fatty acids. *Immunology*. 1997;92(2):234–243.
145. Schley PD, Field CJ. The immune-enhancing effects of dietary fibres and prebiotics. *Br J Nutr*. 2002;87(Suppl 2):S221–S230.
146. Halas D, Hansen CF, Hampson DJ, Mullan BP, Wilson RH, Pluske JR. Effect of dietary supplementation with inulin and/or benzoic acid on the incidence and severity of post-weaning diarrhoea in weaner pigs after experimental challenge with enterotoxigenic Escherichia coli. *Arch Anim Nutr*. 2009;63(4):267–280.
147. Bunce TJ, Howard MD, Kerley MS, Alee GL, Pace LW. Protective effect of fructooligosaccharide (FOS) in prevention of mortality and morbidity from infectious E. coli K:88 challenge. *J Anim Sci*. 1995;63(Suppl 1):69.
148. Stuyven E, Cox E, Vancaeneghem S, Arnouts S, Deprez P, Goddeeris BM. Effect of beta-glucans on an ETEC infection in piglets. *Vet Immunol Immunopathol*. 2009;128(1–3):60–66.
149. Kim K. Jinno C, Ehrlich A, Li X, Joergensen JN, Raff L, Liu Y. Effects of *Bacillus subtilis* on growth performance, diarrhea, and gut health of weanling pigs experimentally infected with F18 *Escherichia coli*. *J Anim Sci*. 2018;96(Suppl 2):68–69.
150. Goodridge HS, Wolf AJ, Underhill DM. β-glucan recognition by the innate immune system. *Immunol Rev*. 2009;230(1):38–50.
151. Hoekstra JH, Szajewska H, Zikri MA, et al. Oral rehydration solution containing a mixture of non-digestible carbohydrates in the treatment of acute diarrhea: a multicenter randomized placebo controlled study on behalf of the ESPGHAN working group on intestinal infections. *J Pediatr Gastroenterol Nutr*. 2004;39(3):239–245.
152. Bruzzese E, VOLPICELLI M, Salvini F, et al. Early administration of GOS/FOS prevents intestinal and respiratory infections in infants. *J Pediatr Gastroenterol Nutr*. 2006;42(5):E95.
153. Lohner S, Kullenberg D, Antes G, Decsi T, Meerpohl JJ. Prebiotics in healthy infants and children for prevention of acute infectious diseases: a systematic review and meta-analysis. *Nutr Rev*. 2014;72(8):523–531.

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154. Bettler J, Euler AR. An evaluation of the growth of term infants fed formula supplemented with fructo-oligosaccharide. *Int J Prob Preb*. 2006;1(1):19–26.
155. Lonnerdal B. Biochemistry and physiological function of human milk proteins. *Am J Clin Nutr*. 1985;42(6): 1299–1317.
156. Wiesner J, Vilcinskas A. Antimicrobial peptides: the ancient arm of the human immune system. *Virulence*. 2010;1(5): 440–464.
157. Masschalck B, Michiels CW. Antimicrobial properties of lysozyme in relation to foodborne vegetative bacteria. *Crit Rev Microbiol*. 2003;29(3):191–214.
158. Ibrahim HR, Thomas U, Pellegrini A. A helix-loop-helix peptide at the upper lip of the active site cleft of lysozyme confers potent antimicrobial activity with membrane permeabilization action. *J Biol Chem*. 2001;276(47): 43767–43774.
159. Laible NJ, Germaine GR. Bactericidal activity of human lysozyme, muramidase-inactive lysozyme, and cationic polypeptides against Streptococcus sanguis and Streptococcus faecalis: inhibition by chitin oligosaccharides. *Infect Immun*. 1985;48(3):720–728.
160. Ibrahim HR, Matsuzaki T, Aoki T. Genetic evidence that antibacterial activity of lysozyme is independent of its catalytic function. *FEBS Lett*. 2001;506(1):27–32.
161. Ellison RT, Giehl TJ. Killing of gram-negative bacteria by lactoferrin and lysozyme. *J Clin Invest*. 1991;88(4): 1080–1091.
162. Gordon LI, Douglas SD, Kay NE, Yamada O, Osserman EF, Jacob HS. Modulation of neutrophil function by lysozyme. Potential negative feedback system of inflammation. *J Clin Invest*. 1979;64(1):226–232.
163. Vakil JR, Chandan RC, Parry RM, Shahani KM. Susceptibility of several microorganisms to milk lysozymes1,2. *J Dairy Sci*. 1969;52(8):1192–1197.
164. Lönnerdal B, Erdmann P, Thakkar SK, Sauser J, Destaillats F. Longitudinal evolution of true protein, amino acids and bioactive proteins in breast milk: a developmental perspective. *J Nutr Biochem*. 2017;41(Supplement C):1–11.
165. Meyer F, Erhardt G, Senft B. Environmental and genetic aspects of lysozyme in cow's milk. *Zuchtungskunde*. 1981;53: 17–27.
166. Chandan RC, Parry RM, Jr., Shahani KM.Lysozyme, lipase, and ribonuclease in milk of various species. *J Dairy Sci*. 1968;51(4):606–607.
167. Tong J, Wei H, Liu X, et al. Production of recombinant human lysozyme in the milk of transgenic pigs. *Transgenic Res*. 2011;20(2):417–419.
168. Bezirtzoglou E, Tsiotsias A, Welling GW. Microbiota profile in feces of breast- and formula-fed newborns by using fluorescence in situ hybridization (FISH). *Anaerobe*. 2011;17 (6):478–482.
169. Fallani M, Young D, Scott J, et al. Intestinal microbiota of 6-week-old infants across Europe: geographic influence beyond delivery mode, breast-feeding, and antibiotics. *J Pediatr Gastroenterol Nutr*. 2010;51(1):77–84.
170. Benno Y, Sawada K, Mitsuoka T. The intestinal microflora of infants: composition of fecal flora in breast-fed and bottle-fed infants. *Microbiol Immunol*. 1984;28(9):975–986.
171. Zavaleta N, Figueroa D, Rivera J, Sanchez J, Alfaro S, Lonnerdal B. Efficacy of rice-based oral rehydration solution containing recombinant human lactoferrin and lysozyme in Peruvian children with acute diarrhea. *J Pediatr Gastroenterol Nutr*. 2007;44(2):258–264.
172. Garas LC, Cooper CA, Dawson MW, Wang JL, Murray JD, Maga EA. Young pigs consuming lysozyme transgenic goat milk are protected from clinical symptoms of enterotoxigenic Escherichia coli infection. *J Nutr*. 2017;147(11): 2050–2059.
173. Maga EA, Desai PT, Weimer BC, Dao N, Kültz D, Murray JD. Consumption of lysozyme-rich milk can alter microbial fecal populations. *Appl Environ Microbiol*. 2012;78(17): 6153–6160.
174. Lu D, Liu S, Shang S, et al. Production of transgenic-cloned pigs expressing large quantities of recombinant human lysozyme in milk. *PLoS One*. 2015;10(5):e0123551.
175. Huang G, Li X, Lu D, et al. Lysozyme improves gut performance and protects against enterotoxigenic Escherichia coli infection in neonatal piglets. *Vet Res*. 2018;49(1):20.
176. Conrad MS, Dilger RN, Johnson RW. Brain growth of the domestic pig (Sus scrofa) from 2 to 24 weeks of age: a longitudinal MRI study. *Dev Neurosci*. 2012;34(4):291–298.
177. Rothkotter HJ, Sowa E, Pabst R. The pig as a model of developmental immunology. *Hum Exp Toxicol*. 2002;21(9–10):533–536.
178. Roura E, Koopmans SJ, Lalles JP, et al. Critical review evaluating the pig as a model for human nutritional physiology. *Nutr Res Rev*. 2016;29(1):60–90.
179. Lei S, Ramesh A, Twitchell E, et al. High protective efficacy of probiotics and rice bran against human norovirus infection and diarrhea in gnotobiotic pigs. *Front Microbiol*. 2016; 7:1699.
180. Yang X, Wen K, Tin C, et al. Dietary rice bran protects against rotavirus diarrhea and promotes Th1-type immune responses to human rotavirus vaccine in gnotobiotic pigs. *Clin Vaccine Immunol*. 2014;21(10):1396–1403.
181. Kamada N, Chen GY, Inohara N, Nunez G. Control of pathogens and pathobionts by the gut microbiota. *Nat Immunol*. 2013;14(7):685–690.
182. Brundige DR, Maga EA, Klasing KC, Murray JD. Lysozyme transgenic goats' milk influences gastrointestinal morphology in young pigs. *J Nutr*. 2008;138(5):921–926.
183. Cooper CA, Garas Klobas LC, Maga EA, Murray JD. Consuming transgenic goats' milk containing the antimicrobial protein lysozyme helps resolve diarrhea in young pigs. *PLoS One*. 2013;8(3):e58409.
184. Liu P, Piao XS, Thacker PA, et al. Chito-oligosaccharide reduces diarrhea incidence and attenuates the immune response of weaned pigs challenged with Escherichia coli K88. *J Anim Sci*. 2010;88(12):3871–3879.
185. Krause DO, Bhandari SK, House JD, Nyachoti CM. Response of nursery pigs to a synbiotic preparation of starch and an anti-Escherichia coli K88 probiotic. *Appl Environ Microbiol*. 2010;76(24):8192–8200.
186. Daudelin JF, Lessard M, Beaudoin F, et al. Administration of probiotics influences F4 (K88)-positive enterotoxigenic Escherichia coli attachment and intestinal cytokine expression in weaned pigs. *Vet Res*. 2011;42:69.
187. Kiarie E, Bhandari S, Scott M, Krause DO, Nyachoti CM. Growth performance and gastrointestinal microbial ecology responses of piglets receiving Saccharomyces cerevisiae fermentation products after an oral challenge with Escherichia coli (K88). *J Anim Sci*. 2011;89(4):1062–1078.
188. Kiarie E, Scott M, Krause DO, Khazanehei H, Khafipour E, Nyachoti CM. Interactions of Saccharomyces cerevisiae fermentation product and in-feed antibiotic on gastrointestinal and immunological responses in piglets challenged with Escherichia coli K88+. *J Anim Sci*. 2012;90(Suppl 4):1–3.

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189. Lee JS, Awji EG, Lee SJ, et al. Effect of Lactobacillus plantarum CJLP243 on the growth performance and cytokine response of weaning pigs challenged with enterotoxigenic Escherichia coli. *J Anim Sci*. 2012;90(11):3709–3717.
190. Yang KM, Jiang ZY, Zheng CT, Wang L, Yang XF. Effect of Lactobacillus plantarum on diarrhea and intestinal barrier function of young piglets challenged with enterotoxigenic Escherichia coli K88. *J Anim Sci*. 2014;92(4):1496–1503.
191. Devi SM, Lee SI, Kim IH. Effect of phytogenics on growth performance, fecal score, blood profiles, fecal noxious gas emission, digestibility, and intestinal morphology of weanling pigs challenged with Escherichia coli K88. *Pol J Vet Sci*. 2015;18(3):557–564.
192. Li H, Zhao P, Lei Y, Li T, Kim I. Response to an Escherichia coli K88 oral challenge and productivity of weanling pigs receiving a dietary nucleotides supplement. *J Anim Sci Biotechnol*. 2015;6:49–57.
193. Amalaradjou MA, Venkitanarayanan K. Proteomic analysis of the mode of antibacterial action of trans-cinnamaldehyde against Cronobacter sakazakii 415. *Foodborne Pathog Dis*. 2011;8(10):1095–1102.
194. Ankri S, Miron T, Rabinkov A, Wilchek M, Mirelman D. Allicin from garlic strongly inhibits cysteine proteinases and cytopathic effects of Entamoeba histolytica. *Antimicrob Agents Chemother*. 1997;41(10):2286–2288.
195. Devi KP, Nisha SA, Sakthivel R, Pandian SK. Eugenol (an essential oil of clove) acts as an antibacterial agent against Salmonella typhi by disrupting the cellular membrane. *J Ethnopharmacol*. 2010;130(1):107–115.
196. Diao W-R, Hu Q-P, Zhang H, Xu J-G. Chemical composition, antibacterial activity and mechanism of action of essential oil from seeds of fennel (Foeniculum vulgare Mill.). *Food Control*. 2014;35(1):109–116.
197. Focke M, Feld A, Lichtenthaler K. Allicin, a naturally occurring antibiotic from garlic, specifically inhibits acetyl-CoA synthetase. *FEBS Lett*. 1990;261(1):106–108.
198. Gill AO, Holley RA. Mechanisms of bactericidal action of cinnamaldehyde against Listeria monocytogenes and of eugenol against L. monocytogenes and Lactobacillus sakei. *Appl Environ Microbiol*. 2004;70(10):5750–5755.
199. Gill AO, Holley RA. Inhibition of membrane bound ATPases of Escherichia coli and Listeria monocytogenes by plant oil aromatics. *Int J Food Microbiol*. 2006;111(2):170–174.
200. Rabinkov A, Miron T, Konstantinovski L, Wilchek M, Mirelman D, Weiner L. The mode of action of allicin: trapping of radicals and interaction with thiol containing proteins. *Biochim Biophys Acta*. 1998;1379(2):233–244.
201. Rai D, Singh JK, Roy N, Panda D. Curcumin inhibits FtsZ assembly: an attractive mechanism for its antibacterial activity. *Biochem J*. 2008;410(1):147–155.
202. Shlar I, Droby S, Rodov V. Modes of antibacterial action of curcumin under dark and light conditions: a toxicoproteomics approach. *J Proteomics*. 2017;160:8–20.
203. Trombetta D, Castelli F, Sarpietro MG, et al. Mechanisms of antibacterial action of three monoterpenes. *Antimicrob Agents Chemother*. 2005;49(6):2474–2478.
204. Upadhyay A, Johny AK, Amalaradjou MA, Ananda Baskaran S, Kim KS, Venkitanarayanan K. Plant-derived antimicrobials reduce Listeria monocytogenes virulence factors in vitro, and down-regulate expression of virulence genes. *Int J Food Microbiol*. 2012;157(1):88–94.
205. Anadón A, Martínez-Larrañaga MR, Ares I, Martínez MA. Prebiotics: safty and toxicity considerations. In: Gupta RC, ed. *Nutrceuticals: Efficacy, Safety, and Toxicity*. Cambridge, MA: Academic Press; 2016:757–775.

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Review Article

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Immune Relevant Models for Ocular Inflammatory Diseases

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Abstract

Ocular inflammatory diseases, such as dry eye and uveitis, are common, painful, difficult to treat, and may result in vision loss or blindness. Ocular side effects from the use of antiinflammatory drugs (such as corticosteroids or nonsteroidal antiinflammatories) to treat ocular inflammation have prompted development of more specific and safer medications to treat inflammatory and immune-mediated diseases of the eye. To assess the efficacy and safety of these new therapeutics, appropriate immune-relevant animal models of ocular inflammation are needed. Both induced and naturally-occurring models have been described, but the most valuable for translating treatments to the human eye are the animal models of spontaneous, immunologic ocular disease, such as those with dry eye or uveitis. The purpose of this review is to describe common immune-relevant models of dry eye and uveitis with an overview of the immuno-pathogenesis of each disease and reported evaluation of models from small to large animals. We will also review a selected group of naturally-occurring large animal models, equine uveitis and canine dry eye, that have promise to translate into a better understanding and treatment of clinical immune-relevant ocular disease in man.

Key words: animal models; dry eye; immune-relevant; inflammatory; naturally-occurring; ocular; uveitis

Introduction

Blindness or low vision affects approximately 1 in 28 Americans older than 40 years of age, the underlying causes of which are commonly noninfectious immune-mediated diseases, including dry eye and uveitis.[1–3] Dry eye symptoms are experienced by 20% of adults over 45 years old, and uveitis is a leading cause of blindness in the United States.[1–6] Dry eye and uveitis are also common causes of blindness in domestic animals, and uveitis is the leading cause of blindness in horses worldwide.[7–12] There are no known cures for immune-mediated ocular diseases, and current treatment regimens are costly, require multiple daily applications, are poorly effective, and have adverse side effects. Therefore, new treatments to address these diseases are needed and for further development, there is a need for accurate and translatable immune-relevant models of ocular disease.

The eye, like the brain and the uterus in pregnancy, is considered an immune privileged site.[13,14] An active suppression of the immune response to endogenous and exogenous antigens occurs in the eye, as overt inflammation may compromise vision. The relative lack of antigen-presenting and MHC II-expressing cells and natural tissue barriers (i.e., the blood–ocular barrier) that physically separate ocular tissues from the systemic immune response contribute to the immune tolerance in the eye.[15] With dry eye and uveitis, the normal ocular tolerance is lost (from several initiating causes) and the physical barriers become disrupted, allowing an influx of inflammatory cells. In addition, proinflammatory mediators induce T-helper cells to proliferate, activate antigen-presenting cells, expand auto-reactive B and T cell populations, and ultimately release proinflammatory and proapoptotic peptides.[16,17] Current treatments for dry eye and uveitis are

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nonspecific and require frequent use of topical medications that may have severe ocular and systemic side effects.[18–20] Furthermore, these medications are life-long therapies and patient compliance is commonly poor, leading to treatment failures, worsening of disease, and in some cases, blindness.[21]

When testing effectiveness of therapeutics on models of ocular disease, there are two separate but important testing goals. The first question is whether the drug is effective in the ocular disease state that is being studied. For this goal, usually rats or mice are evaluated and dosed by a nonocular route, for example, orally, subcutaneously, or intraperitoneally. These studies help determine pathogenesis of disease-drug mechanisms; therefore, the wide array of reagents and genetically modified mice and rats are a major asset. Determination of the appropriate dose (i.e., dose ranging studies) is usually also performed in these first sets of studies. The second goal is to determine if an appropriate dose can reach the ocular target tissue and be effective in the eye using a dosing route and frequency that is clinically feasible. These studies would determine the pharmacokinetics and pharmacodynamics of a specific route of administration of a drug, typically in a normal eye, then repeated using the optimal dosing and routes in eyes of models of the disease state. For this second group of studies to be clinically valid in most instances, the animal models would have to have eyes anatomically similar to the target species and in the case of humans, use of the rabbit, dog, pig, or primate eye would be most appropriate. Finally, when selecting the appropriate animal model, the target tissue and disease state has to be paired with the most appropriate route of therapy. This determination is important for pharmacokinetic, toxicologic, and efficacy studies.

Although there are many disease conditions of the human eye thought to have an immunologic pathogenesis, including allergic conjunctivitis, corneal transplant rejection, and age-related macular degeneration, as examples, the purpose of this review is to describe common immune-relevant models of dry eye and uveitis with an overview and assessment of models from small to large animals. We will also review a selected group of naturally-occurring large animal models, equine uveitis and canine dry eye, which have promise to translate into a better understanding and treatment of clinical immune-relevant ocular disease in man.

Review of Commonly Used Animal Models in Inflammatory Ocular Disease

Ocular Surface Disease Immune-Relevant Models

Dry Eye Disease

Dry eye disease (DED) is one of the most common ocular abnormalities and has multiple underlying causes. Dry eye is a disease of the tear film and ocular surface that results in symptoms of discomfort and visual disturbance with potential damage to the ocular surface.[22] In one study, nearly one-half of patients claimed to have symptoms of dry eye with a negative effect on quality of life, including ocular pain, decreased activities requiring visual attention (e.g., reading, driving), and reduced productivity in the workplace.[21] Dry eye develops from a deficiency of the aqueous portion of the tear fluid as a result of reduced lacrimal aqueous tear secretion or a result of increased evaporation of tears, such as the result of Meibomian gland deficiencies.[23] Decreased aqueous production of the tears results in an increase of tear electrolytes (i.e., increased tear osmolality), proteins, and inflammatory mediators, resulting in damage to the surface ocular tissues, decreased visual acuity, and ocular discomfort. The relative decrease in aqueous tears on the ocular surface in patients with DED causes chronic irritation to ocular surface that disrupts the normal ocular immune tolerance.[24] With breakdown of ocular surface tolerance and immune-homeostasis, autoimmunity develops through activation of NK cells and Toll-like receptors, followed by release of proinflammatory factors such as interleukin (IL)-1α, IL-1β, tumor necrosis factor α, and IL-6. These mediators amplify, activating antigen-presenting cells, which internalize autoantigens and migrate to the draining cervical lymph node where autoreactive Th1 cells, Th17 cells, or B cells (i.e., in Sjogren's syndrome) undergo expansion. Efferent trafficking of these autoreactive T cells to the ocular surface is directed by adhesion molecules (e.g., LFA-1) and chemokine receptors. Autoreactive T-cells in ocular surface tissues potentiate the chronic autoimmune response, resulting in epithelial cell apoptosis, reduced goblet cell density, and squamous metaplasia of epithelium.[17,24,25]

Current treatments for DED rely on frequently applied artificial tears, punctal plugs, topical tetracycline antibiotic, and omega fatty acids, all of which provide only temporary relief of dry eye.[26] Chronic DED is commonly treated with antiinflammatory medications and immunosuppressants, the latter being the mainstay of treatment in the United States.[27,28] Topical cyclosporine, an immunosuppressant, used with or without corticosteroids, is effective in DED through inhibition of T-cell activation and reduction of proinflammatory cytokines.[29] A recently approved topical immunosuppressive for treatment of DED, lifitegrast, is an integrin inhibitor that prevents binding of LFA-1 to ICAM-1, which is upregulated in DED. Lifitegrast thus blocks T-cell efferent recruitment to ocular tissues and reduces inflammatory cytokines.[30–32] However, both cyclosporine and lifitegrast must be administered indefinitely twice daily by the patient and are associated with burning sensation after application, leading to reduced patient compliance and hence poor treatment efficacy and success. Therefore, an effective, long-term, well-tolerated, and convenient therapy for DED is needed.

There are numerous models of ocular surface disease and dry eye, but to be immune relevant, there needs to be evidence of an immuno-pathogenesis in the disease process. There are several mouse models of dry eye disease, the most common of which is a model induced by low humidity and high air flow environments, with or without the additional use of scopolamine (Table 1).[33–35] The extended environmental irritation to the surface of the eye of these mice disrupts the normal ocular immune tolerance and immunohomeostasis,[24] as described previously. These mice models have been used to study the immuno-pathogenesis of dry eye and the initial evaluation of therapeutics. Another described model is the use of repeated application of topical benzalkonium chloride to the mouse or rabbit eye. This produces chronic irritation that may develop immunopathology and chronic ocular surface disease.[36,37] Other induced models of DED in rodents, which may be less immunopathologic in origin, include lacrimal gland excision or injections of toxins or antigens such as botulinum toxin[38] or concanavalin A.[39] Genetic models, such as the MRL/lpr mouse, manifest multiple autoimmune disorders and can be helpful to study diseases such as systemic lupus erythematosus and Sjorgren's syndrome (Table 1).[40] Another example of genetic DED are neurturin-deficient mice, which may develop dry eye and serve as models for neurotrophic keratoconjunctivitis sicca, since this model lacks lacrimal innervation (Table 1).[41] There are numerous other knockout and transgenic mice strains that are commonly studied that may develop DED; however, many of these models do not develop clinical signs of DED observed in large animal models, but instead develop histologic or other features characteristic of human DED.[42]

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Table 1 Selected immune-models of dry eye disease

Animal	Method	Advantages	Disadvantages	Reference
Rodent				
Mice	Environmental chambers ± scopolamine patch	Reproducible, economical	Small eye, anatomic differences	30
	Botulinum toxin injection into lacrimal gland	Reproducible, economical	Above, and toxin present	29,38
	Intraorbital injection of concanavalin A		Above, possible orbital inflammation	39
	Topical administration of benzalkonium chloride	Economical	Above, time to develop, may not be immunologic	36
	Extraorbital lacrimal gland excision ± scopolamine	Surgically induced desiccating irritation	Initiates an abnormal Th1/Th17 T cell response, exogenous antigens	43,44
	Neurturin-deficient (NRTN(−/−))			41
	MRL/lpr	Sjogrens-like dry eye, autoimmune pathogenesis	Chronic model, systemic disease	40,45–47
Rat	Extraorbital lacrimal gland excision ± scopolamine		May not be immunologic	48,49
Rabbit	Activated autologous lymphocytes injected into lacrimal gland	Sjögren's-like autoimmune dacryoadenitis	Specialized, difficult model	50
	Topical administration of benzalkonium chloride	Economical	Above, time to develop, may not be immunologic	37
	Lacrimal gland excision		May not be immunologic	51
Canine	Naturally-occurring	Immunologic, translatable	Availability	9–11

In rats, the most commonly described dry eye model is the extraorbital lacrimal gland excision model (with or without use of scopolamine).[48,49] Like other models of induced dry eye, this rat lacrimal-excision model likely does not develop, substantially, an immunologic pathogenesis and therefore may not be as effective for evaluation of immunosuppressive therapies as naturally-occurring models of dry eye.[33,49] All of these rodent models of dry eye are similar in that they can be used to determine proof of principal of therapeutic response to a drug, but all have similar disadvantages of having orbital and lacrimal anatomy and eye size that differs from the human eye. Rabbit or dog models are more commonly used to evaluate dry eye signs and response to therapy, because they have easily measured decreased tear production and develop ocular surface changes.[42] Therefore, larger animal dry eye models are needed (see later description of canine dry eye).

Rabbits are commonly used as models of ocular disease and for pharmacokinetic studies because of their relatively large eye, compared to rodents, while still being a common and economical laboratory animal. However, there are few true immune-relevant models of DED in rabbits. Most described models induce dry eye signs, but not likely an immunopathogenesis, by use of a topical irritant, such as benzalkonium chloride, or by short-term reduction in lacrimal secretion using parasympathomimetic drug, such as atropine.[37,52,53] A very promising model of autoimmune dacryoadenitis in rabbits that produces a Sjögren's-like keratoconjunctivitis is created by an intra-lacrimal or subcutaneous injection of autologous peripheral blood lymphocytes activated by purified rabbit lacrimal epithelial cells.[54,55] This rabbit autoimmune dacryoadenitis model has been used to effectively evaluate immunomodulatory treatments for dry eye, including topical cyclosporine and lacrimal gland adeno-associated virus (AAV) mediated-IL-10 gene therapy.[50,56]

Naturally-Occurring Keratoconjunctivitis Sicca in Canines

Domestic canines develop spontaneous dry eye that clinically and immunopathologically is similar to dry eye in humans (Table 1).[10] Not only do dogs spontaneously develop dry eye symptoms of ocular discomfort, conjunctival hyperemia, and corneal scarring, these symptoms correlate directly with reduced aqueous tear production, a reduction readily measured using a standard Schirmer tear test strip (Figure 1). Furthermore, dogs with dry eye have a reduced tear breakup time and increased corneal staining, all abnormalities also observed in humans with DED.[10] Like humans, canine dry eye is typically bilateral, develops in middle age, is more common in female dogs and in certain breeds, such as the American Cocker spaniel, Bulldog, and West Highland white terrier.[57] The pathogenesis of dry eye in dogs appears similar to that of humans, where an apparent immunologic inflammation occurs with progressive lymphocytic infiltration and damage to the lacrimal gland with subsequent decreased production of the aqueous tear film.[58,59] With chronicity, the ocular surface becomes progressively more dessicated and inflamed, the cornea vascularizes and scars, and ultimately the dog may lose vision.[9,57] Initial proof of concept of commonly used immunosuppressive eye drops was first demonstrated to be effective in this spontaneous dog model, including topical cyclosporine, tacrolimus, and LTF-1 inhibitors.[9,10,12,60,61]

Uveitis Disease Models

Uveitis is inflammation of the iris, ciliary body, and choroid and is associated with both infectious and noninfectious causes. Uveitis is estimated to be the third leading cause of preventable

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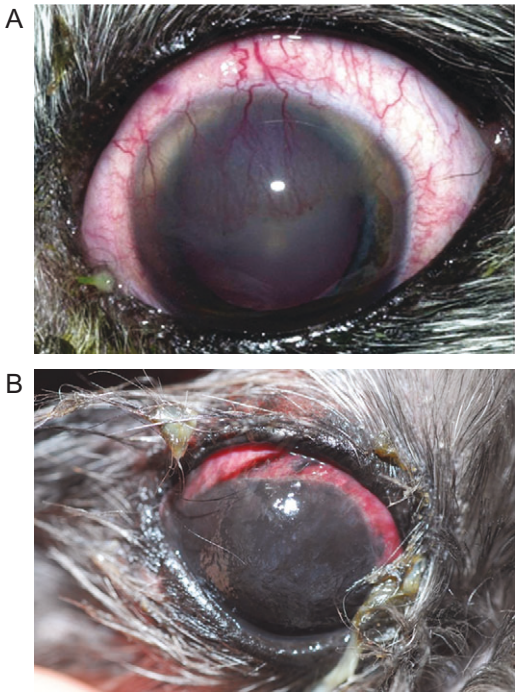

Figure 1 Naturally-occurring dry eye in a dog. (A) Moderate dry eye disease in a dog resulting in conjunctival hyperemia, corneal vascularization, and corneal opacity. (B) Chronic dry eye disease in a dog with mucopurulent ocular discharge, hyperpigmented cornea, and conjunctival hyperemia.

blindness worldwide.[62] In the United States, the incidence of uveitis was estimated to be approximately 58 to 69 cases/100,000 people;[1,6] however, another study estimated that the rate of uveitis, especially anterior uveitis, was approximately 3 times higher and it increased with increasing age of patients.[4] The most common causes of uveitis in humans are human leukocyte antigen (HLA)-B27 related uveitis, acute anterior uveitis in herpes zoster disease, toxoplasmosis, sarcoidosis, and pars planitis.[63]

Uveitis results from several causes. The uveal tract supplies blood to the eye and is in direct contact with peripheral vasculature; therefore, diseases of the systemic circulation (e.g., septicemia, bacteremia, infection, activated lymphocytes, immune diseases, etc.) will disrupt the *blood-ocular barrier*.[64,65] The blood-ocular barrier prevents large molecules and cells from entering the eye and thus limits the immune response to intraocular antigens. With trauma or inflammation, this barrier can be disrupted, allowing blood products and cells to enter the eye, resulting in the clinical signs typical of uveitis, such as flare, cell accumulation, and vitreous haze. Disruption of the barrier enables activation of various host immune responses, including antibody production to self-antigens that are not normally recognized by the immune system, as well as antibody production to foreign antigens inside the eye.

As a result of the blood-ocular barrier, lack of lymphatics, and the presence of limited numbers of resident leukocytes, the eye is considered to have immune privilege. Naïve T cells cannot cross the normal blood-retinal barrier due to the lack of fenestration in the retinal vessels and the lack of appropriate adhesion molecules.[66] Expression of chemokines in inflammation and activated T cells in the ciliary epithelium may play a role in recruitment and activation of leukocytes in diseased eyes.[67] As in other autoimmune disorders, infections may trigger events, either by antigenic mimicry with a pathogen's antigen or as a bystander effect due to the general systemic or local immune stimulation by the pathogen. Uveitogenic retinal proteins documented in experimental animals include retinal arrestin, interphotoreceptor retinoid-binding protein (IRBP), rhodopsin, recoverin, phosducin, and retinal pigment epithelium derived RPE-65.[62,68–70] Irrespective of the eliciting antigen, available experimental evidence suggests that the immunological mechanisms driving the resultant disease are similar.[16] Following disruption of the blood-ocular barrier, large amounts of predominantly CD4+ T cells enter the eye and secrete proinflammatory cytokines such as IL-2 and interferon γ.[71] Auto-reactive effector CD4+ T cells have been associated with the pathogenesis of inflammatory and autoimmune disorders including uveitis. Naıve CD4+ T cells differentiate into effector subsets depending on the nature of the environment in which exposure to the antigen occurs.[66] Several T cell effector phenotypes have been defined, known as T helper 1 (T_H1), T_H2, or T_H17. Early studies suggested that the interferon-γ-producing T_H1 and IL-17-releasing T_H17 subsets are responsible for the pathology of uveitis, with the latter being associated with development of autoimmune disease.[16] Additionally, clinical uveitis frequently develops spontaneous recurrent or relapsing bouts of inflammation, likely from T cells recognizing additional autoantigens in the ocular tissue.[72] Resolution of uveitis is dependent on the presence of T regulatory cells (Tregs) that are labeled as $CD4^+Foxp3^+$ cells. When $Foxp3^+$ T cell percentages in uveitis increase to approximately 10% of the total $CD4^+$ cells, the acute inflammation rapidly resolves. Therefore, $Foxp3^+$ Tregs are important to induce spontaneous resolution and in maintaining remission of uveitis.[73]

Multiple models have been developed to evaluate the immuno-pathogenesis of uveitis and recurrent uveitis, including identification of autoantigens. Most of these models are rodent based. Other models, including those that are acute, chronic, and recurrent in nature, have been developed to evaluate therapeutics (Table 2). Large animal models, such as uveitis induced in rabbits and pigs, have been evaluated to test therapeutics in larger eyes to help translate these treatments to humans (Table 2).

Rodent Models of Uveitis

Endotoxin-induced uveitis A commonly used model of induced uveitis in rodents is the endotoxin-induced uveitis (EIU) model (Table 2).[74,75,89] The uveitis in this model is primarily an acute anterior uveitis (i.e., iris, ciliary body) that is thought to be driven by the innate immune system (Table 2).[16,76] Following intraparentoneal, subcutaneous, or hind footpad injection of endotoxin (lipopolysaccharide; 100 μg or 500 μg) in Lewis or Sprague-Dawley rats or various mouse strains (C3H),[16] ocular inflammation develops within hours of injection characterized by a breakdown of the blood-aqueous barrier and the development of clinical disease. Clinical and histopathologic abnormalities peak at 24 hours and resolve by 48 to 72 hours.[74,75]

Experimental autoimmune uveitis (or uveoretinitis) Experimental autoimmune uveitis (EAU) is a primarily posterior uveitis (or panuveitis [i.e., inflammation of the iris, ciliary, and choroid]) that is induced by immunizing susceptible rodents with retinal antigens (e.g., S-antigen [S-ag], IRBP, recoverin, rhodopsin/opsin); while experimental melanin–protein induced uveitis, a predominantly anterior uveitis, is elicited by immunization with melanin (from RPE) or tyrosinase-related proteins 1 and 2 (Table 2).[16] The predominant animal model is the Lewis rat, but other animals such as the guinea pig or mice have also been described.[76,90] Injection of autoantigens into rodents, combined with bacterial adjuvants, results in EAU; EAU does not develop without the use of adjuvants. The use of complete Freund's adjuvant (*Mycobacterium* cell

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Table 2. Models of uveitis

Type of uveitis	Species/Strains	Agents	Type of inflammation	Advantages	Disadvantages	Reference
Rodent						
EIU	Lewis rat: Harlan Sprague Dawley Mice: (C3H and other strains)	Endotoxin	Anterior uveitis	Rapid onset, predictable	Nonimmunologic	74,75
EAU	Rat	Melanin from bovine RPE Tyrosinase-related proteins 1 and 3	Anterior uveitis	Immunologic		76
Experimental autoimmune uveoretinitis	Mice Rat	Retinal arrestin (S-Ag), IRBP, recoverin, phosducin, rhodopsin/opsin	Posterior segment	Immunologic		73,77–79
Spontaneous	Mice	RBP T cell receptor transgenic mice (R161H) Autoimmune Regulator (AIRE)(−/−) mice Adoptive transfer	Retinal degeneration and persistent cellular infiltrates and lymphoid aggregation, multi-focal infiltrates and severe choroidal inflammation.			77,79
Rabbit						
EIU	NZW Dutch belted	Endotoxin	Anterior and posterior segment	Rapid onset, predictable	Nonimmunogenic	80,81
Recurrent uveitis	NZW	*Mycobacterium tuberculosis* H37Ra antigen Ovalbumin	Anterior (intracameral) or posterior (intravitreal) segment	Immunologic and recurrent		82–85
Cytokine induced uveitis	NZW	IL-1 TNF-alpha		Acute onset (6 hours)	Nonimmunologic	80,86
Porcine						
EIU	Various	Endotoxin	Posterior	Large eye	Nonimmunogenic	87
Horse						
Recurrent uveitis	Various	Spontaneous	Anterior and posterior	Large eye; immunologic; recurrent	Nonstandard research animal Cost	7,68,88

EAU, experimental autoimmune anterior uveitis; EIU, endotoxin-induced uveitis; IL, interleukin; NZW, Zealand White.

wall product) or pertussis toxin is necessary to stimulate the innate immune response and develop inflammation[73,76,78] that ultimately generates activated antigen-presenting cells capable of presenting the injected autoantigen with the coactivation factors required to activate T cells capable of recognizing the antigen. Severe EAU was induced in B6 mice by adoptive transfer of IRBP-specific T cells.[79]

Most of the rodent experimental models of uveitis are not recurrent. They often elicit a single, albeit chronic course of uveitis that eventually resolves. Therefore, the immunologic pathways involved in the development of these rodent models may not be the same as in naturally occurring uveitis. Spontaneous uveitis has been observed in various mouse models, including IRBP T cell receptor transgenic mice (R161H) and autoimmune regulator (AIRE)(-/-) mice.[78] These mouse models have a gradual onset of chronic ocular inflammation that ultimately leads to retinal degeneration.[78]

Despite limitations, these rodent experimental models offer great insight into the pathogenesis and immunopathogenesis of uveitis. These models have been critical in evaluation of therapies, particularly broader immunosuppressive therapies, for treating uveitis.

Rabbit Models of Uveitis

Two rabbit models of uveitis have been most commonly evaluated, including the acute uveitis induced by injection of endotoxin[84,91–93] and the recurrent uveitis induced by tuberculosis antigen.[82,84,94] Other uveitis models in rabbits include those following intravitreal injection of human interleukin 1 alpha,[86] TNF-alpha,[80] or ovalbumin in animals previously ovalbumin-immunized,[85] among others (Table 2).

An advantage of rabbit models of uveitis over rodent models is that the rabbit eye is more similar in size to the human eye, and therefore, more pharmacologically valid when evaluating routes of therapy. In the endotoxin-induced uveitis, after 10 to 100 ng of LPS is injected intracamerally[92] or intravitreally,[80,81] aqueous flare and iridal hyperemia develop within 6 hours, suggesting rapid disruption of the blood–aqueous barrier.[91] The LPS induces inflammation by activating a Toll-like receptor 4-initiated signaling cascade. The inflammatory response peaks at approximately 24 hours after injection, then rapidly declines.[92] Like the rat model of EIU, this endotoxin rabbit model of uveitis is not considered to have a predominantly immunopathogenesis.

Experimental uveitis can be induced by unilateral intravitreal or intracameral injection of *Mycobacterium tuberculosis* H37Ra antigen (50 μg; 1 μg/L) in preimmunized rabbits, typically 7 to 14 days after initial subcutaneous injection.[82–84,95] To simulate chronic recurrent inflammation, eyes are re-challenged with intravitreal antigen every 14 to 21 days.[96] This model has advantages similar to the endotoxin model; however, it is predominantly a T-cell lymphocyte-mediated uveitis that can be induced to be recurrent and therefore, more closely simulate endogenous human uveitis.[95]

Porcine Models of Uveitis

The pig has been used as a large animal model of uveitis (Table 2), which, similar to the rabbit model, has an eye similar in size to the human eye; however, unlike the rabbit, it has a retinal vascular anatomy similar to humans.[97] An acute model of uveitis has been used in the pig to evaluate novel therapeutics and routes of administration. In this model, similar to endotoxin uveitis in rabbits, endotoxin is injected intravitreally and the eye is monitored for up to 72 hours following injection.[87] Like rodent EIU and endotoxin uveitis in rabbits, the endotoxin porcine model of uveitis is not considered to have an immunopathogenesis.

Naturally-Occurring Uveitis Models

Equine Recurrent Uveitis Horses spontaneously develop severe, immunologic uveitis called equine recurrent uveitis (ERU) that is frequently recurrent and chronic (Figure 2).[98] ERU is the most common cause of blindness in horses.[7,8,98] Spontaneous bouts of uveitis develop and blindness may occur after multiple recurrent episodes of uveitis. The immunopathology of ERU has been extensively studied and has demonstrated that T cells are the predominant mononuclear inflammatory cells infiltrating ocular tissues in horses with naturally occurring chronic uveitis, with a significant number of CD4+ cells.[71,72,99] Recruitment of proinflammatory cells as well as autoreactive lymphocytes may be in part driven by the expression of the chemokine RANTES in the ciliary body.[67]

Study of this common, spontaneous uveitis in horses has helped understand the pathogenesis of uveitis in humans, especially the identification of autoantigens and how recurrence of uveitis develops immunologically.[7,69,70,72,100–103] Several potential autoantigens have been identified in horses that could play a role in the development of autoimmune uveitis. T cells isolated from the eyes of horses with ERU proliferate in response to two common autoantigens in rodents: retinal S-Ag and IRBP.[69] In addition, several additional potential autoantigens were identified by analyzing antibodies in the sera of ERU horses that reacted with retinal proteins. These include recoverin, cellular retinaldehyde-binding protein, and malate dehydrogenase.[101] While all these potential autoantigens are capable of inducing experimental uveitis in rodent models, only cellular retinaldehyde-binding protein and IRBP consistently produce uveitis in outbred horses.[70,100]

Additionally, studies of horses with ERU have also helped elucidate how *Leptospira* infections induce immunological uveitis, specifically autoimmune uveitis.[104–106] Field studies of horses in the 1950s after an outbreak of acute leptospirosis caused by *L. interrogans* serogroup Pomona demonstrated that one of the six horses (17%) developed intraocular inflammation during acute leptospiral disease, and all horses developed ERU 18 to 24 months after the initial infection. Subsequent studies demonstrated cross-reactivity between equine ocular tissues and *Leptospira* antigens,[104,105] and horses with uveitis associated with *Leptospira interrogans* infections had high levels of IgA and IgG in their intraocular fluids that reacted to two *Leptospira* lipoproteins, LruA and LruB.[106,107] These antibodies were also subsequently discovered in the serum of human leptospiral uveitis patients.[108]

Studies of spontaneous ERU have helped elucidate the immunopathogenesis of recurrent uveitis. In autoimmune disease, several autoantigens, or epitopes, participate in the immunopathogenesis; *epitope spreading* is accountable for disease induction, progression, and inflammatory relapses.[72] Epitope spreading is defined as the diversification of epitope specificity from the initial focused, dominant, epitope-specific immune response, directed against a self or foreign protein to cryptic epitopes on that protein (intramolecular spreading) or other proteins (intermolecular spreading).[72] The shifts in immunoreactivity, or epitope spreading, have been documented in ERU and are thought to be responsible for the recurring character of ERU.[72]

ERU, as a model of spontaneous immune-mediated uveitis, has also led to the study of promising therapeutics. For

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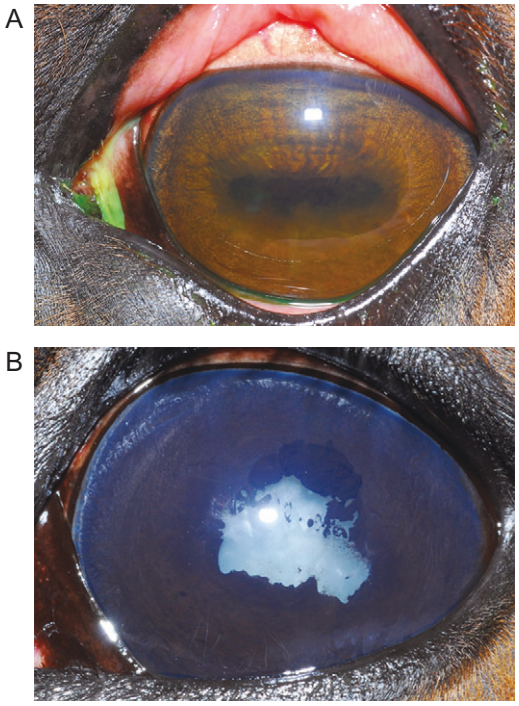

Figure 2 Naturally-occurring uveitis in a horse. (A) Acute active uveitis in a horse, with a miotic pupil and anterior chamber opacity. (B) Chronic uveitis in a horse, from equine recurrent uveitis, with dyscoria, synechiae, and cataract.

example, several sustained release ocular implants have shown much promise in the treatment of ERU.[109,110] Evaluation of drug delivery to the suprachoroidal space has been shown to control ERU and prevent recurrences.[111,112] Triamcinolone injections into the suprachoroidal space are currently under development for treatment of human uveitis.[113] Further study of ERU and its treatment will translate well to improving the understanding and treatment of human autoimmune uveitis.

Next Steps

As further therapeutics are developed that more specifically target immune-mediated diseases, evaluation of these treatments in spontaneous or naturally-occurring models of ocular disease will be needed to provide proof of concept and help translate these therapies to humans. Excellent examples of developing, targeted therapies include gene therapy (especially gene addition therapy) and stem cell therapy. Our laboratory and collaborators at the Gene Therapy Center at the University of North Carolina have developed AAV delivery of immunosuppressive proteins, such as HLA-G, for suppression of ocular surface inflammation and vascularization.[114] Target ocular surface diseases for AAV-HLA-G gene therapy are DED and for prevention of corneal graft rejection.[114]

Autologous stem cell therapy, or use of stem cell supernatant extracts, also shows much promise for immunomodulation in the eye. Effectiveness of topical ocular mesenchymal stem cell therapy was initially demonstrated in dry eye models in mice.[39] Locally injected fat-derived mesenchymal stem cells near the lacrimal glands of dogs with advanced dry eye demonstrated clinical improvement and increased tear production.[115] These results in a naturally-occurring model provides evidence of possible clinical translation to humans with severe dry eye.

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References

1. Acharya NR, Tham VM, Esterberg E, et al. Incidence and prevalence of uveitis: Results from the Pacific Ocular Inflammation Study. *JAMA Ophthalmol*. 2013;131(11):1405–1412. doi:10.1001/jamaophthalmol.2013.4237.
2. Schaumberg DA, Sullivan DA, Buring JE, Dana MR. Prevalence of dry eye syndrome among US women. *Am J Ophthalmol*. 2003;136(2):318–326. doi:10.1016/S0002-9394(03)00218-6.
3. Schaumberg DA, Dana R, Buring JE, Sullivan DA. Prevalence of dry eye disease among US men: Estimates from the Physicians' Health Studies. *Arch Ophthalmol*. 2009;127(6):763–768. doi:10.1001/archophthalmol.2009.103.
4. Gritz DC, Wong IG. Incidence and prevalence of uveitis in Northern California: The Northern California Epidemiology of Uveitis Study. *Ophthalmology*. 2004;111(3):491–500. doi:10.1016/j.ophtha.2003.06.014.
5. Merrill PT, Kim J, Cox TA, Betor CC, McCallum RM, Jaffe GJ. Uveitis in the southeastern United States. *Curr Eye Res*. 1997;16(9):865–874. doi:10.1076/ceyr.16.9.865.5048.
6. Suhler EB, Lloyd MJ, Choi D, Rosenbaum JT, Austin DF. Incidence and prevalence of uveitis in Veterans Affairs Medical Centers of the Pacific Northwest. *Am J Ophthalmol*. 2008;146(6). doi:10.1016/j.ajo.2008.09.014.
7. Deeg CA, Hauck SM, Amann B, et al. Equine recurrent uveitis—A spontaneous horse model of uveitis. *Ophthalmic Res*. 2008;40(3–4):151–153. doi:10.1159/000119867.
8. Gerding JC, Gilger BC. Prognosis and impact of equine recurrent uveitis. *Equine Vet J*. 2015;48(3):290–298. doi:10.1111/evj.12451.
9. Gilger BC, Wilkie DA, Salmon JH, Peel MR. A topical aqueous calcineurin inhibitor for the treatment of naturally occurring keratoconjunctivitis sicca in dogs. *Vet Ophthalmol*. 2013c;16(3):192–197. doi:10.1111/j.1463-5224.2012.01056.x.
10. Kaswan RL, Salisbury MA, Ward DA. Spontaneous canine keratoconjunctivitis sicca. A useful model for human keratoconjunctivitis sicca: Treatment with cyclosporine eye drops. *Arch Ophthalmol*. 1989;107(8):1210–1216. doi:10.1001/archopht.1989.01070020276038.
11. Moore CP, McHugh JB, Thorne JG, Phillips TE. Effect of cyclosporine on conjunctival mucin in a canine keratoconjunctivitis sicca model. *Invest Ophthalmol Vis Sci*. 2001;42(3):653–659. http://www.ncbi.nlm.nih.gov/pubmed/11222523.
12. Murphy CJ, Bentley E, Miller PE, et al. The pharmacologic assessment of a novel lymphocyte function-associated antigen-1 antagonist (SAR 1118) for the treatment of keratoconjunctivitis sicca in dogs. *Investig Ophthalmol Vis Sci*. 2011;52(6):3174–3180. doi:10.1167/iovs.09-5078.
13. Niederkorn JY, Wang S. Immune privilege of the eye and fetus: Parallel universes? *Transplantation*. 2005;80(9):1139–1144. doi:10.1097/01.TP.0000173828.78382.4F.
14. Stein-Streilein J. Immune regulation and the eye. *Trends Immunol*. 2008;29(11):548–554. doi:10.1016/j.it.2008.08.002.
15. Niederkorn JY. See no evil, hear no evil, do no evil: The lessons of immune privilege. *Nat Immunol*. 2006;7(4):354–359. doi:10.1038/ni1328.
16. Caspi RR. Immunological disorders and autoimmunity animal models of autoimmune and immune-mediated uveitis. 2006;3(1):3–9. doi:10.1016/j.ddmod.2006.03.005.

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17. Stern ME, Pflugfelder SC. Pathogenesis: Emphasis on dry eye and the role of the lacrimal functional unit in Sjögren's syndrome. In: Fox R, Fox C, eds. *Sjögren's Syndrome*. New York, NY: Springer; 2011. doi:10.1007/978-1-60327-957-4_13.
18. Carnahan MC, Goldstein DA. Ocular complications of topical, peri-ocular, and systemic corticosteroids. *Curr Opin Ophthalmol*. 2000;11:478–483. doi:10.1097/00055735-200012000-00016.
19. Fraunfelder FT, Sciubba JJ, Mathers WD. The role of medications in causing dry eye. *J Ophthalmol*. 2012;2012:285851. doi:10.1155/2012/285851.
20. Sen HN, Vitale S, Gangaputra SS, et al. Periocular corticosteroid injections in uveitis: Effects and complications. *Ophthalmology*. 2014;121(11):2275–2286. doi:10.1016/j.ophtha.2014.05.021.
21. Uchino M, Schaumberg DA. Dry eye disease: Impact on quality of life and vision. *Curr Ophthalmol Rep*. 2013;1(2):51–57. doi:10.1007/s40135-013-0009-1.
22. DEWS. The definition and classification of dry eye disease: report of the Definition and Classification of the Dry Eye WorkShop (2007). *Ocul Surf*. 2007;5(2):75–92. doi:10.1080/09273940701486803.
23. Lemp MA, Crews LA, Bron AJ, Foulks GN, Sullivan BD. Distribution of aqueous-deficient and evaporative dry eye in a clinic-based patient cohort. *Cornea*. 2012;31(5):472–478. doi:10.1097/ICO.0b013e318225415a.
24. Barabino S, Chen Y, Chauhan S, Dana R. Ocular surface immunity: Homeostatic mechanisms and their disruption in dry eye disease. *Prog Retin Eye Res*. 2012;31(3):271–285. doi:10.1016/j.preteyeres.2012.02.003.
25. Stern ME, Schaumburg CS, Dana R, Calonge M, Niederkorn JY, Pflugfelder SC. Autoimmunity at the ocular surface: Pathogenesis and regulation. *Mucosal Immunol*. 2010;3(5):425–442. doi:10.1038/mi.2010.26.
26. Gayton JL. Etiology, prevalence, and treatment of dry eye disease. *Clin Ophthalmol*. 2009;3(1):405–412.
27. Avunduk AM, Avunduk MC, Varnell ED, Kaufman HE. The comparison of efficacies of topical corticosteroids and non-steroidal anti-inflammatory drops on dry eye patients: A clinical and immunocytochemical study. *Am J Ophthalmol*. 2003;136(4):593–602. doi:10.1016/S0002-9394(03)00326-X.
28. Sall K, Stevenson OD, Mundorf TK, Reis BL. Two multicenter, randomized studies of the efficacy and safety of cyclosporine ophthalmic emulsion in moderate to severe dry eye disease. CsA Phase 3 Study Group. *Ophthalmology*. 2000;107:631–639. doi:S0161642099001761 [pii].
29. Lekhanont K, Leyngold IM, Suwan-Apichon O, Rangsin R, Chuck RS. Comparison of topical dry eye medications for the treatment of keratoconjunctivitis sicca in a botulinum toxin B-induced mouse model. *Cornea*. 2007;26(1):84–89. doi:10.1097/01.ico.0000240079.24583.a1.
30. Keating GM. Lifitegrast ophthalmic solution 5%: A review in dry eye disease. *Drugs*. 2017;77(2):201–208. doi:10.1007/s40265-016-0681-1.
31. Sheppard JD, Torkildsen GL, Lonsdale JD, et al. Semba CPOPUS-1 Study Group.Lifitegrast ophthalmic solution 5.0% for treatment of dry eye disease: Results of the OPUS-1 phase 3 study. *Ophthalmology*. 2014;121:475–483. doi:10.1016/j.ophtha.2013.09.015.
32. Tauber J, Karpecki P, Latkany R, et al. Semba CPOPUS-2 Investigators. Lifitegrast ophthalmic solution 5.0% versus placebo for treatment of dry eye disease results of the randomized phase III OPUS-2 study. *Ophthalmology*. 2015;122(12):2423–2431. doi:10.1016/j.ophtha.2015.08.001.
33. Barabino S, Chen W, Dana MR. Tear film and ocular surface tests in animal models of dry eye: Uses and limitations. *Exp Eye Res*. 2004;79(5):613–621. doi:10.1016/j.exer.2004.07.002.
34. Barabino S, Shen LL, Chen L, Rashid S, Rolando M, Dana MR. The controlled-environment chamber: A new mouse model of dry eye. *Investig Ophthalmol Vis Sci*. 2005;46(8):2766–2771. doi:10.1167/iovs.04-1326.
35. Daull P, Feraille L, Barabino S, et al. Efficacy of a new topical cationic emulsion of cyclosporine A on dry eye clinical signs in an experimental mouse model of dry eye. *Exp Eye Res*. 2016;153:159–164. doi:10.1016/j.exer.2016.10.016.
36. Lin Z, Liu X, Zhou T, et al. A mouse dry eye model induced by topical administration of benzalkonium chloride. *Mol Vis*. 2011;17:257–264.
37. Xiong C, Chen D, Liu J, et al. A rabbit dry eye model induced by topical medication of a preservative benzalkonium chloride. *Investig Ophthalmol Vis Sci*. 2008;49(5):1850–1856. doi:10.1167/iovs.07-0720.
38. Zhu L, Shen J, Zhang C, et al. Inflammatory cytokine expression on the ocular surface in the Botulium toxin B induced murine dry eye model. *Mol Vis*. 2009;15:250–258. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2633460&tool=pmcentrez&rendertype=abstract.
39. Lee MJ, Ko AY, Ko JH, et al. Mesenchymal stem/stromal cells protect the ocular surface by suppressing inflammation in an experimental dry eye. *Mol Ther*. 2015;23(1):139–146. doi:10.1038/mt.2014.159.
40. Jabs DA, Lee B, Burek CL, Saboori AM, Prendergast RA. Cyclosporine therapy suppresses ocular and lacrimal gland disease in MRL/Mp-lpr/lpr mice. *Investig Ophthalmol Vis Sci*. 1996;37(2):377–383.
41. Song XJ, Li DQ, Farley W, et al. Neurturin-deficient mice develop dry eye and keratoconjunctivitis sicca. *Investig Ophthalmol Vis Sci*. 2003;44(10):4223–4229. doi:10.1167/iovs.02-1319.
42. Schrader S, Mircheff AK, Geerling G. Animal models of dry eye. *Dev Ophthalmol*. 2008;41:298–312. doi:10.1159/000131097.
43. Guzmán M, Keitelman I, Sabbione F, Trevani AS, Giordano MN, Galletti JG. Mucosal tolerance disruption favors disease progression in an extraorbital lacrimal gland excision model of murine dry eye. *Exp Eye Res*. 2016;151:19–22. doi:10.1016/j.exer.2016.07.004.
44. Stevenson W, Chen Y, Lee S, et al. Extraorbital lacrimal gland excision: A reproducible model of severe aqueous tear-deficient dry eye disease. *Cornea*. 2014;33(12):1336–1341. doi:10.1097/ICO.0000000000000264.
45. Gao J, Morgan G, Tieu D, et al. ICAM-1 expression predisposes ocular tissues to immune-based inflammation in dry eye patients and Sjögrens syndrome-like MRL/lpr mice. *Exp Eye Res*. 2004;78(4):823–835. doi:10.1016/j.exer.2003.10.024.
46. Jie G, Jiang Q, Rui Z, Yifei Y. Expression of interleukin-17 in autoimmune dacryoadenitis in MRL/lpr mice. *Curr Eye Res*. 2010;35(10):865–871. doi:10.3109/02713683.2010.497600.
47. Ma X, Zou J, He L, Zhang Y. Dry eye management in a Sjögren's syndrome mouse model by inhibition of p38-MAPK pathway. *Diagn Pathol*. 2014;9(1):5. doi:10.1186/1746-1596-9-5.
48. Fujihara T, Murakami T, Fujita H, Nakamura M, Nakata K. Improvement of corneal barrier function by the P2Y(2) agonist INS365 in a rat dry eye model. *Invest Ophthalmol Vis Sci*. 2001;42(1):96–100. http://www.ncbi.nlm.nih.gov/pubmed/11133853.
49. Meng ID, Barton ST, Mecum NE, Kurose M. Corneal sensitivity following lacrimal gland excision in the rat. *Investig Ophthalmol Vis Sci*. 2015;56(5):3347–3354. doi:10.1167/iovs.15-16717.

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50. Thomas PB, Samant DM, Selvam S, et al. Adeno-associated virus-mediated IL-10 gene transfer suppresses lacrimal gland immunopathology in a rabbit model of autoimmune dacryoadenitis. *Investig Ophthalmol Vis Sci*. 2010;51(10):5137–5144. doi:10.1167/iovs.10-5423.
51. Gilbard JP, Rossi SR, Gray KL, Hanninen LA, Kenyon KR. Tear film osmolarity and ocular surface disease in two rabbit models for keratoconjunctivitis sicca. *Investig Ophthalmol Vis Sci*. 1988;29(3):374–378.
52. Burgalassi S, Panichi L, Chetoni P, Saettone MF, Boldrini E. Development of a simple dry eye model in the albino rabbit and evaluation of some tear substitutes. *Ophthalmic Res*. 1999;31(3):229–235. doi:10.1159/000055537.
53. Li C, Song Y, Luan S, et al. Research on the stability of a rabbit dry eye model induced by topical application of the preservative benzalkonium chloride. *PLoS One*. 2012;7(3). doi:10.1371/journal.pone.0033688.
54. Thomas PB, Zhu Z, Selvam S, et al. Autoimmune dacryoadenitis and keratoconjunctivitis induced in rabbits by subcutaneous injection of autologous lymphocytes activated ex vivo against lacrimal antigens. *J Autoimmun*. 2008;31(2):116–122. doi:10.1016/j.jaut.2008.04.019.
55. Zhu Z, Stevenson D, Schechter JE, Mircheff AK, Atkinson R, Trousdale MD. Lacrimal histopathology and ocular surface disease in a rabbit model of autoimmune dacryoadenitis. *Cornea*. 2003;22(1):25–32. doi:10.1097/00003226-200301000-00007.
56. Thomas PB, Samant DM, Zhu Z, et al. Long-term topical cyclosporine treatment improves tear production and reduces keratoconjunctivitis in rabbits with induced autoimmune dacryoadenitis. *J Ocul Pharmacol Ther*. 2009;25(3):285–291. doi:10.1089/jop.2008.0138.
57. Sanchez RF, Innocent G, Mould J, Billson FM. Canine keratoconjunctivitis sicca: Disease trends in a review of 229 cases. *J Small Anim Pract*. 2007;48(4):211–217. doi:10.1111/j.1748-5827.2006.00185.x.
58. Izci C, Celik İ, Alkan F, Erol M, Sur E. Clinical and light microscopic studies of the conjunctival tissues of dogs with bilateral keratoconjunctivitis sicca before and after treatment with topical 2% cyclosporine. *Biotech Histochem*. 2015;90(3):223–230. doi:10.3109/10520295.2014.930177.
59. Kaswan RL, Martin CL, Chapman WL. Keratoconjunctivitis sicca: Histopathologic study of nictitating membrane and lacrimal glands from 28 dogs. *Am J Vet Res*. 1984;45(1):112–118.
60. Barachetti L, Rampazzo A, Mortellaro CM, Scevola S, Gilger BC. Use of episcleral cyclosporine implants in dogs with keratoconjunctivitis sicca: Pilot study. *Vet Ophthalmol*. 2015;18(3):234–241. doi:10.1111/vop.12173.
61. Berdoulay A, English RV, Nadelstein B. Effect of topical 0.02% tacrolimus aqueous suspension on tear production in dogs with keratoconjunctivitis sicca. *Vet Ophthalmol*. 2005;8(4):225–232. doi:10.1111/j.1463-5224.2005.00390.x.
62. Siddique SS, Suelves AM, Baheti U, Foster CS. Glaucoma and uveitis. *Surv Ophthalmol*. 2013;58(1):1–10. doi:10.1016/j.survophthal.2012.04.006.
63. Jabs DA. Epidemiology of uveitis. *Ophthalmic Epidemiol*. 2008;15(5):283–284. doi:10.1080/09286580802478724.
64. Generali E, Cantarini L, Selmi C. Ocular involvement in systemic autoimmune diseases. *Clin Rev Allergy Immunol*. 2015;49(3):263–270. doi:10.1007/s12016-015-8518-3.
65. Levitt AE, McManus KT, McClellan AL, Davis JL, Goldhardt R, Galor A. Ocular inflammation in the setting of concomitant systemic autoimmune conditions in an older male population. *Cornea*. 2015;34(7):762–767. doi:10.1097/ICO.0000000000 000437.
66. Caspi RR. Understanding autoimmune uveitis through animal models the Friedenwald lecture. *Investig Opthalmol Vis Sci*. 2011;52(3):1873. doi:10.1167/iovs.10-6909.
67. Gilger BC, Yang P, Salmon JH, Jaffe GJ, Allen JB. Expression of a chemokine by ciliary body epithelium in horses with naturally occurring recurrent uveitis and in cultured ciliary body epithelial cells. *Am J Vet Res*. 2002;63(7):942–947. doi:10.2460/ajvr.2002.63.942.
68. Deeg CA. Ocular immunology in equine recurrent uveitis. *Vet Ophthalmol*. 2008;11(Suppl 1):61–65. doi:10.1111/j.1463-5224.2008.00625.x.
69. Deeg CA, Kaspers B, Gerhards H, Thurau SR, Wollanke B, Wildner G. Immune responses to retinal autoantigens and peptides in equine recurrent uveitis. *Investig Ophthalmol Vis Sci*. 2001;42(2):393–398.
70. Deeg CA, Pompetzki D, Raith AJ, et al. Identification and functional validation of novel autoantigens in equine uveitis. *Mol Cell Proteomics*. 2006b;5(8):1462–1470. doi:10.1074/mcp.M500352-MCP200.
71. Gilger BC, Malok E, Cutter KV, Stewart T, Horohov DW, Allen JB. Characterization of T-lymphocytes in the anterior uvea of eyes with chronic equine recurrent uveitis. *Vet Immunol Immunopathol*. 1999;71(1):17–28. doi:10.1016/S0165-2427(99)00082-3.
72. Deeg CA, Amann B, Raith AJ, Kaspers B. Inter- and intramolecular epitope spreading in equine recurrent uveitis. *Investig Ophthalmol Vis Sci*. 2006a;47(2):652–656. doi:10.1167/iovs.05-0789.
73. Silver P, Horai R, Chen J, et al. Retina-specific T regulatory cells bring about resolution and maintain remission of autoimmune uveitis. *J Immunol*. 2015;194(7):3011–3019. doi:10.4049/jimmunol.1402650.
74. Cousins SW, Guss RB, Howes EL, Rosenbaum JT. Endotoxin-induced uveitis in the rat: Observations on altered vascular permeability, clinical findings, and histology. *Exp Eye Res*. 1984;39:665–676. doi:10.1016/0014-4835(84)90065-4.
75. Li Q, Peng B, Whitcup SM, Jang SU, Chan C-C. Endotoxin induced uveitis in the mouse: Susceptibility and genetic control. *Exp Eye Res*. 1995;61(5):629–632. doi:10.1016/S0014-4835(05)80056-9.
76. Agarwal RK, Silver PB, Caspi RR. Rodent models of experimental autoimmune uveitis. *Methods Mol Biol*. 2012;900:443–469. doi:10.1007/978-1-60761-720-4_22.
77. Chen J, Qian H, Horai R, Chan CC, Falick Y, Caspi RR. Comparative analysis of induced vs. spontaneous models of autoimmune uveitis targeting the interphotoreceptor retinoid binding protein. *PLoS One*. 2013;8(8). doi:10.1371/journal.pone.0072161.
78. Chen J, Qian H, Horai R, Chan C-C, Caspi RR. Mouse models of experimental autoimmune uveitis: Comparative analysis of adjuvant-induced vs spontaneous models of uveitis. *Curr Mol Med*. 2015;15(6):550–557. http://www.ncbi.nlm.nih.gov/pubmed/26238369.
79. Shao H, Liao T, Ke Y, Shi H, Kaplan HJ, Sun D. Severe chronic experimental autoimmune uveitis (EAU) of the C57BL/6 mouse induced by adoptive transfer of IRBP1-20-specific T cells. *Exp Eye Res*. 2006;82(2):323–331. doi:10.1016/j.exer.2005.07.008.
80. Fleisher LN, Ferrell JB, McGahan MC. Ocular inflammatory effects of intravitreally injected tumor necrosis factor-alpha and endotoxin. *Inflammation*. 1990;14(3):325–335. doi:10.1007/BF00915816.

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81. Goldblum D, Fausch K, Frueh BE, Theurillat R, Thormann W, Zimmerli S. Ocular penetration of caspofungin in a rabbit uveitis model. *Graefe's Arch Clin Exp Ophthalmol*. 2007;245(6):825–833. doi:10.1007/s00417-006-0460-x.
82. Ang M, Ng X, Wong C, et al. Evaluation of a prednisolone acetate-loaded subconjunctival implant for the treatment of recurrent uveitis in a rabbit model. *PLoS One*. 2014;9(5). doi:10.1371/journal.pone.0097555.
83. Edmond M, Yuan A, Bell BA, et al. The feasibility of spectral-domain optical coherence tomography grading of anterior chamber inflammation in a rabbit model of anterior uveitis. *Investig Ophthalmol Vis Sci*. 2016;57(9):OCT184–OCT188. doi:10.1167/iovs.15-18883.
84. Ghosn CR, Li Y, Orilla WC, et al. Treatment of experimental anterior and intermediate uveitis by a dexamethasone intravitreal implant. *Invest Ophthalmol Vis Sci*. 2011;52(6):2917–2923. doi:10.1167/iovs.10-5939.
85. Neumann R, Jabbur NS, Vickers F, Foster SC. Topical diclofenac sodium, dexamethasone and placebo compared in a model of immunogenic uveitis in rabbits. *Ocul Immunol Inflamm*. 1993;1(1–2):87–98. doi:10.3109/09273949309086543.
86. Tilden ME, Boney RS, Goldenberg MM, Rosenbaum JT. The effects of topical S(+)-ibuprofen on interleukin-1 induced ocular inflammation in a rabbit model. *J Ocul Pharmacol*. 1990;6(2):131–135. http://www.ncbi.nlm.nih.gov/pubmed/2394932.
87. Gilger BC, Abarca EM, Salmon JH, Patel S. Treatment of acute posterior uveitis in a porcine model by injection of triamcinolone acetonide into the suprachoroidal space using microneedles. *Invest Ophthalmol Vis Sci*. 2013b;54(4):2483–2492. doi:10.1167/iovs.13-11747.
88. Gerding JC, Gilger BC. Prognosis and impact of equine recurrent uveitis. *Equine Vet J*. 2016;48(3):290–298. doi:10.1111/evj.12451.
89. Altinsoy A, Dileköz E, Kul O, et al. A cannabinoid ligand, anandamide, exacerbates endotoxin-induced uveitis in rabbits. *J Ocul Pharmacol Ther*. 2011;27(6):545–552. doi:10.1089/jop.2011.0049.
90. Wacker WB. Autoimmune uveitis (Choroiditis) in the guinea pig sensitized with homologous uvea and its differentiation from that following sensitization with homologous retina. *Int Arch Allergy Immunol*. 1972;43(1):39–52. doi:10.1159/000230819.
91. Allen JB, McGahan MC, Ferrell JB, Adler KB, Fleisher LN. Nitric oxide synthase inhibitors exert differential time-dependent effects on LPS-induced uveitis. *Exp Eye Res*. 1996;62:21–28.
92. Nussenblatt RB, Calogero D, Buchen SY, Leder HA, Goodkin M, Eydelman MB. Rabbit intraocular reactivity to endotoxin measured by slit-lamp biomicroscopy and laser flare photometry. *Ophthalmology*. 2012;119(7). doi:10.1016/j.ophtha.2012.04.004.
93. Rafie F, Javadzadeh Y, Javadzadeh AR, et al. *In vivo* evaluation of novel nanoparticles containing dexamethasone for ocular drug delivery on rabbit eye. *Curr Eye Res*. 2010;35(12):1081–1089. doi:10.3109/02713683.2010.508867.
94. Mruthyunjaya P. Efficacy of low-release-rate fluocinolone acetonide intravitreal implants to treat experimental uveitis. *Arch Ophthalmol*. 2006;124(7):1012. doi:10.1001/archopht.124.7.1012.
95. Jaffe GJ, Yang CS, Wang XC, Cousins SW, Gallemore RP, Ashton P. Intravitreal sustained-release cyclosporine in the treatment of experimental uveitis. *Ophthalmology*. 1998;105(1):46–56. doi:10.1016/S0161-6420(98)91176-9.
96. Jaffe GJ, Martin D, Callanan D, Pearson PA, Levy B, Comstock T. Fluocinolone acetonide implant (retisert) for noninfectious posterior uveitis. Thirty-four-week results of a multicenter randomized clinical study. *Ophthalmology*. 2006;113(6):1020–1027. doi:10.1016/j.ophtha.2006.02.021.
97. Gilger BC, Abarca E, Salmon JH. Selection of appropriate animal models in ocular research: Ocular anatomy and physiology of common animal models. In: Brian C. Gilger, ed. *Ocular Pharmacology and Toxicology*. Springer Scientific; 2014. ISBN 978-1-62703-744-0 [2013a].
98. Wollanke B, Brem S, Meyer P, et al. Prophylaxis of equine recurrent uveitis (ERU): First results with a leptospiral vaccine in horses. *Pferdeheilkunde*. 2004;20(5):447–454.
99. Kleinwort KJH, Amann B, Hauck SM, Feederle R, Sekundo W, Deeg CA. Immunological characterization of intraocular lymphoid follicles in a spontaneous recurrent uveitis model. *Investig Ophthalmol Vis Sci*. 2016;57(10):4504–4511. doi:10.1167/iovs.16-19787.
100. Deeg CA. A proteomic approach for studying the pathogenesis of spontaneous equine recurrent uveitis (ERU). *Vet Immunol Immunopathol*. 2009;128(1–3):132–136. doi:10.1016/j.vetimm.2008.10.302.
101. Deeg CA, Raith AJ, Amann B, et al. CRALBP is a highly prevalent autoantigen for human autoimmune uveitis. *Clin Dev Immunol*. 2007;2007. doi:10.1155/2007/39245.
102. Degroote RL, Hauck SM, Amann B, Hirmer S, Ueffing M, Deeg CA. Unraveling the equine lymphocyte proteome: Differential septin 7 expression associates with immune cells in equine recurrent uveitis. *PLoS One*. 2014;9(3). doi:10.1371/journal.pone.0091684.
103. Zipplies JK, Hauck SM, Schoeffmann S, et al. Serum PEDF levels are decreased in a spontaneous animal model for human autoimmune uveitis. *J Proteome Res*. 2009;8(2):992–998. doi:10.1021/pr800694y.
104. Lucchesi PMA, Parma AE. A DNA fragment of Leptospira interrogans encodes a protein which shares epitopes with equine cornea. *Vet Immunol Immunopathol*. 1999;71(3–4):173–179. doi:10.1016/S0165-2427(99)00084-7.
105. Lucchesi PMA, Parma AE, Arroyo GH. Serovar distribution of a DNA sequence involved in the antigenic relationship between Leptospira and equine cornea. *BMC Microbiol*. 2002;2:3. doi:10.1186/1471-2180-2-3.
106. Verma A, Artiushin S, Matsunaga J, Haake DA, Timoney JF. LruA and LruB, novel lipoproteins of pathogenic Leptospira interrogans associated with equine recurrent uveitis. *Infect Immun*. 2005;73(11):7259–7266. doi:10.1128/IAI.73.11.7259-7266.2005.
107. Verma A, Kumar P, Babb K, Timoney JF, Stevenson B. Cross-reactivity of antibodies against leptospiral recurrent uveitis-associated proteins A and B (LruA and LruB) with eye proteins. *PLoS Negl Trop Dis*. 2010;4(8). doi:10.1371/journal.pntd.0000778.
108. Verma A, Rathinam SR, Priya CG, Muthukkaruppan VR, Stevenson B, Timoney JF. LruA and LruB antibodies in sera of humans with leptospiral uveitis. *Clin Vaccine Immunol*. 2008;15(6):1019–1023. doi:10.1128/CVI.00203-07.
109. Gilger BC, Malok E, Stewart T, et al. Effect of an intravitreal cyclosporine implant on experimental uveitis in horses. *Vet Immunol Immunopathol*. 2000;76(3–4):239–255. doi:10.1016/S0165-2427(00)00219-1.
110. Gilger BC, Wilkie DA, Davidson MG, Allen JB. Use of an intravitreal sustained-release cyclosporine delivery device for treatment of equine recurrent uveitis. *Am J Vet Res*. 2001;62(12):1892–1896. doi:10.2460/ajvr.2001.62.1892.
111. Gilger BC, Salmon JH, Wilkie DA, et al. A novel bioerodible deep scleral lamellar cyclosporine implant for uveitis. *Invest Ophthalmol Vis Sci*. 2006;47(6):2596–2605. doi:10.1167/iovs.05-1540.

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112. Gilger BC, Wilkie DA, Clode AB, et al. Long-term outcome after implantation of a suprachoroidal cyclosporine drug delivery device in horses with recurrent uveitis. *Vet Ophthalmol*. 2010;13(5):294–300.
113. Moisseiev E, Loewenstein A, Yiu G. The suprachoroidal space: From potential space to a space with potential. *Clin Ophthalmol*. 2016;10:173–178. doi:10.2147/OPTH.S89784.
114. Hirsch ML, Conatser LM, Smith SM, et al. AAV vector-meditated expression of HLA-G reduces injury-induced corneal vascularization and immune cell infiltration. *Sci Rep*. 2017;7(1):17840.
115. Villatoro A, Fernández V. Use of adipose-derived mesenchymal stem cells in keratoconjunctivitis sicca in a canine model. *Biomed Res Int*. 2014;2015(2015):10. doi:10.1155/2015/527926.

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