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The future of amphibian immunology: Opportunities and challenges

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ABSTRACT

Historically, amphibians have been essential to our understanding of vertebrate biology and animal development. Because development from egg to tadpole to adult frog can be directly observed, amphibians contributed greatly to our understanding of not only vertebrate animal development but also the development of the immune system. The South African clawed frog (*Xenopus laevis*) has been key to many of these findings. For example, using *Xenopus* as a model, the comparative immunology community learned about the contribution of hematopoietic stem cells to development of the immune system and about the diversity of antibodies, B cells, T cells and antigen presenting cells. Amphibians offer many advantages as unique potential model systems to address questions about immune skin interactions, host responses to mycobacteria, the diverse functions of interferons, and immune and mucosal interactions. However, there are also many challenges to advance the research including the lack of specific reagents and well annotated genomes of diverse species. While much is known, many important questions remain. The aim of this short commentary is to look to the future of comparative immunology of amphibians as a group. By identifying some important questions or "information-deficit" areas of research, I hope to pique the interest of younger developing scientists and persuade funding agencies to continue to support comparative immunology studies including those of amphibians.

1. Introduction: the future of amphibian immunology

In the past fifty years, the science of immunology and the branch of immunology termed comparative immunology have both flourished. Based primarily on studies of humans and mice, immunology has become a mature science. We now understand the complexity of the immune system and the development of immune responses in mammalian species in amazing detail. Immunology as a science is like a giant sturdy tree with growing branches that help to guide the development of better treatments for human diseases. Comparative immunology is one small branch of the studies that shape immunology, and amphibian immunology is a smaller branch of the larger field of comparative immunology. So, do we now understand immunity in all vertebrate species based on the studies of mammalian species? Is it important to keep studying new species including new amphibians? Is the immune system of amphibians just a variation of what later evolved in mammals? Why should the world and funding agencies care?

My personal view is that we should care because amphibians are central to many ecosystems. They consume vast numbers of insects as adults, and are, in turn, consumed as prey by snakes, birds, and mammals. Amphibians are at a continuing risk of declines due to chytridiomycosis caused by the chytrid fungi *Batrachochytrium dendrobatidis* (*Bd*) (Berger et al., 1998; Longcore et al., 1999) and *Batrachochytrium salamandrivorans* (*Bsal*) (Martel et al., 2013), as well as diseases caused by ranaviruses (Chinchar et al., 2017) and the protistan parasites Perkinsea (Chambouvet et al., 2015; Smilansky et al., 2021; reviewed in Smilansky and Richards, 2023). The importance of amphibians as a group can be illustrated by the loss of amphibians in Panama and Costa Rica due to the chytrid fungus, *Bd.* When amphibians declined, declines in snake fauna followed (Zipkin et al., 2020) and an increased incidence of malaria was noted (Springborn et al., 2022). In addition to their importance to ecosystems, amphibians provide unique and important research models because of their external development, reorganization of the immune system during metamorphosis, development as diploid or triploid individuals allowing tracking of hematopoietic cell populations, and hairless skin covered by a mucus that may facilitate uncovering fundamental immunological processes at the skin environment interface.

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2. Research questions, challenges, and opportunities

In the following paragraphs, I have tried to highlight a limited number of subject areas, in no specific order of importance, that I believe deserve additional attention from future amphibian immunologists.

2.1. Diversity of lymphocytes, monocytes, macrophages and antigen presenting cell populations in amphibians

One important set of challenges for future amphibian immunologists includes discovering greater diversity of immune cell subsets. In addition to the currently described subsets of lymphocytes (see Lopez Ruiz and Robert, 2023) including T cells, B cells, NK cells, γδ T cells, and innate T cells, much more lymphocyte subset diversity is yet to be discovered. Will we be able to distinguish subsets of T helper cells such as Th17 cells? Can we develop better reagents to distinguish regulatory T cells (Tregs)?

Critical for the development of effective immune responses are the phagocytic macrophages and antigen presenting cells such as dendritic cells. Yet to date, comparative immunologists have described only two subsets of macrophages and one bi-functional dendritic cell (Neely et al., 2018) in amphibians. Differentiation of the two known subsets of macrophages from stem cells is driven by distinct cytokines, colony stimulating factor-1 or interleukin 34 (Grayfer and Robert, 2014). These subsets of macrophages have different functions in response to ranavirus infection (Grayfer and Robert, 2014; Yaparla et al., 2018). Furthermore, we know very little about the characteristics of tissue-specific subsets of macrophages such as those that reside in the peritoneum. An understanding of the characteristics and functions of these immune accessory cells would allow for development of better strategies to understand how specific pathogens may evade detection and clearance. Further development of monoclonal antibody reagents to distinguish additional immune subsets is a major challenge for discovery of more diversity of immune cell subsets in amphibians.

2.2. Mucosal immunity in amphibians

Mucosal barriers are essential for protection from all pathogens, and they are difficult to study in mammalian systems because they are lining the inside of the airways, mouth, and digestive tract. Amphibians offer excellent models to study mucosal immunity because they wear their mucosal surfaces on the outside. Although much has been learned about the mucosal defenses of amphibians because of the tragedy of chytrid fungal infections, we still have a poor understanding of how the microbiome may shape skin immunity and the identity and functions of immune cells that interact with the mucus. For example, enrichment of mast cells in the skin enhanced mucus production and limited the skin damage due to infection by the chytrid fungus, *Bd.* Mast cell enrichment also seemed to limit changes in the skin microbiome resulting from *Bd* infection (Hauser et al., 2024). Disruption of skin cells in chytrid infections should activate an epithelia "alarm" response, immigration of macrophages and neutrophils and Th-17 helper cells to release factors that protect the skin and promote healing. Yet we have limited understanding of macrophage and neutrophil functions in the skin and whether a Th-17 response develops in amphibians after exposure to skin pathogens. Although antibodies to chytrid fungal pathogens have been detected in the mucus of *X. laevis* after recovery from infections (Ramsey et al., 2010), we have no clear understanding of how those antibodies are transported from a B cell compartment in the skin to the mucus.

2.3. IL-17 and fungal immunity

Interleukin 17 family members (IL-17A/F, IL-17B, and IL-17D) have been detected in *X. laevis* with variable expression between tissues (Jackson et al., 2012). In mammals, IL-17 is produced by a subset of T cells designated Th17 as well as by γδ-T cells and NK cells, and it is important for the control of fungal infections (reviewed in Amatya et al., 2017; Gaffen and Moutsopoulos, 2020). IL-23 and pathogen-associated molecular patterns (PAMPs) are necessary for induction of IL-17 production (Gaffen and Moutsopoulos 2020). One important mechanism by which IL-17 results in pathogen clearance is by the induction of antimicrobial peptide (AMP) synthesis. Given that amphibian skin is often a rich source of (AMPs) (Rollins-Smith, 2023), it would be of great interest to investigate whether an IL-23, IL-17, and the AMP axis might exist and have a role to play in control of chytridiomycosis.

2.4. Immunity to diseases such as chytridiomycosis, ranavirosis, and severe perkinsea

Because amphibians continue to decline on a global scale (Scheele et al., 2019), continued funding to understand the pathophysiology of amphibian diseases and the immune mechanisms by which diverse amphibian species become tolerant or resistant should remain a priority.

Immune defenses against chytridiomycosis caused by both *Bd* and *Bsal* remain poorly understood. Because the infection remains in the skin compartment, studies have focused on skin defenses including antimicrobial peptides (AMPs, reviewed in Rollins-Smith, 2023) and the skin microbiome (reviewed in Rollins-Smith and Le Sage, 2021). Amphibian skin is critical for water balance, ion exchange, and respiration, and thus, disruption of the skin caused by severe infections leads to death (Voyles et al., 2009; Savage et al., 2020). If the infection burden is low, the default response may be a sort of "immunological ignorance" or immunological tolerance. In the skin, *Bd* interacts with keratinocytes and other epithelial cells or with skin-resident antigen presenting cells. At least two subsets of macrophages are present in the skin of the South African clawed frog, *Xenopus laevis* (Grayfer and Robert, 2015; Popovic et al., 2019). Activation of the more proinflammatory subset designated IL-34 may be beneficial to halt infection or harmful if excessive, whereas activation of the more immunosuppressive subset designated CSF-1 may permit a more limited infection and eventual clearing and healing. The production of immunosuppressive factors by the chytrids (reviewed in Rollins-Smith and Le Sage, 2021) may also deter rapid and effective clearance. Although, *Bsal* has not yet been detected in North America, there is great concern that accidental introduction would have severe impacts on unique endemic salamander species in North America. Many of these species have been shown to be vulnerable in experimental trials (Gray et al., 2023). Furthermore, *Bsal* introduction would likely occur in locations in which *Bd* is enzootic. Co-infection by both chytrids appears to be more harmful than single infections by either species (Longo et al., 2019; McDonald et al., 2020). Thus, further studies of the possible immune responses to dual infections by both chytrid species are needed. In general, further comparative studies in both anuran species and urodeles could provide greater understanding of whether variation in the inflammatory responses and macrophage activation following chytrid infection could explain the wide variation in susceptibility among species.

Ranaviruses are large double-stranded DNA viruses with broad host range (reviewed in Chinchar, 2002). They infect not only amphibians but also reptiles and fish (reviewed in Chinchar et al., 2017). Amphibian immune defenses against ranaviruses have mostly been studied in *X. laevis*. In this species, tadpoles are very susceptible, but adults can survive and limit viral replication (reviewed in Chen and Robert, 2011; Grayfer et al., 2012). Immune defenses include both T cell-mediated defenses and antibody defenses (reviewed in Grayfer et al., 2015). Because ranaviruses infect and cause mortality in many ranid species, further studies in other anurans such as the wood frog (*Rana sylvatica*) have been suggested. The wood frog is highly susceptible, and many populations are at risk for declines because infected adults may return from overwintering sites to the same water bodies (site fidelity). If they carry infections each year, the tadpole populations are at risk for die-offs and eventual population loss (reviewed in Douglas and Katzenback, 2023). Further research on wood frog immune defenses against

ranaviruses would benefit from the development of new research tools including an annotated genome, additional transcriptomes, additional cell lines, and additional reagents such as specific antibodies and cytokines (reviewed in Douglas and Katzenback, 2023).

Amphibian Perkinsea is an alveolate protist associated with amphibian population declines in wide geographic regions including North America (reviewed in Chambouvet et al., 2015, 2020). It has only recently been recognized as an important pathogen of amphibians (Chambouvet et al., 2020). Because this pathogen is not well characterized, studies have been limited by availability of reagents and the number of investigators involved in these studies. In comparison with studies of chytridiomycosis and ranavirosis, almost nothing is known about immune defenses against the pathogen. The limited studies so far suggest that Perkinsea infection prevalence and intensity are greater in ranid frogs than other anuran families and greater in tadpoles than adult frogs (Karwacki et al., 2018, 2021) A recent study links tadpole mortality to co-infections with both ranaviruses and Perkinsea (Atkinson and Savage, 2023). The pathogen seems to target liver and kidneys, but also spleen, pancreas, gills, gastrointestinal tract, muscle, and skin (reviewed in Isidoro-Ayza et al., 2017). Because most Perkinsea infections are detected in tadpoles rather than adults, it is thought that the adult immune system may be able to limit or control infections (Isidoro-Ayza et al., 2017). Taken together, the alarming number of tadpole-associated die-offs and the association with ranavirus infections argue that many basic aspects of the immune defenses of amphibians against Perkinsea need to be examined.

2.5. Salamander immune defenses (order urodela)

Declines of salamander species in Belgium, the Netherlands, Germany and other locations in Europe due to a new emerging chytrid species, *Bsal*, (Martel et al., 2013, 2014) is the impetus for further studies of this fungus and immune defenses of urodele species in general. North American is home to a wide variety of unique endemic species that will be threatened with extinction if *Bsal* is accidently introduced into North America (Gray et al., 2023; Olson et al., 2024).

2.6. Caecilian immune defenses (order gymnophiona)

The least studied order of amphibians (Gymnophiona) contains the limbless worm-like caecilians. Most live in moist soils where they would likely need to defend themselves against diverse bacteria, fungi, and protozoa. Why might they be of interest? Two recent *in silico* studies of the AMP defenses of caecilians discovered a number of candidate proteins and peptides that would likely have the three-dimensional characteristics of AMPs, yet these AMPs are highly different from the AMP families found in other amphibians possibly due to the long evolutionary separation of caecilians from the other orders (Torres-Sánchez et al., 2020; Benítez-Prián et al., 2024). These examples of novel AMPs are of great scientific interest to amphibian biologists, but they may also have potential medical applications.

2.7. Autoimmune regulator (aire) and immunological tolerance

Immunological tolerance to self-antigens is critical for survival. The discovery of the autoimmune regulator (aire) (reviewed in Anderson and Su, 2011) suggested an elegant mechanism by which this transcription factor promotes "promiscuous" expression of a diverse array of tissue-specific antigens by medullary thymic epithelial cells. If the expressed antigens bind with high affinity to developing T cells in the thymus, the T cells undergo negative selection allowing for development of tolerance to most self-antigens. *X. laevis* and *X. tropicalis* encode an aire protein that is highly expressed in the thymus (Saltis et al., 2008), but the amphibian *aire* gene lacks an important region [plant homeodomain (PHD)-2] required for tolerance induction in mammals (Yang et al., 2013). Thus, a mechanism critical for development of T cell tolerance likely arose early in vertebrate evolution, yet the amphibian *aire* may function somewhat differently in amphibians than in mammals. Interesting questions that remain are when in ontogeny is *aire* expressed. Since tadpoles don't need to be tolerant of organ-specific antigens that won't appear until after metamorphosis and the numbers of thymocytes drops dramatically during metamorphosis (reviewed in Rollins-Smith, 1998), is expression of the *aire* gene delayed until after metamorphosis?

2.8. The value and challenges of next generation sequencing

Some of our forward-thinking comparative immunology colleagues argued that the power and decreasing cost of whole genome sequencing and transcriptomics would open the door to understanding the evolution of the immune system, reveal differing and shared components of the immune systems, and reduce the need for model species (Dheilly et al., 2014). They were correct, and there are many examples of amphibian immune molecules such as cytokines and their receptors that have been revealed due to the presence of full amphibian genomes. At present, the full genomes of at least 34 amphibian species have been collected (https://www.genomeark.org), and there are 111 listed reference genomes at scaffold level or higher available on the National Center for Biotechnology Information (NCBI) genome database. Furthermore, there is a newly formed international consortium, the Amphibian Genomics Consortium (AGC) that aims to support genomics sequencing projects and genomics-driven research (https://mvs.unimelb.edu.au/amphibian-genomics-consortium). However, the value of these genomes is limited by the lack of good annotation (Dimitrakopoulou et al., 2023).

3. Final thoughts

The future of comparative immunology lies in the creativity and persistence of students and their mentors who recognize the value of amphibians and wish to better understand the unique as well as shared components and functions of the immune system and immune defenses. Abundant opportunities to better understand the evolution of vertebrate immune systems and to shape the future of comparative immunology exist along with many exciting challenges.

CRediT authorship contribution statement

Louise A. Rollins-Smith: Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization.

Data availability

No data was used for the research described in the article.

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