



Comparative models for human nasal infections and immunity

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ABSTRACT

The human olfactory system is a mucosal surface and a major portal of entry for respiratory and neurotropic pathogens into the body. Understanding how the human nasopharynx-associated lymphoid tissue (NALT) halts the progression of pathogens into the lower respiratory tract or the central nervous system is key for developing effective cures. Although traditionally mice have been used as the gold-standard model for the study of human nasal diseases, mouse models present important caveats due to major anatomical and functional differences of the human and murine olfactory system and NALT. We summarize the NALT anatomy of different animal groups that have thus far been used to study host-pathogen interactions at the olfactory mucosa and to test nasal vaccines. The goal of this review is to highlight the strengths and limitations of each animal model of nasal immunity and to identify the areas of research that require further investigation to advance human health.

1. Introduction

Olfaction is one of the most ancient and conserved senses among animals (Ache and Young, 2005) and it is fundamental for animals to perceive and react to their world (Ache and Young, 2005; Leinwand and Chalasani, 2011). The main function of the olfactory system is the detection of chemical stimuli present in the host environment and the transduction of these stimuli into electrical signals that reach the higher regions of the brain. One of the unique features of the olfactory system is that the sensory neurons that make up the olfactory neuroepithelium are constantly exposed to pathogens and symbionts, establishing the most intimate interactions between neurons and microorganisms found in the human body.

The anatomical organization of the human olfactory system makes it an attractive site for pathogens to gain entry into the host. First, the olfactory system is directly connected to the central nervous system (CNS) via the olfactory bulb and therefore numerous neurotropic agents including parasites, bacteria and viruses can reach the CNS via transport along the olfactory nerve. Examples of neurotropic human pathogens that exploit the nasal route of infection include viruses such as herpes simplex type 1 (HSV-1), coronavirus (HCoV), vesicular stomatitis virus (VSV) and rabies virus; bacteria such as *Streptococcus pneumoniae*, *Burkholderia pseudomallei*, *Listeria monocytogenes*; and the protozoan *Naegleria fowleri* (Dando et al., 2014) (Table 1). Second, the olfactory system is part of the upper respiratory tract in mammals and therefore, pathogens can reach other parts of the respiratory system once they successfully invade the olfactory mucosa. Examples of respiratory

pathogens that are known to infect the human olfactory organ include influenza virus, respiratory syncytial virus (RSV), rhinovirus, *Staphylococcus aureus*, *S. pneumoniae*. Third, the upper respiratory system is also connected to the gastrointestinal tract via the esophagus and therefore it is possible for pathogens that cause gastric infection to cause nasal diseases. Although this route is less well studied, some examples may include human bocavirus (HBoV), human rotavirus (HRV), Epstein-Barr virus (EBV) and *Salmonella enterica* (Table 1).

The human nasopharyngeal cavity has a nasopharynx-associated lymphoid tissue (NALT) that consists of both organized lymphoid structures (O-NALT) and diffuse NALT (d-NALT). D-NALT is found in all vertebrates whereas O-NALT may have first appeared in sarcopterygian fish (Tacchi et al., 2015; Sepahi et al., 2016). Human O-NALT is formed by a series of tonsils (palatine, nasopharyngeal and lingual) arranged in a circular fashion in the oropharyngeal cavity that form the Waldeyer's ring (Fig. 1A) (Zaman et al., 2013). The human Waldeyer's ring is an induction site for mucosal immune responses (Zaman et al., 2013). As we will discuss later, O-NALT anatomy differs among different vertebrate groups and a Waldeyer's ring similar to that of humans is not always present.

In order to establish models for the study of human nasal pathogens, it is important to understand the parallelisms and differences that exist among the olfactory systems and NALT of different species at the cellular, molecular and functional level. In this review, we summarize the main differences in the anatomical organization of NALT and the nasal immune responses in different vertebrate groups as a way to identify species that are more or less suitable for the investigation of specific

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Table 1
Human pathogens entering via the nasal route.

Neurotropic agents	Disease caused	Type of microbe	References
<i>Coronaviruses (HCoV)</i>	Common cold, pneumonia	Virus	(Antoine et al., 2014; Desforages et al., 2014)
<i>Herpes Simplex Virus Type 1 (HSV-1)</i>	Cold sores, encephalitis, corneal blindness, peripheral nervous system disorders	Virus	(Dando et al., 2014; Steiner et al., 2007; Wilson and Mohr, 2012)
<i>Nipah virus (NiV)</i>	Encephalitis	Virus	Munster et al. (2012)
<i>Hendra virus (HeV)</i>	Encephalitis, Pneumonia	Virus	Dups et al. (2012)
<i>Vesicular Stomatitis virus (VSV)</i>	Flu-like illness	Virus	(Dando et al., 2014; Durrant et al., 2016)
<i>West Nile virus (WNV)</i>	Encephalitis and West Nile fever	Virus	(Colpitts et al., 2012; Durrant et al., 2016)
<i>Japanese encephalitis virus (JEV)</i>	Encephalitis	Virus	Durrant et al. (2016)
<i>Rabies virus (RABV)</i>	Rabies	Virus	(Garcia et al., 2018; Schnell et al., 2010)
<i>Burkholderia pseudomallei</i>	Melioidosis	Bacteria	(Dando et al., 2014; Hsueh et al., 2018)
<i>Listeria monocytogenes</i>	Listeriosis, sepsis, meningitis, encephalitis, spontaneous abort	Bacteria	(Farber and Peterkin, 1991; Inoue et al., 2017; Koopmans et al., 2017)
<i>Streptococcus pneumoniae</i>	Pneumonia, meningitis, sepsis, osteomyelitis, endocarditis	Bacteria	(Bogaert et al., 2004; Varon, 2012)
<i>Naegleria fowleri</i>	Primary Amoebic Meningoencephalitis (PAM)	Parasite	(Heggie, 2010; Linam and Cope, 2017)
Respiratory tract			
<i>Respiratory Syncytial Virus (RSV)</i>	Lower respiratory tract infection	Virus	Makris et al. (2016)
<i>Influenza A virus</i>	Flu	Virus	Yusuf and Kett (2017)
<i>Coronavirus (HCoV)</i>	Common cold, pneumonia	Virus	(Antoine et al., 2014; Desforages et al., 2014)
<i>Rhinovirus</i>	Common cold	Virus	Desforages et al. (2014)
<i>Staphylococcus aureus</i>	Staphylococcal Pneumonia	Bacteria	(Parker and Prince, 2013; Schleimer, 2017)
<i>Streptococcus pneumoniae</i>	Pneumonia, meningitis, sepsis, osteomyelitis, endocarditis	Bacteria	(Bogaert et al., 2004; Varon, 2012)
<i>Acanthamoeba</i> spp.	Granulomatous amoebic encephalitis, Rhinosinusitis	parasite	Nachega et al. (2005)
Gastrointestinal tract			
<i>Human Bocavirus (HBoV)</i>	Influenza-like symptoms and diarrhea	Virus	Lekana-Douki et al. (2018)
<i>Epstein-Barr virus (EBV)</i>	Mononucleosis, Nasal polyps	Virus	(Sugden, 1982; Costa et al., 2014)
<i>Human rotavirus (HRV)</i>	Severe childhood diarrhea with nasal discharge	Virus	(Bartfeld, 2016; Yin et al., 2015)
<i>Helicobacter pylori</i>	Chronic rhinosinusitis. Acute gastritis	Bacteria	Nikakhlagh et al. (2015)
<i>Salmonella enterica</i>	Salmonellosis, Maxillary sinusitis	Bacteria	(Horvath et al., 2016; Report, 2018)

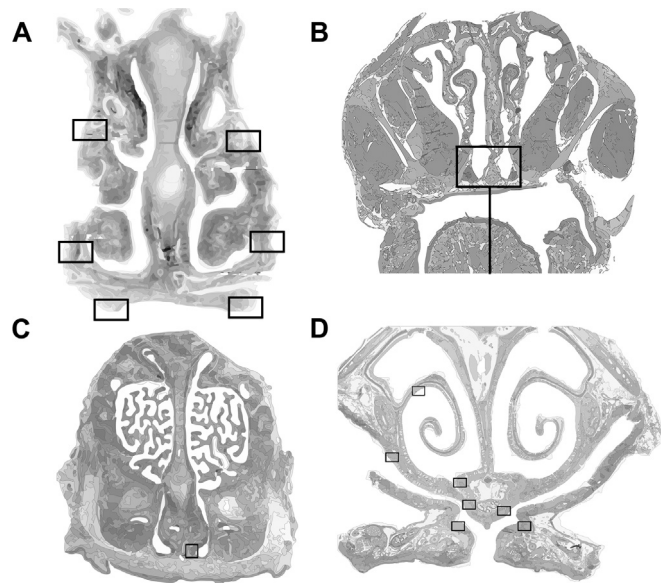


Fig. 1. Anatomy of O-NALT in human (A), mouse (B), rabbit (C) and chicken (D). Black boxes indicate the position of organized lymphoid tissue. Note the presence of six tonsils in human forming the Waldeyer's ring and the absence of ring in mouse, rabbit and chicken.

diseases.

Finally, several human nasal diseases appear to originate from members of the nasal microbiome, which harbors numerous pathogens including *S. aureus*. Although the study of host-microbiota interactions in the olfactory organ is still at its infancy, we will review established and emerging models that could help advance this field.

2. Evolution of olfactory systems: anatomical features

Invertebrates do not have an anatomical structure that forms an olfactory organ. Instead, a network of sensory neurons is found in their

body. For example, the *C. elegans* nervous system is composed of 302 neurons which allow the animal to respond adequately to a variety of environmental stimuli and/or stressors such as chemotaxis, thermotaxis, mechanosensation, navigation, learning and olfactory sensation (Bargmann et al., 1993; Ha et al., 2010; Iino and Yoshida, 2009; Kato et al., 2013; Troemel et al., 1997). Together with AWB, two other pairs of neurons ASH and ADL are responsible for the avoidance behavior. ASH neurons mainly respond to high osmolarity, heavy metals, bitter alkaloids, detergents, and light touch to the tip of the nose (Active et al., 2001; Hilliard et al., 2004; Kaplan and Horvitz, 1993). ADL neurons, in turn, respond to social feeding behavior (Bono et al., 2002). Therefore, *C. elegans* has four neuronal cells (2 AWA and 2 AWC) to sense and discriminate a variety of volatile attractants. As in humans and other animals, *C. elegans* is also able to respond differently to the same odorants at different concentrations. Whereas high concentrations of an odorant result in repulsive behavior, low concentrations of the same odorant trigger attraction behavior (Yoshida et al., 2012).

The first olfactory system may be found in the protochordate amphioxus, where only a rudimentary olfactory bulb was identified by the presence of dopaminergic neurons in the anterior nerve cord (Lacalli, 2004; Satoh, 2005).

Both cartilaginous and bony fish have an olfactory system which largely resembles the overall organization of its mammalian counterpart. In elasmobranchs, the olfactory organ is characterized by a wide epithelial surface giving sharks the name of “swimming noses” (Yopak et al., 2015). Interestingly, it appears that these large surfaces do not provide cartilaginous fish with more sensitivity or lower threshold odor discrimination compared to teleost fish (Meredith and Kajiura, 2010).

The olfactory organ in teleost fish is located in the nasal cavity on the dorsal surface of the head to the front and medial position of the eyes (Kasumyan, 2014) and is composed of two paired olfactory rosettes containing neurons that express vomeronasal receptors (VNR) as well as olfactory receptors (ORs) and therefore it may be considered as both the main and accessory olfactory systems found in tetrapods.

During evolution, lungfish appear to be the most ancient species in which compartmentalization between the main olfactory epithelium (MOE) and vomeronasal organ (VNO) occurs (Nakamuta et al., 2013;

Table 2
Summary of the advantages and disadvantages of different model to study human nasal pathologies.

Model organism	Advantages	Disadvantages
<i>C. elegans</i>	Low cost Rapid reproductive rate Simple organism Transparent Mapped nervous system Allows high throughput screening Used to study behavioral response and neuroimmune responses during infection	Few human pathogens can be studied in <i>C. elegans</i> Lack of anatomical olfactory structures No adaptive immunity (antibody based) so evaluation of vaccines is not possible
Fish	Low costs Small size Rapid reproductive rate Transparent embryos Well characterized d-NALT Easy to dissect (external) olfactory organ Genetic manipulation tools available: i.e. CRISPR, morpholinos Nasal cavity separated from the gastrointestinal tract Used to study human infections, cancer, development, hematopoiesis and in the early stages of vaccine development	Anatomically and physiologically distant from human (no tonsils present; no connection with respiratory surfaces) Ectothermic: temperature effects on the immune response Most zebrafish studies use larvae with no developed adaptive immune system Lack of models using human nasal pathogens
Avian	Nasal immunity extensively studied Nasal vaccination common in industry Model for human-threatening pathogens, such as avian influenza virus, IBV and NDV	Anatomically and physiologically distant from human No Waldeyer's ring present
Pigs	Respiratory system similar to human Well-established model for nasal immunity against influenza	Lack of palatine tonsils Large body size High maintenance in terms of costs and space
Horses	The tonsils organization is similar to the human Waldeyer's ring Nasal immune responses well studied Susceptible to upper respiratory tract infections	Large body size High maintenance in terms of costs and space
Ferret	Medium size Easy to handle Largely used to investigate human respiratory infections lung physiology similar to human Presence of coughing sneezing reflex Parallel symptoms to human for the study of respiratory diseases Highly susceptibility to influenza Used to evaluate human nasal vaccines	Lack of immune reagents No Waldeyer's ring present
Rabbit	Medium size Easy to handle Rapid reproductive rate Established model to study rhinosinusitis Sinus anatomy similar to human and easy accessible	Few studies and models of infection No Waldeyer's ring present
Guinea pig	Medium size Easy to handle Largely used for human bacteria infectious studies due to the high susceptibility to pathogens with symptoms mirroring human Used to study Zika virus, Ebola, Influenza A and B	Long gestation period Limited immunological tools Macrophages do not produce NOS No Waldeyer's ring present
Rat	Medium size Rapid reproductive rate Easy to handle Extensively used Immune response similar to human Used to evaluate viral human respiratory infections	No coughing sneezing reflex Differences in metabolic functions compared to human No Waldeyer's ring present
Mouse	Low costs Rapid reproductive rate Genetic manipulation tools available Vast amount of transgenic lines available Used to study human infection and in the early stages of vaccine development O-NALT can be surgically removed	No coughing sneezing reflex Low susceptibility to human viruses Macromatic Has a functional VNO No Waldeyer's ring present
Non-human Primate	Anatomy and physiology similar to human (Waldeyer's ring is present) Susceptible to the majority of human pathogens	Severely regulated due to ethic issues High maintenance in terms of costs and needs Large body size

Northcutt and Rink, 2012). In amphibians, reptiles and mammals the olfactory organ presents a much more complex structure with a clear separation between the MOE and the VNO. Although found in two distinct anatomical regions, recent findings have highlighted the co-operative role of MOE and VNO in the olfactory perception and the regulation of social communications in mammals (Ache and Young, 2005; Baum and Kelliher, 2009; Kelliher, 2007).

Importantly, the VNO of humans is not functional in adulthood. In the human embryo, the VNO is similar to that of others species, with bipolar neurons similar to vomeronasal sensory neurons. During

development, the VNO is reduced to a more simplified structure until it disappears at 19 weeks (Meredith, 2001). Similarly, other tetrapod species have also lost functional VNOs including cetaceans, some bats, crocodiles and primates (Mucignat-Caretta et al., 2012). This is not the case of mice, in which the VNO is functional throughout life and it plays a vital role in the induction of neuro-hormonal changes due to direct contact with pheromonal stimuli (Mucignat-Caretta et al., 2012).

3. Evolution of olfactory systems: cellular and molecular organization

One of the major differences between the invertebrate and vertebrate sensory neurons is that most vertebrate olfactory sensory neurons (OSNs) express only one functional receptor gene for each neuron. *C. elegans* neurons, in turn, express several functional receptor genes at the same time (Axel, 2005). Due to the limited number of neurons in the *C. elegans* body, odorant recognition power is limited (Active et al., 2001). The fact that each neuron expresses several G-protein-coupled receptors (GPCRs) allows the worm to respond to multiple stimuli using only few neuronal cells (Zhang et al., 2016).

In the majority of vertebrates, the olfactory epithelium is composed by three major cell types: the OSNs, responsible for odor recognition, the sustentacular cells, that provide OSNs with support and other utilities, and the basal cells, which are the stem cells of the olfactory epithelium. As mentioned earlier, one of the most prominent characteristics of OSNs is their ability to express only one type of OR. Additionally, all OSNs expressing the same receptor converge in the same glomerular area in the olfactory bulb (Gao et al., 2000). Several molecular features of sensory systems are not only conserved within vertebrates but are also found in invertebrates. Among these commonalities is the presence of GPCRs, expressed by the OSNs, as the main odorant receptors in worms, flies, several fishes (i.e. trout and zebrafish), mouse and human (Mori and Sakano, 2011). In mouse, for example there are more than 1000 different ORs and each OSN express only one OR gene. Neuropeptides are also conserved in many species including nematodes, flies and mammals and are important modulators of odorant responses in the OSNs. In fact, neuropeptides may facilitate or reduce the activity of OSNs and this regulation helps the animal to localize odors and food sources with great accuracy (Leinwand and Chalasani, 2011; Zhang et al., 2014).

4. The *C. elegans* model for neuroimmune interactions in sensory neurons

Perhaps the best described invertebrate model system in neuroimmunology is *C. elegans*. *C. elegans* feed on bacteria and therefore are attracted to bacterial-derived food odorants (Stensmyr et al., 2012). Additionally, several laboratories have identified pathogenic bacteria that infect *C. elegans* including *Pseudomonas aeruginosa* and *Serratia marcescens* (Pradel et al., 2007). Pathogenic bacteria and some of their metabolites induce aversive behaviors in *C. elegans* and this behavior is mediated by chemosensory neurons (Beale et al., 2006; Schulenburg and Ewbank, 2007). Moreover, elegant studies have demonstrated the key role that sensory neurons play in the regulation of the inflammatory response of worms against pathogens. The *C. elegans* model has therefore demonstrated that animals have the ability to discern between pathogenic and non-pathogenic bacteria using sensory neurons (Pradel et al., 2007). Additionally, microsporidia, natural viruses and non-natural viruses including VSV (Balla and Troemel, 2014), can infect *C. elegans* and these models are readily available for the study of neuroimmune responses during infection. Thus, the detailed map of the *C. elegans* nervous system and the conserved nature of the chemosensory transduction machinery between *C. elegans* and vertebrates, makes the worm a suitable laboratory model for the understanding of how animals respond to microorganisms. Additional strengths of this model include: high throughput screening protocols, established behavioral assays and availability of bacterial and viral infection models. However, *C. elegans* lacks a complex neuroepithelium similar to the vertebrate olfactory epithelium as well as a CNS. Thus, using this model, the immunological contributions of different cell types to pathogens as well as the cross-talk between the peripheral and CNS during the course of an infection cannot be investigated (Table 2).

5. Bony fish: an emerging model for the study of nasal immunity

The teleost olfactory organ is not connected with the oral cavity or respiratory tract, offering a unique and elegant model for the study of human nasal pathologies that have a CNS component.

Teleost fish have also recently emerged as a great model to investigate nasal immunity. The discovery of teleost NALT a few years ago (Salinas, 2015; Tacchi et al., 2014) revealed the conserved nature of this lymphoid tissue in vertebrates. Since teleost NALT only contains d-NALT (Tacchi et al., 2014), teleost fish can now be used to investigate d-NALT responses to pathogens without the use of transgenic animals or surgical procedures. Teleost NALT is composed of B cells (IgM⁺ and IgT⁺ B cells) that are scattered throughout the olfactory epithelium and are mostly located intraepithelially (Tacchi et al., 2014). Additionally, two different CD8 α ⁺ T cell populations have been described in trout NALT, one in the lateral sensory epithelium and one in the apical mucosal epithelium (Sepahi et al., 2016). CD4⁺ T cells have not been investigated in teleost NALT thus far. Finally, abundant MHC-II⁺ antigen presenting cells are found in both the sensory and mucosal epithelium in the trout olfactory organ, but those located in the mucosal tips are found more apically and therefore closer to luminal antigens (Sepahi et al., 2016). Nasal immune responses of rainbow trout against neurotropic viruses such as rhabdoviruses (Tacchi et al., 2014; Larragoite et al., 2016) and protozoan parasites such as *Ichthyophthirius multifiliis* (Yu et al., 2018) have been described. These studies indicate that NALT mounts strong innate and adaptive immune responses upon vaccination or infection in bony fish. Importantly, nasal delivery of a live attenuated viral vaccine not only causes local nasal immune responses and systemic immune responses (Tacchi et al., 2014) but also immune responses in the CNS (Larragoite et al., 2016). Interestingly, these responses are recorded even in the absence of virus in CNS tissues (Larragoite et al., 2016; Sepahi et al., 2018). With regards to specific antibody responses, it appears that IgT is the main antibody isotype involved in specific immune responses against protozoan parasites causing chronic infections in trout NALT (Yu et al., 2018). These findings confirmed that IgT is the functional equivalent to mammalian IgA in nasal secretions and that nasal IgT responses are likely the gold-standard to evaluate specific immune responses, at least in chronic infection models.

Thus, rainbow trout has recently emerged as a model to understand neuroimmune interaction from the olfactory periphery to the CNS. Although to date only the effects of fish pathogens on nasal immune responses have been investigated, we anticipate that bony fish models will be soon adapted to the study of human nasal pathologies. Finally, since the presence of NALT in teleosts meant the development of nasal vaccines for use in aquaculture, we can now exploit this model to better understand the mechanisms of protection induced by nasal vaccines (Salinas et al., 2015; Sepahi et al., 2017, 2016).

To date, the zebrafish (*Danio rerio*) model has been widely used for the study of many human pathogens (Antoine et al., 2014; Harris and Harris, 2015; Medina and Luis, 2013; Neely et al., 2002; Santoriello and Zon, 2012; Swaim et al., 2006). Zebrafish is largely used due to the small body size, rapid life cycle, transparency at the early stages of life and the large numbers of eggs available every week. Moreover, zebrafish resilience and amenability to genetic manipulation techniques have allowed the generation of a broad number of transgenic lines used for studying hematopoiesis, aging, development or cancer. Complex host-microbe interactions can be investigated in real time using life imaging and fluorescently labeled pathogens. Finally, germ-free (GF) zebrafish provide a platform for the identification of microbial contributions to disease onset and progression (Kostic et al., 2013; Melancon et al., 2017; Rawls et al., 2004).

Despite the fact that these infection models have been optimized, to date there are no studies evaluating the effects of any pathogen on the olfactory organ of zebrafish. This may be partly due to the fact that NALT has not been characterized in zebrafish yet, although

comparative histological studies across teleost suggest that NALT is present in most if not all bony fish (Tacchi et al., 2014). With regards to human nasal pathogens, zebrafish has been used to study the neurotropic mammalian VSV and embryos showed 80% mortality (Guerra-Varela et al., 2018). Additionally, the zebrafish model has been widely used to study influenza virus A, (Goody et al., 2014; Gabor et al., 2014), herpes simplex virus 1 (Antoine et al., 2014) and *L. monocytogenes* (Levraud et al., 2009).

As summarized in Table 2, zebrafish offers many advantages for the study of human pathogen-host interactions but there are some caveats to this model too. One of the limiting factors of the zebrafish model is the difference in temperature between zebrafish and the majority of the human virulent pathogens. In fact, zebrafish requires a temperature of 28 °C, while human pathogens require a temperature of 37 °C at which the development as well as the stress and immune response of zebrafish are significantly altered from those at 28 °C (Duggan and Mostowy, 2018; Long et al., 2012).

6. Avian models of nasal immunity

Aves have both O-NALT and d-NALT (Fig. 1D). O-NALT consists of lymphoid nodules at the root of the nasal septum and the dorsal side of the choanal cleft and does not form a Waldeyer's ring (Kang et al., 2013). These lymphoid nodules are well-organized with defined B and T cell areas and support the formation of germinal centers. Developmentally, O-NALT in the chicken begins to form 7 days post-hatch and is fully formed by day 14. Chicken NALT and its capacity to uptake different classes of antigen was thoroughly studied by Kang et al. (2013). With regards to B cell composition, both in chicken O-NALT and d-NALT, IgY B cells are the most abundant whereas IgM and IgA B cells are less frequent (Ohshima and Hiramatsu, 2000).

In ducks, similar to chicken, both O-NALT and d-NALT are found. Duck O-NALT has been described as several lymphoid aggregates found in the ventral wall of the nasal cavity near the choanal cleft and on both sides of the nasal septum (Kang et al., 2014).

The study of nasal immune responses in birds is extensive, not as models of human disease, but rather due to the important threatening pathogens that infect the respiratory tract of birds including avian influenza virus, infectious bronchitis virus (IBV) and Newcastle disease virus (NDV) (de Geus et al., 2012). As a consequence, oculo-nasal vaccination is one of the most common routes of vaccination in poultry farms (Nochi et al., 2018). For instance, MSD manufactures oculo-nasal spray vaccines for IBV and NDV. Intranasal vaccination induces specific IgA responses locally and in serum in chickens (Zhao et al., 2016) and ducks (Kang et al., 2012), making the induction of specific IgA titers a common readout following nasal vaccination both in humans and birds (Table 2). Comparisons between the nasal immune response of chickens and ducks to influenza virus are interesting because ducks are asymptomatic and chickens are highly susceptible accidental hosts (Chaise et al., 2014).

7. Pigs as models of nasal immunity

Pigs have a respiratory system that largely resembles the human respiratory system (Meurens et al., 2012). Pigs, like other farmed animals, present a Waldeyer's ring of lymphoid aggregates. In pigs, the palatine tonsil is not present (Casteleyn et al., 2011; Horter et al., 2003; Liu et al., 2012). It appears that porcine tonsils are richer in B cells than in T cells (Yang and Parkhouse, 1996). Importantly, nasal vaccination of pigs results in local and systemic IgA and IgG specific antibodies (Li et al., 2015; Dhakal et al., 2018), highlighting the value of this vaccination route and the commonalities between the antibody responses of pigs and other species such as chickens, mice and human.

Pigs have been proposed as potential models for the study of human influenza H1N1 virus (Qiu et al., 2015). In support of this model, avian and mammalian influenza viruses infect pig epithelial cells in the

respiratory tract, H1N1 swine influenza virus can be transmitted to humans and the cytokine responses in bronchoalveolar lavage (BAL) fluid from infected pigs are identical to those observed for nasal lavage of infected humans (Khatri et al., 2010; Hayden et al., 1998). Pigs can be intranasally infected with influenza viruses (Qiu et al., 2015) and therefore they represent a well-established model of influenza nasal immunity (Table 2).

Although the delivery of nasal probiotics and other immunostimulants has rarely been investigated, pigs have been used for testing nasal immunostimulants such as *Bacillus subtilis* (Yang et al., 2018).

8. Horses as models of nasal immunity

Horses have five tonsils that form a Waldeyer's ring similar to that of humans (Liebler-Tenorio and Pabst, 2006). The nasal immune responses of horses to bacteria and viruses have been well studied. Some pathogens such as *Streptococcus equi*, cause severe upper respiratory infections in horses since they attach to the equine tonsils (Sweeney et al., 2005). Nasal secretions of horses infected with *S. equi* or intranasally vaccinated with the USDA-approved *S. equi* live attenuated vaccine had specific IgA and IgG antibodies as well as systemic IgA titers (Galan and Timoney, 1985; Delph et al., 2018).

Horses are however not considered model organisms of human disease due to the large size, maintenance costs and need for large enclosures where pathogen containment is hard to achieve (Table 2).

9. Ferrets as models of nasal immunity

Of all model organisms, ferret (*Mustela putorius furo*) is the elective model to study multiple human respiratory infections including avian influenza virus, morbillivirus, coronavirus and others (Belser et al., 2011). Apart from the small size, human and ferrets have similar lung physiological characteristics, and when intranasally inoculated with influenza virus, ferrets showed human clinical symptoms such as fever, nasal secretion, coughing and sneezing; which are not present in the classical mouse model (Belser et al., 2011; Zitzow et al., 2002) (Table 2). Importantly, ferrets have pharyngeal tonsils which are often analyzed in experimental infection models (van den Brand et al., 2012; Lipatov et al., 2009). Moreover, an aged ferret model has been developed to better investigate the effects of aging on the immune responses to influenza A H1N1 strain (Paquette et al., 2014). The high susceptibility to influenza along with the ability to infect healthy ferrets, make these animals a suitable model to follow host-virus interactions (Belser et al., 2011). Along with the influenza infection model, ferrets have been used to evaluate the effectiveness of novel vaccine candidates including FluMist and M2SR. M2SR evokes a wide antibody immune response in ferrets both locally and systemically. Moreover, this vaccine has a protective effect against the drifted viruses H3N2 and H1N1 in both ferrets with and without pre-existing immunity. Thus, M2RS have been considered as a suitable candidate for a large spectrum human vaccine suitable for all ages (Hatta et al., 2018).

10. Rabbits as models for nasal immunity

Rabbits only have palatine tonsils (Fig. 1C) and therefore a full Waldeyer's ring similar to that of humans is not present (Casteleyn et al., 2011). Rabbits have been used as a model for paranasal sinuses fungal infections such as aspergillosis (Chakrabarti et al., 1997) as well as a model for rhinosinusitis (Al-sayed et al., 2017; Dao-yu et al., 2014). Strengths of the rabbit model include the anatomical similarities of the rabbit sinus with that of humans, the easy access to this area in rabbits and the fact that immune responses are largely conserved between rabbits and humans (Table 2).

Domestic rabbits are commonly infected by *Pasteurella multocida* and *Bordetella bronchiseptica*. At weaning, about 25% of rabbits were

found to have nasal infections with *P. multocida* and 75% had infections with *B. bronchiseptica* (Deeb et al., 1990). Intranasal immunization of rabbits with *P. multocida* toxin induces specific IgA responses in the nasal and bronchoalveolar lavage (Jarvinen et al., 1998) indicating again that, similar to human, IgA responses are the gold-standard for evaluation of nasal vaccine efficacy in several animal models including rabbits.

11. Rats and guinea pigs as models of human nasal diseases

Rats do not have any tonsils and therefore lack the Waldeyer's ring (Casteleyn et al., 2011). Rats, however, have O-NALT structures that resemble those found in mice. The cotton rat (*Sigmodon hispidus*) model has traditionally been used to understand many viral human respiratory infections including influenza. Both innate and adaptive immune responses and disease pathogenesis were characterized in cotton rats intranasally infected with influenza virus A and B (Eichelberger, 2007). In particular, cotton rats were infected with several influenza virus A strains including H1N1, H3N2 and influenza virus B to determine the infectivity as well as the pathogenesis. Interestingly, all the viruses were able to replicate in the nose and lung. Moreover, concomitant to the lung infection with influenza virus A, a strong immune response characterized by IFN α , IFN γ , TNF α , IL-1 α and IL-6 was detected (Eichelberger, 2007).

However, similar to mice, cotton rats are macroscopic animals and do not have a sneezing impulse. The latter is a limiting factor in the study of disease propagation. In contrast, cotton rats offer many advantages to the study of RSV pathology because they are more permissive to RSV infection than mice and they show human-like disease symptoms upon infection (Boukhvalova et al., 2010) (Table 2). Thus, cotton rats offer a valid model to study the pathogenesis of RSV and to develop and test vaccines and therapies against this virus (Citron et al., 2018; Sami et al., 1995). As an example, intramuscular immunization with virus-like particles containing the pre-fusion protein F of RSV (Pre-F/F VLPs), induced high titers of neutralizing antibodies and protected the cotton rat lung and nasal cavity from viral replication. Interestingly, animals previously infected and then challenged with RSV had undetectable viral loads in the nose. Hence, intranasal immunization with VLPs may lead to better stimulation of mucosal immune responses and greater protection, compared to other routes of immunization (Cullen et al., 2015).

Finally, the cotton rat model is of particular interest to understand the biology and pathogenesis of *S. aureus*. Nasal carriage of *S. aureus* is an important risk factor for *S. aureus* infections in both adults and children (Kluytmans and Verbrugh, 1997; Mulcahy and McLoughlin, 2016). Cotton rats can successfully be infected with *S. aureus* by nasal instillation of live bacteria and therefore they have been used to assess the efficacy of therapies to control *S. aureus* infections in humans (Kokai-Kun, 2008). Gene expression profile was analyzed during the initial phases of the nasal colonization with a human isolate of *S. aureus* (Burian et al., 2010). Within the genes identified, *S. aureus* adhesin molecules (*tagO*, *tarK*, *clfB* and *isdA*) and virulence factors (*walkR*, *sak*, *sceD*) were fundamental for the nasal colonization of *S. aureus* (Burian et al., 2010). Based on the higher level of IgG against four *S. aureus* antigens, two iron-responsive surface determinants IsdA and IsdH were selected as vaccine candidates against *S. aureus*. In fact, *S. aureus* IsdA adhesin molecule is responsible for the binding to human squamous nasal epithelial cells and clumping factor B (ClfB), playing a complementary role in such binding. When tested in cotton rats, both antibodies raised against IsdA antigen conferred protection against nasal carriage (Clarke et al., 2006). Although the full protective mechanism is still not totally understood, this vaccination strategy may be a promising prophylactic tool in humans (Clarke et al., 2006).

A caveat to this model, however, is that the nasal microbial community of the cotton rat is uniquely composed of several bacterial species that do not necessarily reflect the microbial complex

interactions that occur in human anterior nares (Chaves-Moreno et al., 2015) (Table 2). This is important since previous studies have shown that the nasal microbiota regulates nasal immune responses following intranasal vaccination (Henriksson et al., 2004). Thus, careful evaluation of nasal microbial communities may be critical when performing nasal vaccination trials.

Guinea pigs (*Cavea porcellus*) were one of the first animal models used to study human infectious diseases, such as diphtheria and tuberculosis (Padilla-Carlin et al., 2008). Guinea pigs O-NALT consists of 10–20 lymphoid follicles at the junction of the nasal cavity and the nasopharyngeal duct (Okada et al., 1995). Interestingly, guinea pigs are more susceptible than mice to *L. pneumophila* (McDade et al., 1977). Thus, this model organism was the first to be used to isolate *L. pneumophila* and to determine its virulence factors. Moreover, guinea pigs present all the symptoms present in humans infected with *L. pneumophila*. Guinea pigs can be intranasally infected with *L. pneumophila* (Padilla-Carlin et al., 2008) but studies pertaining nasal immune responses to *Legionella* in guinea pigs are missing.

Susceptibility to viral nasal infection, especially for human influenza viruses A and B (Palese et al., 2007), and Ebola (Wong et al., 2015) was investigated in guinea pigs. In the case of H5N1 avian influenza viruses, the guinea pig was also used as a model to investigate the origin of viral resistance in this species (Zhang et al., 2017). Interesting, at 1 day post infection the viral titer in the nasal wash and lung were already very low and proteomic profiling of the lung showed that more than 100 IFN-stimulated genes (ISGs) were activated during the infection. Thus, unique and potent NALT and lung responses against H5N1 may confer protection against this virus in guinea pigs. Guinea pigs have also been intranasally infected with ZIKA virus (ZIKAV). Nasal infection with ZIKAV results in infection of the brain, parotid glands, tears, saliva and sera but NALT immune responses were not evaluated (Deng et al., 2017). Of interest, nasal vaccination of guinea pigs with a genital herpesvirus HSV2 vaccine results in protection to genital herpes and elicits systemic specific IgG but not IgA titers (Persson et al., 2016). Overall, guinea pigs continue to be used as models for the study of nasal pathogens and can illuminate mechanisms by which nasal vaccines confer protection at distant mucosal sites such as the vaginal tract. However, more detailed characterization of the local nasal immune responses against relevant human nasal pathogens in this species is still needed.

12. Mouse models of human nasal diseases: what are the limitations?

Mice are one of the most widely used models to study human infections. This organism offers many advantages including relative low cost, easy access to reagents and transgenic mice with specific gene manipulation including over-expression or gene disruptions. However, human nasal pathogens do not always replicate efficiently in mice. Apart from the abovementioned examples of RSV and *L. pneumophila*, human influenza virus does not replicate efficiently in mice. Mice are also known to be resistant to many upper respiratory infections and they do not exhibit sneezing reflex, which is an important factor in the study of the viral transmission (Grievess et al., 2015). A final consideration is that mice, like dogs, have a very sensitive sense of smell and therefore they are considered macrosmatic. Primates, on the other hand, are generally considered microsmatic, although this classification may not always be the most adequate depending on the primate species (Smith and Bhatnagar, 2004) (Table 2). Thus, evolutionary speaking, microsmatic and macrosmatic mammals have likely co-opted different strategies to detect and defend their olfactory organs against pathogen infection.

Unlike humans, rodents do not have the ring of tonsils that forms the Waldeyer's ring. Rodents have O-NALT structures described as bell-shaped paired lymphoid aggregates in the floor of the nasal cavity at the entrance of the pharyngeal duct (Fig. 1B) (van der Ven and Sminia,

1993). Murine O-NALT is considered the functional equivalent to the human Waldeyer's ring. Murine NALT is well organized with B and T cell areas and a modified epithelium covering the lymphocytes (Asanuma et al., 1997). Apart from O-MALT, d-NALT is also present in rodents. Detection of specific antibodies in nasal washes, NALT explant culture and phenotyping of NALT leucocytes are all standard techniques in the evaluation of nasal immune responses to vaccination in mice (Cisney et al., 2012). Surgical ablation of O-NALT structures can be performed in mice allowing the investigation of the contributions of d-NALT to vaccination responses (Cisney et al., 2012).

Nasal neuroepithelial responses following multiple poly I:C instillations were studied in mice. Poly I:C administration resulted in infiltration of neutrophils, macrophages and T cells into the olfactory mucosa and presence of caspase-3 positive apoptotic OSNs (Kanaya et al., 2014). The same study observed TLR3 expression in the apical part of the supporting cells and in the cytoplasm of acinar cells of Bowman's glands (Kanaya et al., 2014) suggesting that collaborations between multiple cell types of the olfactory epithelium likely take place upon nasal viral infections. As an example of this cooperation, several studies have shown the innate immune responses of olfactory ensheathing cells (OECs), which are unique glial cells of the MOE, against pathogenic bacteria. In this rat model, the olfactory mucosa is first damaged by irrigation with zinc sulfate or Triton X-100 and then bacteria are administered. OECs are robust iNOS producers (Vincent et al., 2007; Harris et al., 2009). Finally, OECs appear to respond to several PAMPs and express TLR4 (Vincent et al., 2007).

Intranasal administration of LPS in mice induces persistent rhinitis (Hasegawa-Ishii et al., 2017) with a pathology that is characterized by leukocyte infiltration as well as the production of the pro-inflammatory cytokine IL-1 β . Interestingly, loss of OSNs in the main olfactory mucosa as well as synaptic loss in the olfactory bulb have been reported in this model (Hasegawa-Ishii et al., 2017).

Mice have also been extensively used to study CNS neuroinflammation in different disease models (Manghani and McGavern, 2018). VSV, which is not lethal to mice, can be nasally delivered as a model to track CNS immune responses. Recent studies have shown the role of T cells in this response and the presence of local myeloid cells that cross-present antigen in the CNS (Moseman et al., 2018). Thus, similar to fish, the nasal VSV mouse model has evolved as a platform to study neuroimmune interactions.

Finally, mice have been used for the evaluation of several human nasal vaccine formulations including vaccines against influenza viruses, *S. aureus* or ZIKAV (Brown et al., 2014; Schaffer et al., 2006; Sumathy et al., 2017). Similar to birds and other mammals, induction of specific local IgA titers and systemic IgG titers are usually detectable in mice following intranasal vaccination depending on the antigenic model (Kurono et al., 1999; Hirano et al., 2006). *S. aureus* nasal colonization in mice was reduced by the immunization with killed *S. aureus* and/or the recombinant clumping factor B (rClfB) (Schaffer et al., 2006). ClfB is known to bind mouse and human cytokeratin 10 and human desquamated nasal epithelial cells allowing *S. aureus* to colonize the nasal mucosa. Moreover, ClfB is also conserved among *S. aureus* strains, hence is a suitable candidate for a multi-strain vaccine. Both intranasal immunizations showed protection against *S. aureus* along with a reduction of nasal colonization (Schaffer et al., 2006).

The immunocompromised mouse strain A129 that lacks interferon- α/β receptor24, was intranasally inoculated with ZIKAV and all mice showed a prolonged viremia. After 7 days post inoculation, IgM and IgA antibodies were detected, followed by IgG. Thus, a strong infection in the nasal mucosa was established in A129 mice (Deng et al., 2017). A vaccine against the envelope protein E of ZIKAV was tested in this mouse strain and production of neutralizing antibodies was able to confer protection against ZIKAV and other Flaviviridae (Sumathy et al., 2017). Overall, mice continue to be the model of choice for many nasal vaccine trials despite the drawbacks summarized in Table 2.

13. Primates as models for the study of human nasal diseases and immunity

Due to anatomical and immunological similarities, the use of non-human primate models confers great advantages in the evaluation of therapeutic drugs, treatments and vaccines. Of particular interest for the study of nasal immune responses and human nasal diseases, primates have tonsils arranged in a Waldeyer's ring similar to humans (Ludlow et al., 2013).

Macaques are often used because they are easy to breed compared to other primates. Several macaque species have been used in pre-clinical evaluations of infectious agents and in vaccine development studies including *Streptococcus A* (GAS), influenza A virus, measles virus, HIV, Ebola, variola major, hRSV and ZIKAV (Brown et al., 2010; Rivera-Hernandez et al., 2014; Lucas-hourani et al., 2018; Sumathy et al., 2017; Ludlow et al., 2013). In the case of GAS, an intranasal infection model in monkeys was used to evaluate the transcriptomic GAS profile during different phases of infection and the grade of inflammation caused to the animals (Virtaneva et al., 2005).

Primates, like the rest of the endotherms discussed in this review, mount specific IgA antibody responses in the nasal mucosa following intranasal infection or vaccination (Komada et al., 1989; Mizuno et al., 2016). Cynomolgus monkeys, also known as crab-eating macaques (*M. fascicularis*), have been used to evaluate the effects of age on immune responses following intranasal infections or intranasal vaccination (Mizuno et al., 2016; Lucas-hourani et al., 2018). These types of studies are very important when making decisions about vaccination programs in children and adults.

Even if non-human primates are the closest model species to human and maybe the most reliable to study host-pathogen interactions, the similarities between humans and monkeys have often raised ethical issues and public outrage against scientists. Other caveats of this model include the difficult genetic manipulation, the intense husbandry demand and the high costs to maintain animals physically and mentally healthy (Table 2). For all these reasons, the use of non-human primates in research is highly regulated and often used as a “last resource” when any other model cannot be applied to the particular research question.

14. Organoids as models for the study of human nasal diseases

In this final section, we want to introduce the concept of organoids as a model to study human nasal diseases. One of the bottlenecks for the investigation of host-microbe interactions is the complexity of the microbial communities and the lack of *in vitro* models to study one-to-one cause-effect mechanisms for each microbe.

In general, organoids are considered miniaturized and simplified organs. Although organoids are very similar to the real organs, due to the lack of vascularization, structure and sometimes mature function, organoids remain an imperfect model and they are still under development. Therefore, organoids are used along with more classical approaches such as *in vivo* studies. The first organoid was created 9 years ago from a single intestinal crypt cell; where even in the absence of a non-epithelial cellular niche, a single cell was able to self-renew and differentiate, generating a villus-like epithelial structure in which all the different cell types present in the real organ were found (Sato et al., 2009). Since then several “mini-tissue” have been reproduced successfully in many animals including human pancreas, kidney, thyroid, liver, inner ear, retina, skin tissue with follicular hair and brain (Barkauskas et al., 2017; Lee et al., 2018; Koehler et al., 2017; Orsini et al., 2018; Ramachandran et al., 2015; Turksen, 2016; Ueda et al., 2018).

Nasal organoids were also created from nasal human turbinate epithelia (MOCs) almost 10 years ago (Kleinsasser et al., 2009). Although nasal organoids have been used for genotoxicity studies, their full potential has not been exploited to study human nasal diseases and host-microbe interactions at the human nasal mucosa. Due to the impervious and sometimes inaccessible airway anatomy, it is often difficult to determine the contribution of NALT in the cascade of events that

lead to respiratory pathologies. Here, we propose the use of nasal organoids as a model for human nasal infections and other diseases, such as nasal polyps, rhinosinusitis, anosmia, allergy or aspergillosis. Organoids could also constitute a suitable model to study nasal-specific microbial interactions helping to identify virulence factors required for invasion and infection. Additionally, nasal organoids could shed light on potentially favorable competition between commensals and specific pathogens involved, for example, *S. pneumoniae* rhinosinusitis.

15. Concluding remarks

Many animal models have helped unveil host-pathogen interactions at the nasal mucosa but many questions remain to be answered. Overall, nasal mucosal immune responses are not well understood compared to other mucosal immune responses. This is despite the large variety of pathogens that exploit the nasal route to gain entry to the host. Understanding nasal immunity in different animal models is vital for establishing models of human disease, but our current view of the diversity of nasal immune systems is patchy and reflects specific research efforts rather than unbiased comparisons. Although specific antibody responses following intranasal vaccination are a common readout in most animal experimental models as well as humans, very little is known about the early immune responses that halt pathogen entry or the contributions and cross-talk between d-NALT and O-NALT. Additionally, new insights in teleost fish suggest that nasal immunization triggers distant immune responses in the CNS suggesting tight neuroimmune regulation in the olfactory-CNS axis.

Vertebrates have a wide diversity of olfactory systems and the anatomical differences found in NALT across species makes the translation of animal studies to human difficult. Anatomically, the O-NALT of primates and farmed animals is the closest to human O-NALT, however these models also present their own limitations. Some of most refined animal models, such as the pig nasal influenza model, illustrate the importance of validating human disease symptoms, disease progression and immune responses in the laboratory setting. Further efforts are necessary to exploit the best attributes of each animal model and to translate laboratory findings into better therapies and vaccines that advance human health.

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