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## The evolution of nasal immune systems in vertebrates

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### Abstract

The olfactory organs of vertebrates are not only extraordinary chemosensory organs but also a powerful defense system against infection. Nasopharynx-associated lymphoid tissue (NALT) has been traditionally considered as the first line of defense against inhaled antigens in birds and mammals. Novel work in early vertebrates such as teleost fish has expanded our view of nasal immune systems, now recognized to fight both water-borne and air-borne pathogens reaching the olfactory epithelium. Like other mucosa-associated lymphoid tissues (MALT), NALT of birds and mammals is composed of organized lymphoid tissue (O-NALT) (i.e., tonsils) as well as a diffuse network of immune cells, known as diffuse NALT (D-NALT). In teleosts, only D-NALT is present and shares most of the canonical features of other teleost MALT. This review focuses on the evolution of NALT in vertebrates with an emphasis on the most recent findings in teleosts and lungfish. Whereas teleost are currently the most ancient group where NALT has been found, lungfish appear to be the earliest group to have evolved primitive O-NALT structures.

### Keywords

Evolution; Nasal immunity; NALT; Mucosal immunity; Vertebrates; Teleost fish

### 1. Historical aspects

Tonsillectomy or the surgical removal of tonsils is a very ancient practice. Approximately 2000 years ago, Aulus Cornelius Celsus, a Roman writer and physician, described tonsil surgery by using his fingers to remove tonsils (Koempel et al., 2006; Younis and Lazar, 2002). Today, 530,000 children under the age of 15 have their tonsils or adenoids removed in the US every year and it is still one of the most common surgical procedures in children in this country (Cullen et al., 2009; Roland et al., 2011).

The first attempt to nasally vaccinate humans against smallpox was reported in the Golden Mirror of Medicine, Chinese medical text in 1742. Nasal vaccination was done by using powdered scabs that were blown to the nose or filling the nose with a vesicle smeared cotton (Plotkin, 2014). Thus, tonsillectomy and nasal vaccination precede our understanding of nasal immune systems.

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Anatomically, the human Waldeyer's ring was first described in 1884 by von Waldeyer-Hartz as a ring of lymphoid tissue in the pharyngeal wall (Cocquyt et al., 2005; Perry and Whyte, 1998). Nasopharynx-associated lymphoid tissue (NALT) was first described as a paired of lymphoid cells accumulations in the nasal passage of rat in 1947 (Kelemen, 1947) whilst the mouse NALT was first described few decades later (Belal et al., 1977). In the subsequent years, the NALT of other mammals such as monkeys (Harkema et al., 1987; Loo and Chin, 1974) and horses (Mair et al., 1987, 1988) were described. However these studies did not include functional aspects of the nasal immune system (Kuper et al., 1992).

One of the major breakthroughs in nasal immunity field took place in the early 2000, when the first nasal vaccine for use in humans against influenza virus was licensed in the USA (FluMist) (Chen et al., 2006). To date, this vaccine remains the only nasal vaccine licensed for human use. The effectiveness and availability of this vaccine has helped the NALT community to expand basic scientific knowledge on nasal immune responses. Intranasal vaccination offers a number of advantages over other vaccination routes (Neutra and Kozlowski, 2006). Apart from the fact that is needle free and requires small amounts of antigen, intranasal delivery has been shown to stimulate not only local nasal immunity but also systemic immune responses as well as mucosal immune responses in distant mucosal sites (Fukuyama et al., 2012; Lycke, 2012; Neutra and Kozlowski, 2006; Pabst, 2015).

## 2. Anatomy of NALT

In endotherms, mucosa-associated lymphoid tissues (MALT) comprise a network of secondary lymphoid tissues that contain both well-organized lymphoid structures (organized MALT, O-MALT) and scattered or disseminated lymphoid cells (diffuse MALT, D-MALT) (Brandtzaeg and Pabst, 2004). Examples of O-MALT include the Peyer's patches in the gut or the tonsils in the nasopharyngeal cavity. Generally speaking we know very little about NALT (both organized and diffuse) compared to the gut-associated lymphoid tissue (GALT). Furthermore, most mammalian studies focus on organized NALT (O-NALT), whereas diffuse NALT (D-NALT) has received very little attention. It is worth noting that the term "non-NALT" can also be found in the literature instead of D-NALT (Asanuma et al., 1997; Lee et al., 2015; Tamura et al., 1998). Additionally, "nasal passage (NP) leucocytes" is another term that can be found in the mammalian literature referring to D-NALT (Hiroi et al., 1998; Rodríguez-Monroy et al., 2007; Shikina et al., 2004). Despite the fact that the Society for mucosal immunology does not recommend the use of O-MALT and D-MALT (Brandtzaeg et al., 2008), numerous mammalian immunologists continue to use this terminology. Moreover, from the evolutionary immunologist point of view, O-NALT and D-NALT are useful terms considering that early vertebrates lack O-NALT (Fig. 1). In the next part of this review, we will summarize the basic anatomical aspects of NALT in different vertebrate groups.

### 2.1. Rodents and humans

The anatomy and structure of O-NALT have been widely studied in rodents (Mestecky et al., 2005). Murine NALT is composed of a pair of organized mucosal organs located on the roof of the soft palate at the entrance to the nasopharyngeal duct (Liang et al., 2001). NALT in

mouse is considered by some to be analogous to the Waldeyer's ring in human which consists of the adenoids and tonsils (Kuper et al., 1992). Thus, in both mice and humans, O-NALT is strategically placed in the upper airways to combat air-borne antigens. Perhaps, this anatomical observation historically hindered the investigation of NALT in non-terrestrial vertebrates.

It is important to highlight that O-NALT of rodents and humans appears to be significantly different anatomically speaking. In rodents, NALT is located in a single localization bilateral at the entrance of the nasopharyngeal duct whereas in human studies conducted in children, O-NALT was mostly found in the middle concha and it consisted of disseminated lymphoid, subepithelial follicles (Debertin et al., 2003). The results from this study indicated that children have O-NALT structures in addition to a Waldeyer's ring (Debertin et al., 2003). Additionally, these differences under-score the fact that mice may not be the best models for human nasal immunity studies.

Similar to the Peyer's patches in the gut, O-NALT structures are located underlying specialized portions of the epithelium known as follicle-associated epithelium (FAE). Additionally, high endothelial venules (HEVs) control lymphocyte trafficking into O-NALT (Kiyono and Fukuyama, 2004). O-NALT structures also have distinct B-lymphocyte and T-cell zones (Bailey et al., 2005; Brandtzaeg and Pabst, 2004). Germinal center formation occurs in O-NALT in response to infection or antigenic stimulation (Zuercher et al., 2002).

As mentioned earlier, both mice and humans also possess diffuse lymphoid cells situated on the mucosa of the nasal passages called (D-NALT) (Liang et al., 2001). D-NALT includes myeloid cells and lymphoid cells (both B and T cells). The similarities and differences between mammalian O-NALT and D-NALT are summarized in Table 1.

## 2.2. Other mammals

In this section, we are going to focus on reports pertaining four groups of mammals: cattle, sheep, canines and rabbits. The studies in these species have been motivated by the importance of nasal vaccination in veterinary medicine.

In cattle, the tonsil (O-NALT) was first described in 1992 (Schuh and Oliphant, 1992). Cattle tonsils are located at the entry of the pharynx and are equivalent to the Waldeyer's ring in humans (Rebelatto et al., 2000). During development, adenoid can be detected at 95 days of gestation. Moreover, ciliated, microvillus cells and a loose accumulation of mononuclear cells in lamina propria is visible at 120–150 days of gestation. Small lymphoid follicles form at 4–5 months of gestation following by the appearance of goblet cells after 5 months of gestation (Schuh and Oliphant, 1992). Tonsils are well developed at birth in cattle. However, germinal center formation and increase in MHC class II expression only occur in the late natal and early post-natal period (Schuh and Oliphant, 1992). Moreover, cattle infected with foot-and-mouth disease virus (FMDV) showed increased expression of TLR-4 in NALT, indicating the importance of type I IFN responses in NALT against FMDV (Zhang et al., 2006).

Sheep O-NALT structures, similar to horse O-NALT, are clustered posterior to the opening of the Eustachian tube (Mair et al., 1988; Stanley et al., 2001). Thus, ovine NALT is highly organized and consists of discrete B and T cells areas similar to those found of humans and rodents. Furthermore, it has been shown that sheep NALT is covered by ciliated and non-ciliated cells which play an important role in antigen uptake and processing (Stanley et al., 2001).

Peeters et al. reported the absence of typical O-NALT structures in the nasal mucosa of dogs without respiratory disease (Peeters et al., 2005). Waldeyer's ring in the dog consists of the lingual tonsil, the palatine tonsils, the soft palate tonsil and the pharyngeal tonsil or adenoid (Billen et al., 2006). The nasopharyngeal mucosa in dogs appears uniform and the nasopharyngeal tonsil is not obvious (Billen et al., 2006). The latter might be due to the fact that dogs breathe through both the nose and the mouth; therefore, exposure of the canine nasal and nasopharyngeal mucosa to inhaled antigens is decreased. This may explain why the pharyngeal tonsil is less developed in dogs than horses, cattle, sheep and pigs (Billen et al., 2006).

Casteleyn et al. (2010) histologically examined the presence of NALT in rabbits by sectioning the nasal cavity. Rabbits appear to have well organized NALT in their nasal cavities including clustered I and II lymphoid follicles separated by interfollicular regions as well as isolated lymphoid follicles. Interestingly, in the middle third of rabbit nasal cavity, NALT occupied the largest space. The rabbit and human nasal cavities occupy a similar volume considering their respective body masses. However, in comparison with rodents, O-NALT in the rabbit is more abundant. Therefore the similarities between human and rabbit nasal cavities suggest that the rabbit is a better model for intranasal vaccine development than rodents are (Casteleyn et al., 2010). Additionally, rabbits have a D-NALT characterized by intraepithelial and lamina propria lymphocytes (Casteleyn et al., 2010).

### 2.3. Birds

Most of our knowledge on avian NALT comes from studies in chicken and duck. The nasal cavity in chickens is cone-shaped and separated into the right and left sides by a cartilaginous nasal septum. The majority of the nasal cavity is occupied by the turbinates which play a major role in preventing the entry of dust and microbes (Kang et al., 2013). Lymphoid nodules are the main O-NALT structure in chickens. Nodules are made of B cells with developed germinal centers, surrounded by a coat of CD4<sup>+</sup> T cells.

Chicken D-NALT, on the other hand, consists mostly of CD8<sup>+</sup> T cells that can be found in the epithelium and in the lamina propria of the nasal mucosa (Kang et al., 2013; Ohshima and Hiramatsu, 2000). Additionally, scattered lymphoid cells are found in the paranasal organs of chickens (nasolacrimal ducts, lateral nasal glands and their ducts) (Bang and Bang, 1968; Kang et al., 2013).

NALT appears to be an important inductive site for the chicken's mucosal immune system, however, the low absorption rates of antigen by the nasal mucosa may limit induction of effective nasal immune responses (Kang et al., 2013).

The nasal cavity in duck is cone-shaped and separated into right and left sides by a nasal septum (Kang et al., 2014). At the caudal regions of the nasal cavity there are two pairs of symmetrical lymphoid aggregates; one pair located on the dorsal side of the choanal cleft and the other pair on both sides of nasal septum (Kang et al., 2014). The FAE found in duck O-NALT, unlike that of rodents, contains almost no goblet cells. The use of liquid vaccines for ducklings and dry powder vaccine sprays for adult ducks has been recommended because liquid vaccines are unlikely to reach the NALT located on the nasal septum in adult ducks (Kang et al., 2014).

Like the chicken, ducks have diffuse lymphoid tissue and intraepithelial lymphocytes located within their nasal walls and turbinaria randomly (Kang et al., 2014).

#### 2.4. Reptiles and amphibians

Unfortunately, NALT has been studied neither in reptiles nor in amphibians. This is despite the fact that chelonids are affected by upper respiratory tract disease (URTD) caused by *Mycoplasma agassizii* (Brown et al., 1999). The infection results in nasal discharge, nasal wheeze, conjunctivitis and inflammation of the oral mucosa. The contribution of NALT responses in *M. agassizii* infected chelonids should be investigated

Amphibians are known to have both diffuse and organized GALT and NALT (Goldstine et al., 1975). Tonsil-like structures were found in the lingual and sublingual regions of anuran amphibians (Myers, 1928). We further identified lymphoid aggregates associated with the olfactory epithelium of *Lithobates sp.* Tadpoles (Tacchi et al., 2015). Based on histological examination, these were present immediately underneath the olfactory epithelium but did not appear as organized as those found in lungfish. Contrary to birds and mammals, amphibian O-MALT is characterized by lymphoid nodules or aggregates that lack B and T cell zones or GC formation (Goldstine et al., 1975).

#### 2.5. Sarcopterygian fishes

Sarcopterygian fish such as lungfish are the closest living sister species to all tetrapods (Zardoya and Meyer, 1996). Moreover, lungfish represent the transition from aquatic to terrestrial environments during vertebrate evolution and are exposed to both air-borne and water-borne antigens. We recently discovered that African lungfish (*Protopterus sp.*) may be the most ancient vertebrate where O-MALT, including O-NALT, is found (Fig. 1). O-NALT structures in lungfish are FAE-associated or embedded in the mucosa of the upper and lower jaw (Fig. 2). They are mostly composed of lymphocytes both B and T cells and do not display GC formation (Tacchi et al., 2015). In response to bacterial infection, lungfish O-NALT architecture and cellular composition changes, with an increase in the percentage of T and B cells compared to uninfected controls. Although not covered in the same study, we anticipate the presence of D-NALT in lungfish, but their characteristics need to be investigated. The discovery of these primordial forms of O-NALT in lungfish along with the ability to infect these animals intranasally in the laboratory makes this species an interesting system for investigating the structure, organogenesis and function of O-MALT in ectotherms.

## 2.6. Teleosts

Until recently, three MALT have been characterized in teleost fish: gut-associated lymphoid tissue (GALT), skin-associated lymphoid tissue (SALT) and gill-associated lymphoid tissue (Salinas et al., 2011). However, we have discovered that NALT is likely conserved in all jawed vertebrates as it was found in teleost fish. Teleost NALT resembles other teleost MALT in many regards such as the presence of diffuse lymphoid cells with no organized structures present, a predominant constitutive presence of IgT<sup>+</sup> B cells and secreted IgT compared to systemic lymphoid tissues and the presence of an associated microbiota coated by mucosal immunoglobulins (Tacchi et al., 2014). This discovery broke the previous paradigm of nasal immune systems being a first line of defense against inhaled antigens.

Histological examination of the NALT of four different families of teleost fish showed that teleost NALT harbors diffuse lymphoid cells but lacks O-NALT (Tacchi et al., 2014). One of the key features of teleost MALT is the preponderant presence of IgT<sup>+</sup> B cells and secreted IgT into mucosal secretions (Zhang et al., 2010). Trout D-NALT contains abundant B cells, 48.5% are IgM<sup>+</sup> B cells and 51.5% are IgT<sup>+</sup> B cells, following the canonical B cell composition of other trout MALT. Similarly, the ratio of secreted IgT and secreted IgM in nasal mucus is much higher than that in plasma, again highlighting that trout NALT shares the main features of other MALT in teleosts. In gut and skin, previous studies have shown that specific IgT but not IgM responses occur in the local mucosal environment in response to parasitic infections in rainbow trout (Xu et al., 2013; Zhang et al., 2010). Although we detected high levels of IgT in the nasal secretion of trout in the absence of antigenic stimulation, specific IgT responses in the olfactory organ of teleosts in response to infection or vaccination are yet to be investigated. We have demonstrated striking protection levels conferred by nasal vaccines against viral and bacterial pathogens of fish in rainbow trout however, the mechanisms underlying the observed protection are not well understood and may include both humoral and cellular immunity (LaPatra et al., 2015; Tacchi et al., 2014).

Using a live attenuated infectious virus (IHNV) model of nasal vaccination, we were able to show that trout NALT mounts strong innate and adaptive immune responses over the course of a 28 day experiment. Whereas the expression of innate immune genes peaked at day 4 post vaccination, expression of adaptive immune genes peaked at day 14. Additionally, nasal vaccination resulted in stimulation of immune genes not only in NALT but also in the head-kidney (Tacchi et al., 2014).

Altogether, our results clearly reveal that early bony vertebrates evolved olfactory organs with strong defense functions that serve as a primary mucosal immune barrier against pathogens. Moreover, the discovery of teleost NALT and detection of nasal immune responses in trout in response to vaccination underscores the fact that strong nasal immune responses in vertebrates can take place in the absence of O-NALT. Currently, surgical ablation of O-NALT in mice is used as a tool to evaluate the specific contribution of O-NALT to mammalian nasal immune responses. This method is complex and may result in incomplete removal of all O-NALT cells. Thus, we propose that teleost NALT is an excellent model for the study of D-NALT and can provide a unique tool to understand, for instance, how tonsillectomized humans respond to nasal vaccines.

### 3. Evolution of NALT-specific molecules in vertebrates

The mucosal immune system has evolved complex suites of immune molecules as well as cell adhesion and trafficking molecules that separate it from the systemic immune system. For instance, trafficking of lymphocytes to NALT in mice is governed by the PNAd-L-selectin pair (Csencsits et al., 1999; Fukuyama et al., 2002), whereas the  $\alpha 4\beta 7$ -MAdCAM-1 pair governs trafficking of lymphocytes to the gut (Berlin et al., 1995).

The development of lymphoid tissues mainly relies on the lymphotoxin-driven expression of homeostatic chemokines such as CXCL13 (Ansel et al., 2000; Randall and Mebius, 2014). CXCL13 also plays an important role in maintaining the lymphoid architecture of NALT such as formation of germinal centers (Rangel-Moreno et al., 2005). In NALT, however, additional chemokines like CCL20 and CCL9 which are important in dendritic cell and B cell migration are required for development (Zhao et al., 2003). Impaired expression of CCL19/CCL21 results in defects in influenza-specific CD8 T cells responses in NALT of lymphotoxin- $\alpha$ -deficient ( $Lta^{-/-}$ ) and  $plt/plt$  mice (Rangel-Moreno et al., 2005).

We know very little about the role of chemokines in the nasal immune system of vertebrates other than mice and humans. In trout, we found CCL19 expression was upregulated around 50-fold at 4 days post vaccination with live attenuated IHNV suggesting a conserved role of this chemokine in vertebrate nasal immunity (Tacchi et al., 2014).

With respect to O-MALT evolution, it has been proposed that organization of lymphocytes in structures such as lymph nodes or O-MALT is an endotherm-restricted innovation. Lymphocyte organization requires the presence of certain molecular signals that create the adequate microenvironment for B and T cells. Most of the molecules that play a role in lymphocyte organization belong to the tumor necrosis factor superfamily (TNFSF) (Tacchi et al., 2015). In this respect, we recently provided new insights into the evolution of TNFSF and showed that it greatly diversified in African lungfish. Among TNFSF members, lymphotoxins are of particular relevance due to their function in the organization of lymphoid tissues. Interestingly, where lymphotoxin beta receptor (LTBR) is found from teleosts to humans, lymphotoxin alpha (LTA) and beta (LTB) are absent in teleosts but found in lungfish. In other words, TNFSF members known to be essential in lymphocyte organization in mammals first appeared in lungfish and are absent from teleost genomes. Moreover, a number of these TNFSF members were found to be expressed in African lungfish O-NALT (Tacchi et al., 2015).

ID2 is a transcription factor known to be essential for O-NALT formation. ID2 is involved in the induction of CD3<sup>-</sup>CD4<sup>+</sup>CD45<sup>+</sup> cells (Yokota et al., 1999) and ID2 deficient mice could not develop NALT after birth (Fukuyama et al., 2002; Yokota et al., 1999). We found ID2 expressed both in trout and lungfish NALT and its expression was significantly modulated upon nasal vaccination with a viral vaccine or nasal bacterial infection, respectively (Tacchi et al., 2014). Important molecules in the formation of NALT in mice are summarized in Table 2.

#### 4. Nasal microbiota and immunity in vertebrates

Every animal mucosal surface is colonized by millions of microorganisms forming a very ancient and successful symbiosis between prokaryotes and metazoans. The microbiota controls the immunological development of the host by different mechanisms such as inhibition of pathogen colonization on mucosal surfaces and stimulation of the host immune system (Buffie and Pamer, 2013; Kiyono and Fukuyama, 2004; Randall and Mebius, 2014). Although the number of studies revealing the importance of the microbiota in GALT development and function is vast, information regarding its effects on NALT is limited.

The nasal microbiome of human was sequenced and characterized in 2010 and further studies continue to determine its role in health and disease (Dewhirst et al., 2010). In humans, over 96% of the nasal bacterial microbiome belongs to three phyla: Actinobacteria, Firmicutes and Proteobacteria (Yan et al., 2013). In other mammalian species such as pigs, the nasal microbiome is Proteobacteria, Firmicutes and Spirochaetes (Weese et al., 2014). Nasal-associated microbiota are also present in aquatic vertebrates as evidenced by our studies in rainbow trout (Tacchi et al., 2014). The bacterial community associated with the trout olfactory organ is very diverse. 16s rDNA pyrosequencing revealed the presence of 18 total bacterial phyla, the highest number of phyla present among all body sites. The bacterial community was dominated by Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes with the class Betaproteobacteria accounting for 15.1–53.6% of all sequences (Lowrey et al., 2015). Thus, although limited pyrosequencing data is available, it appears that Proteobacteria and Firmicutes may be the most dominant and conserved taxa among nasal microbiomes in vertebrates, further studies will confirm or reject this hypothesis. The trout nasal microbiome composition is distinct from that of any other trout mucosal surface but most closely resembles that of the skin.

The presence of unique bacterial communities in association with each mucosal anatomical site suggests a tight cross-talk between the microbiota and MALT in vertebrates. One of the ways by which the microbiota and the host immune system interact is by means of secretory immunoglobulins that help anchor microorganisms to the mucus layer, while tagging them in a process known as immune exclusion. As an example, trout nasal-associated bacteria, similar to gut and skin bacteria, are coated by secretory immunoglobulins. Particularly, in trout skin and gut mucus, the immunoglobulin isotype IgT has specialized in the role of bacteria coating (Xu et al., 2013; Zhang et al., 2010) with IgM coating significantly lower numbers of bacteria. In nasal mucus, however, an equal proportion of bacteria were coated with IgM or IgT and the proportion of bacteria coated with both isotypes was higher than that found in the gut and skin (Tacchi et al., 2014).

From mammalian studies it appears clear that the microbiota plays a key role in the form and function of NALT. For instance, the microbiota was shown to modulate local nasal T cell responses against *Mycoplasma pulmonis* infection (Henriksson et al., 2004). On the other hand, the composition of the nasal microbiome of humans is a determinant of *Staphylococcus aureus* colonization and carriage (Frank et al., 2010; Yan et al., 2013) and, in mice, it regulates the immune responses against respiratory influenza A virus infection (Ichinohe et al., 2011). Additionally, it has been shown that the number of M cells in NALT



of specific pathogen free rats is lower compared to normally reared rats (Jeong et al., 2000) and the numbers of T and B cells in NALT are 2–3 times greater after an experimental infection (Asanuma et al., 1997). NALT, like most of the lymphoid organs, can develop in germ free mice. NALT development CXCL5-deficient mice in sterile conditions takes about 20 weeks, however by repeated intranasal application of heat inactivated *Propionibacterium acnes* suspension, NALT can be induced after 8 weeks (Krege et al., 2009). In the future, the specific roles of the nasal microbiota on the development and function of vertebrate NALT should be further elucidated.

## 5. How old is NALT?

Our current view of nasal immunity is limited and mostly restricted to mammalian studies. We provided the first evidence supporting the idea that nasal immunity is an ancient arm of the mucosal immune system of vertebrates. Furthermore, our studies underscore that D-NALT, as anticipated, precedes the appearance of O-NALT during evolution (Fig. 1). Currently, we can state that D-NALT is at least 380 MY old, although we anticipate the cartilaginous fish and even agnathans may be equipped with an equivalent immune system associated with their olfactory organs. How old is O-NALT is a separate question. We have provided evidence of primitive O-NALT structures in the African lungfish, indicating that the ancestor to all tetrapods first acquired this immunological innovation (Tacchi et al., 2015). Further studies should address the immune function of primitive O-NALT in ectotherms.

## 6. Concluding remarks

Olfaction is one of the most ancient and conserved sensory systems in vertebrates. It seems that the successful structure and function of olfactory organs evolved to not only provide the exquisite detection of odorants but also to ensure that pathogens do not gain entry into hosts via this route. We hope that our pioneer studies in NALT evolution motivate more studies in non-mammalian models that complete our understanding of nasal immune systems and their evolution. Future studies in teleosts may address whether specific IgT is the main immunoglobulin isotype in nasal adaptive immune responses and whether nasal vaccination can lead to protection in other distant MALT. Lungfish studies, on the other hand, should try to address what evolutionary advantages lymphocyte organization confers at mucosal sites in the absence of germinal center formation.

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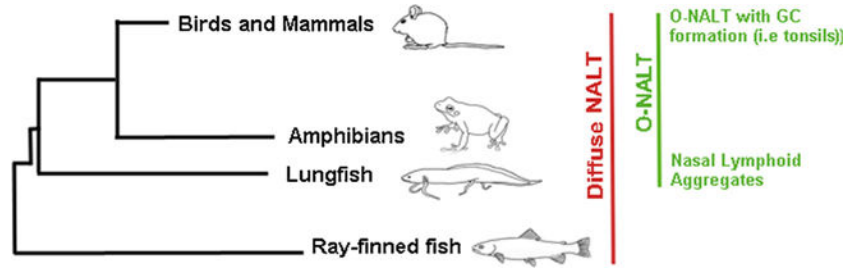
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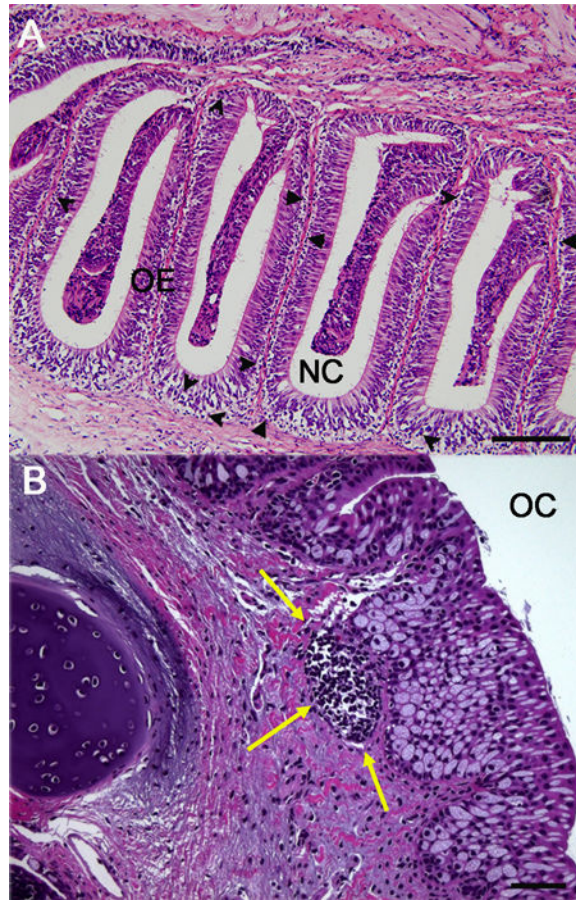
**Fig. 1.** Schematic diagram of the evolution of nasal immune systems in vertebrates.

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**Fig. 2.** (A) Light micrograph (hematoxylin and eosin stain) of paraffin sections of the olfactory organ of an Australian eel (*Anguila australis*) showing the presence of diffuse NALT in the form of scattered lymphocytes (black arrow heads). (B) Light micrograph (hematoxylin and eosin stain) of the olfactory organ of the African lung-fish (*Protopterus dolloi*) showing the presence of a primordial lymphoid aggregate (yellow arrows). NC: nasal cavity; OC: oral cavity; OE: olfactory epithelium. Scale bar= 100  $\mu$ m.



**Table 1**

The differences between mice O-NALT and D-NALT.

	O-NALT	D-NALT	References
Number of lymphocytes	+	++	Rodríguez-Monroy et al. (2007)
T/B cells ratio	0.76–1.2	0.33–1.0	Asanuma et al. (1997), Rodríguez-Monroy et al. (2007)
Percentage of B cells <sup>a</sup>	47–79%	55–74%	Liang et al. (2001), Rodríguez-Monroy et al. (2007)
Plasma cells	+	++	Rodríguez-Monroy et al., (2007)
IgM+ B cells	47 or 85%	0.5–9%	Rodríguez-Monroy et al. (2007), Shikina et al. (2004), Smith et al. (2013)
IgA+ B cells	1–1.6%	7.7–10.8%	
B220 <sup>hi</sup> B cells	Present	Present	Rodríguez-Monroy et al. (2007)
B220 <sup>hi</sup> B220 <sup>low</sup> B cells	Absent	Present	
IgM/IgG/IgA secreting cells ratio (Uninfected)	10/3/3	10/3/3	Asanuma et al. (1997)
IgM/IgG/IgA secreting cells ratio (infected with influenza) <sup>b</sup>	4/50/40	1/25/10	
IgM/IgG/IgA secreting cells ratio(immunized with Cry1Ac protoxin) <sup>c</sup>	1/80/125	1/125/350	Rodríguez-Monroy and Moreno-Fierros (2010)
IgM/IgG/IgA secreting cells ratio(immunized with cholera toxin)	1/30/65	1/90/160	
IgA isotype class switching	Present	Absent	Shikina et al. (2004)
Class switch recombination-associated molecules	Present	Absent	
Long-lasting, specific effector antibody response <sup>e</sup>	Absent	Present	Liang et al. (2001)
Frequency of AFCs <sup>e</sup>	+	++	Liang et al. (2001)
Generation of virus-specific antibody forming cells (AFCs) <sup>e</sup>	+	+	
Infected/uninfected IFN- $\gamma$ production ratio <sup>d</sup>	~1000	~700	Asanuma et al. (1997)
Infected/uninfected IL4 production ratio	1	2	
CD3 <sup>+</sup> T cells	30–40%	13–20%	Rodríguez-Monroy et al. (2007), Smith et al. (2013)
CD4 <sup>+</sup> /CD8 <sup>+</sup> T cells ratio	3–4.4	1.5–6.4	Asanuma et al. (1997), Heritage et al. (1997), Rodríguez-Monroy et al. (2007), Smith et al. (2013)
$\alpha\beta$ / $\gamma\delta$ of CD3 <sup>+</sup> T cells ratio	49–100	2.5–3	Heritage et al. (1997)
$\alpha\beta$ / $\gamma\delta$ of CD4 <sup>+</sup> CD8 <sup>+</sup> T cells ratio	49–100	49–100	
$\alpha\beta$ / $\gamma\delta$ of CD4 <sup>+</sup> CD8 <sup>+</sup> T cells ratio	49–100	9–19	
$\alpha\beta$ / $\gamma\delta$ of CD4 <sup>+</sup> CD8 <sup>+</sup> T cells ratio	0–1	0–1	
Type of CD4 <sup>+</sup> T cells	Th <sub>0</sub>	Th <sub>2</sub>	Hiroi et al.(1998), Liang et al. (2001)

Summary of major immune characteristics of mammalian organized and diffuse NALT in mice: ++ = high, + = low.

<sup>a</sup>C57BL/6 mice have higher B cells percentage compared to BALB/c mice.

<sup>b</sup>7 days post infection (dpi) with influenza.

<sup>c</sup>BALB/c mice immunized intranasally.

<sup>d</sup>7 dpi with influenza.

<sup>e</sup> shows responses after influenza virus infection.

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**Table 2**

Requirements for the formation of NALT in mice.

KO mice with	Organogenesis of NALT	References
Id2	Absent	Fukuyama et al. (2002), Yokota et al. (1999)
Ror- $\gamma$ t	Not required/well developed	Harmsen et al. (2002), Sun et al. (2000)
Lta	Disorganized but developed with fewer number of lymphocytes	De Togni et al. (1994), Fukuyama et al. (2002), Harmsen et al. (2002)
aly/aly	Disorganized but developed with fewer number of lymphocytes	Fukuyama et al. (2002), Miyawaki et al. (1994)
IL-7ra	Disorganized but developed with decreased number of lymphocytes	Adachi et al.(1998), Fukuyama et al. (2002), Harmsen et al. (2002)
plt/plt	Developed with decreased number of lymphocytes	Fukuyama et al. (2006), Rangel-Moreno et al. (2005)
Microbiota	Developed with delay	Randall and Mebius (2014)
LT $\beta$	Developed but disorganized	Fukuyama et al. (2002), Koni et al. (1997)
TRANCE	Developed but disorganized	Ache and Young (2005), Harmsen et al. (2002)
CXCL13/CCL19/CCL21	Developed but disorganized	Ansel et al. (2000), Fukuyama et al. (2006), Rangel-Moreno et al. (2005)