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RESEARCH ARTICLE

Morphology of mucosa-associated lymphoid tissue in odontocetes

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Abstract

This study describes the mucosa-associated lymphoid tissue (MALT) in odontocetes from the Brazilian coast and freshwater systems. Seven species were evaluated and tissue samples were analyzed by light, scanning and transmission electron microscopy, and immunohistochemistry. Laryngeal tonsil was a palpable oval mass located in the larynx, composed of a lymphoepithelial complex. Dense collections of lymphocytes were found in the skin of male fetus and calf. Clusters of lymphoid tissue were found in the uterine cervix of a reproductively active juvenile female and along the pulmonary artery of an adult female. Lymphoid tissues associated with the gastrointestinal tract were characterized by diffusely arranged or organized lymphocytes. The anal tonsil was composed of an aggregate of lymphoid tissue occurring exclusively in the anal canal, being composed of squamous epithelium branches. MALT was present in different tissues and organic systems of cetaceans, providing constant protection against mucosal pathogens present in their environment.

KEYWORDS

cetaceans, immunology, lymphoid system, MALT

1 | INTRODUCTION

The mucosal epithelium lining the internal surfaces of the body and the mucus it produces is the only physical barrier against invasion of tissues by potential pathogens or commensal microorganisms, which may become harmful in immunodeficiency cases (Parham, 2009). Since these surfaces need constant protection and this thin epithelial layer can be easily broken, the body has other mechanisms to provide additional protection, such as immune cells associated with mucosal epithelium (Murphy, 2011).

Thus the mucosa-associated lymphoid tissue (MALT) extends throughout the body and covers about 50% of the immune system lymphocytes (Cesta, 2006; Croitoru & Bienenstock, 1994). In aquatic and terrestrial mammals, MALT is classified according to its location, the best known categories being: the gastrointestinal tract-associated lymphoid tissue (GALT); the bronchus-associated lymphoid tissue (BALT); the nasal-associated lymphoid tissue (NALT); the conjunctiva-associated lymphoid tissue (CALT); the skin-associated lymphoid tissue (SALT); and the vascular-associated lymphoid tissue (VALT) (Azzali, 2003; Brandtzaeg, Kiyono, Pabst, & Russell, 2008; Samuelson, 2006).

In aquatic mammals, reports on the importance and relevance of MALT are scarce, being mostly related to the macroscopic description of GALT, particularly Peyer's patches (Arvy, 1976; Cave, 1980; Cowan & Smith, 1999; Romano, Felten, Olschowka, & Felten, 1993; Simpson & Gardner, 1972), with no major morphological details. Oropharyngeal (Smith, Turnbull, & Cowan, 1999; Cowan & Smith, 1999) and anal tonsils (Cowan & Smith, 1995, 1999) are found in *Tursiops truncatus* and considered of utmost immune importance in cetaceans due to its strategic location in anatomical sites, working as a barrier to continuous antigen presentation.

It is believed that cetaceans have large amounts of MALT scattered in their body, which can be of utmost importance since these animals are constantly exposed to possible contaminants in their habitat (Beineke et al., 2005). However, in terms of size and function, these data remain imprecise and, due to the physiologically critical role and extensive exposure to antigens of this mucosal tissue, a more detailed study of the MALT morphology in cetaceans is necessary, describing its location sites and microscopic characteristics. Thus, the present study aimed to describe the mucosa-associated lymphoid tissues in species of odontocetes of occurrence in the Brazilian coast and freshwater systems.

2 | MATERIALS E METHODS

2.1 | Animals

Animals evaluated in this study were derived from biological collections from Research Institutions Fundação Mamíferos Aquáticos – FMA (Sergipe), Instituto Biota de Conservação – BIOTA (Alagoas), Associação de Pesquisa e Preservação de Ecossistemas Aquáticos – AQUASIS (Ceará) and Instituto de Desenvolvimento Sustentável Mamirauá – IDSM (Amazonas) (Sisbio Permit 37369-1, Animal Bioethics Protocol 2571/2012).

A total of 44 specimens, 24 guiana dolphins (*Sotalia guianensis*), six tucuxis (*Sotalia fluviatilis*), four clymene dolphins (*Stenella clymene*), six pink river dolphins (*Inia geofrensis*), two melon-headed whales (*Peponocephala electra*), and two short-finned pilot whales (*Globicephala macrorhynchus*), of both sexes and different age groups (calf:juvenile:adult; Table 1) were evaluated. These groups were defined based on dentition and body measurements when teeth analyses were not possible to be performed (Jefferson, Leatherwood, & Webber, 1993).

All specimens stranded in the northeastern coasts of Brazil, and freshwater systems of the Amazon. Causes of these strandings were highly variable, ranging from trauma, death by hypothermia or accidental entanglement in fishing nets (Carvalho, personal communication). Animals showing any signs of infection (viral, bacterial, or fungal), respiratory or skin diseases and/or inflammation were excluded from this study.

2.2 | Samples

Carcasses were assigned to one of five categories to define their state of decomposition (CODES 2–5; Geraci & Lounsbury, 2005). Only specimens CODES 2–3 were used.

During necropsy, thoracic and abdominal cavities were examined, allowing the exposure of the entire intestine, as well as identifying any mucosa-associated lymphoid tissue: nasopharyngeal, skin, blood vessels, respiratory system, gastrointestinal tract, and oropharyngeal and anal tonsils. These were photographed, dissected, and intestinal mucosal samples were taken randomly at different sections of each intestinal third.

For didactic reasons, we chose to divide the intestine in thirds: initial (proximal) and middle (medial), defined as the small intestine due to its thinner structure; and the final (distal), defined as the large intestine, since its epithelium was differentiated compared to the other intestinal thirds. The anal canal was defined as the segment composed of stratified squamous epithelium.

2.3 | Microscopic analysis

Samples were washed and fixed in 10% formalin solution for light and scanning electron microscopy. After triage, only samples of tonsils were considered suitable for transmission electron microscopic analysis. Thus, samples of tonsils were fixed in modified Karnovsky solution (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium phosphate buffer solution).

For light microscopy, samples were rinsed in distilled water, dehydrated in increasing ethanol solutions (70–100%), diaphanized in xylene and embedded in Paraplast®. Sections (5 μm) were stained with Hematoxylin-Eosin and Masson's Trichrome and examined in a light microscope (Nikon Eclipse E-800).

For scanning electron microscopy, samples were rinsed in distilled water (3× 10 min, under-rotation) and subsequently dehydrated in ascending series of ethanol [70% (1 × 1 min), 80% (1 × 15 min), 90% (1 × 15 min), and 100% (3 × 30 min), under-rotation]. Afterward, they were critical-point dried, mounted on metal bases (*stubs*) and coated with gold by sputting (Balzers-040 SDC). Samples were analyzed by using a scanning electron microscope (LEO 435 VP).

Samples for transmission electron microscopy were post-fixed in 1% osmium tetroxide solution with sodium phosphate buffer solution (2 h, 4°C). Then, they were dehydrated in increasing series of alcohols and embedded in a propylene oxide solution and Spurr resin (1:1). Later, this solution was replaced by pure resin; samples were placed in a rubber mold and placed in an oven for polymerization (36 h). After the procedure, 1–3 μm sections were obtained on an ultramicrotome (Ultra ®), stained with Toluidine blue and examined under a light microscope (Nikon Eclipse E-800) for location of areas of interest and images capture.

2.4 | Immunohistochemistry analysis

For labeling T lymphocytes using CD3 immunohistochemistry analyses, 5 μm sections were deparaffinized and rehydrated prior to the protocol. Tissues were pretreated with a Tris-EDTA-Tween Buffer Antigen Retrieval (20 min, 96–99°C). After cooling at room temperature (RT), slides were washed in TBS-HCl/Tween buffer, which was used to rinse all slides during the entire analyses.

After recovery, the blockage of endogenous peroxidase (3% H_2O_2 for 15 min at room temperature) was performed. Slides were then washed in tap water, followed by distilled water and TBS/Tween. For blocking non-specific protein, a 2.5% Goat Serum solution (Kit ImmPRESS HRP Anti-Rat Ig, mouse adsorbed, Vector Laboratories) was used (20 min, room temperature). After incubation, slides were washed in TBS/Tween for application of the primary antibody (1:200; MCA1477 - Rat Anti Human CD3, ABDSerotec), remaining in a humid chamber overnight at 4°C.

Slides were then washed in TBS/Tween prior to their incubation using an Immpress Reagent Kit (ImmPRESS HRP Anti-Rat Ig Mouse Adsorbed, Vector Laboratories) for 20–30 min at room temperature. After, slides were washed in TBS/Tween and the reaction was performed using a chromogen kit (Chromogen IMMPACT ™ DAB, Vector Laboratories). Subsequently they were washed in tap water, counterstained with Hematoxylin, and mounted with Permount.

3 | RESULTS

3.1 | Laryngeal tonsil

The laryngeal tonsil (LT) was found in juvenile (J) and adult (A) specimens of *S. guianensis* (1 J, 1 A), *S. fluviatilis* (2 J), *S. clymene* (1 A), *P. electra* (2 J), and *G. macrorhynchus* (1 J). Macroscopically it presented as an oval palpable mass, located in the ventral opening of cricoid cartilages of the larynx. Its structure is well-defined, highly trabeculated due to the thickening of the mucosa, with the presence of small pits and not deep crevices (Figure 1A). In juvenile animals, the LT was more prominent and evident when compared to adult animals (six juveniles/two adults; 30%). It was not possible to evaluate the differences between sexes since the number of specimens available for such analysis was limited.

The LT surface formed invagination, originating crypts and folds (Figure 1B). Microscopically the LT was composed of a lymphoepithelial complex, consisting of a pseudostratified columnar epithelium (Figure 1C). Along these crypts, lymphocytes aggregations were present, shaped in nodules with germinal centers (Figure 1B–D), and disorganized or diffuse (D-MALT) where the lymphocytic infiltrate was permeated by the connective tissue, making it difficult to visualize the boundary between the connective and lymphoid tissues (Figure 1C and D). At the base of crypts there were numerous mucus-secreting glands and T lymphocytes close to the lining epithelium of a crypt, marked through immunohistochemistry analysis (Figure 1E and F).

TABLE 1 List of samples by species, according to sex and age group[a] (calf:young:adult)

Species	Number of males/age group	Number of females/age group	Total number of animals evaluated
Sotalia guianensis[b]	4:4:2	3:6:4	24
Sotalia fluviatilis	0:2:0	0:4:0	6
Stenella clymene	0:0:2	0:0:2	4
Inia geoffrensis	1:0:3	1:0:1	6
Peponocephala electra	0:2:0	–	2
Globicephala macrorhynchus	0:1:1	–	2
Total	23	21	44

[a]Age groups were defined according to the described by Jefferson et al. (1993).
[b]A male fetus in the final third of gestation was evaluated, totaling 24 specimens of this species and 44 in total.

3.2 | Associated lymphoid tissue to the skin

Dense collections of lymphocytes were found in the skin of three male specimens: a fetus and a juvenile *S. guianensis* and a juvenile *G. macrorhynchus*. All lymphoid clusters were located between the epidermis and dermis, near the lamina propria (Figure 2A, E, and F). CD3 T lymphocytes were stained in a brownish color surrounded by B lymphocytes (Figure 2E and F).

3.3 | Lymphoid tissue associated with the reproductive system

Clusters of lymphoid tissue near the lamina propria of the uterine cervix of a juvenile female *S. guianensis*, at reproductive age, were found. The cervix pleated surface was formed by a mucosa layer lined with simple columnar epithelium, mucous, and serous layers (Figure 2B).

3.4 | Associated lymphoid tissue to blood vessels (VALT)

Along the pulmonary artery of an adult female *S. guianensis*, clusters of lymphoid tissue were observed (Figure 2C). Lymphocytes were organized, being surrounded by a layer of collagen fibers, these being disorganized so as not to characterize a capsule.

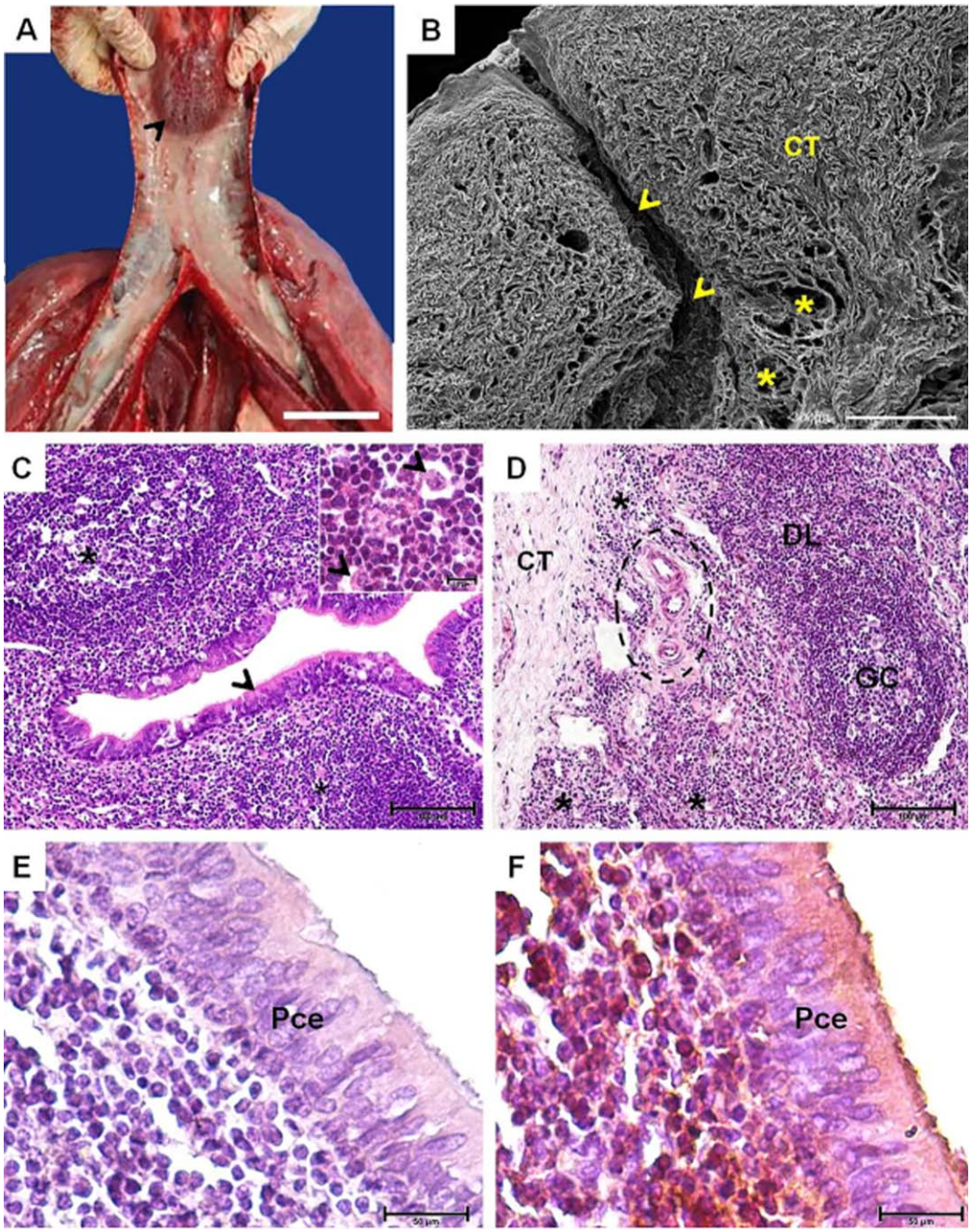

FIGURE 1 A. Laryngeal tonsil (arrowhead) of a juvenile *Peponocephala electra*. Bar: 1.5 cm. B–D. Photomicrographs of Laryngeal tonsil of a *Sotalia guianensis* calf. B. Scanning electron microscopy. Duct of a crypt (arrowheads) surrounded by connective tissue (CT). Crypt covered by clusters of lymphocytes (*). C. Pseudostratified columnar epithelial tissue (arrowhead) in the light of a tonsillar pit. Lymphocytes arranged in a lymphatic nodule (*). Hematoxylin-Eosin (HE). ×40. Highlighted area. Lymphoblasts indicated by arrowheads. HE. ×100. D. Lymph node with evident germinal center (GC). Presence of disorganized lymphocytes (DL), densely concentrated near the GC or diffusely entering the adjacent connective tissue (*). Blood vessels highlighted. HE. ×20. E, F. Immunohistochemistry analysis. E. Negative control. F. T lymphocytes CD3 marked in a brownish color, near the pseudostratified columnar epithelium (EPC) lining the crypt. Counterstaining with Hematoxylin. ×40. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

3.5 | Lymphoid tissue associated with the gastrointestinal tract (GALT)—Peyer's patches and diffuse lymphoid tissue

Lymphoid tissues associated with the gastrointestinal tract (GALT) were characterized by diffuse and follicular lymphocytes within the intestinal mucosa and submucosa. In Figure 3, clusters of lymphocytes were present in the intestinal mucosa near its lumen (Figure 3B), being organized in a defined region (O-GALT; Figure 3C) or diffusely arranged (D-GALT; Figure 3D). They were observed in all species studies and age groups, with no difference between calf, juvenile and adult animals.

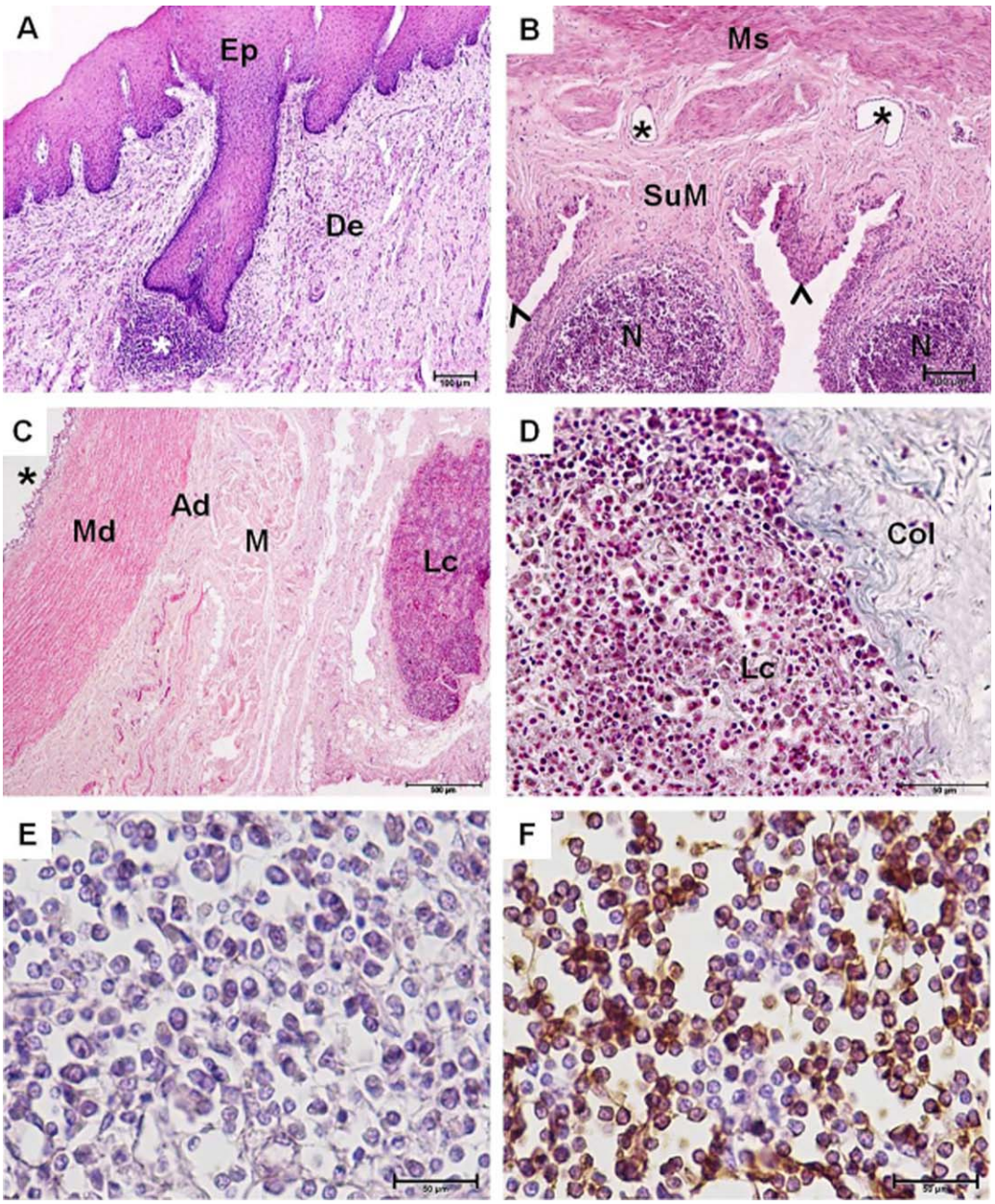

FIGURE 2 Photomicrograph of lymphoid tissues associated with mucosa. A. Skin of a *Sotalia guianensis* calf. Epithelium (Ep) of an elongated epidermal ret peg ending in a lymphoid cluster (*) in the dermis (De). Hematoxylin-Eosin (HE). B. Uterine cervix of a juvenile *Sotalia guianensis*. Presence of folds (arrowheads) on its surface. Blood vessels (*) in the submucosa layer (SuM), followed by muscular layer (Ms). (HE). ×10. C. Pulmonary artery of an adult *Sotalia guianensis*. Lumen (*), middle layer (Md) and adventitia (Ad). Lymphoid cluster (Lc) adjacent to the heart muscle layer (M). HE. ×4. D. Lymphoid cluster (Lc) at higher magnification surrounded by collagen fibers (Col). Masson's Trichrome. ×40. E, F. Immunohistochemistry analysis. Lymphoid cluster present in the skin of a juvenile *Globicephala macrorhynchus*. E. Negative control. F. CD3 T lymphocytes stained in a brownish color surrounded by B lymphocytes. Counterstaining with Hematoxylin. ×40. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Peyer's patches (PP) was considered as aggregations of lymphoid tissue, presenting a distinct appearance, characterized by true aggregates of lymphocytes that often formed a protrusion, which often entered the intestinal lumen (Figure 4A). PP presented themselves as round, oval or irregular-shaped structures (Figure 4B and C), where lymphocytes formed a lymphoid cluster.

PP varied in size and number and were present in greater amounts in the middle and final thirds of intestines, especially in juvenile animals when compared to adults (10 juveniles/6 adults; 40%). No difference related to gender regarding the amount or size of GALT was observed.

In some calves lymph nodules had a more weakly stained central region, representing a germinal center with lymphocyte proliferation.

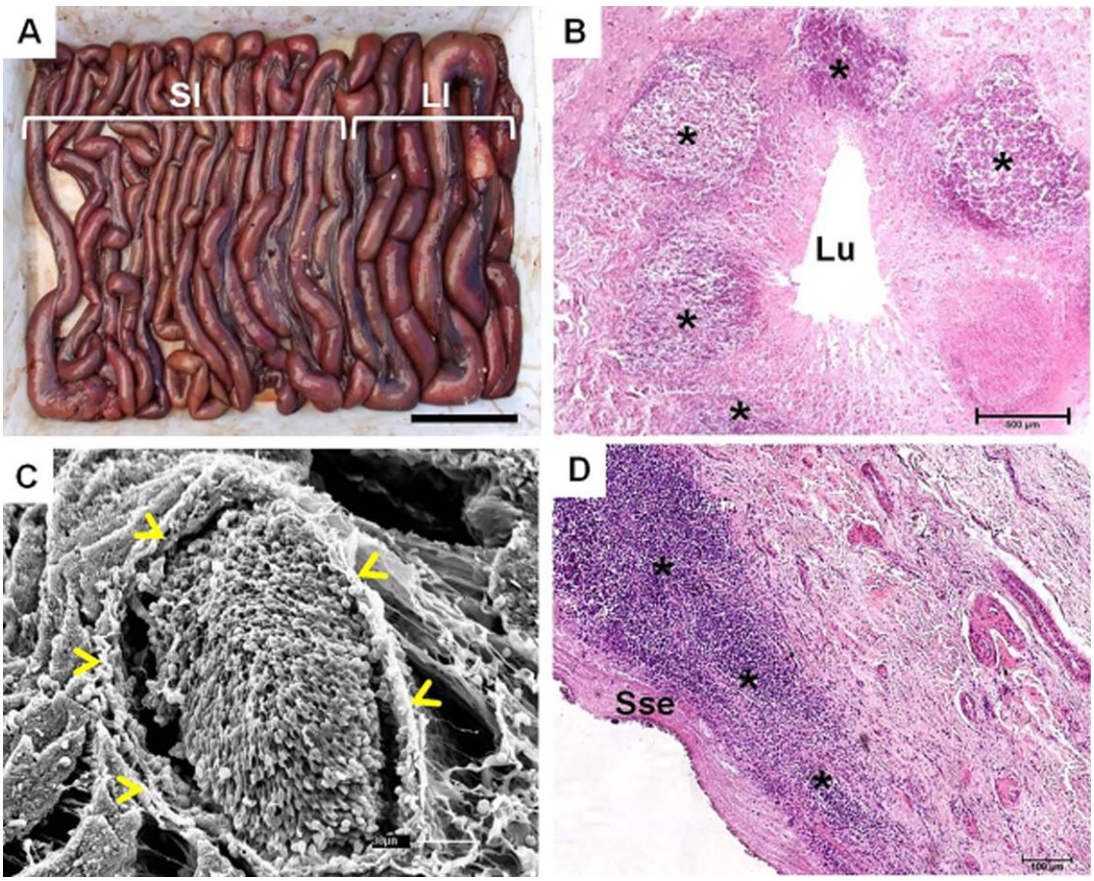

FIGURE 3 A. Estimated delimitation of small and large intestines (SI and LI, respectively) of a juvenile *Sotalia guianensis*. Bar: 3 cm. B–D. Photomicrographs of different portions of the odontocetes intestines. B. Initial third. Juvenile *Sotalia guianensis*. Aggregation of lymphocytes, organized in nodules (*) surrounding the intestinal lumen (Lu). Hematoxylin-Eosin (HE). ×4. C. Scanning electron photomicrograph of the middle third. Juvenile *Inia geoffrensis*. Lymphoid tissue bordered by densely arranged collagen fibers, indicated by arrowheads. D. Final Third. Adult *Sotalia guianensis*. Stratified squamous epithelium (Sse), followed by a layer of diffuse lymphoid tissue (*). HE. ×10. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

This weak staining was due to the presence of several lymphoblasts, proliferating cells which have larger and less heterochromatic nuclei compared to lymphocytes (Figure 4D). These lymph nodules were not encapsulated, being enclosed by densely arranged collagen fibers (Figure 4F).

PP were present as solitary lymph nodules (Figure 5A and B) near each other (Figure 5C and D) or linked to each other (Figure 5D). In some cases the intestinal villi were absent in the site where these nodules "flowed" into the mucosa surface towards the intestinal lumen (Figure 5B, C, and F).

3.6 | Anal tonsil

Anal tonsil (AT) was a cluster of lymphoid tissue, discreet in size when compared to the laryngeal tonsil (LT). As the LT, the AT had crypts and/or crevices that occurred exclusively in the anal canal (Figure 6A), extending into the anus. AT was found in specimens of *S. guianensis*, *S. fluviatilis*, *S. clymene*, *S. geoffrensis*, and *P. electra* from all age groups.

Histologically, AT was composed by branches of squamous epithelium which penetrate the intestinal mucosa and originated crypts of different depths. Clusters of lymphocytes, diffuse (Figure 6B) or organized (Figure 6C), covered some regions of crypts, often penetrating into the lamina propria (Figure 6B). These clusters could also be organized in lymph nodules (Figure 6D), some of which were enclosed by a network of densely arranged collagen fibers (Figure 6E and F).

4 | DISCUSSION

The immune system associated to the mucosa is the largest in the body considering its size and function, since the mucosal tissue largely covers all the body tissues of humans and terrestrial and aquatic mammals. Thus, the production of lymphocytes is essential in this tissue to produce a fast and efficient immune response (Murphy, 2011).

Although MALT sites are anatomically separated, they remain functionally connected since lymphocytes present in a specific location stimulate a generalized immune response, leading to the production of IgA in the mucosa of several organs (Nochi & Kiyono, 2006). Thus, the mucosal immune system produces an independent cellular immune response and a systemic acquired immune response (Janeway & Medzhitov, 2002).

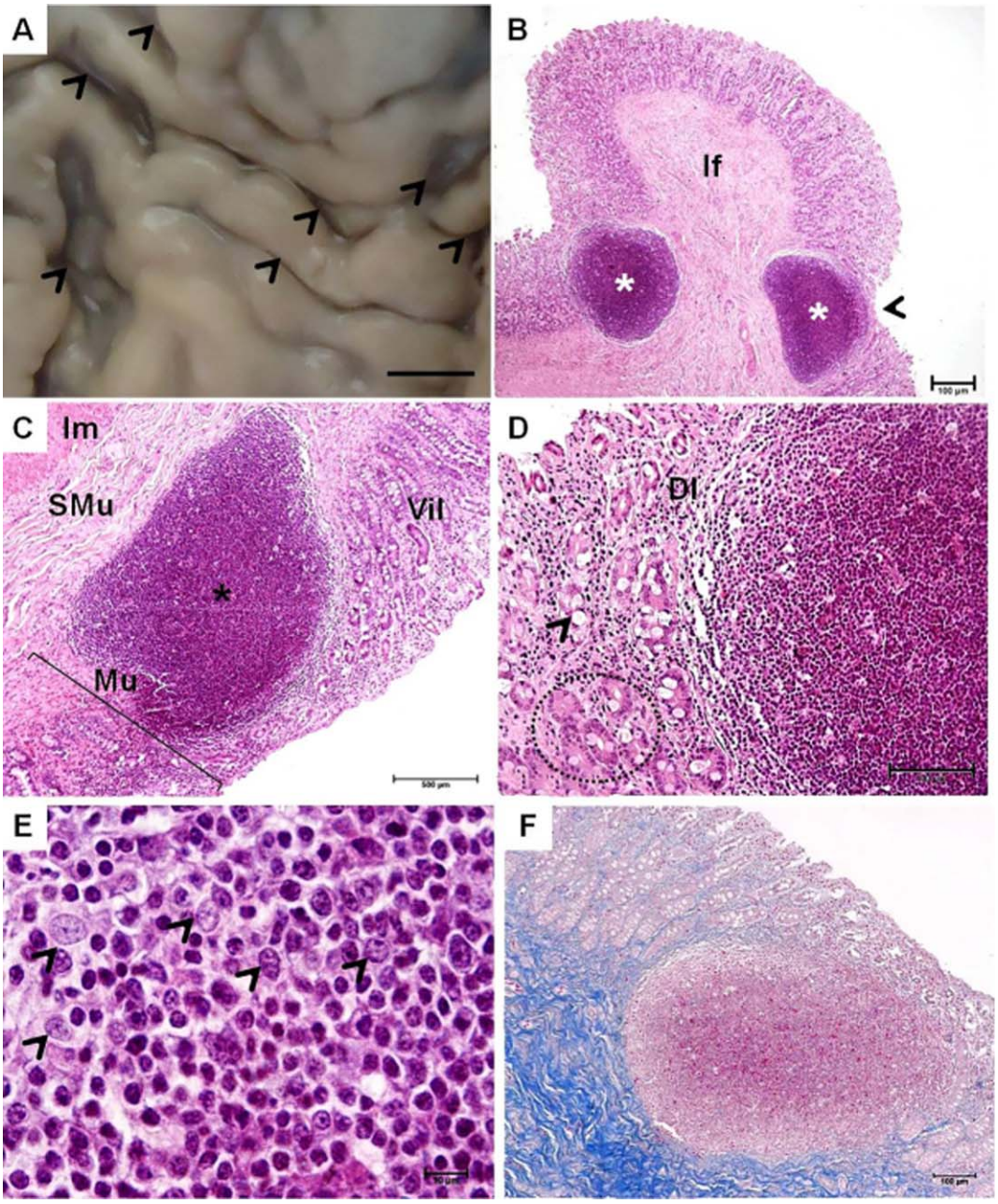

FIGURE 4 Photomicrograph of odontocetes intestines. A. Juvenile *Sotalia fluviatilis*. Internal mucosa, indicating the presence of clusters of lymphoid tissue, forming folds and grooves, indicated by arrowheads. Bar: 0.5 cm. B–D. Peyer's Patches, initial (B, D, F) and median (E) thirds of an juvenile *Inia geoffrensis*. B. Intestinal fold (If), with the presence of two isolated lymph nodules (*). Area with absence of villi in the intestinal lumen indicated by arrowhead. Hematoxylin-Eosin (HE). ×10. C. Lymph nodule (*). Villi (Vil), mucosa (Mu), submucosa (SMu), and inner muscular layer (Im). HE. ×10. D. Diffuse lymphoid tissue (Dl) close to the lymph nodule (*) and the intestinal lumen. Secretory units (mucous glands) highlighted. Goblet cell indicated by arrowhead. HE. ×20. E. Lymphoblasts indicated by arrowheads, surrounded by lymphocytes, with darker nuclei and smaller in size. HE. ×100. F. Lymph nodule (*), delimited by collagen fibers, with no capsule. Masson's Trichrome. ×10. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

As described in the literature (Brandtzaeg et al., 2008), we verified the presence of two specific types of MALT structure, an organized (O-MALT) delimited by organized collagen fiber tissue, and a disorganized or diffuse (D-MALT), comprising populations of lymphocytes from the lamina propria and the base the epithelial lining.

Laryngeal tonsils (LT) have been described in *Tursiops truncatus* (Cave, 1980; Cowan & Smith, 1999) and *Delphinapterus leucas* (Romano et al., 1993). In our study, LT were described for the first time in *S. guianensis*, *S. fluviatilis*, *S. clymene*, *P. electra*, and *G. macrorhynchus*. Thus, further studies are required in order to ascertain if they are present in other species, since it is not clear if the absence of this anatomic structure is species related, if they have an unusual location and are not well known by researchers working in the area, or if there is any correlation

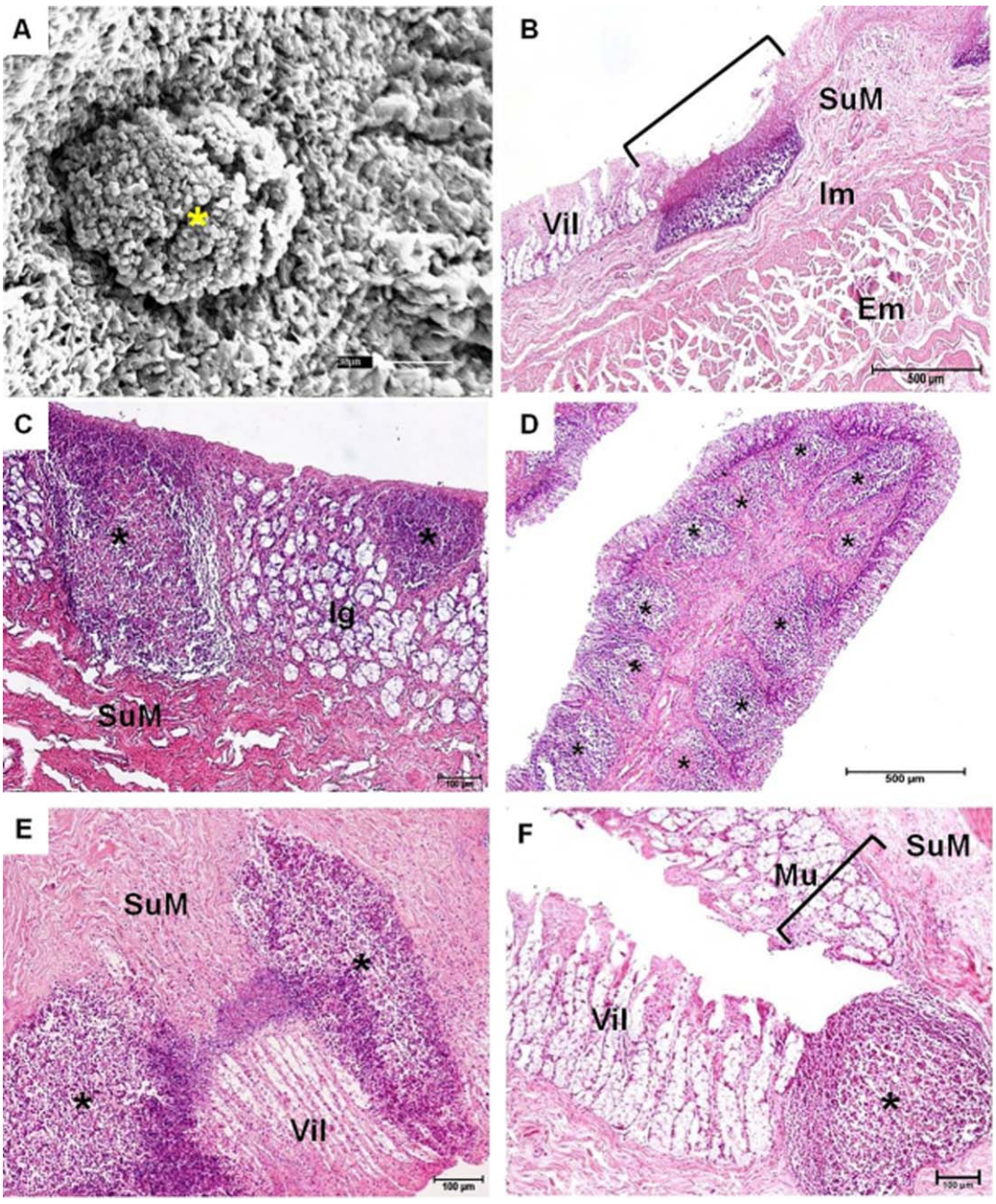

FIGURE 5 Photomicrograph the final third of the intestine. A. Scanning electron photomicrograph of a juvenile *Peponocephala electra*. Protruding lymph nodule (*), after freeze-fracture fragment of the intestine. B–D. Juvenile *Sotalia guianensis*. B. Presence of a lymphoid cluster in the transition site from glandular epithelium and stratified epithelium (highlighted). Villi (Vil), Submucosa (SuM), inner muscular layer (Im), and external muscular layer (Em). Hematoxylin-Eosin (HE). ×10. C. Intestinal glands (Ig) in evidence, surrounding the lymph nodules (*). Submucosa layer (SuM). HE. ×20. D. Solitary lymph nodules of very different sizes in a long intestinal fold. HE. ×20. E. *Stenella clymene*. Lymph nodules communicating through a "bridge" of diffuse lymphoid tissue, indicated by arrowhead. Villi (Vil) and submucosa layer (SuM). HE. ×10. F. Juvenile *Globicephala macrorhynchus*. Lymph nodule (*) present in the pit formed by intestinal folds. Villi (Vil), mucosa (Mu) and submucosa (SuM). HE. ×10. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

between species/age group that justifies the occurrence or absence of such structures.

Smith et al. (1999) believe that the location of LT expose cetaceans to an imminent danger in cases of infection or inflammation due to its location in the respiratory tract. However, these researchers, while studying *T. truncatus*, found no significant change to occlude the airways of animals evaluated, suggesting that the immune response of this lymphoid organ is somehow segregated from the lymph nodes present in this region such as the cervical lymph nodes (Silva et al., 2014). This was confirmed in the present study, where there was no increase in size of the cervical lymph nodes in any of the animals studied.

The adaptive function of this structure in cetaceans remains uncertain for many researchers. Due to the large separation

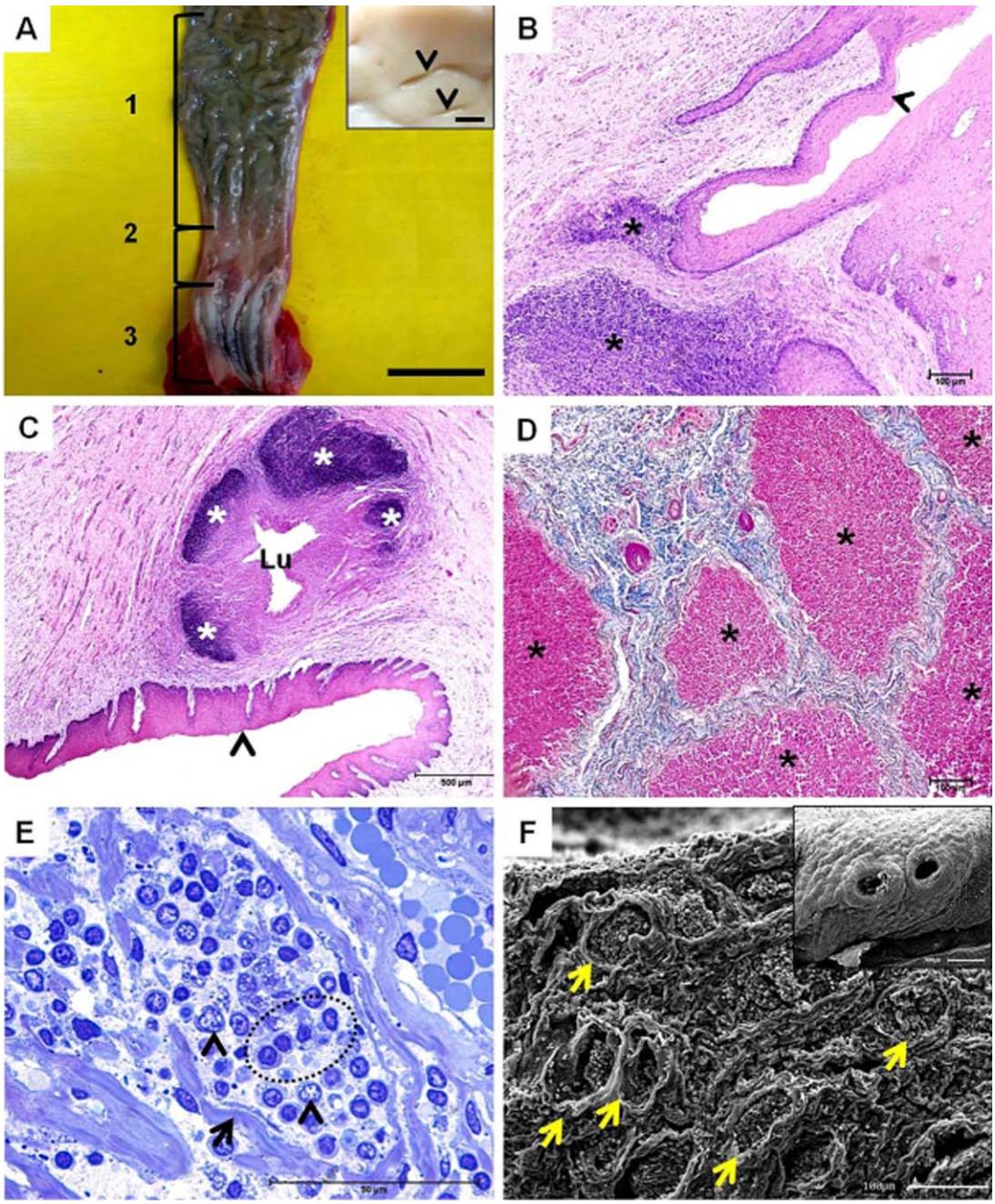

FIGURE 6 Photomicrographs of anal tonsil. A–D. *Sotalia guianensis* calf. A. Distal segment of the intestine. 1. Pleated mucosa containing lymphoid aggregates; 2. Epithelium without lymphoid tissue; 3. Anal canal. Bar: 2 cm. Presence of crevices and crypts in the area at higher magnification. Bar: 0.5 cm. B–C. Crypt lined by squamous epithelium (arrowhead). Clusters of lymphocytes (*) surrounding the lumen (Lu) of the crypt. Hematoxylin-Eosin. B. ×10. C. ×20. D. Lymph nodes (*) surrounded by collagen fibers stained in blue. Masson's Trichrome. ×10. E. Juvenile *Peponocephala electra*. Lymph nodule, delimited by densely arranged collagen fibers (arrow). Presence of small lymphocytes (highlighted area) and lymphoblasts (arrowheads). Semi-thin cut. Toluidine blue. ×8.900. F. Juvenile *Stenella clymene*. Scanning electron photomicrograph. Lymph nodules, delimited by collagen fiber, indicated by arrows. At higher magnification: surface of the tonsil showing the crypts openings. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

between the respiratory system and digestive tract in these animals, the inspired air is conducted directly to the larynx, trachea and lungs, unlike in terrestrial mammals. According to our histological findings, we believe that LT can be compared to human pharyngeal tonsil, since they have the same lymphoid characteristics (Grist, 1990). We also verified the presence of an irregular surface structure, full of crypts and crevices, which suggests a physical barrier to allow the apprehension of foreign bodies.

The skin has a number of unique immunological mechanisms since it is under constant physicochemical stress. Streilein (1989) proposed that the skin-associated lymphoid tissue (SALT) comprises a single collection of cells that are organized according to need to protect the

animal against pathogens. In our study mature lymphocytes were found in skin fragments in three animals evaluated, (3/44; 6.85%), corroborating to the described by Streilein (1983).

Lymph nodules present in the uterine cervix do not have any similar description in the literature of cetaceans. This type of MALT is normally present in sites in which antigen contact is constant, such as urogenital, respiratory and digestive systems (Banks, 1998). Wira, Fahey, Sentman, Pioli & Shen (2005) believed that their presence is closely linked to cell migration, activated by hormonal changes during the menstrual cycle. Lima & Alves (2008) state that the cervix would be the main site for an immune response since in this region there is a large concentration of intraepithelial lymphocytes with the potential for activation. This corroborates with the findings of this study, since the female with MALT in its cervix was on reproductive age.

As observed in the skin, the intestine is constantly challenged by antigens throughout the animal's life, making it highly vulnerable to infections (MacDonald, 2003). The integrity of its mucosa is maintained by numerous defense cells, which may be structurally organized or not. In cetaceans, Cowan & Smith (1999) described the presence of a continuous plate of lymphoid tissue in the lamina propria of the mucosa and submucosa of juvenile *Tursiops truncatus* intestines. The same was observed in our study, with the presence of continuous and discontinuous plates of lymphoid tissue in the proximal and medial portions of all intestines evaluated.

Anal tonsils (AT) were described in several species of cetaceans, such as *Physeter catodon* (Uys & Best, 1966), *Eschrichtius robustus* (Cowan & Brownell, 1974), *Platanista gangetica* (Yamasaki, Komatsu, & Kamiya, 1977), *S. coeruleoalba* (Komatsu, 1979), and *Tursiops truncatus* (Cowan & Smith, 1995). However, studies developed by Cave (1980), Yamasaki, Takahashi, & Kamiya (1975), Yamasaki et al. (1977), and Romano et al. (1993) did not report the occurrence of AT in the evaluated species, different from our study where these tonsil were found in five of the seven species studied (*S. guianensis*, *S. fluviatilis*, *S. clymene*, *S. geoffrensis*, and *P. electra*).

We cannot say that this structure is universal in cetaceans, since the reports vary between species. We strongly believe that, as the observed in OT, AT occur in greater numbers than found in the literature, a fact inherent to the difficulty of identifying such structures during macro and microscopic examinations of the intestine, since observation of their presence or absence in a given species is only possible through the opening and evaluation of the entire distal portion of the intestine.

Cowan & Smith (1999) consider the AT of utmost immune importance in cetaceans due to its location at a strategic anatomical site, working as a barrier to antigen presentation in cases of reflux during diving (Beineke, Siebert, Wohlsein, & Baumgärtner, 2010). This is confirmed in our study, since T lymphocytes, fundamental cells for antigen presentation, were present in all AT evaluated.

The positive staining of CD3 T lymphocytes observed in this study, using a specific anti-rat antibody to human with cross-reactivity with *P. phocoena*, corroborates with the verified by Beineke et al. (2001), who used an anti-rabbit antibody. These findings show that it is possible to use leukocyte antigens from different species to determine the phenotype of lymphocytes in lymphoid tissues of cetaceans.

Given our findings, we conclude that the mucosa-associated lymphoid tissue in cetaceans is similar to that observed in terrestrial mammals, with adaptations inherent to the aquatic environment, such as the presence of laryngeal and anal tonsils, ensuring a more efficient immune response in face of constant antigenic challenges. Thus, it is suggested that this segment of the lymphoid system is essential for the animal protection against any aggressive agent in their habitat, working as potential environmental indicators.

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REFERENCES

Arvy, L. (1976). The unknowns of the lymphatic system in Cetacea. In G. Pilleri (Ed.), *Investigations on Cetacea* (Vol. 7, pp. 169–177). Switzerland: Der Bund.

Azzali, G. (2003). Structure, lymphatic vascularization and lymphocyte migration in mucosa-associated lymphoid tissue. *Immunological Reviews*, *195*, 178–189.

Banks, W. J. (1998). *Histologia veterinária aplicada (629 p)*. São Paulo: Editora Manole.

Beineke, A., Siebert, U., Wünschmann, A., Stott, J. L., Prengel, I., Kremmer, E., & Baumgärtner, W. (2001). Immunohistochemical Investigation of the Cross-reactivity of Selected Cell Markers from Various Species for Characterization of Lymphatic Tissues in the Harbour Porpoise (*Phocoena phocoena*). *Journal of Comparative Pathology*, *12*, 311–317.

Beineke, A., Siebert, U., McLachlan, M., Bruhn, R., Thron, K., Failing, K., … Baumgärtner, W. (2005). Investigations of the potential influence of environmental contaminants on the thymus and spleen of harbor porpoises (*Phocoena phocoena*). *Environmental Science and Technology*, *39*, 3933–3938.

Beineke, A., Siebert, U., Wohlsein, P., & Baumgärtner, W. (2010). Immunology of whales and dolphins. *Veterinary Immunology and Immunopathology*, *133*, 81–94.

Brandtzaeg, P., Kiyono, H., Pabst, R., & Russell, M. W. (2008). Terminology: Nomenclature of mucosa-associated lymphoid tissue. *Mucosal Immunology*, *1*, 31–37.

Cave, A. J. E. (1980). Note on *Tursiops* visceral histology. In G. Pilleri (Ed.), *Investigations on Cetacea* (Vol. *11*, pp. 11–113). Switzerland: Der Bund.

Cesta, M. F. (2006). Normal structure, function, and histology of mucosa-associated lymphoid tissue. *Toxicol Pathology*, *34*, 599–608.

Cowan, D. F. & Brownell, R. L. Jr. (1974). Gut-associated lymphoepithelial organ ('anal tonsil') in the gray whale. In R. J. Harrison (Ed.), *Functional anatomy of marine mammals* (pp. 321–327). New York: Academic Press.

Cowan, D. F. & Smith, T. L. (1995). Morphology of complex lymphoepithelial organs of the anal canal ('anal tonsil') in the bottlenose dolphin, *Tursiops truncatus*. *Journal of Morphology*, *223*, 263–268.

Cowan, D. F. & Smith, T. L. (1999). Morphology of the lymphoid organs of the bottlenose dolphin, *Tursiops truncatus*. *Journal of Anatomy*, *19*, 505–517.

Croitoru, K. & Bienenstock, J. (1994). Characteristics and functions of mucosa-associated lymphoid tissue. In P. L. Ogra, J. Mestecky, M. E. Lamm, W. Strober, J. R. McGhee, & J. Bienenstock (Eds.), *Handbook of mucosal immunology* (pp. 141–151). San Diego: Academic Press.

Geraci, J. R. & Lounsbury, V. J. (2005). *Marine mammals ashore: a field guide for strandings (371 p)*. National Aquarium in Baltimore: Baltimore.

Grist, W. J. (1990). The tonsils and pharynx. In H. K. Walker, W. D. Hall, & J. W. Hurst (Eds.), *Clinical methods: the history, physical, and laboratory examinations* (pp. 626–627). Boston: Butterworths.

Janeway, C. A. Jr. & Medzhitov, R. (2002). Innate immune recognition. *Annual Review of Immunology*, *20*, 197–216.

Jefferson, T. A., Leatherwood, S., & Webber, M. A. (1993). *FAO species identification guide. Marine mammals of the world (320 p)*. FAO: Rome.

Komatsu, S. (1979). The anal tonsil in the striped dolphin, *Stenella coeruleoalba*. *Journal of Liberal Arts and Sciences, Sapporo Medical College*, *20*, 53–56.

Lima, Y. A. R. & Alves, M. F. C. (2008). O sistema imune da mucosa do trato genital feminino e o impacto das doenças sexualmente Transmissíveis. *Revista de Patologia Tropical*, *37*, 295–309.

MacDonald, T. T. (2003). The mucosal immune system. *Parasite Immunology*, *25*, 235–246.

Murphy, K. (2011). *Janeway's immunobiology (888 p)*. New York: Garland Science.

Nochi, T. & Kiyono, H. (2006). Innate immunity in the mucosal immune system. *Current Pharmaceutical Design*, *12*, 4203–4213.

Parham, P. (2009). *The immune system (608 p)*. New York: Garland Science.

Romano, T. A., Felten, S. Y., Olschowka, J. A., & Felten, D. L. (1993). A microscopic investigation of the lymphoid organs of the beluga, *Delphinapterus leucas*. *Journal of Morphology*, *215*, 261–287.

Samuelson, D. A. (2006). *Textbook of veterinary histology (560 p)*. New York: Saunders Elsevier.

Silva, F. M. O., Guimarães, J. P., Vergara-Parente, J. E., Carvalho, V. L., Meirelles, A. C. O., Marmontel, M., ... Miglino, M. A. (2014). Morphological analysis of lymph nodes in odontocetes from north and northeast coast of Brazil. *Anatomical Record (Hoboken)*, *297*, 939–948.

Simpson, J. G. & Gardner, M. B. (1972). Comparative microscopic anatomy of selected marine mammals. In S. H. Ridgway (Ed.), *Mammals of the sea. Biology and medicine* (pp 298–418). Springfield: Charles C. Thomas Publisher.

Smith, T. L., Turnbull, B. S., & Cowan, D. F. (1999). Morphology of the complex laryngeal gland in the Atlantic bottlenose dolphin, *Tursiops truncatus*. *Anatomical Record*, *254*, 98–106.

Streilein, J. W. (1983). Skin-associated lymphoid tissues (SALT): Origins and functions. *Journal of Investigative Dermatology*, *80 Suppl*, 12s–16s.

Streilein, J. W. (1989). Skin-associated lymphoid tissue. *Immunology Series*, *46*, 73–96.

Uys, C. J. & Best, P. B. (1966). Pathology of lesions observed in whales flensed at Saldanha bay, south Africa. *Journal of Comparative Pathology*, *76*, 407–415.

Wira, C. R., Fahey, J. V., Sentman, C. L., Pioli, P. A., & Shen, L. (2005). Innate and adaptive immunity in female genital tract: Cellular responses and interactions. *Immunological Reviews*, *206*, 306–335.

Yamasaki, F., Takahashi, K., & Kamiya, T. (1975). Digestive tract of La Plata dolphin, *Pontoporia blainv*illei. II. Small and large intestines. *Okajimas Folia Anatomica Japonica.*, *52*, 1–26.

Yamasaki, F., Komatsu, S., & Kamiya, T. (1977). A comparative morphology of anal tonsils in Platanistidae. *The Scientific Reports of the Whales Research Institute*, *29*, 95–100.