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Expression of Langerin/CD207 in airways, lung and associated lymph nodes of a stranded striped dolphin (*Stenella coeruleoalba*)

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ABSTRACT

The airways and lungs of vertebrates are an entrance way for several microbial pathogens. Cetaceans present an upper and lower respiratory anatomy that allows the rapid flow of large air volumes, which may lead to high susceptibility to respiratory infections. Mortality and stranding rate of Cetaceans increased dramatically, so wide the knowledge about the immune system and specific antibodies identifying immune cells populations, is of fundamental importance to monitor and document cetacean health.

The aim of this study was to identify the localization of dendritic cells marked by Langerin/CD207 in airways, lungs and associated lymph nodes, of the striped dolphin *Stenella coeruleoalba*. Samples of trachea, bronchi, lungs and lung-associated lymph nodes were obtained from a stranded adult male of *Stenella coeruleoalba*. Our results showed abundant lymphoid aggregates (LAs) in the lung of *S. ceruleoalba*. Langerhans-like dendritic cells were well distributed along the epithelium and interstitium of respiratory tract and in associated lymph nodes.

The present study deepens the knowledge about the cetacean's immune system and report about the exploitability of a commercial antibody (Langerin/CD207) for cetacean species.

1. Introduction

The respiratory system of cetaceans presents upper and lower airways. Upper airways have been well described in different studies (Costidis and Rommel, 2012; Kooyman, 1973; Piscitelli et al., 2013; Racicot and Berta, 2013). Lower airways located within the thorax, form the respiratory tree that is composed as follow: trachea bifurcates into two main bronchi and lungs with terminal bronchi, alveolar sacs, and alveoli.

The histology of some cetaceans respiratory system such as *Phocoena phocoena, Stenella longirostris, Lagenorhynchus obliquidens* has been studied by Piscitelli et al. (2013). Murata (1951) described the lungs histology of some species of Mystacoceti and Odontoceti including the striped dolphin, *Stenella coeruleoalba*. In all studied cetaceans species, the pseudostratified ciliated columnar epithelium with serous and mucous glands, lining trachea and extrapulmonary bronchi (Haldiman et al., 1984; Smith et al., 1999). Intrapulmonary bronchi present a simple cuboidal epithelium with Clara-type cells and submucosal secreting cells (Fanning and Harrison, 1974). In terminal bronchi the capillaries are in close contact with simple squamous epithelium, in order to perform the gas exchange function (Fanning and Harrison, 1974; Wislocki, 1929). Alveolar epithelium consists of type I cells, which create a barrier between the alveolar lumen and septal wall comprising part of the capillary membrane (Ross and Pawlina, 2006), and type II cells which secrete surfactants within the air–liquid interface of the conducting airways and alveoli (Reid et al., 2005).

The mucosal surface of the cetaceans respiratory tract is similar to terrestrial mammals and forms a fragile interface between the immune system and the potential pathogens or commensal microorganisms present in external environment.

Immune cells associated with mucosal epithelium constitute a constant protection for this thin epithelial layer (Holt et al., 2005; Silva et al., 2016). Langerhans cells (LCs) are considered an immature state of Dendritic cells (DCs) which maintain immunological homeostasis within the skin, mucosae and lungs. In respiratory tract of terrestrial mammals, Langerhans cells are typically located within airway

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epithelium, submucosa and associated lung parenchymal tissue under resting conditions, where they form a rich network comparable to the Langerhans cells population in the epidermis and display rapid and dramatic response kinetics to inflammatory challenge (Holt and Schon-Hegrad, 1987; Holt and Stumbles, 2000; Sertl et al., 1986; Stumbles et al., 2003). Langerin is a c-type lectin expressed by different types of DCs: skin's Langerhans cells, dendritic cells present in stratified epithelial surfaces and by a subset of dendritic cells present in most connective tissues, including the dermis, lung, kidney and liver; this type II transmembrane protein recognizes glycosylated patterns of pathogens such as mycobacteria (Mayer et al., 2007). Within the bronchial epithelium, DCs show typical features of LCs, as presence of Birbeck granules, and expression of CD1a and Langerin/CD207 (Vermaelen and Pauwels, 2004).

For most cetaceans, immunological reports are old and scarce. Immune system studies are often limited to a single species. Despite this topic is of fundamental importance, and all efforts to deepen the knowledge about the immune system of cetaceans, much still remains unknown (Cowan and Smith, 1999; Romano et al., 1993; Shea et al., 1972; Vuković et al., 2005). Recently Díaz-Delgado et al. (2015) have tested various commercial primary antibodies to test cross-reactivity against leukocytes and cytokines in lymphoid tissues (thymus, lymph nodes and spleen) of Franciscanas (*Pontoporia blainvillei*) with immunohistochemical techniques. In an our previous study the presence of Langerhans cells and dermal dendritic cells, characterized using immunohistochemical staining for anti-TLR2 and S-100 antibodies, have been reported in the skin of *Stenella coeruleoalba* (Lauriano et al., 2014).

In the present study, for the first time, Langerhans-like dendritic cells langerin/CD 207 positive, have been characterized in respiratory tract and lung-associated lymph nodes of the stranded striped dolphin (*Stenella coeruleoalba*).

2. Materials and methods

2.1. Tissue preparation

An adult male of *Stenella coeruleoalba* (length: 197 cm) was found live stranded, on April 2011 at the Apulian coast (Adriatic Sea). After death, the dolphin, was kept on ice and transported for the necropsy and dissection of tissues and organs to be studied. Authors got the CITES permission for collection of tissue samples. CITES n° 3535/2011/EA. Fragments of lungs, lung-associated lymph nodes, trachea and bronchi, were dissected and treated for histological examinations as described in previous works (Lauriano et al., 2019, 2014). Samples were then fixed in 4 % paraformaldehyde in phosphate buffered saline (PBS) 0.1M (pH7.4) for 12–18 h, dehydrated trough graded ethanol series, clarified in xylene, for Paraplast® (McCormick Scientific LLC, St. Louis, MO, USA) embedding. Included tissues were then cut in to 5–10 µm sections.

2.2. Histology and immunohistochemistry

For analysis of cell components and tissue structures, serial Sections (5 µm thick) were stained with the routinely histological stains: hematoxylin and eosin (H&E), Masson's trichrome, Mallory's trichrome and Alcian blue-periodic acid Schiff (AB-PAS; pH 2.5) and were examined with a Zeiss Axioskop 2 plus microscope equipped with a Digital Camera (Sony DSC-85).

2.3. Immunoperoxidase method

Indirect method of peroxidase was used to carry out immunohistochemical investigations. Serial sections were 0.3 % H_2O_2 (PBS) incubated 30 min to prevent the activity of endogenous peroxidase; then, to rinsed sections was added normal goat antiserum (1:20; Sigma, St. Louis, MO, USA) was added. Serial sections were incubated overnight at 4∘C in a humid chamber with polyclonal antibody to Langerin/CD207 (Santa Cruz Biotechnology, Inc AB_2074213 1:100). The sections were then washed in PBS and incubated for 2 h with a goat anti rabbit IgG-peroxidase conjugate (1:100, Sigma–Aldrich, St. Louis, MO, USA). Peroxidase activity was visualized by incubation of the sections for 1–5 min at room temperature in a solution 0.02 % diaminobenzidine (DAB) and 0.015 % hydrogen peroxide. After rinsing in PBS, sections were dehydrated, mounted and examined under an Axiophot Zeiss microscope equipped with a Sony Digital Camera DSC-85. Control experiments were performed excluding primary antibody.

2.4. Confocal immunofluorescence

Serial sections were deparaffinized and rehydrated, rinsed several times in PBS and blocked in 2.5 % bovine serum albumin (BSA) for 1 h. Langerin/CD207, rabbit antibody (Santa Cruz Biotechnology, Inc AB_2074213 1:100) was used to characterize Dendritic cells. The secondary antiserum was the Alexa Fluor 594 donkey anti-rabbit IgG TRITC conjugated. After washing, the sections were mounted with Vectashield (Vector Labs, Burlingame, CA, USA) to prevent photobleaching, and cover slipped. Control experiments excluding primary antibody were performed (data not shown).

2.5. Laser confocal immunofluorescence

Sections were analyzed and images acquired using a Zeiss LSMDUO confocal laser scanning microscope with META module (Carl Zeiss Micro Imaging GmbH, Germany). Helium-neon (He- Ne) laser emitting at 543 nm has been used for excitation of Alexa Fluor. More details about the instrument settings are reported in Zaccone et al. (2015). Each image was rapidly acquired in order to minimize photodegradation. Digital images were cropped and the figure montage prepared using Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA, USA).

3. Results

3.1. Airways and lungs

Trachea and bronchi epithelium *of Stenella coeruleoalba*, as for other studied cetaceans, consisted of a pseudostratified ciliated columnar epithelium (Fig. 1A and D) with abundant mucous goblet cells scattered among the ciliated cells. These cells with Alcian Blue pH 2.5 PAS stain, showed positivity to acidic mucins (Fig. 1B). Basal cells, resting on the basement membrane, were found as scattered individual cells, not forming a continuous monolayer. Epithelium and connective tissue were separated by the basement membrane. Connective tissue was rich in blood vessels and seromucous glands (Fig. 1A and D). The immunoreactivity of langerin/DC 207 revealed a massive presence of dendritic cells along the airway mucosa in both epithelium and connective tissue (Fig. 1C and E).

3.2. Alveolar epithelium and lung parenchyma

Alveolar epithelium was constitute by cuboidal cells, with associated capillaries. Inflammatory infiltrates were present with erythrocytes, eosinophils and alveolar macrophages (Fig. 2A).

Lymphoid aggregates (LAs) were observed in the lung parenchyma, with several alveolar macrophages, eosinophils, lymphocytes, presumptive plasma cells (Fig. 2B and C) and abundant langerin/CD207 positive cells (Fig. 2D and E).

3.3. Lung-associated lymph nodes

Sections of lung-associated lymph nodes, stained with Mallory's trichrome, presented a thick capsule, consisting of dense connective

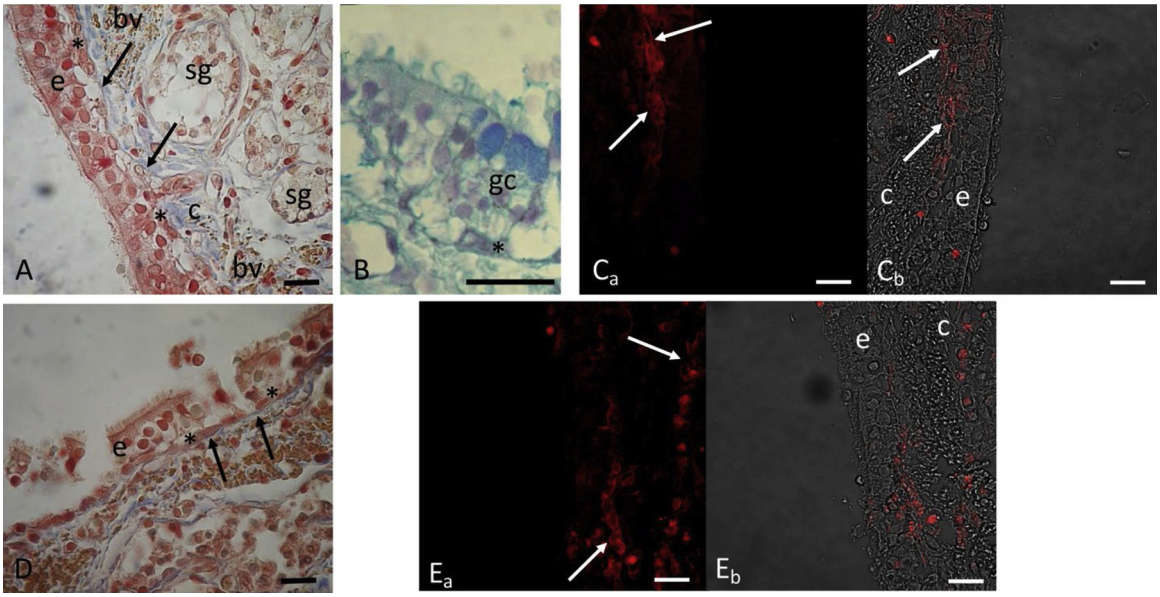

Fig. 1. Histological sections of trachea (A–C) and bronchi (D and E) epithelium, of *Stenella coeruleoalba*; Figures A and D show section Masson's trichrome stained of pseudostratified ciliated columnar epithelium (e), arrows indicate the basal lamina in which non-contiguous basal cells are visible (*). In the deeper part of the connective tissue layer (c) several blood vessels (bv) and seromucous glands (sg) are easily distinguishable; in figure B goblet cells (gc) with acidic content are highlighted by Alcian Blue pH 2.5 PAS stain, acid mucins are known to play an important role in protecting the mucosa against toxins pathogens and physical or chemical damage (Lauriano E. R. et al., 2019;); asterisks (*) indicate basal cells. In figures C and E abundant dendritic cells (white arrows) located in close to the airway epithelium (e) and in the subepithelial zone of the airway wall (c) are immunostained with anti-langerin/DC 207 antibody in immunofluorescence. Figures C and E are given with their respective bright-field C_b and E_b, where tissue background is clearly visible. Magnifications: 1A-1D (40x) -1B 100x, 1C-1E 40 × . Scale bars: 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

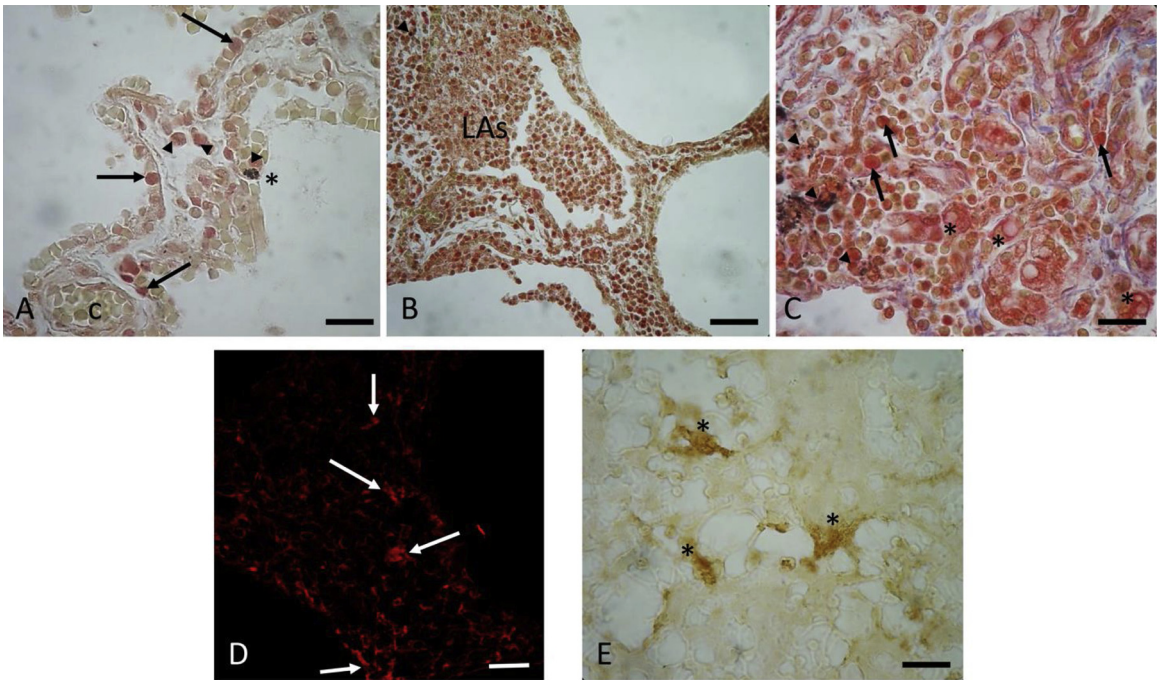

Fig. 2. Histological sections of alveolar epithelium (A) and lung parenchyma (B–E); fig. A, cuboidal epithelium (black arrows) with associated blood vessels (c); numerous granular eosinophils (arrowheads) are present and an alveolar macrophage (dust cell) including black particles in the cytoplasm, is clearly visible between the epithelial cells (*) Masson's trichrome. Fig. B, Lymphoid aggregates (LAs) are clearly visible in transvers section of lung tissue stained with Masson's trichrome. In fig. C, aggregates of immune cells form a lymphoid tissue associated with lung parenchyma, lymphocytes (black arrows), alveolar macrophages (arrow heads), and presumptive plasma cells (*) Masson trichrome. Figures D and E show the presence of several langerin/CD207 immunopositive cells in both confocal immunofluorescence (white arrows) and immunoperoxidase (asterisks) methods respectively in lung parenchyma. Magnifications: A-C-E 100x, B 20x -D 40 × . Scale bars: 2A-C-D-E (2μm), 2B (50 μm).

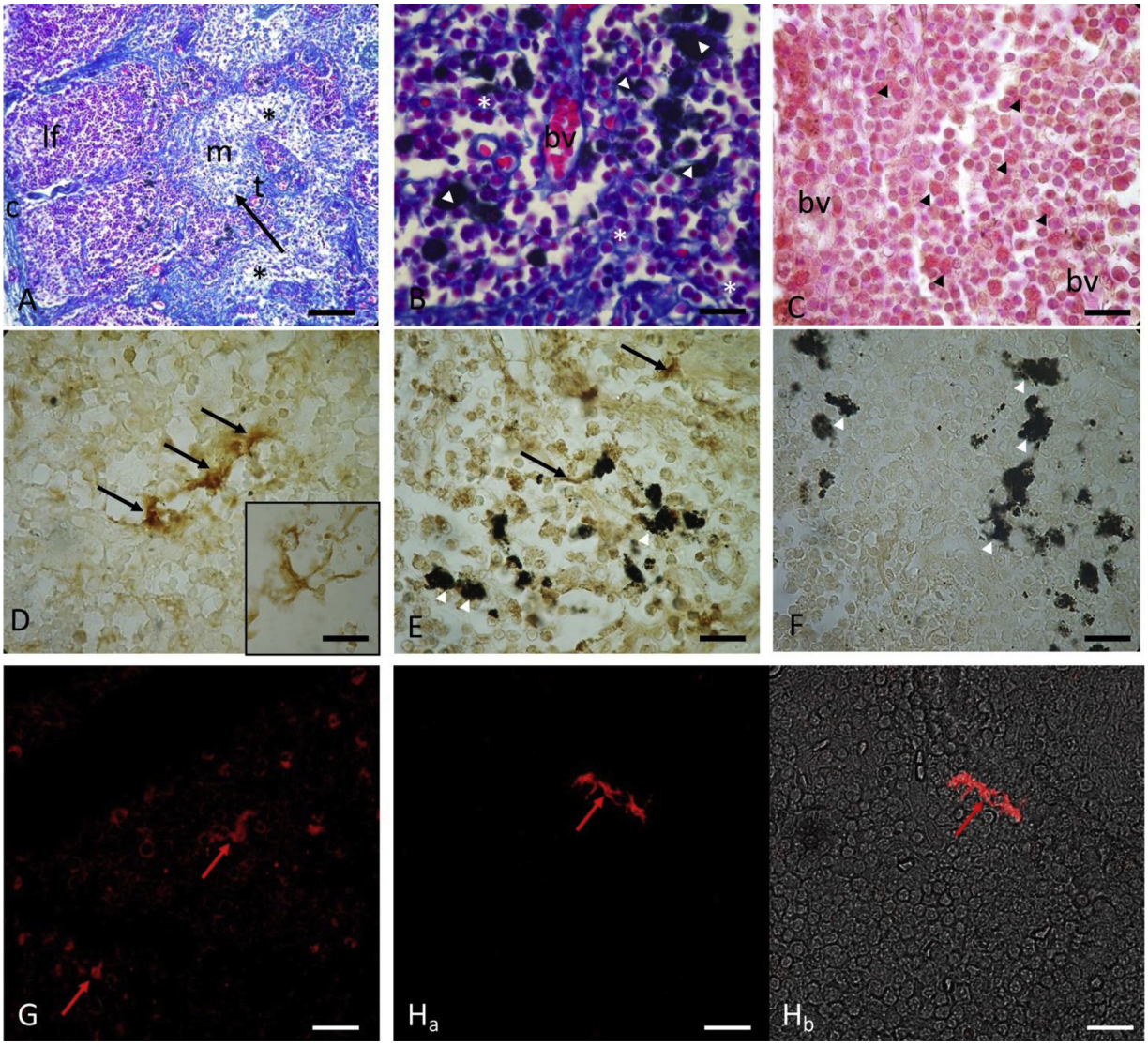

Fig. 3. Lung-associated lymph nodes of *S. coeruleoalba*; fig A, lymph nodes parenchyma stained with Mallory's trichrome, showing capsule (c), consisting of dense connective tissue layer and smooth muscle cells, with projection of highly vascularized collagenous septa infiltrating towards the lymph node centre dividing the parenchyma in lymphoid follicles (lf) in the cortex. Medulla (m) present a low immune cell density (*) separated by sinusoids (black arrow) and trabeculae (t). Fig. B, alveolar macrophages (white arrowheads) and lymphocytic cords (white asterisks) are evident in lymphoid follicles (Mallory's trichrome). Fig. C, show numerous granular eosinophils (black arrowheads) and blood vessels (bv), H/E staining. Fig. D, immunoperoxidase staining with anti-langerin/DC 207 antibody highlighted the presence of dendritic cells (black arrows and inset). Fig. E, dendritic cells (black arrow) in close contact with alveolar macrophages (white arrowheads). Figures G–H, confocal immunofluorescence reaction using the same antibody confirmed the presence of dendritic cells (red arrows) in the cortical region of dolphin lymph nodes. Figure H is given with brightfield background also (H_b). Fig. F Control experiments excluding primary antibody. Magnifications: A 20x; B-C-D-E-F 100x, G–H 40 × . Scale bars: 3A (50 μm), 3B-C-D-E-F-G-H (20 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

tissue and smooth muscle cells; collagenous septa highly vascularized, departing from the capsule constituted a well-developed trabeculae framework dividing the lymph node parenchyma (Fig. 3A). Lymphoid follicles in the cortical region (cortex) were evident. This zone, resulted to be dark-stained for the presence of small lymphocytic cords; furthermore, several alveolar macrophages were observed (Fig. 3B); the sections stained with hematoxylin/eosin showed abundant eosinophils and blood vessels (Fig. 3C). The medulla was a light-staining tissue reflecting the low cell density. In the medulla, medullary cords and sinuses were evident (Fig. 3A). Immunohistochemistry and/or immunofluorescence demonstrated that antibody against Langerin/CD 207 labeled a large number of dendritic cells often in contact with alveolar macrophages in the cortical region (Fig. 3D–H). In Fig. 4, the localization of the antibody Langerin/CD 207 showed the presence of Langerhans cells in *Stenella coeruleoalba* skin.

4. Discussion

The present study reports, for the first time, the localization and presence of Langerhans-like dendritic cells (DCs), highlighted using the langerin/CD207 antibody, in lung, airways and associated lymph nodes of *S. coeruleoalba*.

DCs showed their "classical" dendritic morphology, spreading their cellular processes between keratinocytes, acting as sentinel cells in recognition and endocytosis against foreign and self-antigens (Kubo et al., 2009). Langerin/CD207 is a C-type lectin high-expressed by LCs of the epidermis. However, langerin has been also detected in DCs from dermis, heart, liver, lung, and lymphoid tissue such as spleen, thymus and lymph nodes (Bigley et al., 2015; Pergolizzi et al., 2018; Takahara et al., 2002). DCs present antigenic peptides in secondary lymphoid organs after migration. Tissue inflammation induces the migration of

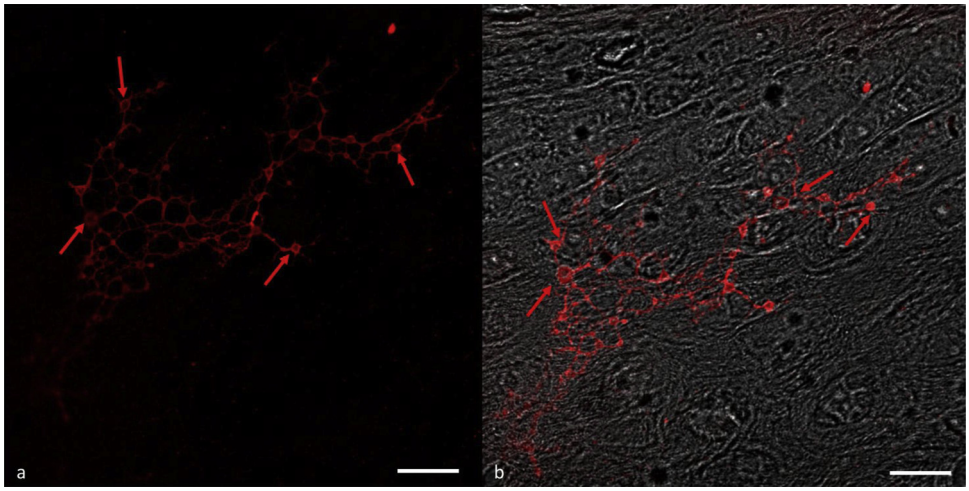

Fig. 4. Confocal immunolocalization of anti-langerin/DC 207, in *Stenella coeruleoalba* skin showing a framework of Langherans cells, magnification 40 × . Fig. 4 is given with brightfield background also (b). Scale bars: a–b (20 μm).

positive Langerin cells, to lymph nodes (Al-Ashmawy, 2018; Flacher et al., 2008). Condon et al. (2011) reviewed the reports of DCs in human, rat and other mammalian species, normally forming a rich network comparable to the Langerhans cells (LCs) population in the epidermis (GeurtsvanKessel et al., 2008; MacPherson, 1989; Steinman, 1991; Steinman and Cohn, 1973), as also in fish species (Lauriano et al., 2018); these cells are distributed within the epithelium overlying the mucosa of the lung parenchyma and conducting airways, in particular at the interseptal junctions between adjacent alveolar units (Inaba et al., 1992; Sallusto et al., 1998; Steinman, 1991). Density of these cells can increase markedly during local inflammatory challenge (Randall, 2010). It can be supposed that the expression of Langerin could confer DCs functions in both antigen presentation and pathogen innate clearance (Flacher et al., 2008). In this study, we observed abundant Langerhans-like dendritic cells located along the airway (trachea and bronchi) epithelium, in the subepithelial zone of the airway wall and in close juxtaposition with alveolar macrophages (also named dust cells). These cells interacting with other ones (stromal, epithelial, and immune cells) in the lung, maintain alveolar integrity arranging responses to environmental challenges. Our results showed alveolar macrophages that present a swollen cytoplasm with black particles in the alveolar epithelium and in lymph nodes.

The distribution and morphological features of alveolar macrophages observed in *S. ceruleoalba* were in agreement with those reported by Kawashima et al. (2004) in some species of Odontoceti. Alveolar macrophages are the first phagocytes that come in contact with inhaled particulate like coal, silica, asbestos, tissue debris. These cells migrating through lymphoid vessels, from the alveolar spaces to the lung lymph nodes, contribute to antigen transport (Claassen et al., 1993; Corry et al., 1984; Harmsen et al., 1985; Kirby et al., 2009; Pierre et al., 1997). Dendritic cells and macrophages are cells which can act in several ways as for example in exo- and endocytosis, digestion of foreign substances, antigen presentation to naïve T cells and cytokine production. These functions occur in both healthy and pathological conditions (Nagy et al., 2016). In our previous paper, we have described TLR2 immunopositive Dendritic cells in the skin of the *S. coeruleoalba* specimen (Lauriano et al., 2014). It is widely known that TLRs recognize a high number of molecules of bacterial, viral, or parasitic origin (Lauriano et al., 2016; Marino et al., 2015, 2013), demonstrating in this specimen a likely inflammation.

The results of this study could confirm an inflammatory state of this stranded specimen, considering the abundant infiltrate of granular eosinophils, lymphocytes and Langerhans-like dendritic cells in respiratory tissues; furthermore dendritic cells in close contact with alveolar macrophages revealing that in lung and airway, dendritic cells may interact with local tissue macrophages acquiring processed antigens (Holt and Stumbles, 2000). The immune system associated to the mucosa (MALT), covers all the body tissues of humans and terrestrial and aquatic mammals (Maheshwari et al., 2011). Despite this, reports on the importance and relevance of aquatic mammals MALT are scarce, 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; Silva et al., 2016).

For the first time, in this study, we have observed abundant lymphoid aggregates (LAs) in the lung of *S. ceruleoalba*, with several presumptive plasma cells, lymphocytes, alveolar macrophages and langerin/CD207 positive cells.

Moreover, Langerhans cells immunoreactive to the Langerin/CD 207 observed also in *Stenella coeruleoalba* skin, validate the localization of the antibody in cells of dolphin respiratory tract and lung-associated lymph nodes.

Future studies are needed to deepen the information about the processes of maturation and activation of lung DCs and their complex interactions with other cells. Furthermore, the immune system study, of wild cetaceans species could be useful to monitor the conservation status of their habitat, as these marine mammals are considered sentinel species.

Concluding, the present study expands the knowledge about the cetacean's immune system and suggests the possibility to use the commercially available antibody (anti-Langerin/CD 207) for cetacean species.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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