COMPARATIVE IMMUNOLOGY OF MARINE MAMMALS

EDITED BY: Giovanni Di Guardo, Michael F. Criscitiello, Eva Sierra and Sandro Mazzariol

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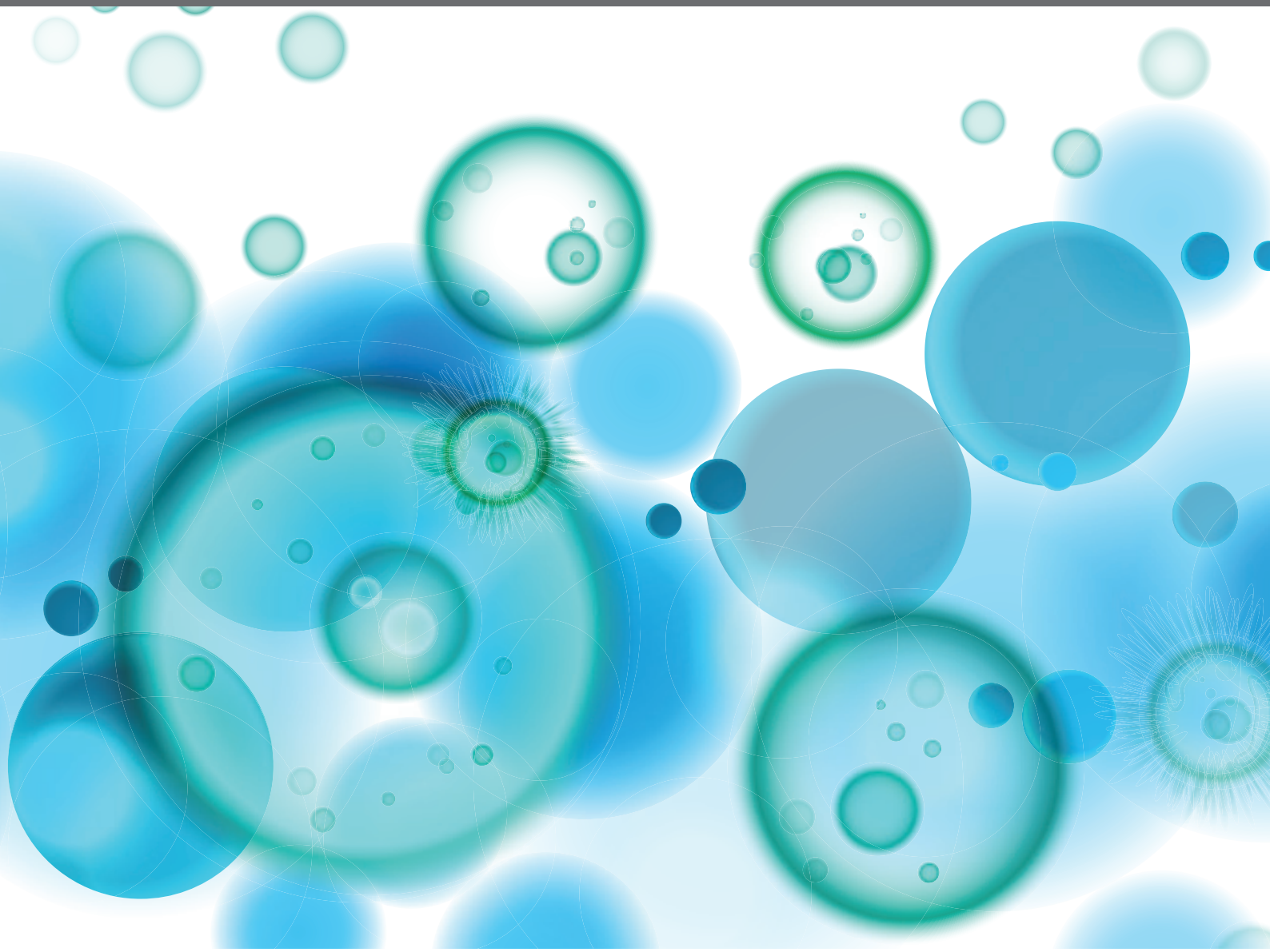

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COMPARATIVE IMMUNOLOGY OF MARINE MAMMALS

Topic Editors:
Giovanni Di Guardo, University of Teramo, Italy
Michael F. Criscitiello, Texas A&M University, United States of America
Eva Sierra, University of Las Palmas de Gran Canaria, Spain
Sandro Mazzariol, University of Padova, Italy

Marine mammals (MMs) are regarded as valuable bioindicators with tremendous potential for public health. However, many aspects of their immune system remain poorly understood. Monitoring immune responses of MMs is pivotal for the health assessment of both individuals and populations, as well as providing the scientific basis for analyzing the anthropogenic environmental impact on marine ecosystems and marine-terrestrial interphases. For instance, the increasing susceptibility of Mediterranean whale and dolphin populations to various diseases has been linked to a possible negative influence of multiple environmental factors on the immune system of MMs.

The currently limited knowledge on MM immunology has mainly centered on: (i) lymphocyte transformation assays; (ii) natural killer cell activity; (iii) phagocytic activity and respiratory burst; (iv) humoral immune responses; (v) cytokines and (vi) acute phase immune responses. Therefore, further research is essential for deepening our understanding of the specificity of the host immune response in MMs, with a particular emphasis on the genesis and dynamics of (i) cytokine 'networks' or 'signatures'; (ii) transcriptional regulation of immune cells and (iii) major immunomodulators. High-throughput molecular techniques, such as transcriptomic analysis and RNA sequencing, may enable the characterization of immune gene responses at the transcriptomic level. This integrative and holistic approach requires sophisticated tools and methods capable of unveiling the diversity of immune cells and immunologically relevant molecules that orchestrate environmental adaptation and immune protection against pathogens in MMs.

This Research Topic aims to provide a comprehensive overview of the current knowledge of MM immunology with a particular emphasis on structural and functional studies at the protein and cellular level. We wish to encourage and coordinate studies and investigations in order to fill gaps of knowledge in this field. This article collection aims to help gain more data regarding: a) The characterization of the immune system in several species of MMs, i.e. cetaceans, pinnipeds and sirenians; b) The interplay between the host immune system and the most relevant pathogens, e.g., Morbillivirus, Brucella, Toxoplasma gondii and c) The possible interplay between the immune system and contaminants.

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EDITORIAL
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Editorial: Comparative Immunology of Marine Mammals

Giovanni Di Guardo[1]*, *Michael Frederick Criscitiello*[2], *Eva Sierra*[3] and *Sandro Mazzariol*[4]

[1] Faculty of Veterinary Medicine, University of Teramo, Teramo, Italy, [2] Department of Veterinary Pathobiology, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX, United States, [3] Faculty of Veterinary Medicine, Instituto Universitario de Sanidad Animal y Seguridad Alimentaria, University of Las Palmas de Gran Canaria, Las Palmas de Gran Canaria, Spain, [4] Department of Comparative Biomedicine and Food Science, University of Padua, Padua, Italy

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Editorial on the Research Topic

Comparative Immunology of Marine Mammals

Marine mammals rank among the most charismatic creatures inhabiting our planet. Yet despite their long evolutionary history, we have a limited knowledge of their biology, ecology, and behavior, including their morphofunctional adaptations to sea life. This is also frustrating for translational studies, as gas and fat embolic syndrome, a pathologic condition of *Ziphiidae* (deep-diving cetaceans), mimics decompression sickness (DCS) in human divers (1). Indeed, the development of a DCS-like condition in animals living exclusively in a marine environment for such a long evolutionary history was totally unpredictable!

Marine mammals, comprising cetaceans, pinnipeds, manatees, sea otters, and polar bears, are all included in the International Union for the Conservation of Nature (IUCN) Red List, being increasingly threatened worldwide by several anthropogenic and natural factors, often acting synergistically with each other, as in the case of infectious agents and immunotoxic environmental pollutants (2), high levels of which may be found in tissues from stranded cetaceans, especially dolphins, given their recognized role of "apex predators" (3). Despite the increased efforts made by the scientific community, a considerable knowledge gap still exists on many aspects of marine mammals' biology. This is especially true regarding their immune response(s) at both individual and population levels, an absolute prerequisite to understand their immunopathological counterparts (i.e., immunodeficiencies, hypersensitivity reactions, and autoimmunity), impaired by a substantial amount of data coming from studies performed on stranded animals, which are "not normal" by definition. Within such context, primary fibroblast cell cultures from skin biopsies of free-living pinnipeds and cetaceans have marked a significant progress in our capability of monitoring the effects of a wide range of persistent environmental pollutants on marine mammals' health, including their stress and immune response genetic control (4).

Still noteworthy, aquatic mammals are increasingly threatened by several infectious *noxae* like morbilliviruses, herpesviruses, *Brucella ceti*, *B. pinnipedialis*, and *Toxoplasma gondii*, just to cite a few (2). Indeed, cetacean morbillivirus (CeMV), phocine distemper virus (PDV), and canine distemper virus (CDV) have recently caused several mass die-offs among dolphin, whale, and seal populations across the globe (5, 6). Consequently, characterizing the host- and the virus-related factors driving the occurrence, the behavior and persistence of morbilliviral infection(s) inside a given marine mammal species and/or population are of paramount importance (7), together with the study of host innate and acquired antiviral immunity.

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Edited and reviewed by:
Brian Dixon,
University of Waterloo, Canada

***Correspondence:**
Giovanni Di Guardo
gdiguardo@unite.it

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No information is available, for instance, on the pathogenetic evolution of CeMV, PDV, and CDV infections in Th1-dominant vs. Th2-dominant individuals within susceptible dolphin, whale, and seal species (8). In this respect, *ad hoc* investigations on human immunodeficiency virus have clearly shown that Th2-dominant patients are much more prone than Th1-dominant individuals to develop full-blown AIDS (9). Studying the host's antiviral immunity could also shed light on the "transmission barrier's jump," which has been repeatedly observed across the Western Mediterranean, in recent years, from CeMV-infected "donor" species (like striped dolphins, *Stenella coeruleoalba*) to "recipient" species like fin whales (*Balaenoptera physalus*) (10), sperm whales (*Physeter macrocephalus*) (11), Cuvier's beaked whales (*Ziphius cavirostris*) (12), and even common seals (*Phoca vitulina*) (13) and Eurasian otters (*Lutra lutra*) (14). Within such context, the possibility that CeMV infection may also spread transplacentally is of concern, given the less efficient immune response during pregnancy (15). A defective functioning of innate and acquired immunity could also represent a key factor underlying viral persistence inside the host's brain, exemplified by the peculiar "brain-only" forms of morbilliviral disease reported in CeMV-infected striped dolphins, which share neuropathologic similarities with subacute sclerosing panencephalitis in measles virus-infected humans and with old dog encephalitis in CDV-infected canines (8). Deepening our knowledge on the comparative immunology of marine mammals may additionally provide valuable insights into the interplay between the infectious pathogens of concern to and the microbiota of pinnipeds, cetaceans, and sea carnivores, on one side, and a wide range of immunotoxic pollutants, which may heavily accumulate inside their tissues, on the other (2, 3). These high contaminant tissue loads could also exert a cell-transforming activity, as clearly shown in the beluga whale (*Delphinapterus leucas*) population residing in the St. Lawrence River Estuary, Canada, in which neoplastic disease was found in association with high tissue burdens of organochlorines, heavy metals, and benzo-a-pyrene. A direct and an indirect carcinogenic activity could have both been involved in the development of the aforementioned neoplasms, together with an antitumor immune surveillance deficiency (16).

A critical component of all studies on the comparative immunology of marine mammals is that the vast majority of the laboratory reagents available on the market have been developed and "validated" only in "conventional" species. Therefore, *ad hoc* immunologic tools, specifically developed and/or validated also for marine mammals, are urgently needed.

The aforementioned issues represent the focus, along with several others, of the present Research Topic on the comparative immunology of marine mammals, hosting eight original contributions from leading scientists and scientific groups worldwide. In more detail, the monograph's contents address T-helper lymphocytes and other cellular and molecular effectors of cetaceans' immune response, acute-phase proteins and class II major histocompatibility complex in marine mammals, along with antioncogenic virus immune surveillance in California sea lions (*Zalophus californianus*), cetacean host–pathogen interaction(s), and the comparative immunopathology of CeMV infection in free-ranging dolphins. In this respect, we would like to express our most sincere feelings of appreciation and gratitude to Prof. Miki Nakao, chief editor of *Frontiers in Immunology*, who enthusiastically accepted our proposal to host this monographic collection in such an outstanding and prestigious Journal, as well as to Dr. Tara Sugrue, Research Topic team leader, Dr. Carmen E. Flores Nakandakare, and all the eminent scientists, associate editors, and reviewers who provided their valuable contributions for the present Research Topic.

AUTHOR CONTRIBUTIONS

GD wrote the first draft of the present Guest Editorial, which was subsequently revised by MC as well as by ES and SM, who provided their valuable and precious comments, remarks, and suggestions. The final Editorial's draft and the current Editorial's text were entirely agreed and approved by all the 4 Guest co-Editors of the present Research Topic, dealing with the Comparative Immunology of Marine Mammals.

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OPINION
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Cetacean Host-Pathogen Interaction(s): Critical Knowledge Gaps

Giovanni Di Guardo[1]*, *Cinzia Centelleghe*[2] and *Sandro Mazzariol*[2]

[1] Faculty of Veterinary Medicine, University of Teramo, Teramo, Italy, [2] Department of Comparative Biomedicine and Food Science, University of Padua, Padova, Italy

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Edited by:
Fabrizio Ceciliani, Università degli Studi di Milano, Italy

Reviewed by:
Francisco Javier Aznar, University of Valencia, Spain

***Correspondence:**
Giovanni Di Guardo
gdiguardo@unite.it

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Within the broad range of viral and non-viral pathogens infecting cetaceans, *Cetacean Morbillivirus* (CeMV), *Herpesvirus* (HV), *Brucella ceti,* and *Toxoplasma gondii* are of special concern, due to their impact(s) on the health and conservation of free-ranging cetacean populations worldwide (1). The most "paradigmatic" example in this direction is represented by CeMV, which throughout the last 3 decades has caused more than 10 mass mortality outbreaks among different cetacean species and populations across the globe (2, 3).

Cetaceans live permanently in the marine environment, a peculiar feature differentiating them from pinnipeds, that are also susceptible to morbilliviral infections. This has been clearly shown, for instance, by the dramatic *Phocine/Phocid Distemper Virus* (PDV) and *Canine Distemper Virus* (CDV) epidemics among North Sea common seals (*Phoca vitulina*) and Lake Bajkal seals (*Pusa siberica*) as well as Caspian seals (*Pusa caspica*), respectively (4). Due to their "in-water-only" life, stranded cetaceans play a key role as "sentinels" (potentially) able to "recapitulate" the "natural history, evolution, ecology, epidemiology, and encounters" of infectious *noxae*, on one side, and cetacean hosts, on the other. Consequently, based upon their crucial relevance as "health and conservation biomonitors" for their increasingly threatened "conspecifics and heterospecifics" living in the open sea, a detailed *post-mortem* examination of stranded cetaceans on behalf of specifically trained veterinarians is mandatory!

These concepts are nicely exemplified by the *motto* "*Hic est locus ubi mors gaudet succurrere vitae.*" This phrase, written in the nineteenth century by Luciano Armanni—the co-discoverer of "*Armanni-Ebstein Diabetic Nephropathy*" (5) -, stands at the entrance of the autopsy room of "Ospedale degli Incurabili" in Naples, Italy. Literally translated, it means "*This is the place where death is pleased to support life.*"

Taking into consideration all the above, we believe there are still a number of critical "knowledge gaps" regarding "cetacean host(s)-pathogen(s) interaction(s)," with special emphasis on the 4 herein dealt infectious *noxae* and, most likely, also in relation to any other pathogen infecting wild cetaceans.

These "knowledge-deficient areas" may be identified as follows: (1) characterization of the cell receptor(s) allowing infection; (2) interaction(s) and effects of chemical pollutants on the expression levels of the aforementioned cell receptors; (3) pathogenetic evolution of the concerned infections in T helper 1 (Th1)-dominant versus (vs.) Th2-dominant cetacean individuals; (4) effects of pregnancy-associated immune *status* on the infectious potential of the herein dealt pathogens; (5) usefulness of cetaceans and their pathogens as models for human disease.

The present Opinion Article, after a brief introduction on these 5 issues, will critically address each of the aforementioned knowledge gaps.

As far as issue (1) is specifically concerned, CeMV, HV, *B. ceti,* and *T. gondii* may infect several cetacean hosts, with the latter 2 agents also carrying a zoonotic potential (1). Since different tissues from susceptible cetaceans may be colonized by the 4 herein dealt pathogens (1, 2), a detailed characterization of the cell receptor(s) allowing their entry into (and subsequent shedding from) host's tissues would be of paramount relevance. In this respect, as with other animal and human morbilliviruses, the lymphotropic behavior typically displayed by CeMV is specified by the lymphoid cell receptor "*Signaling Lymphocyte Activation Molecule*" (SLAM/CD150) (2). Notwithstanding the above, the cell receptor(s) targeted by this lympho-epithelio-neurotropic virus within the central nervous system (CNS) from susceptible cetacean hosts is/are still unknown (6, 7).

Noteworthy, striped dolphins (*Stenella coeruleoalba*) infected by *Dolphin Morbillivirus* (DMV, a CeMV strain) may occasionally develop a peculiar, "brain-only" form of infection sharing neuropathologic similarities with "*Subacute Sclerosing Panencephalitis*" (SSPE) and "*Old Dog Encephalitis*" (ODE) in *Measles Virus* (MeV)-infected humans and CDV-infected canines, respectively (2, 8–10). Despite the recent characterization of the neuronal and non-neuronal cell populations residing in the brain from striped dolphins affected by such neuropathy (11), the receptor(s) allowing viral persistence and spread within their brains—as well as in those from SSPE-afflicted patients and ODE-affected dogs—is/are also undetermined (6, 7, 12).

Identical considerations apply to HV, *B. ceti,* and *T. gondii*, provided that the cell receptor(s) allowing their entry into and subsequent dissemination throughout cetacean hosts' tissues have not been yet identified, to the best of our knowledge. Making specific reference to *B. ceti*, the host's cellular prion protein (PrP^C) has been recently hypothesized to serve as a neuronal cell receptor for this zoonotic microorganism causing fatal neurobrucellosis in striped dolphins (13). Indeed, PrP^C had been previously recognized as a receptor for *B. abortus* heat shock protein (HSP)60—a member of the GroEL family of chaperonins—on murine macrophages, thereby allowing bacterial internalization and establishment of *B. abortus* infection inside these cells (14).

As far as concerns issue (2), it is well established that a wide range of persistent environmental pollutants may heavily accumulate in cetacean tissues, especially in those of "top predators" like dolphins and other Odontocetes, with simultaneous "biomagnification" processes additionally occurring in most cases (15). Many of these pollutants, as in the case of lipophilic polychlorinated biphenyls (PCBs), dioxins and dioxin-like substances, along with methylmercury (MeHg), have also been shown to exert powerful immunotoxic effects (16). Notwithstanding the above, we are not aware of any study investigating the relationship(s), if any, between pollutant-related immunotoxicity, on one side, and the tissue expression profiles, on the other, of given cell receptors (e.g., SLAM/CD150) for highly immunosuppressive agents like CeMV (6). This should be regarded as another critical knowledge gap within the general framework of "cetacean host(s)-pathogen(s) interaction dynamics." Within such context, the growing concerns over the exponentially increasing plastic pollution of oceans and seas across the entire globe should be also taken into account. As a matter of fact, relevant health- and conservation-related issues arise for fish, birds and aquatic mammals, due to their prolonged exposure to micro-nanoplastics through the marine food web(s). Furthermore, the documented roles of "plastic debris" as an "attractor and concentrator" for many persistent pollutants like PCBs, dioxins and other organochlorine (OC) and non-OC compounds (15), as well as for a huge number of invertebrate organisms (17), would deserve special consideration. In this respect, plastics/micro-nanoplastics-based "rafts" have been recently hypothesized to play a role also in the ecology and epidemiology of *T. gondii* infection (18). This could be of interest, provided that the Scientific Community has not yet clarified by which modalities and dynamics striped dolphins and other typically "pelagic" or "offshore" cetaceans may acquire an "oro-faecally transmitted infection" characterized by a "land-to-sea flow," as in the case of that caused by the zoonotic protozoan *T. gondii* (19).

As far as issue (3) is specifically concerned, among the many lessons provided by natural history of *Human Immunodeficiency Virus* (HIV) infection in mankind, we have learned that Th2-dominant patients are much more prone than their Th1-dominant "counterparts" to develop "full-blown AIDS" ("*Acquired Immunodeficiency Syndrome*") in the time course of HIV infection (20). In this respect, while in recent years we have also learned quite a bit on the pathogenetic evolution of other human and animal viral and non-viral infections in Th1-dominant vs. Th2-dominant individuals, we are unaware, on the contrary, of any published reports dealing with the pathogenetic behavior of CeMV infection—as well as of HV, *T. gondii,* and *B. ceti* infections—among Th1-dominant vs. Th2-dominant cetacean hosts.

This could of relevance also in relation to issue (4), given that a reduced efficiency of host's immune response is physiologically observed during pregnancy (21). Indeed, several cases of DMV infection have been recently described both in newborns and in cetacean fetuses (6, 22–24). This concern and its related knowledge gap are additionally amplified by the documented occurrence of cases of DMV infection in cetacean species—mainly from the Western Mediterranean Sea—into which the virus has apparently "jumped," most likely as the result of recent "spillovers" from DMV-infected striped dolphins (22–24).

Finally, with specific reference to the last of the 5 herein dealt issues, the potential role of cetaceans as "*models for human disease*" should be also taken into account (25). In fact, as recently reported for Alzheimer's disease-related neuropathology in striped and bottlenose dolphins (*Tursiops truncatus*) (26, 27), and as previously described for the aforementioned "brain-only" forms of DMV infection among striped dolphins (6, 8–10), cetaceans and—more in general—aquatic mammals could serve as valuable "models" also in "*Comparative Immunology and Immunopathology.*"

This could apply, in parallel with the herein dealt "host(s)-pathogen(s) interaction dynamics," also to the "ontogeny" and "evolutionary phylogeny" of cetaceans' immune response as well

as to further key issues like "immune tolerance," "autoimmunity," and "immune surveillance against neoplasia," just to cite a few.

In conclusion, deepening our understanding of "host(s)-pathogen(s) interaction(s)" in cetaceans and, more broadly, in marine mammals, may provide not only a very useful and insightful "set of tools" to monitor and protect their increasingly threatened health and conservation, but also a reliable and precious source of knowledge highlighting the simultaneous role of cetaceans as putative "*models for human (and animal) disease*" as well as for "*Comparative Immunology and Immunopathology.*"

AUTHOR CONTRIBUTIONS

GDG wrote the first draft as well as the first revision's draft of the present Opinion Article, to be included in the Research Topic *Comparative Immunology of Marine Mammals*, with CC and SM subsequently integrating and providing a thorough and critical revision of both the aforementioned manuscript's drafts.

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Transcriptional Profiles of California Sea Lion Peripheral NK and CD^{+8} T Cells Reflect Ecological Regionalization and Infection by Oncogenic Viruses

Ignacio Peñín[1], *Mónica E. Figueroa-Cabañas*[1], *Fabiola Guerrero-de la Rosa*[1], *Luis A. Soto-García*[1], *Roberto Álvarez-Martínez*[1], *Adriana Flores-Morán*[1] *and Karina Acevedo-Whitehouse*[1,2]*

[1] *Unit for Basic and Applied Microbiology, School of Natural Sciences, Autonomous University of Queretaro, Santiago de Queretaro, Mexico,* [2] *The Marine Mammal Center, Sausalito, CA, United States*

The California sea lion is one of the few wild mammals prone to develop cancer, particularly urogenital carcinoma (UGC), whose prevalence is currently estimated at 25% of dead adult sea lions stranded along the California coastline. Genetic factors, viruses and organochlorines have been identified as factors that increase the risk of occurrence of this pathology. Given that no cases of UGC have as yet been reported for the species along its distribution in Mexican waters, the potential relevance of contaminants for the development of urogenital carcinoma is highlighted even more as blubber levels of organochlorines are more than two orders of magnitude lower in the Gulf of California and Mexican Pacific than in California. *In vitro* studies have shown that organochlorines can modulate anti-viral and tumor-surveillance activities of NK and cytotoxic T-cells of marine mammals, but little is known about the activity of these effectors in live, free-living sea lions. Here, we examine leukocyte transcriptional profiles of free-ranging adult California sea lions for eight genes (Eomes, Granzyme B, Perforin, Ly49, STAT1, Tbx21, GATA3, and FoxP3) selected for their key role in anti-viral and tumor-surveillance, and investigate patterns of transcription that could be indicative of differences in ecological variables and exposure to two oncogenic viruses: sea lion type one gammaherpesvirus (OtHV-1) and sea lion papillomavirus type 1 (ZcPV-1) and systemic inflammation. We observed regional differences in the expression of genes related to Th1 responses and immune modulation, and detected clear patterns of differential regulation of gene expression in sea lions infected by genital papillomavirus compared to those infected by genital gammaherpesvirus or for simultaneous infections, similar to what is known about herpesvirus and papillomavirus infections in humans. Our study is a first approach to profile the transcriptional patterns of key immune effectors of free-ranging California sea lions and their association with ecological regions and oncogenic viruses. The observed results add insight to our understanding of immune competence of marine mammals, and may help elucidate the marked difference in the number of cases of urogenital carcinoma in sea lions from US waters and other areas of their distribution.

Keywords: California sea lion, cytotoxicity, NK, CD^{8+} T cells, otarine gammaherpesvirus, sea lion papillomavirus

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***Correspondence:**
Karina Acevedo-Whitehouse
karina.acevedo.whitehouse@uaq.mx

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INTRODUCTION

The California sea lion (*Zalophus californianus*) is one of the few wild mammals prone to develop cancer under natural conditions. Since the initial report in the early 80's, the prevalence of urogenital carcinoma has remained consistently high, with *post mortem* examination of dead individuals revealing a prevalence of up to 25% in adult sea lions necropsied after stranding along the California coast (1). The high incidence of such an aggressive and fatal pathology in a long-lived top predator of the coastal marine ecosystem warrants studies to increase our understanding of the factors that contribute to its occurrence.

As is the case for most cancers, sea lion urogenital carcinoma appears to be multifactorial, and various risk factors have been identified. These factors include an oncogenic genital gammaherpesvirus, named OtHV-1 (2, 3) and genetic components (4–6). Furthermore, high concentrations of organochlorines have been detected in the blubber of sea lions with urogenital carcinoma (7). This latter association is particularly relevant as studies conducted in laboratory animals have shown that organochlorines can induce carcinogenesis, either directly at high concentrations (8) or indirectly by modulating immune responses, particularly when exposure is low (9–11). *In vitro* experiments with different marine mammal cells have shown that organochlorines modulate NK and cytotoxic T-cell activity (12–14). Based on their known anti-viral and tumor surveillance activity (15–17), and the evidence of organochlorine-induced modulation, it is parsimonious to speculate that NK and cytotoxic T-cells play an important role in preventing the development of urogenital carcinoma in the California sea lion, and that these immune effectors are sensitive to extrinsic and intrinsic factors.

Despite its high prevalence in California, urogenital carcinoma has not been observed in sea lions inhabiting the Gulf of California, in spite of systematic surveys of the breeding colonies by researchers and park managers. However, pre-cancerous transformation of the genital epithelium, including binucleation and koilocytes, do appear to be relatively common in California sea lions from the Gulf of California (18). In humans, the presence of these cellular phenotypes is considered the first step toward carcinogenesis if the abnormal cells are not promptly detected and destroyed by tumor-surveillant and cytotoxic immune cells (19). Interestingly, compared to values reported for sea lions in California (7), blubber PCB levels are three orders of magnitude lower (20) in sea lions from the Gulf of California, and two orders of magnitude lower in sea lions from the Mexican North Pacific (21). This implies that there could be differences in NK and cytotoxic T-cell activity (12–14), which could, in turn, result in differences in oncogenesis.

Within the Gulf of California, 13 sea lion breeding colonies are spread along 177,000 km^2, from the northernmost colony, Rocas Consag, located at less than 100 km from the Colorado River Delta, to the southernmost colony, Islotes, 29 km from the city of La Paz, in the tip of the peninsula of Baja California. Oceanographic and ecological differences among zones have led to regionalization of the Gulf of California, and colonies are grouped in four main regions (22), largely defined by upwelling and phytoplankton profiles (23) that influence the availability of resources (24, 25). Sea lion colonies vary per region in terms of population trends (26), genetic substructure (27, 28) and pathogen exposure (29). In terms of pollutants, there is a marked latitudinal gradient, with the northern region being more polluted than the southern region due to deposition from the Colorado River Delta (30).

Based on the species' genetic substructure in the Gulf of California, and on spatial differences in oceanographic and ecological factors, it is plausible to assume that California sea lions experience intra- and interregional differences in terms of their exposure to organochlorine pollutants and other extrinsic factors that could impact their tumor surveillance and cytotoxic capability, as well as induce chronic inflammation. Furthermore, as disposable energy in a region is limited by its productivity, prey availability, and feeding range (25), it is unlikely that a sea lion's investment of resources for immune activities will be independent of its environment. Some evidence for this phenomenon has already been reported for this species (31).

Lymphocyte development and activation are characterized by marked changes in gene expression (32). Thus, we hypothesized that transcription of key genes relevant to immune surveillance of tumors and cytotoxic responses of California sea lions differs spatially and is related to genital infection by two oncogenic viruses, sea lion type one gammaherpesvirus (OtHV-1) and sea lion papillomavirus type 1 (ZcPV-1), and systemic inflammation. To challenge our hypotheses, we examined transcription patterns of key genes expressed by NK, CD^{8+} T cells and by T regulatory cells in the blood of apparently healthy adult females sampled at breeding colonies within the Gulf of California and the Mexican North Pacific.

MATERIALS AND METHODS

Collection of Samples

During the summer of 2016 we visited 12 California sea lion breeding colonies in the Gulf of California. Based on the ecological regionalization proposed (22), we sampled sea lions in three colonies in the Northern region (Rocas Consag, San Jorge, and Lobos), three in the North-Central (Midriff) region (Granito, Cantiles, and Los Machos) five in the Central region (Partido, El Rasito, San Esteban, San Pedro Mártir, and San Pedro Nolasco), and one (Los Islotes) in the far South region (**Figure 1**).

Samples of peripheral blood were collected from 54 apparently healthy adult female California sea lions, which had been captured using hoop nets, and manually restrained before using inhaled anesthesia (Isoflurane). Once anesthetized, a trained veterinarian examined the sea lions in order to determine their general health, and to monitor their heart rate and respiratory frequency throughout the procedure, which lasted 10–12 min. All sea lions were considered to be in good body condition, with at least 20 mm of lateral blubber depth (skin fold thickness measured with calipers). Two 7-ml blood samples were collected from the caudal gluteal vein of each sea lion with vacuum tubes (Vacutainer), one of them coated with sodium heparin and one with EDTA. Genital epithelial swabs were also collected. Briefly, a sterile genital speculum was introduced to visualize the cervix

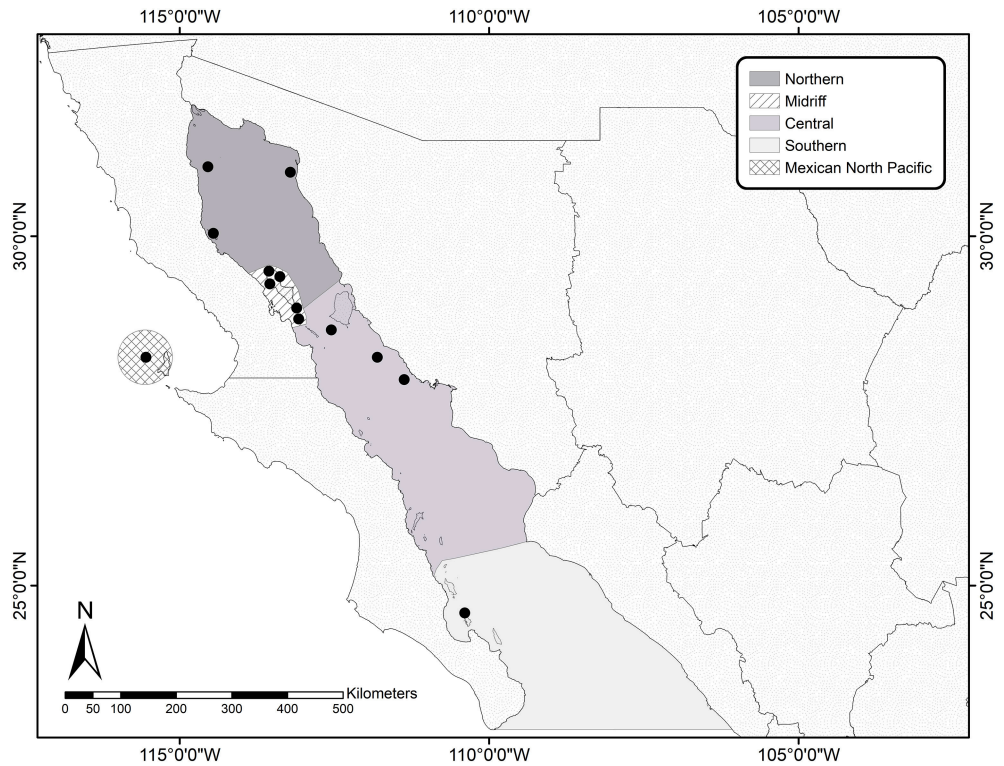

FIGURE 1 | Location of the sampling sites within the Gulf of California and Mexican North Pacific. The map shows the ecological regionalization proposed by Stzeren and Aurioles (22) for the Gulf of California and the San Benito Archipelago on the west coast of the Baja California Peninsula, in the Mexican North Pacific. Black dots indicate the colonies where the sea lions were sampled.

and the cervical mucosa was scraped with a sterile cytobrush. The swabs were stored in a vial containing 96% ethanol and were protected from sunlight until staining in the laboratory. The blood samples were centrifuged using a clinical centrifuge (Clay Adams compact II, Daigger Scientific, USA) for 10 min at a fixed speed of 3,200 rpm within 3 h after collection. The buffy coat was separated using a sterile Pasteur pipette and stored immediately in cryogenic tubes prior to snap-freezing in liquid nitrogen, where kept until processing. EDTA-preserved blood was used to total and differential leukocyte counts. Based on the hematological information, we determined the neutrophil to lymphocyte ratio (NLR) as a measure of chronic systemic inflammation and stress (33), which has been shown to be a useful marker in the California sea lion (34). We also had equivalent samples from nine adult female sea lions, which had been captured during the summer of 2014 at a colony situated in the Archipelago of San Benito, in the Mexican North Pacific (see **Figure 1**). Sampling and processing of these samples was performed as described above. Adult males were not included in our study due to the difficulty and extremely high-risk of capturing and restraining these individuals in the field.

All procedures were approved by the Bioethics committee of the Universidad Autónoma de Queretaro (Mexico), and were conducted under permits SGPA/DGVS/11744/13 (for samples collected in 2014) and SGPA/DGVS/09004/15 (for samples collected in 2016) issued by the Secretariá de Medio Ambiente y Recursos Naturales through the Direccioń General de Vida Silvestre in Mexico.

Relative Quantification of Gene Expression in Lymphoid Subpopulations

We extracted total RNA from the buffy coat samples using Trizol (Sigma-Aldrich, USA) as per the manufacturer's instructions. We treated the solubilized RNA with DNA-free^{TM} DNA removal kit (Thermo Fisher Scientific, USA) as per the manufacturer's instructions. RNA integrity was assessed by electrophoresis in a 1% agarose gel stained with ethidium bromide and purity was determined by Nanodrop spectrophotometry (Qiagen, USA). Good quality samples were those that had no evidence of RNA degradation, showed clear 28S and 18S rRNA bands (see **Figure S1**), and whose A_{260}/A_{280} ratio was between 1.75 and 2.10. Five of the RNA samples did not clearly show the two rRNA bands despite having a good A_{260}/A_{280} ratio. In these cases, we re-extracted the RNA and reassessed the quality. In all of these cases, we were able to confirm that quality was adequate to proceed with reverse transcription (see **Supplementary Material** for more details).

Reverse transcription was performed for each sample with a QuantiTect Reverse Transcription Kit (Qiagen, USA) using 200 ng of RNA in 20 μL reactions. As per the manufacturer's

instructions, the procedure included a 2 min. incubation at 42°C with gDNA wipeout buffer to further ensure the elimination of genomic DNA contamination. cDNA was frozen in aliquots to avoid repeated freeze-thaw cycles.

The levels of transcription of eight genes were assessed by real time quantitative PCR (RT-qPCR). The genes were selected *a priori* to represent the activity of different lymphocyte subpopulations and immune activities relevant to antiviral and antitumor activities (see **Table 1** for genes and primers). RPS5 and HpRT were used as reference (housekeeping) genes as they are expressed in all nucleated cells, and we have previously shown their transcription levels to be stable in California sea lion peripheral white blood cells (35). In order to comply with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (36), we used a subset of 10 samples to run RT-qPCR to evaluate expression stability. This allowed us to confirm that both genes were appropriate to use as reference genes (see details and **Figure S2**). All primers were designed to span exon-exon junctions. All primer pairs were evaluated for their efficiency. The efficiency of the primers ranged between 90 and 112%, and the coefficient of determination (R^2) was >0.95 (E and R^2 results for all the primers used for RT-qPCR can be seen as **Figures S3, S4**).

RT-qPCR reactions contained 4 microliters of cDNA (1:4 to 1:16 of template generated by retrotranscription of 10 ng/μL of RNA), 0.15 μL of each primer (at 0.2 μM each), 5 μL SYBR® Green master mix (Thermo Fisher), and 0.7 μL of water to reach 10 μL of final volume. The reactions were run on a CFX Connect™ Real-Time PCR Detection System (BioRad, USA) as follows: 95°C for 15 min, followed by 40 cycles of 15 s at 95°C, 1 min at 55C (during which the plate was read) and 72°C for 1 min. The ending cycle was kept at 95°C for 15 s and a final step for the melting curve at 60 to 90°C (0.5°C increase and 15 s of wave length measurement for each temperature). Reaction specificity was monitored by melting curve analysis using a final data acquisition phase of 60 cycles of 65°C for 30 s. The threshold was established manually after amplification take-off. Optimal results were achieved when using 1:2–1:8 of cDNA. For each primer pair, a reaction mixture containing water, but no cDNA, was used as a non-template control (NTC) to monitor contamination and primer dimer formation; a no-reverse transcriptase (-RT) mixture was included as a control to monitor DNA contamination. Gene expression levels were determined by relative quantification (i.e., transcription of the target gene relative to the average of the reference genes) as per the ΔCt method (37). The data were considered reliable if the difference between replicates was below one cycle. If the reliability of the reference genes failed, the samples were rerun for all the primer pairs of a plate. Optimal results were achieved when using 1:2–1:8 of cDNA.

Molecular Detection of Oncogenic Viruses

Genomic DNA was extracted from the ethanol-preserved genital swabs using a routine proteinase K digestion followed by a phenol-chloroform protocol and isopropanol precipitation. DNA was quantified and quality was assessed using a spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific, USA). We amplified a 210 bp fragment of the DNA polymerase (Dpol) gene of OtVH-1 in genital swabs. Primers used were Dpol 697 5′- GCGGGAACGCAACTATATCCT and Dpol 65 5′-TCT TCGTCCAGTATCATTG; (38). For ZcPV-1, we used GenBank sequence NC_015325 (*Zalophus californianus* papillomavirus 1) as a reference to design a pair of primers that amplified a 450 bp fragment of gene L2 (ZcPV_F5957-ATACAGGACGGGGACA TGG, ZcPV_R6495-TCATATTCCTCAGCGTGCCT).

All 12.5 μl PCR reactions were performed on an ABI 3100 thermal cycler (Applied Biosystems, Inc.) and were conducted in duplicate. Cycling conditions were 95°C for 15 min, 30 cycles of 94°C for 40 s, 52°C (OtHV-1) or 53°C (ZcPV-1) for 30 s and 72°C for 40 s, and a final extension step at 72°C for 10 min. A template free (template free reaction) was included with every PCR. Amplified products were electrophoresed on an ethidium bromide stained 1.8% agarose gel and visualized on a UV transilluminator. To ensure the amplified products were not the result of non-specific amplification, for each pathogen, two bands selected at random were gel excised, column purified (QiaQuick, Qiagen, USA), cloned and bi-directionally sequenced for confirmation. Each sequence was visually inspected and compared to those reported in GenBank (http://www.ncbi.nlm.nih.gov/genbank/).

Statistical Analyses

Before beginning the analyses, we examined the normality and homoscedasticity of the relative level of transcription of each target gene with Shapiro and Bartlett tests, respectively. None of the gene expression levels showed deviation of normality or heteroscedasticity. We used Spearman correlations to identify relationships between gene transcripts. The *GATA3* to *Tbx21* (hereafter Ga/Tb) ratio, a measure of Th1/Th2 profile (39) deviated from expectations of normality and, based on a Cullen and Frey graph, appeared to follow a beta distribution. Goodness of fit was examined with a Kolmogorov test.

To challenge our hypotheses, we first built generalized linear models (GLM) for each of the genes, and the Ga/Tb ratio defining the error family as per the distribution of the error (40), and indicating the region where each sea lion was sampled as the explanatory variable. We next used independent GLMs to examine whether the transcription level of each gene and the Ga/Tb ratio was affected by the presence of OtHV-1, ZcPV-1, or simultaneous infection by both viruses in the genital epithelium, and whether gene transcription levels were influenced by systemic inflammation, as assessed by the NLR of the sea lions. For genes whose transcription levels varied among regions, we included this variable in the respective models. We used a top-down strategy to determine which variables explained a significant fraction of the data (40), and we examined the residual distribution by inspecting Q-Q plots, Cook distance and plots of the adjusted residuals *vs.* the obtained residuals to validate the model. As lower ΔCt values represent higher levels of expression, interpretation was made easier by using a negative transformation of the response variable (–log ΔCt). To account for differences in prevalence of OtVH-1, ZcPV-1 and simultaneous infection among regions, we built contingency

TABLE 1 | Genes selected as markers of NK and CD^{8+} cytotoxicity, Th2 responses and immunomodulation.

Gene	Encoded protein and function	Primers (5′-3′)
Ly49	Inhibitory receptor of NK cells.	F. TGTCAGAGAGGAAATGAAGGCA R. TGGCAAGTCTGTTTACATCCGT
Granzyme B	Cytotoxic serum protease that induces apoptosis of target cells. Exerts antiviral and antitumoral responses.	F. CACTCTGCAAGTAGTGAGGCT R. CAGCTGAATGGTGTGGTCGTA
Perforin	Glycoprotein responsible for forming pores in the cell membrane of target cells. Mediator required for apoptosis; Relevant for antiviral and antitumoral responses.	F. CCTGCTGCAGTTCTTCCAAC R. CTGGCACTGACCGACTGG
Eomes	Eomesodermin (T-box brain protein 2). Leads activation and differentiation of TCD^{8+}; reflects antitumoral responses.	F. TCAGTCCTTCTCCCGGAGC R. GGTTGACCACCTTTCGTTCTG
STAT-1	Signal transducer and activator of transcription 1. Mediates responses to interferon and cytokines; induces antiviral state.	F. GGTGAACTGGACCCCAGTCT R. CTATGGGACCGCACCTTCAA
Tbx21	T cell associated transcription factor (Tbet). Member of the T-box family of transcription factors expressed in Th1 cells. Directs T-cell differentiation and represses Th2 responses.	F. GAGGCTGAGTTTCGAGCAGT R. AGTAGGACATGGTGGGTCCG
GATA-3	Trans-acting T-cell-specific transcription factor. Promotes secretion of anti-inflammatory cytokines by Th2 cells; inhibits the expression of IFNγ; suppresses differentiation of naïve T-helpers to Th1 cells. Expressed by T cells, NK cells and CD1-restricted NKT cells.	F. CATGACACGCTGGAGGACTT R. AGGGAGGTCATGTGTCTGGA
FoxP3	Transcription factor forkhead box protein P3. Anti-inflammatory and anti.apoptotic role. Shapes immune tolerance.	F. TGCAGTCTCTGGAACAGCAG R. TTTGGTCAGGGCCATCTTCC

tables and ran Fisher exact tests. In all cases we considered results statistically significant if the *p*-value was less than 0.05.

Our second approach was to analyze gene transcription profiles with no pre-defined regionalization to allow natural clustering of immune profiles. For this, data were subjected to hierarchical clustering to generate heat maps and dendrograms of the gene transcripts according to the degree of similarity of the transcriptional profiles. Multiple imputation chain equation was used to account for missing values. We first used the full set of target genes, as the "complete profile" of each individual. Next, we used the relative expression of genes representative of responses relevant to this study: (i) NK and CD^{8+} activity and differentiation (*Ly49*, *perforin*, *granzyme, STAT-1*, *Tbx21*, and *Eomes*), (ii) Th2 and immune modulation (*GATA3* and *FoxP3*), and (iii) Th1/Th2 ratio (*Tbx21* and *GATA3*). The number of natural clusters was determined according to a rarefaction curve and two dendrograms were initially created for each set of genes. Algorithms used were Ward.D2 and Average (41). Goodness of fit of the models was based on the cophenetic correlation coefficient, with an optimality value of $\geq$0.8. As values were lower than 0.8 for all models, we used an Euclidean approach to build the final dendrogram (42). All statistical analyses were conducted in R v3.4.2 (43) using core packages as well as *cluster*, *dendextend*, *factoextra*, *fitdistrplus*, *mice*, *prevalence* and *ggplot2*.

RESULTS

NK and CD^{8+} Activity

Most of the transcription levels of the six genes involved with NK and CD^{8+} activity were significantly correlated to each other (**Table 2**), with correlation coefficients ranging from 0.75 to 0.31. The exceptions were *Ly49* and *perforin*, both of which were not correlated to *STAT-1*.

Transcription levels of *Ly49*, *perforin* and *granzyme B* did not vary significantly among regions [GLM; *Ly49*: $F_{(4,49)} = 0.21$, $p = 0.932$, **Figure 2C**; *perforin*: $F_{(4,31)} = 1.44$, $p = 0.244$, **Figure 2B**; *granzyme B*: $F_{(4,34)} = 1.26$, $p = 0.304$; **Figure 2A**]. In nearly all sea lions, *granzyme B* was upregulated more than 2-fold with respect to the reference genes, while *perforin* showed the inverse pattern, being downregulated on average 2.73-fold; *Ly49* was slightly downregulated in all but a few individuals, with only a few sea lions showing upregulation.

TABLE 2 | Spearman's correlation coefficients of genes expressed by NK and CD^{8+} T cells.

	Eomes	***STAT-1***	***Tbx21***	***Granzyme B***	***Perforin***	***Ly49***
Eomes	–	0.55	0.83	0.63	0.70	0.36
STAT-1	0.0008	–	0.62	0.33	0.30	0.17
Tbx21	0.0000	0.0000	–	0.37	0.58	0.40
Granzyme B	0.0000	0.0452	0.0240	–	0.50	0.44
Perforin	0.0000	0.0880	0.0004	0.0021	–	0.34
Ly49	0.0412	0.2316	0.0028	0.0059	0.0477	–

The bottom half of the table shows p-values.

Transcription of the three transcription factors involved with Th1 development varied among regions [GLM; *Eomes*: $F_{(4,30)} = 2.64$, $p = 0.049$, **Figure 2D**; *Tbx21*: $F_{(4,49)} = 6.22$, $p = 0.016$, **Figure 2E**; *STAT-1*: $F_{(4,49)} = 3.58$, $p = 0.012$; **Figure 2F**]. *Eomes* was slightly downregulated (average: 0.93-fold) with respect to the reference genes in the majority of sea lions from colonies within the midriff and central regions, and upregulated in the northern and southern regions, as well as in the Mexican North Pacific (average: 0.38-fold). *Tbx21* was mostly downregulated, and levels increased across samples collected from north to south within the Gulf of California, reaching the highest values in animals from the Mexican North Pacific (Adj. $R^2 = 0.09$, $df = 52$, $p = 0.016$; **Figure 2E**). In contrast to the other genes indicative of Th1 development, *STAT-1* was generally upregulated, and levels of transcription also exhibited a north to south trend within the Gulf of California, reaching the highest values in the sea lions

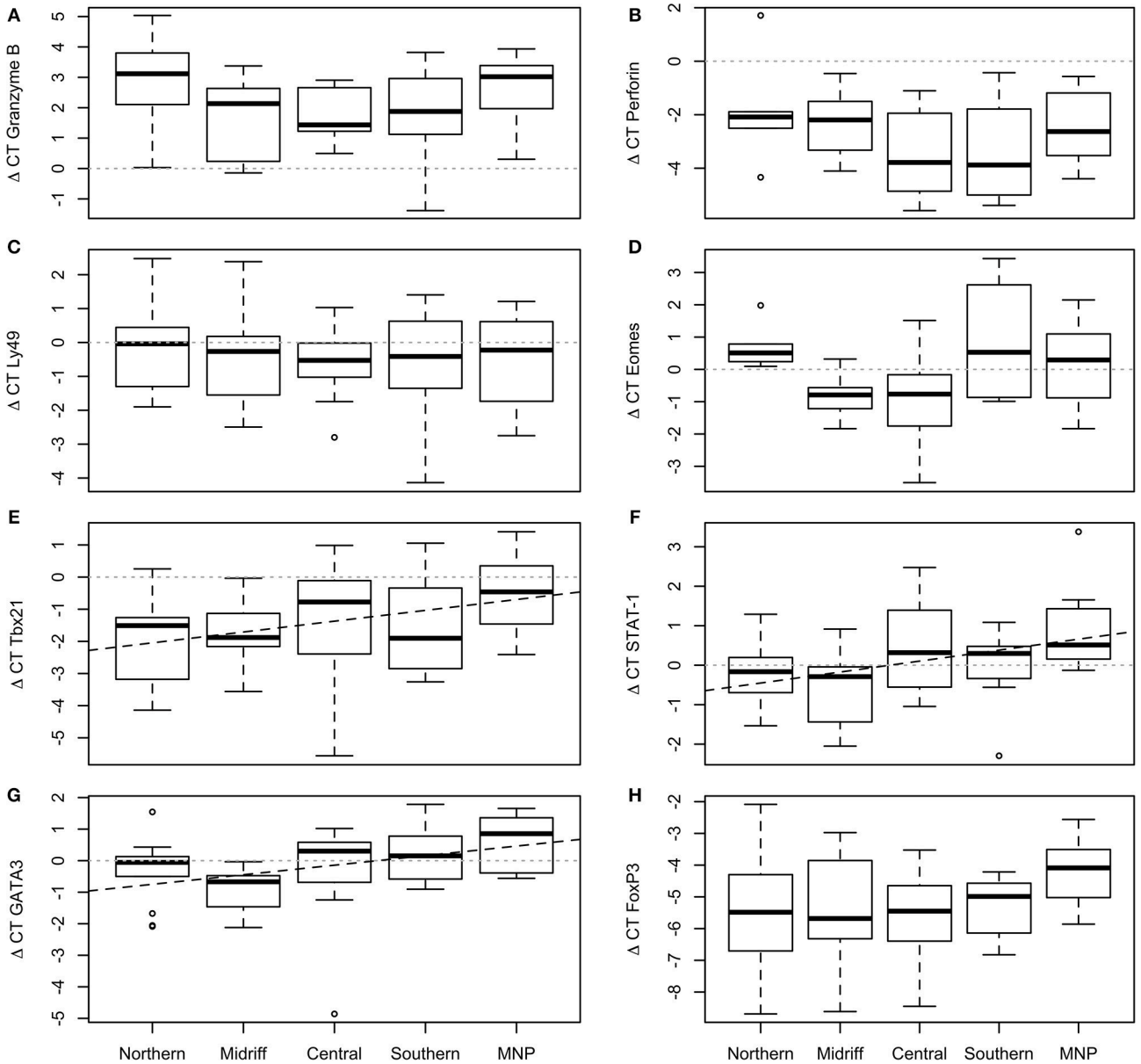

FIGURE 2 | Transcription levels of selected genes in California sea lion blood sampled at different regions. **(A)** *Granzyme B*, **(B)** *Perforin*, **(C)** *Ly49*, **(D)** *Eomes*, **(E)** *Tbx21*, **(F)** *STAT-1*, **(G)** *GATA3*, **(H)** *FoxP3*. *Ly49*, *perforin*, *granzyme B* and *FoxP3* did not vary significantly among regions. *Eomes* was slightly downregulated in sea lions from the midriff and central regions, and upregulated in the northern and southern regions, and the Mexican North Pacific. *Tbx21*, *STAT-1* and *GATA3* increased from north to south within the Gulf of California and reached highest levels in the Mexican North Pacific. The plot shows the median (thick line), first and third quartile (box), and 95% confidence interval of the median (whiskers).

from the Mexican North Pacific (Adj. $R^2 = 0.11$, $df = 52$, $p = 0.007$; **Figure 2F**).

Th2 and Immune Modulation

GATA3 and *FoxP3* were significantly correlated ($r = 0.39$, $p = 0.006$). Transcription of *GATA3* varied significantly among regions [GLM; $F_{(4,49)} = 3.19$, 0.017], with a north-to-south increasing gradient within the Gulf of California, and showing the highest levels of transcription in sea lions from the Mexican North Pacific (Adj $r^2 = 0.12$, $df = 52$, $p = 0.006$; **Figure 2G**). *FoxP3* was consistently downregulated (average: 5.29-fold), with no significant differences among regions [GLM; $F_{(4,44)} = 1.35$ $p = 0.268$; **Figure 2H**].

Th1/Th2 Ratio

There was a wide variation in Ga/Tb, with values ranging to 45.29 to −1.49 (mean = 1.64), and there was no evidence of differences among regions [GLM; $F_{(4,49)} = 0.81$, $p = 0.528$].

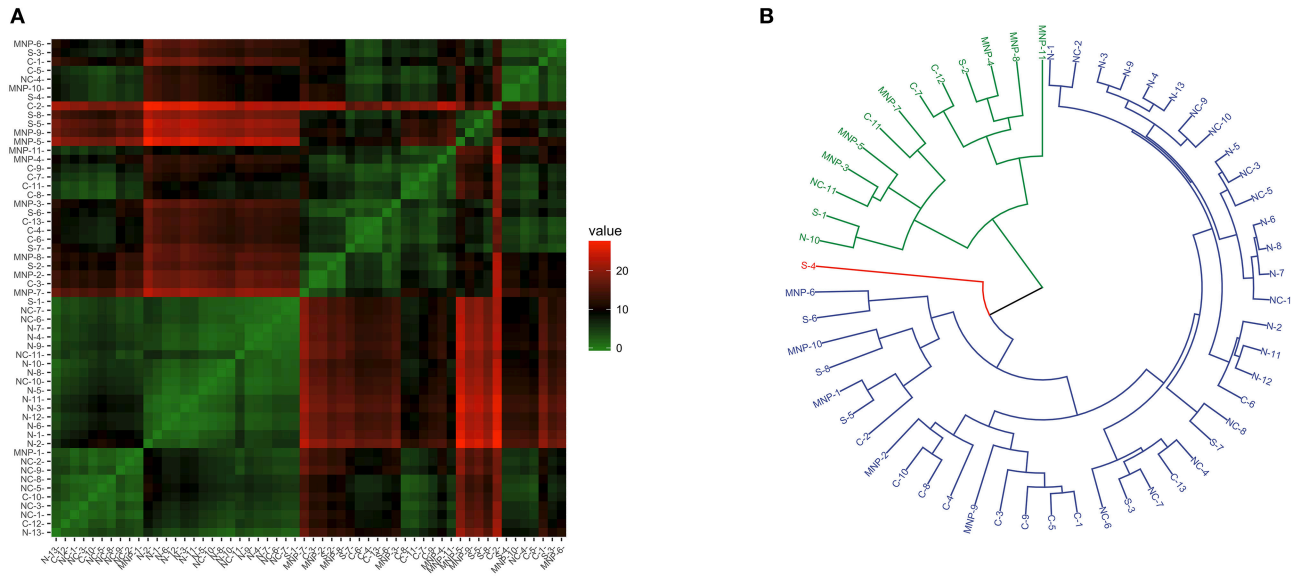

FIGURE 3 | Similarity of NK and CD^{8+} T cell transcription (*Ly49*, *perforin*, *Granzyme B, STAT-1*, *Tbx21*, and *Eomes*) in blood of California sea lions sampled at different regions. **(A)** Heatmap shows similarity clustering of the gene profiles. Green indicates low dissimilarity and red indicates high dissimilarity. **(B)** Dendrogram of gene transcription profile clustering. The tree was built using an Euclidean algorithm. S, South; C, Central; NC, North-Central (Midriff); N, North; MNP, Mexican North Pacific.

Patterns of Gene Transcription Levels Using no Pre-defined Regionalization

When considering transcriptional patterns of all target genes together, no clear grouping emerged (see **Figure S5**). A few sea lions had a distinct pattern, and each belonged to a different region. The transcriptional profile related to the NK and CD^{8+} cells (considering *Ly49*, *perforin*, *granzyme, STAT-1*, *Tbx21*, and *Eomes* transcription levels) showed some heterogeneity and clustering among sea lions (**Figure 3A**), and the best dendrogram separated the samples in three main branches (**Figure 3B**). One of the branches harbored 92% (12/13) of the sea lions sampled in the northern region, and 90.1% (10/11) of the sea lions sampled in the midriff region. Furthermore, sea lions from these regions were grouped into smaller clusters, separating them from samples from the central and southern regions, as well as from the Mexican North Pacific. The second main branch grouped only one sea lion from the northern Gulf of California, two from the midriff region, and a mixture of sea lions from southern and central regions, and from the Mexican North Pacific. The third main branch separated a single sea lion sampled in the southern Gulf of California.

The clustering analysis of Th2 and immune modulation profiles revealed some heterogeneity and clustering (**Figure 4A**), and the dendrogram separated the samples in three main branches (**Figure 4B**); the first branch grouped all of the sea lions from the northern region, 91% (10/11) of the sea lions from the midriff region and a few sea lions from the other regions; namely, four from the central region, one from the southern region and two the Mexican North Pacific. The second branch harbored sea lions from the southern (7/8) and central (8/11) regions, and from the Mexican North Pacific (8/11). One sea lion from the central region was separated from the rest in its own branch.

Clustering patterns of Th1/Th2 profiles were less clear than observed for the other profiles (**Figure 5A**). The dendrogram separated the samples in four main branches (**Figure 5B**); one grouping 92% (12/13) of the sea lions sampled in the northern region, 91% (10/11) of the sea lions from the midriff region, 42% (5/12) of the sea lions sampled in the central region, and 18% (2/11) of the sea lions from the Mexican North Pacific. The second and third branches grouped a mixture of sea lions from the central and south regions, and from the Mexican North Pacific. The fourth main branch separated a single sea lion sampled in the central region.

Gene Transcription and Genital Infection by Oncogenic Viruses

The prevalence of genital viral infections varied significantly across regions (**Figure 6**). Genital ZcPV-1 infections were highest in the Mexican North Pacific followed by the midriff and southern Gulf of California (Pearson's $Chi^2 = 11.32$, $df = 4$, $p = 0.042$), while OtHV-1 infections were highest in the central region, followed by the northern and midriff regions (Pearson's $Chi^2 = 26.39$, $df = 4$, $p = 2.71 \times 10^{-06}$). The prevalence of simultaneous infections by both viruses also varied among regions, with a similar pattern to that observed for OtHV-1 ($Chi^2 = 11.757$, $df = 4$, $p = 0.0231$).

Infection status impacted transcription of four genes related to cytotoxicity, even when accounting for spatial differences [GLM; *Eomes*: $F_{(3,29)} = 4.21$, $p = 0.015$, **Figure 7A**; *perforin*: $F_{(3,30)} = 4.743$, $p = 0.008$, **Figure 7B**; *Tbx21*: $F_{(3,48)} = 4.16$, $p = 0.012$, **Figure 7C**] granzyme B: $F_{(3,33)} = 3.39$, $p =$

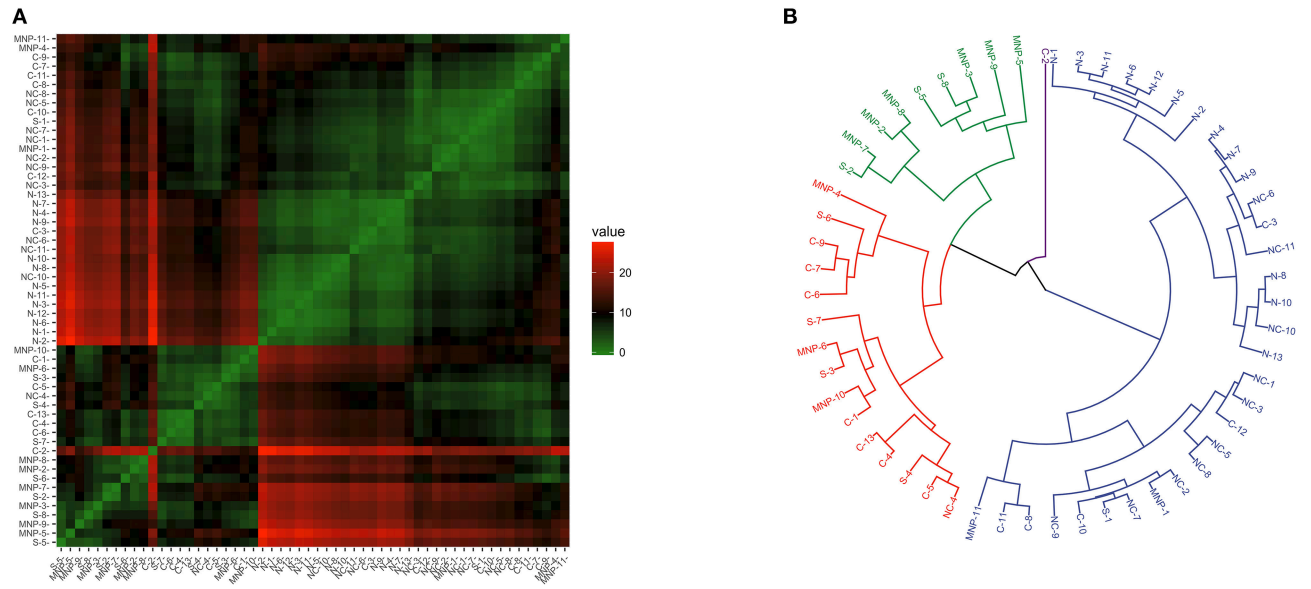

FIGURE 4 | Similarity of Th2 and immunomodulation (*GATA3* and *FoxP3* gene expression) in blood of California sea lions. **(A)** Heatmap shows similarity clustering of the gene profiles. Green indicates low dissimilarity and red indicates high dissimilarity in relative transcription levels of the six genes. **(B)** Dendrogram of gene transcription profile clustering. The tree was built using an Euclidean algorithm. S, South; C, Central; NC, North-Central (Midriff); N, North; MNP, Mexican North Pacific.

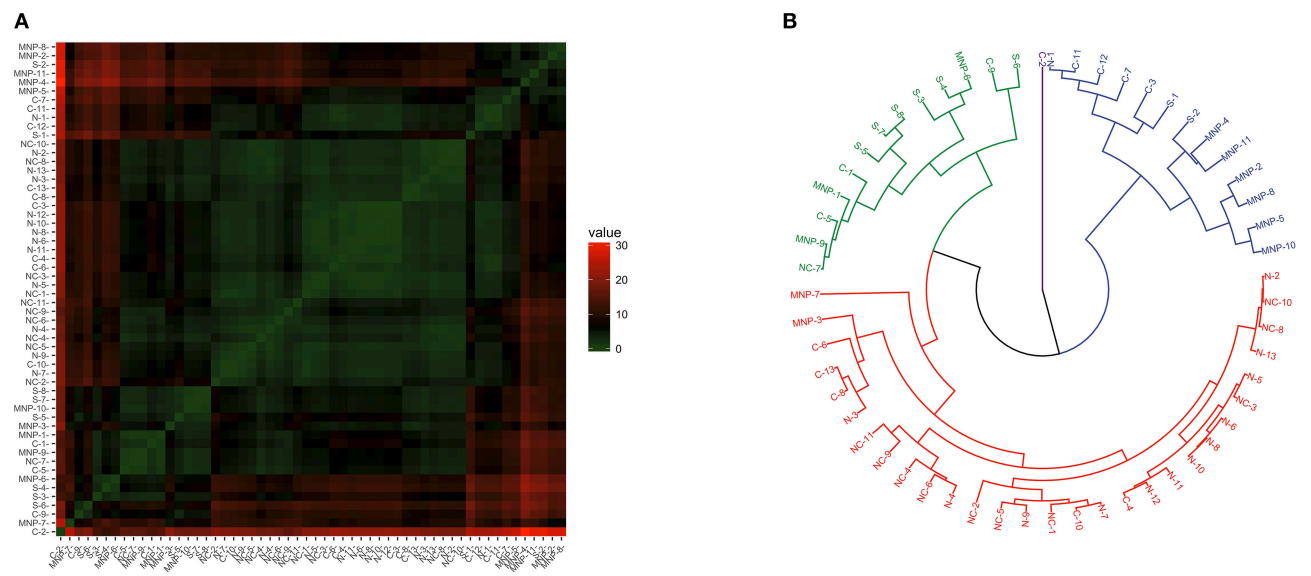

FIGURE 5 | Similarity of Th1/Th2 profile (*GATA3* and *Tbx21* gene expression) in blood of California sea lions. **(A)** Heatmap shows similarity clustering of the gene profiles. Green indicates low dissimilarity and red indicates high dissimilarity in relative transcription levels of the six genes. **(B)** Dendrogram of gene transcription profile clustering. The tree was built using an Euclidean algorithm. S, South; C, Central; NC, North-Central (Midriff); N, North; MNP, Mexican North Pacific.

0.036, **Figure 7D**. The pattern was consistent and contrasting between viruses. Namely, sea lions infected by OtHV-1 had transcription levels similar to or lower than those of non-infected sea lions and sea lions simultaneously infected by both viruses. In contrast, sea lions infected by ZcPV-1 had significantly higher transcription levels. None of the other target genes were significantly influenced by genital infection status.

In order to examine whether the expression levels of *Tbx21* and *Eomes* in sea lions with single infections (either ZcPV-1 or OtHV-1) or with concomitant infections were associated with the up-regulation of inhibitory receptors or suggested a skewed maturation phenotype, we examined the relationship of transcription levels of these genes with those of the inhibitory receptor *Ly49*. We found no correlation for sea lions infected

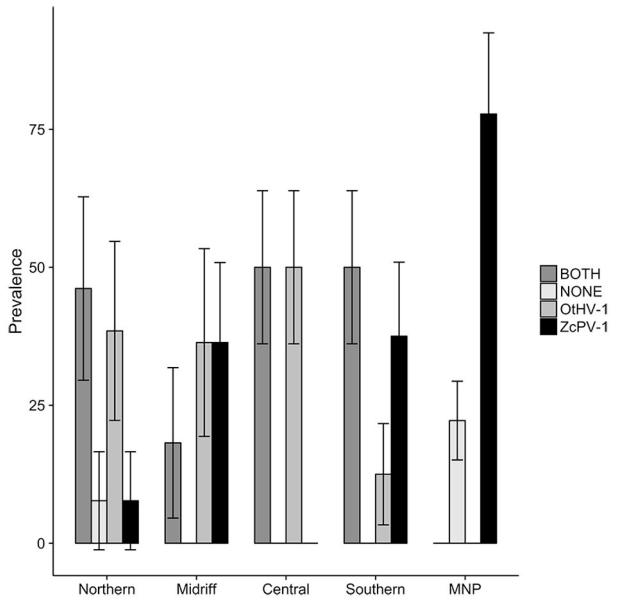

FIGURE 6 | Prevalence of infection by oncogenic viruses in the genital epithelium of California sea lions according to ecological regions within the Gulf of California and the Mexican North Pacific (MNP). ZcPV-1 infections were highest in the Mexican North Pacific followed by the midriff and southern Gulf of California, OtHV-1 infections were highest in the central region, followed by the northern and midriff regions, and concurrent infections by both viruses varied significantly among regions, following the pattern observed for OtHV-1. Bars = ± s.e.

simultaneously by both viruses ($r^2 = 0.04$, $p = 0.52$), nor was there a significant relationship for sea lions that were infected only by OtHV-1 ($r^2 = 9.11$, $p = 0.43$) or only by papillomavirus ($r^2 = 0.12$, $p = 0.26$; see **Figure S6**).

Gene Transcription and Inflammation

The marker of chronic systemic inflammation and stress here used, NLR, did not vary significantly across regions. However, the variance differed significantly [*F*-test; $F_{(39,55)} = 0.37$, $p = 0.002$], being larger for sea lions from the northern and midriff regions (**Figure 8A**). Eight percent of the variation in transcription levels of *Ly49* was influenced by the NLR, and higher NLR values were associated with lower levels of transcription [$F_{(1,38)} = 4.19$, $p = 0.048$, **Figure 8B**]. None of the other target genes were significantly impacted by the NLR of the sea lions sampled.

DISCUSSION

Our study examined the transcriptional levels of key genes related to cytotoxic responses to viruses and tumor cells, as well as those that are involved with immune modulation in free ranging California sea lions. We found indication of regional differences in the expression of genes related to Th1 responses and immune modulation, and detected different transcription levels in sea lions infected by genital papillomavirus compared to those infected by genital gammaherpesvirus. Furthermore, when analyzing the gene expression profiles according to functional groupings, a north to south division became apparent, where sea lions from the northern and midriff regions were clustered together, while most sea lions from the central and southern regions, as well as from the Mexican North Pacific, clustered together in another group. As is unavoidably the case when studying free-ranging species, our results are correlative, and impede the inference of direct causation. Furthermore, given that we focused on gene transcription rather than on protein expression, we cannot make inferences regarding the expression of the gene products themselves. Nonetheless, the patterns observed were consistent with what is known for herpesvirus and papillomavirus infections in humans and model species, and the relationships in gene expression levels between the markers selected are strongly suggestive of differential ecological impacts on immune effectors. Owing to the paucity of information regarding pinniped immunity, we will discuss our findings in light of what is known for humans and model animals.

Transcription of two genes, *granzyme B* and *perforin*, directly involved with cytotoxic responses did not vary amongst ecological regions. *Granzyme B* and *perforin* are expressed by NK and CD^{8+} T cells, and are crucial to induce apoptosis of transformed or virus-infected cells (44). Both genes are induced progressively upon activation by antigen-presenting cells. *Granzyme B* encodes a serine protease (granzyme B) that is released together with perforin, a glycoprotein encoded by the *perforin* gene. Perforin forms a pore in the membrane of the target cell, allowing granzyme B to enter the target cell and trigger apoptosis via the activation of various caspases, generation of mitochondrial reactive oxygen species, and DNA fragmentation (45). The nearly 5-fold difference in transcription levels between both genes implies that individual sea lion NK and CD^{8+} T cells transcribe different combinations of *perforin* and *granzyme B*, and that each gene is differentially regulated at the single cell level (46). It is possible that, as has been reported for humans (47), granzyme B could exhibit some perforin-independent activity in the California sea lion, Alternatively, it is also plausible that the results indicate that for every transcribed mRNA of *perforin*, more *granzyme B* transcription is required.

We also selected *Ly49* as a marker of NK function, and found its expression to be slightly downregulated with respect to the reference genes in nearly all of the sea lions. *Ly49* encodes functional inhibitory NK cell transmembrane receptors in the California sea lion (48). Inhibitory NK receptors are essential for detecting missing or altered MHC class I molecules in target cells (49). The low levels of expression of *Ly49* could reflect that the adult sea lions have a small population of peripheral mature NK cells that are undergoing *Ly49* gene activation. If so, most of the *granzyme B* and *perforin* would have been transcribed by peripheral CD^{8+} cells, as they tend to be more abundant than NK cells in blood (50). This possibility is strengthened by the loose correlation observed between the transcription levels of *Ly49* and those of *granzyme B* and *perforin*. Future studies should aim to explore the functional role of other NK cell transmembrane receptors, such as the killer cell lectin like receptor 1 gene (*nkg2d*), whose role for modulating cytotoxicity has been associated with susceptibility to papillomavirus-related cancers in humans (51).

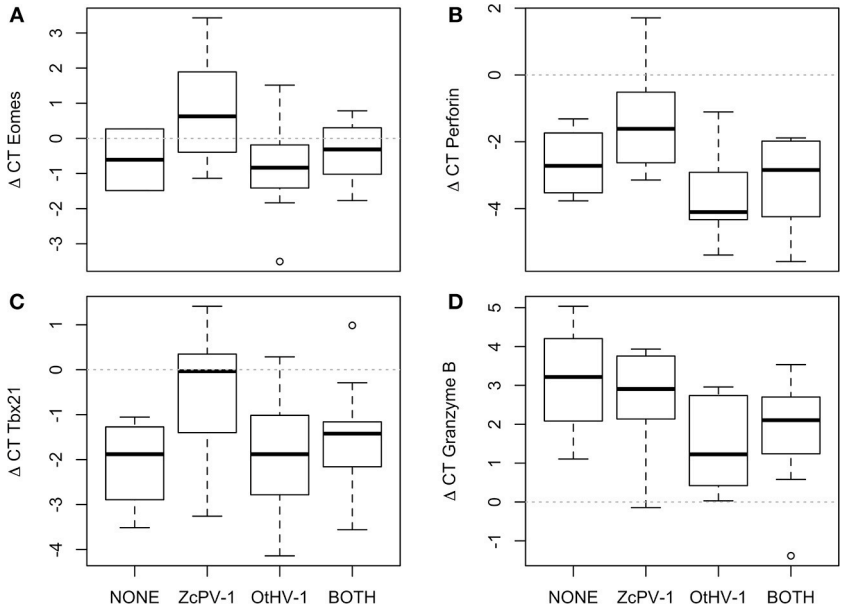

FIGURE 7 | Gene transcription levels varied according to genital infection status. **(A)** *Eomes*, **(B)** *Perforin*, **(C)** *Tbx21*, **(D)** Granzyme. The plot shows the median (thick line), first and third quartile (box), and 95% confidence interval of the median (whiskers).

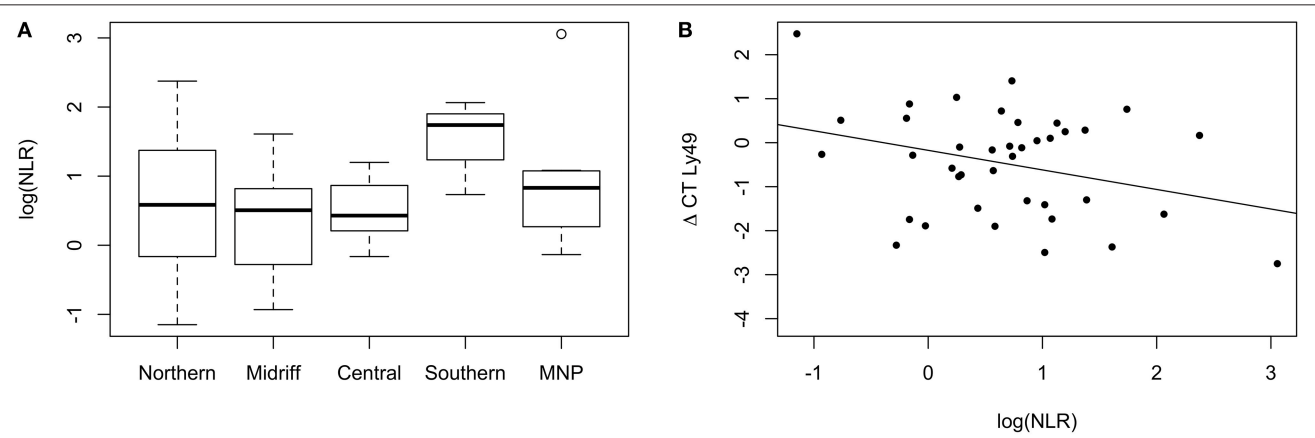

FIGURE 8 | (A) Neutrophil to Lymphocyte Ratio (NLR) of California sea lions from different regions. The variance of NLR was larger in sea lions from the northern and midriff regions. The plot shows the median (thick line), first and third quartile (box), and 95% confidence interval of the median (whiskers). MNP: Mexican North Pacific. **(B)** Relative transcription levels of Ly49 are explained by the NLR of California sea lions. Higher NLR values were associated with lower levels of transcription (Adjusted $R^2 = 0.078$).

Relative expression levels of *Eomes*, *Tbx21*, and *STAT-1*, genes also expressed by NK and CD^{8+} T cells (52), was closely correlated, as would be expected in a Th1 (pro-inflammatory) environment (53, 54). Furthermore, these genes exhibited regional variation in transcription. *Eomes* was downregulated in most sea lions from the midriff and central regions, but upregulated in sea lions from the northern and southern regions, as well as in the Mexican North Pacific, while *Tbx21* and *STAT-1* transcription levels showed a clear north-to-south increase within the Gulf of California and reached highest levels in the Mexican North Pacific. When considering the transcriptional profile of NK and CD^{8+} cells, the hierarchal clustering analysis revealed two main profiles. Sea lions from the northern and midriff regions exhibited one of them, while most sea lions from the southern region and the Mexican North Pacific exhibited the other. Sea lions from the central region exhibited one or the other profile, and a few sea lions from the southern region and the Mexican North Pacific exhibited a more "northern" transcriptional profile. This suggests a spatial difference in the prevalence of intracellular pathogens or differential exposure to other factors that could cause cellular insults.

The encoded products of *Eomes*, *Tbx21*, and *STAT-1* (Eomesodermin, T-bet, and STAT-1, respectively) are essential to Th1 cell differentiation, and are consequently important for downstream anti-viral and anti-tumoral responses (16). *Eomes* gene expression can be upregulated by type I interferon signaling in CD^{8+} T cells (55), while *Tbx21* is upregulated in response to stimulation by antigens and macrophage-derived cytokines (56). STAT-1 regulates *Tbx21* transcription, and in turn, high levels of *Tbx21* influence the transcription of *perforin* and *granzyme B* in antigen-stimulated cells (57). Furthermore, T-bet promotes and sustains virus-specific CD^{8+} responses, particularly during chronic infections (58). The relatively higher transcription levels observed in sea lions from the southern Gulf of California and Northern Pacific could indicate increased exposure to intracellular pathogens or anti-tumoral activity. Two results add support for this proposed explanation. First, sea lions with genital infections by papillomavirus had increased expression of *Eomes*, *Tbx21*, and *perforin*, and second, papillomavirus infections were significantly higher in the southern Gulf of California and Mexican North Pacific. Research on human papillomavirus has shown that the oncogenic proteins E6 and E7 can induce T-cell responses in individuals with a functional immune system. The responses, characterized by higher densities of T cells that express eomesodermin (16), T-bet (17), and perforin, among other markers help clear, or avoid, the occurrence of cervical neoplasia (17). Based on our results, we suggest that ZcPV-1 induces systemic immune responses in otherwise healthy California sea lions.

An opposite pattern was observed for OtHV-1. Here, genetic expression of *Eomes*, *Tbx21*, and *perforin* was lower in infected sea lions. In marked contrast to papillomavirus, whose tropism is restricted to the basal layer of the epithelium (59), gammaherpesviruses have a wider tropism that includes epithelial cells and B-lymphocytes (60). In humans, active gammaherpesvirus infections are most often associated with severe immunosuppression, and the viruses can switch to latency, establishing lifelong persistent infections, which can then lead to lymphomas and carcinomas, as well as lymphoproliferative disorders of NK and T cells (61). Immune control of gammaherpesviruses infections is complicated, as they have evolved various immune evasion strategies (62). Unfortunately, knowledge about OtHV-1 pathogenesis is currently limited, but based on our results, it is tempting to speculate that detectable infections in the genital tract will be much more common in sea lions with a suboptimal immune status, and that infections will, in turn, avoid cytotoxic immune responses, further increasing the risk of malignant transformation of infected cells. This hypothesis is strengthened by two findings: one, a relative decrease in lymphocyte counts was observed in herpesvirus-infected sea lions compared to sea lions with papillomavirus or those with no apparent infections (see **Figure S7**); and two, when comparing levels of transcription of genes related to cytotoxicity among apparently uninfected sea lions, sea lions infected by OtHV-1, ZcPV-1, and by both viruses simulaneously, levels of *Eomes*, *perforin*, and *tbx21* of sea lions infected by OtHV-1 were equal to or lower than uninfected sea lions. Interestingly, simultaneous infection of the genital epithelium by OtHV-1 and ZcPV-1 resulted in a pattern of transcription similar to that of OtHV-1 alone. This was unexpected, and implies that co-infections might be an important risk factor for transformation of the genital epithelium, owing to a reduction in anti-viral and anti-tumor activity. A recent study reported coinfection by herpesviruses and papillomaviruses in genital tumors of Atlantic bottlenose dolphins, *Tursiops truncatus* (63) and it has already been shown that co-infections by oncogenic papillomaviruses and herpesviruses increase the risk of cervical carcinoma in humans (64). Based on our results, we believe that the potential role of papillomavirus as a co-factor in for sea lion urogenital carcinoma should be reconsidered [see (3)].

Transcription levels of the two genes selected as markers of Th2 responses and immune modulation varied among regions, once again with a north to south gradient. *GATA3* was downregulated in sea lions from the northern and midriff Gulf of California, and upregulated in sea lions from the central and southern Gulf of California and Mexican North Pacific, where levels were the highest. *FoxP3* mirrored the spatial pattern, although transcription levels of this gene were, on average 4.5-fold lower than that of *GATA3*. Both genes were correlated, as would be expected since *GATA3* modulates *FoxP3* expression and activity (65), however, *GATA3* only explained 13% of the variation in *FoxP3* levels, and a number of individuals deviated greatly from the predicted regression. *GATA3* is a transcription factor expressed by mature CD^{8+} T cells, and it plays a key role in leading to the differentiation of Th2 cells, regulating T cell development, proliferation, metabolism, and maintenance. In particular, it promotes development of anti-inflammatory Th2 and Th9 cells, and suppresses pro-inflammatory Th1 and Th17 differentiation as well as B cell development (65). In turn, *FoxP3* is highly expressed by CD^{4+} T regulatory (Treg) cells, and it is essential to ensure immune homeostasis by suppressing (or destroying) active leukocytes via stimulating direct cytotoxicity, depleting growth factors in the extracellular environment, and secreting anti-inflammatory cytokines and co-inhibitory molecules that can modulate the activity of antigen-presenting cells (65). Expression of *FoxP3* is particularly important to control excessive anti-viral responses and limit the extent of immunopathology (66). Based on the fact that (i) several of the genes involved with promoting pro-inflammatory and cytotoxic responses were upregulated in sea lions from the central and southern Gulf of California and Mexican North Pacific, and (ii) these are the regions where transcription of *GATA3* was increased, and that expression of *GATA3* is known to be tightly regulated by TCR and pro-inflammatory cytokine stimulation, which in turn responds to active CD^{8+} and NK activity (67), it is possible that sea lions in these regions are experiencing active responses to one or more viruses, and are, at the same time, experiencing some level of immunomodulation to avoid damage associated with these responses. The hierarchal analysis showed some support for this explanation, as the dendrogram revealed two large groups that separated sea lions from the northern and midriff region from those from the central and southern Gulf of California, and the Mexican North Pacific. Thus, it is likely that differences in viral exposure, as well as other as yet unidentified environmental and ecological variables are impacting sea lion immune modulation in addition to their cytotoxic immune activity. Having found that the NLR was higher in sea lions from

the northern and midriff region implies that these individuals are undergoing chronic inflammation. However, as our study focused exclusively on assessing gene transcription levels, rather than proteins, this possibility will need to be examined in more detail in the future by quantifying cytokine levels in the blood.

The ratio of *GATA3* to *Tbx21* (here Ga/Tb) is an indirect measure of the Th1/Th2 profile of an individual (39). Both transcription factors cross-regulate each other, as T-bet modulates GATA-3 function and Th2 cytokines block Th1 differentiation (66). The interrelationship of these molecules and the effector responses that they regulate define the host response and, therefore, influence the outcome of a given infection. While mean Ga/Tb did not vary among regions, when analyzing the Th1/Th2 profile by hierarchal clustering, a north to south pattern emerged once again, with one group comprised by sea lions from the midriff, northern, and one comprised by sea lions from the southern region. As observed for transcriptional markers of cytotoxicity, sea lions from the central region exhibited either one the responses.

The observed patterns in the transcriptional profiles of genes related to cytotoxicity, Th2 and immunomodulation, and Th1/Th2 balance were congruent. In all cases there appeared to be a distinction between patterns exhibited by sea lions from the northern and midriff regions and those exhibited from the southern region and the Mexican North Pacific. While differences in transcription levels do not necessarily indicate differences in the synthesis of the proteins of interest due to transcriptional and post-transcriptional regulation (68), the congruence in the transcriptional profiles observed cannot be ignored. In particular, these profiles appear to be related to genital infection by two oncogenic viruses, and are likely to reflect other ecological and environmental factors, such as differences in contaminant levels (7, 20, 21) and availability of energetic resources (25) to implement immune responses (32). These possibilities will need to be addressed in future studies. Given the role of NK and CD8+ cells for tumor surveillance and anti-viral responses (15–17), the significance of OtHV-1 in the development of sea lion urogenital carcinoma (2, 3), the potential importance of ZcPV-1 in oncogenesis (63), and the stark difference in the prevalence of urogenital carcinoma between sea lions from the US and Mexican waters (18), the patterns observed are likely to be biologically significant.

Marine mammals, as apex predators, are continuously exposed to xenobiotics that can impact their immune competence. This is particularly problematic, given the growing number of emerging pathogens in the marine environment and the rise in disease conditions and unusual mortality events in the past decades. In this context, it is relevant and timely to increase our understanding of the factors that can hinder different immune effectors of these species (69), particularly of those that are prone to develop chronic and deadly diseases, such as urogenital carcinoma, which could be considered sentinels of immune competence (70). Our study is a first approach to profile the transcriptional patterns of key immune effectors of free-ranging California sea lions, and their association with ecological regions and infection by oncogenic viruses. The observed results and suggested patterns add insight to our understanding of immune competence of marine mammals, and may help elucidate the marked difference in the number of cases of urogenital carcinoma in sea lions from US waters and other areas of their distribution.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

KA-W conceived, designed and coordinated the study. IP conducted gene expression assays, statistical analyses, and drafted the manuscript. AF-M conducted hematological analysis; MF-C and RÁ-M assisted with statistical design, analysis and interpretation of data. FG-dlR and LS-G performed pathogen detection assays. All authors read and commented the final draft of the manuscript and gave final approval for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00413/full#supplementary-material

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Comparative Immunopathology of *Cetacean morbillivirus* Infection in Free-Ranging Dolphins From Western Mediterranean, Northeast-Central, and Southwestern Atlantic

Josué Díaz-Delgado[1], Kátia R. Groch[1], Rodrigo Ressio[2], Isis P. J. Riskallah[2,3], Eva Sierra[4], Simona Sacchini[4], Óscar Quesada-Canales[4], Manuel Arbelo[4], Antonio Fernández[4], Elitieri Santos-Neto[5], Joana Ikeda[5], Rafael Ramos de Carvalho[5], Alexandre de Freitas Azevedo[5], Jose Lailson-Brito Jr.[5], Leonardo Flach[6], Cristina T. Kanamura[2], Natália C. C. A. Fernandes[2], Bruno Cogliati[3], Cinzia Centelleghe[7], Sandro Mazzariol[7], Ludovica Di Renzo[8], Gabriella Di Francesco[8], Giovanni Di Guardo[9] and José Luiz Catão-Dias[1]*

[1] Laboratory of Wildlife Comparative Pathology, Department of Pathology, School of Veterinary Medicine and Animal Science, University of São Paulo, São Paulo, Brazil, [2] Pathology Center, Adolfo Lutz Institute, São Paulo, Brazil, [3] Laboratory of Morphologic and Molecular Pathology, Department of Pathology, School of Veterinary Medicine and Animal Science, University of São Paulo, São Paulo, Brazil, [4] School of Veterinary Medicine, Institute for Animal Health and Food Safety, University of Las Palmas of Gran Canaria, Arucas, Spain, [5] Laboratory of Aquatic Mammals and Bioindicators: Profa Izabel M. G. do N. Gurgel' (MAQUA), Faculty of Oceanography, Rio de Janeiro State University, Rio de Janeiro, Brazil, [6] Instituto Boto Cinza, Mangaratiba, Rio de Janeiro, Brazil, [7] Department of Comparative Biomedicine and Food Hygiene (BCA), University of Padova, Legnaro, Italy, [8] Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale", Teramo, Italy, [9] Faculty of Veterinary Medicine, University of Teramo, Teramo, Italy

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Brian Dixon,
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***Correspondence:**
Josué Díaz-Delgado
josue.diaz101@alu.ulpgc.es

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Cetacean morbillivirus (CeMV; *Paramyxoviridae*) causes epizootic and interepizootic fatalities in odontocetes and mysticetes worldwide. Studies suggest there is different species-specific susceptibility to CeMV infection, with striped dolphins (*Stenella coeruleoalba*), bottlenose dolphins (*Tursiops truncatus*), and Guiana dolphins (*Sotalia guianensis*) ranking among the most susceptible cetacean hosts. The pathogenesis of CeMV infection is not fully resolved. Since no previous studies have evaluated the organ-specific immunopathogenetic features of CeMV infection in tissues from infected dolphins, this study was aimed at characterizing and comparing immunophenotypic profiles of local immune responses in lymphoid organs (lymph nodes, spleen), lung and CNS in CeMV-molecularly (RT-PCR)-positive cetaceans from Western Mediterranean, Northeast-Central, and Southwestern Atlantic. Immunohistochemical (IHC) analyses targeted molecules of immunologic interest: caspase 3, CD3, CD20, CD57, CD68, FoxP3, MHCII, Iba1, IFNγ, IgG, IL4, IL10, lysozyme, TGFβ, and PAX5. We detected consistent CeMV-associated inflammatory response patterns. Within CNS, inflammation was dominated by $CD3^+$ (T cells), and $CD20^+$ and $PAX5^+$ (B cells) lymphocytes, accompanied by fewer $Iba1^+$, $CD68^+$, and $lysozyme^+$ histiocytes, mainly in striped dolphins and bottlenose dolphins. Multicentric lymphoid depletion was characterized by

reduced numbers of T cells and B cells, more pronounced in Guiana dolphins. Striped dolphins and bottlenose dolphins often had hyperplastic (regenerative) phenomena involving the aforementioned cell populations, particularly chronically infected animals. In the lung, there was mild to moderate increase in T cells, B cells, and histiocytes. Additionally, there was a generalized increased expression of caspase 3 in lymphoid, lung, and CNS tissues. Apoptosis, therefore, is believed to play a major role in generalized lymphoid depletion and likely overt immunosuppression during CeMV infection. No differences were detected regarding cytokine immunoreactivity in lymph nodes, spleen, and lung from infected and non-infected dolphins by semiquantitative analysis; however, there was striking immunoreactivity for IFNγ in the CNS of infected dolphins. These novel results set the basis for tissue-specific immunophenotypic responses during CeMV infection in three highly susceptible delphinid species. They also suggest a complex interplay between viral and host's immune factors, thereby contributing to gain valuable insights into similarities, and differences of CeMV infection's immunopathogenesis in relation to body tissues, CeMV strains, and cetacean hosts.

Keywords: *Cetacean morbillivirus*, immunopathogenesis, neuroimmunopathology, lymphocytes, histiocytes, apoptosis, cytokines

INTRODUCTION

Cetacean morbillivirus (CeMV; genus *Morbillivirus*, family *Paramyxoviridae*) has caused multiple outbreaks of lethal disease in odontocetes and mysticetes worldwide. Interepizootic or endemic morbidity and mortality is also recorded (1). There are three well characterized CeMV strains (porpoise MV, dolphin [D]-MV, and pilot whale MV) mainly in northern hemisphere, and three novel strains, one of them detected in Brazil, i.e., Guiana dolphin (GD)-MV, which is also considered the first reported example of CeMV infection among cetaceans from South America (2, 3). Studies suggest there is different species-specific susceptibility to CeMV infection with bottlenose dolphins (*Tursiops truncatus*), striped dolphins (*Stenella coeruleoalba*), and Guiana dolphins (*Sotalia guianensis*) ranking among the most susceptible cetacean hosts, with fatal epizootics (1, 3, 4). CeMV may cause severe lymphoid, respiratory, and neurologic disease in susceptible species, leading to stranding and death. Four major presentations of CeMV-associated pathology (CeMV-AP) are currently recognized, which bear resemblance to the pathologic features of measles virus (MeV) and canine distemper virus (CDV) infections, the major morbilliviral diseases in humans and dogs, respectively (5, 6).

The pathogenesis of infections by terrestrial morbilliviruses (TMVs) involve initial replication in lymphoid tissues, followed by viral dissemination in infected lymphocytes through the lymphatics and blood stream ("leukocyte trafficking"), and eventual spread to epithelial and nervous cells (6, 7). Immunohistochemical (IHC) studies on naturally occurring CeMV infections in cetaceans support the above pathogenesis, with a predominant aerogenous transmission (1). Several studies have focused on the immunophenotypic characterization of local inflammatory responses (LIRs) in TMV infections, especially in measles (in both humans and non-human primates) and distemper (in canids and susceptible non-canid carnivore species), with major emphasis on lymphoid tissues, lung, and central nervous system (CNS) (8, 9). In measles and distemper, various lymphocytic, histiocytic, and cytokine patterns have been shown to vary depending on disease chronology and other factors. Furthermore, cytokine imbalance in Th1 and Th2 immune responses (early and advanced disease stages, respectively) plays a major role in disease susceptibility and progression in MeV- and CDV-infected individuals (5, 8, 9). Nevertheless, there are no published studies focused on the pathogenetic evolution of CeMV infection in Th1-dominant vs. Th2-dominant cetacean hosts (10). Immunophenotypic studies on LIR during CeMV infection are also lacking, except for a previous study focused on peripheral blood leukocytes (PBLs) in a set of bottlenose dolphins with subclinical infection (11). In order to partially fill in this knowledge gap, the present study was aimed at characterizing and comparing the immunophenotypic profiles of CeMV-associated LIR in lymphoid, lung and CNS tissues of infected cetaceans from Western Mediterranean (Italy), Northeast-Central (Canary Islands), and Southwestern Atlantic (Brazil).

MATERIAL AND METHODS

Data and Sample Collection

The marine mammal databases and tissue banks of collaborative research institutions, namely the "Laboratory of Wildlife Comparative Pathology—LAPCOM" (São Paulo, Brazil), the "Laboratory of Aquatic Mammals and Bioindicators Profa. Izabel M. G. do N. Gurgel—MAQUA" (Rio de Janeiro, Brazil), the "Institute for Animal Health and Food Safety—IUSA" (Canary Islands, Spain), the "Department of Comparative Biomedicine and Food Science of the Faculty of Veterinary

Medicine of the University of Padua (Legnaro, Italy)," the "Laboratories of Histopathology and Immunohistochemistry of Istituto Zooprofilattico Sperimentale dell'Abruzzo e Molise G. Caporale," and the "Faculty of Veterinary Medicine of the University of Teramo (Teramo, Italy)," were queried based upon the following criteria: "*Sotalia guianensis,*" "*Stenella coeruleoalba*," "*Tursiops truncatus,*" "CeMV reverse transcription polymerase chain reaction (RT-PCR)-positive" "*Toxoplasma gondii* PCR-negative." Only individuals in a "fresh" (code 2) *post mortem* preservation *status*, or in a "moderate *post mortem* autolysis" (code 3) condition (12), which could also warrant a sufficient amount of formalin-fixed, paraffin-embedded (FFPE), and frozen tissues for extensive analysis, including immunohistochemical (IHC) and cytokine gene expression investigations (parallel manuscript) on target organs (lymph nodes, spleen, lung, brain), were included. Additionally, tissues from three CeMV-negative dolphins including one striped dolphin (Canary Islands), one bottlenose dolphin (Italy), and one Guiana dolphin (Brazil) that were fresh, in good body condition and died as result of bycatch and/or traumatic interaction(s) and lacked morphological and molecular evidence of any infectious etiology were used as "controls" for IHC comparison purposes. The tissue samples came from complete standard necropsies. Epidemiologic and biologic data, along with necropsy reports, photographic material, and ancillary diagnostic techniques were retrieved and further analyzed. Required permissions for the management of tissues from cetaceans found stranded along the coasts of Brazil, the Canarian archipelago, and Italy were issued by the respective official authorities. All dolphins had spontaneous naturally occurring CeMV infection and no experiments were performed on live animals. Detailed comparative histopathologic investigations and viral IHC results for these animals will be published elsewhere.

Immunohistochemistry

Selected FFPE tissues including lymph nodes (mediastinal/tracheobronchial, pulmonary, mesenteric, prescapular) and spleen, lung, and CNS (cerebrum, cerebellum, brain stem, spinal cord) were subjected to IHC using the following primary antibodies (pAbs): cleaved caspase 3 (CAS3) [final apoptosis pathway], cluster of differentiation (CD)-3 [T cell], CD20 [B cell], CD57 [natural killer cell], CD68 [histiocyte], Forkhead Box (Fox)-P3 [regulatory T cell], human leukocyte antigen (HLA-DRα; *syn*. major histocompatibility complex II, MHCII) [antigen presenting cell], ionized calcium binding adaptor molecule 1 (Iba1) [histiocyte/microglia], interferon gamma (IFNγ), immunoglobulin (Ig)-G, interleukin (IL)-4, IL10, lysozyme, transforming growth factor beta (TGFβ), and paired box protein (PAX)-5 [B-cell]. Most of this IHC panel was standardized with successful cross-reactivity in cetacean tissues (13). Further details on the IHC protocol are recorded in **Table 1**. Briefly, serial sections at 3 μm-thick were cut and collected onto coated slides. Antigen retrieval was followed by endogenous peroxidase blocking and nonspecific binding blocking with normal serum of same species where pAbs were raised. PAbs were incubated overnight (18 h, 4C°). Amplification and visualization was achieved by the HiDef DetectionTM HRP Polymer System (Cell Marque, Rocklin, California, USA) followed by diaminobenzidine (DAB D-5637; Sigma, St. Louis, Missouri, USA) chromogen and counterstaining with Harris' haematoxylin. Normal human, mouse and franciscana (*Pontoporia blainvillei*) lymph node, spleen, lung, and brain were used as positive controls (13). Tissue sections in which the pAbs were replaced by non-immune homologous serum served as negative controls.

For immunophenotypic characterization of local inflammatory cell populations, the number of immunopositive cells was semiquantitatively evaluated for each marker in lymphoid (lymph nodes, spleen), lung, and CNS tissues according to following score: –, no; +, <10%; ++, 10–50%; +++, 51–90%, and ++++, >90% immunopositive cells, in 10 high-power (400x) fields. The histo-anatomical compartments analyzed were: lymph nodes (primary and secondary follicles, paracortex, medullary cords and cortical, paracortical, and medullary sinuses; afferent/efferent lymphatics); spleen (follicles, perifollicular zone, periarteriolar lymphoid sheath [PALS], red pulp sinuses, and cords); lung (bronchial/bronchiolar mucosa and submucosa including glands and associated lymphoid tissue [BALT]; alveolar spaces and septa; interstitium; pleura; vasculature including lymphatics); brain (cerebral cortex, cerebellum, brain stem, spinal cord) (13, 14). Additionally, IHC expression intensity was subjectively scored (increasing intensity) as 1, 2, and 3. The results were compared between CeMV-infected and non-infected "control" dolphins.

RESULTS

Twenty-seven CeMV-positive dolphins, including 13 striped dolphins, 11 Guiana dolphins, and 3 bottlenose dolphins met the inclusion criteria. Guiana dolphins were infected by GD-CeMV (2, 3), while striped dolphins and bottlenose dolphins were infected by DMV (15–18). Epidemiologic and biologic data as well as CeMV-RT-PCR-positive tissues are recorded in **Table 2**. CeMV-positive animals included: calves ($n = 2$), juveniles ($n = 11$), and adults ($n = 14$). All CeMV-negative dolphins were calves. Detailed gross and microscopic pathologic findings with most probable cause(s) of stranding and/or death for CeMV-positive are published elsewhere and for CeMV-negative dolphins are recorded in **Supplemental Table S1**.

Consistent immunolabeling (with variations according to LIR) was detected for CAS3, CD3, CD20, CD57, CD68, FoxP3, MHCII, Iba1, IFNγ, IgG, IL4, IL10, lysozyme, TGFβ, and PAX5 in all organs/tissue sections examined. The following alterations in immunophenotypic profiles of LIRs are referred as to comparisons to "control" animals. "Normal" or "physiologic" antigen cell distribution and intensity for the pAbs aforementioned in control animals (cases 28, 29, and 30) were similar to those previously reported (13) (**Supplemental Figure**).

Lymphoid Tissues: Lymph Nodes, Spleen

CeMV-LIR often overlapped focally with verminous lymphadenitis-associated LIR, especially in Guiana dolphins. The following changes had somewhat similar distribution patterns in all lymph nodes (mediastinal/tracheobronchial, pulmonary,

TABLE 1 | Tested antigen, clone, species of origin, clonality, antigen retrieval methods, working dilutions for primary antibodies and amplification and visualization method for immunohistochemistry of selected immune components in formalin-fixed, paraffin-embedded lymphoid tissues of striped dolphins (*Stenella coeruleoalba*), bottlenose dolphins (*Tursiops truncatus*), and Guiana dolphins (*Sotalia guianensis*).

Antigen	Clone	Species	Clonality	Retrieval	[Antibody]	Amplification/visualization
Caspase 3[a]	Asp175	Rabbit	Pol	pH6	1:200	PoH/DAB[i/j]
CD3[b]	A0452	Rabbit	Pol	pH9	1:1000	PoH/DAB[i]
CD20[c]	RB9013P	Rabbit	Pol	pH9	1:100	PoH/DAB[i]
CD57[d]	NKNE1	Mouse	Mon	pH6	1:400	PoH/DAB[i]
CD68[b]	KP1	Mouse	Mon	pH6	1:3000	PoH/DAB[i]
FoxP3[a]	D2W8E	Mouse	Mon	pH9	1:10	PoH/DAB[i]
HLA-DRα[b]	TAL1B5	Mouse	Mon	pH9	1:400	PoH/DAB[i]
Iba-1[e]	NCNP24	Rabbit	Pol	pH9	1:500	PoH/DAB[i]
IFNγ[f]	DBNE1	Mouse	Mon	pH6	1:1000	PoH/DAB[i]
IgG[b]	A0423	Rabbit	Pol	pH9	1:1000	PoH/DAB[i]
IL4[g]	ab9811	Rabbit	Pol	pH9	1:400	PoH/DAB[i]
IL10[g]	ab34843	Rabbit	Pol	pH9	1:400	PoH/DAB[i]
Lysozyme[b]	A0099	Rabbit	Pol	pH6	1:3000	PoH/DAB[i]
TGF-β[g]	213NE4.4	Mouse	Mon	pH9	1:200	PoH/DAB[i]
PAX-5[h]	BC/24	Mouse	Mon	pH9	1:300	PoH/DAB[i]

CD, cluster of differentiation; FoxP3, Forkhead Box P3; HLA, human leukocyte antigen (synonym major histocompatibility complex); IFN, interferon; Ig, immunoglobulin; IL, interleukin; TGF, transforming growth factor; Pax-5, paired box 5; Mon, monoclonal; Pol, polyclonal; PK, proteinase K; NE: not evaluated; PoH; HiDef Detection™ Polymer System; DAB, Diaminobenzidine. Best antigen retrieval and primary antibody concentrations are indicated in bold. pH6, citrate; pH9, EDTA, Ethylenediamine tetraacetic acid. [a]Cell signaling (Danvers, MA, USA), [b]Dako-Agilent (Santa Clara, CA, USA), [c]Thermo (Fremont, CA, USA), [d]Novocastra-Leica (Nussloch, Germany), [e]Wako Pure Chemical (Richmond, VA, USA), [f]Bio-Rad (Hercules, CA, USA), [g]Proteintech (Rosemont, IL, USA), [h]Biocare (Pacheco, CA, USA), [i]Cell Marque (Rocklin, CA, USA), [j]DAB, 3,3′-Diaminobenzidine (D-5637; Sigma, St. Louis, Missouri, USA).

mesenteric, prescapular) and spleens evaluated (**Table 3**). There was an overall increased CAS3 expression in mononuclear cells (MNCs), including lymphocytes and histiocytes (often with engulfed apoptotic debris) in the cortex and paracortex, and histiocytes of subcortical sinuses and medullary cords (**Figure 1A**) in lymph nodes of CeMV-infected dolphins. While there was a consistently decreased number of CD3+ (**Figure 2A**), CD20+ (**Figure 3A**), and PAX5+ (**Figure 4A**) lymphocytes (hereafter, T cells, and B cells, respectively) in lymph nodes of most Guiana dolphins, striped dolphins, and bottlenose dolphins often had cortical, paracortical, and medullary cord expansion of T cells and B cells in addition to milder multifocal depletion phenomena. MHCII expression varied between CeMV-infected and uninfected animals. Approximately half of the dolphins, more predominantly in Guiana dolphins, infected by CeMV had reduced MHCII expression associated with diminished B-cells; however, there were increased MHCII+ histiocytes (see below) influx (**Figure 5A**). Fewer MHCII+ MNCs were detected in the paracortex and subcortical medullary cords. Iba1+ histiocytes (macrophages, monocytes, dendritic cells) (**Figure 6A**) correlated with CD68+ (**Figure 7A**) and lysozyme+ (**Figure 8A**) histiocytes; however, Iba1+ tended to label a greater number of histiocytic cells. Increased histiocytes (dendritic cells and circulating monocytes/macrophages) were common in depleted follicles, paracortex, and sinuses of infected dolphins. Occasional syncytia were Iba1+ and CD68+. CD57+ MNCs in lymph nodes varied slightly among CeMV-infected dolphins but overall did not differ considerably from "control" dolphins, with only few scattered immunoreactive cells in cortex and paracortex. Furthermore, the number of IgG+ lymphocytes varied among CeMV-infected dolphins; however, they tended to be slightly more numerous than in "control" animals, being mostly detected in germinal centers (**Figure 9A**) and paracortex and to a lesser extent in medullary cords and occasionally within sinuses. Scattered FoxP3+ lymphocytes were seen in the cortex, paracortex, and medullary cords of CeMV-infected and control dolphins with no apparent differences from "control" dolphins.

In the spleen, there was increased CAS3 expression in MNCs of white pulp and to a lesser extent in red pulp (**Figure 1B**). There was a consistently reduced number of T cells (**Figure 2B**) and B cells (**Figures 3B**, **4B**), especially prominent in Guiana dolphins; however, striped dolphins and bottlenose dolphins occasionally presented "reactive (regenerative) hyperplasia" in addition to multifocal lymphoid depletion. As observed in lymph nodes, infected dolphins tended to have reduced expression of MHCII in B cell areas (**Figure 5B**); however, increased MHCII+ histiocytes contributed to overall similar semiquantitative results for MHCII expression in spleen of "control" animals. Iba1+ (**Figure 6B**) histiocytes correlated to CD68+ (**Figure 7B**) and lysozyme+ (**Figure 8B**) histiocytes, and were more numerous in the red pulp. Rare CD57+ MNCs were detected in the white pulp with no evident difference regarding "control" dolphins. IgG+ lymphocytes were more common in the white pulp and to a lesser extent in the red pulp (**Figure 9B**) of infected dolphins, particularly in striped dolphins from the Canary Islands and Italy, in contrast to Guiana dolphins. No differences were detected regarding FoxP3+ lymphocytes in CeMV-infected and uninfected "control" dolphins.

TABLE 2 | Epidemiologic and biologic data of Guiana dolphins (*Sotalia guianensis*), striped dolphins (*Stenella coeruleoalba*), and bottlenose dolphins (*Tursiops truncatus*) included in this study.

No	Species	Stranding	Coordinates (country)	BL (cm)	Age	Sex	NS	DC	SC	CeMV chronicity
1	*S. guianensis*[a]	09-Nov-2017	23°10′6″S; 44°20′82″W (BR)	177	Ad	Fe	Po	2	D	AS
2	*S. guianensis*[a]	14-Nov-2017	23°00′47″S; 44°26′32″W (BR)	94	Ca	Ma	Mo	2	D	AS
3	*S. guianensis*[a]	17-Dec-2017	22°56′27″S; 43°59′34″W (BR)	164	Ju	Ma	Mo	3	D	SS
4	*S. guianensis*[a]	17-Dec-2017	23°03′08″S; 44°04′13″W (BR)	93	Ca	Fe	Go	3	D	AS
5	*S. guianensis*[a]	23-Dec-2017	22°58′43″S; 43°57′46″W (BR)	149	Ju	Ma	Mo	2	D	AS
6	*S. guianensis*[a]	25-Dec-2017	23°00′11″S; 43°56′46″W (BR)	125	Ju	Ma	Po	3	D	AS
7	*S. guianensis*[a]	26-Dec-2017	22°59′49″S; 43°55′12″W (BR)	188	Ad	Fe	ND	3	D	AS
8	*S. guianensis*[a]	27-Dec-2017	22°56′47″S; 44°00′35″W (BR)	176	Ad	Ma	Mo	3	D	AS
9	*S. guianensis*[a]	27-Dec-2017	22°56′50″S; 44°02′16″W (BR)	183	Ad	Ma	Mo	3	D	AS
10	*S. guianensis*[a]	27-Dec-2017	23°01′08″S; 43°54′06″W (BR)	186	Ad	Ma	Po	3	D	AS
11	*S. guianensis*[a]	15-Jan-2018	22°56′45″S; 43°54′26″W (BR)	130	Ju	Fe	Po	2	D	SS
12	*S. coeruleoalba*[a]	13-Nov-2002	28°9′2″N; 15°32′8″W (SP)	224	Ad	Ma	Go	2	A	CS
13	*T. truncatus*[a]	18-Jul-2005	29°7′41″N;13°27′58″W (SP)	250	Ju	Fe	Mo	2	A	SS
14	*S. coeruleoalba*[a]	16-Aug-2005	28°0′24″N; 15°22′35″W (SP)	168	Ju	Fe	Go	2	A	AS
15	*S. coeruleoalba*[a]	16-Apr-2007	28°33′28″N; 16°20′1″W (SP)	195	Ju	Ma	Po	2	D	AS
16	*S. coeruleoalba*[a]	02-May-2008	28°29′53″N; 13°50′59″W (SP)	194	Ju	Fe	Po	3	D	BOFDI
17	*S. coeruleoalba*[a]	22-Jan-2009	28°28′2″N; 13°51′37″W (SP)	212	Ad	Fe	Go	2	D	BOFDI
18	*S. coeruleoalba*[a]	10-Feb-2011	28°54′18″N; 13°44′20″W (SP)	215	Ad	Fe	Go	2	D	CS
19	*S. coeruleoalba*[a]	28-Apr-2012	28°55′57″N; 13°49′46″W (SP)	203	Ju	Ma	Mo	2	D	AS
20	*S. coeruleoalba*[a]	04-Jul-2011	40°06′38.5″N 15°13′10.1″E (IT)	205	Ad	Ma	Mo	3	D	BOFDI
21	*S. coeruleoalba*[a]	20-Oct-2013	40°54′15.4″N 14°01′47.3″E (IT)	NR	Ad	Ma	ND	3	D	AS
22	*S. coeruleoalba*[a]	02-Feb-2013	40°38′04.6″N 14°49′47.3″E (IT)	NR	Ad	Fe	ND	3	D	CS
23	*T. truncatus*[a]	20-Mar-2013	43°09′48.9″N 10°32′20.9″E (IT)	203	Ju	Ma	ND	2	D	AS
24	*S. coeruleoalba*[a]	05-Feb-2013	38°12′57.1″N 15°13′50.8″E (IT)	202	Ad	Ma	ND	2	D	CS
25	*T. truncatus*[a]	30-Jun-2011	41°37′32.6″N 12°27′18.2″E (IT)	297	Ad	Ma	Mo	2	A	SS
26	*S. coeruleoalba*[a]	12-Oct-2017	42°28′05″N 14°13′27″E (IT)	200	Ad	Fe	Mo	2	D	AS
27	*S. coeruleoalba*[a]	10-Nov-2017	42°10′37″N 14°41′33″E (IT)	188	Ju	Fe	Mo	3	D	SS
28	*S.coeruleoalba*[b]	29-Apr-09	28.002990, −15.373500 (SP)	105	Ca	Fe	Go	2	D	Not infected
29	*T. Truncatus*[b]	15-Oct-2008	44.006247, 12.662941 (IT)	118	Ca	Ma	Mo	2	D	Not infected
30	*S. guianensis*[b]	26-Nov-17	23°00′57″S; 43°55′23″W (BR)	89	Ca	Ma	Mo	2	D	Not infected

[a]*CeMV-positive;* [b]*CeMV-negative; NR, not recorded; Ca, calf; Ju, juvenile; Ad, adult; Fe, female; Ma, male; NS, nutritional status; Po, poor; Mo, moderate; G, good; DC, decomposition code (2, fresh; 3, moderate autolysis); SC, stranding condition (A: alive; D: dead). AS, acute systemic; SS, subacute systemic, CS, chronic systemic; BOFDI, brain only form of DMV infection.*

Approximately a third of the infected dolphins had increased IFNγ immunoreactivity in lymph nodes and/or spleen tissue compared to uninfected dolphins. IFNγ was variably expressed in MNCs (lymphocytes, macrophages), rare syncytia and extracellularly in lymph nodes, and spleen (**Figures 10A,B**). Except for few dolphins, most infected dolphins showed no immunohistochemically evident difference regarding TGFβ, IL4, and IL10 immunoreactivity in lymph nodes and spleen. Occasional MNCs expressed TGFβ, rarely expressed also by syncytia (case 25). Scattered IL4+ and IL10+ MNCs along with occasional extracellular labeling were noted in cortex and paracortex (lymph nodes) and white pulp (spleen), and to a lesser extent in lymph node and spleen sinuses.

Lung

CeMV-LIR often overlapped focally with verminous pneumonia-associated LIR, especially in Guiana dolphins. IHC results in lung tissue are recorded in **Table 4**. There was increased CAS3 expression in circulating, infiltrating, and exocytosing/exudating MNCs as well as luminal cell debris (**Figure 1C**). There was a mildly to moderately increased number of T cells (**Figure 2C**) and B cells (**Figures 3C**, **4C**) in alveolar septa, interstitium, and bronchial/bronchiolar mucosa/submucosa including BALT. Lung from "control" dolphins had scattered detectable T cells and B cells. Case 25 (with concomitant septicemia by *Staphylococcus aureus*) had abundant T cell and B cell infiltrates around bronchi/bronchioles, and alveoli (**Figure 2C**). Overall, the number and intensity of CAS3+ and T cells and B cells was greater in parasitic inflammatory foci. MHCII immunoexpression was consistently increased and involved presumed resident and circulating MNCs, including putative pulmonary intravascular macrophages (PIVMs), and infiltrating inflammatory MNCs (**Figure 5C**), being more abundant in foci of parasitic injury. Histiocytes were increased

TABLE 3 | Summary of the results for selected immunomarkers in lymph nodes and spleen of striped dolphins (*Stenella coeruleoalba*), bottlenose dolphins (*Tursiops truncatus*), and Guiana dolphins (*Sotalia guianensis*) included in this study.

No	Organ	CAS3	CD3	CD20	CD57	CD68	FoxP3	HLA-DRα	Iba1	IFNγ	IgG	IL4	IL10	Lysozyme	TGFβ	PAX-5
1	LNs	++/3	+/3	+/3	–/0	++/2	+/2	++/3	+/3	+/3	+/3	+/3	+/2	++/3	+/2	+/2
	Spleen	+ + +/3	+/3	+/3	+/3	++/3	–/0	+,++/3	++/3	+/3	++/3	+/2	++/2	++/3	+/2	+/2
2	LNS	++/2	+/3	+,++/3	–/0	+,++/2	+/2	++,+++/3	+/3	+/3	+/3	+/2	+/2	++/3	+/3	+,++/2
	Spleen	+/2	+/3	+,++/3	+/2	+,++/2	–/0	++/3	++/3	+/2	+/3	+/3	+/2	++/3	+/3	+,++/3
3[a]	LNs	+/2	+/3	+/3	+/3	++/3	+/2	+,++/3	+/2	++/2	+/3	+/2	+,++/2	+,+ + +/3	+/2	+/2
	Spleen	+/2	+/2	+/3	–/0	+/2	–/0	+/3	+,++/3	+/2	+/2	+/3	+/2	++/3	+/3	+/2
4	LNs	+ + +/2	+/3	+/3	+/2	+,++/2	+/3	++,+++/3	+,++/3	+/2	+/2	+/2	+/2	++,+++/3	+/2	+/2
	Spleen	+/2	+/3	+/3	–/0	+,++/2	+/2	++/3	++/3	NE	NE	NE	NE	+,++/3	NE	+/2
5	LNs	+ + +/3	++/3	+,++/3	–/0	+/2	+/3	++/3	+,++/3	+/2	+,++/3	+,++/3	+,++/2	++/3	+/3	+/3
	Spleen	+/2	+/3	+,++/3	–/0	++/3	+/2	+,++/3	+ + +/2	+/3	+,++/2	+/2	+,++/2	++,+++/3	+/2	+,++/2
6	LNs	+/2	+/2	+/3	+/3	+/3	+/2	++/3	++/2	+/2	+/2	+/2	+/2	+/3	+/2	+/2
	Spleen	+/2	+/2	+/3	+/2	+/3	+/2	+,++/3	+,++/3	–/0	+/2	+/2	+/2	++/3	+/2	+/3
7	LNs	+/3	+/3	+/3	NE	NE	+/2	NE	+,++/3	NE	NE	+/2	+/2	NE	NE	+/3
	Spleen	+/3	+/2	+/3	NE	NE	+/2	NE	+,++/3	NE	NE	NE	NE	NE	NE	+/3
8	LNs	+/3	+/3	+/3	+/2	++/3	+/2	+,++/3	+/3	+/3	+/3	+/3	+/3	+,++/3	+/2	+/3
	Spleen	++/3	+/3	+/3	+/2	++/3	–/0	++/3	+/3	+/2	+/2	+/3	+/2	++,+++/3	+,++/2	+/3
9	LNs	++/3	++/3	+,++/3	+/3	++/3	+/3	++/3	+/3	NE	+/3	+/3	NE	++,+++/3	NE	+/2
	Spleen	+/2	++/2	+,++/3	+/3	++/2	+/3	+/2	+/3	+/3	+/3	+/2	+/2	++/3	+/2	+,++/2
10	LNs	+ + +/3	++/3	+,++/3	+/2	++/2	+/3	+,++/3	+/3	+/2	+/3	+/2	+/3	++/3	+/2	+,++/2
	Spleen	+/2	++/3	+/3	+/2	++/3	+/2	+/3	++/2	+/2	+/3	+,++/2	+,++/2	++,+++/3	+/2	+/2
11[b]	LNs	+/2	+/3	+/3	+/2	+/3	+/2	+/3	+/3	+3	+,++/3	+/3	+/2	++/3	+/2	+/2
	Spleen	+/2	+/3	+/3	–/0	++/3	–/0	+/3	+,++/3	+/3	+,++/3	+/2	+,++/3	+/3	+,++/2	+/2
12	LNs	+/3	++,+++/3	+,++/3	+/3	+,++/2	+/3	++/3	++,+++/3	+/3	++,+++/3	+/3	+/2	+,++/3	+/3	+,++/3
	Spleen	++/2	++/2	++/3	+/3	+,++/3	+/3	++,+++/2	++,+++/3	+,++/3	+,++/3	+/2	+/3	++/3	+/2	+/3
13[c]	LNs	++/3	+/3	+,++/3	+/3	++/2	+/3	++,+++/3	++,+++/3	+/3	++/3	+/2	+/3	+,++/3	+/2	+/2
	Spleen	++/2	+/3	+/3	+/2	+,++/3	+/3	++/3	++,+++/3	+/3	++/3	+/3	+/3	++/3	+/3	+/2
14	LNs	+ + +/2	+ + +/2	++/3	+/2	+,++/2	+/3	++,+++/3	++,+++/3	+/3	+/2	+/2	+/2	+,++/3	+/2	++/2
	Spleen	++/3	++,+++/3	++/3	+/2	+,++/2	+/3	++,+++/3	++,+++/3	+/3	+/3	+/2	+/2	++/3	+/2	++/2
15	LNs	+ + +/3	+,++/3	+,++/3	+/3	++/2	+/3	++/3	++/3	+,++/3	+,++/3	+/3	+/3	++/2	+,++/2	+,++/2
	Spleen	+ + +/3	++/2	++/3	+/3	+/2	+/3	++/3	++,+++/3	+,++/3	++/3	+/2	+/3	++/2	+/2	++/2
16	LNs	++/3	+/2	+,++/3	+,++/3	+/3	+/2	+,++/3	++/3	+,++/2	++/2	+/2	+/3	+,++/2	+/3	+,++/2
	Spleen	++/3	++/3	+,++/3	+/3	+,++/2	+/2	+,++/3	++/3	+/3	+/3	+/3	+/2	+/2	+/2	+,++/2
17	LNs	+ + +/3	++/3	++,+++/3	+/3	+,++/3	+/3	+,++/3	++/3	+/3	+,++/3	+/3	+/3	+,++/3	+/3	+,+ + +/3
	Spleen	++/3	++/3	+,++/3	–/0	+,++/2	+/3	++/3	++/3	+/3	++/3	+/3	+/3	+,++/3	+/2	+,++/3
18	LNs	++/3	++/3	+,++/3	+/3	+,++/2	+/3	++/3	++/3	+,++/3	+/3	+/3	+/3	++/3	+/2	+,+ + +/3
	Spleen	++/3	++,+++/3	+,++/2	+/2	+/2	+/3	++/3	++/3	+/3	+,++/3	+/2	+/2	++/2	+/2	+,+ + +/3
19	LNs	+ + +/2	+ + +/2	+,++/3	++/3	++3	+/3	++,+++/3	++,+++/3	+,++/3	+/3	+/3	+,++/3	++,+++/3	+/2	++/2

(Continued)

TABLE 3 | Continued

No	Organ	CAS3	CD3	CD20	CD57	CD68	FoxP3	HLA-DRα	Iba1	IFNγ	IgG	IL4	IL10	Lysozyme	TGFβ	PAX-5
	Spleen	++/2	++/3	+/2	+,++/3	+,++/3	+/3	++,+++/3	++,+++/3	+/3	+,++/3	+/2	++/2	++/3	+/2	+/2
20	LNs	+/3	+/3	+,++/3	+/2	+,++/2	–/0	+,++/3	++/3	NE	NE	NE	NE	NE	NE	+/2
	Spleen	+/3	++/2	++/3	–/0	+/2	+/3	+,++/3	++/2	+/2	+/2	+/2	+/2	++/3	+/2	+/2
21	LNs	++/3	++/2	++/3	+/2	NE	+/3	+,++/3	++/3	NE	NE	NE	NE	NE	NE	++/2
	Spleen	+++/3	++,+++/2	++,+++/3	+/3	++/3	+/2	+,++/3	+,++/3	+/3	+/3	+,++/2	+/3	++/3	+,++/2	++/2
22	LNs	++/3	+++/3	++,+++/3	+/2	++/2	+/3	++/3	+,++/2	+/2	++,+++/3	+/3	+/2	+ to ++/3	+/2	++,+++/3
	Spleen	++/3	++/3	++,+++/3	+/2	+,++/2	+/3	++/3	+,++/2	++/3	++/3	+/3	+/2	++/3	+/2	++/2
23	LNs	++/2	+++/2	++,+++/3	+/3	+/3	+/2	++/2	++/3		+/2	+/2	+/2	+/3	+/2	++/2
	Spleen	++/2	++/2	++/3	+/2	NE	+/2	+,++/2	++,+++/2	+,++/2	+/2	NE	NE	++/3	NE	++/2
24	LNs	++/2	++/3	++/3	+/3	++,+++/2	+/2	+,++/3	++/3		NE	+,++/2	NE	NE	NE	++/3
	Spleen	++/2	++/3	++/3	+/3	+,++/3	+/3	+,++/3	+++/2	+/3	+,++/3	+/3	+/2	++/3	+/3	++/3
25[d]	LNs	++/3	++,+++/3	+,++/3	+,++/3	++,+++/2	+/3	++/3	++/2	+,++/2	++/3	++/2	+/3	++/+++/3	+/3	+,++/2
	Spleen	+++/3	++/2	++/3	+/3	++,+++/3	+/3	++/3	++/3	+/2	+/3	+/2	+/3	++/3	+/2	++/2
26	LNs	+/3	+/3	+/3	NE	NE	+/2	++/3	++/3	NE	NE	NE	NE	NE	NE	+/3
	Spleen	+/3	+/3	++/3	NE	NE	+/2	++/3	++/3	NE	NE	NE	NE	NE	NE	+/3
27[e]	LNs	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE
	Spleen	+/3	+/3	++/3	NE	NE	+/2	++/3	++,+++/3	NE	NE	NE	NE	NE	NE	NE
28	LNs	+/3	++,+++/3	++,+++/3	+/3	–/0	+/3	++,+++/3	++,+++/3	–/0	+/2	+/2	+/2	+/3	–/0	++,+++/2
	Spleen	+/3	++/3	++,+++/3	+/2	+/2	–/0	+,++/3	++/2	–/0	+/2	+/2	+/2	+/2	+/2	++,+++/2
29	LNs	+/3	++,+++/3	++,+++/3	++/2	–/0	+/2	++,+++/3	++/3	+/3	+/2	–/0	+,++/2	+/3	+/2	++,+++/2
	Spleen	+/3	++/3	++,+++/3	+/2	–/0	–/0	+,++/3	++/3	+/3	+/2	+/2	+/2	+/2	+/2	++,+++/2
30	LNs	+/3	++,+++/3	++,+++/3	+/2	+/2	+/3	++/3	++/3	+/3	+/2	+/2	+,++/2	+/3	+/3	++,+++/2
	Spleen	+/3	++/3	++,+++/3	+/2	+/2	+/2	++,+++/3	++/3	+/2	+/2	+/2	+/2	+/2	+/2	++,+++/2

CD, cluster of differentiation; FoxP3, Forkhead Box P3; HLA, human leukocyte antigen (synonym major histocompatibility complex); IFN, interferon; Ig, immunoglobulin; IL, interleukin; TGF, transforming growth factor; Pax-5, paired box 5; NE, not evaluated. Semiquantitative analysis of immunopositive cells: –, no; +, <10%; ++, 10–50%; +++, 50–90%, and ++++, >90% immunopositive cells. Subjective labeling intensity score of immunopositive cells: 0, 1, 2, and 3.

[a] *Multisystemic hyphate mycosis (lung, kidney).*

[b] *Hyphate mycosis (lung).*

[c] *Suspect Brucella coinfection (primarily CNS lesions).*

[d] *Sepsis by Staphylococcus aureus (particularly severe CNS and lung lesions).*

[e] *Multisystemic hyphate mycosis (lung, cerebrum).*

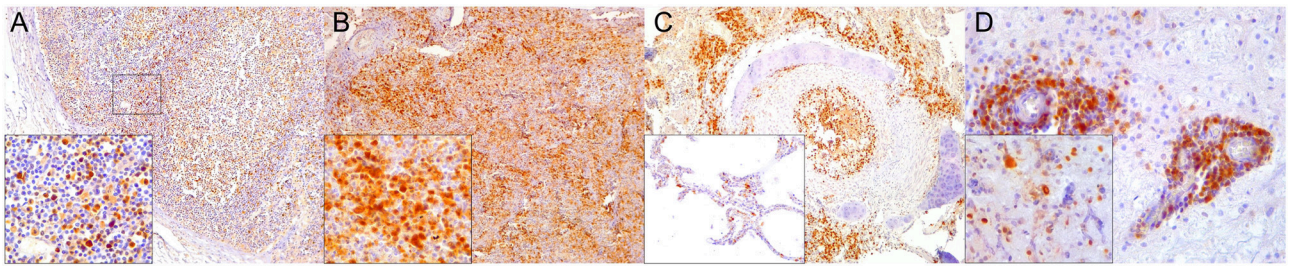

FIGURE 1 | Caspase 3 (CAS3) immunohistochemical findings. **(A)** Lymph node (case 22). Increased expression of CAS3 throughout the cortex. 100×. Inset: lymph node (case 22). Detail of increased CAS3 expression in mononuclear cells (MNCs) of mantle and paracortex. 400×. **(B)** Spleen (case 21). Increased expression of CAS3 throughout the white and red pulp. 100×. Inset: spleen (case 21). Detail of increased expression of CAS3 in white and red pulp. 400×. **(C)** Lung (case 18). Increased expression of CAS3 in proliferative and necrotizing bronchiolitis. 100×. Inset: lung (case 21). Detail of CAS3+ circulating MNCs in alveolar capillaries. 200×. **(D)** Cerebrum (case 24). Increased CAS3 expression in inflammatory cell infiltrates in Virchow-Robins space and adjacent neuroparenchyma. 400×. Inset: cerebrum (case 26). Detail of CAS3$^+$ neurons, neuroglia, and infiltrating MNCs. 400×.

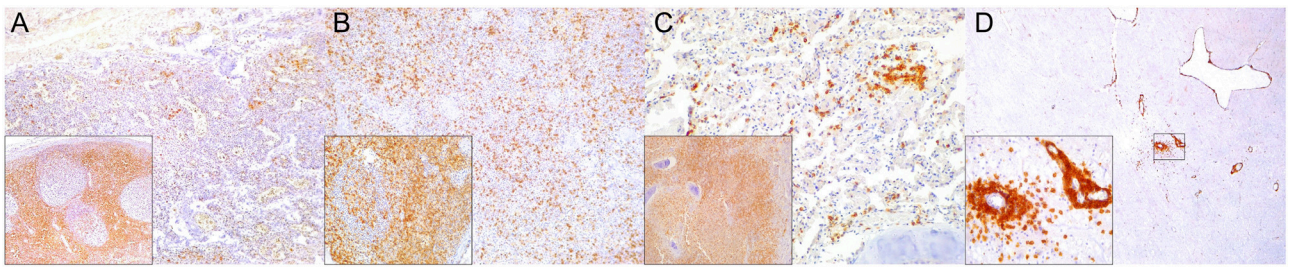

FIGURE 2 | Cluster of differentiation (CD)-3 immunohistochemical findings. **(A)** Lymph node (case 4). Markedly depleted CD3$^+$ lymphocytes of cortex and paracortex. 100×. Inset: lymph node (case 22). CD3$^+$ lymphocyte hyperplasia in paracortex (interfollicular). 400×. **(B)** Spleen (case 24). Reduced CD3$^+$ lymphocytes through white pulp. 100×. Inset: spleen (case 17) CD3$^+$ hyperplasia in white pulp and red pulp districts. 200×. **(C)** Lung (case 17). Mild, multifocal circulating and infiltrating CD3$^+$ lymphocytes in alveolar walls and interstitium. 200×. Inset: lung (case 25). Marked CD3$^+$ lymphocyte bronchiolar hyperplasia. 100×. **(D)** Cerebrum (case 24). Multiple Virchow-Robin spaces are expanded by CD3$^+$ lymphocytes, which also infiltrate the adjacent parenchyma. 40×. Inset: cerebrum (case 24). Detail of abundant CD3$^+$ lymphocytes perivascular and neuroparenchymal infiltration. 400×.

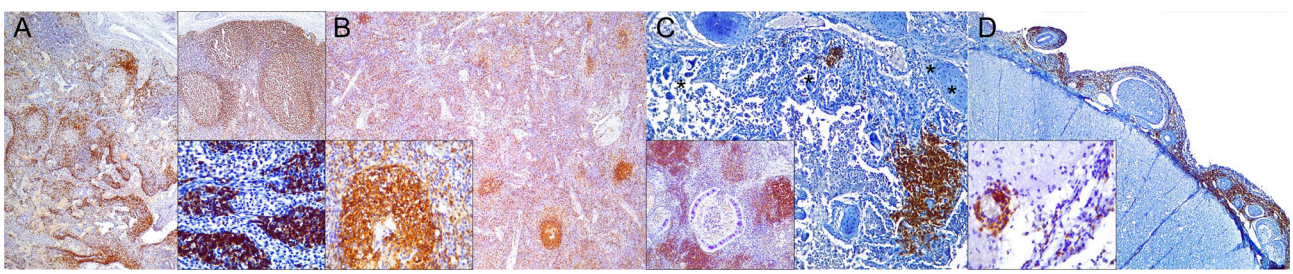

FIGURE 3 | Cluster of differentiation (CD)-20 immunohistochemical findings. **(A)** Lymph node (case 18). Moderate depletion of CD20 lymphocytes in cortical areas. 40×. Upper inset: lymph node (case 22). CD20$^+$ lymphocyte hyperplasia. 40×. Lower inset: lymph node (case 18). CD20$^+$ lymphocyte hyperplasia in medullary cords. 400×. **(B)** Spleen (case 21). Mild to moderate multifocal CD20-expressing lymphocyte depletion in white pulp. 40×. Inset: spleen (case 21). Detail of relatively normal CD20$^+$ lymphocyte number in follicle. **(C)** Lung (case 13). Multifocal parabronchiolar, interstitial/alveolar septa CD20$^+$ lymphocytic infiltrates. 100×. Inset: lung (case 25). Marked, multifocal peribronchiolar CD20$^+$ lymphocytic infiltrates. 100×. **(D)** Spinal cord (case 13). Marked CD20$^+$ leptomeningeal infiltration. 40×. Inset: cerebrum (case 26). Subpial CD20 perivascular and leptomeningeal CD20$^+$ infiltrates. 400×.

in CeMV-infected dolphins. Iba1+ histiocytes were seen circulating as well as infiltrating the bronchial/bronchiolar mucosa/submucosa, BALT, interstitium, and exocytosing and filling the alveolar lumina (**Figure 6C**). CD68+ (**Figure 7C**) and lysozyme+ (**Figure 8C**) histiocytes paralleled Iba-expressing cells yet they were often less numerous. Histiocytes and lysozyme+ neutrophils were overall more abundant in parasitic inflammatory foci. Hyperplastic (type II) pneumocytes and alveolar exudates expressed lysozyme in many animals, including parasitic and fungal pneumonia cases. CD57+ MNCs were

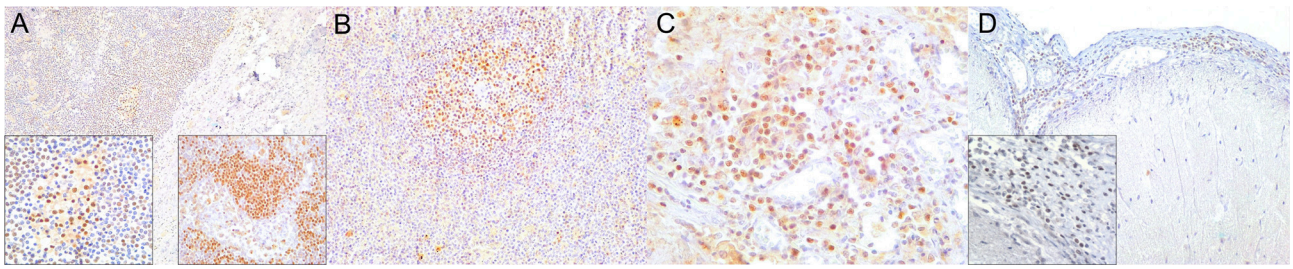

FIGURE 4 | Paired box protein **(**PAX)-5 immunohistochemical findings. **(A)** Lymph node (case 17). Mild to moderate PAX5$^+$ lymphocyte depletion in cortical area. 100×. Left inset: lymph node (case 17). Detail of mild PAX5$^+$ lymphocyte depletion in follicle. 400×. Right inset: lymph node (case 18). PAX5$^+$ lymphocyte hyperplasia in medullary cord. 400×. **(B)** Spleen (case 17). Mild PAX5$^+$ lymphocyte follicular depletion. 200×. **(C)** Lung (case 18). Increased numbers of PAX5$^+$ lymphocytes in lung interstitium. 400×. **(D)** Cerebrum (case 13). Moderate infiltration of PAX5 lymphocytes in leptomeninges. 200×. Inset: cerebrum (case 13). Detail of leptomeningeal infiltrating PAX5$^+$ lymphocytes. 400×.

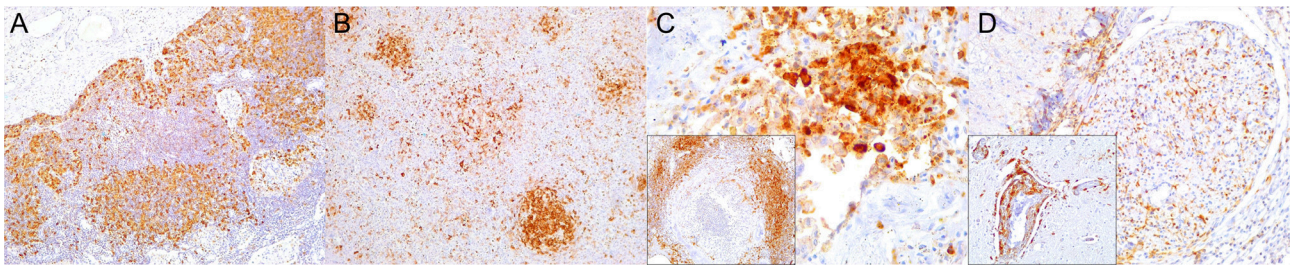

FIGURE 5 | Major histocompatibility (MHC) class II immunohistochemical findings. **(A)** Lymph node (case 12). MHCII expression in cortical cell elements mainly B-cell areas and sinuses. 100×. **(B)** Spleen (case 2). MHCII expression mostly in white pulp cell elements and red pulp. 100×. **(C)** Lung (case 9). Increased expression of MHCII in inflamed alveolus and exudative cells including reactive and binucleated histiocytes and syncytia. 400×. Inset: lung (case 12). Abundant MHCII-expressing inflammatory cells in inflamed distal bronchus. 100×. **(D)** Spinal cord (case 13). Overexpression of MHCII in spinal nerve root cell elements and infiltrating inflammatory cells. 200×. Inset: cerebrum (case 18). Overexpression of MHCII in perivascular inflammatory cells and vascular and neuroparenchymal cell elements. 200×.

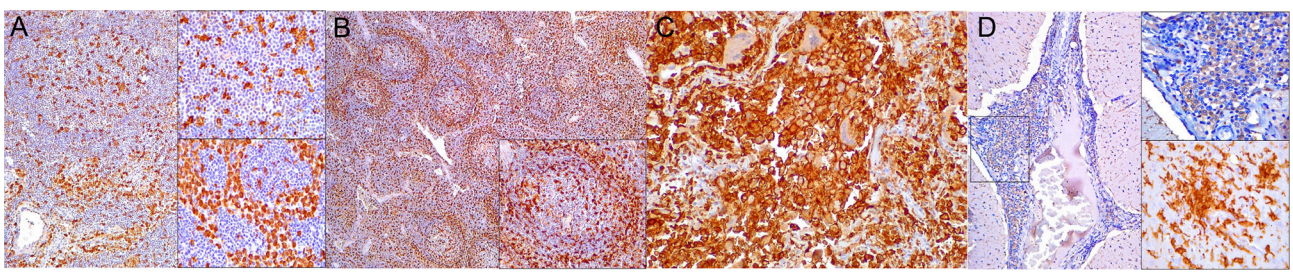

FIGURE 6 | Ionized calcium binding adaptor molecule (Iba)-1 immunohistochemical findings. **(A)** Lymph node (case 17). Small to moderate amounts of Iba1-expressing histiocytes (including intra- and interfollicular dendritic cells, and sinus histiocytes) in cortex/paracortex interface and medullary cords. 100×. Upper inset: lymph node (case 17). Detail of Iba1$^+$ follicular dendritic cells. 400×. Lower inset: lymph node (case 18). Detail of Iba1-expressing sinus histiocytes and hyperplastic medullary cords. 400×. **(B)** Spleen (case 15). Abundant expression of Iba1$^+$ in histiocytic populations in red and white pulp. 100×. Inset: spleen (case 15). Detail of Iba1-expressing histiocytic cells in follicle and perifollicular zone. 400×. **(C)** Lung (case 19). Striking alveolar Iba1$^+$ histiocytic inflammatory response including reactive, binucleated, and multinucleate macrophages/syncytia. 400×. **(D)** Cerebellum (case 25). Marked infiltration of Iba1$^+$ histiocytes in interfoliar leptomeninges. 100×. Upper inset: cerebellum (case 25). Detail of Iba$^+$ meningeal inflammatory infiltrate. 400×. Lower inset: prominent perivascular histiocytic infiltration including macrophages and microgliosis. 400×.

rare in the lung; no differences were noted compared to control animals. IgG+ lymphocytes were increased in CeMV-infected dolphins and were detected mainly in BALT (**Figure 9C**) and to a lesser extent in alveolar septa and interstitium; they were especially prominent in parasitic LIR. Intra-alveolar syncytia were generally Iba1+. Rare FoxP3+ lymphocytes were seen in lung of some CeMV-infected dolphins; by contrast, no FoxP3-expressing lymphocytes were seen in control dolphins.

All "control" dolphins had scattered MNCs immunoreactive and multifocal extracellular signal for IFNγ, IL4, IL10, and TGFβ in lung tissue. However, even though these cytokines appeared

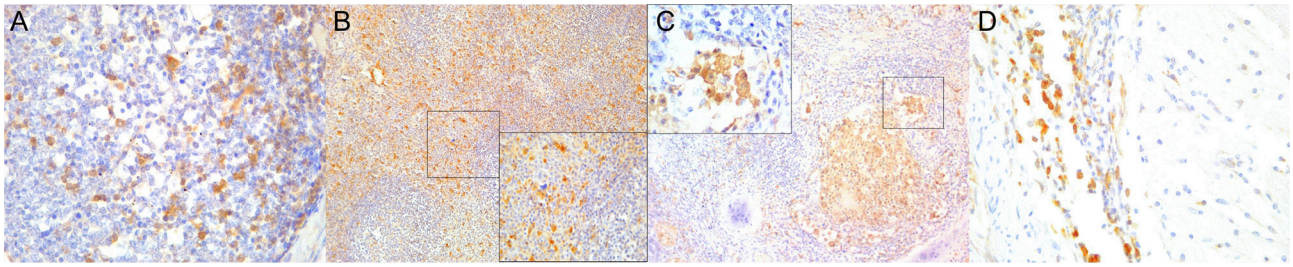

FIGURE 7 | Cluster of differentiation (CD)-68 immunohistochemical findings. **(A)** Lymph node (case 25). Detail of $CD68^+$ follicular and interfollicular dendritic (histiocytes) cells. 400×. **(B)** Spleen (case 25). Abundant sinus histiocytic cells expressing CD68. 100×. Inset: spleen (case 25). Detail of perifollicular $CD68^+$ histiocytic cells. 400×. **(C)** Lung (case 25). Abundant intrabronchiolar and intraalveolar histiocytes express CD68. Inset: lung (case 25). Detail of intraalveolar histiocytes expressing CD68. 400×. **(D)** Cerebellum (case 25). Detail of $CD68^+$ histiocytes infiltrating the leptomeninges. 400×.

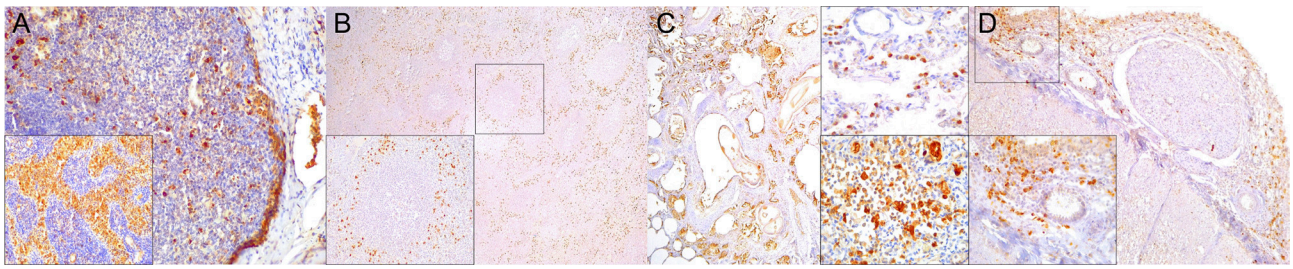

FIGURE 8 | Lysozyme immunohistochemical findings. **(A)** Lymph node (case 25). $Lysozyme^+$ histiocytic cells in cortical and paracortical area. 200×. Inset: lymph node (case 25). Abundant intrasinus lysozyme-expressing histiocytes. 200×. **(B)** Spleen (case 14). $Lysozyme^+$ histiocytes through the red pulp. 40×. Inset: spleen (case 14). Detail of lysozyme histiocytes in red pulp. 400×. **(C)** Lung (case 11). Overexpression of lysozyme by inflammatory cells and exudate in inflamed bronchioles and alveoli associated with CeMV and concomitant verminous pneumonia. 40×. Upper inset: lung (case 13). Circulating MNCs (including presumed pulmonary intravascular macrophages) are $lysozyme^+$. 400×. Lower inset: lung (case 21). Increased lysozyme+ (mainly histiocytes) cells within inflammatory focus. 400×. **(D)** Spinal cord (case 13). Moderate number of lysozyme+ histiocytes in spinal cord leptomeninges. 100×. Inset: spinal cord (case 13). Detail of lysozyme+ histiocytic meningeal infiltrate. 400×.

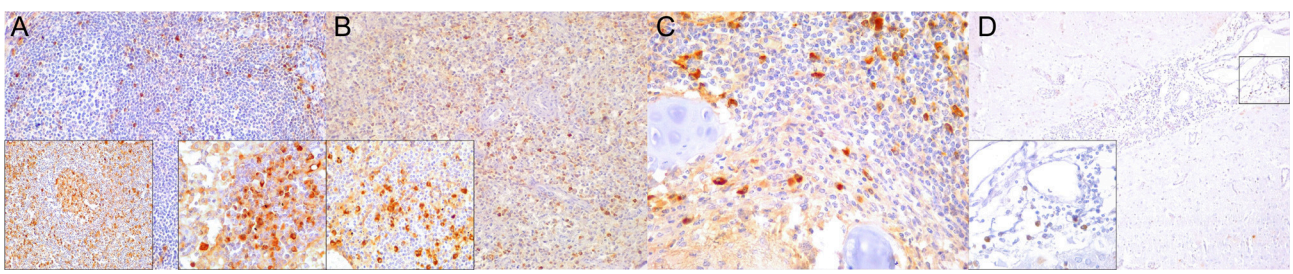

FIGURE 9 | IgG immunohistochemical findings. **(A)** Lymph node (case 18). Scattered IgG^+ cells in follicular and parafollicular areas. 200×. Left inset: lymph node (case 15). Increased IgG-expressing cells in follicular and parafollicular areas. 200×. Right inset: lymph node (case 12). IgG^+ cell hyperplasia in medullary cord. 400×. **(B)** Spleen (case 13). Scattered IgG+ cells at white/red pulp interface. 200×. Inset: spleen (case 17). Increased IgG^+ cells in white/red pulp interface. 400×. **(C)** Lung (case 12). IgG^+ cells in hyperplastic BALT of inflamed bronchiole. 400×. **(D)** Cerebrum (case 14). Scattered IgG^+ cells in leptomeninges. 400×. Inset: cerebrum (case 14). Detail of IgG^+ cells in meningeal infiltrates. 400×.

expressed by a slightly greater number of MNCs in lung of infected dolphins they represented <10% of the inflammatory cells (including BALT and interstitium); thus, no IHC evident differences were noted based on the proposed semiquantitative approach. IFNγ was mainly expressed by exudating MNCs including alveolar macrophages and syncytia (**Figure 10C**), and fewer infiltrating macrophages and lymphocytes, and occasional epithelial cells with an extracellular signal in inflammatory foci. Both, IL4 and IL10 were expressed by infiltrating and circulating MNCs compatible with histiocytes and lymphocytes, and extracellularly. TGFβ+ cells were less numerous and typically involved alveolar macrophages, bronchial/bronchiolar epithelia, exocytosing MNCs, and occasional mesenchymal cells.

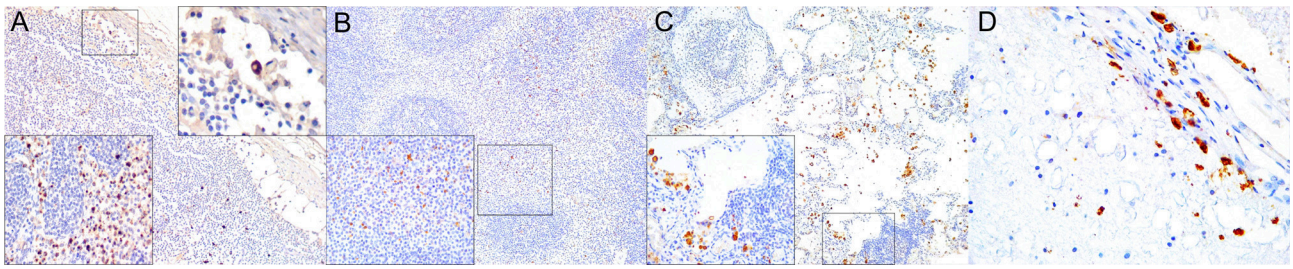

FIGURE 10 | Interferon (IFN)-γ immunohistochemical findings. **(A)** Lymph node (case 17). Scattered MNCs including lymphocytes and histiocytes express IFNγ in cortex, paracortex and subcapsular/marginal sinus (square). Right inset: lymph node (case 17). IFNγ-expressing histiocyte within subcapsular/marginal sinus. 400×. Left inset: lymph node (case 17). Moderate number of IFNγ-expressing histiocytes and lymphocytes within paracortical/medullary sinuses. **(B)** Spleen (case 22). Scattered IFNγ-expressing cells in white and red pulp. Detail of IFNγ-expressing cells. 100×. Inset: 22. 400×. **(C)** Lung (case 23). IFNγ-expressing cells mainly include alveolar macrophages and scattered interstitial mononuclear cells. 100×. Inset: lung (case 23). Detail of IFNγ-expressing cells within perialveolar lymphocytic aggregate and rare alveolar macrophage. 400×. (D) Cerebrum (case 9). Meningeal and neuroparenchymal IFNγ-expressing cells. 400×.

Central Nervous System

IHC results are recorded in **Table 5**. CAS3+ MNCs were seen in meningeal, parenchymal, Virchow-Robin spaces (VRSs), and neuroparenchymal inflammatory infiltrates as well as in circulating MNCs (**Figure 1D**). In areas of marked neurodegeneration, neurons and neuroglia occasionally expressed CAS3. In decreasing order, T cells (**Figure 2D**) and B cells (**Figures 3D**, **4D**) predominated in meningeal, VRSs, and neuroparenchymal inflammatory infiltrates in striped dolphins and bottlenose dolphins with varying degrees of CNS inflammation. B-cells were comparatively more numerous in case 13, a case with a suspect *Brucella* co-infection based on cellular inflammatory components and neuroanatomical distribution of lesions. Rare T cells were seen circulating and/or in meningeal perivascular spaces in three Guiana dolphins, likely representing early CNS inflammation. CAS3+ cells appeared to involve mostly T and B cells. MHCII was widely overexpressed, involving MNCs either circulating or expanding the VRS and/or infiltrating the neuroparenchyma, along with vascular endothelial cells (**Figure 5D**). Occasionally, neuroglia (microglia and astrocytes) expressed MHCII. Few animals additionally exhibited MHCII labeling in roots of spinal nerves and meningeal mesenchyme. Inflamed CNS tissues often harbored Iba1+ histiocytes, either circulating or located within the vessel walls, expanding the VRSs and infiltrating the neuroparenchyma (**Figure 6D**). Microglia was consistently labeled by Iba1. In few cases, degenerating neurons expressed Iba1+. Lysozyme+ MNCs were rare in the CNS, except for cases 13 and 25 (**Figure 7D**). CD68+ MNCs were rarely seen in CNS inflammatory foci (**Figure 8D**). CD57+ cells were not detected in CNS tissues examined. Small numbers of IgG+ lymphocytes were common in meningeal inflammatory infiltrates and VRS (**Figure 9D**) of striped dolphins. Case 13, a bottlenose dolphin with a suspect coinfection by *Brucella* sp. had greater numbers of IgG-expressing cells and histiocytes. Only cases 13 and 14 showed rare FoxP3+ cells intermingled with perivascular inflammatory infiltrates in CNS tissue sections examined.

Brain tissue from "control" dolphins had no immunoreactivity to any of the cytokines evaluated (IFNγ, IL4, IL10, TGFβ); however, CeMV-infected striped dolphins and bottlenose dolphins had consistent IFNγ (**Figure 10D**) and occasional IL4 immunoexpression in CNS tissue sections examined. Overall, there was a more consistent number of IFNγ+ cells in all these cases, but scattered IL4+ cells could be also observed. IFNγ was mainly expressed by MNCs and neuroparenchymal elements. IL4 was expressed by scattered circulating MNCs. Five striped dolphins and one bottlenose dolphin had detectable TGFβ and/or IL10 immunoexpression. The latter involved MNCs in inflamed choroid plexus of fourth ventricle (case 25).

DISCUSSION

Comparative analyses of LIRs are of paramount relevance as they help unraveling local immunopathogenetic mechanisms of disease processes. While in humans and in given domestic and livestock animal species, as well as in laboratory animals there is a considerable body of knowledge, the immunopathogenetic bases of disease including LIRs in cetaceans remain largely unknown (11). We have herein characterized, by means of a set of lymphocytic, histiocytic, antigen-presenting cell-, cell death-related and cytokine immunomarkers, LIRs among CeMV-infected dolphins from the Western Mediterranean as well as from the Northeast-Central and the Southwestern Atlantic, with special emphasis on their lymphoid, pulmonary, and CNS tissues. There are no previous data on the LIRs in cetaceans infected by CeMV, except for a previous study focused on PBLs in a set of free-ranging bottlenose dolphins with subclinical infection (11). In that study, dolphins with subclinical CeMV infection had elevated lysozyme concentrations, marginally significant increases in monocytic phagocytosis, reduced mitogen-induced T lymphocyte proliferation responses, and marginally significant CD4+ decreases in PBLs compared to seronegative dolphins (11). We will discuss our results tissue by tissue and correlate them with the tissue-specific immunopathology and immunopathogenesis of MeV (6) and CDV infections (5) whenever appropriate. This IHC-based approach was coupled with inferred CeMV disease presentation forms for all animals included in this study (data published elsewhere).

TABLE 4 | Summary of results for selected immunomarkers in lung of Guiana dolphins (*Sotalia guianensis*), striped dolphins (*Stenella coeruleoalba*), and bottlenose dolphins (*Tursiops truncatus*) included in this study.

No	CAS3	CD3	CD20	CD57	CD68	FoxP3	HLA-DRα	Iba1	IFNγ	IgG	IL4	IL10	Lysozyme	TGFβ	PAX-5
1	+/2	+/2	+/3	–/0	+/2	–/0	+/3	+/3	+/2	+,++/3	+/3	+/2	+/3	+/2	+/2
2	+/2	+/3	+/3	+/3	+/3	–/0	+/3	+/3	+/3	+,++/3	+/3	+/2	+/3	+/2	+/2
3[a]	++/2	+/3	+/3	–/0	++/2	+/2	+,++/3	+/3	+,++/2	+,++/3	+/3	+,++/2	++,+ + +/3	+/2	+/2
4	+/2	+/3	+/3	–/0	++/2	–/0	+/3	+/3	++/3	+/3	+/3	+/2	+/3	+/2	+/2
5	+/3	+/3	+/3	–/0	+/2	+/2	+/3	++/3	+/3	+/2	+/2	+,++/2	++/3	+/2	+/3
6	+/2	+/2	+/3	+/3	+/3	–/0	+/3	+/2	+/2	+/3	+/2	+/2	+/3	+/2	+/2
7	+/2	+/3	+/3	+/2	+/3	+/2	+/3	+/2	+/3	+/2	+/2	+/2	+/3	+/2	+/2
8	+/2	+/3	+/3	–/0	+/3	+/3	+/2	+/3	+/3	+,++/3	+/2	+/2	+,++/3	+/2	+/2
9	++/3	++,+ + +/3	+/3	–/0	+/3	+/3	++/3	++/3	+/2	+,++/3	+,++/2	+,++/2	+/3	+/2	+/2
10	++/2	+/3	+/3	–/0	+/3	+/3	+/3	+/3	+/2	+/3	+/2	+/2	++/3	+/2	+/2
11[b]	++/2	+/2	+/3	–/0	+/2	+/2	+/3	++/3	+/3	+/3	+,++/2	++,+ + +/3	++,+ + +/3	+/2	+/2
12	+/3	+/3	+,++/3	+/3	++/3	+/3	+,+ + +/3	++/3	+/3	++/3	+/3	+,++/3	+/3	+/3	+/3
13[c]	++/3	+/2	+,++/3	–/0	++/3	+/2	+,++/3	++/3	+/3	+/3	+/3	++,+ + +/3	++,+ + +/3		++/2
14	++/3	+/2	+/2	–/0	+/3	+/2	+/3	+,++/3	+/3	+/2	+/2	+/3	+/3	+/2	+/2
15	+/3	+/3	+,++/3	+/3	+/3	+/3	++/3	+,++/3	+,++/3	+,++/3	+/3	++,+ + +/3	++/2	+/2	+/3
16	++/3	++/3	+/3	–/0	+/3	+/2	+/3	+,++/3	+/3	+/2	+/2	+/2	+/3	+/2	+/3
17	++/3	++/3	++/3	+/2	+/3	+/3	+,++/3	+,++/3	+,++/3	+/3	+/3	+,++/2	+,++/2	+/3	+/2
18	++/3	++/3	++/3	+/3	++/3	+/3	++,+ + +/3	++/3	+/3	++/3	+/2	+,++/3	++/3	+/2	+,++/3
19	+ + +/3	++/3	+,++/2	+/2	+/2	+/3	+,++/3	+ + ++/3	+ + +/3	++/3	+,++/3	++,+ + +/2	+,++/3	+/3	+/2
20	+/3	+/2	+/3	–/0	+/2	+/3	+/3	+,++/3	+/3	+/3	+/3	+/3	+,++/3	–/0	+/2
21	+/3	+/3	+/3	+/2	+/2	+/2	+/3	+/3	+/3	+/3	+/2	+/3	+,++/3	+/2	+/2
22	++/3	+,++/3	+/3	+/2	+/2	+/2	+,+ + +/3	+/3	+/2	+/2	+,++/2	+/2	+/3	+/2	+/3
23	+/2	+/3	+/3	+/2	+/3	+/2	+,++/3	+/3	+,++/3	+/3	+/2	+/3	+/3	+/2	+/2
24	+/3	+/3	+/3	–/0	+/3	+/2	+/3	+/3	+/2	+/3	+/3	+/3	+/3	+/2	+/2
25[d]	++/3	+ + ++/3	++,+ + +/3	+/3	++,+ + +/3	+/3	+,++/3	+/3	+/3	+,++/3	++,+ + +/2	+/3	++,+ + +/3	+/2	+,++/3
26	+/3	+/3	+/3	NE	NE	NE	NE	+/3	+/3	+/3	NE	NE	NE	NE	NE
27[e]	+/3	+/2	+/3	–/0	+,++/3	+/2	+/2	+/2	+/2	+/3	+/2	+/2	+,++/3	+/2	+/2
28	+/3	+/3	+/3	–/0	–/0	–/0	+/3	+/3	–/0	–/0	+/2	+/2	–/0	–/0	+/3
29	+/3	+/3	+/3	–/0	–/0	–/0	+/3	+/3	+/3	+/2	+/2	+,++/2	–/0	+/3	+/3
30	+/3	+/3	+/3	–/0	–/0	–/0	+/3	+/3	–/0	+/2	+/2	+/2	+/2	+/2	+/3

CD, cluster of differentiation; FoxP3, Forkhead Box P3; HLA, human leukocyte antigen (synonym major histocompatibility complex); IFN, interferon; Ig, immunoglobulin; IL, interleukin; TGF, transforming growth factor; Pax-5, paired box 5; NE, not evaluated. Semiquantitative analysis of immunopositive cells: –, no; +, <10%; ++, 10–50%; + + +, 50–90%, and + + ++, >90% immunopositive cells. Subjective labeling intensity score of immunopositive cells: 0, 1, 2, and 3.

[a] *Multisystemic hyphate mycosis (lung, kidney).*

[b] *Hyphate mycosis (lung).*

[c] *Suspect Brucella coinfection (primarily CNS lesions).*

[d] *Sepsis by Staphylococcus aureus (particularly severe CNS and lung lesions).*

[e] *Multisystemic hyphate mycosis (lung, cerebrum).*

TABLE 5 | Summary of results for selected immunomarkers in cerebrum, cerebellum, brain stem, and spinal cord of Guiana dolphins (*Sotalia guianensis*), striped dolphins (*Stenella coeruleoalba*), and bottlenose dolphins (*Tursiops truncatus*) included in this study.

No	CAS3	CD3	CD20	CD57	CD68	FoxP3	HLA-DRα	Iba1	IFNγ	IgG	IL4	IL10	Lysozyme	TGFβ	PAX-5
1	+/2	–/0	–/0	–/0	–/0	–/0	+/3	–/0	+/3	–/0	–/0	–/0	+/3	–/0	–/0
2	+/2	–/0	–/0	–/0	–/0	–/0	+/3	+/2	–/0	–/0	–/0	–/0	+/3	–/0	–/0
3[a]	+/3	–/0	–/0	–/0	–/0	–/0	+/3	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0
4	–/0	–/0	–/0	–/0	–/0	–/0	+/3	+/2	–/0	–/0	+/2	–/0	–/0	–/0	–/0
5	–/0	–/0	–/0	–/0	–/0	–/0	+/3	–/0	–/0	–/0	–/0	–/0	+/3	–/0	–/0
6	+/2	–/0	–/0	–/0	+/2	–/0	+/3	+/3	–/0	–/0	–/0	–/0	+/3	–/0	–/0
7	+/3	+/3	–/0	–/0	–/0	–/0	+/3	–/0	+/3	–/0	+/2	–/0	+/2	–/0	–/0
8	+/3	–/0	–/0	–/0	–/0	–/0	+/3	–/0	–/0	–/0	–/0	–/0	+/3	–/0	–/0
9	+/2	+/3	–/0	–/0	–/0	–/0	+/3	+/3	+/3	+/2	–/0	–/0	+/3	–/0	–/0
10	+/3	+/3	–/0	–/0	+/2	–/0	+/3	+/3	+/3	–/0	+/2	–/0	+/3	–/0	–/0
11[b]	+/2	–/0	–/0	–/0	+/2	–/0	+/3	+/3	–/0	–/0	–/0	–/0	+/3	–/0	–/0
12	+/3	++/2	+/2	–/0	+/2	–/0	+/2	+/3	+/3	+/3	–/0	+/2	+/3	–/0	+/2
13[c]	+/3	++/2	++,+ + +/3	+/3	+/2	+/3	+ + +/3	++,+ + +/3	++/3	++/3	+/2	–/0	+,++/3	–/0	++/3
14	+/3	++/2	+,++/2	–/0	+/2	+/2	++/3	+/3	+/3	+/3	+/2	+/3	+/3	–/0	+/2
15	+/2	+/3	+/3	–/0	+/2	–/0	++/3	+/3	+/3	+/3	+/2	–/0	+/2	–/0	+/2
16	+/2	++/2	+/2	–/0	+/2	–/0	++/3	+/3	+/2	+/2	+/2	–/0	+/3	–/0	+/2
17	+/2	++/3	+/2	–/0	+/2	–/0	++/3	+/3	+/3	+/3	+/3	–/0	+/3	–/0	+/2
18	+/2	++/3	+,++/3	–/0	+/2	–/0	++/3	+,++/3	+/2	+/3	+/3	–/0	+/3	–/0	+/2
19	++/3	++/3	+,++/3	–/0	+/2	–/0	+ + +/3	++/3	+/3	+/2	+/3	+/2	+/3	–/0	+/2
20	+/2	+/2	+/2	–/0	–/0	–/0	+/3	+/2	+/2	+/2	+/3	–/0	+/2	–/0	–/0
21	+/2	+/3	+/2	–/0	+/3	–/0	+/2	+/3	+,++/3	–/0	+/2	–/0	+/3	–/0	–/0
22	+/2	+/3	+/3	–/0	+/2	–/0	+/2	+/3	+/3	+/3	–/0	–/0	+/2	+/2	+/2
23	+/3	+/3	+/3	–/0	+/2	–/0	+/3	+/2	+/3	+/2	+/2	–/0	+/3	–/0	–/0
24	+,++/3	+ + +/3	+/3	–/0	+/3	–/0	+,+ + +/3	+/2	+/3	+/2	+/2	–/0	+/3	–/0	+/2
25[d]	+,++/2	+,+ + +/3	++/3	+/3	+/3	–/0	++/3	+,+ + +/3	+/3	+,++/3	+/2	+/3	+,+ + +/3	+/3	+/2
26	++/3	+ + +/3	+/3	+/3	+/2	–/0	+,++/3	++/3	+,++/2	+/3	+/2	–/0	+/3	–/0	+/2
27[e]	++/3	+/3	+/2	–/0	+/2	–/0	+/3	+/2	–/0	+/3	+/3	–/0	+,++/3	–/0	–/0
28	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0
29	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0
30	+/3	–/0	–/0	–/0	–/0	–/0	+/2	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0

CD, cluster of differentiation; FoxP3, Forkhead Box P3; HLA, human leukocyte antigen (synonym major histocompatibility complex); IFN, interferon; Ig, immunoglobulin; IL, interleukin; TGF, transforming growth factor; Pax-5, paired box 5; NE, not evaluated. Semiquantitative analysis of immunopositive cells: –, no; +, <10%; ++, 10–50%; + + +, 50–90%, and + + + +, >90% immunopositive cells. Subjective labeling intensity score of immunopositive cells: 0, 1, 2, and 3.

[a] *Multisystemic hyphate mycosis (lung, kidney).*

[b] *Hyphate mycosis (lung).*

[c] *Suspect Brucella coinfection (primarily CNS lesions).*

[d] *Sepsis by Staphylococcus aureus (particularly severe CNS and lung lesions).*

[e] *Multisystemic hyphate mycosis (lung, cerebrum).*

Humoral and cellular immune responses to MeV (6, 19) and CDV (20–23) are crucial for viral clearance and recovery, as well as for the establishment of a "lifelong" protective immunity. However, they are also the pathological basis of measles and distemper morbidity and mortality (5, 9). In both, early immunosuppression is associated with viremia and lysis of lymphocytes and macrophages (6, 24). B-cells, follicular dendritic cells, and T-cells, especially CD4+, and CD8+ cells, are initially targeted by MeV and CDV resulting in generalized lymphoid depletion in lymph nodes and spleen, mucosa-associated lymphoid tissue (MALT), and tonsils (5, 6). Hyperplasia of reticular cells (sinus histiocytosis) in the medullary region of lymph nodes typically accompanies this early phase of infection (24, 25). In this study, we observed consistently decreased numbers of T cells and B cells in Guiana dolphins, striped dolphins, and bottlenose dolphins; however, lymphoid cell depletion appeared to be more severe in Guiana dolphins and in acute/subacute cases from the Canary Islands (cases 13, 14, and 19) and Mediterranean Sea (cases 21, 25, 26, and 27). Additionally, we observed increased sinus histiocytosis (Iba1-, CD68-, and lysozyme-expressing cells) in these dolphins, recapitulating features observed in acute/subacute measles and distemper (5, 9).

CAS3 is a frequently activated death protease, catalyzing the specific cleavage of many key cellular proteins and playing a major role in apoptosis (26). In MeV and CDV infections, upregulation/overexpression of CAS3 is commonly observed in infected lymphocytes and uninfected lymphocytes, suggesting the existence of virus-dependent, and virus-independent mechanisms of apoptosis (27–29). We detected an overall increased CAS3 expression mainly in lymphocytes and histiocytes of cortex and paracortex, but to a lesser extent in histiocytes of subcortical sinuses and medullary cords in lymph nodes. These findings suggest apoptosis is a major cell death mechanism (30) also in the time course of CeMV infection, as it is in measles and distemper (27, 31, 32). Although in these cases viral-induced apoptosis may be the major triggering factor (27, 31, 33), the pathogenetic intricacies of such phenomenon, including viral-independent apoptosis pathways remain undetermined in CeMV infection. Altogether, the above findings concerning lymphocytic and histiocytic disarrangements and generalized immunoexpression of CAS3 in CeMV-infected dolphins provide compelling morphological and IHC evidence of a compromised immune response capacity in these animals.

If the host survives, incipient MeV and CDV infections are generally followed by regeneration of lymphoid organs (5, 6). Repopulation and germinal center formation in lymphoid tissues from persistently infected and convalescent hosts are common in this stage (5, 6). Detection of such "persistently infected" and/or "convalescent" dolphins is complicated; however, we observed similar regenerative findings in several cases from the Canary Islands and the Mediterranean Sea largely involving those animals with chronic systemic and chronic localized "brain only forms" of CeMV infection [supported by IHC and PCR results [data published elsewhere]] (1, 14). These changes were characterized by follicular, paracortical, and medullary cord expansion (reactive hyperplasia), typically encompassing distorted or poorly delineated lymphocytic growths including T cells and B cells accompanied by slightly increased numbers of IgG+ cells. Thus, we detected IHC evidence of a somewhat similar immune response progression in CeMV-infected dolphins compared to MeV- and CDV-infected individuals (5, 6). Nonetheless, further conclusions are limited by the lack of other means of infection's chronology surrogates, including serological profiling. Future studies addressing these limiting factors would allow more accurate comparisons with TMVs. Interestingly, morphologically intact appearing compartments after lymphoid repopulation and virus clearance from lymphoid tissues does not necessarily result in complete functional regeneration of the immune response (22, 34, 35). This may also apply to CeMV infection, so that apparently "normal" lymphocytic density and distribution patterns in lymphoid districts from CeMV-infected cetaceans may not necessarily be associated with appropriate immunological fitness. In chronic measles and distemper, often this multicentric repopulation is accompanied by CD4- and CD8-dominated inflammatory responses in the CNS (22, 36, 37). This remains to be evaluated in cetaceans.

Mammals differ in their expression of MHCII molecules (38). In this study, we observed MHCII expression was largely confined to MNCs consistent with B-lymphocytes, dendritic cells, and macrophages (especially sinus histiocytes) which are considered professional antigen-presenting cells (38). Also, there were small numbers of MHCII-expressing MNCs in the paracortex and subcortical medullary cords, diminishing centripetally. This distribution pattern is consistent with reports in other species, including rodents (38); however, more accurate inferences may be obtained by double immunolabeling analyses. We suspect certain T-cell lymphocytes could also express MHCII in CeMV-infected animals; however, greater T cell numbers, including resting ones, as reported in pigs, dogs, cats, mink, and horses (38), were not a feature in CeMV-infected or "control" animals. No significant alterations in MHCII immunoexpression were observed between infected and control animals on the basis of semiquantitative comparative analysis.

Comparatively, fewer studies have addressed in detail the immune disarrangements in the spleen of MeV and CDV cases (5, 6). Typically, there is a generalized depletion of T and B cell compartments in the spleen (5, 22). In our study, most findings observed in lymph nodes were paralleled to some extent by those detected in the spleen, with divergences dependent upon T-cell and B-cell topographic locations. There was an overall decreased number of T cells and B cells in PALS and follicles, respectively, along with an increased CAS3 immunoreactivity and increased number of histiocytes. These findings are in agreement with observations in MeV and CDV infections (5, 6, 22). IgG-expressing cells were slightly increased, as reported in measles (39).

There is relatively limited knowledge on cytokine networks and interplay in LIR in TMV infections, especially concerning IHC investigations. In cetaceans, there is a complete lack of information on "*in situ*" cytokine expression. Th1 responses evoke cell-mediated immunity and phagocyte-dependent inflammation. Th2 cells evoke strong antibody responses

and eosinophil accumulation, but inhibit several functions of phagocytic cells (phagocyte-independent inflammation). Furthermore, Th1/Th2 balance can be evaluated by the *ratio* of their polarizing cytokines (i.e., IFNγ/IL4), and animals with imbalanced Th1/Th2 response may be more susceptible to certain kinds of infections. Since cytokine imbalance is implicated in the pathogenesis and outcomes of MeV and CDV infections, we aimed at evaluating, for the first time, Th1 and Th2 *in situ* cytokine immunoexpression and potential associations with CeMV-AD. We employed a set of commercially available non-cetacean-specific but cross-reactive proinflammatory cytokines to evaluate Th1 cells (IFNγ-secreting) and Th2 cells (IL4-, IL10-secreting) (13). In distemper, a lack of detectable cytokine expression in peripheral blood leukocytes is associated with a high viral load and viremia, indicating that an overwhelming virus infection may suppress cytokine production in lymphoid cells (40, 41). Plasma IFNγ levels (consistent with a predominant Th1 immune response) are increased during the acute phase of measles, whereas, a subsequent Th2 response promotes the development of protective MeV-specific antibodies and is characterized by high concentrations of IL4, IL10, IL13, and IL17 (9). This shift may promote B cell maturation and contribute to the continued production of antibody-secreting cells (9). In the present study, no IHC-based differences were detected regarding cytokine immunoreactivity in lymph nodes and spleen between infected and uninfected dolphins. Severe cytokine "storms" are often ascribed as to the cause of multiorgan dysfunction and death in infectious diseases. IHC analysis may not be as sensitive and specific as PCR-based mRNA transcript quantification; therefore, ongoing studies are aimed at quantifying cytokines by molecular techniques to better address this issue.

Few studies have evaluated the LIR to MeV infection in lung (42) and presumably no information is available on CDV-associated LIR (5). MeV-infected children may display severe depletion of CD4+, CD20+, CD68+, NK+, and S100+ cells in alveoli and BALT without depletion of CD8+ T-cells (42). Also, there is prominent apoptosis of dendritic, CD4+ and NK cells (42). In our study, comparisons made with "control" dolphins—all of which were regarded as calves—revealed mild to moderate increase in T cells and B cells and histiocytes in lung of CeMV-infected dolphins, which also contrasts with observation in children with measles (42). Further studies involving "age-matched control" dolphins, an extremely difficult setting when dealing with free-ranging cetaceans, may help elucidate potential age-related bias in CeMV-LIRs, including lung tissue. Furthermore, there were increased CAS3+ cells in CeMV-infected dolphins mainly involving inflammatory MNC populations and epithelial cells (27). The number and intensity of CAS3-expressing cells was greater in parasitic inflammatory foci. This novel finding also adds to the limited available knowledge on the immunopathogenesis of verminous pneumonia in dolphins (43). Cases 3, 11, and 27 had concomitant pulmonary hyphate mycosis, which accounted for increased numbers of lymphocytes, macrophages, and exudating neutrophils and necrosis, primarily associated with fungal elements. Fungal coinfections are common and exacerbate pneumonia in measles (44), distemper (45), and CeMV infections (46–48). Case 25 presented a consistently expanded BALT and alveolar septa harboring abundant T cells and B cells. To the authors' knowledge, this inflammatory pattern is unusual in dolphins, yet it recapitulates features of *Mycoplasmataceae* infection ("cuffing pneumonia") in other *Cetartiodactyla* members. The etiopathogenesis of this finding remains unknown. Furthermore, we detected increased expression of MHCII chiefly involving circulating MNCs, including presumed PIVMs, and infiltrating inflammatory MNCs (lymphocytes and histiocytes) and exocytosing/exudating MNCs. This was more prominent in parasitic LIRs. Putative PIVMs appeared to consistently express Iba1, lysozyme and CD68; these results broaden the repertoire of immunomarkers for PIVMs in cetacean species (49). IgG+ lymphocytes were mainly around bronchioles/bronchi (in BALT) and scattered in the interstitium, especially in dolphins with concomitant verminous pneumonia. No evident differences were detected for CD57- and FoxP3-expressing cells in lymphoid organs between infected and uninfected cetaceans.

Scarce studies on measles lung LIR indicated depletion of IL-10+ and IL-12+ cells in infected children; however, there was a greater number of IL-1+, IFN+, and IL-4+ cells (42). Recently, IFNγ-secreting cells were shown to be more abundant early and IL-17+ cells late in lung of rhesus macaques (*Macaca mulatta*) experimentally infected with wild type MeV. Both CD4+ and CD8+ T cells were sources of IFNγ (19). In the present study, we detected some "basal" immunoreactivity for IFNγ, IL4, IL10, and TGFβ in lung tissue of "control" dolphins and even though these cytokines appeared expressed by a slightly greater number of MNCs in lung of infected dolphins we did not find evident differences based on the proposed IHC semiquantitative approach. To better address potential cytokine differences in lung between CeMV-infected and uninfected dolphins we are currently focusing on quantitative molecular cytokine analysis.

The three main MeV-CNS complications include: acute disseminated encephalomyelitis, measles inclusion body encephalitis, and subacute sclerosing panencephalitis (SSPE). CDV-CNS disease may show distinctive manifestations: acute fulminant encephalopathy and encephalitis, post-vaccinal encephalitis, old dog encephalitis, inclusion body polioencephalitis, and demyelinating leukoencephalitis (CDV-DL) (5, 50). Their etiopathogenesis is known to vary and except for SSPE and CDV-DL, their LIR remain largely undetermined (51–53). In this study, we observed varying degrees of CNS inflammation in CeMV-infected dolphins, predominantly in striped dolphins and bottlenose dolphins with DMV. Overall, the LIR in these cases was dominated by (in decreasing order) by T cells, B cells, and histiocytes accompanied by scattered IgG+ plasma cells, regardless of the CeMV-associated presentation. These findings, particularly concerning lymphocytic LIR in CeMV-infected dolphins resemble CNS lymphocytic LIR in measles (53) and distemper (54); however, an in-depth analysis of CD4+ and CD8+ lymphocytes (both subpopulations representing CD3+ cell subtypes) is hampered by the lack of reliable CD4 and CD8 markers applicable in cetacean FFPE tissues (13). Divergences of CeMV-CNS LIR were evident in cases 13 and 25. The former was a suspect case of CeMV and

Brucella coinfection. In this case, a greater number of histiocytes, multifocally characterized by a granulomatous phenotype and B cells were observed. Case 25 had a confirmed septicemia by *Staphylococcus aureus*, with major CNS involvement (16). Immunophenotypic divergences in the latter included greater number of histiocytes, along with the presence of a suppurative exudate compared to the other herein investigated genuine cases of CeMV infection with CNS involvement. CD57- and FoxP3-expressing cells were very rarely detected in CNS tissue sections.

We observed consistently increased numbers of CAS3+ in CNS tissue, mainly involving lymphocytes and histiocytes circulating and infiltrating the meninges, expanding the VRS and infiltrating the neuroparenchyma. Occasionally, neurons and neuroglia expressed CAS3 in areas of marked neurodegeneration. These findings are in agreement with previous observations in MeV- and CDV-neurologic disease (55, 56). MHCII was only expressed in CeMV-infected dolphins, thereby involving a consistent fraction of the aforementioned inflammatory cells and occasionally vascular cells. Neuroglial cells, namely microglia and astrocytes, rarely expressed MHCII. MHCI, a major viral antigen-presenting molecule, awaits development of reliable antibodies for use in FFPE cetacean tissues (13). Overall, these findings are in agreement with previous reports of MeV- and CDV-associated neurological disease (8, 39, 51, 53, 57).

Previous studies suggested there is a cytokine imbalance in SSPE. INFγ and TNFα are overexpressed in endothelial and glial cells from SSPE-affected patients (51). Furthermore, in CDV-DL, there is IHC evidence of increased pro-inflammatory cytokines such as IL1, IL6, IL8, IL12, and TNF in early stages, whereas, IL1, IL6, and IL12 would predominate in advanced diseases stages (58). By contrast, the expression of anti-inflammatory cytokines, e.g., IL-10 and TGF-β appears to be restricted to animals with inactive or chronic disease stages (59). Interestingly, the cerebrospinal fluid of naturally infected dogs may contain detectable levels of TNFα and IL-6 mRNA as well as of IL10 and TGFβ RNA transcripts simultaneously, so that the staging of the disease becomes troublesome (5). In the present study, CeMV-infected striped dolphins and bottlenose dolphins had consistent IFNγ and occasional IL4 immunoexpression in CNS tissue sections examined. Overall, there was greater number of IFNγ+ cells in all these cases, suggesting a Th1-CNS polarization at the time of death, regardless acute or chronic CeMV-AD presentation. By contrast, brain tissue from "control" dolphins appeared immunologically quiescent (60). IFNγ is pivotal in the CNS-MeV infection (61). Deficient CNS-MeV IFNγ responses render individuals highly susceptible (62). The IFNγ immunoreactivity observed in the present study suggests IFNγ plays a role in the infection's neuropathogenesis also in CeMV-infected dolphins. The detection of occasional simultaneous IL4+ cells suggests Th1/Th2 cytokine interplay during the course of CeMV. Finer quantitative methodologies and *in vitro* analyses may allow better assessment of the roles of these two "mutually inhibitory" cytokines in CNS-CeMV infection. The participation of IL10+ and TGFβ+ cells, detected in much smaller numbers in CNS tissue sections, remains unclear. Further studies are necessary to understand the neuroimmunopathogenesis of CeMV infections, with special emphasis on cytokine networks.

To the best of our knowledge, this study represents the first attempt to characterize and compare the LIR in cetaceans infected with CeMV other than in PBLs (11). It would be appropriate to comment on various limiting factors of the present study, some inherent to dealing with carcasses of free-ranging cetaceans and some inherent to laboratory diagnostics. Although we prioritized fresh carcasses, some of the tissues showed mild *post-mortem* autolysis/decomposition *phenomena* that could have negatively interfered with IHC reactions. Additionally, although we used pAbs standardized for their use in cetacean species and we based our judgment(s) of suitable cross-reactivities upon cellular morphologic features and cellular immunostaining patterns similar to human and mouse and other cetacean control tissues (13), future studies involving cetacean-specific antibodies should revisit these findings. A relevant factor in this study concerned the "control" group. In this respect, it is extremely difficult to find "perfectly healthy" or "gold-standard" control animals in natural settings, with special reference to free-ranging cetaceans; however, the application of strict inclusion criteria (targeting at least one individual for each species) retrieved three calves. Based on histologic analysis and IHC results, these animals had developed lymphoid systems and did not show overt variations in comparison to a previous report including a female juvenile and two male calves (13). Thus, no evident age-related bias was readily apparent in these animals and comparisons with CeMV-infected dolphins were deemed to be appropriate.

These results indicate a complex interplay between lymphocytic, histiocytic, antigen-presenting cell-, cell death-related, and cytokine elements in LIRs to DMV and GD-CeMV infections in striped dolphins, bottlenose dolphins, and Guiana dolphins, respectively. We detected consistent CeMV-associated inflammatory response patterns with some similarities and few differences between DMV-infected striped and bottlenose dolphins, and GDCeMV-infected Guiana dolphins. These are summarized as follows. In the lymphoid system (lymph nodes, spleen), (a) there was multicentric lymphoid depletion, characterized by reduced numbers of T cells and B cells in all three species infected by CeMV; however, lymphoid depletion phenomena were more pronounced in Guiana dolphins infected with GDCeMV; (b) striped dolphins and bottlenose dolphins, infected with DMV, often had hyperplastic (regenerative) phenomena involving the aforementioned cell populations, particularly chronically infected animals; (c) there was generalized increased expression of caspase 3 in all three species; and (d) no differences were detected regarding cytokine immunoreactivity. In the lung, (a) there was a mild to moderate increase in T cells, B cells, and histiocytes in all three species; and (b) no differences were detected regarding cytokine immunoreactivity. Concerning the CNS: (a) inflammation was a consistent feature in DMV-infected striped and bottlenose dolphins in contrast to Guiana dolphins infected by GDCeMV; (b) inflammation was dominated by T cells and B cells, accompanied by fewer Iba1+, CD68+, and lysozyme+ histiocytes; (c) there was increased expression

of caspase 3; and (d) no differences were detected regarding cytokine immunoreactivity except for IFNγ in the CNS of infected dolphins of all three species.

In conclusion, these novel results delineate the local immunophenotypic response during CeMV infection in three highly susceptible delphinid species. They also suggest a complex interplay between viral and host's immune factors, thereby contributing to gain valuable insights into similarities and differences of CeMV infection's immunopathogenesis in relation to body tissues, CeMV strains and cetacean hosts. Finally, the herein presented IHC investigation results may help elucidating the immunopathogenetic bases, including the kinetics of LIRs in other infectious and non-infectious disease processes in cetaceans, with major applications in ecotoxicological pathology.

DATA AVAILABILITY

All data for this study are included in the manuscript and/or the supplementary files.

AUTHOR CONTRIBUTIONS

JD-D, KG, and JC-D contributed conception and design of the study. JD-D, KG, ES, SS, ÓQ-C, MA, AF, ES-N, JI, RC, JL-B, LF, CC, SM, LD, GDF, GDG, and JC-D contributed to organization of the databases and/or collected samples for histopathological, immunohistochemical, and molecular analyses. JD-D, KG, RR, IR, CK, NF, and BC contributed to immunohistochemical analyses and laboratorial resources. JD-D, KG, ES, CC, LD, and GDF conducted molecular analyses. JD-D wrote the first draft of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00485/full#supplementary-material

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The Marine Mammal Class II Major Histocompatibility Complex Organization

André Luiz Alves de Sá[1,2]*, *Breanna Breaux*[3], *Tibério Cesar Tortola Burlamaqui*[4], *Thaddeus Charles Deiss*[3], *Leonardo Sena*[5], *Michael Frederick Criscitiello*[3]* and *Maria Paula Cruz Schneider*[2]

[1] Laboratory of Applied Genetics, Socio-Environmental and Water Resources Institute, Federal Rural University of the Amazon, Belém, Brazil, [2] Laboratory of Genomics and Biotechnology, Biological Sciences Institute, Federal University of Pará, Belém, Brazil, [3] Comparative Immunogenetics Laboratory, Department of Veterinary Pathobiology, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX, United States, [4] Center for Technological Innovation, Evandro Chagas Institute, Belém, Brazil, [5] Center of Biodiversity Advanced Studies, Biological Sciences Institute, Federal University of Pará, Belém, Brazil

Sirenians share with cetaceans and pinnipeds several convergent traits selected for the aquatic lifestyle. Living in water poses new challenges not only for locomotion and feeding but also for combating new pathogens, which may render the immune system one of the best tools aquatic mammals have for dealing with aquatic microbial threats. So far, only cetaceans have had their class II Major Histocompatibility Complex (MHC) organization characterized, despite the importance of MHC genes for adaptive immune responses. This study aims to characterize the organization of the marine mammal class II MHC using publicly available genomes. We located class II sequences in the genomes of one sirenian, four pinnipeds and eight cetaceans using NCBI-BLAST and reannotated the sequences using local BLAST search with exon and intron libraries. Scaffolds containing class II sequences were compared using dotplot analysis and introns were used for phylogenetic analysis. The manatee class II region shares overall synteny with other mammals, however most *DR* loci were translocated from the canonical location, past the extended class II region. Detailed analysis of the genomes of closely related taxa revealed that this presumed translocation is shared with all other living afrotherians. Other presumptive chromosome rearrangements in Afrotheria are the deletion of *DQ* loci in Afrosoricida and deletion of *DP* in *E. telfairi*. Pinnipeds share the main features of dog MHC: lack of a functional pair of *DPA/DPB* genes and inverted *DRB* locus between *DQ* and *DO* subregions. All cetaceans share the Cetartiodactyla inversion separating class II genes into two subregions: class IIa, with *DR* and *DQ* genes, and class IIb, with non-classic genes and a *DRB* pseudogene. These results point to three distinct and unheralded class II MHC structures in marine mammals: one canonical organization but lacking *DP* genes in pinnipeds; one bearing an inversion separating IIa and IIb subregions lacking *DP* genes found in cetaceans; and one with a translocation separating the most diverse class II gene from the MHC found in afrotherians and presumptive functional *DR*, *DQ,* and *DP* genes. Future functional research will reveal how these aquatic mammals cope with pathogen pressures with these divergent MHC organizations.

Keywords: molecular evolution, genomics, marine mammals, manatee, MHC, immunogenetics, pinnipeds, cetaceans

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Brian Dixon,
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Reviewed by:
Unni Grimholt,
Norwegian Veterinary Institute, Norway
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Institut National de la Recherche Agronomique (INRA), France
Ben F. Koop,
University of Victoria, Canada

***Correspondence:**
André Luiz Alves de Sá
andre.luiz.sa@hotmail.com
Michael Frederick Criscitiello
mcriscitiello@cvm.tamu.edu

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INTRODUCTION

The transition from terrestrial to aquatic habitat has occurred in several terrestrial vertebrate lineages. In mammals, early after the Cretaceous period, independent ancestral lineages of afrotherian, cetartiodactyl, and carnivore would begin their path to return to aquatic environments which would lead to current sirenian, cetacean, and pinniped species of marine mammals. Those three lineages have further undergone adaptive radiation and their descendants are found in both oceanic and freshwater habitats.

The order *Sirenia* is represented by one species of dugong (*Dugong dugon*) and three species of manatees (*Trichechus manatus*, *T. senegalensis,* and *T. inunguis*), all of them exclusively herbivorous and whose closest living relatives are the elephants. The order Cetacea have approximately 89 species divided into two suborders: Odontoceti (toothed whales) and Mysticeti (baleen whales), and is closely related to hippopotamuses. Pinnipedia comprises a carnivore suborder with three families (Otaridae, Phocidae, and Odobenidae) of around 34 species of aquatic fin-footed mammals (seal, sea-lions, and walrus), closely related to bears and musteloids (e.g., raccoons and skunks), which are still dependent on the land to live, in contrast to sirenians and cetaceans, which are totally adapted to the aquatic environment. Aquatic mammals share several convergent traits selected for fresh water and marine habitats, including morphologic and genetic traits (1–4).

Living in water poses new challenges not only for locomotion and feeding but also for combating new pathogens. How the three major independent aquatic mammal lineages just detailed dealt with the genetic constraints of their ancestry and to what extent their recent history in similar habitats led to convergent evolution in their immune system is not clear. Several marine mammals lack major predators in the adult phase, so infectious disease may be an important cause of mortality (5). This may make the immune system of these aquatic lineages particularly important for their fitness and fecundity. Compared to their terrestrial relatives, marine mammals face distinct diversity of pathogens, disease ecology, and epidemiology (6–8), which may create distinct selective pressures on immune genetic systems, including the Major Histocompatibility Complex (MHC).

The MHC encodes many immune (and many non-immune) genes, canonically divided in class I, II, and III regions in vertebrates. The class II region includes classical (e.g., *DR*, *DQ*, *DP*) MHC, non-classical (e.g., *DO*, *DM*) MHC, antigen processing (e.g., *TAP*, *PSMB*) and other genes. Classical class II alpha and beta genes encode a protein heterodimer that presents antigens for T lymphocytes to detect infections and other danger signals. Classical MHC genes are highly polymorphic, confer resistance or susceptibility to diseases, and may be used as genetic markers for species conservation (9, 10). Several studies have reported the diversity of class II genes in cetaceans (11–19) and pinnipeds (5, 20–25). Past evidence also showed that class II MHC genes may be important genetic markers for survival in a seal species (5). Despite its proposed importance for marine mammals, so far only a representative of cetacean has had their class II MHC organization characterized (26). Therefore, we aimed to compare the genomic organization and evolution of the MHC class II region in sirenians, cetaceans, and pinnipeds, using genome assemblies from representatives of these groups available in public databases. We also included other mammals from different eutherian lineages for a better understanding of the evolution of marine mammals and the eutherian class II MHC region.

MATERIALS AND METHODS

MHC Class II Genes Identification and Reannotation

The marine mammals investigated in this research were: the sirenian Florida manatee (*Trichechus manatus latirostris)*; the cetaceans minke whale (*Balaenoptera acutorostrata scammoni*), sperm whale (*Physeter catodon*), baiji (*Lipotes vexillifer*), beluga whale (*Delphinapterus leucas*), finless porpoise (*Neophocaena asiaeorientalis*), bottlenose dolphin (*Tursiops truncatus*), Pacific white-sided dolphin (*Lagenorhynchus obliquens*), and killer whale (*Orcinus orca*); and the pinnipeds walrus (*Odobenus rosmarus*), Northern fur seal (*Callorhinus ursinus*), Hawaiian monk seal (*Neomonachus schauinslandi*), and Weddell seal (*Leptonychotes wedelli*). We included in the analysis other mammals as outgroups and representatives of other major eutherian branches. A summary of the assembly reports from each analyzed species is provided in **Supplementary File S1**.

Preliminary search on the NCBI database identified annotated MHC class II genes in the genomes of cetaceans, afrotherians, and pinnipeds. All predicted mRNA gene sequences were aligned to their human homologs. We selected presumptive well-annotated classical genes based on the presence of full-length sequences, presence of all exons, and no evidence of pseudogene misidentification (presence of stop codons and lack of homology in any exons). Those predicted genes and their human homologs were used to perform megablast and discontiguous megablast searches in the genomes of marine mammals and other mammals representative of the main eutherian branches. Gene references used were: *DMA*, NM_006120.3; *DMB*, NM_002118.4; *DOA*, NM_002119.3; *DOB*, NM_002120.3; *DRA*, NM_019111.4 and XM_007951302.1; *DRB*, NM_002124.3 and XM_003423461.2; *DPA*, NM_001242525.1 and XM_006882197.1; *DPB*, NM_002121.5 and XM_012559980.1; *DQA*, NM_002122.3 and XM_003421050.1; *DQB*, NM_001243961.1.

We checked all predicted class II gene and pseudogene sequences for proper annotation using Geneious 9 (27). MAFFT (28) alignments with the predicted coding sequence were used to check for missing or poorly annotated exons. We constructed local BLAST libraries containing exons and introns for each gene and performed blast on scaffolds containing class II sequences. Nomenclature used for class II genes of non-model species included a prefix formed by the first two letters of the genus and species (i.e., *Loxodonta africana*, Loaf; *Trichechus manatus*, Trma; *Orycteropus afer*, Oraf; *Elephantulus edwardii*, Eled; *Chrysochloris asiatica*, Chas; *Echinops telfairi*, Ecte; *Dasypus novemcintus*, Dano; *B. acutorostrata*, Baac; *D. leucas*; Dele; *L. vexillifer*, Live; *N. asiaeorientalis*, Neas; *O. orca*, Oror; *L. obliquidens*, Laob;

T. truncates, Tutr; *P. catodon*, Phca; *C. ursinus*, Caur; *N. schauinslandi*, Nesc; *L. weddelli*, Lewe; *O. rosmarus*, Odro; *Pteropus alecto*, Ptal; *Equus caballus*, Eqca) (29, 30); BoLA, DLA, H2 and HLA were used for bovine, dog, mouse and human genes, respectively.

Predicted coding sequences with no stop codons or frameshift mutations were presumed to be functional and annotated as genes (including incomplete coding sequence due to assembly gaps); sequences with at least one stop codon or frameshift mutations were annotated as pseudogenes. Thus, for clarity, in this manuscript "locus/loci" will be used when broadly referring to sequences from a gene family, including both presumed genes and pseudogenes; "gene" will be used when referring only to presumed functional sequences; and "pseudogene" will be used for presumed non-functional sequences. New annotations and reannotation CDS, as well as the summary of the loci used in this study are provided here as **Supplementary Files S2**,**S3**.

Comparative Genomics Analysis

To construct dot plot graphs, the manatee, Northern fur seal and sperm whale were chosen as representatives of the main marine mammal branches. Scaffolds containing class II genes were first submitted to RepeatMasker (31) and resulting masking files were used along with sequence and gene annotations on PipMaker (32). For the manatee, scaffolds covering the class II MHC region were concatenated. Part of the annotations and sequences from the extended class II region were removed to provide a better view of the identity of key regions.

TABLE 1 | Number[a] of MHC class II genes and pseudogenes in each marine mammal scaffolds.

Species	Scaffold	DRA	DRB	DQA	DQB	DPA	DPB	DOA	DOB	DMA	DMB	DYA/DYB
T. manatus	NW_004444001	0	0 (1ψ)	0	0	0	0	0	0	0	0	0
	NW_004444197	0	0	0	0	0 (1ψ)	0 (4ψ)	0	0	0	0	0
	NW_004444318	0	0	0	0 (2ψ)	1	1	1	1	1	1	0
	NW_004444391	1	0	1	1 (1ψ)	0	0	0	0	0	0	0
	NW_004444463	0	0 (1ψ)	0	0	0	0	0	0	0	0	0
	NW_004444511	0	0	0	0	0	1	0	0	0	0	0
	NW_004444627	0	0	0	0	1	0	0	0	0	0	0
	NW_004446990	0	0 (1ψ)	0	0	0	0	0	0	0	0	0
B. acutorostrata	NW_006728570	0	0 (1ψ)	0	0	0	0 (1ψ)	1	1	1	1	0
	NW_006732678	1	1 (1ψ)	0	0	0	0	0	0	0	0	0
	NW_006731889	0	1	0	0	0	0	0	0	0	0	0
D. leucas	NW_019160881	1	2 (1ψ)	1	1	0	0	1	1	1	1	0
L. vexillifer	NW_006778796	0	0 (1ψ)	1	1	0	0	0	0	0	0	0
	NW_006787800	0	0 (1ψ)	0	0	0	0	1	1	1	1	0
	NW_006786873	1	1 (1ψ)	0	0	0	0	0	0	0	0	0
N. asiaeorientalis	NW_020174277	1	1 (1ψ)	1	1	0	0	0	0	0	0	0
	NW_020175393	0	0 (1ψ)	0	0	0	0	1	1	1	1	0
O. orca	NW_004438437	0	0 (1ψ)	0	0	0	0	1	1	1	1	0
	NW_004438672	1	2	1	1	0	0	0	0	0	0	0
L. obliquidens	NW_020837975	1	1 (2ψ)	1	1	0	0	1	1	1	1	0
T. truncatus	NW_017842945	0	0 (1ψ)	0	0	0	0	0	1	1	1	0
	NW_017844288	1	2 (1ψ)	1	1	0	0	0	0	0	0	0
P. catodon	NW_019873557	1	2 (1ψ)	1	1	0	0	1	1	1	1	0
C. ursinus	NW_020313370	1	1 (1ψ)	1	1	0 (1ψ)	0 (2ψ)	1	1	1	1	0
	NW_020319034	1	0	0	0	0	0	0	0	0	0	0
	NW_020321179	1	0	0	0	0	0	0	0	0	0	0
N. schauinslandi	NW_018734297	0	0	1	1	0 (1ψ)	0 (1ψ)	1	1	1	1	0
	NW_018734368	0 (1ψ)	1	0	0	0	0	0	0	0	0	0
L. weddellii	NW_006383774	1	1	0	0	0	0	0	0	0	0	0
	NW_006383968	0	1	1	1	0 (1ψ)	1	0	1	1	1	0
	NW_006386795	0	0	0	0	0	0	1	0	0	0	0
O. rosmarus	NW_004450609	0	1	0	0	0 (1ψ)	0 (1ψ)	1	1	1	1	0
	NW_004450757	2	1 (1ψ)	0	0	0	0	0	0	0	0	0
	NW_004452682	0	1	1	1	0	0	0	0	0	0	0

[a] *The number of presumptive functional genes are outside of brackets, and number of pseudogenes (ψ) between brackets.*

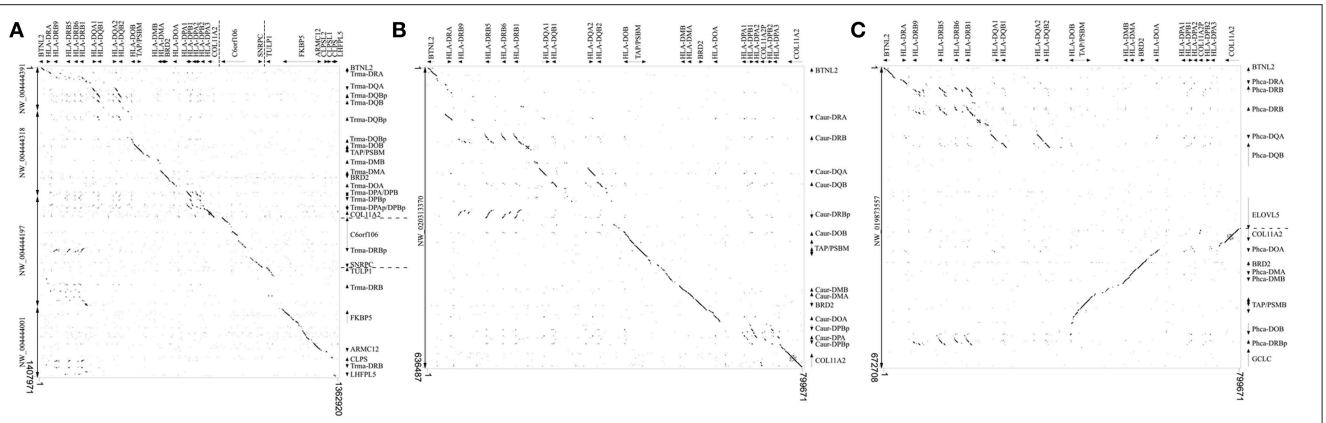

FIGURE 1 | Dot plot analyses of **(A)** *Trichechus manatus*, **(B)** *Callorhinus ursinus*, and **(C)** *Physeter catodon* scaffolds containing MHC class II genes, compared to the same region of the human genome. Human gene annotations are shown on the top of the graphs. For clarity purposes, extended class II region was reduced to include only key regions containing class II sequences; dashed lines on the annotation represent gaps. *T. manatus* sequences were in different scaffolds and were therefore concatenated for this analysis.

Phylogenetic Analysis

We constructed phylogenetic trees using the exons and introns from classical MHC class II genes and pseudogenes (when they could be accurately determined). We chose to use only loci located in scaffolds that allowed us to determine their location. Introns were separately aligned using MAFFT online service (33) and the alignments cleaned in GBlocks (34) under default settings and allowing gaps in all sequences. Intron alignments were then concatenated for the rest of the analysis. Exons were also aligned using MAFFT. Best-fit partition scheme and corresponding nucleotide substitution model was checked on PartitionFinder (35); each intron was discriminated for the search of all possible partition schemes and each codon position was treated as a partition. Maximum likelihood trees were constructed in CIPRES (36) using RAxML (37), with 1,000 bootstrap iterations. All phylogenetic tests were performed in triplicate. Trees were constructed on iTOL (38). Exon phylogenies did not change the main conclusions of this study, therefore we present only intron phylogenies since they are more comprehensive.

RESULTS

The Marine Mammal Genomes and Class II MHC Synteny

All marine mammal genomes in our analysis were *de novo* assembled using varying assembly methods (**Supplementary File S1**). The manatee genome was made using Illumina Hi-seq technology with a 150x coverage; pinniped genomes were also made using Illumina reads with coverage ranging from 27.44x to 200x; and cetacean genomes were made using Illumina or BGISEQ-500 technology, with coverage ranging from 35.68x to 248x (**Supplementary File S1**).

The manatee class II sequences were distributed over eight scaffolds, while other marine mammals had their class II MHC distributed on 1–3 scaffolds (**Table 1**). Overall, we were able to locate one copy of each non-classical gene in all marine mammals, whereas the classical genes varied across taxa. MHC class II genes showed conservation in sequence length and number of exons when compared to human homologs, although many entries were only partial due to gaps in the genome or difficulty in determining exon boundaries.

We chose a representative of each marine mammal lineage to construct dot plot graphs against the human MHC. The manatee class II region maintain the overall synteny compared to human, but all *DRB* loci are located after the extended class II region (**Figure 1A**). The Northern fur seal class II region also have the main features of the human MHC class II organization; however it lacks conservation in the *DP* subregion and possesses an inverted *DRB* pseudogene between its *DQ* and *DO* subregions (**Figure 1B**). The sperm whale class II region is divided in two subregions due to an inversion separating the *DR* and *DQ* genes from the non-classic genes (**Figure 1C**); the cetacean also lacks identity in the *DP* subregion and possesses a *DRB* pseudogene between *DOB* and *GCLC* (**Figure 1C**).

Main Features of the Marine Mammal MHC Class II Region

Due to the fragmentation of the manatee class II region in distinct scaffolds, we turned our attention to other afrotherians to understand their organization. The genomes investigated here were all sequenced and assembled by the Broad Institute. *L. africana* was the first sequenced *afrotherian* genome, assembled with Sanger reads; other *afrotherian* genomes were sequenced by NGS Illumina Hi-seq technology. All genomes were *de novo* assembled, with coverage ranging from 44x to 150x for the Illumina assembled genomes (**Supplementary File S1**). *C. asiatica* and *E. edwardii* have all class II loci in the same scaffold, evidence that class II sequences lie in the same chromosome. All analyzed afrotherian share the presumptive translocation of *DR* loci (**Figure 2**); this translocation was not found in other boreoeutherian or xenarthran genomes analyzed here (data not shown). Other presumptive chromosomal rearrangements were

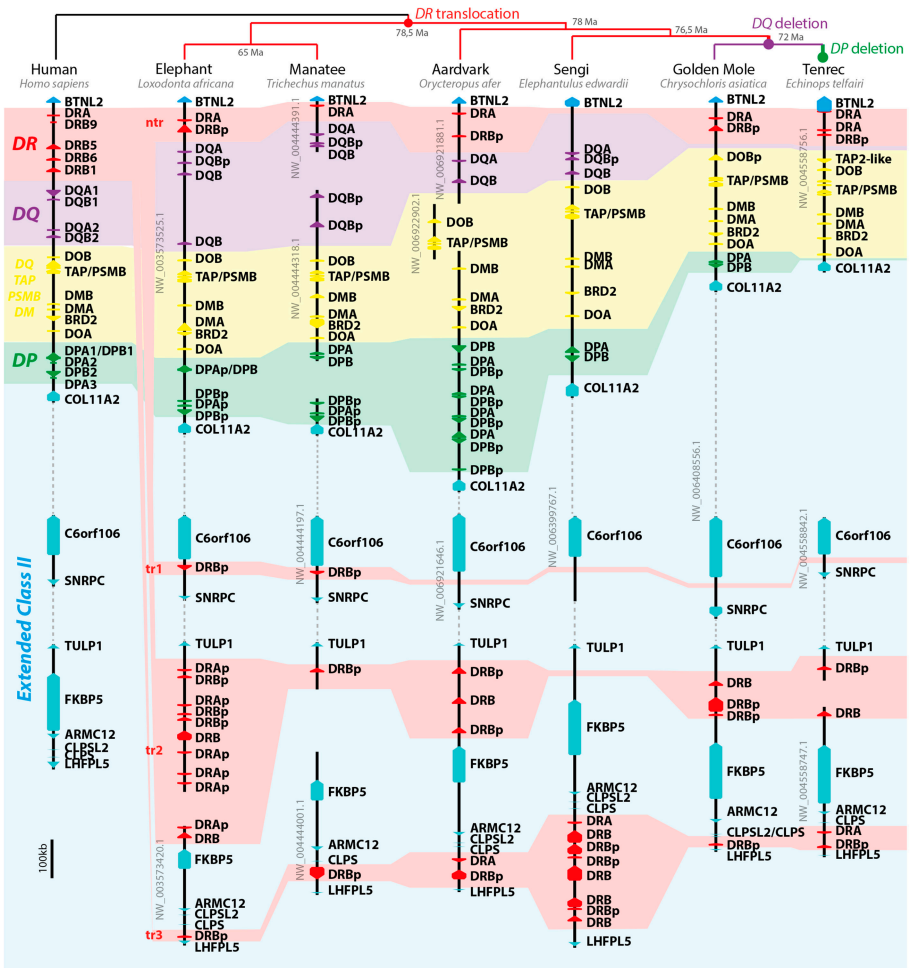

FIGURE 2 | Model of afrotherian class II MHC evolution. On the top of image, the afrotherian phylogeny and divergence time (Ma, million years before present) proposed by Springer et al. (39) and presumptive evolutionary events leading to current afrotherian MHC structure. On the left the human class II MHC region is depicted as an outgroup and model of the mammalian genome organization. Dashed lines represent regions of the scaffolds excluded for clarity purposes, which are not to scale. Arrows represent genes and pseudogenes (shown as "p" in the end of the gene's name). Only informative scaffolds of the MHC structure are displayed. In color, a schematic view of class II loci helps to understand the evolution of class II loci (*DR*—red; *DQ*—purple; *DP*—green; *DO/TAP/PSMB/DM* —yellow). *TAP/PSMB* represents *TAP1*, *TAP2*, *PSMB8*, *PSMB9*.

the deletion of *DQ* in the ancestor of *Afrosoricida* (i.e., *C. asiatica* and *E. telfairi*) and deletion of *DP* in *E. telfairi* (**Figure 2**).

The pinniped class II region has the same composition found in the dog DLA (**Figure 3**). Like the Northern fur seal, walrus and Weddell seal also possess *DRB* loci between *DOB* and *DQB*. Pinniped genomes have varied numbers of *DR* loci but a single pair of presumed *DQ* genes. Most *DP* loci seem to be pseudogenes in pinnipeds (**Figure 3**).

All cetaceans share with terrestrial Cetartiodactyla the inversion separating class II genes in two subregions: IIa, including *DR* and *DQ* loci; and IIb, including non-classic genes (**Figure 4**). Cetaceans have one *DRA* gene and up to three *DRB* loci in class IIa and a presumed *DRB* pseudogene next to *DOB* on class IIb region. Despite lying in the same location occupied by *DYB* and *DSB* in cattle, those sequences share a higher homology with *DRB* exons and introns and therefore were annotated as such. Most cetaceans have only one pair of *DQ* genes, whereas no *DQ* loci was found in the minke whale genome. Like cattle, cetaceans seem to have lost *DP* loci altogether, with only remnants of a *DPB* pseudogene found in the minke whale class IIb region. No *DY* loci was found in any cetacean (**Figure 4**).

Non-classical Class II and Antigen Processing Genes

Overall, non-classical genes were already annotated in the genomes analyzed here, but some entries needed a refined prediction of exon boundaries. The gene content and organization across marine mammals is highly conserved, as found in other mammals. Notably, *D. leucas* and *E. telfairi* *DOB* had to be separated from the *TAP2* gene annotations. *L. vexillifer* and *C. asiatica* *DOB* have a stop codon at exon 5, therefore were annotated as presumed pseudogene despite

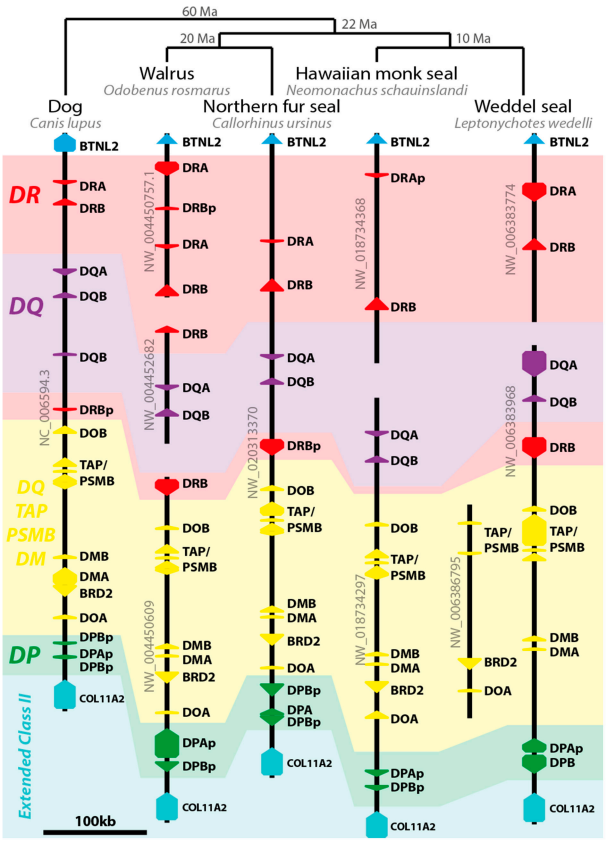

FIGURE 3 | Model of pinniped class II MHC evolution. On the top of image, the pinniped phylogeny and divergence time (Ma, million years before present) proposed by Nyakatura and Bininda-Emonds (40). On the left, dog class II MHC region is depicted as an outgroup and model of the Carnivora genome organization. Arrows represent genes and pseudogenes (shown as "p" in the end of the gene's name). Only informative scaffolds of the MHC structure are displayed. In color, a schematic view of class II loci helps to understand the evolution of class II loci (*DR*—red; *DQ*—purple; *DP*—green; *DO/TAP/PSMB/DM*—yellow). *TAP/PSMB* represents *TAP1*, *TAP2*, *PSMB8*, *PSMB9*.

being the only predicted *DOB* locus in their genomes. Protein alignments showed conservation of exon sizes, with most differences related to missing exons due to gaps in assembly (**Supplementary Files S4–S7**).

Classical Class II Genes

DR Loci

Most *DR* loci had to be reannotated, especially the small exons 5 and 6 from *DRB*. Protein alignments of *DR* genes are provided in **Supplementary Files S8, S9**. In the three orders of marine mammals we were able to find *DRA* and *DRB* genes, despite manatee having most of its sequences outside the canonical class II region. The translocation of *DR* loci in *Afrotheria* split sequences into four subregions (**Figure 2**): within the canonical class II region (not translocated, "nt"), between *C6orf106* and *SNRPC* (translocation 1, "tr1"; ∼2.3 Mb distant from nt), between *TULP1* and *FKBP5* (tr2; mean ∼3.2 Mb distant from nt), and between *CLPS* and *LHFPL5* (tr3; mean ∼3.9Mb distant from nt). The manatee has presumptive functional *DRA* and *DRB* genes at the nt and tr2 region, respectively, this *DRB* gene has a 16 codon gap in exon 3 but was considered a functional gene since no stop codons were found. The *DR* subregion in manatee have several assembly gaps, which hinders a clearer definition of number of genes. Tr1 was only found in *Paenungulata* (i.e., *L. africana* and *T. manatus*). All afrotherians seem to have lost functional *DRB* from nt, whereas *E. edwardii* lack all *DR* loci in the region (**Figure 2**). Afrotherian species seem to maintain only one subregion with presumed functional *DRB* genes, either tr2 (Paenungulata, *O. afer,* and *Afrosoricida*) or tr3 (*E. edwardii*).

Pinnipeds possess *DRB* and *DRA* loci; walrus have an *in tandem* duplication of *DR* loci in the nt. The only *DRA* locus from Hawaiian monk seal has a 1-bp deletion in exon 2 leading to several stop codons, and therefore was annotated as a pseudogene. However, this species has a presumptive functional *DRB* gene. Pinnipeds (except the Hawaiian monk seal) also share with dog a *DRB* locus between *DQB* and *DOB* (termed "nt2" region) that seems to be functional in walrus and Weddell seal (**Figure 3**).

Cetaceans have one bona fide *DRA* gene and one to three *DRB* loci (**Figure 4**). The cetaceans and cattle share a one codon deletion on the first exon of *DRA* genes (**Supplementary File S8**). The cetacean *DRB* pseudogene in the class IIb region lies in a location similar to *DSB* in cattle (**Figure 4**). The position and direction of class IIb *DRB* pseudogenes are compatible to that of nt2 *DRB* in the non-inverted class II region of other mammals.

DRA phylogenies formed well-supported clusters separating Carnivora, Cetartiodactyla and Afrotheria loci (**Figure 5A**). The afrotherian translocated loci from different locations did not form a well-supported cluster on the phylogenies; overall, *DR* loci grouped by species and not by genomic position on the phylogenetic trees. The only evidence of orthology from different afrotherian species occurred in the Paenungulata *DRA* nt genes (**Figure 5**). Carnivora formed 2 well-supported clusters in *DRB* phylogeny separating nt from nt2 sequences, although horse nt2 loci did not cluster with the Carnivora nt2 loci (**Figure 5B**). Similarly, cetacean IIb loci formed a well-supported cluster apart from nt2 loci from horse and carnivores (**Figure 5B**). Cetacean IIa loci formed two clusters separating most *DRB* pseudogenes from the genes, and therefore the ancestor of cetaceans probably had one *DRB* gene and one pseudogene.

DQ Loci

The marine mammals have a similar *DQ* subregion, with at least a pair of *DQA* and *DQB* functional genes annotated in most species analyzed here. The manatee genome has one *DQA* gene and four *DQB* loci, although only one seems to be functional. Most afrotherians also have a single *DQA* gene, while the number of *DQB* loci varied across taxa. The only species analyzed with multiple presumptive functional *DQB* genes is *L. africana*. We could not find any *DQ* loci in the genome of *C. asiatica* and *E. telfairi*. Pinnipeds and cetaceans have a pair of *DQA* and *DQB* genes, however, no *DQ* loci was located in the minke whale genome, possibly due to the abundance of assembly gaps in

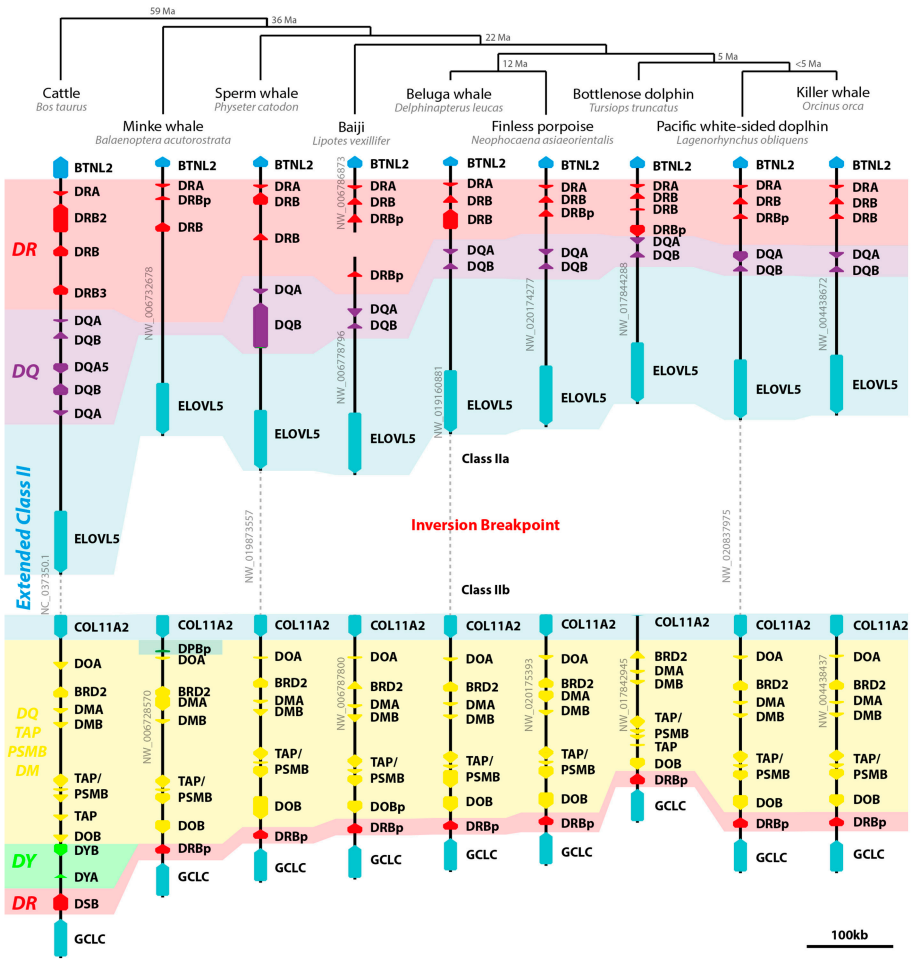

FIGURE 4 | Model of cetacean class II MHC evolution. On the top of image, the Cetartiodactyla phylogeny and divergence time (Ma, million years before present) proposed by Zurano et al. (41). On the left, cattle class II MHC region is depicted as an outgroup and model of the terrestrial Cetartiodactyla genome organization. Dashed lines represent regions of the scaffolds excluded for clarity purposes, which are not to scale. Arrows represent genes and pseudogenes (shown as "p" in the end of the gene's name). Only informative scaffolds of the MHC structure are displayed. In color, a schematic view of class II loci helps to understand the evolution of class II loci (*DR*—red; *DQ*—purple; *DP*—green; *DY*—light green; *DO/TAP/PSMB/DM*—yellow). *TAP/PSMB* represents *TAP1*, *TAP2*, *PSMB8*, *PSMB9*.

the *DQ* subregion. *DQ* genes maintained overall conservation of exon length, with differences only in exon 1 of *DQB* (**Supplementary Files S10**, **S11**). Most manatee loci clustered with elephant sequences in the phylogenies (**Figure 6**), but one manatee *DQB* pseudogene clustered with *P. alecto* pseudogene, suggesting this pseudogene was present in the ancestor of eutherians (**Figure 6B**). Carnivora and Cetartiodactyla *DQA* and *DQB* genes clustered inside their groups; cetacean *DQA* is orthologous to BoLA-*DQA2* (**Figure 6A**).

DP Loci

We reannotated most *DP* loci, mainly due to difficulty in assigning exon 1 for *DPA* and exon 5 for *DPB*. Most *DPA* loci were annotated with a small exon 1, because the start codon seemed to have mutated (coding for a valine instead of methionine). Protein alignments are provided in **Supplementary Files S12**, **S13**. Among the marine mammals, the only species with a pair of presumptive functional *DPA* and *DPB* genes is the manatee (**Supplementary File S3**, **Table 1**); the manatee possesses three *DPA* and four *DPB* loci, but only two *DPA* and one *DPB* are presumptive genes. Inside Afrotheria, *O. afer* possesses four *in tandem* duplications of the *DP* loci, while *E. telfairi* lost all *DP* loci (**Figure 2**). Most pinniped's *DP* loci are pseudogenes, except one *DPB* gene in Weddell seal and one *DPA* in Northern fur seal (**Figure 3**); *Caur-DPA* lacks homology in the end of exon 4 and *Lewe-DPB* is a partial sequence including only exons three and four. Cetaceans lack *DP* loci altogether, with the exception of a remnant of a *DPB* pseudogene (homology only to the exon 3) found in minke whale (**Figure 4**).

Four *DPA* genes (three from *O. afer* and one from *E. edwardii*) and two pseudogenes (one from *O. afer* and one from *L. africana*) possess a distinctive three codon insertion in exon 3, which may be evidence of an ancestral form of *DPA* in the afrotherian lineage (**Supplementary Files S3**, **S12**). However, sequences with this insertion did not form a well-supported cluster in the phylogenies (**Figure 6C**). *DPA* showed

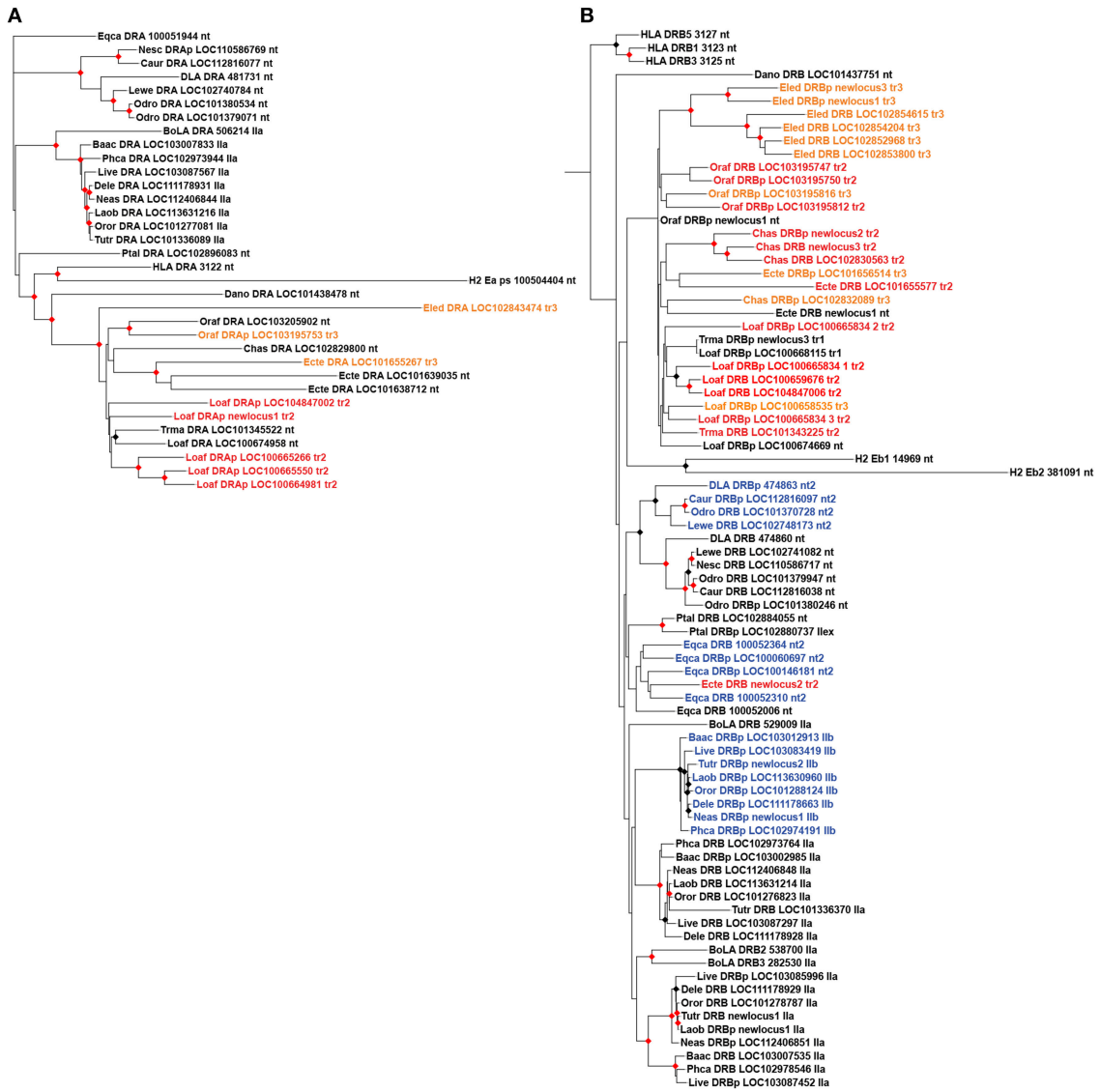

FIGURE 5 | Maximum likelihood phylogenetic trees of *DR* MHC class II genes and pseudogenes. **(A)** *DRA* introns 1, 2, and 3 phylogeny; **(B)** *DRB* introns 1, 2, 3, 4, and 5 phylogeny. On the sequence positions: "nt" refers to not translocated; in blue, "nt2" refers to genes between *DQ* and *DO*, and IIb to genes located on class IIb region in Cetartiodactyla, in blue; "tr1" refers to translocated between C6orf106 and SNRPC; "tr2" between TULP1 and FKBP5, in red; "tr3" between CLPS and LHFPL5, in orange; "p" refers to pseudogenes. Black and red circles indicates >80 and >95% support values, respectively. *L. africana*, Loaf; *T. manatus*, Trma; *O. afer*, Oraf; *El edwardii*, Eled; *C. asiatica*, Chas; *E. telfairi*, Ecte; *D. novemcintus*, Dano; *B. acutorostrata*, Baac; *D. leucas*; Dele; *L. vexillifer*, Live; *N. asiaeorientalis*, Neas; *O. orca*, Oror; *L. obliquidens*, Laob; *T. truncates*, Tutr; *P. catodon*, Phca; *C. ursinus*, Caur; *N. schauinslandi*, Nesc; *L. weddelli*, Lewe; *O. rosmarus*, Odro; *P. alecto*, Ptal; *E. caballus*, Eqca; *B. taurus*, BoLA; *H. sapiens*, HLA; *M. musculus*, H2; *C. l. familiaris*, DLA.

signs of orthology in Paenungulata; *DPB* pseudogenes showed signs of orthology between Paenungulata and *O. afer* (**Figure 6**). The ancestor of eutherians seems to have had two *in tandem* duplications of DP loci, one with functional genes and the other with pseudogenes; the manatee has loci from both clusters (**Figure 6**). Most Carnivora loci grouped inside pseudogene clusters; the presence of *Caur-DPA* and *Lewe-DPB* inside this cluster of Carnivora pseudogenes suggests both may not be functional (**Figure 6**).

DISCUSSION

Here we report the organization of the marine mammal class II MHC and the first model for the evolution of this region in afrotherians, *sirenians* and pinnipeds, including species from the families Otaridae, Phocidae, and Odobenidae. We also expanded the number of class II MHC organization reports in cetaceans, including species from Mysticeti and Odontoceti lineages. We found that the manatee MHC includes the main classical

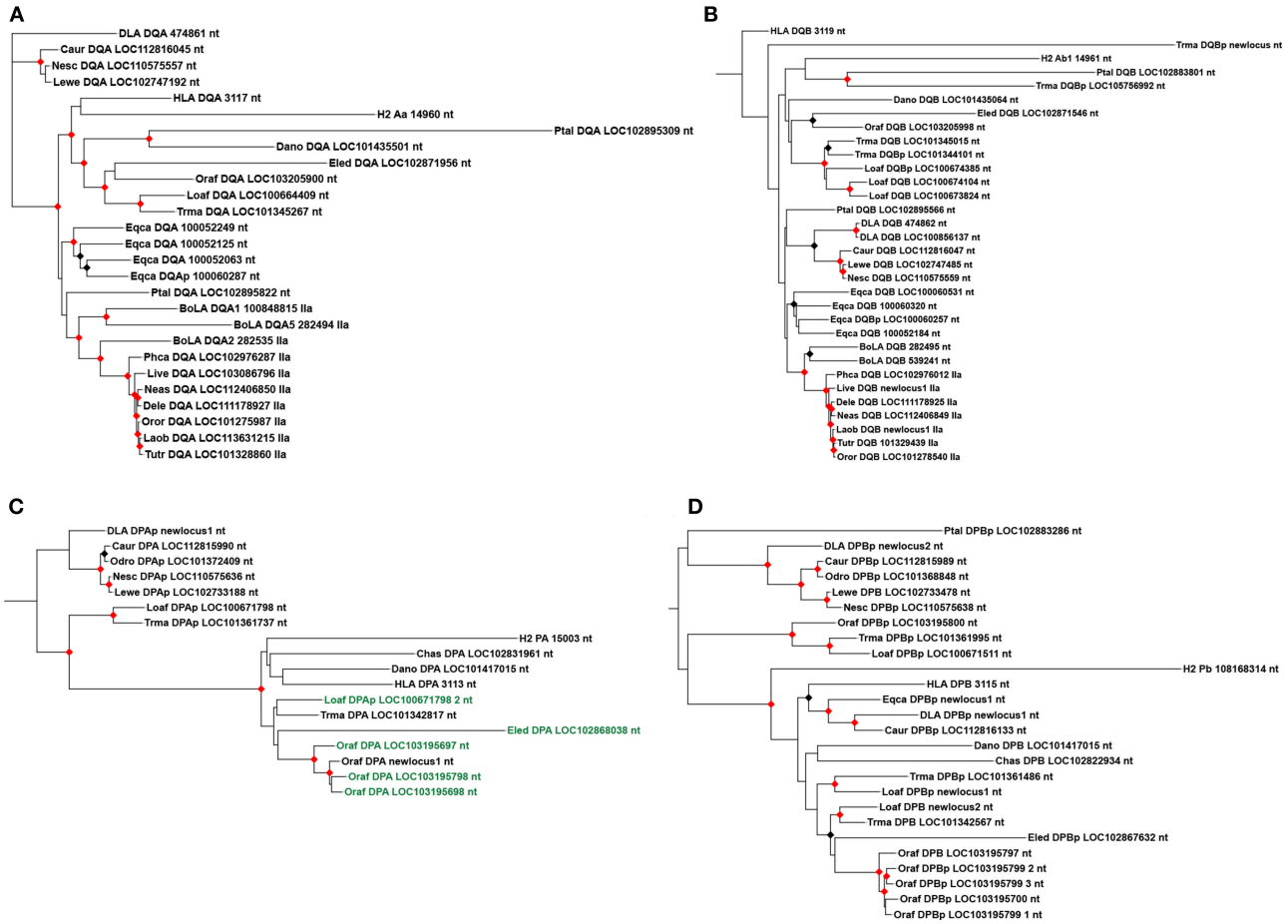

FIGURE 6 | Maximum likelihood phylogenetic trees of *DQ* and *DP* MHC class II genes and pseudogenes. **(A)** *DQA* introns 1, 2, and 3 phylogeny; **(B)** *DQB* introns 1, 2, 3, 4, and 5 phylogeny; **(C)** *DPA* introns 1, 2, and 3 phylogeny; **(D)** *DPB* introns 1, 2, 3, and 4 phylogeny. In **(C)**, sequences with a 3-codon insertion on exon 3 are in green. "p" refers to pseudogenes. Black and red circles indicates >80 and >95% support values, respectively. *L. africana*, Loaf; *T. manatus*, Trma; *O. afer*, Oraf; *El edwardii*, Eled; *C. asiatica*, Chas; *E. telfairi*, Ecte; *D. novemcintus*, Dano; *B. acutorostrata*, Baac; *D. leucas*; Dele; *L. vexillifer*, Live; *N. asiaeorientalis*, Neas; *O. orca*, Oror; *L. obliquidens*, Laob; *T. truncates*, Tutr; *P. catodon*, Phca; *C. ursinus*, Caur; *N. schauinslandi*, Nesc; *L. weddelli*, Lewe; *O. rosmarus*, Odro; *P. alecto*, Ptal; *E. caballus*, Eqca; *B. taurus*, BoLA; *H. sapiens*, HLA; *M. musculus*, H2; *C. l. familiaris*, DLA.

mammalian class II genes while most *DR* loci were translocated—a feature manatee shares with the other afrotherians analyzed here. Both pinnipeds and cetaceans have presumptive functional *DQ* and *DR* genes, probably lost functional *DP* genes, and have one *DRB* locus lying next to *DOB*. These findings fill a gap in the study of marine mammal immunogenetics and eutherian MHC evolution, providing evidence of new chromosomal rearrangements events that led to changes in the organization of the mammalian MHC.

The afrotherian MHC is poorly studied as a whole—to our knowledge, the only reports on afrotherian MHC are two studies on the *DQA* polymorphisms in elephant and wooly mammoth (42, 43). Based on genomic resources analyzed here, the manatees share with other afrotherians a unique *DR* translocation separating it from the core class II region. Despite manatee genes being distributed over four main scaffolds, all class II MHC sequences, including translocated *DR* loci, presumably lie on the same chromosome, based on other afrotherian class II regions and data from chromosome painting in manatee (44). The similarity between the manatee and elephant class II organization suggests that elephant may serve as a model for understanding the manatee MHC function. Antigen presentation in manatee presumably uses *DR*, *DQ*, and *DP*, with evidence of *DQ* duplications. Future research addressing the expression and polymorphism of class II genes in both species is needed. Afrotherians also have other unique features: a three-codon insertion on exon 3 in some of the *DPA* loci and deletion of *DQ* and *DP* loci during Afrosoricida (tenrec and golden mole) evolution. It is important to notice that tenrec (*E. telfairi)* seem to have one of the simplest mammalian MHC class II regions. We found three *DRA* genes and only one *DRB* gene on the tenrec assembly (**Figure 2**; **Supplementary File S3**), which may represent a mammalian "minimal essential" MHC class II, like in chickens—with only two classical class II genes, coding the alpha and beta peptides (45).

Despite several reports on class II gene polymorphisms in pinnipeds, to our knowledge this is the first analysis of MHC structure in this clade. The class II MHC of pinnipeds is similar to the DLA organization (46). The lack of a pair of presumed functional *DPA* and *DPB* genes in pinnipeds suggests their MHC may function similarly to cetaceans—using primarily *DR* and *DQ* molecules—and may provide opportunities to investigate convergence in class II evolution in both clades. Most pinnipeds also have an inverted *DRB* locus between *DQB* and *DOB*, which is not only present in carnivores such as dogs (46) and cats (47) but also in horses (48). This inversion event is thought to have occurred in the ancestor of Laurasitheria (48) and this *DRB* may be functional in walrus and Weddell seal.

Despite several studies focused on class II gene polymorphism in cetaceans, their MHC structure was only recently characterized (26). Most cetaceans analyzed here and the previously reported Yangtze finless porpoise MHC (26) have one *DRA* and two *DRB* loci in class IIa, a *DRB* pseudogene in class IIb, a single *DQ* pair, and lack *DP* and *DY* genes. On the other hand, cattle have *DYA*, *DYB*, *DSB,* and duplicated *DQ* genes; therefore using cattle as a model for cetacean immunogenetics should be cautionary until further characterization of expression and MHC haplotype variation in cetaceans. Notably, the only *Mysticeti* species analyzed here lacks *DQ* loci in its assembly, probably due to the large assembly gaps in this region, since there are reports of *DQ* polymorphisms in baleen whales (16, 19).

The difficulties of assembling the MHC region is widely known, due to extensive variation in gene sequence and haplotype composition of multigene families. However, due to increasing availability of good non-model species genomes, researchers have started using publicly available genomes to analyze the MHC region (49–56). Despite the challenges, to our knowledge, there are no reports that such difficulties resulted in artifactual chromosomal rearrangements of MHC loci, such as the translocations seen in Afrotheria. This mutation event is supported by the fact that two different sequencing technologies (long-reads from Sanger and short-reads from Illumina) and two different *de novo* assembly algorithms [ARACHNE and ALLSPATH (57, 58)] resulted in the same translocated subregions across the afrotherian genomes. Thus, if the translocation was an assembly artifact, the same misassembly would have to be repeated six times independently with datasets from different species, generated with different sequencing methodologies and different assembly algorithms. It is important to note that no other mammals analyzed here or elsewhere had similar events. The MHC organization of the other analyzed marine mammals were highly consistent with the organization of their eutherian lineages. Similarly, the deletion of *DQ* in Afrosoricida is supported by the evolutionary relationship of *C. asiatica* and *E. telfairi*—a deletion shared by two independent assemblies of animals from the same lineage and by the overall reduction in MHC size in the species scaffolds especially in the *DQ/DP* subregion. Another way to provide physical evidence for the translocation or deletion would be to use fluorescent *in situ* hybridization or sequence BAC libraries containing MHC genes, which was beyond the scope of the present study.

The *DR* phylogenies clustered sequences by species, although we expected that all translocated loci across afrotherians would form a well-supported cluster in the phylogenies, separated from non-translocated sequences. *DRB* loci in the nt2-IIb region and *DPA* with a three-codon insertion also did not cluster in the phylogenies. Previous research, using class II MHC genes from laurasitherians, also had similar results (48). Orthologous relationships were particularly observed inside Cetacea and Carnivora in which species diverged < 60 Ma (40, 41). A better resolution of orthology among translocated loci would probably be achieved using less divergent taxa, since the clades analyzed here diverged early in afrotherian evolution. For instance, one of the closest pair of species studied here is the African elephant and the Florida manatee, with estimates of 70∼65 million years of divergence (39, 59). The non-translocated *DRA* from manatee indeed clustered with the non-translocated elephant homolog, but the lack of other sequences (i.e., translocated *Trma-DRA* and non-translocated *Trma-DRB*) presently hinders a more comprehensive analysis.

In a simplistic scenario of the ancestral Afrotheria MHC, the translocated loci would diverge, and a phylogenetic analysis would separate loci from both regions (**Figure 7A**). However, in a realistic scenario, birth-and-death evolution (see below), natural selection, occasional gene conversion-like events and recombination may blur the evolutionary relationship among loci (**Figure 7B**). Those evolutionary processes may result in no true orthologs for MHC genes between distantly related taxa (60, 61). Similar processes may also explain why nt2 *DRB* loci and *DPA* loci with a three-codon insertion did not form a well-supported group in the phylogeny (since it is unlikely that both are cases of homoplasy).

The birth-and-death model of evolution for gene families—in which duplication, deletion and pseudogenization of genes lead to expansion and contraction of gene families (61)—affects both MHC class I and II genes but is more pronounced in the former, which usually results in lack of orthology when comparing animals from different families/orders (60). It has been proposed that the class I region evolves faster in eutherians due to its separation from the antigen processing genes (62); in addition, teleost classical class II genes are separated from the rest of the MHC and evolve similarly to eutherian class I genes (62). The variation in the number of *DR* loci in the afrotherian translocated regions suggests that the separation of *DR* loci from the class II region may have allowed genes to evolve faster, which could account for the loss of orthology seen in the phylogenies. Even though the translocation separated two DR subregions, the coded proteins still must form a functional heterodimeric class II protein that can interact with the TCR/CD4 complex of T lymphocytes. Thus, alpha and beta *DR* genes may coevolve and converge irrespective of their position in the genome, which again may impact phylogenies.

The clustering of genes related to antigen processing and presentation in the MHC and their conserved organization in eutherians is thought to be of functional importance (63). In mammals, early evidence of disruption in this organization was found in ruminants, in which an inversion split their class II region into two subregions (64, 65), an event now known to

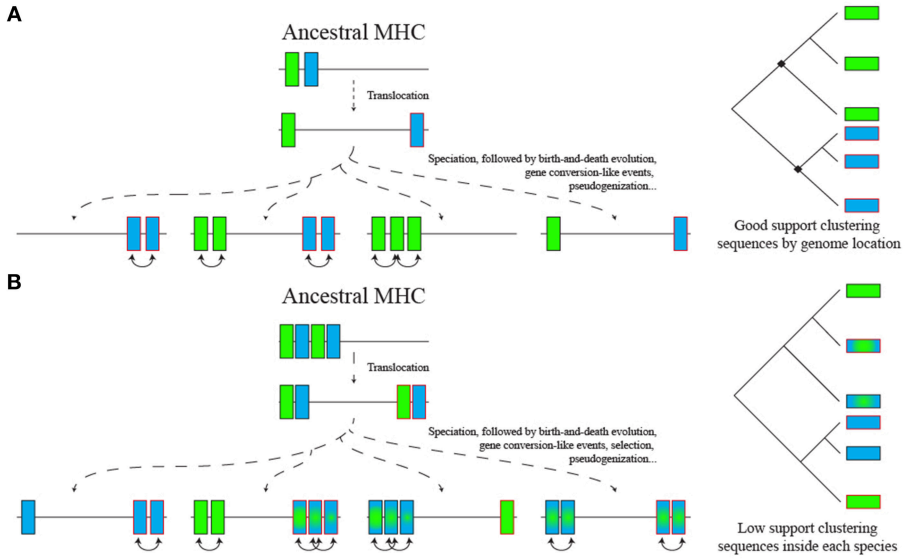

FIGURE 7 | Two scenarios for ancestral MHC class II region evolution and impact on phylogenetic analysis. **(A)** A simple model with only two paralogs separated by a translocation event. **(B)** A complex model with *in tandem* duplication of two loci separated by a translocation event. In the first scenario, translocated genes from current species would cluster together in the phylogeny, while in the second scenario a birth-and-death model of evolution, including gene conversion-like events and differential loss of tandemly duplicated loci, results in no true orthologs across distantly related taxa. Selection acting on both translocated and non-translocated regions can also blur the phylogenetic signal in both scenarios both for exonic and intronic sequences.

have taken place in the Cetartiodactyla ancestor (26). Since then, other studies revealed additional events disrupting this seemingly conserved organization: an inversion on distal class I region and loss of functional *DQ* and *DP* in felines (66); loss of *DR* in mole rats (67); disruption of the MHC organization and pseudoautosomal localization in monotremes (68); and several rearrangements in class I and II regions in wallaby (69). Those reports, including ours, provide opportunities to investigate how the MHC function evolves in different genomic landscapes and may challenge the functional importance of conserving the MHC organization (70). We also note that no afrotherian or xenarthran mammals had their entire MHC organization characterized, therefore our results show that the separation of class I and class II genes took place in the ancestor of all living eutherians after the split with marsupials [~170 million years ago (59, 71)], as suggested by Belov et al. (72).

The comparative study of the MHC in marine mammals may address how each lineage dealt with unique pathogen pressures in marine environments with the proposed distinct MHC class II genomic organization: the sirenians, with three classical gene families and the presumptive afrotherian translocated *DR* loci; the pinnipeds, with two classical gene families and inverted *DRB* loci; and the cetaceans, with two classical gene families and the cetartiodactyl inversion separating class IIa and IIb. Due to the lack of gene expression studies, the presumed annotation of genes and pseudogenes presented here should be interpreted with caution. Therefore, our results mandate future studies focusing upon in depth characterization of the structure, function and expression of the MHC as well as other important immunogenetic systems—such as TLR, Ig, and TCR, already in progress for some lineages and genes (73–78)—in the three marine mammal lineages. Direct sequencing and transcriptomic data will help clarify which sequences are functional, the degree of polymorphisms and any functional specialization of duplicated or translocated loci. Future research may use data provided here to carefully design amplification schemes that target canonical, translocated, inverted or IIb *DRB* loci.

Taken together, our results indicate a unique class II MHC architecture in each major marine mammal lineage. The evidence presented here also shows a sequential loss of two classical class II genes during Afrosoricida evolution, which may have resulted in the simplification of the class II region in *E. telfairi*, with only one classical class II protein encoded. Those results point to the separation of MHC class I and II regions in the ancestor of all living eutherians and reiterates the challenges to uncovering evolutionary relationships between MHC genes in distantly-related taxa. The occurrence of rearrangements in the mammalian MHC suggests this highly conserved system may be more malleable than once thought.

AUTHOR CONTRIBUTIONS

AS, LS, MC, and MS designed the study. AS performed all analysis and prepared figures. AS, BB, and TD reannotated the class II genes and performed dot plot analysis. AS and TB performed the phylogenetic analysis. LS, MC, and MS supervised all analysis. AS, BB, LS, and MC prepared the manuscript. All authors reviewed and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00696/full#supplementary-material

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Insights Into Dolphins' Immunology: Immuno-Phenotypic Study on Mediterranean and Atlantic Stranded Cetaceans

Cinzia Centelleghe[1], Laura Da Dalt[1], Letizia Marsili[2], Rossella Zanetti[1], Antonio Fernandez[3], Manuel Arbelo[3], Eva Sierra[3], Massimo Castagnaro[1], Giovanni Di Guardo[4] and Sandro Mazzariol[1]*

[1] Department of Comparative Biomedicine and Food Science, University of Padova, Legnaro, Italy, [2] Department of Physical Sciences, Earth and Environment, University of Siena, Siena, Italy, [3] Institute of Animal Health and Food Safety, Universitad de Las Palmas de Gran Canaria, Las Palmas, Spain, [4] Faculty of Veterinary Medicine, University of Teramo, Teramo, Italy

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***Correspondence:**
Cinzia Centelleghe
cinzia.centelleghe@gmail.com

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Immunology of marine mammals is a relatively understudied field and its monitoring plays an important role in the individual and group management of these animals, along with an increasing value as an environmental health indicator. This study was aimed at implementing the knowledge on the immune response in cetaceans stranded along the Italian coastline to provide a baseline useful for assessing the immune status of bottlenose (*Tursiops truncatus*) and striped (*Stenella coeruleoalba*) dolphins. In particular, since the Mediterranean Sea is considered a heavily polluted basin, a comparison with animals living in open waters such as the Atlantic Ocean was made. Formalin-fixed, paraffin-embedded spleen, thymus, and lymph node tissues from 16 animals stranded along Italian and 11 cetaceans from the Canary Island shores were sampled within 48 h from death. Information regarding stranding sites, gender, and age as well as virologic, microbiological, and parasitological investigations, and the cause and/or the death mechanism were also collected in order to carry out statistical analyses. Selected tissues were routinely stained with hematoxylin-eosin (H&E) and with immunohistochemical techniques (IHC). For IHC analysis, anti-human CD5 monoclonal mouse antibody to identify T lymphocytes, CD20 monoclonal mouse antibody for the identification of mature B lymphocytes and HLA-DR antigen (alpha-chain) monoclonal mouse antibody for the identification of the major histocompatibility complex type II were previously validated for both species by Western-blotting technique. *T*-test method applied to quantitative evaluation of IHC positive cells showed a significant relationship between the number of (expression) of CD20 stained lymphocytes and normal and hypoplastic lymph nodes, respectively. No other significant correlations were noticed. Analyses for organochlorines (OC) compounds were performed in animals (n°5) having frozen blubber tissue available. A simple linear regression was calculated to predict if the amount of OCs could influence

the number of inflammatory cell subpopulations and a moderate negative correlation was found between the presence of high quantity of contaminants and the number of T lymphocytes. Future analysis should be aimed to understand the effect of the major immunomodulatory pathogens on sub-populations of B and T cells.

Keywords: striped dolphin, bottlenose dolphin, immunohistochemistry, lymphocytes, organochlorine

INTRODUCTION

Investigating the lymphoid tissues and, more in general, the immunology, and immunopathology of whales and dolphins is relevant to understand the delicate host-pathogen interactions in some of the most relevant infections of cetaceans. For example, Cetacean Morbillivirus (CeMV) enters the immune system and induces an extensive lymphocytolysis causing a generalized lymphoid cell depletion in lymphoid organs. The subsequent immune impairment can then result in an increased susceptibility to secondary infections (1). Beside the direct action of this viral pathogen, a contaminant-induced immunosuppression as a trigger for infectious disease susceptibility's enhancement has been frequently advocated (2–5), highlighting the possible hazards associated with exposure to environmental contaminants, in particular persistent organochlorine (OC) pollutants bioaccumulating in these top predators (6, 7).

Despite the relevance of these studies, descriptions regarding relevant features of the lymphoid system are scanty, fragmented and generally dated, with the first record of lymphoid organs' microscopic anatomy in selected species by Simpson and Gardner (8). Notable exceptions include belugas (*Delphinapterus leucas*) and bottlenose dolphins (*Tursiops truncatus*), in which the morphological architecture of lymphoid organs was extensively investigated (9, 10). Furthermore, an evaluation of cellular and humoral immune responses was carried out again in belugas and harbor porpoises (*Phocoena phocoena*) with an immunophenotyping of their lymphoid cells by an immunohistochemical evaluation of cluster of differentiation (CD) antigens (11).

The specificity of cross-reacting bovine, human, ovine, and murine monoclonal antibodies raised against different leukocyte subsets and the major histocompatibility complex class (MHC) class II antigens of peripheral blood lymphocytes of beluga whales and bottlenose dolphins has been confirmed by immunoprecipitation and flow cytometry (9, 12, 13). Furthermore, cross-reacting antibodies directed against various cell surface antigens of the hematopoietic system, including T cell, B cell, histiocytic, and MHC II antigens have been established for common dolphin (*Delphinus delphis*), striped dolphin (*Stenella coeruleoalba*), bottlenose dolphin, and harbor porpoise lymphoid tissues using immunohistochemistry (14, 15). Similarly, histiocytic cells, particularly resident and inflammatory macrophages can be detected by cross-reacting human antibodies raised against the macrophage-associated antigens CD163, CD204, and lysozyme in short-finned pilot whales (*Globicephala macrorhynchus*) and Risso's dolphins (*Grampus griseus*) using immunohistochemistry (16, 17). Bottlenose dolphin specific monoclonal antibodies for the detection of CD2, CD19, CD21, and CD45R antigens as well as the adhesion molecule beta-2-integrin have been produced and characterized by flow cytometry and immunoprecipitation (13, 18). Furthermore, T cells are recognized by the CD2 marker, while B lymphocytes are predominantly labeled by monoclonal anti-CD19 and -CD21 specific antibodies, using immunohistochemistry (13). Still notably, B cells and a subset of T cells are labeled through the CD45R biomarker (19).

The present investigation was aimed at increasing our knowledge on the immune system of cetaceans, in order to partially fill the existing gaps. Besides characterizing some primary commercial antibodies against cetaceans' leucocytes, this study investigates any possible variations of B and T cells in stranded bottlenose and striped dolphins related both to intrinsic host's factors and to extrinsic insults.

MATERIALS AND METHODS

Animals and Sampling

Formalin-fixed and paraffin embedded lymphoid tissue samples (spleen, lymph nodes, and/or thymus) coming from 27 cetaceans were collected as follow. Tissues from 12 striped dolphins and 4 bottlenose dolphins stranded along the Italian coastlines, or died in aquaria were retrieved from the Mediterranean Marine Mammals Tissue Bank (www.marinemammals.eu). In order to have a comparison with animal coming from different geographical areas, features, 8 striped dolphins, 2 common dolphins, and 1 Risso's dolphin from Canary Island, which succumbed due to ship strikes and with no other pathological findings and/or molecular evidences of infection, were included in the study as "control specimens." Available data concerning all selected animals and their respective data are summarized in **Table 1**.

Briefly, specimens were selected considering the carcass preservation degree [codes 1 and 2 according to Geraci and Loundsbury (21)], along with anamnestic data including sex, age category (calf, juvenile, adult) estimated on total body length and/or on teeth microscopic examination (21), *post mortem* findings. Microbiological and biomolecular investigations for *Morbillivirus* and *T. gondii* were also performed on all major organs (brain, lungs, liver, spleen, lymph nodes, kidneys) according to already published methodologies [respectively (22, 23)]. Finally, 5 out of the 16 Italian dolphins were selected for ecotoxicological analyses (due to the relevant economic costs of such analyses).

TABLE 1 | Data concerning the 27 cetaceans under study*.

ID	Species	Sex	Age class	Stranding place	Conserv. code	Stranding condition	Body condition§	Ongoing infections	Other relevant findings
145	*Tursiops truncatus*	M	Calf	UHC	2	None	Moderate	None	Mild hemorrhagic omphalitis; sub-acute moderate diffuse interstitial pneumonia; moderate to severe multifocal ulcerative gastritis; mild chronic enteritis and moderate chronic colitis with reactive hyperplasia of the GALT.
167	*Stenella coeruleoalba*	M	Adult	Collesalvetti (LI)	2	Dead	Good	None	Nutritional panniculitis, exacerbated by an abnormal localization of Pholeter spp which has significantly reduced the lumen between the first and second gastric chambers.
170	*Stenella coeruleoalba*	F	Adult	Capalbio (GR)	2	Dead	Thin	None	Mild diffuse pyogranulomatous parasitic pneumonia; mild multifocal granulomatous parasitic gastritis associated with a foreign body; acute moderate diffuse catarral enteritis with parasitic infestation.
196	*Tursiops truncatus*	M	Adult	Cervia (RA)	2	Alive	Thin	Toxoplasma spp.	Multifocal moderate pyogranulomatous bronchopneumonia with pulmonary fibrosis and multifocal mild chronic interstitial pneumonia; multifocal severe chronic hepatitis with foci of necrosis and biliary stasis; chronic severe meningo-encephalo-myelitis.
212	*Stenella coeruleoalba*	F	Adult	Livorno (LI)	2	Dead	Moderate	None	Severe diffuse pyogranulomatous pneumonia; mild diffuse chronic endometritis; mild multifocal chronic interstitial nephritis.
214	*Stenella coeruleoalba*	F	Adult	Porto Garibaldi (FE)	3	Dead	Thin	None	Acute moderate multifocal ulcerative esophagitis and stomatitis; severe chronic multifocal parasitic ulcerative gastritis; acute diffuse severe catarrhal gastro-enteritis.
218	*Stenella coeruleoalba*	M	Adult	Lido di Classe (RA)	2	Alive	Good	None	Multifocal mild granulomatous parasitic pneumonia; multifocal moderate ulcerative parasitic gastritis caused by Anisakis spp.; severe diffuse acute catarrhal enteritis; severe multifocal chronic hepatitis.
221	*Stenella coeruleoalba*	M	Adult	Lido di Volano (FE)	2	Alive	Moderate	None	Severe esophageal and gastric food impaction.
229	*Tursiops truncatus*	M	Calf	UHC	1	None	Moderate	None	Moderate multifocal chronic ulcerative gastritis; sub-acute severe diffuse catarral enteritis; meningeal hemorrhages with cerebral edema.
251	*Stenella coeruleoalba*	M	Juvenile	Giugliano (NA)	2	Dead	Good	Morbillivirus	n.a.
255	*Stenella coeruleoalba*	F	Calf	Civitavecchia (RO)	1	Alive	Moderate	None	Moderate multifocal chronic ulcerative stomatitis; severe disseminated granulomatous pneumonia; severe chronic granulomatous parasitic gastritis with partial obstruction of the gastric lumen; severe acute multifocal enteritis.
262	*Stenella coeruleoalba*	M	Juvenile	Napoli (NA)	2	Dead	Moderate	Morbillivirus	n.a.
267	*Stenella coeruleoalba*	F	Adult	Ortoliuzzo (ME)	2	Dead	Good	None	n.a.
273	*Stenella coeruleoalba*	M	Juvenile	Salerno (SA)	2	Dead	Moderate	None	n.a.

(Continued)

TABLE 1 | Continued

ID	Species	Sex	Age class	Stranding place	Conserv. code	Stranding condition	Body condition[§]	Ongoing infections	Other relevant findings
327	*Stenella coeruleoalba*	M	Calf	Brancaleone Marina (RC)	1	Alive	Thin	None	Capture myopathy.
343	*Tursiops truncatus*	M	Calf	UHC	1	None	Good	None	Mild to moderate multifocal chronic bronchitis; diffuse and severe hepatic degeneration with capsular multifocal fibrosis; moderate multifocal chronic interstitial nephritis with segmental membranous glomerulopathy.
CET 131	*Delphinus delphis*	M	Calf	Guía de Isora (Tenerife)	2	Dead	Good	None	Trauma by fishing utensil.
CET 151	*Stenella coeruleoalba*	M	Juvenile	La Graciosa (La Graciosa)	1	Alive	Thin	None	Entanglement.
CET 281	*Stenella coeruleoalba*	F	Adult	Puerto del Carmen (Lanzarote)	2	Dead	Good	None	Trauma due to intra/interspecific interaction.
CET 293	*Stenella coeruleoalba*	M	Adult	Arico (Tenerife)	2	Dead	Good	None	Trauma by fishing utensil.
CET 371	*Stenella coeruleoalba*	F	Adult	Arona (Tenerife)	2	Dead	Good	None	Trauma by fishing utensil.
CET 374	*Stenella coeruleoalba*	M	Adult	Playa Tebeto (Fuerteventura)	2	Dead	Moderate	None	Trauma.
CET 406	*Delphinus delphis*	M	Calf	Santiago (Tenerife)	2	Dead	Moderate	None	Trauma due to intra/interspecific interaction.
CET 483	*Grampus griseus*	M	Adult	Puerto del Rosario (Fuerteventura)	2	Dead	Good	None	Trauma due to intra/interspecific interaction.
CET 606	*Stenella coeruleoalba*	F	Adult	Teguise (Lanzarote)	2	Dead	Good	None	Fishing interaction.
CET 616	*Stenella coeruleoalba*	F	Adult	Mogan (Gran Canaria)	2	Alive	Good	None	Collision with ship.
CET 698	*Stenella coeruleoalba*	F	Adult	Los Giunchos (La Palma)	2	Dead	Moderate	None	Trauma consequent to fishing interaction.

**M, male; F, female; UHC, under human care; n.a., not available.*

[§], Body condition were defined according to Joblon et al. (20).

Western Blotting Analysis

Bottlenose dolphin and striped dolphin (1 g frozen tissue at −80°C) tissue were homogenized using Potter glass (Vetrotecnica, Italia) in 5 ml of buffer A (10 mM Tris-Base, 150 mM NaCl, 5 mM EDTA, pH 7.2 and cocktail inhibitor—Sigma, Milan, Italy) and centrifuged at 10,000 g for 30 min. The supernatant was then centrifuged at 125,000 g for 1 h (Optima L-90K, Beckman, Italy) and the pellet proteins were dissolved in 0.2 ml of buffer B (10 mM Tris, 150 mM pH 7.2 NaCl). Total protein concentration was determined using BCA Protein Assay Kit (Pierce Biotechnology, USA). The samples were diluted 1:1 in 2x Laemmli sample buffer (Sigma–Aldrich, St. Louis, MO, USA), boiled for 5 min at 95°C and separated by 12% SDS–PAGE in a mini-gel apparatus (Hoefer SE 260, GE Healthcare, UK) under denaturing and reducing conditions in according to Laemmli protocol (24). Homogenate of human tonsil was used as a positive control to test the binding with specific antibodies. Following electrophoresis, gels were blotted (350 V, 1 h, 4°C) onto nitrocellulose membranes (0.45 μm; GE Healthcare, UK) in Laemmli transfer buffer (25 mM TRIS-base, 192 mM Glycine and 20% Methanol, pH 8.3) using a trans blot apparatus (Elettrofor, Rovigo, Italy). Membranes were carefully washed in deionized water and blocked overnight at room temperature with 10% skimmed milk and 0.1% Tween-20 (Sigma–Aldrich, St. Louis,

MO, USA). Each single membrane was incubated for 1 h at room temperature using the specific antibody diluted in PBS with 0.1% Tween-20 and 5% skim milk. Membranes were washed three times for 10 min with washing buffer (0.1% Tween-20 in PBS) and then incubated for 1 h at room temperature with a horse radish peroxidase (HRP)-conjugated.

Dilution of anti-CD5 (monoclonal rabbit anti-Human; Biocare Medical, USA), anti-CD20 (monoclonal rabbit anti-Human; Thermo Scientific, UK) and anti-HLA-DR (monoclonal mouse anti-Human HLA-DR antigen, Alpha-Chain; DakoCytomation) antibodies (Abs) were defined after appropriate dilution tests and cross-reaction with the secondary anti-rabbit or anti-mouse antibody horse radish peroxidase-conjugated (GAR-HRP and GAM-HRP; BioRad, USA) (**Table 2**). Finally, the membranes were washed three times with the same washing buffers, and the antigens were visualized by Immobilon Western Chemiluminescent HRP Substrate (MILLIPORE, Billerica, USA) and exposure to autoradiographic films (GE Healthcare, Amersham, UK). Protein bands in autoradiographic films were scanned using an ImageScanner apparatus (Amersham Biosciences, NJ, USA) and analyzed by the software ImageMaster (Total Lab, Amersham Biosciences, NJ, USA).

Microscopic, Immunohistochemical (IHC), and Semi-quantitative Analyses

All the samples were tested by routine microscopic examination and immunohistochemistry (IHC): pre-scapular and/or mediastinal lymph nodes and/or spleen and/or thymus were fixed in 4% buffered formalin, embedded in paraffin and stained for routine microscopic examination using hematoxylin and eosin. In particular, according to Elmore (25) and Valli et al. (26) a lymph node was considered normal if include all the physiological structures (i.e., capsule, subcapsular sinus, cortex, composed by standard predominately B-cell follicles and germinal centers, T-cell-rich paracortical area, medullary sinuses, medullary cords and hilus). Hyperplastic changes were identified by an increase in number and size of follicles and conversion to secondary follicles, involving the B-cell-rich follicles and/or the T-cell-rich paracortex. Reactive follicles were usually larger than the unstimulated ones and had a paler staining germinal center with large lymphoblasts and increased numbers of apoptotic lymphocytes. On the other site, the lack of secondary follicles in the lymph nodes together with the reduced number of primary follicles and the decreased size of their paracortex were indicative of lymph node hypoplasia.

For IHC analysis, staining was performed using an automatic immunostainer (Ventana Benchmark XT, Roche-Diagnostic), which uses a kit with a secondary antibody and with a horseradish peroxidase (HRP)-conjugated polymer that binds mouse and rabbit primary antibodies (ultraViews Universal DAB, Ventana Medical System). All reagents were dispensed automatically except for the primary antibody, which was dispensed by hand. Then we used the anti-CD5 Ab at a dilution of 1:50 and the anti-CD20 Ab at a dilution of 1:800 both incubated for 13 min at room temperature and the anti-HLA-DR, alfa-chain Ab at a dilution of 1:50 incubated for 32 min at room temperature (**Table 3**).

A semi-quantitative analysis was performed using a slide scanner for digital pathology (D-sight, A. Menarini diagnostic). Each IHC-processed section was scanned and immunolabelled cells were counted by two operators within 10 microscopic fields at high power field (40x objective), considered to be representative of the entire lymphoid tissues under investigation. The count was performed manually using an open source image processing program designed for scientific multidimensional images (ImageJ, LOCI, University of Wisconsin-Madison).

Statistical analyses were performed to find possible correlations between the different immune cell populations and independent variables such as species, gender, age class, regional areas, and the presence of ongoing infections.

For statistical analysis the *T*-test was chosen because of the heterogeneity of the samples and the amount of data. A statistically significant threshold was set at a p-value of 0.05; a p-value <0.05 was considered indicative of a strong association.

Toxicological and Statistical Analysis

Analyses for Hexachlorobenzene (HCB), dichlorodiphenyltrichloroethane compounds (DDTs) and polychlorobiphenyl compounds (PCBs) were performed according to methods recommended by the U.S. Environmental Protection Agency (EPA) 8081/8082 with modifications (27) in animals having frozen blubber tissue available at The Mediterranean Marine Mammal Tissue Bank (ID196, ID214, ID218, ID221, and ID229). Blubber samples were lyophilized, and about 1 g each was extracted with n-hexane for gas chromatography (Merck) in a Soxhlet apparatus for analysis of organochlorine compounds. Each sample was spiked with surrogate compound (2,4,6-trichlorobiphenyls—IUPAC number 30) (28) prior to extraction. This compound was quantified and its recovery calculated. Surrogate recovery was reported with the sample results. The samples were then purified. Decachlorobiphenyl (DCBP—IUPAC number 209) was used as an internal standard, added to each sample extract prior to analysis, and included in the calibration standard, constituted by a mixture of specific compounds (Arochlor 1260, HCB and pp'- and op'-DDT, DDD and DDE). The analytical method used was High Resolution Capillary Gas Chromatography with an Agilent 6890 N and a 63Ni ECD and an SBP-5 bonded phase capillary column (30 m long, 0.2 mm i.d.). The carrier gas was nitrogen with a head pressure of 15.5 psi (splitting ratio 50/1). The scavenger gas was argon/methane (95/5) at 40 ml/min.

TABLE 2 | Primary Ab dilutions for Western Blotting analyses*.

		Anti-CD5	Anti-CD20	Anti-HLA-DR
	mw	58	33	33
Primary Ab		1:500	1:2000	1:2000
Secondary Ab GAR-HRP		1:50000	1:50000	
Secondary Ab GAM-HRP				1:8000

mw, molecular weight; GAR-HRP, anti-rabbit antibody horseradish peroxidase-conjugated; GAM-HRP, anti-mouse antibody horseradish peroxidase-conjugated.

TABLE 3 | Primary Abs used for IHC analysis.

Mono/ Polyclonal	Antibody name	Clone	Target cells	Antigen localization
Monoclonal Mouse	Anti-human CD5	4C7	T lymphocytes	Cytoplasmic and cell membrane
Monoclonal Rabbit	Anti-human CD20		B lymphocytes	Cell membrane and cytoplasm
Monoclonal Mouse	Anti-human HLA-DR Alpha-chain	TAL.1B5	Antigen presenting cells	Cell membrane

Oven temperature was 100°C for the first 10 min, after which it was increased to 280°C at 5°C/min. Injector and detector temperatures were 200 and 280°C, respectively. The extracted organic material (EOM%) from freeze-dried samples was calculated in all samples. Capillary gas chromatography revealed op'- and pp'- isomers of DDT and its derivatives DDD and DDE, and about 30 PCB congeners. Total PCBs were quantified as the sum of all congeners. These congeners constituted 80% of the total peak area of PCBs in the samples. Total DDTs was calculated as the sum of op'DDT, pp'DDT, op'DDD, pp'DDD, op'DDE, and pp'DDE. The results were expressed in ng/g lipid weight (ng/g lipid weight).

Linear regression analysis was used to study if there was a relationship between the number of the different cellular populations and organochlorines (OC) amount presents in their tissues.

RESULTS

The present investigation was carried out on a total of 27 dolphins: 20 striped dolphins, 4 bottlenose dolphins, 2 common dolphins and 1 Risso's dolphin; as far as the gender is concerned, 10 individuals were females (37%) and 17 males (63%). Furthermore, 7 of the animals under study (25.9%) were calves, while 4 (14.8%) juveniles and 16 (59.3%) were adults.

Western Blotting Analysis

Western blotting analysis performed to verify possible cross-reactions of CD5, CD20, and HLA-DR antigens against the secondary antibody did not show specific binding in correspondence of the antigens molecular weight (images not showed). The expression of the CD5 and CD20 antigens was visible at 58kDa and 35kDa and antigens were less visible in striped dolphin (SC) than in bottlenose dolphin (TT) (**Supplementary Figures 1A,B**). Anti-HLA-DR Ab reacted to the antigens of SC and TT showing a signal corresponding to proteins with a molecular weight of 33kDa with greater intensity in man (Hu) than in SC and TT. The other visible bands were due to non-specific interactions with proteins with molecular weight >50kDa that did not interfere with the proteins of our interest (**Supplementary Figure 1C**).

The band intensity difference between Hu and SC or TT was probably due to the different antigen concentration of the sample. In the two dolphin's samples there was only cellular membrane extracted and the antigen was more than in the total cellular human tonsil purified.

Microscopic, Immunohistochemical (IHC), and Semi-quantitative Analyses

The normal microscopic architecture of the lymph nodes varied depending upon location: the major differences involved the amounts of muscle tissue within the *capsula* and the *trabeculae*. The pre-scapular lymph nodes generally showed very little smooth muscle in this location, while in the visceral ones (mainly in mediastinal lymph nodes) smooth muscle encapsulated the node itself and in addition to extending along *trabeculae*, formed an interlacing network throughout the node.

The cetaceans' spleen showed a white pulp composed by lymphoid nodules located at the arterial terminals, evenly distributed throughout the red pulp. Lymphoid nodules were composed of small to medium-sized lymphocytes.

The results of microscopic examinations carried out on the lymphoid organs of the selected specimens are summarized in **Table 4**. A total of 11 dolphins showed normal lymphoid tissues, whereas 11 and 5 out of them showed hyperplastic and hypoplastic lymphoid tissues, respectively (**Figure 1**).

More in detail, positive immunolabeling with the anti-human CD20 Ab was evident within lymphocytes located in the germinal centers, as well as in the mantle and marginal zones of the lymphoid follicles (**Figure 2A**). As expected, a positive immunostaining reaction against anti-human CD5 Ab was additionally found in lymphocytes located in the paracortical zone (**Figure 2B**). The medullary area was formed by both CD20 and CD5 positively labeled cells. Cells immunoreactive to anti-human HLA-DR Ab were detected throughout the entire lymph node structure, where antigen-presenting cells were also found to be distributed (**Figure 2C**).

Normal cetacean thymus follows the typical mammalian organization with a cortex, medulla, and Hassall's corpuscles. The presence of T lymphocytes was demonstrated using the monoclonal anti-human CD5 Ab, with T lymphocytes dominating the entire thymic parenchyma. Few scattered cell clusters lacked MHC class II antigen expression in the thymus.

The average number of B lymphocytes (CD20-immunoreactive, IR), T lymphocytes (CD5-IR) and cells presenting the MHC-II membrane antigen was higher in lymph nodes than in spleen. As anticipated during the IHC qualitative analysis, the thymus did not show CD20-IR lymphocyte. The results of the semi-quantitative analyses on cetacean lymphoid tissues are summarized in **Table 5**.

Statistical analysis was performed to evaluate the existence of possible correlations between immune cells populations expression and independent variables such as species, gender, age class, geographic origin/location

TABLE 4 | Results of histological analyses on the lymphoid tissues of the cetaceans under study.

	Total n° of specimen	Animal with ongoing diseases	*Stenella coeruleoalba (20 animal)*	*Tursiops truncates (4 animals)*	Other species *(3 animals)*	Mediterranean basin	Atlantic basin
Normal histology	11	0 (11)	9 (11)	1 (11)	1 (11)	6 (11)	4 (11)
Lymphoid hyperplasia	11	1 (11)	6 (11)	3 (11)	2 (11)	3 (11)	6 (11)
Lymphoid hypoplasia	5	2 (5)	4 (5)	1 (5)	0 (5)	4 (5)	1 (5)

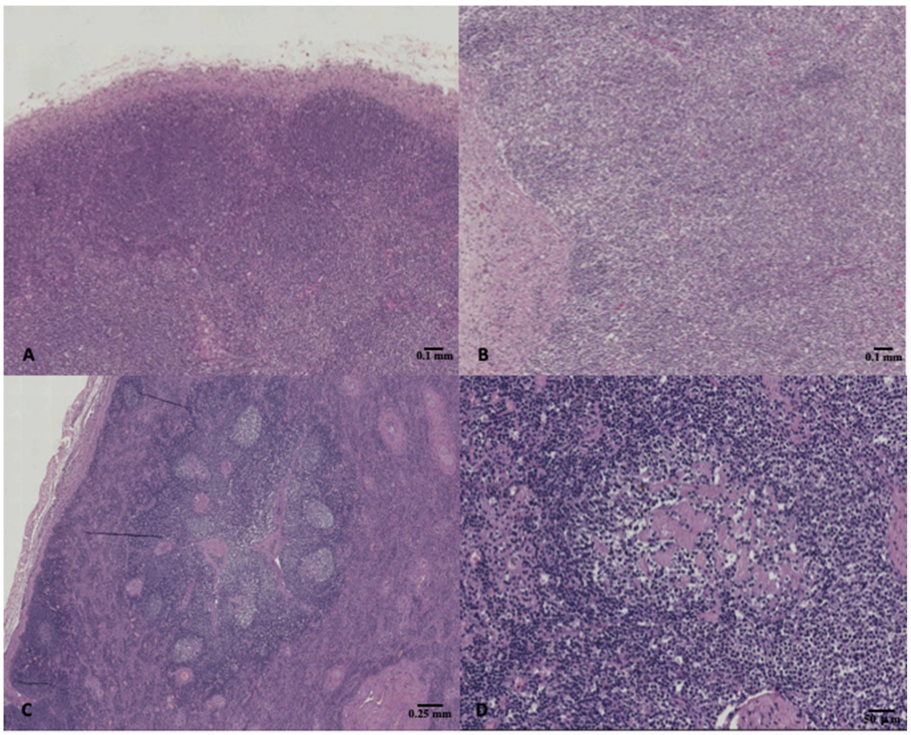

FIGURE 1 | (A) Striped dolphin (*Stenella coeruleoalba*) ID 218, normal lymph node, hematoxylin & eosin (H&E), 4X magnification; **(B)** Bottlenose dolphin (*Tursiops truncatus*) ID 229, lymph node hypoplasia characterized by lack of secondary follicles together with the reduced number of primary follicles, H&E, 4X magnification; **(C,D)** Bottlenose dolphin (*Tursiops truncatus*) ID 196, lymph node presenting hyperplastic changes such as reactive follicles which had a paler staining germinal center, H&E, 2X, and 10X magnification respectively.

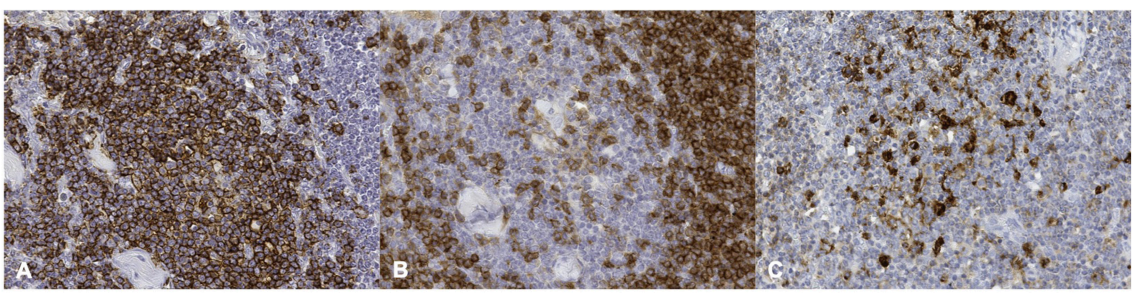

FIGURE 2 | Striped dolphin (*Stenella coeruleoalba*) ID212, lymph node. **(A)** positive cell membrane staining of CD20 antigen; **(B)** positive cell membrane staining of CD5 antigen; **(C)** positive cell membrane staining of HLA-DR antigen (Mayer hematoxylin counterstained; original magnification 40X).

and the presence of ongoing infections. The only value that approached a strong association (p-value <0.05) was obtained comparing the total number of CD20-IR cells (B lymphocytes) in normal and hypoplastic lymph

TABLE 5 | Number of immunoreactive cells in cetacean lymphoid tissues.

	CD5*	CD20*	HLA-DR*
Lymph node	2391.72 ± 1053,96	2398.64 ± 965,97	2158.64 ± 808.91
Spleen	1293.67 ± 877.45	1524.17 ± 336.99	1158.17 ± 438.77
Thymus	2570.67 ± 1694.94	0	3181 ± 1587.98

**Results expressed as mean ± standard deviation of immunoreactive cells per organ.*

nodes (0.09). No other statistically significant correlations were found.

No other factor, either intrinsic or extrinsic, appeared to influence the different lymphoid cell marker expression in the specimens under study.

Toxicological and Statistical Analysis

The blubber organochlorine (OC) levels of IDs 196, 214, 218, 221, and 229 specimens are shown in **Table 6**. HCB was the contaminant present with the lowest levels in all specimens, with the 3 bottlenose dolphins showing greater concentrations of the all striped dolphins. PCBs were generally higher than DDTs, except for samples of IDs 218 and 221. The main DDT component was pp' DDE. The higher levels of DDTs and PCBs were in one bottlenose dolphin (ID 196), which had about 1124 ppm lipid weight of PCBs and about 430 ppm lipid weight of DDTs, which represent very high levels at an absolute level (29). Very low percentages of extracted organic matter, showing a relevant depletion of blubber layer, indicates a high metabolic stress of the specimens that have mobilized many of your fatty acids, probably due to a sudden weight loss. IDs 214 and 299 had the lowest concentrations of chlorinated xenobiotics, and shows much lower levels compared to other specimens. Despite this, PCB levels of all specimens were greater than the estimated toxicity threshold (17 mg/kg l.w.) set by Jepson et al. (30) and Kannan et al. (31) for cetaceans. This could suggest an important toxicological stress for these cetaceans.

A simple linear regression was calculated to predict if the amount of OCs could influence the number of inflammatory cell subpopulations. A moderate negative correlation was found between the presence of high quantity of contaminants (PCBs and DDTs) and the number of T lymphocytes ($r = 0.82$ and 0.80, respectively) (**Figure 3**).

TABLE 6 | Results of toxicological analyses.

Compound	ID 196	ID 214	ID 218	ID 221	ID 229
HCB	334.84	89.48	44.95	6n.r.	439.62
30	451.23	266.09	n.r.	56.28	n.r.
95	16588.07	584.08	469.96	799.87	1106.31
op'DDE	1823.45	342.50	441.47	615.93	306.74
101	7267.45	647.43	623.33	1179.92	1062.45
99	113.34	45.86	21.54	39.93	41.13
pp'DDE	306193.29	13268.39	45267.65	52130.65	13803.44
op'DDD	3848.19	243.82	335.49	534.22	315.88
151	23021.64	416.01	622.48	1042.45	650.70
144+135	11355.44	429.08	616.55	967.36	1359.61
149+118	65791.62	1795.42	3112.24	5411.77	3388.50
pp'DDD	11435.87	298.43	981.60	1480.01	965.84
op'DDT	6239.51	309.64	1146.77	1415.65	364.09
146	21296.80	661.01	1212.64	2056.73	1098.32
153	231180.34	3378.12	7414.99	13113.22	4749.11
141	14616.87	206.97	419.90	731.71	219.55
pp'DDT	8387.76	597.06	1249.44	1979.78	658.78
138	124897.00	1743.53	4229.88	7683.19	3391.53
178	12815.13	198.39	419.35	830.33	956.76
187	64919.21	1207.60	2363.71	4366.82	1017.67
183	23533.58	348.56	645.61	1251.99	167.59
128	11117.99	146.62	428.19	764.03	273.17
174	20942.67	335.64	796.84	1544.77	327.74
177	17088.20	224.54	549.64	1025.84	247.06
156 + 171 + 202	11683.37	200.10	424.70	867.35	171.46
172	3338.75	105.79	199.20	467.22	158.07
180	95382.50	1643.69	3222.03	5842.16	631.96
199	619.97	17.61	42.80	46.62	3.82
170	55203.49	786.33	1766.05	3395.17	280.53
196	16114.29	420.94	490.62	1005.19	92.91
201	14128.10	339.57	390.15	719.23	43.03
195	8113.46	267.99	229.00	540.01	38.68
194	9650.27	235.34	260.04	533.63	196.43
206	1191.47	66.28	n.r.	117.87	n.r.
209	418.35	n.r.	n.r.	45.75	n.r.
PCB tot ps	881971.01	16452.51	30971.45	56344.39	21674.07
DDT tot ps	337928.05	15059.84	49422.41	58156.23	16414.76
OC tot ps	1220233.90	31601.83	80438.82	114560.63	38528.46
EOM%	78.50	88.40	51.00	61.80	76.50
HCB bl	426.55	101.23	88.14	97.09	574.67
PCB tot bl	1123529.95	18611.44	60728.34	91172.15	28332.12
DDT tot bl	430481.60	17036.01	96906.69	94103.94	21457.20
OC tot bl	1554438.10	35748.68	157723.17	185373.18	50363.99

DISCUSSION

The validation of a panel of antibodies reacting against some cetacean antigens opens the possibility of a better understanding of the morpho-functional organization of cetaceans' lymphoid tissues. Although monoclonal antibodies identifying distinct immune cell populations and sub-populations are essential to investigate the role of these cells in the pathogenesis and evolution of different infectious disease conditions, the information about cross-reactivities of antibodies between phylogenetically distant species are important to correctly interpret data obtained from their use (32). Antibodies against human, bovine, mouse and ovine surface antigens such as HLA-DR, CD2, CD4, B cells, and TCR have already been reported to label leukocytes of beluga whale and bottlenose dolphin (9, 13) and different monoclonal antibodies targeting lymphocyte surface antigens for cetaceans were also used in several previous studies aimed at investigating the leukocyte populations residing in cetaceans' lymph nodes (13, 32).

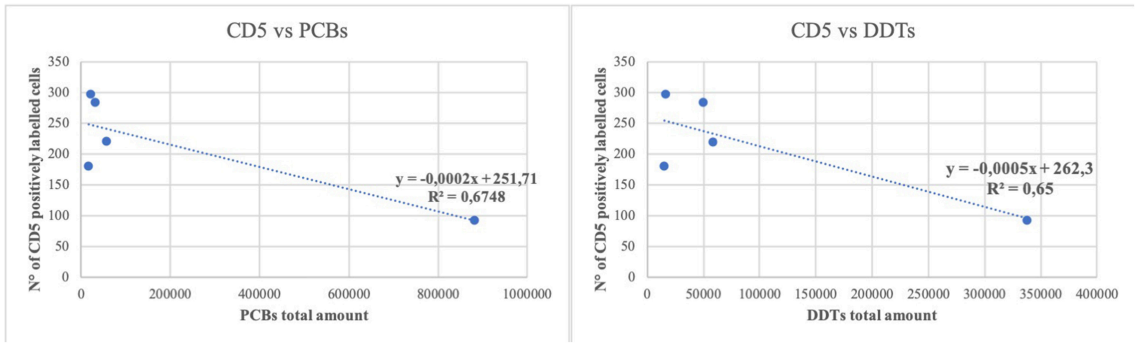

FIGURE 3 | Linear regression between the number of CD5 positively labeled cells and the total amount of polychlorobiphenyl (PCBs) and dichlorodiphenyltrichloroethane compounds (DDTs). Regression lines are reported. R 2 = determination coefficient.

In the present investigation, the antibody specificity validation was achieved following standard guidelines for veterinary laboratories which include, beside the immunoreactivity pattern and the proper use of positive and negative control tissues/matrices, the demonstration of specific immunoreactivity with the respective antigen(s) by Western Blotting analyses (33). The herein investigated Abs resulted valuable for IHC application on formalin-fixed, paraffin-embedded tissues of striped dolphins and bottlenose dolphins, thus mirroring the immunoreactivity patterns observed in other mammalian species (34, 35). Despite the small number of samples, leukocyte subsets' counting and the subsequent statistical analysis revealed that hypoplastic lymphoid tissues were associated with a lower number of B lymphocytes. Interestingly, many animals showing these features (40%) suffered from an ongoing infection, albeit not showing any other morphological alterations.

A possible reason for the decreased B lymphocytes' number could be related to the immune response impairment cause by pathogens like cetacean morbillivirus (CeMV), as already reported in the literature (1). Morbilliviral infections have long been known to result in host immune suppression (36–38), although the most relevant viral effects are a decreased mitogen-induced proliferation and an increase in lysozyme concentration of CD4+ T lymphocytes and a marginally significant increase in monocyte phagocytosis (38).

In this respect, our data seem to be in contrast to those available in the literature, with special reference to CeMV/DMV infection. One of the possible explanation of these apparent discrepancies could be the result of the cetacean host's dominant "immunophenotype." In this respect, however, despite its pivotal role in driving the evolution and the final outcome of many infectious disease processes, as the natural history of *Human Immunodeficiency Virus* (HIV) infection has taught us (39), we are not aware, thus far, of any study focused on the pathogenetic evolution of CeMV/DMV infection—as well as of *Herpesvirus*, *Toxoplasma gondii*, and *Brucella ceti* infections—among T helper 1 (Th1)-dominant vs. Th2-dominant cetacean hosts (40).

In addition to infectious agents, lymphoid cell depletion might be caused by environmental contaminants in cetaceans. Although a strong real effect of xenobiotics on the immune system, especially for the dioxin-like poly-chloro-biphenyl compounds (PCBs), has been well established in laboratory rodents (41). Only few studies are focused on the influence of xenobiotics on the immune function of whales and dolphins. In our investigation, preliminary in-depth ecotoxicological analyses carried out on 5 specimens revealed a moderate negative correlation between high tissue levels of PCBs and DDTs and the number of CD3-IR cells (T-lymphocytes). A reduced mitogen-induced T cell proliferation associated with elevated PCB and p,p0-dichlorodiphenyltrichloroethane (DDT) blood levels has been reported in free-ranging bottlenose dolphins on the coast of Florida, suggestive of a contaminant-induced inhibition of the cellular immune response (42). However, definitive conclusions about the impact of the pollutants under study on the health status were limited by the small number of investigated dolphins and by the lack of controls specimens (42). Lymphoid tissue hypoplasia is primarily associated with elevated polybrominated diphenyl ether (PBDE) tissue levels, while there is no apparent correlation with the health and nutritional *status* in bycaught animals, supporting the hypothesis of a contaminant-induced immune deficiency (43). The immunotoxicity of several xenobiotics on cetacean blood leukocytes at concentrations equivalent to those observed in wild marine mammal populations has been also verified *in vitro*. Noteworthy, DDT and non-coplanar PCB congeners inhibited spontaneous and mitogen-induced proliferation of beluga whale lymphoid cells, while coplanar (dioxin-like) PCB congeners and TCDD failed to modulate leukocyte function (19). Further in *vitro* experiments have confirmed the aforementioned inhibitory effects on phagocytosis of neutrophils and monocytes of bottlenose dolphins and beluga whales. The dominating effect of non-coplanar PCB congeners is suggestive of a modulation of the leukocyte function in an aryl hydrocarbon receptor-independent manner in these marine mammals (44). Similarly to T lymphocytes, mitogen-induced proliferation is mainly modulated by POPs and PCBs also in marine mammal B cells (44, 45), although clear-cut effects have not been defined on either lymphocyte populations.

In this respect, the limited number of the investigated cetacean specimens do not allow definitive conclusions. Indeed, non-uniform results were reported also in human beings after chronic exposure to PCBs. For instance, the consumption of pilot whale's (*Globicephala melas*) meat harboring high PCB levels has been associated, in a cohort of children from the Faroe Islands, with a reduced antibody production in response to vaccination against some infectious pathogens as an indirect indicator of the effects on B cell populations (46). On the other hand, children living in highly polluted areas showed a significant increase in B lymphocytes related to chronic PCB exposure, along with a T cell increase and an NK cell decrease, respectively (47).

Recent publications highlighting the re-emerging threats of these substances increase our concerns for marine mammals' health and conservation (7, 48). Future research on wild cetaceans and, more in general, on free-living aquatic mammals should be aimed at evaluating the effect of environmental pollutants on host-pathogen interaction dynamics, thereby assessing whether contaminant-induced immunotoxicity could be related to a CD4+ T helper cells and/or to a CD8+ cytotoxic T cell reduction, together with a decreased splenic humoral immune response, as it happens in laboratory rodents exposed to lipophilic environmental contaminants (11). Further studies are also needed to precisely define the effective role of CeMV/DMV as an immune-suppressive pathogen, with special emphasis on the viral effects on T and B cell populations, along with its interplay with a progressively increasing number of persistent environmental pollutants.

ETHICS STATEMENT

Tissues for this project have been provided by the Mediterranean Marine Mammal Tissue Bank, Department of Comparative Biomedicine and Food Science, University of Padova, and by Institute of Animal Health (University of Las Palmas de Gran Canaria).

AUTHOR CONTRIBUTIONS

CC and SM performed Mediterranean dolphins' necropsy, tissue sampling, and contributed to manuscript writing. LD performed western blot analysis, while CC and RZ executed IHC analysis and LM toxicological analysis and all of them contributed to manuscript writing. AF, MA, and ES performed Atlantic dolphins' post-mortem examination and tissue sampling. MC and GDG reviewed critically the manuscript. All authors reviewed and agreed on the current version of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00888/full#supplementary-material

Supplementary Figure 1 | Western blot analysis was performed by loading 4 μg of human tonsil (Hu), 2.5 ug of striped dolphin (SC) and bottlenose dolphin (TT) cell membrane extract from lymph node onto an 12% SDS polyacrylamide gel. **(A)** CD5 monoclonal rabbit antibody (Biocare medical, USA product CRM 328) at a dilution of 1:500. **(B)** CD20 polyclonal rabbit antibody (Thermo Scientific product RB-9013) at a dilution of 1:2,000. **(C)** HLA-DR monoclonal mouse antibody (DakoCytomation, Denmark product M0746) at a dilution of 1:2,000.

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MINI REVIEW
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Acute Phase Proteins in Marine Mammals: State of Art, Perspectives and Challenges

Maria Elena Gelain[1]* *and Federico Bonsembiante*[1,2]

[1] Department of Comparative Biomedicine and Food Science (BCA), University of Padova, Padova, Italy, [2] Department of Animal Medicine, Productions and Health, University of Padova, Padova, Italy

The term "acute phase response" (APR) is referred to a nonspecific and complex reaction of an organism that occurs shortly after any tissue damage, such as infection, trauma, neoplasia, inflammation, and stress. The APR can be identified and monitored with some laboratory tests, such as the concentration of several plasma proteins, the acute phase proteins (APPs). The APPs are components of the non-specific innate immune response, and their plasma concentration is proportional to the severity and/or the extent of tissue damage. The evaluation of health status of marine mammals is difficult because the classical clinical signs of illness used for human and domestic animals are difficult to recognize and understand. For this reason, in the past years, several efforts were done to identify laboratory markers of disease in these animals. The APPs have demonstrated their role as early markers of inflammation in veterinary medicine, thus several APPs were tested in marine mammals, such as C-reactive protein (CRP), serum amyloid-A (SAA), and Haptoglobin (Hp). However, the difficulty to extrapolate the knowledge about APPs in one species to another, the lack of specie-specific reagents, the absence of data about negative APPs have hampered their extent use in marine mammals. Herein, the state of art of APPs in marine mammals is reviewed, with particular attention to pre-analytical and analytical factors that should be taken into account in validation and interpretation of APPs assays. Moreover, the current application, potential utility and the future developments of APPs in marine mammals is highlighted and discussed.

Keywords: marine mammals, immune system, acute phase reaction, acute phase proteins, serum proteins

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Mike Criscitiello,
Texas A & M University, United States

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Annalisa Pinsino,
Istituto di Biomedicina e di Immunologia Molecolare Alberto Monroy (IBIM), Italy
Cara Lisa Field,
Marine Mammal Center, United States

***Correspondence:**
Maria Elena Gelain
mariaelena.gelain@unipd.it

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INTRODUCTION

The mammalian immune system includes innate or nonspecific immunity as well as adaptive or specific immunity. The responses of these two different pathways are distinct, but highly interconnected. The first reaction of an organism to different pathological conditions is an innate, non-specific response (1), a more conserved response during evolution which aim is the immediate reaction against pathological stimuli (2). After the initial recognition of pathogens or tissue damages by the tissue-resident macrophages, which express the pattern recognition receptors (PRRs), a variety of different inflammatory mediators are produced by leukocytes, endothelial cells, tissue cells or are derived from plasma proteins. These mediators include different chemokine, cytokine, vasoactive amines and products of the arachidonic acid: their primary effect is to elicit inflammatory response locally and to recruit leukocytes and plasma proteins in the site of injury (3).

At the same time, many of these inflammatory mediators influence the function of many tissues and organs throughout all the organisms, inducing the acute phase response.

The activated macrophages on the site of injury are also responsible of the activation of the adaptive immune response, a more slowly, but more focused response. The expression of major histocompatibility complex (MHC) class II antigens, combined with foreign antigen, by tissue macrophages leads to the antigen-dependent activation of T-cells and start the precise immune response against that specific target. T-cells can differentiate in different kinds of T-helper (Th) lymphocytes with the expression of different cytokine pattern. Th1 cells coordinate the interaction between innate and cell-mediated adaptive response by means of different cytokines, among them IFN-γ and IL-2. Differently, Th2 cells, which produce IL-4 and IL-5, promote humoral immune response, the differentiation of B-cell into plasma cells, but have also an anti-inflammatory activity by producing IL-10 (4).

The term "acute phase response" (APR) refers to a nonspecific and complex reaction that occurs shortly after tissue damage due to several conditions such as infection, trauma, neoplasia, inflammation, and stress (5). The reaction of APR includes systemic consequences, such as fever, leucocytosis, increased release of several hormones and drastic rearrangement of plasma protein synthesis (6). These changes include a decrease of plasma low and high density lipoproteins-bound cholesterol, an increase of ACTH and glucocorticoids, the activation of complement and coagulation system, a decrease of calcium, zinc, iron, vitamin A, and α-tocopherol serum levels. All these changes in blood plasma composition induced by APR are beneficial to the organism because they prevent microbial growth and help restore of homeostasis (1). Moreover, a considerable change in concentration of several plasma proteins, the acute phase proteins (APPs), is evident few hours after an inflammatory stimulus (7).

APPs are a group of blood proteins that change in concentration in animals subjected to external or internal challenges, such as infection, inflammation, trauma or stress, proportionally to the severity of the disorder and/or the extent of tissue damage (8). They are synthesized primarily by hepatocytes stimulated by cytokines or endogenous glucocorticoids (9, 10). APPs are classified based on the direction of change: synthesis of positive APPs is upregulated with a resulting increase in blood concentration, and synthesis of negative APPs is downregulated with a resulting decrease in blood concentration (11). Positive APPs are further classified as major, moderate or minor based on the magnitude of increase: major APPs showed an increase of 10- to 100-fold, moderate APPs showed an increase of 2- to 10-fold, and minor APPs have only a slight increase (5). Also the rate of the concentration increase varies between major, moderate, and minor positive APPs: major APPs usually have a marked increase within 48 h after a pathogen stimulus and a subsequent rapid decline after the cessation of stimulus due to their short half-life (12). Moderate and minor APPs usually have a slower increase, but also a slower decline to normal value and so they usually increase during chronic inflammatory process (5). However, the APPs response varies between species: the major APP in dogs and men is C-reactive protein (CRP) (13), in cats is α1–acid glycoprotein (AGP) (9), in ruminants is Haptoglobin (Hp) (14); in horse is Serum Amyloid A (SAA) (15) and in pigs are Hp, SAA, and major acute phase protein (MAP) (10).

Recently, in veterinary medicine, studies on the role of APPs as markers of infectious, inflammatory and neoplastic diseases have proliferated (16) and at least 40 different plasma proteins have been identified as APPs (8). Their use as marker of homeostasis perturbation provides some advantages compared with traditional parameters like the white blood cell (WBC) counts. Compared to WBC count, the diagnostic sensitivity of APPs is higher and the change in concentration is faster (17). Moreover, their stability in serum/plasma is high, so it is possible to measure APPs in frozen samples (18). One limitation of the APPs is the poor diagnostic specificity, for this reason they cannot be used as primary diagnostic test for a specific disease, but they were successfully used to detect subclinical diseases and to monitor clinical evolution and to assess the response to treatment (5). Additionally, the combined measurement of several APPs provides more information than the evaluation of a single protein: in the "APP value index," proposed by Gruys et al. (1), sensitivity and specificity are improved by combining response of both positive and negative major and minor APPs.

Marine mammals are a group of around 130 mammalian species which depend on water environment for most of their needs. They include 3 orders: Carnivora (pinnipeds, otters, and polar bear), Cetacea (dolphins, whale, and porpoise) and Sirenia (manatee and dugogons). Marine mammals are differently adapted to life in water, with some species, which are fully aquatic (cetaceans and sirenia), and others that spend part of their life on land (pinniped and polar bears) (19). From an immunological point of view, aquatic adaptation caused few differences in distribution and function of immune system between marine and terrestrial mammals (20). However, nowadays the marine mammal' immune system is deeply exposed to environmental pollution because they are a long-lived animals placed at the top of food chain, thus they are exposed to a progressive bioaccumulation of fat-soluble pollutants, such as PCBs, which affect both innate and adaptive immune function (21). For these reasons, increasing knowledge in cellular and humoral immune response is continuously required to understand their immune system and in particular its relationship with infectious pathologies and the environmental pollution (22). Furthermore, marine mammals live also in controlled environment like aquaria, rehabilitation facilities and research center where health assessment is fundamental to evaluate the correct management of animals and to monitor the response to therapy during rehabilitation. From this perspective, the availability of new markers to asses immune functions is fundamental both for medical care and research purpose (20).

ACUTE PHASE REACTION AND ACUTE PHASE PROTEIN IN MARINE MAMMALS

Innate immune response represent the first line of response against pathological stimuli, it's very fast and it's based primarily on effector cells (e.g., mast cells, macrophages, neutrophils) and

antimicrobial substances (e.g., complement, reactive oxygen, and nitrogen species). On the other hand, adaptive immune system is antigen-specific, it takes more days to be effective and it's based on different T-cells response and on B-lymphocytes which are responsible of humoral response mediated by the different subclass of immunoglobulin (IgG, IgM, IgA) (20). Several assays were proposed to evaluate both immune response in marine mammals, generally based on isolated leucocytes with the aim to evaluate the leukocytes response against *in vitro* stimuli (23).

To assess the response pattern of cetaceans' cellular innate immune system, the phagocytosis and the generation of reactive oxygen species of polymorphonuclear leukocytes were investigated. In particular, *in vitro* ingestion of latex beads and hydrogen peroxide production have been evaluated in beluga whales (*Delphinapterus leucas*) and in bottlenose dolphins (*Tursiops truncatus*) (24, 25) whereas phagocytosis and respiratory burst assay, using whole blood from bottlenose dolphins, were used to assess the antimicrobial activity (26). In addition, the investigation of APR by analyzing the cytokine expression gives important information on the functionality of lymphoid cells. The production of specie-specific antibodies allows the development of immunological assays for the quantification of cytokine expression useful to investigate the inflammatory response in whales and dolphins. The coding regions of IL-2, IL-1β, IL-6, and TNF-α gene of the beluga whale have been sequenced, and a cytokine-specific rabbit antisera have been produced (27–29). In harbor porpoise (*Phocoena phocoena*), the quantification of mRNA of IL-1β, IL-2, IL-4, IL-6, IL-10, and TNF-α have been performed by RT-PCR (30), and the increase of IL-10 was seen in harbor porpoises suffering from long lasting infectious (31). Also in bottlenose dolphins, pacific white-sided dolphins (*Lagenorhynchus obliquidens*), and beluga whales, IL-2, IL-4, IL-10, IL-12, IL-13, IL-18, TNF-α, TGF-β, and interferon (IFN)-γ quantification was performed using RT-PCR (32). An IL-2 receptor expression assay and an IL-6 ELISA were developed in bottlenose dolphins and killer whale (*Orcinus Orca*), respectively (33, 34).

Similarly, both innate and the cell-mediated response were studied in pinnipeds. To better understand the innate response, phagocytic activity of isolated peripheral blood leukocytes was evaluated in harbor seal (*Phoca vitulina*), gray seal (*Halichoerus grypus*), and harp seal (*Phoca groenlandica*) pups, in harbor seal female during lactation and in harbor seal pups admitted to rescue center (35, 36). The authors found an age-related variation in both pups and adults: phagocytosis increased with age in gray and harbor seal pups, while in female harbor seals decreased from sub-adult to adulthood. At the same time, pups after rehabilitation showed a decreased phagocytic activity, probably due to the decreased stimulation of innate response after therapy. Also cytokine response was evaluate in harbor seal. Pro-inflammatory cytokine mRNA (IL-1β, IL-6, IL-8, and IL-12) in pups in a rehabilitation center were higher at admission whilst IL-4 was higher before the release (37), demonstrating the recovery from inflammation. Recently, a multiplex canine cytokine assay was validate in harbor, gray and harp seal to measure proteins levels in cell culture supernatant of peripheral blood mononuclear cells (PBMC) (38).

However, all these techniques are not generally applicable in a clinical setting in which the primary goal is a sensitive diagnostic tool with a rapid turnaround, even if give us important information on factors affecting cetaceans' immune system. For this reason, in the past years, several efforts were made to identify laboratory markers of disease in these animals. First parameters tested were WBC and erythrocyte sedimentation rate (39). However, even if they are inexpensive and rapid, they lack specificity and sensitivity. Moreover, changes in WBC occur after several hours after inflammatory stimuli. Thus, efforts were directed to identify inflammation at earlier stage (40). To examine the humoral response, species-specific antibodies against IgG were produced and used to evaluate serum IgG levels in killer whale by radial immunodiffusion assay (41) and by competitive ELISA in bottlenose dolphins (42, 43). The determination of IgG baseline values in free-ranging and in managed dolphins revealed higher levels of immunoglobulin in the first group with several values over the accurate range of the assay, probably due to the higher parasitic load in free-ranging dolphins (43).

Serum total protein, albumin, globulin and albumin:globulin ratio (A:G) are undoubtedly among the most measured markers in basic health assessment in domestic animals as well as in marine mammals. Serum protein electrophoresis is also broadly applied in veterinary medicine and it has the advantage to produce an accurate measurement of albumin and the visualization of globulin fractions (44). The interpretation of total proteins values and electrophoretic pattern of serum proteins is receiving increased attention also in marine mammals in which a typical pathologic pattern could be identified in inflammatory diseases (40). Reference intervals for these markers are available for free-ranging bottlenose dolphins (45) and, compared to these, recently data on managed dolphins showed slightly lower values of TP, α-globulins, and γ-globulins and higher albumin and albumin/globulins ratio (46).

It's interesting to note that Hp, α1-antitrypsin, α1-antichymotripsin, and α2-macroglobulin migrate in the α-globulins fraction, while the IgG and CRP migrate in the γ-globulins fraction. Albumin acts as a negative acute phase protein since the synthesis of this protein is decreased during an inflammation (47). Thus, the lower concentration of positive APPs associated to a higher concentration of albumin and the consequent higher albumin/globulins ratio could reflect lower antigenic stimuli in managed population compared to the free-ranging populations (36). Serum total protein analysis were used to assess health status in several cetaceans species such as pantropical spotted dolphins (*Stenella attenuata*) (48), beluga (49), minke whales (*Balaenoptera acutorostrata*) (50) and killer whales (51) as well as in other marine mammals, like harbor seals (*Phoca vitulina*) (52) and walruses (*Odobenus rosmarus*) (53). In all these species, serum total protein analysis was demonstrated to be one of the most used and commonly accepted marker of inflammation.

However, specific APPs have demonstrated their superior role as early markers of inflammation, so based on the results obtained in humans and companion animals, several positive APPs were tested in marine mammals (**Table 1**). Published

TABLE 1 | Acute phase proteins: site of production, role and marine mammal species in which they are validated.

Acute phase protein	Site of production	Role	Positive/ negative	MAJOR APP IN	Validated in	References
CRP	Hepatocytes	Complement activation and opsonisation; modulation of monocytes and macrophages; cytokine production; binding of chromatin; prevention of tissue migration of neutrophils	Positive	DOG, HUMAN	Bottlenose dolphin; Harbor porpoise; Harbor seal	Cray et al. (54); Fonfara et al. (37); Müller et al. (55)
SAA	Hepatocytes	Transport of cholesterol from dying cells to hepatocytes; inhibitory effect on fever; inhibitory effect on the oxidative burst of neutrophilic granulocytes; inhibitory effect on *in vitro* immune response; chemotaxic effect on monocytes; polymorphonuclear leucocytes and T lymphocytes; induction of calcium mobilization by monocytes; inhibition of platelet activation	Positive	HORSE, PIG	Bottlenose dolphin; West Indian manatee	Cray et al. (54); Harr et al. (56); Cray et al. (57)
Hp	Hepatocytes	Binds hemoglobin dimers so that iron is not available to organisms; bacteriostatic effect; stimulation of angiogenesis; role in lipid metabolism; immunomodulatory effect; inhibition of neutrophil respiratory burst activity	Positive	COW, PIG	Bottlenose dolphin; Harbor porpoise; Harbor seal; Stellar sea lion; Ringed seal; West Indian manatee	Cray et al. (54); Frouin et al. (35); Fonfara et al. (37); Harr et al. (56); Müller et al. (55); Zenteno-Savin et al. (58); Rosenfeld et al. (59)
AGP	Hepatocytes	Several anti-inflammatory activities	Positive	CAT		Not validated
fibrinogen	Hepatocytes	Hemostasis	Positive		Bottlenose dolphin; West Indian manatee	Terasawa et al. (60); Harr et al. (56)
albumin	Hepatocytes	Major contributor to oncotic pressure, transports Ca2+, Mg2+, unconjugated bilirubin, fatty acids, thyroxine, and many other substances	Negative		Bottlenose dolphin; Spotted dolphin; White whales; Minke whales; Killer whales; Harbor Seals; North Atlantic walrus	Schwacke et al. (45); Gili et al. (46); St. Aubun et al. (48); Tryland et al. (49); Tryland and Brun (50); Robeck and Nollens (51); Greig et al. (52); Tryland et al. (53)
PON	Hepatocytes	Protection against oxidative stress; protection against organophosphate compounds	Negative			Not validated

works had the primary aims to evaluate the feasibility of the assays to measure the APPs, to validate the antibody-based assay and to determine the RIs. In bottlenose dolphins three APPs (CRP, SAA, and Hp) were tested, even if not always complete validation studies were performed (54, 61). For these APPs, the authors established the RIs in free-ranging and managed dolphins using automated assays (54) and they found significantly lower SAA and higher Hp levels in free ranging animals. The only clinical significance of these alteration was a higher ability to detect chronic inflammation for Hp. Regarding Hp, Segawa and colleagues validated commercially available Hp-ELISA and Hp-hemoglobin binding assay in bottlenose dolphins with ''acceptable" intra- and inter-assay imprecision (CV: 3.3% healthy dolphins and 3.5% inflamed dolphins; CV: 10.4% healthy dolphins and 21.7% inflamed dolphins) and demonstrated that Hp levels in the serum increase under inflammatory conditions (62).

Positive APPs were tested also in Florida manatees (*Trichechus manatus latirostris*) to define the more accurate marker of inflammation. Five different APPs were tested: AGP, CRP, Hp, fibrinogen, and SAA. SAA showed the highest diagnostic sensitivity and specificity (90% for both sensitivity and specificity) in the detection of inflammatory diseases, the diagnostic specificity of Hp and fibrinogen were 93 and 95%, respectively, while their diagnostic sensitivity were 60 and 40%, respectively, (56). When used in stranded manatee suffering from cold stress and trauma, SAA showed 93% of sensitivity and 98% of specificity in detecting diseased animals (57). By contrast, the Abs used for the determination of AGP and CRP did not cross-react in this species (56).

In harbor seal, an Ab anti-CRP and a competitive immunoassay was produced (63), but Hp is probably the APP most used in pinnipeds. A multispecies assay based on hemoglobin binding capacity was used to demonstrate as Hp is a sensitive marker of the health vs. disease status in harbor seal (64). In seal pups admitted in a rescue center. Hp, total protein, IgG and globulin values correlated positively, but Hp levels increased during the hospitalization, probably reflecting age-related changes (35). Hp is considered a health marker also in Steller sea lions (*Eumetopias jubatus*): significantly higher levels of Hp were found in declining population compared to more stable ones (58). However, also genetic differences between

distant and isolated population of wild animals could be the causes of this difference, not only a pathological condition.

If some data on marine mammals positive APPs are available in literature, quite surprising are the lack of data available on negative APPs. For these reasons, the possibility to evaluate the usefulness of an "APP value index" is still far from being applied.

ACUTE PHASE PROTEIN IN MARINE MAMMALS: CHALLENGES AND FUTURE DEVELOPMENTS

The availability of sensitive markers of inflammation both for free-ranging and managed marine mammals is nowadays considered fundamental to evaluate the health status and, in rehabilitation setting, to monitor the response to therapy and to define the prognosis. As serum markers, the APPs have several advantages: they have longer stability compared to other blood component such as WBC; they can be performed on frozen serum, thus the samples can be shipped to references laboratories; some assays can be automated to obtain results in an excellent turnaround time.

However, is important to consider that the knowledge about APPs in one species cannot be readily generalized to another species, in which healthy levels, response to inflammation or infection, and prognostic significance may be different (65). Moreover, the evolution of marine mammals and their adaptation throughout the millennia to an aquatic environment had led to a different physiology and metabolism compared to terrestrial mammals. Thus, the understanding of the genetic, phenotypical and biochemical properties of marine mammals APPs are essential prior to using them as a new biomarker.

An example of how the biochemical properties influence the analytic method is paroxonase-1 (PON1), a HDL-bound esterase which protects against organophosphate compounds, acts as negative APP and as oxidative stress marker. PON1 is usually assessed by enzymatic method and, based on the different PON1 functions, several substrates have been identified to evaluate serum PON1 activities. Nevertheless, both in humans as in some terrestrial mammals, PON1 gene polymorphisms highly influence the enzymatic activity toward different substrate: the single-nucleotide polymorphisms (SNPs) Leu55Met and Gln192Arg increase the paraoxonase activity (66) in humans and different PON1 genotypes influence activities toward paraoxon and phenyl-acetate in rabbit (67). Also in cows, some SNPs in the promotor region of PON1 gene are associated with serum PON1 activity (68). Recently, a phylogenetic study on convergent functional losses across marine mammals, has identified a PON1 functional loss in marine mammals, probably related to their different lipid metabolism and fatty acid oxidation due to adaptation to the marine environment and a high concentration of ω-3 fatty acids on their diet. As a consequence, in several marine mammals species paroxonase activity is very low, while enzymatic activity against other PON1 substrates is still present, such as arylesterase activity (69). For these reasons, the use of classical enzymatic assays is hampered in these animals and further studies are needed to elucidate the role of PON1 as possible negative APP, oxidative stress marker and the consequences of its inability to detoxify organophosphates compounds.

From an analytical point of view, another challenge in the evaluation of APPs in marine mammals is the need of species-specific assays, especially for the immunological assay, such as ELISA or immunoturbidimetry. This means the development of a *de-novo* method, often a time-consuming and expensive approach, or the validation of a commercial available assay used in other species (65). The latter approach is surely the most used in veterinary medicine, in which some human assays were validated for dogs, cats and horses (62). However, even if some APPs appeared highly conserved among species, an accurate validation of antibody cross reactivity is needed as well as species specific standards and control material (54). Among positive APPs, SAA is the most used across different species: it appeared as the most conserved APP in mammals even if some difference in circulating isoforms were reported (61) and it's considered a major APP in all the mammals in which it was investigated (65). Some commercial SAA assays showed good results also in marine mammals, such as bottlenose dolphin, manatee and striped dolphin *(Stenella ceoreloualba)* (54, 56, 70) and its use as diagnostic and prognostic marker appears nowadays the most promising.

To obtain accurate data, all the pre-analytical factors that could influence the results should be taken into consideration. The effect of storage, temperature and different anticoagulant had to be evaluated in a correct validation process as well as the interference of hemolysis and lipemia, as done in other species (5).

The application of a novel biomarker required a full evaluation of all the analytical performances and the clinical value. This process is usually divided in 4 steps: the assessment of analytical features (precision, accuracy, detection limits), the overlap performance (the ability to detect difference between healthy and diseased animals), the assessment of diagnostic capacity (sensitivity, specificity, accuracy, positive, and negative predictive values) and, at the end, the evaluation of the outcome of the new methods (which is the advantage of the test and its influence in the patient management) (65). In veterinary medicine, the validation studies do not always follow all these steps, mainly due to the lack of resources or technical limitation (44, 65). Also in marine mammals, the majority of studies had performed only some steps (44, 54, 56, 61, 62). This is mainly due to the limitation in species-specific reagents, the number of samples from animal with known health status and, last but not least, the capability to generate appropriate reference intervals, hampered the possibility to perform complete validation studies.

Population-based reference intervals derived from an appropriate group of reference individuals are usually required for diagnostic purpose (71). However, a number of biological factors have to be taken in consideration to select the appropriate reference population. Surely, age, sex and pregnancy could be used for partitioning (45, 72), but in marine mammals greater attention should be given to the difference between wild and managed animals. Serum protein electrophoresis values obtained in managed bottlenose dolphins showed lower total proteins and

higher albumin levels compared to reference intervals derived from free-ranging (46) while 9 wild-caught manatees, apparently clinically healthy, had SAA level above reference limit (56). These data could indicate a trend to an inflammatory status or the presence of subclinical inflammation in free-ranging animals which are more exposed to immunological stimuli. In any case, this highlights the need to define appropriate reference intervals for animals living in different environment to have an accurate toll for the evaluation of clinical condition.

Compared to human and companion animals, the use of APPs in marine mammals is just getting started. The increasing need of knowledge on immune system and its response against infectious diseases or chemical pollutants and the request of more sensitive inflammation markers have increased the effort of researchers to study the APR and APPs. Even if APPs are considered a sensitive, but non-specific marker of inflammation, some studies revealed that, in some infectious diseases, APPs showed a specific behavior and biochemical features. One example is the modification of the glycan moiety of AGP in feline infectious peritonitis, FIV and FeLV, influencing the host-pathogens interaction and the immune response (73–75). Currently, some of the greatest threats for wild marine mammals is pathogens, like *Morbillivirus*, *Herpesvirus*, *Brucella ceti,* and *Toxoplasma gondii* (76): the evaluation of APR and APPs patterns during these infectious diseases could lead to the identification of a distinctive response of the immune system and increase the understanding of host-pathogen interaction.

Secondly, for managed or rescued animals, the forthcoming needs are the increase of automated assays, the standardization of procedures across laboratories and the discovery of new markers, for example negative APPs, to generate an APP index also in marine mammals. These new tools will certainly increase the diagnostic and prognostic skills for health assessment and, especially for stranded animals, the development of new "health status" markers will provide valuable resources in evaluating the response to treatment and rehabilitation prior to the release into the wild.

AUTHOR CONTRIBUTIONS

MG and FB analyzed the literature review, designed, and wrote the review.

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Insights Into Cetacean Immunology: Do Ecological and Biological Factors Make the Difference?

Letizia Marsili[1]*, *Giovanni Di Guardo*[2], *Sandro Mazzariol*[3] and *Silvia Casini*[1]

[1] Department of Physical, Earth and Environmental Sciences, University of Siena, Siena, Italy, [2] Faculty of Veterinary Medicine, University of Teramo, Teramo, Italy, [3] Department of Comparative Biomedicine and Food, University of Padua, Padua, Italy

The aim of this study was to evaluate the expression of Major histocompatibility complex (MHC) class I chain-related protein A (MICA) in fibroblast cell cultures of cetaceans (skin biopsies of free-ranging specimens and skin samples of freshly stranded cetaceans) by an immunofluorescence technique and to outline possible variations in MICA expression linked to different ecological and biological factors, while also investigating MICA expression after *in vitro* treatments with different contaminants. Free-ranging or stranded specimens of cetaceans were sampled in the Sea of Cortez (Mexico) (*Balaenoptera edeni, Delphinus capensis,* and *Orcinus orca*) and in the Mediterranean Sea (*Balaenoptera physalus, Physeter macrocephalus, Tursiops truncatus,* and *Stenella coeruleoalba*). Cell cultures were treated with an OC mixture, flame retardants, PAHs, MeHg, and BPA. The three species from the Sea of Cortez showed higher basal activity of MICA and lower levels of DDTs and PCBs than the Mediterranean species. A Pearson's linear coefficient equal to −0.45 also confirmed this tendency to have high levels of MICA and low total OC levels. Treatment of cultured fibroblasts with different contaminants mostly resulted in the upregulation of MICA protein expression by at least one treatment dose; downregulation was also found in some species or treatments. MICA alteration indicates a state of stress of the organism and a modification of the immune system's response and can be proposed as a non-invasive immunological marker that can be measured in skin biopsy samples, thus offering a good alternative to blood measurements.

Keywords: MICA, cetaceans, OCs, PBDEs, PAHs, MeHg, BPA

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***Correspondence:**
Letizia Marsili
letizia.marsili@unisi.it

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INTRODUCTION

Studies on the immune system of cetaceans have followed one another over the years and focused on endpoints of innate and adaptive immunity, such as the evaluation of cellular and humoral immune responses (lymphocyte transformation assay, natural killer cell activity, phagocytotic activity, and respiratory burst humoral immune responses), insights into cytokines, and acute phase responses, immunophenotyping of cetacean lymphoid cells, morphology, and pathology of lymphoid organs, and immunotoxic effects of environmental contaminants (1–4). Almost all investigations have been conducted on blood or on cetaceans found freshly stranded. In bottlenose dolphin, the possibility exists to work on live specimens, not only with captive specimens but also with wild populations, whereas for other cetacean species, carrying out blood sampling is absolutely unthinkable if we exclude the specimens kept in captivity.

Having an in-depth knowledge not only on how the cetacean immune system is composed but also on how it is modulated in the natural environment according to biological and ecological factors, while also considering the effects deriving from environmental pollution, is important.

The information concerning "immunologic baseline data" in natural populations and the possible alterations and/or modulations linked to internal and external factors are lacking. Stranded organisms can have infections and/or suffer from other disease conditions so that the data found cannot always be considered as baseline. In most of the published studies, the immunotoxic effects of several contaminants or mixtures were investigated only *in vitro*.

In this context of objective limitations, only a few studies have provided information on possible diversity in the immune response, for example, between different species, or within the same species among populations living in different habitats. More information can be found in the literature regarding the differential responses to contaminants after *in vitro* leukocyte exposure.

An interesting study was conducted by Levin et al. (5), in which blood was sampled from belugas (*Delphinapterus leucas*) from the Mystic Aquarium (CT) and from bottlenose dolphins (*Tursiops truncatus*) from the U.S. Bottlenose Dolphin Navy Marine Mammal Program (San Diego, CA). Leukocytes were isolated and treated with different coplanar and non-coplanar polychlorobyphenils (PCBs) in different combinations and in combination with tetrachlorodibenzo(P)dioxins (TCDDs). The results showed a significant reduction in the phagocytic capacity of neutrophils and monocytes in both species, with a markedly greater percentage effect in dolphins compared to belugas. The dolphins, moreover, showed a markedly lower percentage of neutrophils and monocytes even in the untreated samples compared to the belugas. Uncertainties remain regarding basic differences between the two species or differences related to the animals' environmental status (captive/non-captive).

A study by Fair et al. (6) found an upregulation of several blood immune system analytes in wild bottlenose dolphins in comparison with under human care dolphins, allowing to hypothesize that variation in environmental conditions (temperature, nutrition, veterinary care, and pathogen/contaminant exposure) can modulate immune responses.

In most cases, contaminants were found to act as immunosuppressors in cetaceans, although some immune responses were also found to be upregulated after *in vitro* exposure to contaminants.

Ritz and Streibig (7) calculated the adverse effects of different concentrations of PCBs, Hg, methylmercury (MeHg) and Cd on lymphocyte proliferation in belugas and bottlenose dolphins; belugas seem less sensitive to PCBs than bottlenose dolphins but more sensitive to Hg and Cd.

Further immunotoxicological studies on the bottlenose dolphin showed that the treatment concentrations considered environmentally realistic for Hg, Cd, and Pb produced a significant reduction in lymphocyte proliferation, whereas Hg, Al, and Cd treatment resulted in decreased lymphocyte phagocytosis. Chromium did not show any effects on any immune assay at the concentrations tested (8).

In a field study conducted by Schaefer et al. (9) in *Tursiops truncatus* from the coasts of Florida and South Carolina, an inverse relationship was found between the Hg concentrations in blood and skin and the absolute numbers of lymphocytes, eosinophils, and platelets.

Following bottlenose dolphin exposure to perfluorinated compounds (PFCs), Fair et al. (10) found statistically significant positive associations between these contaminants and several immunological parameters: absolute numbers of CD2+ T cells, CD4+ helper T cells, CD19+ immature B cells, CD21+ mature B cells, CD2/CD21 ratio, MHCII+ cells, B cell proliferation, serum IgG1, granulocytic, and monocytic phagocytosis. No effects were found on natural killer (NK) cell activity.

In the same species, treatments with coplanar non-ortho-PCB congeners and butyltins (TNT and DBT) significantly reduced the proliferation response of peripheral blood mononuclear cells (11).

No effect was found on bottlenose dolphin natural killer (NK) cell activity and lymphocyte proliferation (T and B cell) after *in vitro* exposure of peripheral blood leukocytes to environmentally relevant perfluorooctane sulfonates (PFOS) and a penta-PBDE mixture (DE-71) concentrations (12, 13).

As previously stated, we presently have a fairly complete picture of the immune system of cetaceans and its interactions with the main classes of contaminants; however, great limitations in the study of natural populations and the modulation of their immune systems persist and are derived essentially from the typology of sampling and related tests developed and applied to date, which include blood sampling and use of freshly stranded animals. *In vitro* experiments are very useful to understand the dynamics of the interaction(s) between contaminants and the immune system but cannot completely illustrate what happens in wild populations. *In vitro* tests, in fact, cannot describe the whole-organism level processes, such as cell interactions and messenger molecules or chemical exposure, absorption, metabolism, and excretion. An urgent need exists, consequently, to search for new non-invasive tests that use more easily sampled tissues such as skin biopsies, which can provide information comparable to those obtained from blood tests but can be applied to all free-ranging cetacean species and populations, allowing for a high number of samples to be analyzed.

In this paper, we propose the use of the Major Histocompatibility Complex (MHC) Class I Chain-Related gene A (MICA) as a potential biomarker for the cetacean immune system. Information on Human MICA is available in the literature but nothing exists regarding its presence and behavior in cetaceans.

The Human MICA encodes for a 62-Kda cell surface glycoprotein, which is expressed on endothelial, dendritic and epithelial cells; as well as on fibroblasts; and on many neoplastic and virus–infected cells, serving as a target for cellular and humoral immune responses in transformed cells. The level of expression of MICA protein in epithelial

tissues is normally low, but upregulation can occur due to several cellular stress stimuli, including heat shock proteins. MICA also functions as a ligand that is recognized by the activating receptor NKG2D, which is expressed on the surface of NK, NKT, CD8+, and TCRγδ+ T cells. Allelic variants of MICA can result in large differences in NKG2D binding. This differential affinity might affect NK cell activation thresholds and T cell modulation in autoimmune diseases and tumors (14, 15). Soluble forms of MICA molecules (sMICA) can also be found, and altered serum levels of sMICA have been reported in multiple health and disease conditions (16).

Zou et al. (17) documented MICA expression on freshly isolated human fibroblasts and a marked decrease when fibroblasts were grown to confluence in culture dishes. By contrast, increased MICA expression was found during the proliferation of fibroblasts, possibly acting as a support for the host response to injury.

Much interest exists in the study of the relationship of MICA with cancer. MICA was found to be expressed on the surface of several neoplastic cells, where it may enhance innate immunity by stimulating NK cells and participating in T cell immunity by costimulation of CD8+lymphocytes. Several tumors can release soluble MICA, and soluble MICA has been reported to inhibit the stimulating pathway mediated by NKG2D.

Cetacean morbilliviruses and papillomaviruses, as well as *Brucella* spp. and *Toxoplasma gondii,* are thought to interfere with population abundance by inducing high mortalities, lowering reproductive success or by synergistically increasing the virulence of other infectious pathogens. Severe cases of lobomycosis and lobomycosis-like disease (LLD) indicate that these infections may contribute to the death of some dolphins. Environmental contamination seems to play a role in these diseases because, as already reported, many pollutants are known as immunosuppressors and can adversely affect the immune status of cetaceans. The cetacean skin is an important tissue district of the immune system, and the MICA protein, which was used in this study as a toxicological stress marker of the immune system, is expressed in fibroblasts.

The aim of this study, in fact, was to evaluate MICA protein expression in fibroblast cell cultures of cetaceans (skin biopsies of free-ranging specimens and skin samples of stranded cetaceans within 2–12 h of death) by an immunofluorescence technique and to outline possible variations in MICA expression linked to differential ecological and biological factors, while also investigating MICA expression after *in vitro* treatments with different contaminants. Free-ranging or stranded specimens of cetaceans were sampled in the Sea of Cortez (Mexico) [Bryde's whale (*B. edeni*), long-beaked common dolphin (*D. capensis*), and killer whale (*O. orca*)] and in the Mediterranean Sea [fin whale (*B. physalus*), sperm whale (*P. microcephalus*), *T. truncatus*, and striped dolphin (*S. coeruleoalba*)], and the cell cultures were treated with organochlorine compound (OC) mixture, flame retardants, polycyclic aromatic hydrocarbons (PAHs), MeHg, and bisphenol A (BPA).

MATERIALS AND METHODS

Sampling Methods

Free-Ranging Cetaceans

Samples of skin biopsies (epidermis, dermis, and blubber) were obtained from free-ranging specimens of long-beaked common dolphin (MDC12) and striped dolphin (STG96) using an aluminum pole armed with biopsy tips (0.7 cm ø, 3.0 cm length), while skin biopsies from free-ranging specimens of Bryde's whale (MBE3), killer whale (MOO12), sperm whale (PMAs1 and PMAs2), fin whale (BPT1), and bottlenose dolphin (TTAs1) were obtained using a Barnett Wildcat II crossbow with a 150-pound test bow, using a biopsy dart with modified stainless steel collecting tip (0.9 cm ø, 4.0 cm length). Biopsy samples were taken in the dorsal area near the dorsal fin, with CITES authorization (CITES Nat. IT025IS, Int. CITES IT 007), in the Sea of Cortez (MDC12, MBE3, and MOO12) and Mediterranean Sea (PMAs1, PMAs2, BPT1, TTAs1, and STG96). A little biopsy fragment was immediately stored in cell medium for cell cultures.

Stranded Cetaceans

Skin tissue of stranded cetaceans (dead for only 2–12 h) was obtained from specimens found dead along the Italian coastline in the period 2005–2009. Samples were taken from beneath the dorsal fin of stranded specimens of fin whale (RT25) and striped dolphin (RT17 and RT23) and immediately placed in cell medium.

Sex Identification

Sex determination in cetaceans was performed by genetic investigations according to Berubè and Palsboll (18). The sex of specimens was as follows: MDC12 female (F), STG96 male (M), MBE3 (F), MOO12 (M), PMAs1 (M), PMAs2 (M), TTAs1 (F), RT25 (M), (BPT1 (F), RT23 (F), and RT17 (M).

Fibroblast Cell Cultures

The development of a non-invasive sampling method for obtaining viable tissue samples for cell cultures from skin biopsies of free-ranging and stranded cetaceans was described by Marsili et al. (19). Successful cell cultures were obtained from all the specimens.

After the animals were sampled, each skin sample was stored in sterile medium MEM Eagle Earle's salts w/L-glutamine and sodium bicarbonate + 10% gamma irradiated fetal calf serum + 1% MEM non-essential amino acids (NEAA) solution 100x + 1% Penicillin/Streptomycin 100x + 0.1% Amphotericin B 100x at room temperature and was processed within 24 h of collection. In the laboratory, each sample was washed with Earle's balanced salt solution (EBSS) containing antibiotic (Penicillin/Streptomycin 100x) and antimycotic (Amphotericin B 100x) solutions. All specimens were handled under strict sterility conditions. First, the collected tissue was cut into small pieces with curved surgical scissors, placed in 30-mm Petri dishes and incubated with Trypsin-EDTA solution 1x for 15 min at 37°C. The biopsy fragments were washed again and then placed in Falcon 25 flasks, moistened with medium. After 24 h at 37°C in an incubator with 5% CO_2, the cultures were covered with 1 ml of medium. Half of the culture medium was replaced every 48 h with fresh medium.

Indirect Immunofluorescence Technique

Fibroblast cell cultures (third generation) were subjected to this experimental protocol for 48 h. The preliminary test that we conducted concerned the treatment of fibroblasts with an inducer and a repressor of the immune system. We used cyclosporine A (CsA), a drug belonging to the category of immunosuppressants, along with β-glucan, a polysaccharide known to increase immune system response. For the main research the different cell lines were exposed as follows: (1) to a mixture of organochlorines (Arochlor 1260, pp′-DDT and pp′-DDE), solubilized in dimethyl sulfoxide (DMSO) (0.05%) (named OCs), at three doses: 0.01, 0.1, and 1 μg/ml, plus a DMSO (0.05%) control; (2) to a mixture of benzo(a)pyrene (1 mM) and beta-naphthoflavone (20 mM), solubilized in acetone (0.1%) (named PAHs), at three doses: C = (0.5 μM BaP + 10 μM BnF), B = (2.5 μM BaP + 50 μM BnF), and A = (12.5 μM BaP + 250 μM BnF), plus an acetone (0.1%) control; (3) to a mixture containing 27 polybrominated diphenyl ethers (PBDEs), from mono- to deca-brominated (BDE-MXE), solubilized in nonane (0.01 μg/ml) (named PBDEs) at three doses: 0.01, 0.05, and 0.1 μg/ml, plus a nonane (0.01 μg/ml) chemical control; 4) to bisphenol A (BPA) solubilized in ethanol (0.1%) (named BPA), at four doses: 0.1, 1, 10, and 100 μg/ml, plus an ethanol (0.1%) control; and 5) to hydrosoluble and therefore solubilized in cultured medium methylmercury (MeHg), at four doses: 0.01, 0.1, 1, and 10 μM.

We applied immunofluorescence to fibroblast cell cultures for a qualitative and semiquantitative analysis of target protein MICA. After fixing and extraction with methanol and acetone at −20°C, we conducted a first reaction with the primary polyclonal antibody (Ab) (rabbit polyclonal anti-MICA Ab; Abcam), the cells were treated with the secondary Ab (Alexa Fluor 568 rabbit anti-goat IgG (H + L) for MICA) and labeled with red-fluorescent Alexa Fluor dye. Immunofluorescence was quantified with a specially designed Olympus Soft Imaging Systems macro, *DetectIntZ,* which works with the image acquisition, processing and analysis system, *analySIS*^{B} (Olympus) (20). The image analysis procedure has the objective of quantifying, with an adimensional index generated for this purpose, the amount of Alexa Fluor localized on the cytoplasmic membrane of the various cell preparations. The cells under study were imaged using DAPI, and this image was presented to the operator for threshold selection of cytoplasmatic and nuclear regions of interest (ROI) across the field. The procedure then utilized these ROI to measure the fluorescence intensity with Alexa Fluor of the different cell samples and the results were summarized in a worksheet. The system generated index values that are unitless until compared with other units, such as number of cells to obtain mean fluorescence per cell or the area in which it was calculated to obtain the mean fluorescence per mm^2. Images were all obtained with a magnification of 20X, a calibration of 0.65 μm/pixel and a resolution of 1,360 × 1,024 x 8 pixel. Exposure times were fixed while reading MICA expression for each treatment. A series of images of each slide was acquired so that a minimum of 250 cells/slide could be counted. The total fluorescence revealed by the program was then divided by the number of cells to obtain an arbitrary unity of fluorescence (AUF) per cell. Several slides for MICA were made for each culture: one was a blank (cells treated only with primary and secondary antibodies), one was a secondary blank (cells treated only with the secondary antibody), one was a chemical blank (cells treated with contaminant carrier), and two were for each treatment dose of all contaminants. The former two blank samples enabled the natural presence of the target proteins in cultured fibroblasts to be checked, while the secondary blank enabled validation of the dose of secondary Abs without cross reaction, as the primary Ab was absent.

Organochlorine Analysis

Analyses for DDTs and PCBs were performed according to methods recommended by the U.S. Environmental Protection Agency (EPA) 8081/8082, with modifications (21, 22). The analytical method used was High Resolution Capillary Gas Chromatography with an Agilent 6890N and a 63Ni ECD and an SBP-5 bonded phase capillary column (30 m long, 0.2 mm i.d.). The carrier gas was nitrogen, with a head pressure of 15.5 psi (splitting ratio 50/1). The scavenger gas was argon/methane (95/5) at 40 ml/min. The oven temperature was 100°C for the first 10 min, after which it was increased to 280°C at 5°C/min. Injector and detector temperatures were 200 and 280°C, respectively. The extracted organic material (EOM%) from the freeze-dried samples was calculated in all samples. Capillary gas chromatography revealed op′- and pp′- isomers of DDT and its derivatives DDD and DDE and revealed ∼30 PCB congeners. Total PCBs were quantified as the sum of all congeners. These congeners represented 80% of the total peak area of the PCBs in the samples. Total DDT was calculated as the sum of op-DDT, pp′-DDT, op′-DDD, pp′-DDD, op′-DDE and pp′-DDE. The results were expressed in nanograms per gram of lipid weight (ng/g l.w.).

Statistical Analysis

Shapiro-Wilks test has been used to evaluate if the population is normally distributed ($p > 0.05$). Findings showed that all the investigated groups were non-normal distributed. Descriptive statistics (mean, standard deviation, minimum, and maximum) was used to summarize the data. The Spearman's rank correlation coefficient (*r*-Spearman) was instead used in order to measure the degree of association between This indicator ranges from +1 to −1. A value of 0 indicates that no association exists between the two variables. A value greater/lower than 0 indicates a positive/negative association between the variables investigated. Finally, the non-parametric tests of Kruskal-Wallis ($p < 0.05$) and Kolmogorov-Smirnov ($p < 0.05$) were used to determine if the levels of MICA, DDTs, and PCBs significantly differ between specimens and if the levels of MICA in the cells significantly differ between doses and type of treatments.

RESULTS AND DISCUSSION

Basal Levels of MICA in Different Species

The basal level of MICA, evaluated with immunofluorescence technique in the fibroblasts of different cetacean species, is the first important result to indicate the immune status of the specimens sampled in different areas. In particular, these data

were compared to DDT and PCB levels detected in the blubber samples of the same specimens. The mean values, expressed as immunofluorescence for cell (AUF/nucleus) for MICA and as ng/g lipid weight for OCs, are presented in **Figure 1**.

The three species from the Sea of Cortez (*B. edeni, D. capensis,* and *O. orca*) exhibited a basal activity of MICA higher than the Mediterranean species, except for the free-ranging striped dolphin STG96, and correspondingly lower levels of DDTs and PCBs. Despite the low total sample number, a Spearman's rank correlation coefficient equal to −0.59 also confirmed this tendency for high levels of MICA and low total OC levels (**Figure 2**). In fact, in the guidelines to interpreting Spearman's rank correlation coefficient, this association is medium. Interestingly, in the Mexican species, the PCB levels did not exceed the estimated toxicity threshold (17 mg/kg l.w.) for deleterious health effects set by Jepson et al. (23) and Kannan et al. (24) (**Figure 1**); this indicates that, in this area the toxicological risk for these marine mammals is low, regardless of their position in the trophic chain. Instead, the basal activity of MICA in species of the Sea of Cortez seems to be related to their different diets, with an enhanced activity parallel to the increase of their trophic level, while noting that *B. edeni* feeds mainly on blue fish and is not strictly plankton-eating as *B. physalus* is in the Mediterranean Sea. However, the total OC levels of *B. edeni* were the lowest, showing that plankton intake is not negligible in the diet. In the Sea of Cortez, the specimens had been sampled while free-ranging, by skin biopsy, so they were all supposedly healthy; consequently, the differences both in the accumulation and in the immune response were most likely linked to ecological and biological factors and not to a more or less compromised health condition, as in the case of stranded specimens. In the Mediterranean Sea, instead, three samples came from specimens found stranded alive and then died (*B. physalus* RT25 and *S. coeruleoalba* RT17 and RT23), while five came from free-ranging specimens (*P. microcephalus* PMAs1 and PMAs2, *T. truncatus* TTAs1, *B. physalus* BPT1, and *S. coeruleoalba* STG96). Regardless of the type of sampling (free-ranging or stranded), the health condition, the position in the trophic chain, and the sex, all the herein investigated specimens had consistent tissue levels of both DDTs and PCBs. The latter xenobiotics far exceeded the toxicity threshold of 17 ppm, except for the free-ranging fin whale BPT1, and the MICA response was globally much lower than in the Sea of Cortez species.

Since MICA has a non-parametric distribution, evaluated with the Shapiro-Wilks test ($p < 0.00156$), a non-parametric statistical analysis was used to compare the different variables. Using the Kruskal-Wallis test and putting the data together according to the sampling area, without considering the single species, we verified that MICA expression was significantly different ($p = 0.0247$) between the Sea of Cortez and the Mediterranean Sea samples. The same occurred with the levels of DDTs and PCBs ($p = 0.0412$).

Based upon the results of the present study, we can affirm that the environment in which the specimens live and, accordingly, the anthropogenic stress "magnitude" to which they are subjected, are key determinants in MICA protein-based immune responses. It is well-known that the Mediterranean Sea is highly contaminated by several anthropogenic activities, such as coastal urbanization and industrialization, oil tanker traffic and general maritime transport, fisheries, and agricultural waste (25). Sea of Cortez instead is considered close to a pristine environment, with a low anthropogenic impact (26). Therefore, it can be also inferred that the lower the anthropogenic stress of the specimens is, the higher is the basal activity of MICA.

Regarding the Mediterranean species, the two fin whale specimens had the lowest OC levels related to other species, and this is in line with what we have always found in this basin, depending on the different trophic position (21, 27). However, the *B. physalus* RT25 concentrations were very high compared to those found in *B. physalus* BPT1 (∼7 times for the DDTs

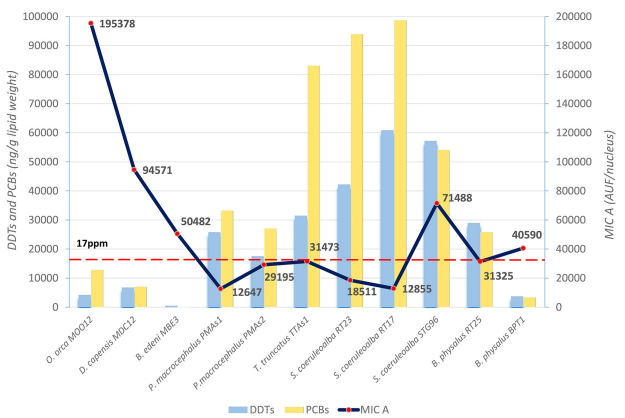

FIGURE 1 | Basal levels of immunofluorescence (AUF/nucleus) of MICA (line) in fibroblast cells and mean levels of DDTs and PCBs (ng/g lipid weight) (histograms) in blubber *of B. edeni, D. capensis, O. orca, P. macrocephalus, T. truncatus, S. coeruleoalba, and B. physalus*. The red dotted line represents the PCB threshold (17 ppm l.w.) indicated to produce deleterious effects in marine mammals.

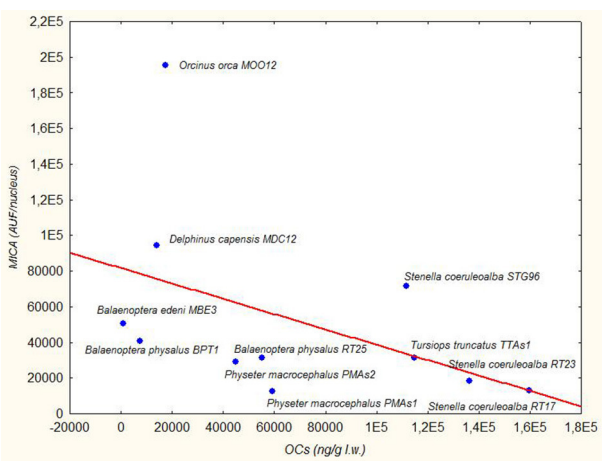

FIGURE 2 | Trend Line (Spearman's rank correlation coefficient $r = -0.59$) between MICA (AUF/nucleus) in fibroblast cells and OCs (ng/g lipid weight) in blubber of *B. edeni, D. capensis, O. orca, P. macrocephalus, T. truncatus, S. coeruleoalba, and B. physalus*.

and PCBs) and in other Mediterranean fin whale specimens, both stranded and free-ranging (25). The mysticete RT25, found stranded in January 2011 along the Tyrrhenian coast of Italy, showed an unprecedented coinfection by *Dolphin Morbillivirus* (DMV) and *Toxoplasma gondii*, together with high OC pollutant concentrations, with special reference to DDTs (28). Therefore, the basal activity of MICA might have been higher than that of a free-ranging fin whale as a function of a response to these multiple stress factors Instead, the free-ranging fin whale BPT1 had a higher MICA value, with considerably lower OC xenobiotic levels. Within such context, it is of interest that the hepatitis B virus (HBV), for example, suppresses the expression of MICA/B on hepatoma cells through the upregulation of transcription factors GATA2 and GATA3 to escape from NK cell immune surveillance (29), and a similar pathogenetic mechanism could also be shared by DMV and *T. gondii*. In addition, for the striped dolphin specimens, we obtained comparable results: RT17 and RT23, both stranded animals, had PCB levels higher than free-ranging STG96, but the opposite was true for MICA. Furthermore, the RT23 individual was also infected by DMV. The other specimens sampled by biopsy, belonging to bottlenose dolphin (TTA1) and sperm whale (PMAs1 and PMAs2) species, had MICA levels like the stranded striped dolphin and fin whale specimens. An explanation could be linked also to interspecies differences or to sex or age variability. In this respect, a non-parametric statistical test (Kolmogorov-Smirnov) showed that no significant differences existed between males and females in MICA levels, without considering the sampling area and the species. In the striped dolphin group, however, we had two specimens of different sex that were both stranded, with an adult and almost certainly sexually mature (190 cm) individual (RT23) and a male subadult (168 cm) (RT17). In these specimens, at similar tissue concentrations of OC pollutants, a similar level of MICA expression was found. All that we have highlighted with these results makes it difficult to explain if the activity of MICA has an upregulation or a downregulation with a toxicological stress. What we know is that, in humans (*Homo sapiens sapiens*), where it is most studied, MICA protein has a low level of expression in healthy epithelial tissues but is upregulated in many tumors (30–32) and under various cellular stress stimuli, including heat shock proteins, DNA damage, and viral infections (33–36). MICA also functions as a ligand recognized by the activating receptor Natural Killer Group 2D (NKG2D), which is expressed on the surface of Natural Killer (NK) cells, Natural Killer T cells (NKT), CD8+ T cells (often called cytotoxic T lymphocytes, or CTLs) and T-cell receptor (TCR)γδ+ T cells. McGilvray et al. (37) reported that NKG2D ligands were highly expressed in lymph node metastasis of stage I colorectal cancer samples, but they were expressed in lower amounts in Stage II, III, or IV tumors. A higher expression of MICA in colorectal cancer patients was associated with a good prognosis (38). MICA molecules exist also in soluble forms (sMICA) and altered serum levels of sMICA have been reported in multiple health and disease conditions. For example, in the case of hepatitis C virus (HCV)-associated hepatocellular carcinoma (HCC) the control serum had soluble MICA values $\leq$ 5 pg/ml, whereas the serum from diseased patients had values $\geq$ 5 pg/ml (39). The higher level of sMICA in the blood serum from HCV-infected, HCC-affected patients highlighted the possible role of MICA as a tumor suppressor. However, the elevation of serum sMICA was shown to be associated with a poor prognosis in various cancer patients (40–43), thereby emphasizing once again the difficulty of interpreting the quantitative response. Nothing is known about the "stress" caused by environmental contaminants, although NKG2D expression has also been shown to be regulated by estradiol (44). Endometrial cells exposed to estradiol upregulated MICA protein expression (45). In this respect, the MICA promoter contains an estrogen receptor response element, suggesting that estrogens may increase MICA expression through transcriptional regulation. Consequently, provided that many of the xenobiotics accumulated by these marine mammals are known as endocrine-disrupting chemicals (EDCs), they could exert biologic activities similar to that of estradiol.

Since MICA expression has been extensively investigated in human studies, a comparison of the basal activity of MICA in cetacean fibroblasts with those of a human biopsy carried out for medical investigations was also deemed of interest. In human fibroblasts, the basal activity of MICA resulted in 11.863 AUF/nucleus and therefore was lower than that of all the herein investigated cetacean specimens. In the biomedical literature, no studies on MICA expression in human fibroblasts have been made available before the present investigation, that was carried out by means of an indirect immunofluorescence technique. Unfortunately, no subcutaneous adipose tissue was sampled in the human biopsy, so that we cannot correlate the levels of DDTs and PCBs with the values of MICA. Regardless of this, we can still infer that the levels of MICA in human fibroblasts are lower than those of all investigated cetacean species but very similar to those of the sperm whale PMAs1 and the striped dolphin RT17 and generally to most of the Mediterranean specimens.

However, given that even in humans, as already mentioned above, whether MICA showed an upregulation or a downregulation in the presence of a "generic" stress(or), is difficult to determine.

The results of each specimen whose cells were treated with the two compounds are expressed as index numbers (AUF Treatment: AUF BA = X Index Number: 100) and shown

TABLE 1 | Immunofluorescence of MICA, expressed as index numbers relative to the blank (BA), revealed in cultured fibroblasts of different cetacean species and humans treated with the immune response activator (β-glucan), and suppressor (Cyclosporine A).

	BA	Inducer 500 μg/ml	Repressor 0.8 μg/ml	Repressor 80 μg/ml
MOO12 (*O. orca*)	100	96	55	143
TTAs1 (*T. truncatus*)	100	61	69	66
RT23 (*S. coeruleoalba*)	100	95	112	All cells died
RT25 (*B. physalus*)	100	56	90	61
MA1 *(Homo sapiens)*	100	9	70	All cells died

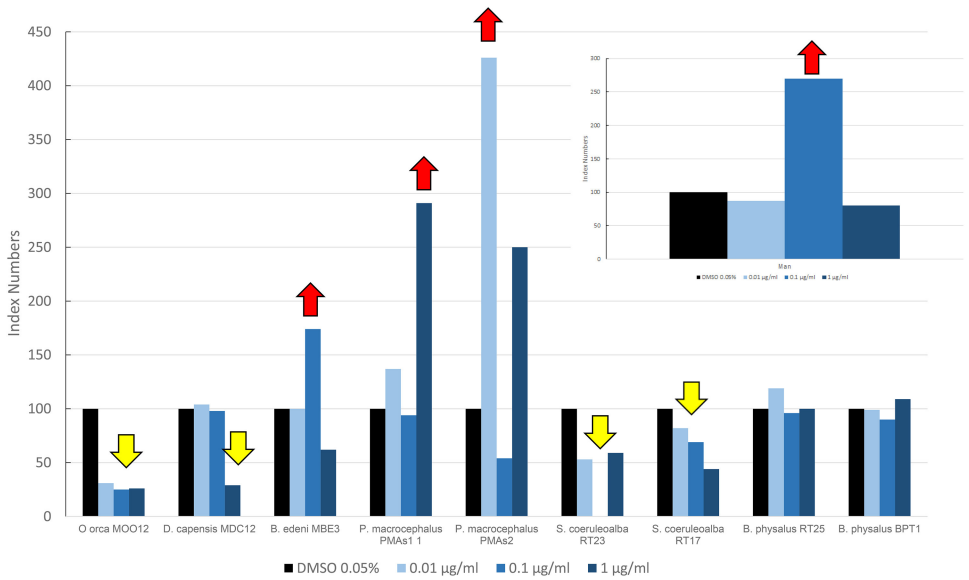

FIGURE 3 | Mean values of immunofluorescence of MICA expressed as index numbers respect to the solvent control (DMSO) revealed in cultured fibroblasts of different cetacean species and in man treated with OCs. Red arrows indicate a significant upregulation respect to DMSO control, yellow arrow a significant downregulation.

in **Table 1**. The sample named BA is the blank or "basal activity sample," which is the cultured fibroblasts treated only with primary and secondary antibodies. The doses applied to induce and repress expression were established according to the literature (46–51).

The results were quite contradictory: in killer whales, in fact, MICA showed a downregulation with both the inducer and the lower dose of the repressor but upregulation with the higher dose of repressor; in striped dolphins, by contrast, the upregulation was already present at the lower dose of the repressor, whereas at the highest dose, we observed a complete cell death, with this same effects also occurring in humans, but with both the inductor and the low-dose repressor, downregulation occurs; finally, in bottlenose dolphins and in fin whales, downregulation occurred with all the treatments. Most likely, the choice of the two compounds, known to have such capabilities in relation to the immune system but not specifically in respect to MICA, should be reassessed.

MICA in Different Species After Treatment With OCs, PBDEs, PAHs, BPA, and MeHg

After the basal activity of the different specimens had been evaluated, trying to evaluate how much MICA was modified following treatment with some of the main environmental contaminants with which these animals come into contact in their living environment was deemed of interest. Since most of these toxicants are not water-soluble and therefore need a carrier (for example, DMSO, or nonane) to treat the fibroblasts, the basal activity with which the different treatments were compared is relative to that given by the carrier. This way of proceeding is correct because upregulation of MICA very often had already occurred with the solvent alone. The toxicity of different solvents to cells was previously demonstrated in other studies (20, 52).

In **Figure 3**, we reported the results of the mean levels of immunofluorescence of MICA, which was revealed in cultured fibroblasts of different species treated with OCs and expressed as index numbers relative to the solvent control (DMSO). According to **Figure 3**, the response was very different between the species and at different doses: killer whale and striped dolphin specimens showed no increase in MICA expression at any dose; in the long-beaked common dolphin, the MICA level remained unchanged until the highest treatment, where we had a downregulation; the two fin whale specimens showed neither upregulation nor downregulation at any dose; and only in the case of the sperm whale and Bryde's whale that we assisted we observed a clear upregulation effect on MICA due to OCs, compared to DMSO. The two specimens of sperm whale showed the same response curve, while the Bryde's whale had an equal response to that of man, that is, a bell-shaped response.

Figure 4 shows results of the mean levels of immunofluorescence of MICA, revealed in cultured fibroblasts of different cetacean species treated with the PBDEs, which are expressed as index numbers relative to the solvent control (nonane). In the sperm whale (PMAs2), striped dolphin, and fin whale specimens, the highest response of MICA was the one related to the flame retardant treatment, with a bell-shaped response in all species; the long-beaked common dolphin showed an upregulation at the highest dose; a discontinuous response was shown by sperm whale (PMAS1), whereas Bryde's whale and the killer whale showed a downregulation. Additionally, in this case, as for the OCs, the two striped dolphins responded in the same way, even if the flame retardants lead to upregulation and the OCs to downregulation.

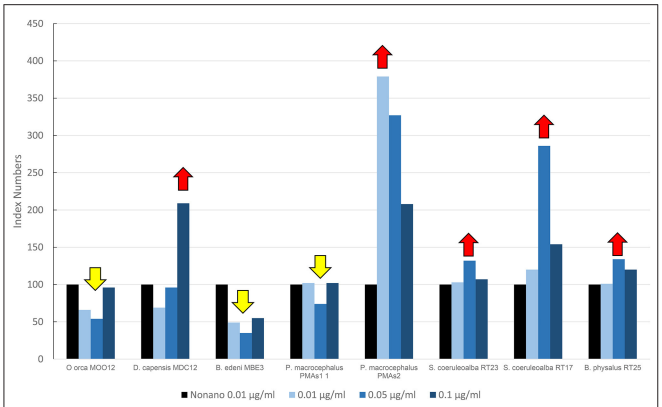

FIGURE 4 | Mean values of immunofluorescence of MICA expressed as index numbers relative to the solvent control (nonane) revealed in cultured fibroblasts of different cetacean species treated with PBDEs. Red arrows indicate a significant upregulation respect to nonano control, yellow arrow a significant downregulation.

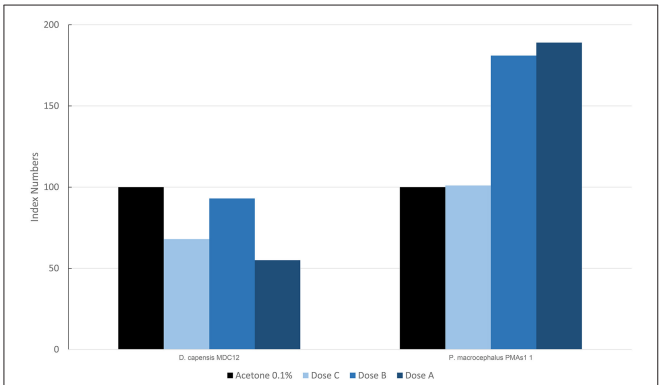

FIGURE 5 | Mean values of immunofluorescence of MICA expressed as index numbers respect to the solvent control (acetone) revealed in cultured fibroblasts of different cetacean species treated with PAHs.

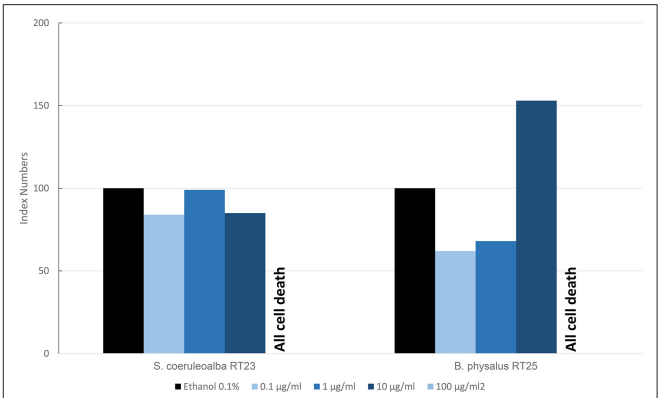

FIGURE 6 | Mean values of immunofluorescence of MICA expressed as index numbers respect to the solvent control (ethanol) revealed in cultured fibroblasts of different cetacean species treated with BPA.

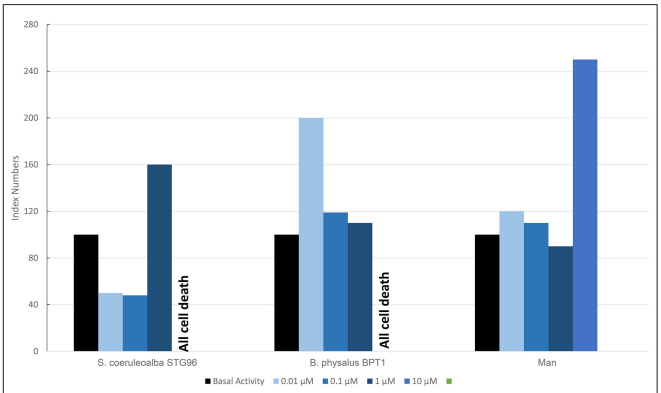

FIGURE 7 | Mean values of immunofluorescence of MICA expressed as index numbers respect to basal activity revealed in cultured fibroblasts of different cetacean species and in man treated with MeHg.

In **Figure 5**, the results are reported for the mean levels of immunofluorescence of MICA, which was detected in cultured fibroblasts of different species treated with PAHs and expressed as index numbers relative to the solvent control (acetone). Only two species, the long-beaked common dolphin and the sperm whale (PMAS1), were exposed to PAHs. In the sperm whale, a significant increase was present in the level of MICA, with a trend in the dose/response type. The long-beaked common dolphin showed a discontinuous response but overall a downregulation.

Figure 6 shows the results of the mean levels of immunofluorescence of MICA, found in cultured fibroblasts of different cetacean species treated with BPA and expressed as index numbers relative to the solvent control (ethanol). We investigated only two specimens: a fin whale and a striped dolphin. Their responses were markedly different, even if the higher dose caused the death of all cells in both animals, thus highlighting its toxicity. Only the fin whale fibroblasts exhibited an upregulation phenomenon compared to the solvent control.

Figure 7 shows results of the mean levels of immunofluorescence of MICA, found in cultured fibroblasts of different cetacean species and man treated with MeHg and expressed as index numbers with respect to the blank and represented by cells treated only with primary and secondary antibodies. Although the responses were different, in the two cetacean species compared to human skin fibroblasts, at least one dose of treatment with MeHg caused upregulation of MICA. Additionally, in this case, as for PAHs in cetaceans, the higher dose caused the death of all cells, while in man with this dose, an evident upregulation of MICA was documented.

Summary of Treatment Results

What we can summarize from all the herein presented results is shown in **Table 2**, where for each specimen, the results of upregulation, downregulation, or no MICA response, following a specific treatment, are reported.

Some specimens had the same response patterns even with different treatments, such as the killer whale (MOO12), which always underwent a downregulation with both OCs and PBDEs. In both cases, the differences between the carrier and the doses were statistically significant with the non-parametric Kruskal-Wallis test ($p < 0.05$). The long-beaked common

TABLE 2 | Summary of response types with different treatments in different specimens.

	OCs	PBDEs	PAHs	BPA	MeHg
Orcinus orca MOO12	↓	↓	n.t.	n.t.	n.t.
Delphinus capensis MDC12	↓	↑	↓	n.t.	n.t.
Balaenoptera edeni MBE3	↑	↓	n.t.	n.t.	n.t.
Physeter macrocephalus PMAs1	↑	↓	↑	n.t.	n.t.
Physeter macrocephalus PMAs2	↑	↑	n.t.	n.t.	n.t.
Stenella coeruleoalba RT23	↓	↑	n.t.	-	n.t.
Stenella coeruleoalba RT17	↓	↑	n.t.	n.t.	n.t.
Stenella coeruleoalba STG96	n.t.	n.t.	n.t.	n.t.	↑
Balaenoptera physalus RT25	-	↑	n.t.	↑	n.t.
Balaenoptera physalus BPT1	-	n.t.	n.t.	n.t.	↑
Man	↑	n.t.	n.t.	n.t.	↑

Different color of box is related to different response of MICA: orange, upregulation; green, downregulation; yellow, no alteration; n.t., no treatment.

dolphin (MDC12) showed a downregulation with both OCs and PAHs, while simultaneously exhibiting an upregulation with PBDEs. Again, these responses were statistically significant. The Bryde's whale (MBE3) showed a MICA upregulation with OCs and a downregulation with PBDEs, both statistically significant. In the two specimens of sperm whale, the predominant response was an upregulation. Downregulation occurred in PMAs1 only for the PBDE treatment, but it was not statistically significant, unlike all others, both in PMAs1 and in PMAs2. RT23 and RT17 specimens of striped dolphins showed the same MICA behavior after treatments, with downregulation with PCBs and upregulation with PBDEs. Whereas, with PCBs, the reaction was statistically significant in both samples, for the PBDEs, it was not. With BPA in the RT23 striped dolphin, we did not observe a different response between the carrier and the doses. The STG96 striped dolphin treated with MeHg showed a statistically significant upregulation. The two specimens of fin whales (RT25 and BPT1) treated with OCs did not show a change in MICA levels between the carrier and the doses, whereas they constantly exhibited a statistically significant upregulation with the other treatments. Finally, in human fibroblast cell cultures, taken as a comparative study model, MICA upregulation occurred with OCs and with MeHg and, in both cases, it was statistically significant.

CONCLUSIONS

In conclusion, we can state that MICA expression has been extensively investigated herein and validated as an important immune response biomarker in cetaceans, with the possibility of being also evaluated in skin biopsies from free-ranging individuals, when it is not possible to have blood as the biological investigation material. The response of upregulation or downregulation in the presence of some of the main environmental contaminants needs to be explored further, both from an ecological and biological standpoint. In fact, factors such as the area in which cetaceans live, coupled with their position(s) within the marine food webs as well as their sex, age, and their general health condition(s) can strongly influence MICA expression patterns. However, equally evident is that MICA expression modifications/alterations occur both with an increase or with a decrease in ecotoxicological stress levels, indicating either a "stress condition" or an immune response alteration/modification. The main result of this study refers, therefore, to "ecotoxicological stress-associated MICA expression's modulation," and this is particularly interesting because these marine mammals are the main accumulators of liposoluble and persistent contaminants. If the accumulation of these substances joins the presence of infectious pathogens, such as DMV or *T. gondii*, these exposures and their deleterious effects could be potentially amplified, making the synergy of the two phenomena inseparable in the evaluation of the conservation status of these highly threatened and vulnerable species.

ETHICS STATEMENT

The cetaceans tissues collection procedure was carried out in strict accordance with the relevant national and international guidelines under CITES permits (CITES Nat. IT025IS, Int. CITES IT 007).

AUTHOR CONTRIBUTIONS

LM designed and provided the resources for the study and performed the sampling, in *vitro* experiments and laboratory analysis and also elaborated the data and wrote, reviewed, and edited the manuscript. GDG and SM provided scientific support in the interpretation of lab data and they also revised and critically reviewed the manuscript. SC collaborated in performing the *in vitro* experiments, lab investigations, and data analysis and also wrote and reviewed the manuscript.

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T Helper Cell Subsets and Their Functions in Common Bottlenose Dolphins (*Tursiops truncatus*)

Sylvain De Guise[1,2]*, *Milton Levin*[1], *Lindsay Jasperse*[1], *Guillermo Risatti*[1] and *Randall S. Wells*[3]

[1] Department of Pathobiology and Veterinary Science, University of Connecticut, Mansfield, CT, United States, [2] Connecticut Sea Grant College Program, University of Connecticut, Groton, CT, United States, [3] Sarasota Dolphin Research Program, Chicago Zoological Society and Mote Marine Laboratory, Sarasota, FL, United States

Considerable efforts have been made to better understand the immune system of bottlenose dolphins in view of the common environmental challenges they encounter, such as exposure to polychlorinated biphenyls, oil spills, or harmful algal bloom biotoxins. However, little is known about the identity and functionality of the Th1, Th2, and Treg T helper cell subsets in bottlenose dolphins. The present study aimed at validating assays and reagents to identify T helper cell subsets and their functions in a subset of dolphins from Sarasota Bay, Florida, USA, which have been long studied and often used as a reference population. A population of CD4+ FOXP3+ lymphocytes was identified representing an average <1% of blood lymphocyte population, which is in the range observed in for Treg cells in other species. The use of porcine reagents to measure TGFß, one of the key Treg cytokines, was further validated using the relatively high-throughput and highly standardized Luminex technology. The proportion of circulating Treg cells was not correlated with the serum concentrations of the Treg effector cytokines TGFß and IL-10, nor could it significantly contribute to predicting the variability of T lymphocyte proliferation, suggesting that not all dolphin circulating Treg cells are functional and active. However, stimulation of dolphin lymphocytes with TGFß and IL-2 increased the expression of the gene for TGFß and IL-10, and stimulation with IL-12 and IFNγ induced a robust increase in the expression of the gene for IFNγ, suggesting the potential for polarization and differentiation of dolphin T helper cells toward a Treg and Th1 response, respectively. The lack of an increase in the expression of the genes for the Th2 cytokines IL-4 and IL-13 upon stimulation with IL-4 may be due to the requirement for IL-2 for a Th2 polarization as described in mice. However, regression analysis and PCA suggested the potential ability of both the Th1 and Th2 response to be triggered upon acute inflammatory signals. These results may be useful in better understanding the mechanisms by which the dolphin immune system is affected upon exposure to environmental challenges and how it responds to pathogen challenges.

Keywords: T helper, Th1, Th2, Treg, cytokines, polarization, dolphin, immunology

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Mystic Aquarium and University of Connecticut, United States

***Correspondence:**
Sylvain De Guise
sylvain.deguise@uconn.edu

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INTRODUCTION

Considerable efforts have been made to better understand the immune system of marine mammals in general and of common bottlenose dolphins (*Tursiops truncatus*) in particular (1), in view of the common environmental challenges they encounter, such as exposure to polychlorinated biphenyls or PCBs (2), oil spills (3), or harmful algal bloom biotoxins (4). However, little is known about the identity and functionality of different T helper cell subsets in these species.

It has long been recognized that naïve T cells can differentiate into T helper 1 (Th1) or T helper 2 (Th2) cells that play an important role in the adaptive immune system. The commitment toward a Th1 response is promoted by interferon gamma (INFγ) and interleukin (IL)-12, and Th1 cells in turn secrete INFγ, which stimulates cell-mediated immunity to help combat intracellular pathogens (e.g., viruses), eliminate cancerous cells, and stimulate delayed-type hypersensitivity (DTH) skin reactions, while at the same time inhibiting Th2 differentiation (5). The commitment toward a Th2 response is promoted by IL-4, and Th2 cells in turn produce IL-4, IL-5, IL-6, and IL-13, which inhibit cell mediated (Th1) immunity and promote humoral (i.e., antibody mediated) immune responses to help combat extracellular pathogens (e.g., extracellular bacteria, parasites) (5). However, important additional Th cell subsets have more recently been recognized.

T regulatory (Treg) cells are specialized CD4+ T cells that function to maintain self-tolerance and immune homeostasis by suppressing the activation, proliferation, and effector functions of various immune cells (6). In humans, alterations in the number and function of Treg cells have been implicated in many diseases such as type I diabetes (7), graft vs. host disease (8), systemic lupus erythematosus (9), and rheumatoid arthritis (10). Treg cells can be identified based upon cell surface expression of $CD4^{+}CD25^{+}CD127^{low}$ or by the intracellular transcription factor Forkhead box P3 (FOXP3) (11). The commitment toward a Treg response is promoted by TGFß, and Treg cells in turn secrete IL-10 and TGFß, which down-regulate immune responses (11, 12).

Several functions of the immune system in general, and of T lymphocytes in particular, are modulated by the balance of cytokines secreted by different T cell subsets. The differentiation and regulation of naive T cells into different T cell subsets with specific functions are in turn modulated by a number of critical signals including cell to cell interactions and cytokines (12). A recent report suggested the potential for dysregulation of the Th1/Th2 balance and changes in T lymphocyte proliferation that might be attributable to effects on Treg in bottlenose dolphins following exposure to oil after the *Deepwater Horizon* oil spill, but more in-depth studies were hindered by the lack of validated methods and reagents in this species.

The present study aimed to identify and assess the functions of Th1, Th2, and Treg cells in bottlenose dolphins. It clearly identified Treg cells from dolphin blood and their serum cytokines, demonstrated the functionality of Th1 and Treg dolphin cells, and assessed relationships among serum cytokines in wild bottlenose dolphins.

MATERIALS AND METHODS

Source of Blood

Twenty long-term resident bottlenose dolphins from Sarasota Bay, FL, United States, were captured in June 2018, sampled, and released as part of health assessment programs (which included the immunological data presented here), as previously described in detail elsewhere (13, 14). The dolphins sampled included 10 males and 10 females, ranging in age from 2 to 48 years old (mean 17 years old), and likely represented a good cross section of the population sampled. Whole blood was collected in Vacutainer tubes (Becton Dickinson, Franklin Lakes, New Jersey, USA) with sodium heparin as part of the routine physical examinations, kept cool on ice packs and shipped overnight for functional immunological assays. In addition, 1 ml serum from each of those 20 dolphins was collected and immediately frozen prior to shipping on dry ice for cytokine analysis. Dolphin samples were collected under National Marine Fisheries Service Scientific Research Permit No. 20455, issued to RSW, as approved by the Mote Marine Laboratory Institutional Animal Care and Use Committee (IACUC). All samples were received and experiments performed following approval from the University of Connecticut IACUC. Human whole blood was purchased from Biological Specialties Corporation (Colmar, PA 18915, USA) and bovine and ovine whole blood were purchased from Lampire Biological Laboratory (Pipersville, PA 18947, USA). Blood purchased from commercial sources was deemed exempt from IACUC oversight by the University of Connecticut IACUC.

Isolation of Peripheral Blood Mononuclear Cells

Dolphin blood samples were processed immediately upon receipt in the laboratory, within 24 h of collection. Blood samples from other species were also processed immediately upon receipt in the laboratory, however the collection time of samples from commercial sources was not known. Dolphin, human, bovine, and ovine whole blood was diluted 1:1 with phosphate buffered saline (PBS) with 2 mM EDTA (Miltenyi, Auburn, CA 95602, USA), layered on top of an equal volume of Ficoll-Paque Plus 1.077 (GE, Pittsburgh, PA 15264, USA), and centrifuged for 40 min at 400 g, as per manufacturer's instructions. The peripheral blood mononuclear cell (PBMC) layer was collected, washed twice with Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Grand Island, NY 14072, USA) supplemented with 1 mM sodium pyruvate, 100 mM non-essential amino acids, 25 mM HEPES, 2 mM L-glutamine, 100 U/mL, penicillin, 100 mg/mL streptomycin, and 0.25 mg/mL Fungizone (all from Thermo Fisher Scientific, Grand Island, NY 14072, USA), along with 10% fetal bovine serum (Hyclone, Logan, UT 84321, USA), hereafter referred to as complete DMEM, and cells were enumerated with their viability assessed using the exclusion dye trypan blue (Life Technologies, Grand Island, NY 14072, USA). Cell viability was typically >90%. PBMC isolation after Ficoll-Paque centrifugation was confirmed using a BD FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ 07417, USA) using forward scatter (relative cell size) and side scatter (relative

cell complexity) settings to assess the proportion of PBMCs in the sample.

Immunophenotyping

Table 1 lists the primary antibodies, as well as the isotype control antibodies, tested on bottlenose dolphin PBMCs. Human, ovine, and bovine PBMCs were used as positive controls to assure that expected labeling was observed in the species against which the antibodies were raised. For CD4 labeling, 1×10^6 PBMCs were labeled with 100 μl of either fluorochrome-conjugated or unconjugated primary antibodies, as well as with the isotype control antibodies (conjugated or unconjugated), at the dilutions listed in **Table 1**, for 30 min at 4°C in the dark. PBMCs were then washed with 1 ml PBS and centrifuged at 400 g for 5 min. For fluorochrome-conjugated primary antibodies, PBMCs were then re-suspended with 200 μl of 1% neutral buffered formalin in PBS. For unconjugated antibodies, PBMCs were re-suspended with the 100 μl of a goat anti-mouse FITC conjugated secondary antibody (Life Technologies, Grand Island, NY 14072, USA) for 30 min at 4°C in the dark. PBMCs were then washed with 1 ml PBS, centrifuged, and re-suspended in 200 μl of 1% neutral buffered formalin in PBS.

For CD25 labeling, PBMCs (2×10^5 cells/well) were plated in 96 well flat bottom plates (Fisher Scientific, Agawam, MA 01001, USA) with either no mitogen (complete DMEM alone) or the T cell mitogen, concanavalin A (ConA) at 1 μg/ml. PBMCs were incubated for 24 h at 37°C and 5% CO_2. Cells were pooled from five replicate wells (for a total of ∼1×10^6 cells) in a 5 ml conical tube. Tubes were centrifuged and the cells were re-suspended in 100 μl of either fluorochrome-conjugated or unconjugated primary antibodies, as well as with the isotype control antibodies (conjugated or unconjugated), at the dilutions listed in **Table 1**, for 30 min at 4°C in the dark. Afterwards, the procedures were the same as with CD4 labeling described above.

The fluorescence of approximately 10,000 lymphocytes was read using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ 07417, USA) and the automated CellQuest software (Becton Dickinson Immunocytometry System, San Jose, CA 95131, USA). Lymphocytes were identified by their relative size (forward-scattered light, FSC) and their complexity (side-scattered light, SCC). The percent of cells that were positive for CD4 or CD25 were compared to the isotype control for CD4 or CD25. Cells were considered positive for CD4 or CD25 if cell fluorescence was above the isotype control.

For intracellular FOXP3 labeling, 1×10^6 PBMCs were incubated with 100 μl of a 1:100 dilution of the CD4 antibody, SIM.4, for 30 min at 4°C in the dark. Cells were then washed with 1 ml of eBioscience™ flow cytometry staining buffer (Life Technologies, Grand Island, NY 14072, USA) and centrifuged. PBMCs were then re-suspended with 100 μl of a goat anti-mouse FITC conjugated secondary antibody (1:100) (Life Technologies, Grand Island, NY 14072, USA) for 30 min at 4°C in the dark and washed with eBioscience™ staining buffer. Intracellular FOXP3 was labeled with FOXP3 Monoclonal Antibody (FJK-16s), APC, and the Foxp3/Transcription Factor Staining Buffer Set Kit (both from Life Technologies, Grand Island, NY 14072, USA), per Thermo Fisher Scientific's Protocol B: one-step protocol (intracellular (nuclear) proteins) instructions. For the isotype control, an APC labeled rat IgG2a kappa isotype control antibody (Life Technologies, Grand Island, NY 14072, USA) was used at the same recommended dilution as for the FoxP3 antibody (1:20).

The fluorescence of approximately 10,000 lymphocytes was read using a BD Biosciences LSRFortessa X-20 Cell Analyzer (Becton Dickinson, Franklin Lakes, NJ 07417, USA) and FACSDiva software (Becton Dickinson Immunocytometry System, San Jose, CA 95131, USA). Lymphocytes were identified by their relative size (forward-scattered light, FSC) and their complexity (side-scattered light, SCC). Two-color flow cytometry was used to identify cells that were positive for both CD4 and FOXP3. The percent of cells that were positive for FOXP3 was compared to the isotype control for FOXP3.

Cytokines

Dolphin serum cytokines were quantified using the Bio-Plex Pro™ Human Cytokine Th1/Th2 Panel (Bio-Rad, Hercules, CA 94547, USA) and the Millipore Porcine 3-plex Panel (Millipore, Billerica, MA 01821, USA), as previously described (3). The Th1/Th2 cytokine kit included antibodies to detect IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IFNγ, TNFα, and GM-CSF. The porcine kit included antibodies to IL-1b, IL-4, and IL-8.

TGFß was quantified using the TGF beta-1 Porcine ProcartaPlex™ Simplex Kit (Thermo Fisher Scientific, Grand Island, NY 14072, USA) or the Bio-Rad Bio-Plex Pro™ TGFß Kit (Bio-Rad, Hercules, CA 94547, USA), according to the manufacturers' instruction. To validate this kit, dolphin and porcine PBMCs were adjusted to 2×10^6 cells/ml and plated in 96 well flat bottom plates, in triplicate wells (2×10^5 cells per well). PBMCs were incubated at 37°C with 5% CO_2 for a total of 48 h with purified *Escherichia coli* 0111:B4 LPS (lipopolysaccharide; Sigma, St. Louis, MO 63118, USA), a mitogen shown to induce secretion of TGFß (15), at a final concentration of 0.1 μg/ml in pigs, a concentration (referred to as suboptimal for graphic display) previously demonstrated to induce cytokine secretion in humans (16) and pigs (17), and 0.05 or 5.0 μg/ml, which induced suboptimal and optimal B lymphocyte proliferation in bottlenose dolphins (3) and bracketed the concentration used for pigs to maximize the likelihood of detecting a response if one existed. In a separate set of wells, PBMCs were incubated with medium alone, i.e., unstimulated cells, to serve as negative control. At the end of 48 h, tissue culture supernatant was harvested and stored in multiple aliquots at −80°C until analysis.

After the incubation and conjugation process, the plates were measured on the Bio-Plex 200 system (Bio-Rad, Hercules, CA 94547, USA), and analyzed using Bio-Rad Manager 5.0. The observed concentration (pg/ml or ng/ml) of each analyte for each sample was calculated using a curve fit generated for each analyte from seven or eight standards (depending on kit). If a sample concentration was extrapolated outside the standard curve and designated as "Value extrapolated beyond standard range" by the software, that sample concentration was accepted as the calculated value. If a sample concentration was reported as "out of range" by the software, that sample concentration was given a 0 pg/ml or 0 ng/ml value or the highest value on the

TABLE 1 | Antibodies tested on bottlenose dolphin mononuclear cells.

Primary antibody (clone)	Vendor	Documented species reactivity	Species tested as positive control	Isotype	Antibody dilution
RPA-T4 (CD4)	Thermo Fisher Scientific	Human	Human	IgG1	1:50
VIT4 (CD4)	Miltenyi	Human	Human	IgG2a	1:11
TR-204-33 (CD4)	Tracy Romano	Dolphin	Dolphin	IgG1	1:124
SIM.4 (CD4)	NIH-AIDS	Human	Human	IgG1k	1:100
CACT108A (CD25)	Kingfisher Biotech	Bovine	Bovine	IgG2a	1:100
CACT116A (CD25)	Kingfisher Biotech	Bison, bovine, caprine, ovine, water buffalo	Bovine	IgG1	1:100
MEM-181 (CD25)	Life Technologies	Human, mouse	Human	IgG1	1:100
MEM-140 (CD25)	Life Technologies	Human	Human	IgM	1:100
HI25a (CD25)	Abbiotic	Human	Human	IgG1	1:100
PC61.5-PE (CD25)	Life Technologies	Human, mouse	Mouse	IgG1	1:1,600
9.14 (CD25)	Bio-Rad	Ovine	Ovine	IgG1	1:100
BC96 (CD25)	eBioscience	Human	Human	IgG1	1:5
4E3 (CD25)	Miltyeni	Human, rhesus monkey, cynomolgus monkey	Human	IgG2b	1:50
FJK-16s (FOXP3)	Life Technologies	Bovine, dog, cat, mouse, pig, rat	Dolphin	IgG2a	1:20
Mouse IgG (isotype control)	Life Technologies	n/a	n/a	IgG	1:100
Mouse IgG1 FITC (isotype control)	Life Technologies	n/a	n/a	IgG1	1:5
Mouse IgG2a FITC (isotype control)	Miltyeni	n/a	n/a	IgG2a	1:11
Mouse IgG2b (isotype control)	Miltyeni	n/a	n/a	IgG2b	1:50
Rat IgG1k PE (isotype control)	Life Technologies	n/a	n/a	IgG1	1:1,600
Rat IgG2a kappa (isotype control)	Life Technologies	n/a	n/a	IgG2a	1:20

standard curve, depending on whether it was below or above the measurable range.

Prior to each use of the Bio-Plex 200 system, an instrument calibration and validation procedure using the Bio-Rad Validation and Calibration kit (Bio-Rad, Hercules, CA 94547, USA) was performed to assure the instrument was performing properly, as per manufacturer's instruction. The instrument passed both calibration and validation tests prior to each use.

Lymphocyte Stimulation With Th1, Th2, or Treg Cytokines

In order to assess ability of dolphin lymphocytes to respond to a Th1, Th2, or Treg stimulus, dolphin PBMCs (2×10^6 cells/ml) were incubated in 96 well flat bottom plates with human recombinant cytokines at concentrations of 0 (unstimulated), 1, 10, and 25 pg/ml for 24 h. To assess a Th1 response, cells were stimulated with IL-12 (Millipore Sigma, Burlington, MA 01803, USA) and IFNγ (Thermo Fisher Scientific, Grand Island, NY 14072, USA) and analyzed for IFNγ expression. To stimulate a Th2 response, cells were stimulated with IL-4 (Millipore Sigma, Burlington, MA 01803, USA) and analyzed for IL-4 and IL-13 expression. To assess a Treg response, cells were stimulated with IL-2 (Thermo Fisher Scientific, Grand Island, NY 14072, USA) and TGFβ (Thermo Fisher Scientific, Grand Island, NY 14072, USA) and analyzed for TGFβ and IL-10 expression. After a 24 h incubation at 37°C and 5% CO_2, cells were collected from the plates, centrifuged at 220 g for 10 min, re-suspended in RNAlater solution (Thermo Fisher Scientific, Grand Island, NY 14072, USA) and stored at 4°C for up to 1 month. RNAlater samples were then moved to −20°C for long-term storage.

TABLE 2 | Sequence of the forward and reverse primers used to amplify housekeeping and cytokine genes.

Cytokine		Sequence: 5′ to 3′	References
S-9	Forward	GAGGATTTCTTGGAGAGACGCCTG	(18, 19)
	Reverse	CTTGCGGACCCTGATATGGCGC	
HPRT1	Forward	GTGGCCCTCTGTGTGCTC	(19)
	Reverse	ACTATTTCTGTTCAGTGCTTTGATGT	
IFNγ	Forward	CAGAGCCAAATAGTCTCCTTCTACTT	(18)
	Reverse	CTGGATCTGCAGATCATCTACCGGAATTTG	
IL-4	Forward	GGAGCTGCCTGTAGAAGACGTCTTTG	(18)
	Reverse	CTTCATTCACAGAACAGGTCATGTTTGCC	
IL-13	Forward	CCTCTACAGCCCTCAAGGAGC	(18)
	Reverse	CTTCCAGGGCTGAACAGTACATGT	
TGFß	Forward	GAGCTGCGCCTGCTGAGGCT	(18)
	Reverse	CCTCTATTTCCTCTCCGTGGGTC	
IL-10	Forward	GACTTTAAGGGTTACCTGGGTTG	(18)
	Reverse	TCCACTGCTTTGCTCTTGTTTTC	

Primers

Primers were chosen based on published bottlenose dolphin primer sequences (18, 19). Primer sequences are reported in **Table 2**.

Gene Expression

RNA was extracted from dolphin PBMCs samples using a RNeasy Mini Kit (Qiagen, Valencia, CA 91355, USA), and genomic DNA was removed using a TURBO DNA-free kit (Thermo Fisher Scientific, Grand Island, NY 14072, USA). RNA concentration was determined using a Qubit fluorometer (Thermo Fisher Scientific, Grand Island, NY 14072, USA). After isolation and quantification, RNA was reverse transcribed into cDNA with 100 ng RNA per reaction using a high capacity cDNA reverse transcription kit (Thermo Fisher Scientific, Grand Island, NY 14072, USA). Real time PCR (qRT-PCR) reactions were performed using SYBR green (Thermo Fisher Scientific, Grand Island, NY 14072, USA) on a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA 94547, USA). All samples were analyzed for housekeeping genes HPRT1 and S-9. Cycling conditions for genes were 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s and annealing at 63°C for 1 min, followed by a dissociation stage. For IL-4 and IL-10, the annealing temperature was 54°C. Reactions containing water, but no cDNA, were used as negative controls. Product specificity was monitored by analysis of melting curves. Gene expression data were analyzed using the Comparative C_T ($\Delta\Delta C_T$) Method. Samples for which the amplification of the housekeeping genes were outside of the expected range were discarded, so as to not misinterpret a change in the expression of a target gene as an inadequate PCR reaction.

Mitogen-Induced T Lymphocyte Proliferation

Lymphocyte proliferation was evaluated as previously described (3). Briefly, lymphocytes were incubated with mitogens for 66 h in flat-bottom 96-well plates (Fisher Scientific, Agawam, MA 01001, USA) at 37°C and 5% CO_2. Mitogens chosen included two T cell mitogens (ConA and phytohemagglutinnin A, or PHA). Mitogens were used at optimal as well as suboptimal concentrations, since suboptimal concentrations of mitogens allowed for higher sensitivity to subtle deficits when optimal concentrations of mitogens did not reveal differences (20). Lymphocyte proliferation was evaluated by the incorporation of 5-bromo-2′-deoxyuridine (BrdU), a thymidine analog, detected with a monoclonal antibody and colorimetric enzymatic reaction (Cell Proliferation ELISA BrdU (colorimetric), Roche Diagnostics GmbH, Mannheim, Germany) as per manufacturer's instructions using an ELISA plate reader (Multiskan EX v.1.0) at 450 nm with a reference wavelength of 690 nm. Results were expressed as optical density (OD).

Cells from mice (one mouse for each experimental day) were assayed concurrently with dolphin samples for quality control, as previously described (2). After field sampling, mouse data were assessed for the presence of outliers using the SPSS software (IBM SPSS Statistics version 21, Armonk, NY 10504, USA). If outliers were detected, it was assumed that normal daily variability for the assay was exceeded, and the corresponding dolphin data for that assay on that day were eliminated from the dataset.

Statistical Analyses

Correlation and regression analyses as well as *t*-tests were conducted using the SPSS software (IBM SPSS Statistics version 21, Armonk, NY 10504, USA) and Principal Component Analysis was performed using the Minitab software v. 18.1 (Minitab, Inc., State College, PA 16801, USA).

RESULTS

In order to identify dolphin Treg cells, we tested a battery of antibodies to CD4, CD25, and FOXP3 (**Table 1**). Immunolabeling was observed for all antibodies in the species of origin (human, bovine, and ovine) used as a positive control (data not shown). A cross-reacting antibody to human CD4 (SIM.4) labeled on average 32% (SD = 8%) of bottlenose dolphin lymphocytes (n = 20), while an antibody generated against cetacean CD4 (TR202) labeled 23 and 36% of bottlenose dolphin peripheral blood lymphocytes in preliminary experiments (n = 2), when the fluorescence of isotype control was subtracted from that of cells labeled with a CD4 antibody (**Figure 1**), while three other antibodies to human CD4 did not cross-react. None of the nine antibodies to CD25 tested cross-reacted with dolphin lymphocytes, with or without ConA stimulation to induce expression as observed in positive control species (data not shown). The antibody to FOXP3 clearly labeled a distinct population of CD4+ lymphocytes not present in cells labeled with the isotype control (**Figure 2**). In Sarasota Bay dolphins, when isotype control was subtracted, 0.67 ± 0.40% of all dolphin lymphocytes were FOXP3+, and 1.93 ± 1.07% of dolphin CD4+ lymphocytes were FOXP3+. The proportions of lymphocytes were FOXP3+ and of dolphin CD4+ lymphocytes were FOXP3+ were not significantly different between males and females, and were not significantly correlated with age (data not shown).

In order to determine the functionality of dolphin Treg cells, we validated the use of human and porcine reagents to measure TGFß, the major Treg effector cytokine. To do so, we stimulated dolphin and porcine PBMCs with LPS *in vitro* to induce the secretion of TGFß in the tissue culture supernatant. Dolphin lymphocytes showed a concentration-response increase in TGFß production upon stimulation with LPS using the porcine reagents (34 and 61% increase in TGFß production with 0.05 and 5.0 μg/ml of LPS, respectively), as did porcine lymphocytes (31% increase in TGFß production with sub-optimal concentration, **Figure 3**). The use of human reagents did not allow the measurement of such an increase in the expression of TGFß in dolphin cells upon stimulation with LPS (data not shown). We therefore concluded that the porcine, but not the human reagents, cross-reacted in a specific manner with dolphin TGFß, and further experiments were performed using the porcine reagents.

The serum concentrations of TGFß in 20 wild bottlenose dolphins from Sarasota Bay ranged from 0 to 868 pg/ml, with an average of 170 pg/ml and a standard deviation of 106, while IL-10 ranged from 0 to 7 pg/ml, with an average of 0.62 pg/ml and a standard deviation of 1.01. The serum concentrations of TGFß were not significantly different between males and females, and were not significantly correlated with age (data not shown). In order to assess if the proportion of peripheral

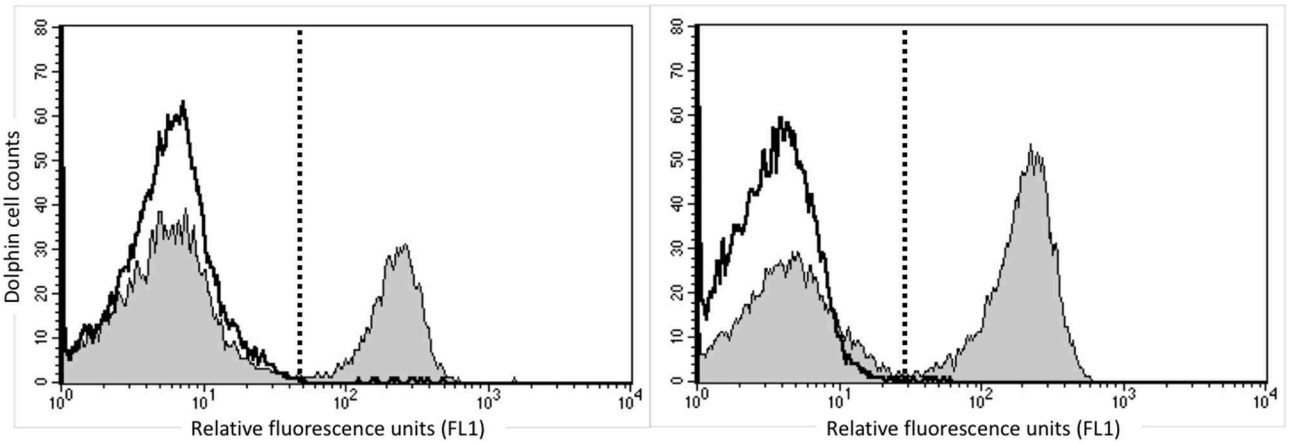

FIGURE 1 | Identification of CD4+ bottlenose dolphin cells using flow cytometry. Dolphin PBMCs were labeled with the commercially available anti-human CD4 SIM.4 (left) or research anti-cetacean CD4 TR202 (right). The white histogram represents isotype control and the gray histogram represents CD4 labeling, and the dashed line discriminates positive (right) from negative (left) labeling, with isotype control consistently including <1% background. Both antibodies labeled ∼30% of bottlenose dolphin peripheral blood lymphocytes.

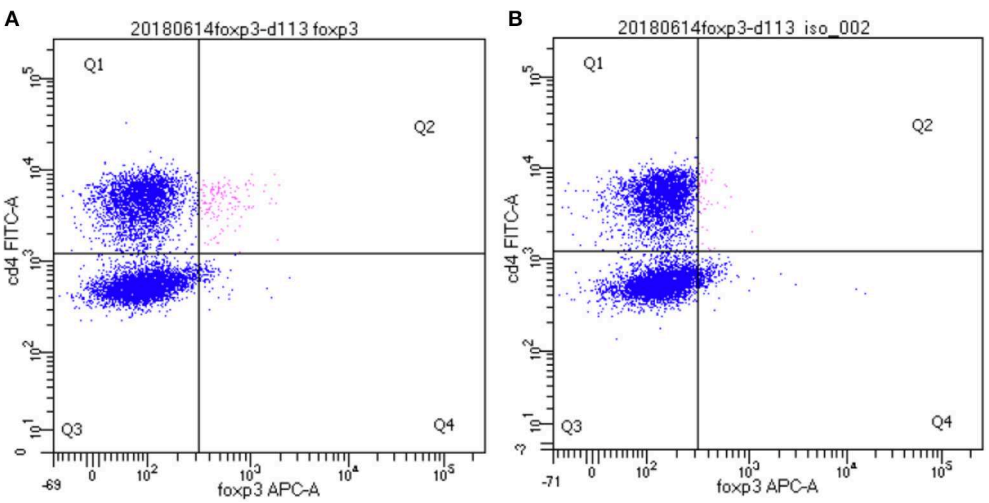

FIGURE 2 | Identification of CD4+/FOXP3+ bottlenose dolphin Treg cells using flow cytometry. A distinct population of CD4+/FOXP3+ cells are observed in the upper right quadrant (Q2, in pink) **(A)** that was absent upon labeling with isotype control for FOXP3 **(B)**.

blood FOXP3+ dolphin lymphocytes were representative of Treg function we tested the hypothesis that dolphins with more FOXP3+ lymphocytes had higher levels of serum TGFß and IL-10, the two major Treg effector cytokines. There was no significant correlation between either dolphin serum TGFß or IL-10 concentrations and the proportion of FOXP3+ lymphocytes, or with the proportion of CD4+ lymphocytes that were FOXP3+ (**Figure 4**).

In order to assess the capacity of dolphin Th cell subsets to respond to a Th1, Th2, or Treg stimulus, we tested the relative expression of effector cytokines upon *in vitro* stimulation with human recombinant cytokines, as previously done on a limited basis in cetaceans (21, 22). A robust 9.7 fold increase in the expression of the gene for IFNγ was observed upon stimulation with 25 pg/ml IL-12 and IFNγ compared to unstimulated cells, with a clear dose-response pattern (**Figure 5**). An increase in Th2 cytokines was not observed upon stimulation with 25 pg/ml IL-4; rather, IL-4 down-regulated the expression of IL-4 and IL-13 to 68 and 44%, respectively, of the levels in unstimulated cells (**Figure 5**). This down-regulation was clearly dose-dependent for IL-13, but not for IL-4. Treg stimulation by IL-2 and TGFß resulted in nearly no change at 1 pg/ml, a modest up-regulation of the genes for TGFß and IL-10 (1.2 and 2.0 fold increase, respectively, at 10 pg/ml, and 1.3 and 1.5 fold increase, respectively, at 25 pg/ml), compared to unstimulated cells (**Figure 5**).

To understand the balance of cytokines in wild dolphins, we measured serum concentrations of 12 cytokines including

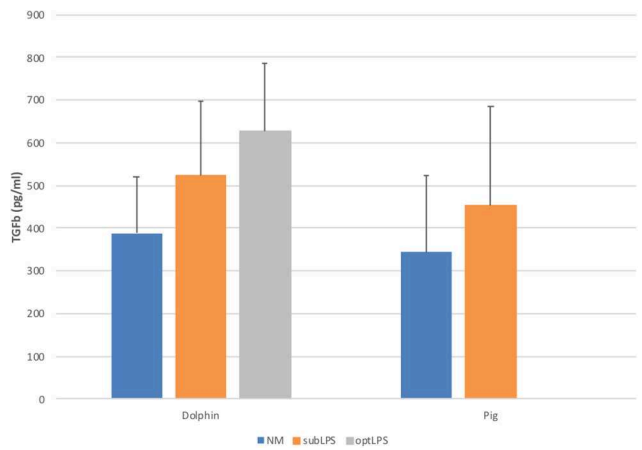

FIGURE 3 | Tissue culture supernatant concentrations of TGFß following a 48 h stimulation of porcine ($n = 3$) or dolphin ($n = 5$) PBMCs with LPS at sub-optimal (subLPS, 0.05 μg/ml for dolphin and 0.1 μg/ml for pigs) and optimal (optLPS, 5.0 μg/ml for dolphin) concentrations, compared to no mitogen stimulation (NM). TGFß concentrations were measured using the Luminex platform with porcine reagents. Data are presented as mean and error bars represent standard error of the mean.

the Th1 cytokines IL-2, IL-12, and IFNγ, the Th2 cytokines IL-4, IL-5, and IL-13, the Treg cytokines IL-10 and TGFß, and the inflammatory cytokines IL-1ß, IL-8, TNFα, and GM-CSF, in 20 wild dolphins and performed Principal Component Analysis (PCA) to see how cytokines clustered (**Figure 6**). The plot of the first two components showed the Treg cytokines IL-10 and TGFß varying in the same general direction, which was also similar to that for IL-8. Further, IL-4 and IL-1ß, and to a lesser extent IL-12, varied in a similar direction, which was different from that for the previous cluster. IL-5 and IL-13 varied in different directions, which were also different from that for IL-4. GM-CSF varied in a direction which was closest to that for IL-13, and IL-2 varied in a direction that was closest to that for TGFß. Note the absence of IFNγ and TNFα that were removed from the analysis by the software as they did not include variance in this dataset.

We further assessed the relationship between serum cytokine concentrations in wild dolphins and the results of *in vitro* mitogen-induced T cell proliferation using regression analysis. The only cytokines that significantly contributed to explaining the variability in T cell proliferation were IL-4 upon sub-optimal and optimal stimulation with ConA and optimal stimulation with PHA, and IL-2, IL-10, and GM-CSF upon sub-optimal stimulation with PHA (**Table 3**). The R^2 for models explaining the variability of T cell proliferation with IL-4 upon sub-optimal and optimal stimulation with ConA and optimal stimulation with PHA were relatively low, whereas the R^2 for the model explaining the variability T cell proliferation with IL-2, IL-10, and GM-CSF upon sub-optimal concentration of PHA was considerably higher. Further, regression analysis did not find a significant contribution of the proportion of FOXP3+ lymphocytes in explaining the variability of mitogen-induced T lymphocyte proliferation using either sub-optimal or optimal concentrations of the mitogens ConA or PHA (data not shown).

DISCUSSION

We have, for the first time to the authors' knowledge, detected the presence of Treg cells in bottlenose dolphin blood, and shown the potential for bottlenose dolphins to mount a Th1, Th2, and Treg response. This represents a significant new step in fundamental and applied dolphin immunology, which will help us better understand the importance of these responses in health and disease.

Antibodies were used to label dolphin cell subsets. Studies requiring the use of antibodies in marine mammals are challenging given the paucity of reagents available, and the lack of commercially available marine mammal specific antibodies. In addition to a small number of antibodies developed against marine mammal leukocyte subset markers (23–29), several studies reported the use of cross-reactive monoclonal antibodies to label leukocyte subsets in marine mammals (30–38). This study used blood samples from original target species or species with documented cross reactivity as positive control, to assure quality control in documenting lack of cross-reactivity with dolphin cells.

We successfully detected a subset of CD4+ lymphocytes in dolphin blood samples. The specificity of SIM.4 for labeling beluga whale CD4 has previously been demonstrated through immunoprecipitation of a 56.4 kDa protein in beluga, in the range of the 53.5 kDa protein in human (30), and the proportion of lymphocytes labeled in bottlenose dolphins ($X = 32.2\%$, $SD = 8.3\%$) was similar to that previously reported in belugas ($X = 29.8\%$, $SD = 6.9\%$). This is not surprising since bottlenose dolphins and beluga whales CD4 molecules share 98% homology at the nucleotide level and 97% at the amino acid level (39). In addition, the proportion of CD4+ cells over the sum of CD2+ T lymphocytes and CD19+ B lymphocytes in "normal" dolphins was also 30% in a field study to quantify the immune changes in bottlenose dolphins with lobomycosis (26). Further, in this study, SIM.4 labeled a relatively similar proportion of lymphocytes as TR202, an anti-CD4 developed specifically against bottlenose dolphin CD4 (26, 27).

This study did not successfully identify CD25+ dolphin lymphocytes, despite the use of unstimulated and stimulated T cells to increase the frequency of those cells. No study has reported cross-reactive antibodies to marine mammal CD25, except for one study that tested one antibody in dolphins and stated that it cross-reacted but was not selected for further use because of "low detectability" without further explanation (40). A separate study also reported the lack of cross-reactivity in dolphin of two antibodies to CD25 (38), although they did not appear to test upon stimulation as we did. An earlier study used fluorochrome-labeled IL-2 to detect the increase in surface expression of IL-2 receptor on the surface of mitogen-activated bottlenose dolphin lymphocytes (41), however this reagent unfortunately appears to be no longer available commercially.

This study successfully identified FOXP3+ dolphin lymphocytes. Treg cells are generally defined as CD4+/CD25+/FOXP3+ lymphocytes (11). While we could not find a cross-reactive antibody to CD25, we observed the presence of a clear and distinct population of CD4+/FOXP3+ Treg cells

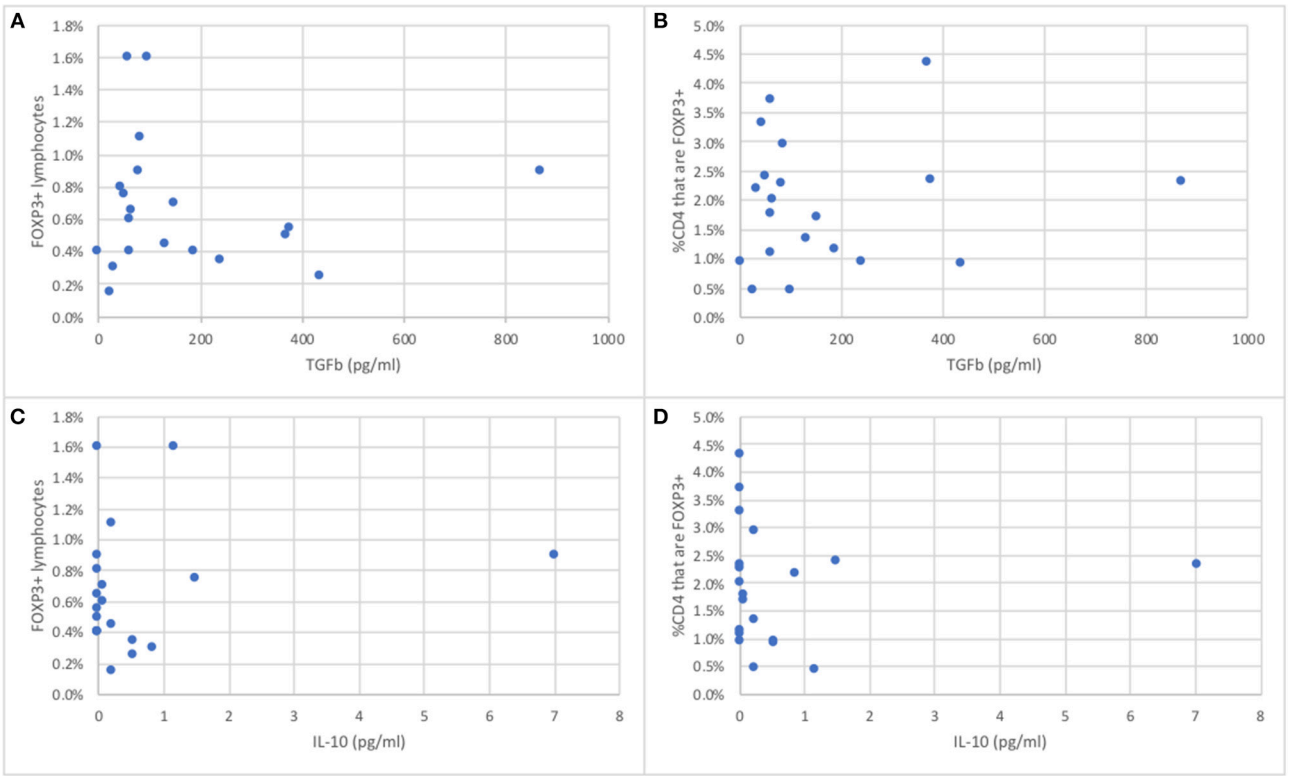

FIGURE 4 | Relationship between the proportion of peripheral blood FOXP3+ dolphin lymphocytes **(A,C)** or the proportion of CD4+ T cells that are FOXP3+ **(B,D)** and serum concentrations of TGFß **(A,B)** or IL-10 **(C,D)**, the major Treg effector cytokines ($n = 20$).

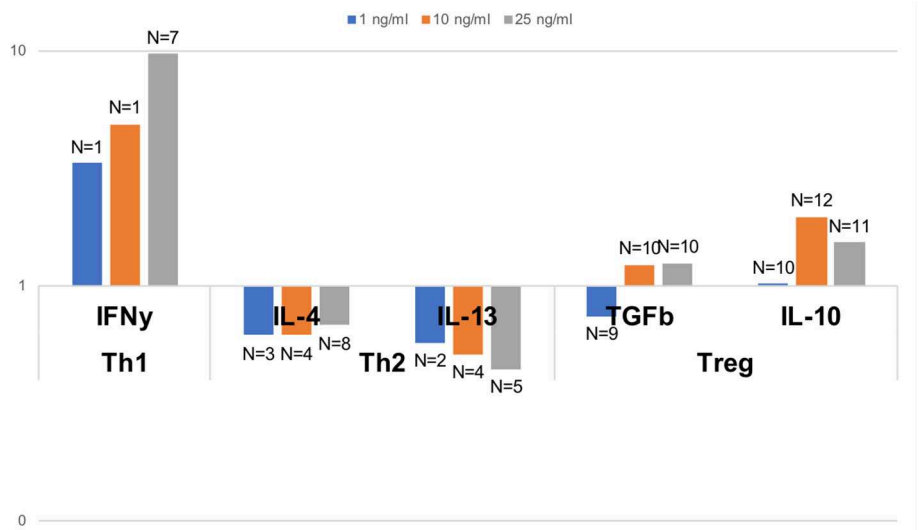

FIGURE 5 | Relative gene expression of effector Th1, Th2, and Treg cytokines upon polarizing stimulus with the Th1 inducing cytokines IL-2 and IFNγ, the Th2 inducing cytokine IL-4, and the Treg inducing cytokines IL-2 and TGFß, all tested at 1, 10, and 25 pg/ml. Data are expressed relative to unstimulated cells (value of 1). Dolphin PBMCs responded most robustly to a Th1 stimulus, and to some extent to a Treg stimulus (at some but not all concentrations), but Th2 polarization with IL-4 resulted in a down-regulation, and not an enhancement, of the expression of the genes for IL-4 and IL-13. The number of dolphins tested for each stimulation condition, once samples with inadequate expression of housekeeping genes were removed, is indicated on the figure over the bars.

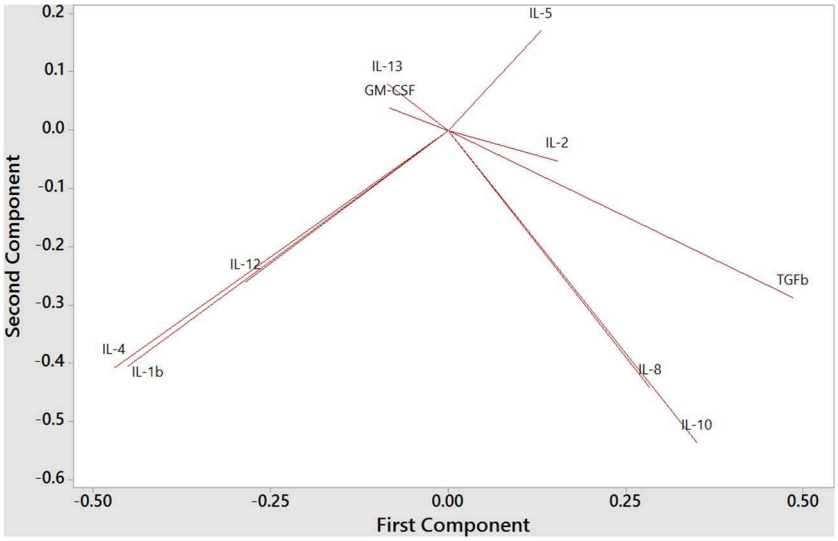

FIGURE 6 | Principal Component Analysis of Th1, Th2, Treg, and inflammatory cytokines in bottlenose dolphin serum ($n = 20$), according to the first two components (first component on the x axis and second component on the y axis).

TABLE 3 | Forward regression analysis to assess which cytokines contribute significantly to explaining the variability of T lymphocyte proliferation ($n = 20$).

Mitogen	Variables	Model R^2	Model p-value
Sub ConA	IL-4	0.284	0.015
Opt ConA	IL-4	0.310	0.011
Sub PHA	IL-2, IL-10, GM-CSF	0.737	0.008
Opt PHA	IL-4	0.264	0.025

in dolphin blood samples. The antibody to FOXP3 used in this study appeared to be broadly cross-reactive, with demonstrated labeling in cows (42), sheep (43), pigs (44), and dogs (45), in addition to the numerous species cited by the manufacturer, including cats, mice and rats (eBioscience/ThermoFisher Scientific). Dolphin CD4+/FOXP3+ Treg cells generally represented <1% of lymphocytes, a proportions very similar to what was observed in cows (∼0.66%) (42), sheep (4.6%), (43), pigs (∼1.6%) (44), and dogs (1–2.6%) (45). The relatively small proportion of Treg cells labeled in this study is not a concern given the sensitivity and precision of the instrument used and the consistent specificity of the labeling (**Figure 2**). While we have not ruled out the possibility that some of the CD4+/FOXP3+ dolphin cells identified as Treg in this study are CD25-, the literature in other species consistently reports the vast majority of CD4+/FOXP3+ cells are CD25+ (42–45). Overall, the labeling pattern limited to CD4+ cells in dolphins, the proportion of PBMCs labeled, and the specificity of FOXP3 labeling for Treg cells (42, 44), altogether suggest the specificity of FJK-16s labeling for dolphin Treg cells.

Cytokines are important mediators of the immune response, and several studies have explored the use of cytokines to relate to health status of marine mammals in different manners. Amplification of mRNA for cytokines using quantitative reverse-transcription polymerase chain reaction (qRT-PCR) to quantify cytokine expression in marine mammals, with or without stimulation of PBMCs, has been used in several studies (18, 46–53). While useful to measure responsiveness to a signal, the quantification of mRNA does not necessarily represent the circulating bioactive protein, which requires important steps, in which translation and secretion need to take place. One study used brefeldin to block secretion and flow cytometry with intra-cellular labeling for cytokines using cross-reactive monoclonal antibodies (54). While powerful at associating cytokine production with specific cell types using simultaneous extra-cellular labeling, this approach is tedious and time consuming, and allows the labeling for a relatively low number of cytokines at a time, in this case only two cytokines. Similarly, immunohistochemistry has been used to detect cytokines in cetacean tissue section (53, 55, 56). While informative on the distribution of the cell types secreting cytokines in different tissues, this method is limited to the use of tissues from dead animals, or biopsies in live animals, which would be a rather invasive procedure. ELISAs have been developed to measure killer whale-specific IL-6 (57) and dolphin-specific IFNγ and TNFα (58) in serum and tissue culture medium. While sensitive and reproducible under stringent laboratory conditions, ELISAs are relatively time consuming and labor-intensive. This study focused on measuring bioactive cytokines circulating in serum using the relatively high-throughput Luminex technology and highly standardized commercial reagents for highly replicable results (3, 59). With the specificity of human and porcine reagents was validated based on the detection of the analytes in dolphin serum and *in vitro* cell responsiveness to stimuli similar to the species of origin (59), this new approach might significantly advance the use of cytokine measurements as part of marine mammal health assessment.

While bottlenose dolphin Treg cells could be identified and quantified, we also wanted to assess their functionality by measuring their effector cytokines. Our lab previously validated the use of human reagents to detect bottlenose dolphin IL-10 using the Luminex platform and human Th1/Th2 reagent kits (3). The present study validated the use of porcine, but not human reagents, to document a <2 fold increase in expression of TGFß upon *in vitro* stimulation with LPS, as seen using porcine PBMCs (**Figure 3**) and goat macrophages (15). This validation allowed the quantification of TGFß in the serum of free-ranging bottlenose dolphins, as a reference for future comparisons.

The evaluation of Treg should include their identification as well as their functions. Our inability to label CD25 on dolphin cells prevented us from sorting live CD4+ $CD25^{hi}$ $CD127^{low}$ Tregs for functional assays, as described by others (60). Intracellular labeling for FOXP3 required cell fixation and permeabilization, and cells could no longer be used in functional assays. However, we could induce the expression of the gene for TGFß and IL-10 upon stimulation with TGFß and IL-2, as expected for functional Tregs (11), demonstrating the functionality of Treg in bottlenose dolphins. Importantly, the clustering of serum IL-10 and TGFß using PCA in our subset of dolphins suggested the potential for actual *in vivo* Treg polarization and differentiation. The proportion of circulating Treg lymphocytes was not correlated with serum concentrations of the Treg effector cytokines TGFß and IL-10, or with mitogen-induced T lymphocyte proliferation, suggesting that not all circulating Treg cells are functional and active. This is not surprising given the demonstration that the proliferation (expansion of the pool) of Treg and their functional suppressive capacity are driven by different pathways (61). Further, while the effector T cell subsets Th1, Th2, and Th17 mostly draw their energy through glycolysis, Treg use fatty acid oxidative pathways for energy (62), and it is possible that qualitative or quantitative differences in serum lipids may affect the functioning of Treg cells in dolphins. With the diversity of emerging receptors and pathways involved in the modulation of Treg cells (63), it is also possible that the proliferation or functions of dolphin Treg may be affected by environmental contaminants such as dioxin-like PCBs via the Ah receptor. It is also understood that the regulatory functions of Treg cells are not solely modulated by their effector cytokines, and in part require cell-cell interactions (6, 64). The proportion of circulating FOXP3+ lymphocytes did not contribute to significantly explaining *in vitro* mitogen-induced T cell proliferation in our study, which may relate to the relatively small number of dolphins used and/or the use of mitogens that stimulate T cell proliferation in a manner that likely exceeds physiological stimuli. It would be valuable to find reagents to sort bottlenose dolphin live Treg cells for use in functional test for suppressive activity as described in humans (60). Additional studies are required to further characterize the functionality of Treg cells in bottlenose dolphins.

There is mounting evidence for the importance of the Th1/Th2 balance and functionality to appropriately respond to immunological challenges. While Th1 and Th2 cells have not been specifically identified or quantified in this study, we demonstrated a robust expression of the gene for IFNγ upon stimulation with IL-12 and IFNγ, as expected for Th1 cells (11), suggesting the potential for Th1 polarization and differentiation in bottlenose dolphin Th cells. Stimulation with IL-4, however, unexpectedly did not induce the expression of the genes for the Th2 cytokines IL-4 and IL-13. This may be due to the requirement for IL-2 for a Th2 polarization as described in mice (65). It is interesting that the Th2 cytokines IL-4, IL-5, and IL-13 did not cluster closely using PCA, suggesting a potential disconnect between the different effector cytokines in the wild bottlenose dolphins sampled. However, IL-4, and to a lesser extent IL-12, clustered closely with the pro-inflammatory cytokine IL-1ß, suggesting the potential ability of both the Th1 and Th2 response to be triggered upon acute inflammatory signals, and the possibility that a Th2 response might be favored. Regression analysis including IL-4 as a significant contributor to explaining the variability in mitogen-induced T lymphocyte proliferation under three out of four scenarios may support a preference for a Th2 over Th1 response in bottlenose dolphin. Continued studies to better understand the relationships between Th1 and Th2 cytokines and immune responsiveness will be critical to better understand the functionality of the immune system in dolphins.

This study focused on live captures of wild dolphins, and the authors acknowledge that the chase and capture, with associated stress response, could have influenced some aspects of the immune functions measured. However, the focus on live captures of wild dolphins will allow direct comparisons with health assessments in other populations of wild dolphins, subjected to challenges such as exposure to environmental contaminants or disease outbreaks, that are also captured using similar methods.

Overall, this study for the first time demonstrated the ability to quantify FOXP3+ Treg cells in bottlenose dolphins, and the potential for polarization and functional differentiation of Th cells toward a Th1 or Treg response. While we have not directly demonstrated the polarization and functional differentiation of Th cells toward a Th2 response, we provided evidence for such potential, and the potential for a preferential Th2 over Th1 response in bottlenose dolphin in a relatively small subset of individuals from a well-studied reference population of bottlenose dolphins. These results may be useful in better understanding the mechanisms by which the dolphin immune system is affected upon exposure to environmental challenges and how it responds to challenges with pathogens.

ETHICS STATEMENT

Dolphin samples were collected under National Marine Fisheries Service Scientific Research Permit No. 20455, issued to RW. This study was carried out in accordance with the recommendations of Institutional Animal Care and Use Committee (IACUC) at Mote Marine Laboratory and at the University of Connecticut. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at Mote Marine Laboratory and at the University of Connecticut. Human, bovine, ovine, and porcine blood purchased from commercial sources was deemed exempt from IACUC oversight by the University of Connecticut IACUC.

AUTHOR CONTRIBUTIONS

SD and ML are responsible for the study design. SD and RW participated in the dolphin captures and sampling, which was overseen by RW. ML and LJ performed the laboratory work, with advice from GR for experiments on gene expression. SD, ML, LJ, GR, and RW participated in the drafting and editing of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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The reviewer TR declared a shared affiliation, with no collaboration, with two of the authors, SD and ML, to the handling editor at the time of review. The reviewer TR provided the authors with a monoclonal antibody but was not otherwise involved in this work, nor in any on-going projects with the authors.

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