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Long-Term Immunological Alterations in Bottlenose Dolphin a Decade after the *Deepwater Horizon* Oil Spill in the Northern Gulf of Mexico: Potential for Multigenerational Effects

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Abstract: Health assessments were conducted on bottlenose dolphins in Barataria Bay, Louisiana, USA, during 2011 to 2018, to assess potential health effects following the *Deepwater Horizon* oil spill, compared to the unoiled Sarasota Bay, Florida, USA, reference dolphin population. We previously reported significant increases in T-lymphocyte proliferation, as well as lower T helper 1 (Th1) cytokines, higher Th2 cytokine IL-4, and lower T regulatory (Treg) cytokine IL-10 in Barataria Bay in 2011 compared to Sarasota Bay, consistent with *Deepwater Horizon* oil exposure. Although values between 2013 and 2016 were more similar to those observed in Sarasota Bay, T-cell proliferation was again elevated and cytokine balance tilted toward Th2 in Barataria Bay during 2017–2018. In 2018, Barataria Bay dolphins had significantly more circulating Treg cells than Sarasota Bay dolphins. Mice experimentally exposed to oil also had significantly increased T-lymphocyte proliferation and circulating Treg cell number, including effects in their unexposed progeny. In vitro stimulation resulted in greater Th2 responsiveness in Barataria Bay compared to Sarasota Bay dolphins, and in vitro oil exposure of Sarasota Bay dolphin cells also resulted in enhanced Th2 responsiveness. Evidence points to Treg cells as a potential target for the immunomodulatory effects of oil exposure. The immunological trends observed in Barataria Bay appeared exaggerated in dolphins born after the spill, suggesting the possibility of continued oil exposure or multigenerational health consequences of exposure to oil, as observed in mice. *Environ Toxicol Chem* 2021;40:1308–1321. © 2021 SETAC

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INTRODUCTION

Although important and interesting, relatively few studies have documented the health effects of environmental contaminants on live marine mammal populations, given the inherent logistical constraints. Some studies have accessed carcasses of beluga whales harvested by Inuvialuit hunters to demonstrate relationships between polychlorinated biphenyls (PCBs) and the expression of relevant genes (Noël et al. 2014). Studies using remote access to live cetaceans, with biopsy techniques, have documented changes in gene expression associated with exposure to PCBs in northeast Pacific killer whales (Buckman et al. 2011). An additional level of logistical complexity emerges with live captures of marine mammals to determine effects of contaminants. For example, live captures of harbor seals in the northeastern Pacific demonstrated correlations between PCB contamination levels and the expression of target genes in skin and blubber biopsies (Noël et al. 2017), as well as disruptions of immune functions (Levin et al. 2005; Mos et al. 2006), thyroid hormones (Tabuchi et al. 2006), and vitamin A (Mos et al. 2007). Live captures of bottlenose dolphins with comprehensive health assessments have documented an association between PCB levels and anemia, hypothyroidism, and immune functions (Schwacke et al. 2012) as well as an association between harmful algal bloom exposure and immune functions (Schwacke et al. 2010). Longitudinal studies add yet one more level of complexity, both logistically and financially. A semi–field study of harbor seals temporarily held in captivity for a feeding study demonstrated immune function impairment associated with ingestion

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of polluted fish from the Baltic Sea (de Swart et al. 1994). Perhaps the best example of longitudinal studies using live captures and comprehensive health assessment is the population of bottlenose dolphins in Sarasota Bay (Wells 2014), although it has not suffered significant health effects from environmental pollutants and is in fact often used as a reference population, including in the present study. The present study focuses on the long-term impacts of the *Deepwater Horizon* oil spill on bottlenose dolphin immune functions over a decade-long longitudinal study.

The explosion of the *Deepwater Horizon* oil platform on 20 April 2010 resulted in an unprecedented release of oil in the Gulf of Mexico, and targeted funding supported broad efforts by the scientific community to understand the ecological and public health consequences associated with the spill. Common bottlenose dolphins (*Tursiops truncatus*) are long-lived apex predators with genetically distinct stocks in the bays, sounds, and estuaries of the northern Gulf of Mexico (National Marine Fisheries Service 2019), some of which were exposed to heavy oiling following the *Deepwater Horizon* spill. Numerous health effects were associated with oil exposure in a relatively well-studied Barataria Bay, Louisiana, USA, population compared to a reference population in Sarasota Bay, Florida, USA, including decreased survival (Lane et al. 2015), abnormal adrenal function (Schwacke et al. 2014; Venn-Watson et al. 2015; Smith et al. 2017), lung disease (Smith et al. 2017), impaired reproduction (Lane et al. 2015; Kellar et al. 2017), and immune system impairments (De Guise et al. 2017). The immune system changes observed in Barataria Bay dolphins sampled in 2011 appeared to resolve over the following years (De Guise et al. 2017). The present longitudinal study investigated the nature and mechanisms of long-term health effects of oil exposure on Barataria Bay dolphins, including impacts on their immune system.

We present results suggesting that immune system impairments in Barataria Bay dolphins similar in nature to those observed 2011 were still present in 2017 to 2018. Results from experimental mouse exposure studies and laboratory exposure of dolphin cells to *Deepwater Horizon* oil further strengthen the weight of evidence that health conditions observed in wild Barataria Bay dolphins are associated with oil exposure. We further provide insights into the potential mechanism involved in immunotoxicity, investigate the potential for multigenerational health effects of oil exposure, and discuss the implications of these findings for other vertebrates.

MATERIALS AND METHODS

Animals

Bottlenose dolphins were temporarily captured, sampled, and released as part of health-assessment programs (which included the immunological data presented in the present study), as previously described in detail elsewhere (Wells et al. 2004; Schwacke et al. 2014). Sampling was conducted at 2 Gulf of Mexico sites following the *Deepwater Horizon* oil spill: Barataria Bay, an area that received prolonged and heavy oiling (Michel et al. 2013), sampled in 2011, 2013, 2014, 2016, 2017, and 2018, and Sarasota Bay, an area where no oil was observed following the *Deepwater Horizon* spill, sampled in 2011, 2012, 2013, 2014, and 2018, with the data from 2011 to 2014 previously reported (De Guise et al. 2017). The well-studied Sarasota Bay population of resident bottlenose dolphins was used as a reference in the present study (Wells et al. 2004). Captures in Sarasota Bay were conducted under National Marine Fisheries Service (NMFS) permits 522-1785, 15543, and 20455, whereas those in Barataria Bay were conducted under NMFS permit 932-1905/MA-009526. Protocols were reviewed and approved by the Mote Marine Laboratory Institutional Animal Care and Use Committee (IACUC; Sarasota Bay) and National Oceanic and Atmospheric Administration's Animal Care and Use Committees (Barataria Bay).

Four-week-old female B6C3F1 mice were purchased from Charles River Laboratory and allowed to acclimate for 10 d prior to experiments. Mice were either experimentally exposed to oil or used as quality control in dolphin functional assays to discriminate between daily variability and true differences between dolphins analyzed at different times (De Guise et al. 2017). All procedures were approved by the IACUC at the University of Connecticut.

Mouse experimental exposure study design

Approximately 5-wk-old female mice were exposed to Louisiana sweet crude oil (MC252, surrogate, SO-20111116-MPDF-003; supplied by British Petroleum; $n = 25$) via gavage needle (21G, 1.5 inch), 3 times per week for 6 wk, at a dose (~0.1% body wt per day) comparable to that which induced immune dysregulation in mink (Schwartz et al. 2004a). Unexposed control mice ($n = 25$) received an equivalent volume of mineral oil (M1180; Millipore Sigma). After 6 wk, mice were euthanized ($n = 8$/group), and spleen and whole blood were collected to measure T-lymphocyte proliferation, the proportion of T regulatory (Treg) cells, and concentrations of plasma cytokines. After exposure (Louisiana sweet crude or mineral oil control), another group of female mice ($n = 17$/group) were mated with 8- to 10-wk-old unexposed male mice at a 2:1 (F:M) ratio. Female mice were weighed once a week after mating. After parturition, all pups (F_1) were counted weekly to document survival. After 21 d, all pups were weaned, sexed, and weighed. A subset of female pups, $n = 17$ from exposed mothers and $n = 17$ from unexposed mothers, were housed for an additional 3 wk. Afterward, all F_1 mice were euthanized, and spleen and whole blood were collected to measure the proportion of Treg cells and concentrations of plasma cytokines to assess the potential for multigenerational effects.

Collection of spleen and blood

Dolphin blood from the fluke blade periarterial venous rete was collected into BD Vacutainer® tubes with sodium heparin (Becton Dickinson) as part of the physical examinations, kept cool, and shipped overnight for functional immunological assays. In addition, blood was collected in serum separator tubes, allowed to clot, and centrifuged to collect serum.

Aliquots of serum (1 mL) were collected and immediately frozen prior to shipping on dry ice for cytokine analysis.

Mouse spleen and whole blood were collected as previously described (Schwacke et al. 2012). Briefly, blood was immediately collected via cardiac puncture, followed by cervical dislocation to ensure death. The spleen was harvested aseptically from each animal, and a single-cell suspension was prepared using 2 pairs of forceps in complete Dulbecco's modified Eagle's medium (DMEM; Gibco). Plasma was collected and frozen for cytokine analysis.

Lymphocyte isolation

Mononuclear cells were isolated by density gradient centrifugation on Ficoll-Paque 1.077 (GE Healthcare Life Sciences) gradient for 35 min at 900 *g* in dolphins and for 15 min at 400 *g* in mice. Mononuclear cells were resuspended in complete DMEM, washed twice, and enumerated with their viability assessed using the exclusion dye trypan blue. Complete DMEM consisted of DMEM supplemented with 1 mM sodium pyruvate, 100 µM nonessential amino acids, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 2 mM l-glutamine, penicillin (100 U/mL), streptomycin (100 µg/mL; all from Gibco, Grand Island, NY), and 10% fetal calf serum (HyClone, GE Healthcare Life Sciences).

Mitogen-induced lymphocyte proliferation

Lymphocyte proliferation was evaluated as previously described (De Guise et al. 2017). Briefly, mouse splenic or dolphin blood lymphocytes (2×10^6 cells/mL) were incubated in triplicate with 2 T-cell mitogens (concanavalin A [ConA] and phytohemagglutinnin A [PHA]; Millipore Sigma) for 66 h in flat-bottom 96-well plates (Fisher Scientific) at 37 °C and 5% CO_2. Mitogens were used at optimal as well as suboptimal concentrations (1 and 0.1 µg/mL for both ConA and PHA) because suboptimal concentrations of mitogens allow for higher sensitivity to subtle deficits and optimal concentrations of mitogens do not always reveal differences (Mori et al. 2006).

Lymphocyte proliferation was evaluated for the incorporation of 5-bromo-2-deoxyuridine, a thymidine analogue, detected with a monoclonal antibody and colorimetric enzymatic reaction (Cell Proliferation ELISA BrdU [colorimetric]; Roche Diagnostics) per the manufacturer's instructions using an ELISA plate reader (Multiskan EX, Ver 1.0) at 450 nm with a reference wavelength of 690 nm. Results were expressed as optical density.

Cytokine concentrations

Dolphin serum cytokines were quantified using the Bio-Plex Pro™ Human Cytokine Th1/Th2 Panel (Bio-Rad), the Millipore Porcine 5-plex Panel, and the TGF beta-1 Porcine ProcartaPlex™ Simplex Kit (Thermo Fisher Scientific), as previously described (De Guise et al. 2017, 2019). Mouse plasma cytokines were quantified using the commercially available MILLIPLEX MAP Mouse High Sensitivity T Cell Panel. Samples were prepared and analyzed according to the manufacturers' instruction, with quality control samples, using the Bio-Plex 200™ System and Bio-Manager 5.0 software. The observed concentration (picograms per milliliter) of each analyte for each sample was calculated using a curve fit generated for each analyte from the 7 standards. Prior to each use of the Bio-Plex 200 system, an instrument calibration and validation procedure using the Bio-Rad Validation and Calibration kit was performed to assure that the instrument was performing properly, per the manufacturer's instruction. The instrument passed both calibration and validation tests prior to each use. All quality control values were within the manufacturer's specified concentration ranges. Samples with measurements below the minimum detection limit were assigned a value of zero.

Treg cell quantification

The proportion of dolphin peripheral blood Tregs was measured as previously described (De Guise et al. 2019), using monoclonal antibodies to cluster of differentiation 4 (CD4; SIM.4, National Institutes of Health) and forkhead box P3 (FOXP3; Life Technologies). The proportion of mouse splenic and peripheral blood Tregs was measured using the Miltenyi Mouse Treg Detection Kit (CD4/CD25/FOXP3), according to the manufacturer's instructions. The fluorescence of approximately 30 000 events was read using a BD Biosciences LSRFortessa X-20 Cell Analyzer (Becton Dickinson) and FACSDiva software (Becton Dickinson Immunocytometry System). Lymphocytes were identified by their relative size (forward-scattered light) and their complexity (side-scattered light), and Treg lymphocytes were defined as $CD4^+$ and $FoxP3^+$ (dolphins) or $CD4^+$, $CD25^+$, and $FoxP3^+$ (mice).

Cytokine gene expression following stimulation with T helper 1, T helper 2, or Treg cytokines

To assess the responsiveness of dolphin lymphocytes to a T helper 1 (Th1), Th2, or Treg stimulus, dolphin lymphocytes were stimulated with human recombinant cytokines and assessed for cytokine gene expression, as previously described (De Guise et al. 2019). Briefly, to assess a Th1 response, cells were stimulated with 25 ng/mL IL-12 (Millipore Sigma) and IFNγ (Thermo Fisher Scientific) and analyzed for IFNγ expression. To assess a Th2 response, cells were stimulated with 25 ng/mL IL-4 (Millipore Sigma) and IL-2 (Thermo Fisher Scientific) and analyzed for IL-4 and IL-13 expression. To assess a Treg response, cells were stimulated with 10 ng/mL IL-2 (Thermo Fisher Scientific) and TGFβ (Thermo Fisher Scientific) and analyzed for TGFβ and IL-10 expression. Peripheral blood mononuclear cells (PBMCs) were incubated with human recombinant cytokines for 24 h, resuspended in RNAlater solution (Thermo Fisher Scientific), kept at 4 °C overnight, and then stored for up to 1 mo at –20 °C. The RNA was extracted from dolphin PBMC samples, and gene expression was assessed using real-time polymerase chain reaction (PCR), as previously described (De Guise et al. 2019). Gene expression data were analyzed using the comparative C_T ($\Delta\Delta C_T$)

method. Samples for which the amplification of the housekeeping genes was 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.

In vitro dolphin lymphocyte oil exposure

To assess the potential of oil exposure to directly affect dolphin lymphocyte activity, Sarasota Bay dolphin lymphocytes were isolated as described and incubated with increasing concentrations of oil high energy medium accommodated fraction (HEMAF), as described (White et al. 2017). Briefly, Louisiana sweet crude oil (1 g/L culture medium) was mixed with DMEM culture medium at low speed for 30 s. The mixture was then transferred to separation funnel for 1 h and the medium removed from the bottom of the funnel, leaving the medium/oil interface undisturbed. As described, DMEM was supplemented, and dolphin lymphocytes were incubated with HEMAF and recombinant cytokines for 24 h prior to assessing for gene expression.

Dolphin age determination

Dolphin age was estimated using life-history data available from photoidentification of individuals to confirm chronological age, given the long-term data sets in the well-studied Sarasota Bay population (Wells 2014). The ongoing photoidentification efforts in Barataria Bay since the *Deepwater Horizon* oil spill have resulted in sufficient information to establish age for many younger animals in that population (Schwacke et al. 2014; Smith et al. 2017; Barratclough et al. 2019). In a subset of the dolphins examined, a tooth was extracted for age determination using the growth layer group method (Hohn et al. 1989). Several dolphins underwent dental radiography for age determination based on pulp-tooth area ratios (Herrman et al. 2020), as validated in humans (Cameriere et al. 2007). In all cases, dolphin ages were assessed using a similar combination of methods in Barataria Bay and Sarasota Bay to determine whether or not they were alive at the time of the spill.

Statistical analysis

Differences between groups (locations and year or HEMAF concentrations) were assessed using one-way analysis of variance (ANOVA), with Dunnett's post hoc test used to compare groups to the reference group (Sarasota Bay or unexposed control) or *t* test to compare Barataria Bay and Sarasota Bay or dolphins alive at the time of the spill and those born after the spill. A 2-way ANOVA was used to assess differences between exposure groups (exposed vs control) and generations (F_0 and F_1) in mice. All analyses were performed using SigmaStat 3.5 (Systat) or SPSS (IBM; Ver 21), with $p < 0.05$ for statistical significance.

RESULTS

As previously reported (De Guise et al. 2017), given the general lack of differences among years in Sarasota Bay, Sarasota Bay data for all years tested were pooled, and Sarasota Bay as a whole was considered as the reference population. Although a previous report documented an increase in T-lymphocyte proliferation in Barataria Bay dolphins in 2011 and in some circumstances in 2013, with an apparent return to normal (no different from Sarasota Bay) in 2014 (De Guise et al. 2017), ConA- and PHA-stimulated T-cell proliferation in Barataria Bay in 2016, 2017, and 2018 was significantly higher than in Sarasota Bay (Figure 1). The increase in Barataria Bay T-cell proliferation in 2016 to 2018 was generally in the range of that observed in Barataria Bay in 2011 (within 8, 5, 26, and 17% of Barataria Bay 2011 values for suboptimal ConA, optimal ConA, suboptimal PHA and optimal PHA, respectively) and was most marked with suboptimal mitogen concentrations.

Mice experimentally exposed to Louisiana sweet crude oil by gavage for 6 wk showed no significant changes in splenocyte T-lymphocyte proliferation compared to control mice gavaged with mineral oil (Figure 2A). However, the unexposed progeny of exposed mice had significantly (15–33%) higher ConA- (suboptimal concentration only) and PHA- (both concentrations) induced T-lymphocyte proliferation compared to the progeny of control mice (Figure 2B).

Serum concentrations of Th1, Th2, and Treg cytokines in Barataria Bay and Sarasota Bay dolphins are presented in Figure 3. Cytokine concentrations were highly variable, as expected for samples from wild populations of outbred animals with different ages and health status, resulting in generally low statistical power. However, serum IFNγ in Barataria Bay-14 and serum IL-5 in Barataria Bay-16 were statistically higher than in Sarasota Bay, whereas serum TGFß in Barataria Bay-13, Barataria Bay-14, Barataria Bay-16, and Barataria Bay-17 was significantly lower than in Sarasota Bay. Furthermore, although not statistically significant, interesting trends mimic findings reported in 2011 (De Guise et al. 2017). The Th1 cytokines IL-2, IL-12, and IFNγ in Barataria Bay-11 were 60, 6, and 37%, respectively, of the concentrations measured in Sarasota Bay, before reaching higher concentrations in 2013 to 2016. However, IL-2, IL-12, and IFNγ in Barataria Bay-17 and Barataria Bay-18 were 2% or less of the concentrations measured in Sarasota Bay. The Th2 cytokine IL-4 in Barataria Bay-11 was 418% of those of Sarasota Bay, lower than Sarasota Bay in Barataria Bay-13 to Barataria Bay-16, and 296 and 227%, respectively, of those of Sarasota Bay in Barataria Bay-17 and Barataria Bay-18. Over the years, IL-5 was variable without a distinct pattern. The concentration of IL-13 in Barataria Bay-11 was 15% of that in Sarasota Bay, before returning to higher concentrations in Barataria Bay-13 to Barataria Bay-16, but Barataria Bay-17 and Barataria Bay-18 were 11 and 2%, respectively, of those in Sarasota Bay. Also, IL-10 followed an interesting trend. Serum IL-10 in Barataria Bay-11 was 3% of that in Sarasota Bay, before returning to higher concentrations in Barataria Bay-13 to Barataria Bay-16, but was 1% or less than that in Sarasota Bay in Barataria Bay-17 and Barataria Bay-18.

There were no significant differences in the plasma concentrations of the Th1 cytokines IL-2, IL-12, and IFNγ; the Th2 cytokines IL-4, IL-5, and IL-13; and the Treg cytokines IL-10 and TGFß between oil-exposed and control F_0 mice and between the unexposed progeny (F_1) of oil-exposed and control mice

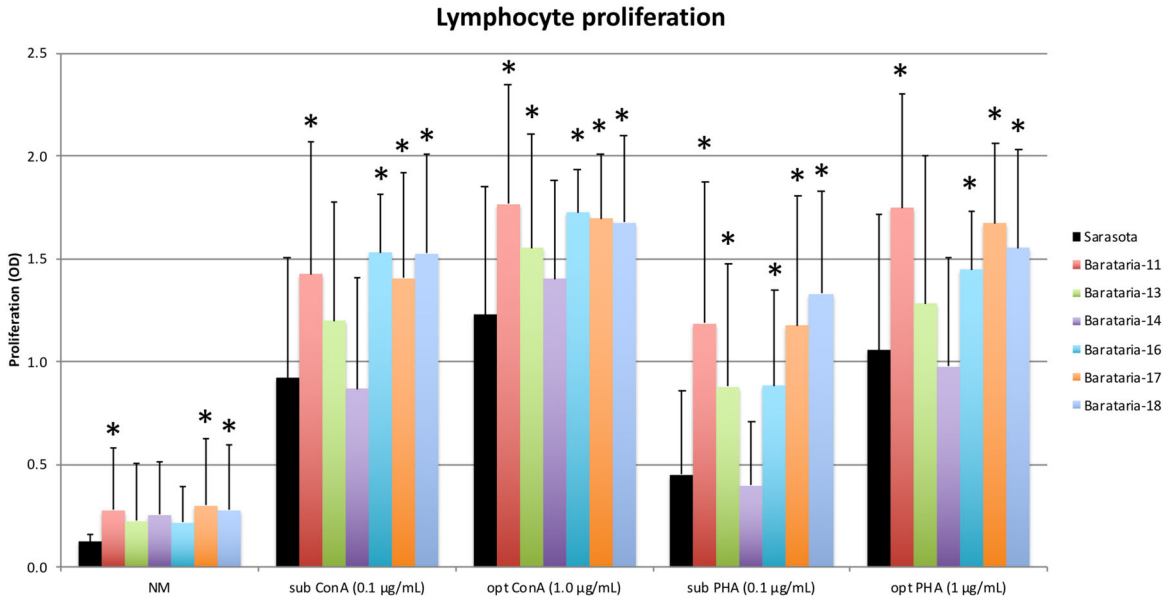

FIGURE 1: Lymphocyte proliferation in blood of bottlenose dolphins upon stimulation with optimal and suboptimal concentrations of mitogens, relative to no mitogen stimulation. Results (means of triplicates) are expressed as optical density. Samples were obtained from the reference population in Sarasota Bay, Florida, USA, in 2011 to 2014 ($n = 60$) and from dolphins exposed to oiling following the *Deepwater Horizon* oil spill in Barataria Bay, Louisiana, USA, sampled in 2011 ($n = 29$), 2013 ($n = 31$), 2014 ($n = 32$), 2016 ($n = 36$), 2017 ($n = 22$), and 2018 ($n = 34$). Results are means, and error bars represent standard deviation. Results for Sarasota Bay and Barataria Bay 2011 to 2014 from De Guise et al. (2017). *Results significantly different from Sarasota Bay ($p < 0.05$). ConA = concanavalin A; PHA = phytohemagglutinnin A; NM = no mitogen; OD = optical density; opt = optimal; sub = suboptimal.

(Figure 4). Plasma cytokines concentrations were generally low and often below the assay detection limit.

The proportion of FOXP3$^+$ Treg cells in the blood of Barataria Bay dolphins sampled in 2018 was significantly (~3 times) higher than that in Sarasota Bay dolphins (Figure 5). Similarly, the proportion of FOXP3$^+$ Treg cells in blood of mice exposed to Louisiana sweet crude oil by gavage for 6 wk was significantly (~2 times) higher than that in control mice exposed to mineral oil (Figure 6). However, there was no significant difference in the proportion of FOXP3$^+$ Treg cells in spleen from the same mice. There was no significant difference in the proportion of FOXP3$^+$ Treg cells in blood or spleen from the unexposed F_1 progeny of exposed versus control mice (data not shown).

In vitro stimulation with recombinant cytokines was performed to assess the responsiveness of Barataria Bay and Sarasota Bay dolphin lymphocytes (Figure 7). Stimulation with recombinant IL-4 resulted in a significantly higher expression of the genes for IL-4 and IL-13 in Barataria Bay dolphins compared to Sarasota Bay (Figure 7A). Stimulation with IL-12 and IFNγ did not result in a significantly different expression of the genes for IFNγ in Barataria Bay compared to Sarasota Bay, nor did stimulation with IL-2 and TGFß significantly change the expression of the genes for TGFß and IL-10. Further, preincubation of Sarasota Bay dolphin lymphocytes with increasing concentrations of HEMAF significantly increased the expression of the gene for IL-13 upon stimulation with IL-4 (Figure 7B), whereas others were not significantly affected.

When available, age determination through photoidentification, tooth growth layer group, or dental radiography was used to assess whether dolphins sampled were alive at the time of the spill or born after the spill. Dolphins that were born after the spill had significantly (19%) higher ConA- (suboptimal concentration) induced T-lymphocyte proliferation than dolphins that were alive at the time of the spill (Figure 8). Although cytokine levels were highly variable, resulting in relatively low statistical power, interesting trends were observed (Figure 9). The concentrations of the Th1 cytokines IL-2, IL-12, and IFNγ in dolphins born after the spill were 42, 49, and 21%, respectively, of those in dolphins alive at the time of the spill. The concentrations of the Th2 cytokines IL-4, IL-5, and IL-13 in dolphins born after the spill were 21, 245, and 54%, respectively, of those in dolphins alive at the time of the spill. The concentrations of the Treg cytokines IL-10 and TGFß were relatively similar between dolphins born after the spill and those alive at the time of the spill. Correlation analysis demonstrated no significant correlation between T-lymphocyte proliferation and age in the reference Sarasota Bay dolphins (data not shown). Similarly, correlation analysis demonstrated no significant correlation between the Th1 cytokines (IL-1, IL-12, and IFNγ), the Th2 cytokines (IL-4, IL-5, and IL-13), the Treg cytokine IL-10, and age in the reference Sarasota Bay dolphins (data not shown). Only the Treg cytokine TGFß was significantly negatively correlated with age ($R = -0.348$, $p = 0.004$, $n = 64$).

There were no significant differences in litter size or weight at weaning between the F_1 progeny of oil-exposed and control mice (data not shown).

DISCUSSION

Long-term immunological alterations

We present evidence that the previously documented immune changes in a population of bottlenose dolphins in

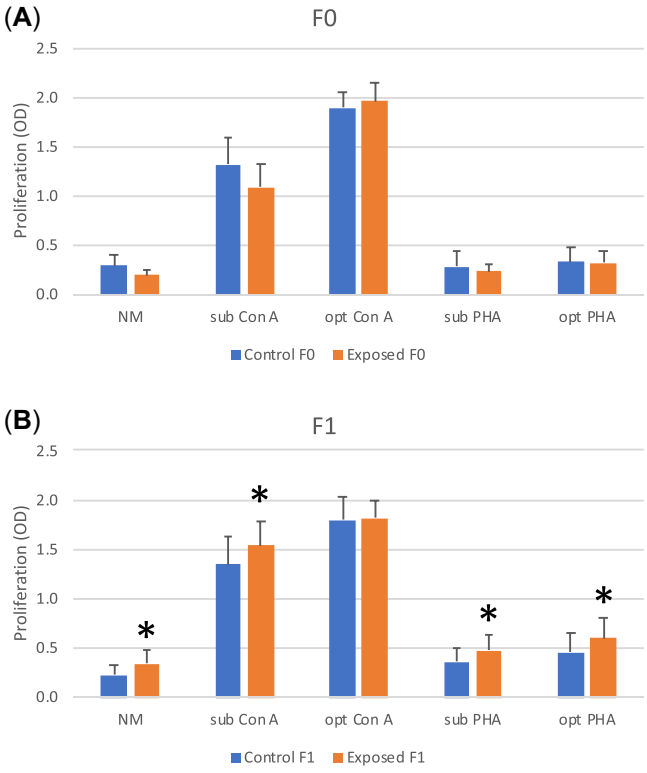

FIGURE 2: Lymphocyte proliferation of mouse splenocytes upon stimulation with optimal and suboptimal concentrations of mitogens, relative to no mitogen stimulation, in (**A**) F0 mice exposed to Louisiana sweet crude oil for 6 wk by gavage ($n = 8$) compared to control mice exposed to mineral oil ($n = 8$) and (**B**) the unexposed F1 progeny of mice exposed to Louisiana sweet crude ($n = 17$) compared to the progeny of control mice exposed to mineral oil ($n = 17$). Results are expressed as optical density and presented as means, and error bars represent standard deviation. *Results significantly different from control mice ($p < 0.05$). ConA = concanavalin A; PHA = phytohemagglutinnin A; NM = no mitogen; OD = optical density; opt = optimal; sub = suboptimal.

Barataria Bay in the footprint of the *Deepwater Horizon* oil spill (De Guise et al. 2017) were still present in 2017 to 2018. The pattern of immune changes reported in Barataria Bay dolphins in 2017 and 2018 (increased T-lymphocyte proliferation, decreased Th1 cytokines, tilt toward a Th2 balance, and affected Treg cytokines, with decreased IL-10 without decreased TGFß) was strikingly similar to that observed in Barataria Bay in 2011 (De Guise et al. 2017). Although sampling in 2013 and 2014 appeared to show a dampening of the immunological effects (De Guise et al. 2017), continued monitoring in 2016 to 2018 suggests a reversal of the trend. It is possible that immune changes persisted over time but that we may not have detected such persistence, given sampling variation and/or potentially annual variation related to environmental conditions and given the relatively small sample size and heterogeneous sampling cohort.

We considered causes other than oil exposure to explain these observations. Morbillivirus infections have been described in marine mammals (Duignan et al. 2014; Van Bressem et al. 2014), and morbilliviruses have the potential to modulate immune functions. Exposure to morbillivirus is episodic in some cetaceans in the Gulf of Mexico (Rowles et al. 2011; Fauquier et al. 2017), and some of the dolphins sampled in Barataria Bay exhibited detectable titers (C. Cloyed et al., Dauphin Island Sea Lab, Dauphin Island, AL, USA, unpublished data). However, high morbillivirus titers in bottlenose dolphins have been shown to decrease T-lymphocyte proliferation (Bossart et al. 2011). Similarly, immunological changes observed in other populations of wild bottlenose dolphins undergoing different environmental stressors, such as exposure to harmful algal bloom toxins (Schwacke et al. 2010) or elevated exposure to PCBs (Schwacke et al. 2012), have been documented; but the patterns of immunomodulation described were qualitatively different from those observed in the present study. Further, comprehensive health assessments in Barataria Bay between 2011 and 2018 did not unveil morbillivirus outbreaks, harmful algal bloom exposure, high exposure to environmental contaminants, or other causes that could explain the changes in immune functions reported in the present study. Finally, it is possible that there is continued exposure to the *Deepwater Horizon* oil that may not have been completely removed from the Barataria Bay ecosystem. In fact, oil in Barataria Bay marsh sediments 8 yr after the *Deepwater Horizon* spill was still 10 times above prespill concentrations, with the authors suggesting "a long-term contamination by oil or oil residues that will remain for decades" (Turner et al. 2019). Of interest is a particular ongoing feeding behavior observed in Barataria Bay dolphins, whereby they appear to "drill into the sediments" (B. Quigley, National Marine Mammal Foundation, San Diego, CA, USA, unpublished data), with the potential to inadvertently ingest and/or resuspend oil constituents and continue their exposure.

Together, our findings present a pattern of immune changes in Barataria Bay in 2017 and 2018 similar to that observed in dolphins sampled in Barataria Bay a year after the *Deepwater Horizon* spill, which was reasonably attributed to the exposure to oil from the spill (De Guise et al. 2017), different from patterns of immunotoxicity observed in association with other stressors, and in the absence of other compelling causes.

While reproductive impairment and immune changes were observed in Barataria Bay dolphins following the *Deepwater Horizon* spill (Lane et al. 2015; De Guise et al. 2017; Kellar et al. 2017), as was the case for mink experimentally exposed to oil (Mazet et al. 2001; Schwartz et al. 2004b), the observation of oil-induced immunological effects in our mouse experimental exposure in the absence of reproductive effects suggests that the immune system may be more sensitive to the adverse impacts of oil exposure. This is compatible with previous reporting that the immune system is among the most sensitive to the effects of xenobiotics (Tryphonas 2001).

Although the cause of health changes in wildlife is difficult to unequivocally attribute to a specific cause, the weight-of-evidence approach can help link health effects to a potential cause. Several studies assessed the immunomodulatory effects of individual polycyclic aromatic hydrocarbons (PAHs), but few involved direct oil exposure. Experimental oil exposure in fish can affect the expression of immune function genes (Jones et al. 2017; Rodgers et al. 2018, 2020) and increase susceptibility to a bacterial challenge (Jones et al. 2017), the ultimate

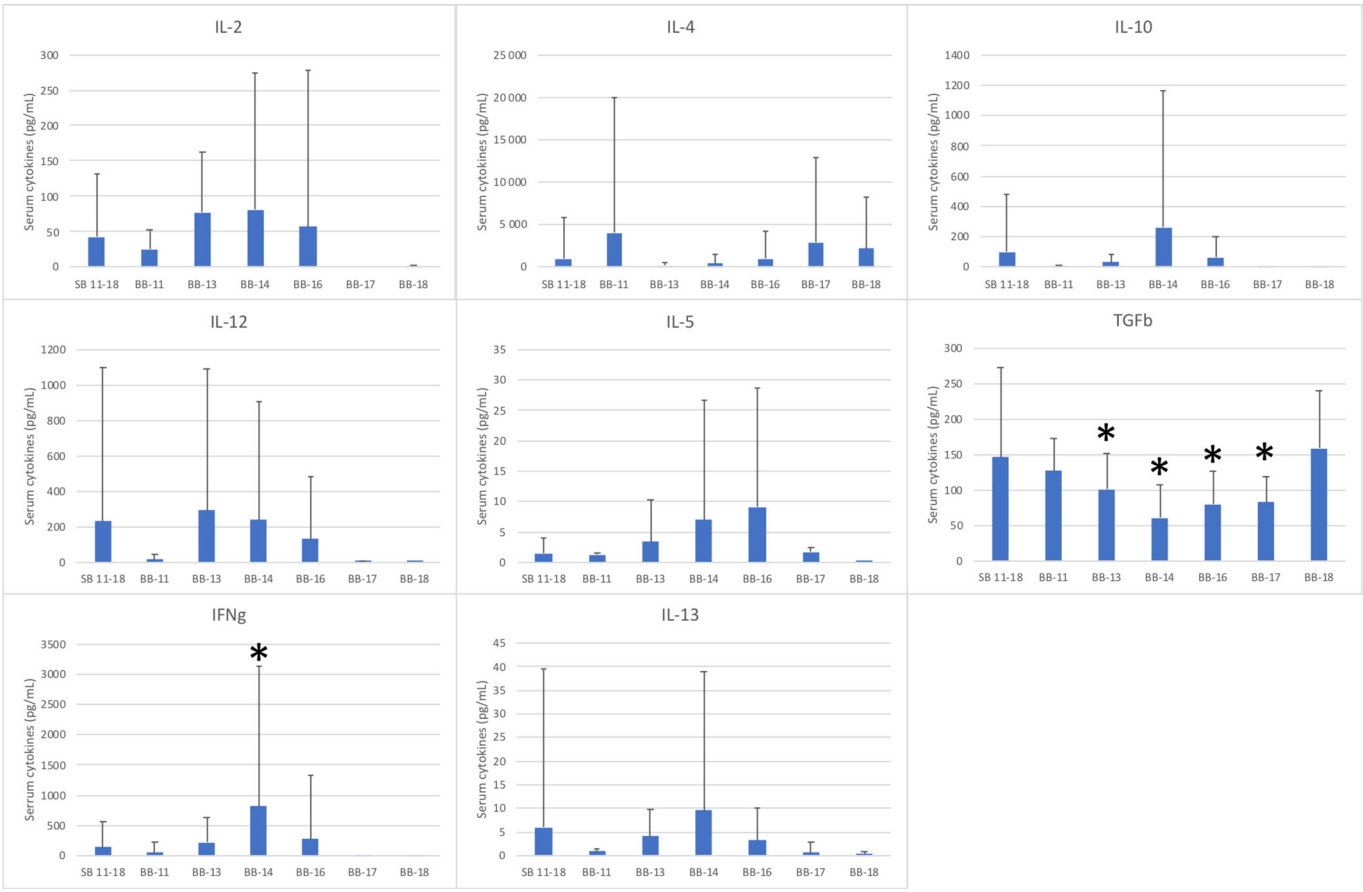

FIGURE 3: Serum concentrations of T helper 1 (Th1; IL-2, IL-12, and IFNγ), Th2 (IL-4, IL-5, and IL-13), and T regulatory (IL-10 and TGFß) cytokines in bottlenose dolphins sampled from the reference population in Sarasota Bay, Florida, USA (SB) in 2011 to 2018 ($n = 68$) and from dolphins exposed to oiling following the *Deepwater Horizon* oil spill in Barataria Bay, Louisiana, USA (BB), sampled in 2011 ($n = 32$), 2013 ($n = 31$), 2014 ($n = 32$), 2016 ($n = 38$), 2017 ($n = 22$), and 2018 ($n = 34$). Results are means, and error bars represent standard deviation. Results for Sarasota Bay and Barataria Bay 2011 to 2014 from De Guise et al. (2017). *Results significantly different from Sarasota Bay ($p < 0.05$).

manifestation of immunotoxicity. In mammals, the observed increase in T-cell proliferation was very similar in nature and magnitude to that observed in mink chronically exposed to bunker C fuel oil (Schwartz et al. 2004b). Further, an increase in T-lymphocyte proliferation was also observed in our mouse experimental oil exposure study, albeit in the F_1 progeny of exposed mice. The lack of direct modulation of T-lymphocyte proliferation in the directly exposed (F_0) mice might be explained by the duration/chronicity of exposure and/or dose because it has been suggested that PAHs might have suppressive effects at high dose and stimulatory effects at lower doses (Burchiel and Luster 2001). Finally, in vitro exposure of bottlenose dolphin lymphocytes to Louisiana sweet crude oil also resulted in a concentration–response increase in T-lymphocyte proliferation (White et al. 2017). The combination of field studies in wild dolphins, mouse experimental exposure, and in vitro oil exposure in dolphin cells supports the potential for oil exposure to affect T-lymphocyte proliferation in dolphins.

Modulation of cytokine balance following various PAH exposures generally involves a downregulation of the Th1 response and favors a Th2 response (reviewed in De Guise et al. 2017). However, none of those studies involve direct oil exposure. Our mouse study failed to demonstrate noticeable changes in the Th1/Th2 cytokine balance in directly exposed (F_0) mice or their progeny (F_1). However, cytokine concentrations were generally low, which is not surprising in laboratory mice kept in environments devoid of environmental challenges. In contrast, in vitro stimulation of dolphin lymphocytes showed a small and nonsignificant reduction in Th1 responsiveness and a significant increase in Th2 responsiveness in previously oil-exposed Barataria Bay dolphins compared to reference Sarasota Bay dolphins. Finally, in vitro exposure of reference Sarasota Bay dolphin lymphocytes to oil confirmed a small and nonsignificant concentration–response reduction in Th1 responsiveness and a significant increase in Th2 responsiveness. Altogether, the combination of wild dolphin population studies and laboratory in vitro exposure experiments supports the potential for oil to modulate the Th1/Th2 balance in favor of a Th2 response in dolphins.

Potential mechanisms of immunotoxicity

With the recent speculation that Treg cells may be involved in the immunotoxicity of oil in dolphins (De Guise et al. 2017) and building on recent validation studies (De Guise et al. 2019),

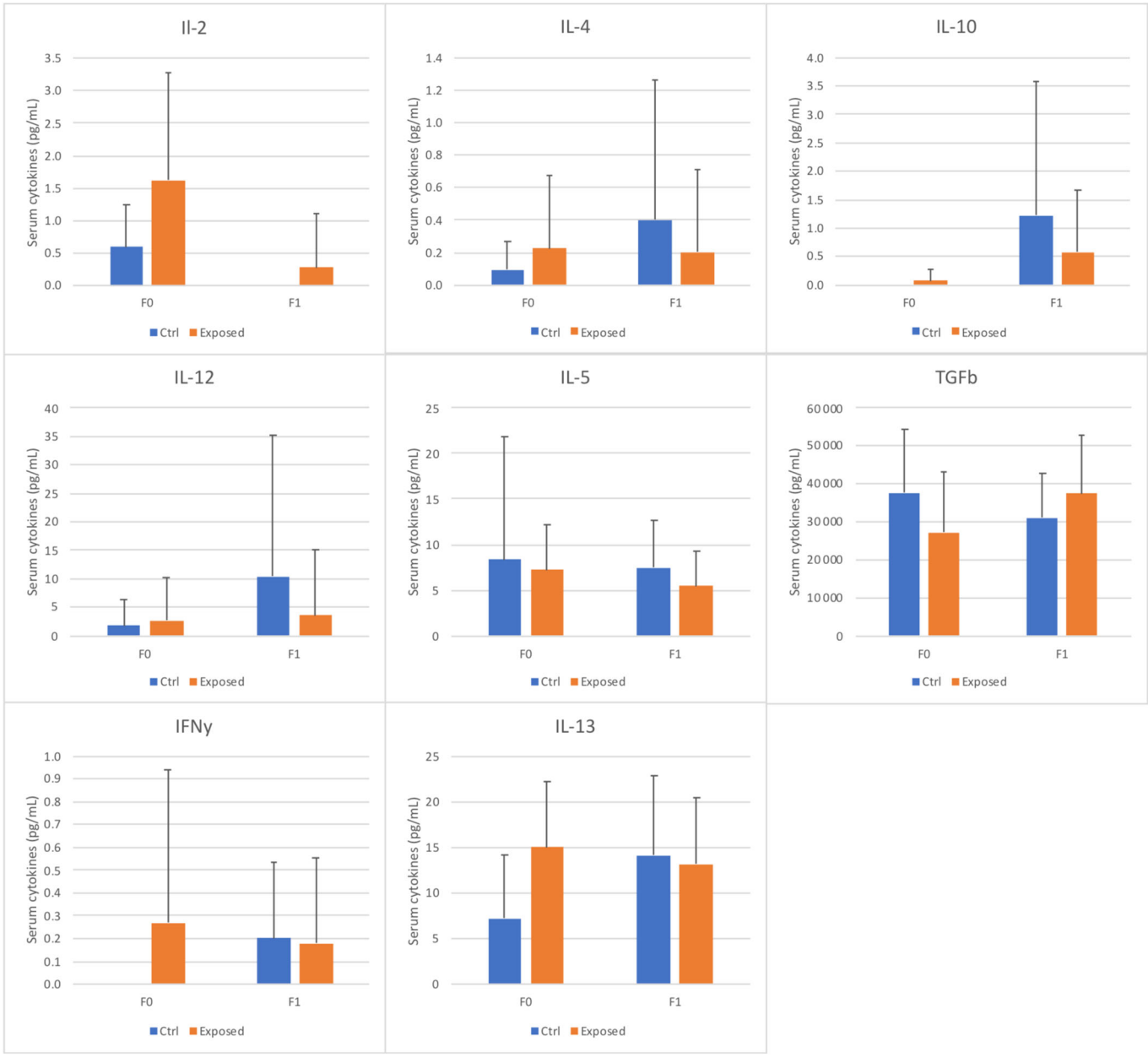

FIGURE 4: Serum concentrations (picograms per milliliter) of the T helper 1 (Th1) cytokines IL-2, IL-12, and IFNγ; the Th2 cytokines IL-4, IL-5, and IL-13; and the T regulatory cytokines IL-10 and TGFß in oil-exposed and control F0 mice ($n = 8$/group) and in the unexposed progeny (F1) of oil-exposed and control mice ($n = 17$/group). Results are means, and error bars represent standard deviation.

the present study quantified Treg cells in Barataria Bay and Sarasota Bay dolphins. Dolphins from Barataria Bay had 3 times as many circulating Treg cells as reference Sarasota Bay dolphins. This closely resembled findings in mice experimentally exposed to oil, which had 2 times as many circulating Treg cells as control mice. A higher proportion of Treg cells in the absence of increased Treg cytokines in mice and dolphins suggests the potential for a functional dysregulation of Treg cells on oil exposure. There are several potential mechanisms by which oil exposure could lead to Treg dysregulation (summarized in Figure 10), informed by a recent review of the metabolic regulation of Treg cells (Chen et al. 2019).

Activation of the aryl hydrocarbon receptor (AhR) with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) expanded the population of $CD4^+CD25^+Foxp3^+$ Treg cells in the pancreatic lymph nodes of mice and suppressed the development of autoimmune type 1 diabetes in nonobese diabetic mice (Kerkvliet et al. 2009). In an experimental colitis mouse model, TCDD attenuated colitis clinical signs with increased differentiation of Tregs and attenuation of Th17 cells, through increased methylation of CpG islands of Foxp3 and demethylation of IL-17 promoters, suggesting epigenetic mechanisms (Singh et al. 2011). These studies led to the search for nontoxic AhR ligands inducing Treg cells for potential therapeutic use in

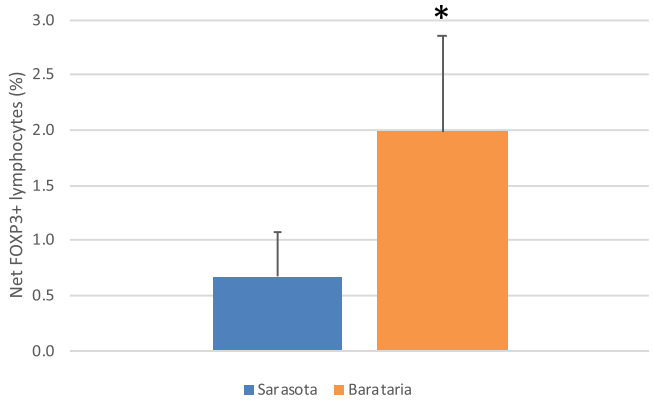

FIGURE 5: The proportion of FOXP3$^+$ T regulatory cells in blood of bottlenose dolphins sampled from the reference population in Sarasota Bay, Florida, USA, in 2018 ($n = 20$) and from dolphins exposed to oiling following the *Deepwater Horizon* oil spill in Barataria Bay, Louisiana, USA sampled in 2018 ($n = 34$). Results are means, and error bars represent standard deviation. *Results significantly different from Sarasota Bay ($p < 0.05$). FOXP3 = forkhead box P3.

preventing graft rejection (Punj et al. 2014), ulcerative colitis (Lv et al. 2018a, 2018b), and arthritis (Tong et al. 2016) models. Activation of AhR in mice by TCDD induced functional Treg cells, whereas AhR activation by 6-formylindolo[3,2-b]carbazole interfered with Treg-cell differentiation and functionality (Quintana et al. 2008). Thus, AhR regulates Treg- and Th17-cell differentiation in a ligand-specific manner. This may be of importance because AhR signaling is potentially important in modulating the effects of PAHs during an oil spill (Bak et al. 2019).

Glycolysis inhibits Treg differentiation and promotes Treg expansion, whereas fatty-acid oxidation promotes Treg differentiation (Chen et al. 2019). If oil exposure affects oxidative stress in dolphins, as was demonstrated in birds (Bursian et al. 2017; Harr et al. 2017) and turtles (Mitchelmore et al. 2015) exposed to oil, it has the potential to disrupt lipid metabolism and potentially Treg metabolism and therefore differentiation.

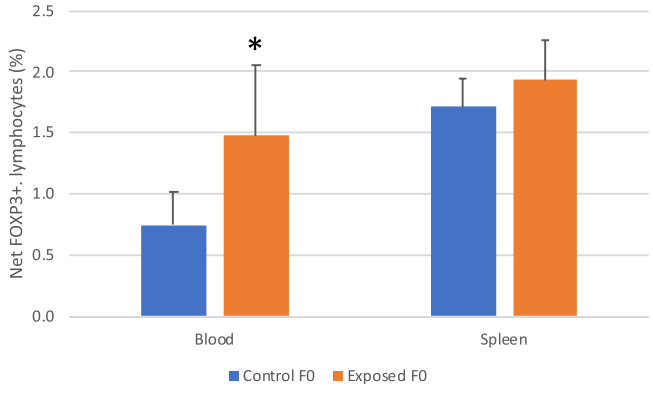

FIGURE 6: The proportion of forkhead box P3 (FOXP3$^+$) T regulatory cells in blood and spleen of mice exposed to Louisiana sweet crude oil for 6 wk by gavage ($n = 8$) compared to control mice exposed to mineral oil ($n = 8$). Results are means, and error bars represent standard deviation. *Results significantly different from control mice ($p < 0.05$).

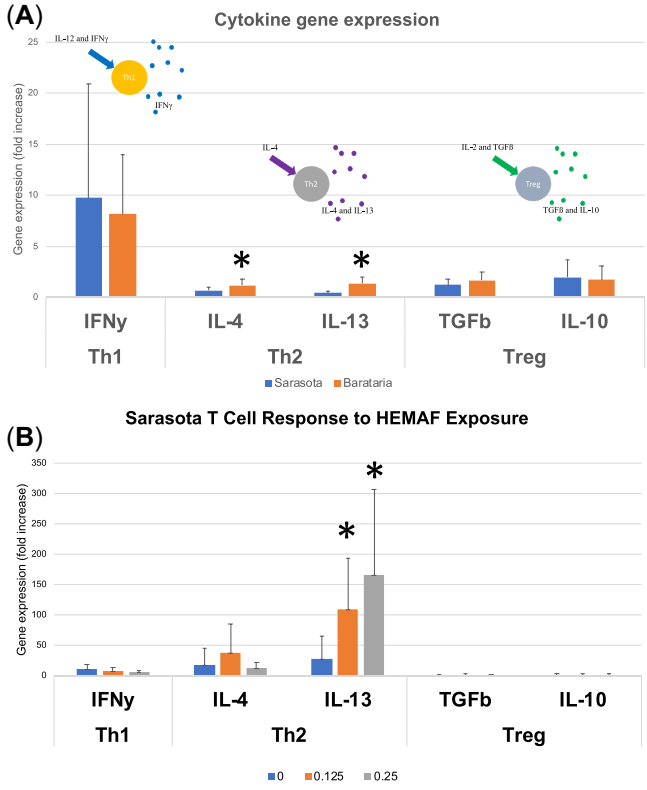

FIGURE 7: Relative gene expression of effector T helper 1 (Th1), Th2, and T regulatory (Treg) cytokines on polarizing stimulus with the Th1-inducing cytokines IL-2 and IFNγ (25 ng/mL), the Th2-inducing cytokines IL-4 and IL-2 (25 ng/mL), and the Treg-inducing cytokines IL-2 and TGFß (10 ng/mL). Data are expressed relative to unstimulated cells (value of 1). The responsiveness was compared (**A**) between bottlenose dolphins sampled from the reference population in Sarasota Bay, Florida, USA, in 2018 ($n = 9$–13 dolphins/cytokine) and dolphins exposed to oiling following the *Deepwater Horizon* oil spill in Barataria Bay, Louisiana, USA, sampled in 2018 ($n = 11$–13 dolphins/cytokine) and (**B**) in Sarasota Bay dolphins upon in vitro exposure to increasing concentrations of high-energy medium accommodation fraction of Louisiana sweet crude oil ($n = 3$–10 dolphins/cytokine). Results are means, and error bars represent standard deviation. *Results significantly different from control mice ($p < 0.05$). HEMAF = high-energy medium accommodation fraction.

Several signaling pathways may influence different aspects of Treg-cell biology (Figure 10). The mammalian target of rapamycin (mTOR) pathway plays an important role in the regulation of Treg cells, with mTOR complexes 1 and 2 having opposite activities on Treg-cell expansion and function (Chen et al. 2019). Toll-like receptor signals promote Treg-cell proliferation but also increase glycolysis and expression of glucose transporter 1 (Glut1), which impaired Treg-cell suppressive capacity; and Glut1 expression was sufficient to increase the number of Treg cells, but it reduced their suppressive capacity and Foxp3 expression (Gerriets et al. 2016). Defects in a kinase controlling the expression of receptors on Tregs impaired their metabolic and functional fitness, leading to an excessive Th2-dominant inflammatory disorder in mice (Yang et al. 2017). Overall, it appears that the differentiation, expansion, and regulatory functions of Treg cells have the potential to be modulated independently, as appears to be the case in our

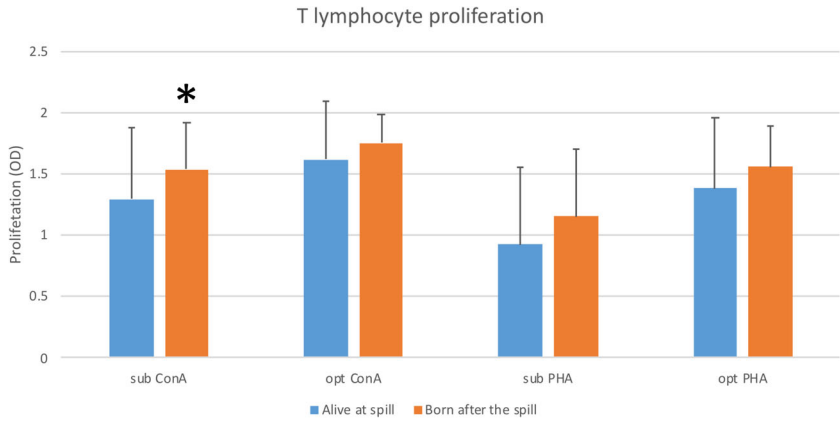

FIGURE 8: Lymphocyte proliferation in blood of Barataria Bay, Louisiana, USA, bottlenose dolphins exposed to oiling following the *Deepwater Horizon* oil spill upon stimulation with optimal and suboptimal concentrations of mitogens, expressed as optical density. Using age estimation (when available) relative to the time of the *Deepwater Horizon* spill, T lymphocyte proliferation was compared between dolphins alive at the time of the spill ($n = 155$) and those born after the spill ($n = 30$). Results are means, and error bars represent standard deviation. *Results significantly different from "alive at spill" ($p < 0.05$). ConA = concanavalin A; PHA = phytohemagglutinnin A; NM = no mitogen; OD = optical density; opt = optimal; sub = suboptimal.

study (more Treg cells without associated increased Treg functionality). It is possible that the delicate interplays between pathways and receptors that regulate Treg differentiation and suppressive functions could be directly or indirectly affected by oil exposure.

Whatever mechanism is involved, dysregulation of Treg cells following oil exposure would be consistent with the observed increase in T-lymphocyte proliferation, with or without mitogen. Interestingly, an increase in T-lymphocyte proliferation at lower doses and a decrease in T-cell proliferation at higher doses in mice exposed to cyclophosphamide were attributed to the higher sensitivity of regulatory T cells at low exposure dose (Luster et al. 1993). Dysregulation of Treg cells would also be consistent with a Th2-biased cytokine pattern, as observed in mice that develop Th2-dominant inflammatory disorders (Yang et al. 2017).

Multigenerational effects

Our mouse study demonstrated the potential for oil exposure to have immunomodulatory effects on directly exposed mice as well as their unexposed progeny. Dolphins in Barataria Bay have been sampled up to 8 yr after the *Deepwater Horizon* spill, affording the opportunity to sample dolphins that were born after the oil spill. To explore the potential for multigenerational effects in dolphins, we compared immune functions in dolphins with a known age estimate relative to the time of the spill. We observed an increase in T-cell proliferation and serum cytokines, suggesting a reduced Th1 response in dolphins that were born after the spill compared to those alive at the time of the spill. The lack of significant correlations between those variables and age in reference Sarasota Bay dolphins suggests that those differences are not biased by the younger age of dolphins born after the spill but rather suggests the potential for multigenerational effects, through parental exposure of the germline that can in turn increase disease susceptibility of subsequent generations of the exposed ancestors through epigenetics (Nilsson et al. 2018) or the potential for continued environmental oil exposure in young dolphins potentially more susceptible to the effects of oil. Several environmental toxicants have been shown to promote the transgenerational inheritance of increased disease susceptibility in a diversity of species including birds, fish, rodents, and pigs (Nilsson et al. 2018); and multigenerational effects have recently been documented in fish following experimental oil exposure, although the specific mechanisms were not documented (Jasperse et al. 2019). The potential for multigenerational effects of oil, irrespective of the mechanisms involved, raises significant concerns for the recovery of dolphin stocks affected by the *Deepwater Horizon* oil spill.

Long-term health implications

Modeling efforts suggest that the *Deepwater Horizon* oil spill resulted in 30 347 lost cetacean yr (the difference between baseline and injured population size, summed over the modeled time period) in Barataria Bay alone, with an estimated 39 yr to population recovery (Schwacke et al. 2017). Rodent literature meta-analyses documented that chemical-induced immunomodulation increases susceptibility to infectious diseases, with the likelihood that the concept applies to natural populations that experience continued background exposure to pathogens (Luster et al. 1993). It is therefore possible that Barataria Bay dolphins sampled in 2017 and 2018 may have increased susceptibility to infectious diseases, therefore increasing the likelihood of disease and possibly death. Such reduction in fitness could further increase the subtle, long-term impact on the population and could slow anticipated population recovery, especially if it continues to be observed across generations.

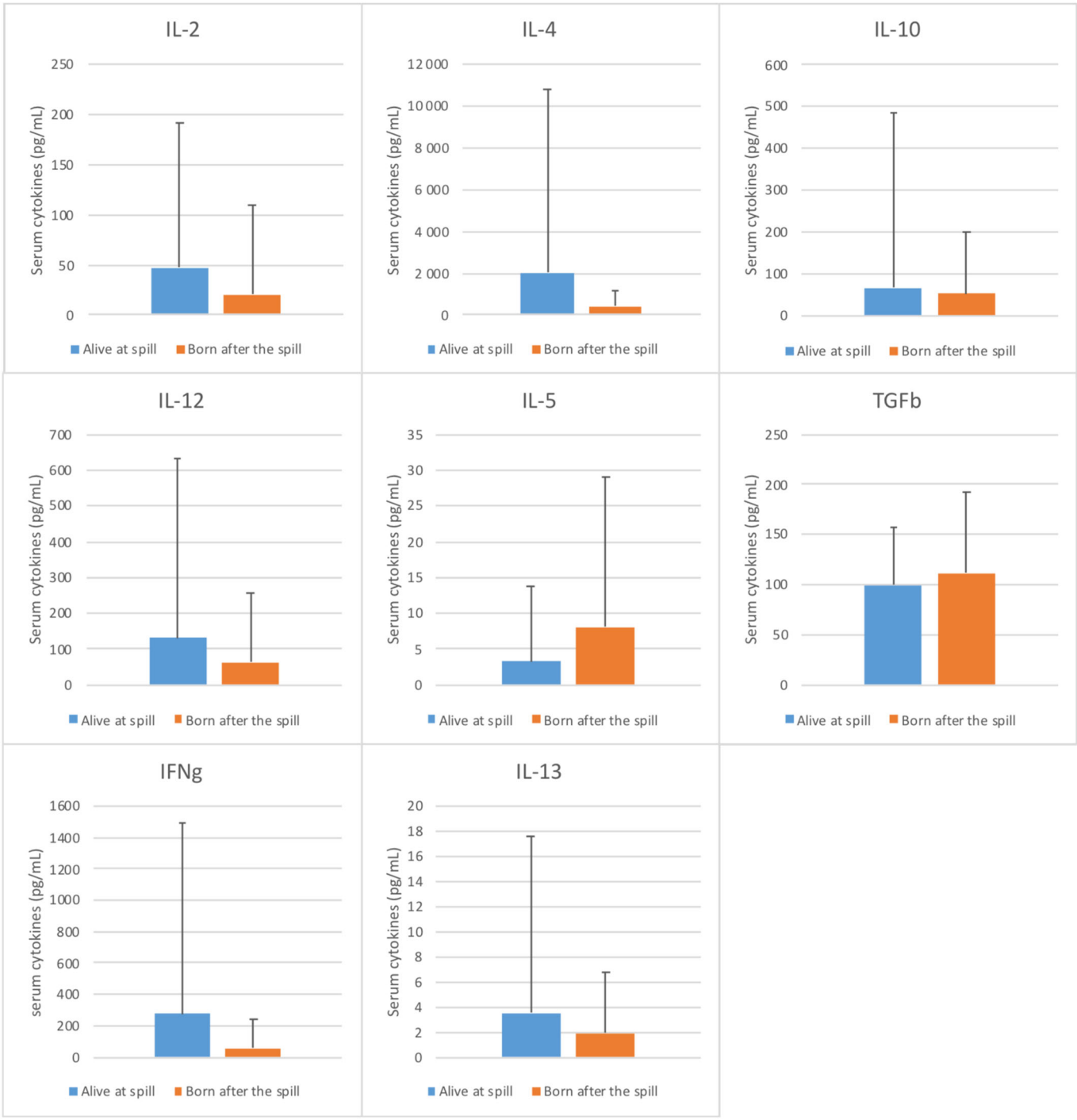

FIGURE 9: Serum T helper 1 (Th1; IL-2, IL-12, and IFNγ), Th2 (IL-4, IL-5, and IL-13), and T regulatory (IL-10 and TGFß) cytokines in Barataria Bay, Louisiana, USA, bottlenose dolphins exposed to oiling following the *Deepwater Horizon* oil spill and sampled in 2011 to 2018. Using age estimation (when available) relative to the time of the *Deepwater Horizon* spill, serum cytokine concentrations were compared between dolphins alive at the time of the spill ($n = 155$) and those born after the spill ($n = 30$). Results are means, and error bars represent standard deviation.

The explosion of the *Deepwater Horizon* oil exploration platform on 20 April 2010 resulted in an unprecedented release of oil in the Gulf of Mexico. Oil spills involve health risks for humans participating in cleanup or inhabiting or otherwise using the surrounding affected area (Laffon et al. 2016). Respiratory problems have been documented in cleanup workers following the *Deepwater Horizon* spill (Alexander et al. 2018; D'Andrea and Reddy 2018). Other human health impacts were recently reviewed following several major crude oil or heavy fuel oil (bunker C) spill accidents (Aguilera et al. 2010; Goldstein et al. 2011; Levy and Nassetta 2011; Laffon et al. 2016). Beyond acute effects (Levy and Nassetta 2011), subtle but potentially important immune effects have been described in cleanup workers, including

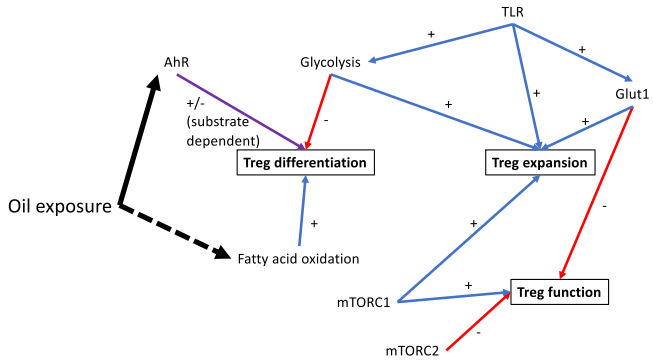

FIGURE 10: Demonstrated pathways for modulation of T regulatory cell differentiation, expansion, and regulatory function can be modulated—positively (blue arrows), negatively (red arrows), or either positively or negatively—depending on the substrate (purple arrow). Oil polycyclic aromatic hydrocarbons have demonstrated effects through the aryl hydrocarbon receptor (black arrow), and oil exposure has been demonstrated to affect fatty acid oxidation in other species (dashed black arrow). AhR = aryl hydrocarbon receptor; Glut1 = glucose transporter 1; mTORC = mammalian target of rapamycin complex; TLR = toll-like receptor; Treg = T regulatory cell.

reduced blood levels of CD4 T lymphocytes, IL-2, IL-4, IL-10, and IFNγ (Gestal Otero et al. 2004) and lower circulating $CD16^{+}56^{+}$ natural killer cells (Laffon et al. 2013). Despite the paucity of studies, the data confirm the potential for oil exposure to adversely affect the immune system in humans and that it may decrease disease resistance.

The observation of long-term health effects in dolphins following the *Deepwater Horizon* oil spill, including the trend in unfavorable prognosis (L.H. Schwacke, unpublished data), and the observation of subtle but potentially impactful effects on the immune system, coupled with the observation of immunological changes in humans exposed to oil and the potential for multigenerational effects, suggest that long-lived bottlenose dolphins may serve as sentinels for the potential environmental risk associated with oil exposure.

CONCLUSIONS

The present study documented immunological alterations in Barataria Bay bottlenose dolphins sampled up to a decade following the *Deepwater Horizon* oil spill that were similar in nature to those associated with and immediately following the spill. The specific nature of the changes, the compatibility of those changes with similar effects observed in a mouse model, and in vitro exposure of dolphin cells point to the potential for continued health effects associated with oil exposure. These effects may result from continued exposure and/or the potential for multigenerational consequences. Our results support a central role for Treg-cell dysfunction as a key mechanism underlying those effects. Long-term consequences of oil exposure on the highly sensitive immune system, with the potential for multigenerational effects, might have significant consequences on the potential for population recovery and raise concerns for other mammalian species.

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Disclaimer—The statements, findings, conclusions, and recommendations are those of the author(s) and do not necessarily reflect the views of Ocean Leadership or the National Marine Mammal Foundation.

Author Contribution Statement—S. De Guise, M. Levin, L. Jasperse: conception and design of experiments; M. Levin, L. Jasperse: performance of experiments; S. De Guise, M. Levin, L. Jasperse, L. Schwacke: statistical analysis; S. De Guise, M. Levin, L. Jasperse: writing; S. De Guise, M. Levin, L. Jasperse, J. Herrman, R. Wells, T. Rowles, L. Schwacke: technical and editorial assistance.

Data Availability Statement—Data are publicly available through the Gulf of Mexico Research Initiative Information & Data Cooperative at https://data.gulfresearchinitiative.org/ (https://doi.org/10.7266/n7-2wtd-hz89, https://doi.org/10.7266/n7-vjnn-yz30, https://doi.org/10.7266/n7-qf5g-zv19, https://doi.org/10.7266/n7-ymp8-x712, https://doi.org/10.7266/n7-np2g-r569).

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