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Environmental perfluorooctane sulfonate exposure drives T cell activation in bottlenose dolphins

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Abstract

Perfluoroalkyl acids (PFAAs) are highly stable compounds that have been associated with immunotoxicity in epidemiologic studies and experimental rodent models. Lengthy half-lives and resistance to environmental degradation result in bioaccumulation of PFAAs in humans and wildlife. Perfluorooctane sulfonate (PFOS), the most prevalent PFAA detected within the environment, is found at high levels in occupationally exposed humans. We have monitored the environmental exposure of dolphins in the Charleston, SC region for over 10 years and levels of PFAAs, and PFOS in particular, were significantly elevated. As dolphins may serve as large mammal sentinels to identify the impact of environmental chemical exposure on human disease, we sought to assess the effect of environmental PFAAs on the cellular immune system in highly

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Conflict of interest

The authors did not report any conflict of interest.

Supporting information

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exposed dolphins. Herein, we utilized a novel flow cytometry-based assay to examine T cell-specific responses to environmental PFAA exposure ex vivo and to exogenous PFOS exposure in vitro. Baseline PFOS concentrations were associated with significantly increased $CD4^+$ and $CD8^+$ T cell proliferation from a heterogeneous resident dolphin population. Further analysis demonstrated that in vitro exposure to environmentally relevant levels of PFOS promoted proinflammatory cytokine production and proliferation in a dose-dependent manner. Collectively, these findings indicate that PFOS is capable of inducing proinflammatory interferon-gamma, but not immunoregulatory interleukin-4 production in T cells, which may establish a state of chronic immune activation known to be associated with susceptibility to disease. These findings suggest that PFOS directly dysregulates the dolphin cellular immune system and has implications for health hazards.

Keywords

perfluorooctane sulfonate (PFOS); perfluoroalkyl acids; immunology; bottlenose dolphin (*Tursiops truncatus*); $CD4^+$ and $CD8^+$; T cell activation; interferon-gamma (IFNγ)

Introduction

Perfluorooctane sulfonate (PFOS) is a persistent organic pollutant consisting of an eight-carbon chain perfluoroalkyl acid (PFAA). PFOS is the principal and final degradation product of several commercial per/polyfluoroalkyl substances (Hansen *et al.*, 2002; Lau *et al.*, 2004). While the production of PFOS and several PFAAs have ceased in the USA and elsewhere, progress on global emission reductions are lagging due to continued production in developing regions and their slow degradation (Lindstrom *et al.*, 2011). An important environmental concern is that PFOS can bioaccumulate and biomagnify in food chains and has been reported in aquatic wildlife and humans (Houde *et al.*, 2006a; Kannan *et al.*, 2004; Olsen *et al.*, 2003). As such, PFOS is the dominant PFAA found in human and wildlife blood (Houde *et al.*, 2011; Lau *et al.*, 2007). Despite reduced production, PFOS continues to be detected globally in biota and the environment and has generated concerns regarding the potential consequences of chronic exposure to wildlife and human health.

PFAA sediment levels in Charleston (SC, USA) were recently found to be higher than any other urban US area with 52% of 36 sites exceeding the median global PFOS sediment concentration (White *et al.*, 2015). Not surprisingly, higher body burdens of specific PFAA compounds are found in Charleston dolphins inhabiting areas with greater developed land use (Adams *et al.*, 2008). Fish are an important dietary source of PFAAs for wildlife and humans (Haug *et al.*, 2010; D'Hollander *et al.*, 2010; Hölzer *et al.*, 2011). As such, a dolphin food web study conducted in 2006 reported significantly higher PFAA concentrations in the Charleston dolphin food web compared to Florida (Houde *et al.*, 2006b). High levels of PFAA have been detected in plasma of resident dolphins inhabiting the estuarine waters of Charleston and in fact, are on the same order of magnitude as that of occupationally exposed humans (Fair *et al.*, 2013; Houde *et al.*, 2005). The Charleston dolphins with their high PFAA levels have served as important sentinels prompting a comprehensive survey of PFAAs in their environment. Dolphins are long-lived, top-level predators in coastal waters

where they are exposed to ecosystem perturbations, including anthropogenic contaminants and natural toxins, through both direct exposure and food web magnification. As environmental sentinels, they may provide insight on the health of coastal marine ecosystems and serve as a proxy for human health.

Numerous reports have focused on the occurrence of PFOS along with other PFAAs and their toxic effects including hepatotoxic, carcinogenic, adverse reproductive and developmental, immune- and neurotoxic effects (Lau *et al.*, 2004, 2007; Kennedy *et al.*, 2004; Stahl *et al.*, 2011). Compared to other toxicological endpoints, the immune system appears to be particularly sensitive to exposure of PFOS and other PFAAs as noted in recent reviews (Corsini *et al.*, 2014; DeWitt *et al.*, 2012). Epidemiology and laboratory studies indicate both cell-mediated and humoral immunotoxic effects, including altered cytokine profiles and reduced antibody production (Corsini *et al.*, 2011, 2012; DeWitt *et al.*, 2009; Dong *et al.*, 2009, 2011; Fair *et al.*, 2011, 2013; Granum *et al.*, 2013; Lv *et al.*, 2015; Peden-Adams *et al.*, 2008). Studies in mouse splenocytes demonstrate that PFOS exposure results in cell cycle dysregulation, NRF2-mediated oxidative stress response and activation of T cell receptor and calcium signaling pathways (Lv *et al.*, 2015). The disruption of both T cell-independent and -dependent IgM antibody responses in mice suggest that B cells, macrophages, or both may be specific targets of PFAAs and that additional studies are needed to clarify mechanisms involved with this effect (Corsini *et al.*, 2014). Although *in vitro* studies have demonstrated that PFAA toxicity occurs, in part, via ligation of peroxisome proliferator-activated receptor alpha (PPARα), few studies have evaluated the ability of PFAAs to modulate directly the inflammatory responses and the specific effects upon T cell activation (Takacs *et al.*, 2007). Mounting evidence suggests that immune effects in laboratory models occur below and within the range of reported levels for highly exposed humans and wildlife (Corsini *et al.*, 2014; DeWitt *et al.*, 2012). In fact, the bioaccumulation of PFAA contaminants in the Charleston dolphins was associated with modulation of their immune responses and thus, may pose a threat to the health of these populations (Fair *et al.*, 2013). Similarly, PFAA exposure has been associated with reduced survival after influenza infection in mice, elevated rates of disease in sea otters, and immune and hematopoietic alterations in dolphin populations (Fair *et al.*, 2013; Guruge *et al.*, 2009; Kannan *et al.*, 2006).

Owing to the limited availability of commercial reagents, direct evaluation of T cell function in dolphins has yet to be examined. Herein, we utilized fluorochrome conjugation methods to label monoclonal antibodies specific for bottlenose dolphin $CD4^+$ and $CD8^+$ T cells appropriate for multiparametric flow cytometry. These reagents were then used to examine T cell responses, including proliferation and cytokine production, from a cohort of native dolphins residing in the estuarine coastal waterways surrounding Charleston. Utilizing flow cytometry for single-cell analysis, we investigated the effects of both baseline toxicant loading and *in vitro* exposure to environmentally relevant concentrations of PFOS on T cell function.

Materials and methods

Sample collections

Blood samples were collected from free-ranging Atlantic bottlenose dolphins (*Tursiops truncatus*) during the Dolphin Health and Risk Assessment Project conducted in August 2013. Dolphins were temporarily captured and released in the estuarine waters of Charleston (SC, USA) to assess their clinical and immune status, disease and contaminant exposure. This study was carried out in strict accordance under National Marine Fisheries permit no. 14352–02 issued to G.D.B. and approved by the Florida Atlantic University Institutional Animal Care and Use Committee. Detailed information pertaining to the study site, methods for capture, sampling and release are described elsewhere (Fair *et al.*, 2005). During the sampling process, each animal received a physical examination, including full body photo-documentation, diagnostic ultrasound, blood and urine collection, blubber and lesion biopsies, and microbiologic and cytologic sampling. Once restrained, blood samples were collected from the periarterial venous rete in the flukes using a 19-gauge needle, 1.9 cm butterfly catheter with a vacutainer attachment. Whole blood was collected in 10 ml ethylenediaminetetraacetic acid vacutainers. Peripheral blood leukocytes (PBL) were isolated following lysis of red blood cells after treatment with Becton Dickinson (BD, San Jose, CA, USA) Pharm Lyse solution as per the manufacturer's instructions. PBLs were isolated within 12 h of sample collection and cryopreserved in freezing media (90% fetal bovine serum, 10% dimethyl sulfoxide [DMSO]). Plasma for PFAA analysis was isolated from whole blood collected in 10 ml sodium heparin tubes. Blood was immediately centrifuged and plasma transferred into polypropylene cryovials, placed in liquid nitrogen Dewar and stored at −80°C until analysis. A tooth was extracted under local anesthesia and sectioned according to established protocols to determine age (Hohn *et al.*, 1989).

Sample perfluoroalkyl acid extraction/instrument analysis

Concentrations of PFAAs in plasma were determined at the Environment Canada's Laboratories (Burlington, ON, Canada). Plasma samples were stored at −80°C. Sample extraction, analysis and quality control procedures have been previously described in detail (Houde *et al.*, 2005). In brief, plasma (0.25 g) was extracted with acetonitrile, followed by cleanup on a Supelco© graphite carbon solid phase extraction column as described by De Silva *et al.* (2011) and Reiner *et al.* (2012). PFAAs were quantified using high-performance liquid chromatography with negative electrospray tandem mass spectrometry. Sixteen PFAA analytes were determined including C_6–C_{14} perfluorocarboxylates, perfluorobutane sulfonate, perfluorohexane sulfonate, perfluorodecane sulfonate, PFOS and perfluorooctane sulfonamide. A detailed list is provided in the supporting information (Tables S1 and S2). Data quality assurance and control measures (Table S2) included both field and laboratory blanks, matrix spikes and a standard reference material injection every 10 samples. Instrumentation consisted of an Agilent 1200 (Santa Clara, CA) series liquid chromatograph coupled to an AB SCIEX© 4000 Q Trap™ tandem mass spectrometer operated in negative electrospray ionization multiple reaction monitoring with a pentafluorophenyl stationary phase (Hypersil Gold PFP 150 × 3 mm, 3 μm; ThermoFisher Scientific, Waltham, MA, USA). Analytes were quantified using C_{13}-labeled surrogates via internal standard calibration curves. The method detection limit was defined as 3× standard deviation the

blanks (ng g^{-1}) assuming average sample weight of 0.25 g. If the blanks had non-detect PFAAs then the instrument detection limit at S/*N*= 10 was used. Results for National Standards and Technology (NIST) 1946 Standard Reference Material (SRM) for PFOS were within 26% of the certified value, while results for other PFAA were generally within the range of consensus values reported by Reiner *et al.* (2012) (Supporting Information, Table S2).

Cell culture and *in vitro* perfluorooctane sulfonate treatment

Cryopreserved PBLs were rapidly thawed, washed and plated at 1–5 × 10^6 cells in supplemented Roswell Park Memorial Institute medium (RPMI-1640 with L-glutamine, 10% fetal bovine serum, 1% non-essential amino acids, 1% sodium pyruvate, 10 mM HEPES, 1% antibiotic/antimycotic [10 000 units ml^{-1} penicillin, 10 000 μg ml^{-1} streptomycin and 25 μg ml^{-1} Gibco (ThermoFisher Scientific) amphotericin B] and 10 μM 2-mercaptoethanol, pH7.4). PBL cultures were exposed to PFOS (perfluorooctane sulfonic acid potassium salt [stated purity >98%]) at concentrations of 0, 0.5 and 5.0 μg ml^{-1}. Exposure concentrations are environmentally relevant levels representing concentrations reported in plasma from Charleston dolphins ranging from 0.5 to 3.1 μg ml^{-1} (Houde *et al.*, 2005) and 0.3 to 6.3 μg ml^{-1} (Fair *et al.*, 2012). As a vehicle control, DMSO was used as in all treatments ensuring all cells were exposed to 0.1% DMSO. Cells were incubated at 37°C, 5% CO_2 for 0, 48 or 96 h for each assay. PFOS and DMSO were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Characterization of dolphin T cells, proliferation and cytokine production by flow cytometry

Phenotypic characterization of $CD4^+$ and $CD8^+$ T cells was performed by flow cytometry as previously described with minor modifications (Soloff *et al.*, 2014). Monoclonal antibodies with specificity against bottlenose dolphin CD4 and CD8 proteins were generated in mice and kindly provided by Dr. Tracy Romano (Mystic Aquarium, Mystic, CT, USA) (Romano *et al.*, 1999). CD4-and CD8-specific antibodies were fluorescently labeled at the Flow Cytometry and Cell Sorting Shared Resource of the Hollings Cancer Center, Medical University of South Carolina (Charleston, SC, USA) using PE-Cy5.5 and APC-Cy7 fluorochrome conjugation kits, respectively, as per manufacturer's instructions (Abcam, Cambridge, MA, USA). In all subsequent flow cytometry assays cultured PBL were labeled to identify dead cells by incubation with amine-reactive LiveDead dye (Blue or Aqua; ThermoFisher Scientific, Waltham, MA, USA) for 15 min at 4°C in PBS and then stained for extracellular expression of CD4 or CD8 for 30 min at 4°C in fluorescence-activated cell sorting staining buffer. To measure T cell proliferation, 1–5 × 10^6 PBL were labeled with 1 μm carboxyfluorescein succinimidyl ester (CFSE) (BioLegend, San Diego, CA, USA) at 37°C for 7 min then thoroughly washed with fluorescence-activated cell sorting staining buffer before *in vitro* culture with or without PFOS as above. Cells were then sequentially stained with LiveDead, then CD4 and CD8 antibodies were immediately analyzed. To detect intracellular cytokine expression, 1–5 × 10^6 PBL were cultured in the presence of 0, 0.5 or 5.0 μg ml^{-1} PFOS with 10 μg ml^{-1} brefeldin A included for the last 12 h. No additional mitogen was included. Cultured cells were subsequently labeled with LiveDead dye followed by staining for extracellular CD4 and CD8 expression. Cells were then fixed and

permeabilized by treatment with BD Cytofix/Cytoperm solution (BD Pharmingen, San Jose, CA, USA) followed by labeling with monoclonal antibodies specific for interferon (IFN) γ (clone CC302, AlexaFluor647; AbD Serotec, Raleigh, NC, USA) and interleukin (IL)-4 (clone CC303, RPE; AbD Serotec) for 30 min at 4°C in the presence of permeabilizing staining buffer. Population gating was performed using a fluorescence minus 1 strategy for all populations. Data were acquired using an LSR Fortessa flow cytometer (BD, San Jose, CA, USA) collecting a minimum of 200 000 events and analyzed using FlowJo V10 (Tree Star, Inc., Ashland, OR, USA).

Statistical analysis

Descriptive statistics were calculated for all demographics and baseline plasma data. PFAA associations between baseline plasma levels of each of the PFAAs and proliferation and cytokine production by cell type over time was evaluated using a series of generalized linear mixed models (GLMMs) assuming a beta distribution with a logit link. The beta distribution was selected, as the outcomes were proportions ranging between 0 and 1. Similarly, the immune parameters for low and high PFOS doses were evaluated using a series of GLMMs assuming a beta distribution and a logit link. All models examining the impact of PFOS perturbation included fixed effects for baseline serum PFOS levels, baseline immune parameters, PFOS treatment level, time and treatment × time interaction. All GLMMs included a random subject effect to account for repeated measurements taken on the same dolphin plasma sample. Model assumptions were checked graphically and transformations and/or quadratic terms were considered in models where linearity assumption did not appear to hold. Using a regression model, we also compared IFNγ production from $CD8^+$ T cells of dolphins considered diseased ($n = 7$) to those classified as possibly diseased ($n = 8$) and healthy ($n = 4$). The health classification was based on Reif *et al.* (2008), and in our study group, the diseased group included two dolphins with genital papillomatosis and five dolphins with several abnormal hematologic and serum chemistry values. The possibly diseased group had less concerning hematologic and serum chemistry alterations but further diagnostic evaluation was recommended. All analyses were conducted in SAS v. 9.3.1 (IBM Corp. 2011, Armonk, NY, USA).

Results

Population demographics and perfluoroalkyl acid exposure

Samples from 19 animals ranging in age from 8 to 35 years with a mean of 24 years were collected during the dolphin capture–release health assessment studies conducted in 2013. Of the animals sampled, 68% were male and 79% were adults. Previously reported, juvenile dolphins have higher concentrations of PFAAs compared to adults (Fair *et al.*, 2012). We were unable to account for age as a possible confounder; however, as juveniles comprised a small portion of the samples ($n = 4$) in this study they likely were not a major contributing factor to the overall outcome. Thus, PFAA concentrations in plasma reported in Table 1 are for all dolphins ($n = 19$).

Environmental perfluoroalkyl acid exposure is associated with enhanced T cell proliferation

We have previously identified an association between increased perfluoroalkyl compounds and altered immune function in the Charleston dolphin population (Fair *et al.*, 2013). To assess the impact of environmental PFAA exposure on the dolphin T cell compartment *ex vivo*, we designed multiparametric flow cytometry-based assays utilizing custom-conjugated monoclonal antibodies specific for dolphin CD4 and CD8 receptors. Flow cytometry-based assays allow for direct measurement of $CD4^+$ and $CD8^+$ T cell responses to stimuli at the single-cell level. Herein, we examined the $CD4^+$ and $CD8^+$ T cell division following culture of the PBLs in the absence of mitogenic stimulation, representing baseline proliferation from PFAA-exposed dolphins. T cell division, detected by flow cytometry via CFSE dilution, was evaluated at day 0 and again at 2 and 4 days following sample culture (Quah *et al.*, 2007). Our initial analysis identified a significant correlation between increased plasma levels of PFOS and increased $CD8^+$ T cell division following 2 and 4 days of culture (Supporting Information, Fig. S1). Similarly, elevated perfluoroheptanoate levels were associated with increased $CD8^+$ T cell division after 2 days in culture (Supporting Information, Fig. S1). Although these findings suggest that elevated PFAA levels affect $CD8^+$ T cell proliferation over time, correlative analysis does not control for repeatedly measuring the same PBL sample at different times. As such, animals were stratified into lower, middle and upper tertiles based on plasma PFAA concentrations. Tests for univariate associations between the T cell division and plasma concentrations for 16 PFAAs found a significant association between increased proliferation among both $CD4^+$ and $CD8^+$ T cells and plasma PFOS and perfluoroheptanoate levels (Fig. 1). Additionally, there was a trend towards greater proliferation with increasing plasma levels of perfluoroundecanoate, in both $CD4^+$ and $CD8^+$ T cells, with increasing levels of perfluorodecane sulfonate in $CD4^+$ cells, and with increasing levels of perfluorodecanoate in $CD8^+$ cells, although none of these relationships achieved statistical significance (data not shown).

Perfluorooctane sulfonate exposure induces profound T cell division

Given the finding that elevated plasma PFAA concentrations in coastal dolphins were associated with increased T cell division *ex vivo*, we next sought to define the role of PFAA exposure on T cell function *in vitro*. As plasma concentrations of PFOS are consistently among the highest PFAAs detected in humans and dolphins, the immunotoxicity of PFOS was evaluated. Furthermore, we have previously observed that PFOS exposure *in vitro* enhances dolphin lymphocyte proliferation in response to mitogenic stimulation, an effect that was positively associated with elevated plasma PFOS levels in wild dolphins and thus levels of environmental exposure (Wirth *et al.*, 2014). Herein, PBLs were cultured in the presence of 0, 0.5 or 5.0 μg ml^{-1} PFOS, without mitogenic stimulation and the rate of $CD4^+$ and $CD8^+$ T cell division was determined via CFSE dilution following 2 and 4 days exposure. We observed robust proliferation in response to PFOS exposure, with 57% ± 4.6% SEM and 82% ± 1.9% SEM cells dividing within the $CD4^+$ and $CD8^+$ T cell subsets, respectively, following 4 days, high-dose co-culture (Fig. 2). To account for variable levels of environmental PFAA exposure in study subjects, T cell division was analyzed using a multivariable GLM regression model, which included day 0 cell division, plasma concentration of PFOS, concentration of PFOS exposure *in vitro*, time post-exposure and

exposure level by time interaction (McCulloch *et al.*, 2008). Additionally, this model included a random sample effect to control for the correlation due to repeatedly measuring the same plasma sample under different PFOS exposure levels and times (McCulloch *et al.*, 2008). Notably, the amount of PFOS exposure, time post-PFOS exposure and the interaction between time and PFOS exposure levels were significantly associated in the final model of cellular division for both $CD4^+$ and $CD8^+$ T cells. We detected a significant dose-dependent effect on lymphocyte proliferation with increased $CD4^+$ and $CD8^+$ T cell division associated with greater exposure to PFOS ($P < 0.001$). Similarly, this effect was enhanced with time, with longer PFOS co-culture resulting in significantly elevated cell division ($P < 0.001$). Additionally, we observed a significant interaction between the level of PFOS exposure and time, collectively, such that cells exposed to higher doses of PFOS had greater proliferative with increasing time relative to samples that received a lower dose of PFOS (Fig. 2). Interestingly, exposure to PFOS had no significant effect on T cell apoptosis, irrespective of PFOS dose or duration of exposure, as determined by amine-reactive viability staining (data not shown).

Perfluorooctane sulfonate promotes a proinflammatory cytokine response in T cells

To assess further the effects of PFOS exposure on T cell function, we examined the ability of exogenous PFOS to induce T cell production of the archetypical T-helper (T_H)1 and T_H2 cytokines, IFNγ and IL-4, respectively. Dolphin PBLs were co-cultured with 0, 0.5 or 5.0 μg ml^{-1} PFOS, and cytokine production was determined via intracellular flow cytometry after 0, 2 and 4 days. As above, results were analyzed using a multivariable GLM including the percentage of $CD4^+$ and $CD8^+$ T cells that produced IFNγ or IL-4 as the outcome, and independent variables were the dose of PFOS exposure and time of post-PFOS exposure in addition to an exposure level by time interaction. Notably, co-culture with exogenous PFOS induced significant production of IFNγ, but only modest IL-4 from both $CD4^+$ and $CD8^+$ T cells compared to cytokine levels detected from PBLs following exposure to media alone (Fig. 3). Within the $CD4^+$ T cell subset, we detected a dose-dependent effect of PFOS exposure upon IFNγ production with increased PFOS levels associated with greater cytokine production at 2 and 4 days co-culture ($P < 0.001$) (Fig. 3). Interestingly, exposure to either low- or high-dose PFOS resulted in a rapid and robust IFNγ response from $CD8^+$ T cells at 2 days co-culture (80.2% ± 1.6% high; 76.8% ± 1.4% low), which was significantly reduced by 4 days post-exposure (45.8% ± 3.4% high; 49.7% ± 2.9% low) (Fig. 3). Nevertheless, peak levels of IFNγ produced by $CD8^+$ T cells were nearly twice that of $CD4^+$ T cells, suggesting that the $CD8^+$ T cell compartment may be more susceptible to the effects of PFOS exposure. As such, we examined the ability of environmentally PFAA-exposed $CD4^+$ and $CD8^+$ T cells to produce IFNγ following polyclonal activation with phorbol myristate acetate (50 ng ml^{-1}) and ionomycin (1 μgml^{-1}) during 6 h culture without additional PFOS exposure. Although the group size for the dolphin health categories was small, it was interesting, when measured at day 0 in the absence of further PFOS exposure, $CD8^+$ T cells from diseased animals produced significantly more IFNγ following polyclonal activation than healthy animals based on beta regression model comparisons (mean 45.0% healthy, 52.4% possible disease, 62.4% diseased) ($P = 0.042$) (data not shown).

Discussion

Free-ranging dolphins are large mammal species that share a marine diet with human coastal inhabitants, representing a unique sentinel population to monitor the effects of environmental contaminant exposure on health outcomes. Owing to the lack of species-specific reagents suitable for the analysis of dolphin leukocytes, our understanding of the cellular immune response in these animals has been limited. In the present study, we utilized multiparametric flow cytometry to examine the effects of PFOS exposure specifically upon the $CD4^+$ and $CD8^+$ T cell populations present in the peripheral blood of native dolphins. These experiments were made possible by advances in fluorochrome conjugation methods, allowing for in-house fluorescence labeling of dolphin-specific antibodies. Flow cytometry provides the ability to examine rapidly large quantities of cells, and importantly, delivers phenotypic and functional data at the single cell level. As such, this study represents the first report detailing the immunotoxic effects of PFOS exposure specifically upon the $CD4^+$ and $CD8^+$ T cell subsets from the dolphin.

Our findings suggest that environmental PFAA exposure dysregulates the cellular immune compartment through driving aberrant T cell activation in the dolphin. Notably, we observed these effects in the absence of antigenic stimulation; thus, measuring a putative major histocompatibility complex independent mechanism. Yet, these data are difficult to interpret in the context of the experimental rodent and *in vitro* human models of PFAA exposure. Signaling via the PPAR family of nuclear receptor proteins has been implicated in regulating the immunotoxic effects of PFAAs, including PFOS (Corsini *et al.*, 2014). PPAR-mediated pathways regulate lipid metabolism and inflammation, and may affect immunity via signaling of the PPAR receptors expressed by T cells or through bystander activation of PPAR pathways in non-leukocyte populations (Clarke, 2002). Species-specific variation of PPAR receptor expression has been documented, with rodents expressing 10-fold greater levels of PPARα mRNA levels in the liver than humans (Kennedy *et al.*, 2004). In addition, mouse and human PPAR receptors exhibit differential activation following PFOS and perfluorooctanoate stimulation, highlighting species-specific discrepancies of PFAA-induced immunoregulation (Takacs *et al.*, 2007). To date, the role of PPAR activation in the dolphin in response to PFAAs has yet to be examined. In conjunction, PFOS exposure increases protein expression of Themis, CD3G and CAMK4 in mouse splenocytes, associated with upregulation of genes mediating T cell receptor signaling and calcium influx necessary for T cell activation (Lv *et al.*, 2015). Accordingly, *in vitro* PFOS exposure in this model increased intracellular calcium levels in splenocytes in a dose-dependent manner (Lv *et al.*, 2015). Collectively, our findings in conjunction with alternate experimental models suggest that PFOS may directly stimulate T cell responses through multiple, albeit incompletely defined, mechanisms that may vary among species.

Cytokine production by T cells is a highly ordered process whereby the host elicits a tailored response suitable to eliminate pathogenic or malignant threats, or re-establish homeostasis upon inflammatory resolution. Herein, we have demonstrated that PFOS exposure is sufficient to induce robust IFNγ production from both $CD4^+$ and $CD8^+$ dolphin T cell populations in the absence of significant quantities of IL-4. It is possible that such a T_H1-dominant cytokine profile will be the result of non-specific PFOS activation of T cells

present within an outbred population of free ranging dolphins. Alternatively, PFOS may directly promote a proinflammatory response in dolphin $CD4^+$ and $CD8^+$ T cells via PPAR and/or yet unknown signaling mechanisms as described above. Interestingly, we observed robust IFNγ production in the $CD8^+$ T cell subset in response to PFOS exposure, which decreased over time (Fig. 3). It is possible that high-level activation rendered the $CD8^+$ T cell compartment refractory to continued PFOS stimulation. Notably, chronic hyperactivation results in T cell exhaustion and cellular immune dysfunction as seen during progressive human immunodeficiency virus infection (Appay & Rowland-Jones, 2002; Day *et al.*, 2006). Although a similar reduction in IFNγ-producing $CD4^+$ T cells was not observed following continued PFOS stimulation, IFNγ responses among $CD4^+$ T cells were markedly lower compared to the $CD8^+$ counterparts, suggesting that $CD4^+$ T cells may be less susceptible to PFOS-mediated activation. These results are in contrast to previous PFOS exposure studies in mice, which have described the development of a T_H2-skewed cytokine profile, with increased IL-4 and decreased IFNγ production from bulk splenocyte cultures (Dong *et al.*, 2011; Zheng *et al.*, 2011). Although these studies did not look at T cells directly, we believe it is likely that species-specific variation in PFOS susceptibility and outbred nature of study populations may account for these differences.

T cell regulation is a critical component to maintaining homeostasis and for proper response to, and resolution of disease. The HIV-mediated elimination of $CD4^+$ T cells results in immunodeficiency, whereas $CD8^+$ T cell recruitment and proliferation have been associated with active multiple sclerosis lesions (Hohlfeld *et al.*, 2016). Yet, reports describing the effects of PFOS on leukocyte proliferation and apoptosis across species remain inconclusive. Whereas oral PFOS exposure resulted in a decreased cell count in the spleens and lymph nodes of mice (Dong *et al.*, 2012), exposure of human $CD4^+$ T cells to PFOS *in vitro* failed to elicit cell death (Midgett *et al.*, 2015). Additionally, we have found that treatment of bulk dolphin PBLs with PFOS *in vitro* further enhanced mitogen-activated T- and B-cell proliferation, with B cells showing statistical association (Wirth *et al.*, 2014). Notably, our previous studies observed a significant positive association between PFOS levels and increased $CD2^+$ total T cells and $CD4^+$ T-helper cell populations in the peripheral blood of wild dolphins (Aheng *et al.*, 2011; Fair *et al.*, 2013). The finding that PFOS promotes T cell proliferation *in vitro* suggests that environmental PFOS exposure may result in aberrant immune activation and dysregulation, resulting in increased susceptibility to disease.

Although there is strong epidemiologic evidence of PFAA immunotoxicity in humans, the specific effects of PFAA immune regulation remain uncertain. Prenatal PFAA exposure was associated with dramatic inhibition of vaccine-induced immunity, with a twofold increase in PFOS exposure resulting in a roughly 39% decrease in diphtheria-specific antibody concentrations in children post-immunization (Grandjean *et al.*, 2012). Similar prospective studies observed an increase in cases of the common cold and gastroenteritis, but not allergy in children associated with high levels of prenatal PFAA exposure (Granum *et al.*, 2013). Studies in mice suggest that ingestion of PFOS at levels relevant to those identified in human populations is capable of inducing immunotoxicity characterized by reduced antigen-specific IgM production and increasing susceptibility to influenza A infection (Guruge *et al.*, 2009; Peden-Adams *et al.*, 2008). In the current study, we describe a state of heightened T cell activation in environmentally PFOS-exposed dolphins, characterized by increased

proliferation *ex vivo* and robust proinflammatory IFNγ production and proliferation following exogenous PFOS exposure *in vitro*. Notably, such chronic activation can lead to T cell exhaustion and state of "frustrated resolution" marked by the inability to resolve chronic inflammation (Fullerton & Gilroy, 2016). Sustained low-level inflammation, termed smoldering inflammation, such as that identified in the current study for our dolphin population is associated with the development of cancer, autoimmune disease and increased susceptibility to infectious disease in humans (Fullerton & Gilroy, 2016; Hinks, 2016; Shalapour & Karin, 2015). In conclusion, the findings reported here suggest that PFOS exposure significantly dysregulates the cellular immune system in wild dolphins, and has consequences for dolphin health. Dolphins represent a worst case model for human health risk assessment due to their constant exposure to contaminants of emerging concern. They represent a prime sentinel species that can be exploited to provide spatial and temporal trends in contaminant exposures and afford early warnings of the potential hazards of environmental exposure to these chemicals. Therefore, the findings reported here suggest that the long-term effects of PFAA exposure on rates of diseases of chronic inflammation in high-risk wildlife and human populations should be examined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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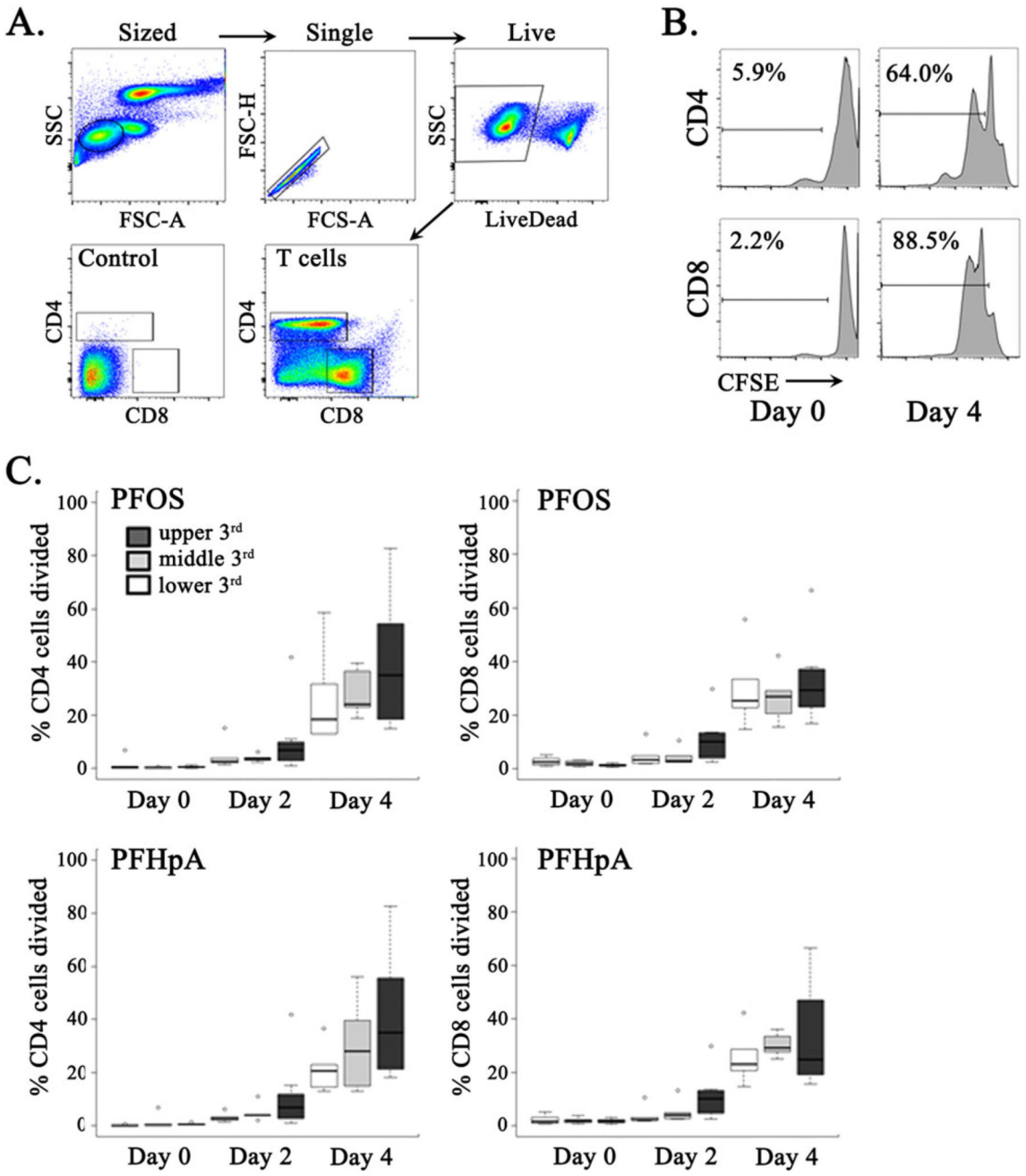

Figure 1.

Environmental PFOS exposure promotes dolphin T cell division. (A) Representative dot plots depicting the gating strategy used to identify sized (SSC vs. FSC-A), single (FSC-H vs. FSC-A), live (LiveDeadneg), $CD4^+$ and $CD8^+$ T cells in dolphin peripheral blood leukocytes. (B) Illustration of CFSE staining profile of $CD4^+$ and $CD8^+$ T cells from peripheral blood leukocytes at day 0 and following 4 days incubation with exogenous PFOS. Numbers represent the percentage of cells in each gate. (C) $CD4^+$ and $CD8^+$ T cell proliferation following 4 days culture in the absence of exogenous PFOS. Extent of $CD4^+$ and $CD8^+$ T cell division is shown in groups of dolphins segregated by tertiles of plasma

PFOS (upper graphs) and PFHpA (lower graphs). CFSE, carboxyfluorescein succinimidyl ester; FSC-A, forward scatter-area; FSC-H, forward scatter-height; PFHpA, perfluoroheptanoate; PFOS, perfluorooctane sulfonate; SSC, side scatter. [Colour figure can be viewed at wileyonlinelibrary.com]

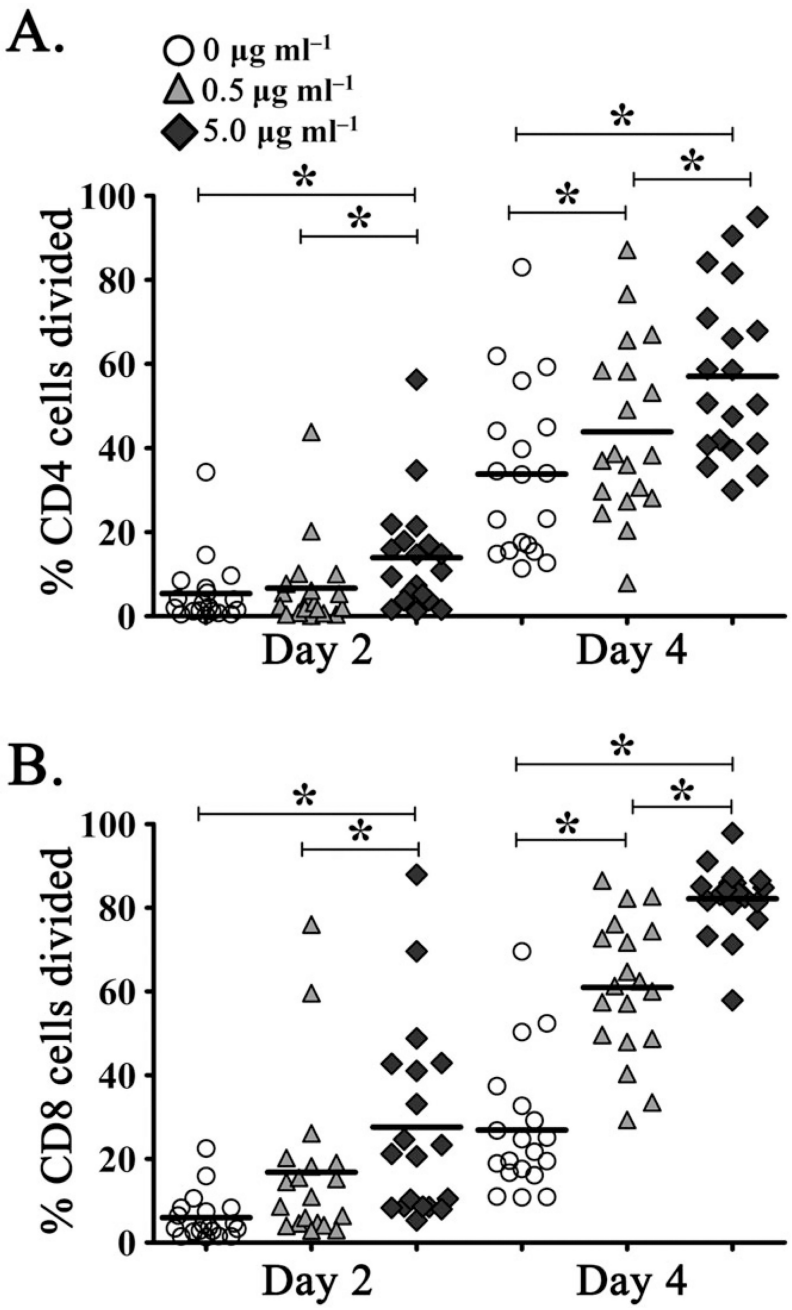

Figure 2.

In vitro exposure to perfluorooctane sulfonate induces dose-dependent $CD4^+$ and $CD8^+$ T cell division in the dolphin. Percentage of (A) $CD4^+$ and (B) $CD8^+$ T cells divided as determined by carboxyfluorescein succinimidyl ester dilution at 2 and 4 days post-culture with 0 µg ml^{-1} (open circles), 0.5 µg ml^{-1} (gray triangles) and 5.0 µg ml^{-1} (dark diamonds) perfluorooctane sulfonate. $*P \leq 0.001$.

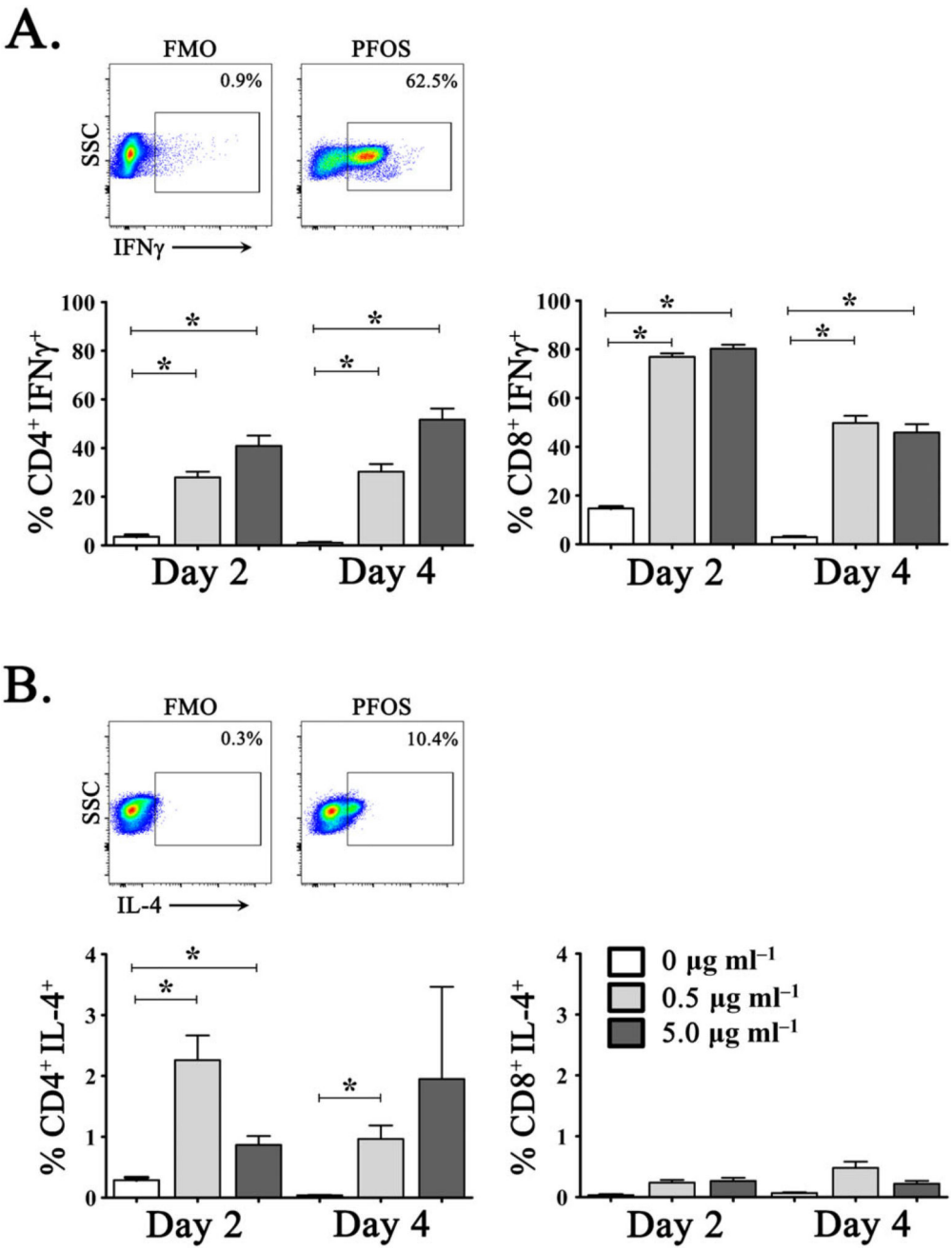

Figure 3.

In vitro exposure to PFOS induces potent IFNγ production from dolphin CD4+ and CD8+ T cells. Production of (A) IFNγ and (B) IL-4 in CD4+ (left) and CD8+ (right) T cells determined by intracellular cytokine staining following *in vitro* culture with 0 μgml^{-1} (open bars), 5 μgml^{-1} (gray bar), or 50 μgml^{-1} (dark bars) exogenous PFOS. Representative images of dolphin peripheral blood T cell production of IFNγ (CD8+) and IL-4 (CD4+) following *in vitro* stimulation with exogenous PFOS. Representative FMO-negative gating controls are illustrated for IFNγ and IL-4 staining. *$P \leq 0.001$. FMO, fluorescence minus 1;

IFNγ, interferon-γ; IL-4, interleukin-4; PFOS, perfluorooctane sulfonate; SSC, side scatter. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 1

Plasma perfluoroalkyl acids concentration (ng g^{-1} wet wt) in dolphins (n = 19) represented as median and range

Chemical	Chemical name	Median concentrations (ng g^{-1} wet wt)
PFHxA	Perfluorohexanoate	0.14 (0.04–0.41)
PFHpA	Perfluoroheptanoate	6.69 (0.93–38.6)
PFOA	Perfluorooctanoate	23.3 (6.34–55.4)
PFNA	Perfluorononanoate	59.9 (18.6–113)
PFDA	Perfluorodecanoate	70.8 (41.4–110)
PFUnA	Perfluoroundecanoate	25.6 (14.8–69.2)
PFDoA	Perfluorododecanoate	7.16 (3.02–36.4)
PFTriA	Perfluorotridecanoate	4.27 (2.02–11.7)
PFTA	Perfluorotetradecanoate	0.70 (0.31–1.93)
PFBS	Perfluorobutane sulfonate	0.04 (0.04–0.18)
PFHxS	Perfluoroheptane sulfonate	11.8 (4.14–45.4)
PFHpS	Perfluorohexane sulfonate	9.47 (2.22–47.6)
PFOS (total)	Perfluorooctane sulfonate	571 (288–1833)
PFOS (linear)	Perfluorooctane sulfonate	535 (262–1409)
PFDS	Perfluorodecane sulfonate	3.85 (2.03–24.0)
PFSOA	Perfluorooctanesulfonamide	5.61 (2.44–14.6)
PFECHS	Perfluoroethylcyclohexane sulfonate	9.90 (3.29–33.5)