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Immunomodulatory Effects of Exposure to Polychlorinated Biphenyls and Perfluoroalkyl Acids in East Greenland Ringed Seals (*Pusa hispida*)

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ABSTRACT

To better elucidate the potential immune-related health effects of exposure to environmentally persistent organochlorine pollutants (POP), such as polychlorinated biphenyls (PCBs) and perfluoroalkyl substances (PFASs), in ringed seals (*Pusa hispida*), a sentinel Arctic species, we assessed 1) associations between mitogen-induced lymphocyte proliferation and *in vivo* tissue contaminant burdens, and 2) the concentration-response effects of *in vitro* exposure to PFASs and PCB congeners on mitogen-induced lymphocyte proliferation. Upon *in vitro* contaminant exposure, the non-coplanar PCB congeners CB 138, 153, and 180, but not the coplanar CB 169, significantly reduced lymphocyte proliferation between 10 and 20 µg g^{-1} ww. The respective *in vitro* EC_{50} values for these congeners were 13.3, 20.7, 20.8, and 54.6 µg g^{-1} ww. Upon *in vitro* exposure to two individual PFASs, PFOS and PFOA, no modulation of lymphocyte proliferation was observed up to a concentration of 1000 ng g^{-1}. In addition, no significant correlations were found between lymphocyte proliferation and any blood or blubber contaminant measured. Taken together, these data suggest this population of ringed seals is not currently at high risk of immunotoxicity from POP or PFAS exposure.

Keywords—Ringed seal, PCB, PFAS, immunotoxicology, lymphocyte proliferation

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1. Introduction

Environmentally persistent organochlorine pollutants (POP), such as polychlorinated biphenyls (PCBs) and perfluoroalkyl substances (PFASs), have been detected in the tissues of numerous marine mammals inhabiting the Arctic, an area remote from major sources (Letcher et al., 2010; Riget et al., 2013; Vorkamp et al., 2011). Effects of these chemicals on the immune system have been well characterized in controlled laboratory experiments, and have been suggested in several wildlife species as well (Letcher et al., 2010). The immune system is a complex network of cells with diverse functions, communicating through a wide array of messenger molecules, which has evolved to protect the host from potentially pathogenic agents including viruses, bacteria, and parasites (Kuby, 2012). An immunomodulatory effect on any number of essential functions within this complex system is likely to have adverse consequences on an organism's survival and fitness, and its potential to handle several environmental stressors. Indeed, contaminant-induced immunomodulation has been suggested as a contributing factor in several morbillivirus-associated marine mammal mass mortality events (Di Guardo et al., 2005; Ross et al., 1996; Van Loveren et al., 2000).

The persistence of PCBs in the environment is due in part to long half-lives and high lipophilicity, leading to bioaccumulation and biomagnification in food webs. The lack of ortho-positioned chlorine atoms allows a congener to produce a coplanar (non-ortho) configuration similar to that of the most toxic of the halogenated aromatic hydrocarbons, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Congeners with two or more chlorines within the ortho positions (di-ortho PCBs) experience steric hindrance resulting in their aromatic rings being offset into separate planes (non-coplanar PCBs). The role of the aryl hydrocarbon receptor (AhR) in dioxin and dioxin-like (co-planar) PCBs immunotoxicity has been well established in

laboratory rodent studies, and is linked in particular to effects on developing lymphoid organs and subsequent cellular and humoral defects (Lawrence and Nancy, 2006). However, non-coplanar PCBs, with little to no affinity for the AhR, were shown to modulate harbor seal (*Phoca vitulina*), dolphin (*Tursiops truncatus*), and beluga (*Delphinapterus leucas*) immune functions upon either *in vitro* or *in vivo* exposure (Levin et al., 2005a; Levin et al., 2004; Levin et al., 2005b; Mori et al., 2008; Mori et al., 2006) and the toxicity pathways are therefore likely very different from those induced by the AhR.

PFASs, such as perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS), have physical and chemical properties that make them ideal surfactants (Lau et al., 2004). Although the manufacturing process results mostly in the production of perfluorooctane sulfonyl fluoride (POSF), PFOS is a common end-stage metabolite and product (Lau et al., 2004). PFOS and PFOA are readily absorbed through the gastro-intestinal tract, distributed in serum and liver, and poorly excreted or metabolized (Seacat et al., 2002). The immunotoxic effect of PFASs was recently reviewed (Dewitt et al., 2012) and involves activation of the alpha isotype of peroxisome proliferator-activated receptors (PPARs), a ligand-activated transcription factor that regulates gene expression (DeWitt et al., 2009). However, some immunotoxic effects may occur independently of the receptor (DeWitt et al., 2009).

To better elucidate the immune effects of POPs and PFASs in an Arctic sentinel species, we collected live immune cells from ringed seals during Inuit subsistence hunting in Scoresby Sound, East Greenland. The ringed seal serves as a key species in the Arctic Monitoring and Assessment Programme (AMAP) already for several decades due to its widespread distribution, its year-round presence and its ecological and nutritional importance within the entire Arctic area (AMAP, 2004). We aimed to test whether select POPs and PFASs are immunotoxic to free-

ranging ringed seals from East Greenland upon either *in vitro* or *in vivo* exposure. More specifically, we aimed at assessing 1) associations between mitogen-induced lymphocyte proliferation and *in vivo* tissue contaminant burdens, and 2) the concentration-response effects of *in vitro* exposure to PFASs and PCB congeners on mitogen-induced lymphocyte proliferation.

2. Materials and methods

2.1 Sample collection

Nine ringed seals were collected between 22 February and 5 March 2012 during the legal local subsistence hunt in the area around Ittoqqorrtoormiit, East Greenland (70° 24' 0" N, 21° 58' 0" W). Seals were either shot while hauled out on ice or in the water or caught in nets under the sea ice. Sections of mesenteric lymph nodes were collected from harvested ringed seals within 10 to 60 minutes of death, under Aarhus University's permits and supervision. Sections of lymph nodes were cut into 5 x 5 mm pieces using a sterile scalpel, and were placed in cold Dulbecco's modified Eagle's medium (DMEM) culture medium prior to preparing single cell suspensions for cryopreservation in the vapor phase of liquid nitrogen, as previously described for field samples of marine mammals (De Guise et al., 1996). Full depth blubber samples, sections of most major organs, and blood were also collected for contaminant analysis and/or other research programs. Tissues were imported into Denmark and further distributed to send to various collaborators including the University of Connecticut (United States; National Marine Fisheries Service Permit #1008-1637-03) and the University of Antwerp (Belgium). All tissue samples were stored at -20 °C while lymph node cells were kept in liquid nitrogen until analytical processing.

2.2 Preparation of immune cells

Cryovials containing lymph node cell suspensions were quickly thawed in a 37°C water bath and the contents were transferred to a conical tube containing 10 times the volume of warm DMEM supplemented with 1 mM sodium pyruvate, 100 µM non-essential amino acids, 25 mM HEPES, 2 mM L-glutamine, 100 U mL^{-1} penicillin, 100 µg/ml streptomycin and 0.25 µg mL^{-1} Fungizone (all obtained from Life, Grand Island, NY, USA), along with 10% fetal bovine serum (Hyclone, Logan, UT, USA), thereafter referred to as complete DMEM. Tubes were centrifuged at 220 g for 10 min at room temperature, and the cell pellet was re-suspended in complete DMEM. After thawing and washing once in DMEM, the cell suspension was passed through a sterile pipette containing sterile nylon wool to remove any residual connective tissue. To remove dead cells, the cell suspension was processed using the MACS Dead Cell Removal Kit (Miltenyi Biotec, Auburn, CA, USA), as per manufacturer's instructions. After collecting and washing the live cells, as confirmed by trypan blue staining (viability above 95%), the cell suspension was adjusted to 2 x 10^6 cells mL^{-1}.

2.3 Mitogen-induced lymphocyte proliferation

Mitogen-induced lymphocyte proliferation, without *in vitro* chemical exposure, was evaluated by methods routinely performed in our laboratory (Levin et al., 2007; Mori et al., 2006). Lymphocytes were plated in 96 well flat bottom tissue culture plates, in triplicate, and incubated at 37 °C with 5% CO_2 for a total of 66 hr with the T cell mitogen concanavalin A (ConA), the B cell mitogen lipopolysaccharide (LPS), or complete DMEM medium alone (no mitogen, unstimulated). Con A and LPS were used at the sub-optimal concentrations of 0.1 µg ml^{-1} and 0.05 µg ml^{-1}, respectively. Sub-optimal concentrations were used as they proved more

sensitive in detecting immunotoxicity (Mori et al., 2006). Lymphocyte proliferation was measured both as the optical density and a stimulation index (SI = mitogen/no mitogen).

Mitogen-induced lymphocyte proliferation with *in vitro* chemical exposure was evaluated as above, though with addition of chemical during incubation. The following chemicals and final concentrations were tested: PFOA (Sigma-Aldrich, St. Louis, MO, USA) was tested at 0 (unexposed control), 1, 3, 10, 30, 100, 300, 1000 ng g^{-1}. PFOS (Sigma-Aldrich, St. Louis, MO, USA) was tested at 0 (unexposed control), 1, 3, 10, 30, 100, 300 ng g^{-1}. PCB congeners 138, 153, 180 and 169 (ULTRA Scientific, N. Kingstown, RI, USA) were tested at 0 (unexposed control), 0.5, 1.0, 2.5, 5, 10, 15, and 20 µg g^{-1}. Both PFASs were tested using the mitogens ConA and LPS, while the PCB congeners were tested with ConA only.

2.4 Analysis of blubber POP concentrations

The analytical protocol was based on the methods described by Weijs et al. (2009) and allowed for the analysis for 42 PCB congeners (CB 18, 28, 31, 44, 47, 49, 52, 66, 70, 74, 87, 95, 99, 101, 105, 110, 118, 128, 132, 138, 146, 149, 151, 153, 156, 158, 170, 171, 172, 174, 177, 180, 183, 187, 194, 195, 196, 199, 203, 205, 206 and 209), several organochlorine pesticides (OCPs), amongst which dichlorodiphenyltrichloroethane (*p,p'*- and *o,p'*-DDT) and its metabolites (*p,p'*- and *o,p'*-DDD and *p,p'*- and *o,p'*-DDE), hexachlorobenzene (HCB), *cis*- and *trans*-chlordanes (CN and TN) and its metabolites oxychlordane (OxC) and *cis*- and *trans*-nonachlor (CN and TN), and several PBDE congeners (BDE 28, 47, 49, 99, 100, 153, 154, and 183). An accurately weighed blubber subsample (median: 0.14 g; range: 0.09-0.23 g) was homogenised and mixed with anhydrous sodium sulfate, spiked with internal standards (CB 143 and BDE 77), and extracted for 2h with hexane:acetone (3:1, v:v) in an automated hot Soxhlet extractor (Büchi

Flawil, CH). The lipid content was determined gravimetrically on 1/10th of the extract (after 1 h at 100 °C) prior to clean-up on 8g acidified silica (44% sulphuric acid). PBDEs, CHLs, and higher PCBs were measured with an Agilent 6890-5973 gas chromatograph coupled to a mass spectrometer system (GC-MS), equipped with a 30 m x 0.25 mm x 0.25 µm DB-5 capillary column (J&W Scientific, Folsom, CA, USA) and operated in electron capture negative ionisation mode. Lower PCBs, DDXs, and HCB were quantified using an identical GC-MS operated in electron ionisation mode and equipped with a 25 m x 0.22 mm x 0.25 µm HT-8 capillary column (SGE, Zulte, Belgium). Procedural blanks, analysed simultaneously with every batch of seven samples, were consistent (RSD<30%) and therefore the mean value was calculated for each compound and subtracted from the sample values. The limit of quantification (MLOQ) was set at 3*SD of the mean blank measurements, or, for analytes not detected in procedural blanks, set at S:N=10:1. Mean±SD recoveries of the internal standards CB 143 and BDE 77 were 86±6% and 93±10%, respectively. The analytical procedure was validated through the analysis of certified material SRM 1945 for which deviations from certified values were less than 10%. This quality control scheme has been positively assessed through participation in the NIST/NOAA Intercomparison Program for Organic and Inorganic Contaminants in Marine Mammal Tissues.

2.5 Analysis of serum PFAS concentrations

The extraction method for PFAS in serum was based on solid phase extraction (SPE) as previously described (Keller et al., 2010). Before extraction, the samples (100 µl volume) were spiked with 1 ng of each of the following ^{13}C-labelled internal standards: $^{13}C_2$-PFHxA, $^{13}C_4$-PFOA, $^{13}C_5$-PFNA, $^{13}C_2$-PFDA, $^{13}C_2$-PFUnA, $^{13}C_2$-PFDoA, $^{13}C_8$-PFOSA, $^{18}O_2$-PFHxS, $^{13}C_4$-PFOS, all obtained from Wellington Laboratories (Guelph, ON, Canada). Instrumental analysis

was performed by liquid chromatography-tandem mass spectrometry (LC-MS-MS) with electrospray ionization (ESI) in negative mode. The samples were extracted and analyzed in batches together with a procedural blank and two control samples, represented by an aliquot of test material previously analyzed in a ring test and for which assigned values for PFOS and PFOA concentrations are available. The detection limits ranged from 0.02 to 0.7 ng ml^{-1}. As an additional quality control procedure, the performance of analytical method is regularly tested three times a year by participating in the Arctic Monitoring and Assessment Programme (AMAP) Ring Test for Organic Pollutants in Human Serum organized by the Institute Nationale de Santé Publique du Québec. Detection limit and precision of the analytical method were determined using spiked rabbit serum (Sigma Aldrich).

2.6 Statistical analysis

Since immune cells from the same individual were divided into several chemical exposure groups (*in vitro* exposure), one-way repeated measures ANOVA with Dunnett's test, using the SigmaStat 3.5 (Jandel Scientific, San Rafael, CA) software, was used to compare the effects at the different *in vitro* concentrations to unexposed control cells. This was done to determine what concentrations significantly affected proliferation, and determine the lowest concentration that was significantly different from control (LOEC: Lowest Observed Effective Concentration). Concentration-response curves were generated by plotting the effects of the different treatment groups as a percentage of the unexposed control cells, and determining mathematically what concentration resulted in a 50% change (decrease or increase) in response (EC_{50}). Correlation analyses using Pearson product-moment correlation were performed using SPSS 16.0 (IBM, New York, USA). For all tests, $p < 0.05$ was used for statistical significance.

A t-test was used to compare contaminant concentrations between males/females and adults/subadults.

3. Results

Table 1 summarizes the biological and contaminant data for the major POPs (*Σ*PCBs, *Σ*DDTs, *Σ*HCHs, *Σ*PBDEs, HCB) and FFASs (PFOS and PFOA) for each ringed seal. Full details of all blubber/serum concentrations are provided in the supplementary information (Suppl 1). Nine adult or sub-adult seals were captured in total, six males and three females, with all but one (ID 43166) in good body condition. Contaminant concentrations were neither significantly different between males and females nor between adults and sub-adults ($p>0.05$ for all contaminants).

No significant correlations existed between lymphocyte proliferation (with and without mitogen) and any tissue contaminant concentration in the nine ringed seals ($p>0.05$ for all comparisons; data not shown).

Exposure to the non-coplanar CB 138, 180 and 153 significantly reduced ConA stimulated lymphocyte proliferation at 10, 15 and 20 µg g^{-1} respectively (Figure 1). Furthermore, the relative magnitude of reduction in lymphocyte proliferation was greater for CB 138 than CB 153 and 180. In contrast, the coplanar CB 169 did not significantly modulate ConA stimulated lymphocyte proliferation at any concentration tested ($p>0.05$; Figure 1). EC_{50} values were determined at 13.3, 20.7, 20.8, and 54.6 µg g^{-1} for CB 138, 153, 180, and 169, respectively (Figure 1). Unstimulated lymphocyte proliferation was not significantly modulated by any PCB congener or concentration tested ($p>0.05$; data not shown). ConA- and LPS-induced lymphocyte

proliferation was not significantly modulated by any concentration of PFOA or PFOS at the concentrations tested ($p>0.05$; data not shown).

4. Discussion

This study utilized blubber, blood, and lymph node immune cells from freshly harvested ringed seal to evaluate the effects of two major classes of environmentally persistent contaminants, i.e. PCBs and PFASs, upon either *in vitro* or natural exposure, on the immune system. No significant associations were found between mitogen-induced lymphocyte proliferation and tissue contaminant concentrations. However, *in vitro* exposure to the non-coplanar PCB congeners 138, 153, and 180 significantly reduced lymphocyte proliferation at 10 μg g^{-1} or above.

The lack of significant relationships between lymphocyte proliferation and tissue contaminant concentrations was not surprising, as the concentrations of tissue contaminants were below concentrations shown to modulate immune functions in other marine mammals and laboratory animals. For example, serum PFOS (mean 57.3 ng mL^{-1}) and serum PFOA (mean 1.8 ng mL^{-1}) measured in these animals were below the lowest observed adverse effect level (LOAEL) in serum that induced immunotoxicity (notably T-cell-dependent IgM antibody response) in laboratory mice (Dewitt et al., 2012), ~92 ng mL^{-1} for PFOS and ~74,000 ng mL^{-1} for PFOA, with one exception, seal ID 43184, which had a serum PFOS concentration of 196.6 ng mL^{-1}.

Blubber ΣPCBs (mean 723 ng g^{-1} lw) measured in this study were below the threshold for physiological effects (1,370 ng g^{-1} lw blubber) as previously reported for aquatic mammals, including ringed seals (Brown et al., 2014; Kannan et al., 2000). Concentrations were, however,

within range of the effect threshold for lymphocyte proliferation recently established for marine mammals, as well as for seals specifically. In their meta-analysis of all available marine mammal immunotoxicology studies (including *in vitro* and *in vivo* data), Desforges et al. (Desforges et al., 2016) found a sigmoidal dose-dependent decrease in lymphocyte proliferation, with seal threshold effect levels of ΣPCBs of 8 ±0.01 ng g^{-1}, respectively. Concentrations in this study were also within range of estimated critical body residues (CBR) for immunosuppression in polar bears, 469 ng g^{-1} lw blubber (Dietz et al., 2015). CBR is defined as the lowest observed total body concentration of a contaminant, which is associated with the occurrence of adverse toxic effects. Although marine mammal CBRs have only been calculated for polar bears, they may serve as a reference for other marine mammals until species-specific CBRs are defined.

Blubber *p,p'*-DDT (mean 133 ng g^{-1} lw) measured in this study was below concentrations found in blubber of harbor seals fed either herring from the 'uncontaminated' Atlantic ocean or 'contaminated' Baltic Sea (mean 306 and 2448 ng g^{-1} lw, respectively). Only seals fed the Baltic Sea herring had a significant reduction in mitogen-induced T lymphocyte proliferation (de Swart et al., 1996), but this was related to TEQ levels in blubber, and not only DDT specifically. In addition, ΣDDXs in this study (mean 652 ng g^{-1} lw blubber) was also 10-fold below the estimated CBR for immunosuppression in polar bears, 6502 ng g^{-1} lw blubber (Dietz et al., 2015).

Blubber ΣHCHs (mean 31.2 ng g^{-1} lw) measured in this study was nearly 42-fold below the estimated CBR for immunosuppression in polar bears, 1302 ng g^{-1} lw blubber (Dietz et al., 2015). Blubber ΣPBDEs (mean 17.2 ng g^{-1} lw) measured in this study was below concentrations (~18 $x10^6$ ng g^{-1} lw liver) found to modulate immune functions (i.e. antibody production) in experimentally exposed mink (Martin et al., 2007). To date, relationships between HCB and changes in marine mammal immune functions have not been documented (Dupont et al., 2013).

Overall, most tissue contaminant concentrations were below concentrations found to induce immunotoxicity in other marine mammals and laboratory animals. The only exception are PCBs where blubber levels appear to fall within range of effect thresholds calculated for marine mammal lymphocyte proliferation (Desforges et al., 2016); thus concentrations in this study may be bordering or just above the NOEL. A larger sample size is however necessary to observe an *in vivo* link to PCB concentrations.

To further examine the effects of contaminants on the immune system of ringed seals, *in vitro* exposure experiments were conducted with four individual PCB congeners, CB 138, 153, 180 and 169, and two PFSAs, PFOA and PFOS. For the PCB congeners, the concentrations chosen (range 0.5 to 20 µg g^{-1} ww in culture media) were within effect levels seen in previous *in vitro* exposure studies (De Guise et al., 1998; Mori et al., 2008; Mori et al., 2006) and above concentrations measured in plasma of ringed seals from the Baltic and Svalbard areas (maximum values of 0.182 and 0.260 µg g^{-1} ww plasma, respectively) (Routti et al., 2008). For PFOA and PFOS, the concentrations chosen (range 1 to 1000 ng g^{-1} ww) were also within and above the concentrations measured in ringed seal serum (range 0.6 to 3.1 ng g^{-1} ww PFOA; range 21.1 to 196.6 ng g^{1} ww PFOS).

In vitro experiments allow for the detection of the direct effects of contaminants on immune functions. When performing the *in vitro* experiments, cells from the same individual serve as the unexposed control for that individual; therefore, it takes into account any previous POP exposure and other confounding factors such as age and sex. Therefore, any modulation of immune function is the result of the actual *in vitro* exposure.

Similar to natural exposure, there was no modulation of mitogen-induced lymphocyte proliferation upon *in vitro* exposure to PFOA or PFOS. For PFOS, the estimated CBR for

immunotoxic effects in polar bears was 9356 ng g^{-1} ww (Dietz et al., 2015), with no value available for PFOA. The LOAEL that induced immunotoxicity in laboratory mice, ~92 ng mL^{-1} serum, was within the range of *in vitro* concentrations tested here, which was not the case for PFOA (Dewitt et al., 2012).

In this study, there was a concentration dependent suppression of T lymphocyte proliferation at concentrations of 10 µg g^{-1} or higher for non-coplanar PCBs. Similar immunomodulatory effects were documented for non-coplanar PCBs in B6C3F1 mouse, beluga, and human T lymphocyte proliferation under comparable *in vitro* conditions (Leibrecht, 2007). For example, suppressed lymphocyte function was found in mice for PCB congeners 138, 153 and 180 and in beluga for CB 138, however, lymphocyte proliferation increased in beluga exposed to CB 153 and 169 and in humans for all four congeners. Not only is the directionality of *in vitro* lymphocyte response variable between species, but the effect concentrations and thresholds differ as well. For example, human lymphocytes were most sensitive to CB 138 with LOEC and EC_{50} values of 5 and 6.9 µg g^{-1}, compared to 10.0 and 13.3 µg g^{-1} for ringed seals, 15.0 and 19.4 µg g^{-1} for mouse, and 18.7 and > 20 µg g^{-1} for beluga.

5. Conclusions

Taken together, these data suggest this population of ringed seals is not currently at high risk of immunotoxicity from contaminant exposure, despite being near critical threshold effect levels. However, under stressful conditions such as nutritional deficiency and reproduction, marine mammals may undergo periods of blubber metabolism and fat mobilization such that blubber PCBs would concurrently mobilize and reach higher levels in circulation (Debier et al., 2006; Yordy et al., 2010). Thus, the higher concentrations in blood may reach levels

demonstrated to temporarily modulate immune functions, potentially increasing an individual's risk for immunomodulation during already stressful periods. In addition, these data provide further 'weight of evidence' that PCBs, especially the non-coplanar congeners (CB 138, 153, and 180), are potentially immunotoxic in marine mammals, although difference among species susceptibility must be considered as previously documented (Mori et al., 2006). At current levels, PFASs appear not to be of concern on some aspects of the immune system. Importantly, measuring other aspects of immune functions, such as key innate and adaptive immune functions, including phagocytosis, respiratory burst, and natural killer (NK) cell activity, will provide a broader and clearer picture on the effects of contaminants on the overall health of ringed seals, as well as other top Arctic predators. Ideally, similar studies as the present should be conducted for marine mammals at risk including seals from the Baltic seals and other marine mammals foraging at higher trophic levels, including e.g. harp seals (*Pagophilus groenlandicus*), hooded seals (*Cystophora cristata*), toothed whales like narwhals (*Monodon monoceros*), beluga (*Delphinapterus leucas*), pilot whales (*Globicephala melas*) and killer whales (*Orcinus orca*) as well as polar bears (*Ursus maritimus*).

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