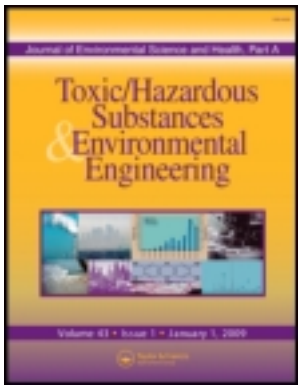

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Perfluorooctane sulfonate (PFOS; 1,1,2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-heptadecafluoro-1-octanesulfonic acid) has been reported to alter humoral immune functions, but inflammatory processes following PFOS exposure have not been fully characterized. Therefore, the current study, assessed TNF-α and IL-6 concentrations in serum and peritoneal lavage fluid, numbers of splenoctyes expressing intracellular TNF-α, IL-6, IL-10 or IL-1, and *ex vivo* TNF-α and IL-6 production by peritoneal macrophages following either *in vivo* or *in vitro* LPS exposure. Adult female B6C3F1 mice were exposed orally for 28 days to 0, 1, 3, or 300 mg PFOS/kg total administered dose [TAD] (e.g., 0, 0.0331, 0.0993 or 9.93 mg/kg/day). Body and spleen masses were significantly reduced in the highest PFOS treatment group compared to the control group, whereas liver mass was significantly increased. Serum TNF-α levels were significantly decreased following exposure to 1 mg PFOS/kg TAD as compared to controls, while serum IL-6 levels were increased. IL-6 concentrations in peritoneal lavage fluid decreased with increasing dose. PFOS treatment did not alter numbers of splenocytes expressing intracellular levels of TNF-α, IL-10 or IL-1. Numbers of splenocytes expressing intracellular levels of IL-6 were significantly decreased in the 3 mg/kg treatment as compared to controls. Overall, these data suggest that PFOS exposure can alter some inflammatory processes, which could potentially lead to misdirected inflammatory responses.

Keywords: PFOS, inflammation, IL-6, TNFα, cytokines, immune, macrophages.

Introduction

Perfluorinated alkyl acids (PFAAs) are used as surface protectors or stain repellants for fabrics, upholstery, carpet and leather and as fire-fighting foams, insecticides and corrosion inhibitors. This class includes the compounds perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA).[1,2] PFAAs are persistent environmental contaminants that bioaccumulate and biomagnify. PFAAs are known to be globally distributed, having been identified in environmental and wildlife samples worldwide, with the specific PFAAs, PFOS and PFOA constituting the highest concentrations.[3–5] Exposure to these PFAA compounds is associated with hepatotoxicity, neurotoxicity, endocrine disruption, immunotoxicity and carcinogenicity.[6–10] Research on the toxicities of these compounds show they are directly responsible for a number of biological alterations including peroxisome proliferation, increased lipid metabolism, altered gap junction communication and increased cell membrane fluidity.[2,11,12]

Recent research has provided evidence that PFAAs may up-regulate a large number of genes involved in the control of lipid and fatty acid metabolism.[2,13] The exact mechanism or mechanisms of action by which PFAAs exert their toxicity is currently unknown; nevertheless, a significant body of recent research suggests that they may act through activation of the peroxisome proliferator-activated receptor-alpha (PPAR-α).[14–19] PFOS and PFOA are known activators of both human and mouse PPAR-α and exposure to PFOS or PFOA results in the induction of endogenous PPAR-α target gene expression.[16,19,20]

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PPAR-α activation triggers a wide array of signaling cascades including those that regulate innate immunity, adaptive immunity, and processes involved in inflammation.[21–24]

Several laboratories have assessed the general immunotoxicity of PFAAs,[10,25–37] as well as their effects on inflammation,[13,17,18,38] with most of the published inflammation studies investigating PFOA. In mouse models of inflammation, PFOA is anti-inflammatory[17,18] and causes the down-regulation of a number of immunoregulatory genes including interleukins,[13] known regulators of inflammatory processes.[39] Guruge et al.[13] identified significant up-regulation of a gene involved in the metabolism of prostaglandin, a known inflammatory mediator, following PFOA exposure *in vivo*. These above studies indicate an inverse relationship exists between PFOA exposure and inflammation. However, in wild bottlenose dolphins, preliminary data suggest a positive correlation between PFOS and PFOA plasma levels and indices of inflammation and immunity (increased CRP, numbers of total lymphocytes, and numbers of B-cells).[40,41] Additionally, mice exposed to PFOA exhibit increased IgE levels that would most likely trigger an increase in inflammation.[29]

Few studies to date have assessed the effects of PFOS on inflammation. Qazi et al.[42] assessed TNF-α and IL-6 production in male C57BL/6 mice exposed for ten days to either 0 or 400 mg PFOS/kg TAD[43] (40 mg/kg/d; 0.02% in diet).[42,43] They report increased *ex vivo* TNF-α and IL-6 production by peritoneal cells and increased TNF-α production in mixed spleen cell suspensions (e.g., not separated by immunophenotype) following *in vitro* LPS stimulation. Qazi et al.[42] also report no change in numbers of macrophages (CD11b+) in the spleen at this PFOS concentration. Additionally, following *in vivo* exposure to PFOS for 10 days and 300 μg LPS for 2 hr (with no *ex vivo* LPS exposure) they observed significant increases in peritoneal cell production of TNF-α and significant decreases in mixed splenocyte TNF-α and IL-6 production, but no effect on peritoneal cell production of IL-6.[42] Thus, the relationship between PFAA exposure and the inflammatory response is currently not fully understood, but species differences, dose and exposure regimen appear to alter the effects observed.

PFOS is the predominate PFAA found in both human and wildlife blood samples,[3,44–47] but little is known regarding its impact on inflammation at environmentally relevant exposure concentrations. TNF-α and IL-6 are known mediators of inflammatory and immunoregulatory responses and are commonly used as indicators of inflammation in mammals.[48–50] Therefore, the current study assessed TNF-α and IL-6 in serum, splenocytes, peritoneal lavage fluid and production by macrophages in a standard 28-day oral exposure[51] in adult female B6C3F1 mice[52,53] using a dose range to include environmentally relevant exposure concentrations.[46,47]

Materials and methods

Materials

Unless otherwise specified, all chemicals and mitogens were purchased from Sigma (St. Louis, MO). Perfluorooctane sulfonic acid potassium salt (stated purity >98%) was obtained from Fluka Chemical (via Sigma, CAS No. 2795-39-3). Non-essential amino acids (NEAA; 10 mM 100X) and sodium pyruvate (100 mM) were obtained from GIBCO Laboratories (Grand Island, NY). RPMI-1640 medium (with L-glutamine and sodium bicarbonate), Dulbecco's phosphate buffered saline (DPBS; without Ca^{+2} and Mg) and penicillin/streptomycin (5,000 I.U./ml; 5,000 μg/ml) were purchased from Cellgro (Mediatech, Herndon, VA). The fetal bovine serum (FBS) was from Gemini Bio-Products (West Sacramento, CA). ELISA plates were from NUNC (Rochester, NY) and BD Biosciences (Franklin Lakes, NJ). The fluorescent antibodies allophycocyanin (APC) conjugated rat immunoglobulin (Ig) G_1 anti-mouse TNF monoclonal antibody, phycoerythrin (PE) conjugated Armenian hamster IgG_1 anti-mouse IL-1α antibody, PE conjugated rat IgG_1 anti-mouse IL-6 monoclonal antibody and APC conjugated rat IgG_{2b} anti-mouse IL-10 monoclonal antibody were purchased from BD Biosciences. GolgiStop protein transport inhibitor and BD OptEIA cytokine ELISA sets were purchased from BD Biosciences. Tissue culture plates, bovine serum albumin (fraction V; BSA), Tris and disposables were purchased from Fisher Scientific (Atlanta, GA). Lipopolysaccharide (LPS) from *E. coli* 0111:B4 was acquired from InvivoGen (San Diego, CA) and paraformaldehyde from Sigma Aldrich Co. (St. Louis, MO). Isoflurane (AErrane) was obtained from Baxter Pharmaceutical Products Inc. (Deerfield, IL).

Animal care

Mice were housed in plastic shoebox cages on corncob bedding with micro-isolator lids in a HEPA filtered ventilated rack system and administered food (TekLab Sterilizable Rodent Diet, formula no. 8656; Harlan-Teklab, Madison, WI) and water *ad libitum*. Prior to starting a particular experiment five, 7- to 8-week-old female B6C3F1 mice (Jackson Laboratory, Bar Harbor, ME or Harlan, Madison, WI) were randomly placed 5 to a cage in a treatment room. Mice acclimated to the conditions of the treatment room (12 hr light/dark cycle, 22 $\pm$ 2°C, 60–65% relative humidity) for 1 week before dosing began. Mice were observed daily and bedding, food and water were changed twice a week.

Animal dosing

PFOS concentrations selected for this study were based on reported mean serum levels in bottlenose dolphin[44] and occupationally exposed humans[46,55] as well as on a

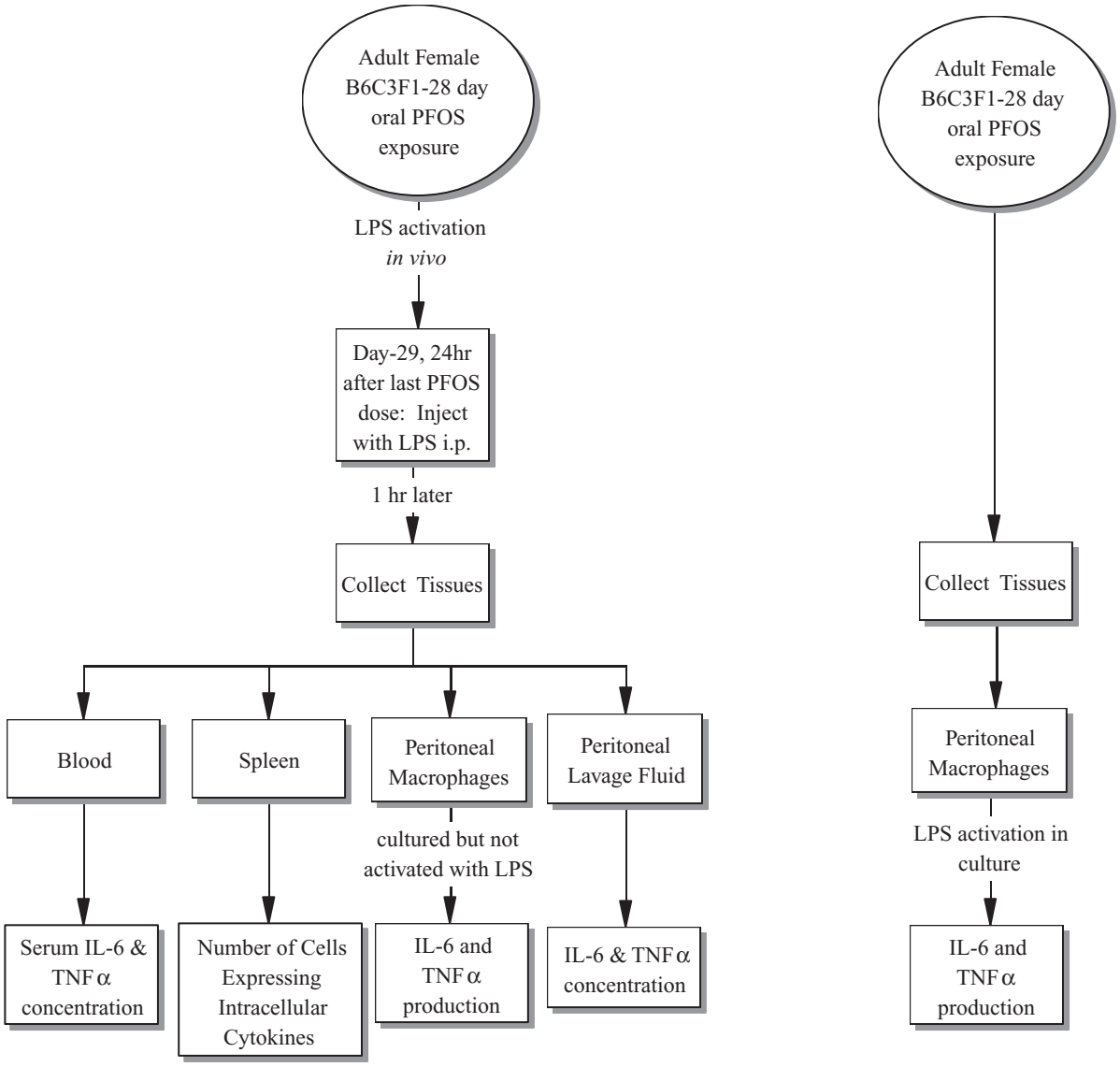

Fig. 1. Schematic of experimental design. Adult female B6C3F1 mice were exposed orally for 28 days to a total of 0, 1, 3, or 300 mg PFOS/kg. The sample size was 5 mice/treatment and all experiments were repeated a minimum of two times unless otherwise stated. PFOS = perfluorooctane sulfonate. i.p. = intraperitoneal (i.p.) injection.

preliminary study with PFOS that examined similar inflammatory endpoints following a 400 mg/kg total dose.[42,43] PFOS was administered via oral gavage in a solution of Milli-Q water containing 0.5% Tween-20.[54] Control mice received Milli-Q water containing 0.5% Tween 20. Mice were dosed daily for 28 days (0, 0.0331, 0.0993 or 9.93 mg PFOS/kg/day) to yield a targeted total administered dose (TAD) over the 28 days of 0, 1, 3, or 300 mg/kg. The daily doses listed here reflect only the concentration of the PFOS ion separate from the potassium salt. When comparing doses or concentrations reported in other studies, it is often unknown whether the potassium mass was removed. When it is not removed, the PFOS concentration is overestimated by 7.3%. When rounded to a single significant digit, the TAD is identical for PFOS potassium salt or the PFOS ion; therefore, these doses are used throughout the paper for simplicity.[10]

In some trials, mice were challenged via intraperitoneal (i.p.) injection with 0.1 mL of a 25 μg/mL LPS solution (ultra pure from *E. coli* 0111:B4 strain)[56,57] 1 hr prior to sample collection, while in other trials the mice were not challenged with LPS (Fig. 1). LPS injection via i.p. exposure is a common model of inflammation.[49] All procedures were approved by the Medical University of South Carolina Institutional Animal Care and Use Committee (IACUC) and conducted in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited facility. Unless otherwise stated, all experiments were conducted at least twice to verify results.

Sample collection and processing

To calculate body mass change over the 28 days, mice were measured one day prior to exposures and at the termination of the experiment (weight change = final mass - initial mass). Mice were then euthanized with CO_2 and spleens and livers were collected. Organ mass was normalized for body weight and reported as a somatic index {(organ weight/body wt) × 100}. Spleens were aseptically processed into single cell suspensions with the use of sterile, frosted microscope slides. Red blood cells were removed by adding red blood cell lysis buffer (0.16 M ammonium chloride, 0.17 Tris, pH 7.6). Viable spleen cell counts were obtained by trypan blue exclusion using a hemacytometer.

Serum cytokines

For detection of inflammatory cytokines, mice were challenged i.p. with 0.1 mL of a 25 μg/mL LPS solution (ultra pure from *E. coli* 0111:B4 strain)[56,57] 1 hr prior to blood collection (Fig. 1). Whole blood was collected retro-orbitally, following anesthetization with isoflurane. Immediately following blood collection, mice were euthanized with CO_2. Blood was permitted to clot for 1 hr. After clot formation, the blood sample was centrifuged for 10 min using a microcentrifuge (Eppendorf, 5415C, 1350g) and serum was transferred into an Eppendorf tube. Serum was frozen at –80°C until analysis. TNF-α and IL-6 were measured by cytokine ELISA sets according to manufacturer's directions. Samples were read at 450 nm with a spectrophotometer (SpectraCount; Packard, Meridian, CT). Sample cytokine concentrations were determined using regression analysis of a 7-point standard curve ranging from 15.6 pg/mL to 1000 pg/mL.

Splenic intracellular cytokine expression

Numbers of splenocytes expressing intracellular TNF-α, IL-1, IL-6 and IL-10 were assessed from PFOS treated mice following *in vivo* LPS challenge (Fig. 1). Spleen cells were diluted to 1×10^6 cells and incubated with GolgiStop protein transport inhibitor for three hours at room temperature to inhibit cytokine transport. Inhibition of transport results in accumulation of intracellular cytokine levels allowing for better detection of cytokine producing cells by flow cytometry. Cytokine secreting cells were analyzed by flow cytometry following incubation of permeabilized splenocytes with antibodies specific for TNF-α, IL-1, IL-6 and IL-10 (Becton Dickinson flow cytometer, FACScalibur; San Jose, CA).

Ex Vivo *production of cytokines by peritoneal macrophages following* In Vitro *or* In Vivo *LPS stimulation*

Peritoneal macrophages were isolated from unchallenged and LPS challenged mice (Fig. 1). Macrophages were obtained by lavage in peritoneal macrophage culture media that consisted of RPMI-1640 supplemented with 1% FBS, 1% non-essential amino acids, 1% sodium pyruvate and 1% penicillin/streptomycin. Peritoneal macrophages (1×10^6 cells/well) were plated in 24-well plates, allowed to adhere for 2 hr and then washed 3 times with culture media to remove nonadherent cells. After washing, cells from unchallenged mice were cultured for 24 hr[58] with 0.1 μg/mL of ultra pure LPS (from *E. coli* 0111:B4 strain).[57] in peritoneal macrophage culture media, while cells from LPS challenged mice were cultured for 24 hr in fresh media without LPS. Following the 24-hr culture period, supernatants were collected and stored (−80°C) for assessment of TNF-α and IL-6. TNF-α and IL-6 were measured by BD cytokine ELISA sets according to manufacturer's directions as described above.

Peritoneal lavage fluid cytokine levels

Following collection of peritoneal macrophages from LPS challenged mice, peritoneal lavage fluid was centrifuged ($500 \times g$) and supernatant was stored at −80°C for TNF-α and IL-6 analysis. TNF-α and IL-6 were measured by BD ELISA as described previously.

Statistics

Data were tested for normality (Shapiro-Wilks W-test) and homogeneity (Bartlett's test for unequal variances) and, if needed, appropriate transformations were made. A one-way ANOVA was used to determine differences among doses for each endpoint using JMP 4.0.2 (SAS Institute Inc., Cary, NC) in which the standard error used a pooled estimate of error variance. When significant differences were detected by the F-test ($P < 0.05$), the student's t-test was used to compare means among treatments. Trend analysis, in selected cases, was performed using Kendall's Tau. Dose-response determinations were assessed using Prism Graph Pad 4.0 (GraphPad Software, Inc.; La Jolla, CA) with a standard curve for calculating dose response according to Equations 1 and 2.

$$Y = \text{Bottom} + (\text{TOP} - \text{Bottom})/(1 + 10^{(X - \log EC_{50})}) \quad (1)$$

$$Y = \text{Bottom} + (\text{TOP} - \text{Bottom})/(1 + 10^{(\log EC_{50} - X)}) \quad (2)$$

Where "Bottom" is the minimum value, "Top" is the maximum and $LnEC_{50}$ is the log of the concentration that gives a half-maximal response. Four of the data sets fit this model and graphs of these are included as insets in the corresponding figures.

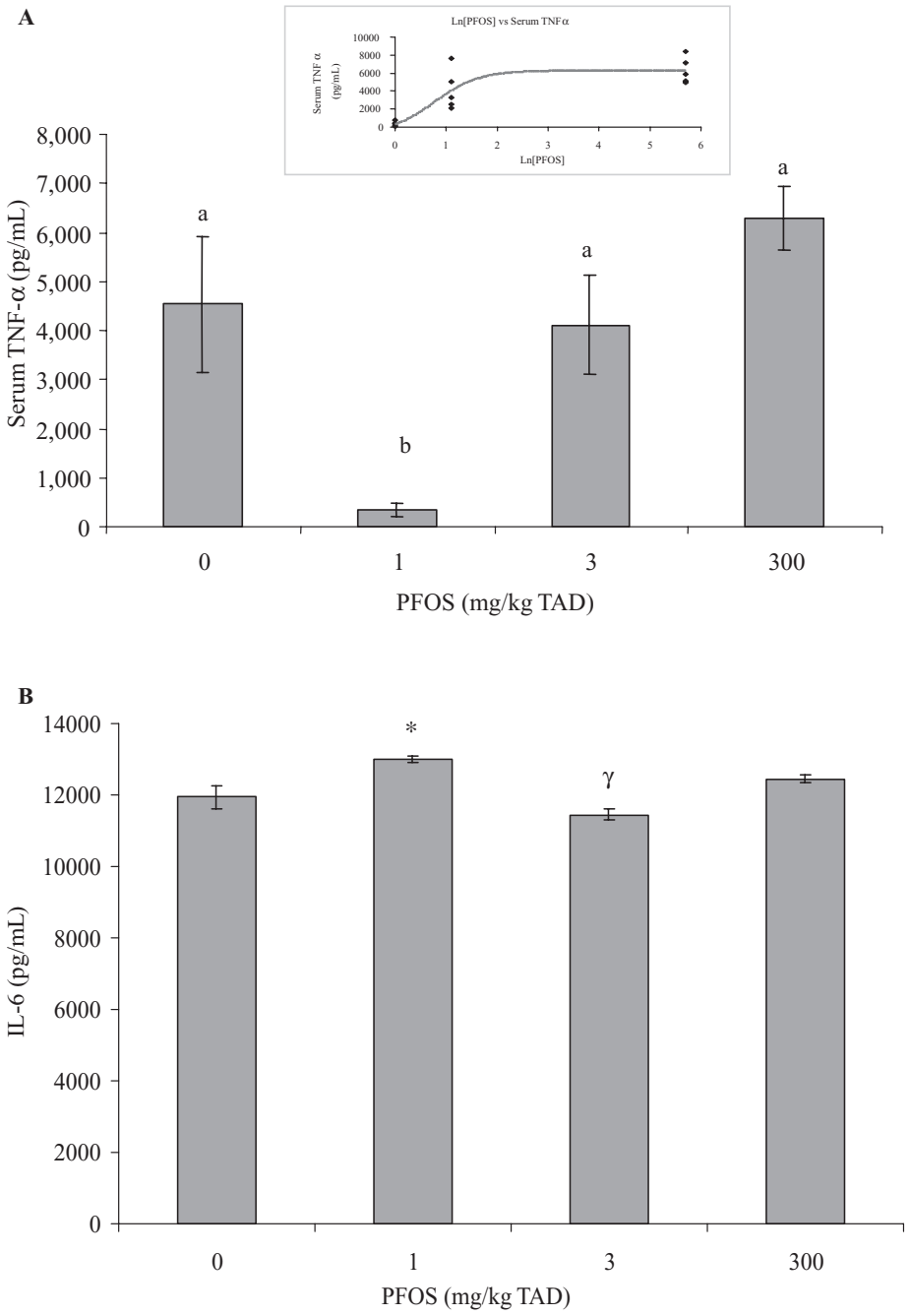

Fig. 2. Serum (A) TNF-α and (B) IL-6 levels in adult female B6C3F1 mice treated with PFOS orally for 28 days. Mice were challenged *in vivo* by intraperitoneal injection with 25 μg lipopolysaccharide (LPS; ultra pure from *E. coli* 0111:B4 strain) 1 hr prior to sample collection. Data are presented as mean ± SEM. Sample size for all treatments is 5. These experiments were conducted three times. Data from a single experiment are shown, as results are representative of experiments. Bars with same letters in (A) are not significantly different from each other, while bars with different letters are significantly different from each other ($P < 0.05$). The inset in (A) indicates a dose-response model based on a standard curve for calculating dose response. The equations used were: Y = Bottom + (TOP-Bottom)/(1+10(X-logEC_{50})) and Y = Bottom + (TOP-Bottom)/(1+10(logEC_{50}X)). Where "Bottom" is the minimum value, "Top" is the maximum and LnEC_{50} is the log of the concentration that gives a half-maximal response. PFOS = perfluorooctane sulfonate. TAD = Total Administered Dose (daily doses are 1/28 of the TAD). * Significantly different from respective control ($P < 0.05$; P-value for serum IL-6 ANOVA was 0.014). $^{\gamma}$Significantly different from IL-6 serum levels at the 1 mg/kg treatment. This increase in serum IL-6 at 1 mg/kg and decrease at 3 mg/kg mirrors the numbers of cells expressing intracellular IL-6 in Table 2 and when graphed in relationship to numbers of cells expressing intracellular IL-6 (Fig. 3) shows an association ($R^2 = 0.46$).

Table 1. Body mass change and organ mass and cellularity in adult female B6C3F1 mice treated with PFOS orally for 28 days.

PFOS mg/kg TAD	*Body Mass Change*[a]	*Normalized Spleen Mass*[b]	*Normalized Liver Mass*[b]	*Total Spleen Cellularity*[c]
0	1.7 ± 0.3	0.40 ± 0.01	5.02 ± 0.05	5.8 ± 0.7
1	1.9 ± 0.3	0.41 ± 0.02	4.91 ± 0.11	4.1 ± 0.4
3	1.4 ± 0.2	0.39 ± 0.01	4.99 ± 0.06	6.1 ± 0.5
300	−0.3 ± 0.4*	0.31 ± 0.02*	11.05 ± 0.09*	5.6 ± 0.4

[a]Final mass (g) – initial mass (g). [b]Calculated as: (organ weight/body weight) × 100. [c]Cells × 10^7. Data are reported as mean ± SEM. Sample size for all groups is 5. *Significantly different from respective control. ($P \leq 0.05$). This experiment was conducted 3 times. Data from a single representative experiment are shown. PFOS = perfluorooctane sulfonate. TAD= Total Administered Dose (daily doses are 1/28 of the TAD).

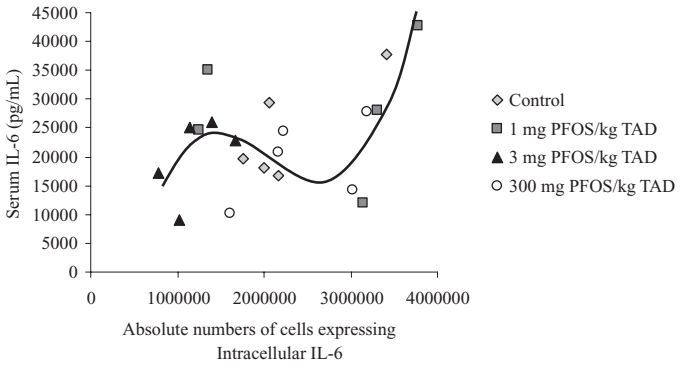

Fig. 3. Scatter plot of serum IL-6 levels and corresponding numbers of splenocytes containing IL-6 in adult B6C3F1 mice treated with PFOS orally for 28 days. Mice were challenged *in vivo* by intraperitoneal injection with 25 μg lipopolysaccharide (LPS; ultra pure from *E. coli* 0111:B4 strain) 1 hr prior to sample collection. ($y = 8E\text{-}^{15}x^3 - 5E\text{-}^{8}x^2 + 0.0881x - 29521$, $R^2 = 0.46$).

Results

Body and organ mass and spleen cellularity

Body mass change over the 28 days was significantly decreased compared to the control in the 300 mg/kg treatment group only (Table 1). In the 300 mg/kg treatment group liver mass was significantly increased (2.2-fold), while spleen mass was significantly decreased by 22% (Table 1). Although spleen mass was decreased significantly in the 300 mg/kg TAD treatment, total spleen cellularity was not altered by any PFOS concentration (Table 1).

Serum and intracellular cytokines

PFOS exposure significantly decreased serum TNF-α concentrations in the 1 mg/kg treatment by 92%, but did not alter serum TNF-α concentrations in the 3 or 300 mg/kg PFOS treatments as compared to control (Fig. 2). Serum TNF-α concentrations in the 3 and 300 mg/kg treatment groups were significantly higher than concentrations in the 1 mg/kg treatment group (Fig. 2). Serum IL-6 levels were significantly increased at the 1 mg/kg dose as compared to control, while levels at the 3 mg/kg dose were significantly different from those at 1 mg/kg (Fig. 2). Absolute numbers of splenocytes containing intracellular IL-1, IL-10 and TNF-α were not significantly affected by PFOS treatment (Table 2). However, numbers of splenocytes expressing intracellular IL-6 were significantly decreased from control in the 3 mg/kg treatment group (Table 2). The pattern of increase at 1 mg/kg and decrease at 3 mg/kg was consistent between serum IL-6 levels and numbers of splenocytes expressing intracellular IL-6 and did exhibit a non-linear relationship ($R^2 = 0.46$; Fig. 3). This relationship fit a third-order polynomial equation indicating a non-random response.

Peritoneal macrophage cytokine production and peritoneal lavage fluid cytokine levels

Ex vivo TNF-α production by *in vitro* LPS-stimulated peritoneal macrophages collected from unchallenged mice (e.g., no LPS injection *in vivo*) from the 300 mg/kg group was

Table 2. Percent and absolute numbers of splenic lymphocytes expressing intracellular IL-1, IL-6, IL-10 or TNF-α following a 28 day PFOS oral gavage in female B6C3F1 mice.

PFOS (mg/kg TAD)	*Percent TNF-α*	*Percent IL-1*	*Percent IL-6*	*Percent IL-10*	*Absolute No. TNF-α Cells ×10⁶)*	*Absolute No. IL-1 (Cells x10⁶)*	*Absolute No. IL-6 (Cells x10⁶)*	*Absolute No. IL-10 (Cells x10⁶)*
0	6.1 ± 0.9	3.8 ± 0.8	5.6 ± 0.7	0.3 ± 0.1	3.8 ± 0.9	1.5 ± 0.3	2.3 ± 0.3	1.1 ± 0.5
1	8.8 ± 2.0	3.9 ± 1.0	6.3 ± 1.3	0.3 ± 0.2	3.4 ± 0.6	1.6 ± 0.4	2.6 ± 0.5	1.4 ± 0.7
3	4.6 ± 0.6	2.3 ± 0.3	3.0 ± 0.4*	0.2 ± 0.1	2.8 ± 0.5	0.7 ± 0.2	1.2 ± 0.2*	0.7 ± 0.4
300	6.1 ± 0.5	4.0 ± 0.9	6.0 ± 0.7	0.3 ± 0.1	3.4 ± 0.3	1.2 ± 0.4	2.4 ± 0.3	1.2 ± 0.4

Mice were challenged i.p. with 25 μg/mL LPS 1 hr prior to sample collection. Data are reported as mean ± SEM. Sample size for all treatment groups is 5. This experiment was conducted three times. Data from a single representative experiment are shown. *Significantly different from respective control ($P < 0.05$). The pattern of response for numbers of cells containing intracellular IL-6 mirrors pattern observed with serum IL-6 levels and when graphed in relationship to serum levels (Fig. 3) shows an association ($R^2 = 0.46$). LPS = lipopolysaccharide. PFOS = perfluorooctane sulfonate. TAD = Total Administered Dose (daily doses are 1/28 of the TAD).

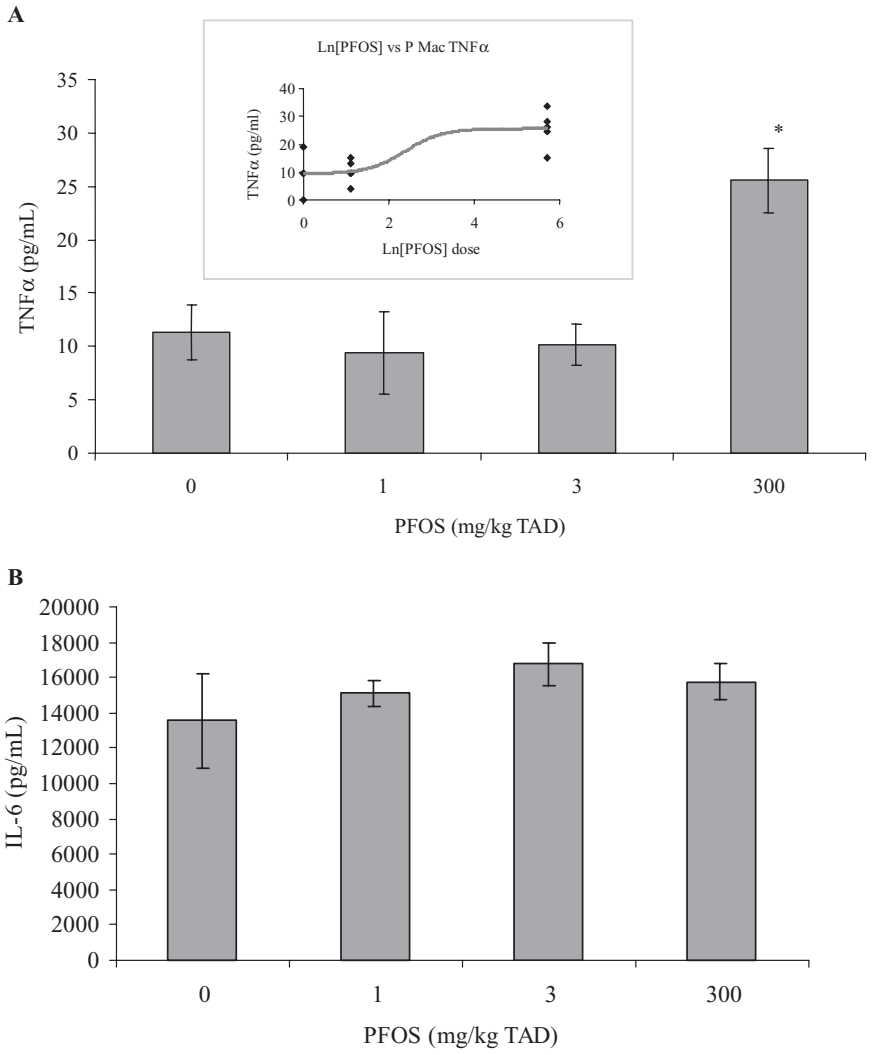

Fig. 4. *Ex vivo* (A) TNF-α and (B) IL-6 production by peritoneal macrophages from adult female B6C3F1 mice treated with PFOS orally for 28 days. Mice were not challenged with lipopolysaccharide (LPS) *in vivo*. Peritoneal macrophages were stimulated with 0.1 μg/mL LPS *in vitro* for 24 hr. Data are presented as mean ± SEM. Sample size for all treatments is 5. *Significantly different from control ($P < 0.05$). This experiment was conducted three times. Data from a single experiment are shown, as results are representative of experiments. The inset in (A) indicates a dose-response model based on a standard curve for calculating dose response. The equations used were: Y = Bottom+(TOP-Bottom)/(1+10(X-logEC50)) and Y = Bottom+(TOP-Bottom)/(1+10(logEC_{50}-X)). Where "Bottom" is the minimum value, "Top" is the maximum and LnEC_{50} is the log of the concentration that gives a half-maximal response. PFOS = perfluorooctane sulfonate. TAD= Total Administered Dose (daily doses are 1/28 of the TAD).

significantly increased by 2.4-fold as compared to control mice (Fig. 4). TNF-α production by peritoneal macrophages following *in vivo* LPS injection, but no LPS stimulation *in vitro*, was not altered compared to control (Fig. 5). IL-6 levels in culture supernatants from the 300 mg PFOS/kg TAD group that received LPS *in vivo* were significantly higher (2.7-fold increase) than control (Fig. 5). Peritoneal lavage fluid, however, exhibited a significant decreasing trend in IL-6 concentration in relation to PFOS dose (Kendall's Tau b= −1.0, p = 0.042; Fig. 6). Decreases in TNF-α and IL-6 in peritoneal lavage fluid were dose responsive and TNF-α exhibited first order exponential decay ([TNF=t_1*exp(−t_2*PFOS)+t_3]; Fig. 6).

Discussion

LPS injection, by either the i.v. or i.p. route, is a common model of inflammation.[57–59] Following ip injection, LPS is absorbed rapidly and distributed throughout the body quickly (with approximately 10% of the LPS administered

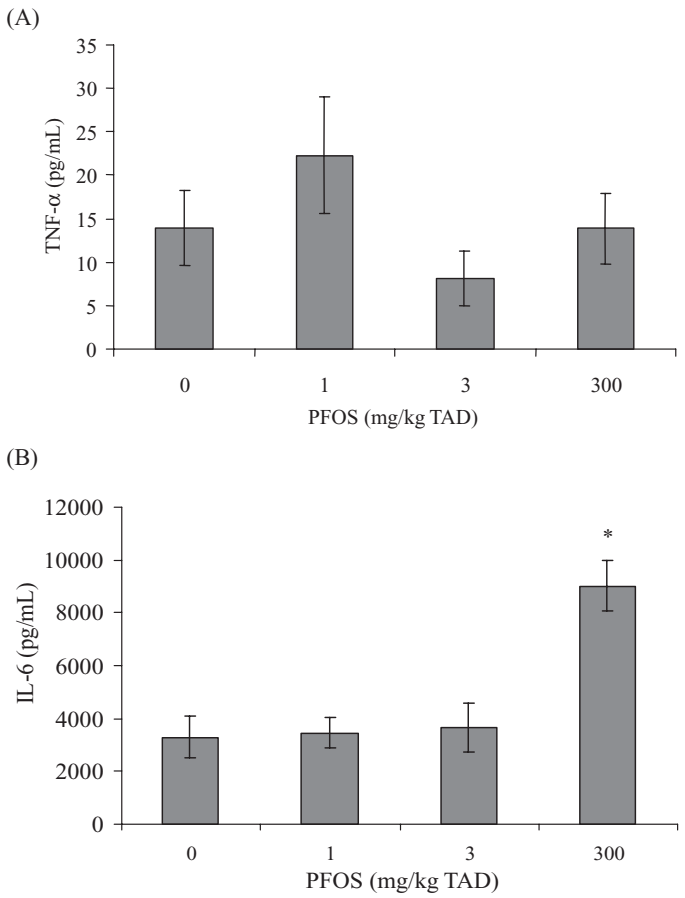

Fig. 5. *Ex vivo* (A) TNF-α and (B) IL-6 production by peritoneal macrophages from adult female B6C3F1 mice treated with PFOS orally for 28 days. Mice were challenged *in vivo* by intraperitoneal injection with 25 μg lipopolysaccharide (LPS; ultra pure from *E. coli* 0111:B4 strain) 1 hr prior to sample collection. Data are presented as mean ± SEM. Sample size for all treatments is five. *Significantly different from control ($P < 0.05$). This experiment was conducted three times. Data from a single experiment are shown, as results are representative of experiments. PFOS = perfluorooctane sulfonate. TAD = Total Administered Dose (daily doses are 1/28 of the TAD).

appearing in circulation within 15 min).[59] When triggered by infection (as mimicked by LPS injection) monocytes and macrophages are the first cell types to respond and TNF-α is the first cytokine released followed shortly by IL-6 and IL-1.[59]

Studies in mice indicate that the kinetics of TNF-α and IL-6 production vary between *in vivo* and *in vitro* LPS exposure.[60] *In vivo* exposure to LPS causes more rapid changes in the production of TNF-α and IL-6 than does *in vitro* exposure, and is potentially influenced by other cell types in the body. *In vitro* exposure to LPS following *in vivo* exposure to PFOS, however, allows isolation of peritoneal macrophages from the system for assessment. In the current study, *in vitro* LPS exposure resulted in increased TNF-α production by peritoneal macrophages in the 300 mg PFOS/kg treatment and IL-6 production was not altered. Conversely, following LPS injection, IL-6 production by peritoneal macrophages was increased in the 300 mg PFOS/kg treatment group, but TNF-α production was not altered.

These results are similar, in part, to those of Qazi et al.[42] where exposure to 400 mg/kg TAD caused an increase in both TNF-α and IL-6 production by peritoneal macrophages following *in vitro* LPS exposure and an increase in TNF-α following LPS injection. As the *in vitro* and *in vivo* exposures to LPS offer different assessments of the macrophages and system, and have differing cytokine kinetic profiles it is not unexpected that the results between the models differed in the current study as also shown by Qazi et al.[42] Taken together, the results of the current study suggest that peritoneal macrophage TNF-α and IL-6 production in response to LPS exposure are not altered at environmentally relevant levels and that the balance of these cytokines remains normal, while at 300 mg/kg TAD the balance of TNF-α and IL-6 seemed to be altered and this alteration varies depending on the LPS exposure model.

Serum cytokine levels reflect systemic production made by various cells types, not just cells of the immune system.[59] PFOS exposure in the current study, at the 1 mg/kg dose, significantly attenuated the increase in serum TNF-α levels that occur following LPS injection. This was not observed at the other doses. The observed increase in mean serum TNF-α levels at 300 mg/kg TAD, suggests a response similar to that seen in a previous study that reported an increase in mean serum concentrations in mice exposed to 400 mg PFOS/kg TAD (40 mg/kg day for 10 days orally [0.02% in diet]).[42,43] At the higher PFOS concentrations hepatotoxicity, as suggested by the increases in liver mass, could have caused increased serum concentrations of TNF-α.[61]

The decreases observed in serum TNF-α at the 1 mg/kg dose could be expected to concurrently occur with decreased serum IL-6 levels since TNF-α production is required, in part, to trigger IL-6 production.[59] However, it appears that PFOS may influence the balance and dynamics of this relationship and that peritoneal macrophages may not be the primary cellular target. Moreover, IL-6 and TNF-α can modulate the levels of each other through negative feedback.[62,63] Qazi et al.[42] report increased serum IL-6 levels in mice exposed to PFOS that were not injected with LPS and no change in serum TNF-α levels, but following LPS injection they report no statistically significant change in either serum TNF-α or IL-6 levels. The increases in serum IL-6 at the 1 mg/kg treatment in the current study do not appear to completely account for the decrease in serum TNF-α levels at this PFOS concentration. Taken together, PFOS seems to elicit the most striking effects on serum TNF-α and IL-6 levels at the lowest concentration used in the current study, indicating assessment of the dose-response between 0 and 1 mg/kg TAD needs to be further characterized. As the 1 mg/kg dose is relevant to reported

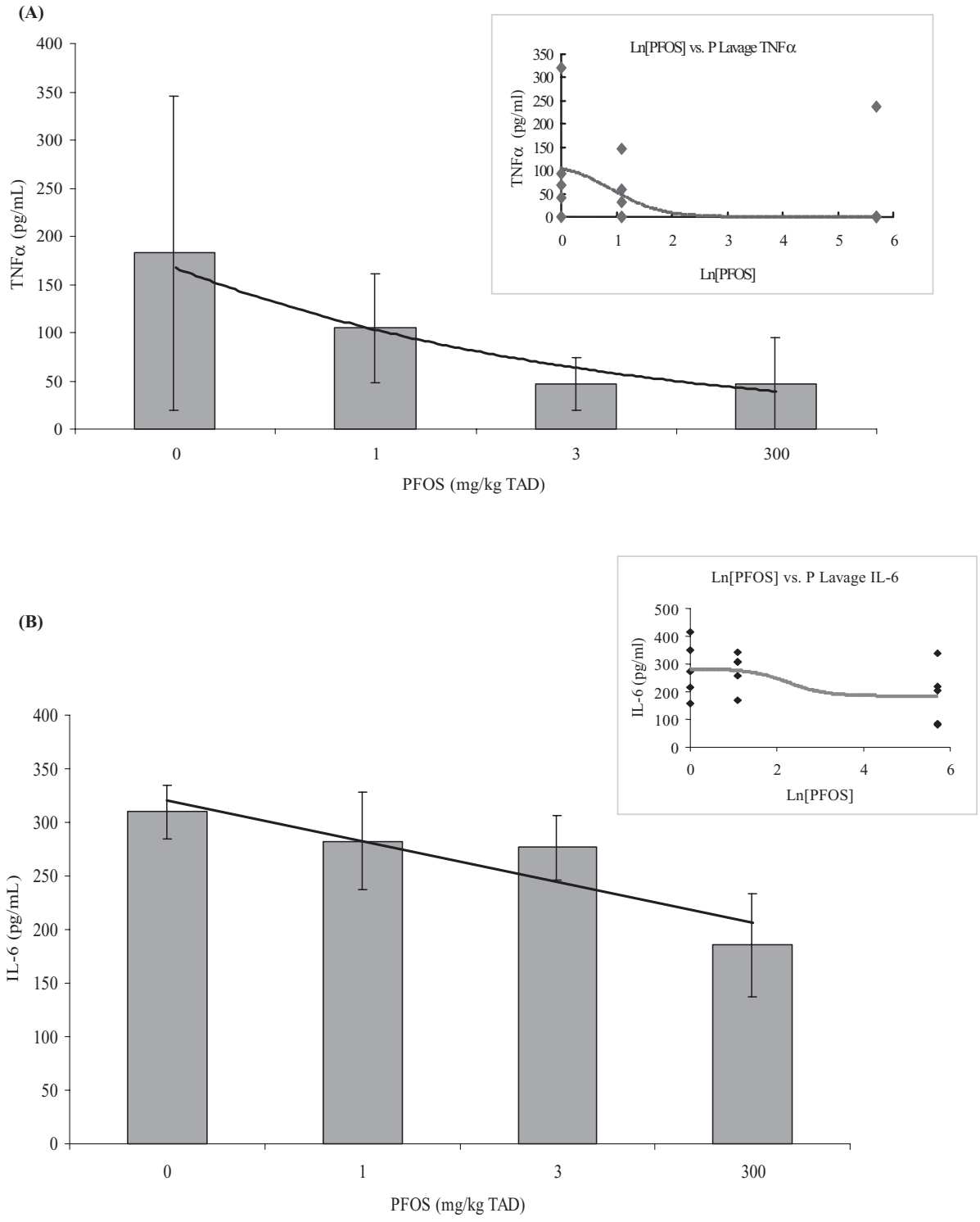

Fig. 6. Peritoneal lavage fluid (A) TNF-α and (B) IL-6 levels in adult female B6C3F1 mice treated with PFOS orally for 28 days. Mice were challenged *in vivo* by intraperitoneal injection with 25 μg lipopolysaccharide (LPS; ultra pure from *E. coli* 0111:B4 strain) 1 hr prior to sample collection. Data are presented as mean ± SEM. Sample size for all treatments is 5. This experiment was conducted 3 times. Data from a single experiment are shown, as results are representative of experiments. The insets in (A) and (B) indicate a dose-response model based on a standard curve for calculating dose response. The equations used were: Y = Bottom + (TOP-Bottom)/(1+10(X-logEC50)) and Y = Bottom + (TOP-Bottom)/(1+10(logEC_{50}-X)). Where "Bottom" is the minimum value, "Top" is the maximum and LnEC_{50} is the log of the concentration that gives a half-maximal response. The decreasing trend in TNFα with increasing dose seen in (A) fits a first-order exponential decay model [TNF = t_1 *exp(-t_2*PFOS)+t_3] where t_1 = 140.16, t_2 = 0.87829 and t_3 = 43.266 and exhibits a decreasing dose-response. The decreasing trend with IL-6 in (B) is significant using Kendall's Tau (Kendall's Tau b = −1.0, P = 0.042) and fits the dose-response model noted here PFOS = perfluorooctane sulfonate. TAD = Total Administered Dose (daily doses are 1/28 of the TAD).

occupational exposures and falls in the upper range of exposures reported in the general population, possible effects in humans cannot be disregarded.

A recent study by Fair et al.[28] demonstrated that *in vitro* LPS-stimulated splenic B-cell IL-6 production from adult female B6C3F1 mice is increased following *in vivo* exposure to 1 mg PFOS/kg TAD (oral, 28-day exposure). The B-cell IL-6 data from Fair et al.[28] corroborates the increases in serum IL-6 levels and numbers of splenocytes expressing intracellular IL-6 observed in the current study at 1 mg/kg TAD following a 1-hr *in vivo* LPS challenge. Moreover, numbers of cells containing intracellular IL-6 appeared related to serum levels of IL-6 in the current study. Serum IL-6 levels seemed proportionate to numbers of cell expressing IL-6 for the spleen. Overall, the pattern of increased IL-6 following exposure to 1 mg/kg TAD was replicated in these studies using both *in vitro* and *in vivo* IL-6 determinations. In light of this observed pattern in PFOS exposure as it relates to serum IL-6 levels and numbers of splenocytes expressing intracellular IL-6, an association between PFOS and IL-6 can not be discounted at this time.

Inflammation, including TNF-α and IL-6 production, is a tightly controlled process in the body that, when modulated, can result in deleterious health effects. The balance between TNF-α and IL-6 is critical to the control of the inflammation process during early stages of infection.[64,65] These processes are dynamic and effects often vary between *in vitro* and *in vivo* LPS exposure.[60] The data assessed in the current study provides only a snapshot of this dynamic process as determination of kinetics was beyond the stated scope of the study. Overall, these data suggest that PFOS, at these exposure concentrations, affects inflammation, but that the effect is not overwhelmingly anti-inflammatory as would be expected with a PPAR-α agonist.[16–20,66–69] Other studies report similar pro- and anti-inflammatory results with other PPAR-α agonists.[66,70] Mice treated with fenofibrate or Wy14,643 exhibited significantly increased plasma levels of LPS-induced TNF-α[61,65] similar to the increase in TNF-α at the highest PFOS concentration tested in the current study. Hill et al.[65] speculate that these disparate inflammatory effects could be the result of a very complicated mixture of systemic PPAR-α agonism effects. This could, therefore, explain the findings in the current study.

Conclusion

To our knowledge, this is the first study to begin to examine the possible effects PFOS may have on inflammation at environmentally relevant exposures (e.g., 1 and 3 mg/kg TAD). Although the TNF-α data suggests possible anti-inflammatory properties at low concentrations, overall the data suggest that PFOS, a known PPAR-α agonist, is not overwhelmingly anti-inflammatory but appears to cause a mixture of both pro- and anti-inflammatory effects. Effects, however, observed in serum TNF-α and IL-6 levels at the lowest concentration, which falls in reported human exposure ranges, suggests possible risk. Based on these data and reported PFOS concentrations in humans and wildlife, continued investigation is warranted to determine the complete range of effects PFOS may have on immune function, the role of PPAR-α, and more specifically assessment of inflammation markers at lower exposure concentrations.

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