Suppression of Humoral Immunity in Mice following Exposure to Perfluorooctane Sulfonate

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Adult male and female B6C3F1 mice were exposed to perfluorooctane sulfonate (PFOS) daily via gavage for 28 days (0, 0.005, 0.05, 0.1, 0.5, 1, or 5 mg/kg total administered dose [TAD]). Following exposure, various immune parameters were assessed and serum PFOS concentrations were determined. Lymphocyte proliferation was not altered in either gender. Natural killer cell activity was increased compared with control at 0.5, 1, and 5 mg/kg TAD in male mice but was not altered in female mice. At these treatment levels, splenic T-cell immunophenotypes were minimally altered in females, but all T-cell subpopulations were significantly modulated in males beginning at 0.1 mg/kg TAD. The sheep red blood cell (SRBC) plaque-forming cell (PFC) response was suppressed in male mice beginning at 0.05 mg/kg TAD and in females at 0.5 mg/kg TAD. Serum trinitrophenyl (TNP)–specific IgM titers were also decreased by PFOS after TNP–LPS (TNP conjugated to lipopolysacharide) challenge suggesting that the humoral immune effects may be attributed to the B-cell rather than T-cell because both T-dependent (SRBC) and T-independent (TI) (TNP–LPS) antigens result in suppressed IgM production. Based on the PFC response, the low observed effect level (LOEL) for males was 0.05 mg/kg TAD (ED_{50} = 0.021 mg/kg TAD) and for females was 0.5 mg/kg TAD (ED_{50} = 0.59 mg/kg TAD). Measured PFOS serum concentrations at these dose levels were 91.5 ± 22.2 ng/g and 666 ± 108 ng/g (mean ± SD), respectively. The male LOEL serum level was approximately 14-fold lower than reported mean blood levels from occupationally exposed humans and fell in the upper range of concentrations reported for the general population. Overall, this study provides a profile of PFOS immunotoxicity showing effects at levels reported in humans and identifies the B-cells as a potential target.

Key Words: PFOS; immunotoxicity; immune; humoral immunity; PFC assay; TNP–LPS; serum levels.

Perfluorinated compounds are carbon chains where hydrogens have been replaced with fluorine and, depending on the intended use of the compound, contain various substitution groups (R-groups) on the terminal end. They have been manufactured for over 50 years and are currently used in a multitude of industrial applications including stain repellents, fire-fighting foams, and as pesticides. Two major classes are the perfluorinated carboxylates (i.e., perfluorooctanoic acid [PFOA]) and the perfluorinated sulfonates (i.e., perfluorooctane sulfonate [PFOS]), which can be grouped as perfluorinated alkyl acids (PFAAs). Because of the strength of the carbon–fluorine bond, some of these perfluorinated compounds are extremely resistant to environmental degradation processes and metabolism. The human health effects of perfluorinated compounds such as PFOS are becoming an increasing concern in the United States and worldwide. Studies have shown comparable plasma concentrations for adults and children from the United States, whereas studies from Japan indicate that levels of these compounds have been increasing in human blood samples over the last 25 years (Harada *et al.*, 2004; Olsen *et al.*, 1999, 2001a, b, 2003a, b).

Initial studies demonstrated that PFAAs cause peroxisomal proliferation, hepatomegaly, altered steroidogenesis, and body weight loss (Biegel *et al.*, 2001; Cook *et al.*, 1992; Kennedy, 1985, 1987; Liu *et al.*, 1996b; Olsen *et al.*, 1999; Pastoor *et al.*, 1987). An ever-increasing amount of research on the toxicity of these compounds shows they are directly responsible for a number of biological alterations including increased lipid metabolism, altered gap junction communication, and increased cell membrane fluidity (Hu *et al.*, 2002, 2003, 2005). The range of effects noted with PFAAs has been well reviewed (Hekster *et al.*, 2003; Kennedy *et al.*, 2004, Lau *et al.*, 2004, 2007). It is widely accepted that PFAAs up regulate a large number of genes involved in the regulation of lipid and/or fatty acid metabolism (Guruge *et al.*, 2006; Hu *et al.*, 2005). Ammonium perfluorooctanoate, the ammonium salt of PFOA, produces increased aromatase activity and plasma estradiol levels, and induces Leydig cell adenomas (Liu *et al.*, 1996a, b). Studies

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also indicate that PFOA suppresses antibody production, causes thymic and splenic atrophy, and alters T-cell populations (DeWitt *et al.*, 2008; Yang *et al.*, 2000, 2001, 2002a). Perfluorodecanoic acid (PFDA) causes decreases in serum levels of IgG 2a specific to keyhole limpet hemocyanin (KLH) (Nelson *et al.*, 1992). Sulfluramid, a perfluorinated pesticide that is metabolized to PFOS, also suppresses antibody production (Peden-Adams *et al.*, 2007).

As PFOA, PFDA, and sulfluramid were shown to be immunotoxic (DeWitt *et al.*, 2008; Nelson *et al.*, 1992; Peden-Adams *et al.*, 2007; Yang *et al.*, 2000, 2001, 2002a), it is reasonable to expect that other perfluorinated compounds like PFOS may act similarly. PFOS is the predominate PFAA found in both human and wildlife blood samples (Giesy and Kannan, 2001; Houde *et al.*, 2005; Keller *et al.*, 2005; Olsen *et al.*, 2003a, b) but little is known regarding its impact on immune function. Therefore, the current study utilized traditional measures of immunotoxicity as outlined by Luster *et al.* (1988, 1992) combined with the 28-day exposure requirements as outlined by USEPA's Immunotoxicity Harmonized Test Guideline (OPPTS 870.7800) to assess the immunotoxicity of PFOS. Targeted total administered dose (TAD) levels at the end of the 28 days were based on the range of blood levels found in humans and wildlife (Giesy and Kannan, 2001; Houde *et al.*, 2005; Keller *et al.*, 2005; Olsen *et al.*, 2003a, b; Table 1). Therefore, daily doses administered for 28 consecutive days represent 1/28 of the final TAD. The results of this study support the fact that PFOS targets humoral immune function at environmentally relevant concentrations.

MATERIALS AND METHODS

Chemicals, antibodies, and supplies. Unless otherwise specified, all chemicals and mitogens used for immune assays were purchased from Sigma (St Louis, MO). Perfluorooctane sulfonic acid potassium salt (stated purity > 98%) used for animal treatments was obtained from Fluka (via Sigma, CAS No. 2795-39-3). Sheep red blood cells (SRBCs) in Alsever's solution were obtained from BioWhitaker, Inc. (Walkersville, MD). Lyophilized guinea pig complement (GPC), GPC restoring solution, nonessential amino acids (NEAA; 10mM

$100\times$) and sodium pyruvate (100mM) were obtained from GIBCO Laboratories (Grand Island, NY). RPMI-1640 medium (with L-glutamine and sodium bicarbonate), phosphate buffered saline (PBS; with or without Ca^{+2} and Mg), and penicillin/streptomycin (5000 IU/ml, 5000 μg/ml) were purchased from Cellgro (Mediatech, Herndon, VA). The radio-isotopes, sodium chromate (^{51}Cr; specific activity 351 mCi/mg^{Cr}), and triitiated thymidine (^{3}H; specific activity 71 Ci/mmol) were acquired from ICN (Costa Mesa, CA). The fetal bovine serum (FBS) was from Hyclone (Logan, UT). The fluorescent antibodies, mouse IgG 2a (isotype control), fluorescein isothiocyanate (FITC) conjugated rat-anti-mouse CD4 (monoclonal), and phycoerythrin (PE) conjugated rat-anti-mouse CD8 (monoclonal) were purchased from Caltag (Burlingame, CA). Luma Plates, Unifilters, and Microscint 20 were procured from Packard (Meriden, CT). Yac-1 cells were purchased from ATCC (Manassas, VA). Isoflurane (AErrane) was obtained from Baxter Pharmaceutical Products, Inc. (Deerfield, IL). Triton X, tissue culture plates, and disposables were purchased from Fisher Scientific (Atlanta, GA).

Animal care. Mice were housed in plastic shoebox cages on corncob bedding and administered food (TekLab Sterilizable Rodent Diet, formula no. 8656; Harlan-Teklab, Madison, WI) and water *ad libitum*. For each treatment group, five 7- to 8-week old female or male B6C3F1 mice (Harlan) were homogeneously distributed by weight into each treatment group. Mice were acclimated to the conditions of the treatment room (12-h light/dark cycle, 22 ± 2°C, 60–65% relative humidity) for 1 week before dosing began. Bedding, food, and water were changed twice a week, and mice were observed daily.

Animal dosing. PFOS was administered via oral gavage in Milli-Q water containing 0.5% Tween 20 (Lau *et al.*, 2003). Control mice received Milli-Q water containing 0.5% Tween 20 only. Mice were dosed daily for 28 days (0, 0.166, 1.66, 3.31, 16.6, 33.1, or 166 μg PFOS/kg body weight/day) to yield a targeted TAD over the 28 days of 0, 0.005, 0.05, 0.1, 0.5, 1, or 5 mg PFOS/kg body weight (Table 2). These doses 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%. All procedures were approved by the institutional animal review board (Institutional Animal Care and Use Commitee) and conducted in an Association for Assessment and Accreditation of Laboratory Animal Care accredited facility.

Sample collection and processing. To calculate mass change over the 28 days, body mass was measured one day prior to exposures and at the termination of the experiment (weight change = final mass − start mass). Whole blood was collected retro-orbitally using glass capillary tubes into nonheparinized eppendorf tubes, following anesthetization with isoflurane. Blood was permitted to clot for 1 h before centrifugation when serum was removed and frozen in polypropylene tubes at −20°C until analysis of lysozyme activity or PFOS concentrations. Spleen, thymus, liver, kidney,

TABLE 1
TAD Concentrations Applied in this Study were based on the following Human and Wildlife PFOS Serum/Plasma Levels

Species	Location	PFOS mean[a] *(ng/ml = ppb)*	Reference
Ringed seal	Baltic Sea	110	Giesy and Kannan, 2001
Polar bear	Beaufort Sea	34	Kannan *et al.*, 2001
Bottlenose dolphin	Charleston, SC, USA	1315.6	Houde *et al.*, 2005
Bald eagle	Midwestern USA	360	Giesy and Kannan, 2001
Loggerhead sea turtle	Southeastern USA	15.7	Keller *et al.*, 2005
Kemp's ridley sea turtle	Southeastern USA	49.2	Keller *et al.*, 2005
Human	USA	43.7	Olsen *et al.*, 2001a
Human	Production Plant Workers, USA	1320	Olsen *et al.*, 2003a

[a]Arithmetic mean.

TABLE 2
Comparison of TAD of PFOS at the End of the 28-Day Exposure in Relation to the Daily Administered dose (μg/kg/day), expected Serum Levels, and Actual Measured Serum Levels in the B6C3F1 Mice from this Study

Administered dose PFOS (mg/kg TAD)[a]	Administered dose PFOS ion (mg/kg TAD)[a,b,c]	Daily administered dose PFOS ion (μg/kg/day)[d,e]	Expected serum levels (PFOS ion) (ppb = ng/g)[f]	Actual serum levels (ng/g)[g] male mice	Actual serum levels (ng/g)[g] female mice
0	0	0	0	12.1 ± 4.64 (5)	16.8 ± 4.31 (5)
0.005	0.00464	0.166	4.64	17.8 ± 4.24[h] (5)	ND
0.05	0.0464	1.66	46.4[i]	91.5 ± 22.2[j] (4)	88.1 ± 10.5 (5)
0.1	0.0927	3.31	92.7	131 ± 15.2 (5)	123 ± 18.7[h] (5)
0.5	0.464	16.6	464	ND	666 ± 108[j] (5)
1	0.927	33.1	927[k]	ND	ND
5	4.64	166	4640[k]	NR	NR

Note. ND = not determined; NR = not reported (over calibration curve); TAD = over the course of 28 days.

[a]Dose concentrations were made by weighing the potassium salt of PFOS and therefore include the salt mass. Target dose is identical for PFOS potassium salt or the PFOS ion when rounded to a single significant digit as shown here (see column titled: administered dose PFOS ion [mg/kg TAD]). These doses (0.005, 0.05, 0.1, 0.5, 1, and 5 mg PFOS/kg body weight) are used throughout the paper for simplicity. For comparison purposes to the measured serum levels, these doses expressed as ng/g are: 5, 50, 100, 500, 1000, and 5000 ng PFOS/g, respectively. Moreover, the PFOS ion TAD is 4.6, 46, 92.7, 464, 927, and 4640 ng PFOS ion/g, respectively; which are the expected serum levels.

[b]Actual TAD after correcting for only the mass of the PFOS ion separate from the potassium salt. Molecular weight PFOS ion = 499.12 g/mol. This was done because when it is not removed, the PFOS concentration is overestimated by 7.3%. Note that when comparing doses or concentrations reported in other studies, it is often unknown whether the potassium mass was removed. For comparison purposes to the measured serum levels in this study, these doses expressed as ng/g are: 4.6, 46, 92.7, 464, 927, and 4640 ng PFOS ion/g, respectively; which are the expected serum levels.

[c]0.0093, 0.093, 0.19, 0.93, 1.9, 9.3 μmol/kg, respectively.

[d](Actual TAD using mass of PFOS ion only/28 days) × 1000.

[e]0.00033, 0.0033, 0.0068, 0.033, 0.068, 0.33 μmol/kg/day, respectively.

[f]Expected concentration in serum is equal to the PFOS ion TAD assuming complete absorption, no elimination or biotransformation, no background levels prior to administration, and equal distribution throughout the body [ppb = (ng/ml ≈ ng/g); because the density of serum is approximately 1 g/ml, the values reported as ng/g in this table are approximately equivalent to ng/ml or ppb]. Therefore, these expected serum levels expressed as ng/g are 4.6, 46, 92.7, 464, 927, and 4640 ng PFOS ion/g, respectively.

[g]Serum levels (ng PFOS ion/g serum) of PFOS ion on day 29 as measured by LC/MS/MS. Data are reported as mean ± standard deviation (sample size). This experiment was only conducted once [(ng/g ≈ ng/ml) = ppb].

[h]NOEL for this study based on the PFC response.

[i]Human serum levels, mean ~43 ppb (Olsen *et al*., 2001a); Kemp's ridley plasma levels, mean ~49 ppb (Keller *et al*., 2005).

[j]LOEL for this study based on the PFC response.

[k]Bottlenose dolphin plasma levels, mean ~1300 ppb (Houde *et al*., 2005); Plant production workers, mean ~1300 ppb (Olsen *et al*., 2003a).

uterus, and testis were collected and weighed following euthanization with CO_2. All balances were calibrated, using standard weights, prior to use. Organ mass was normalized for body weight and reported as a somatic index {(organ weight/body wt) × 100}. Immune organs (spleen and thymus) were aseptically processed into single cell suspensions with the use of sterile, frosted microscope slides for functional immune endpoints and T-cell immunopheno-type determinations. A Coulter Counter (Model ZF; Hialeah, FL) was used to obtain cell counts from these single cell suspensions. Additionally, alterations in cell viability (trypan blue exclusion) following treatment were assessed after red blood cell lysis.

Lymphocyte proliferation. Spleens were processed and diluted to 2×10^6 nucleated cells/ml in supplemented RPMI-1640 medium (RPMI-1640, 10% FBS, 1% NEAAs, 1% sodium pyruvate, 10mM HEPES, 1% pen–strep, and 10μM 2-mercaptoethanol, pH 7.4). One hundred microliter aliquots of the resulting cell suspensions were dispensed into 96-well plates (2×10^5 cells per well) containing triplicate wells of either 2.5 μg/ml concanavalin A (Con A, type IV-S), 5 μg/ml lipopolysaccharide (LPS; *Escherichia coli* 0111:B4), or supplemented RPMI-1640 (unstimulated wells). All mitogen concentrations are expressed in μg/ml of culture (final culture concentration, culture volume was 200 μl). Plates were incubated for 40 h at 37°C and 5% CO_2. After 40 h, 0.5 μCi of tritiated thymidine in RPMI-1640 was added to each well in a volume of 100 μl. Six hours later, cells were harvested onto Unifilter plates using a Packard Filtermate 96-well plate harvester and the plates were allowed to dry.

Once dry, 25 μl of Microscint 20 was added to each well and the samples were analyzed using a Packard Top Count-NXT scintillation counter. Data are reported as the stimulation index (SI = cpm stimulated/cpm unstimulated).

Serum lysozyme activity. Lysozyme activity was assessed using slight modifications of a standard turbidity assay previously described by Demers and Bayne (1997). A 1 mg/ml stock solution of hen egg lysozyme (HEL) was prepared in 0.1M phosphate buffer (pH 5.9), and aliquots were frozen until use. A solution of *Micrococcus lysodeikticus* was prepared fresh daily by dissolving 50 mg of the lyophilized cells in 100 ml of 0.1M phosphate buffer (pH 5.9). HEL was serially diluted in phosphate buffer to produce a standard curve of 40, 20, 10, 5, 2.5, 1.25, 0.6, 0.3, and 0 μg/μl. Aliquots of each concentration (25 μl per well) were added to a 96-well plate in triplicate. For each sample, 25 μl of test plasma was added in quadruplicate to the plate. The solution of *M. lysodeikticus* (175 μl/well) was quickly added to three sample wells and to each of the standard wells. The fourth well containing plasma received 175 μl of phosphate buffer and served as a blank. Plates were assessed for absorbance at 450 nm with a spectrophotometer (SpectraCount; Packard, Meridian, CT) immediately (T0) and again after 5 min (T5). Absorbance unit (AU) values at T5 were subtracted from AU values at T0 to determine the change in absorbance. The AU value for the blank sample well was subtracted from the average of the triplicate sample wells to compensate for any hemolysis or color variation in the samples. The resultant AU value was converted to HEL concentration (micrograms per microliter) via linear regression of the standard curve.

Natural killer cell activity. Natural killer (NK) cell activity was assessed via an *in vitro* cytotoxicity assay using ^{51}Cr-labeled Yac-1 cells as described previously (Duke *et al.*, 1985; Holsapple *et al.*, 1988). To minimize radioactive waste, the procedure was adapted to 96-well plates that were read on a Packard Top Count scintillation counter. Splenocytes were adjusted to 2×10^7 nucleated cells/ml in complete media (RPMI-1640, 10% FBS, 50 IU penicillin and 50 μg streptomycin). Splenocyte and Yac-1 cells were plated, in triplicate wells, in ratios of 200:1, 100:1, 50:1, and 25:1 splenocytes:labeled Yac-1 cell counts, in a final volume of 0.2 ml per well. Maximum release was determined by lysing ^{51}Cr-labeled Yac-1 cells with 0.1% Triton × in complete media. Spontaneous release was determined by incubating Yac-1 tumor cells only in complete media. After a 4-h incubation at 37°C and 5% CO_2, the plates were centrifuged (377 × g, 5 min), and 25 μl of supernatant was then transferred to a 96-well plate containing solid scintillant (LumaPlate). Plates were air dried overnight, and then counted for 5 min, after a 10-min dark delay, using a Packard Top Count-NXT. The results are expressed in lytic units per 10^7 splenocytes using 10% lysis as the reference point (Bryant *et al.*, 1992).

Antibody plaque-forming cell assay (T-dependent IgM production). The number of plaque-forming cells (PFCs) was determined using the Cunningham modification of the Jerne plaque assay (Cunningham and Szenberg, 1968; Jerne and Nordin, 1963). Five days prior to euthanasia, mice were administered 0.1 ml of a 25% SRBC suspension in PBS via ip injection (Harper *et al.*, 1993). All SRBCs for the experiments were drawn from a single donor animal. Splenocytes were diluted to 2×10^6 nucleated cells/ml in supplemented RPMI-1640 medium (RPMI-1640, 10% FBS, 1% NEAAs, 1% sodium pyruvate, 10mM HEPES, 1% pen–strep, and 10μM 2-mercaptoethanol, pH 7.4). Aliquots of the cell suspension were added to eppendorf tubes containing supplemented RPMI-1640 and SRBCs. Lyophilized GPC was reconstituted with GPC restoring solution as per manufacturers directions and was then diluted 1:2 (vol/vol) in GPC restoring solution. Fifty microliters of the diluted, reconstituted lyophilized GPC was then added on a tube by tube basis, and aliquots of the solution were placed in Cunningham chamber slides. The slides were sealed with a wax mixture and were incubated at 37°C and 5% CO_2 for 1 h. Plaques were enumerated microscopically. Results are reported as PFCs/million splenocytes.

Serum anti-trinitrophenyl IgM levels (TI IgM production). In a separate experiment conducted to assess TI antibody production following PFOS exposure, adult female B6C3F1 mice were exposed to the vehicle control (0.5% Tween 20 in Milli-Q water) or 0.334 mg/kg/day PFOS (7 mg/kg TAD) orally for 21 days (10 mice/treatment group). Seven days prior to euthanization mice were injected with trinitrophenyl conjugated to LPS (TNP–LPS) iv (tail vein; 1 μg TNP–LPS/μl of complete Freund's adjuvant in an injection volume of 100 μl). 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 h before centrifugation when serum was removed. Serum was frozen at −20°C until analysis. Serum levels of anti-TNP–specific antibodies (IgM) were determined by enzyme-linked immunosorbent assay (ELISA) as a modification of Stuber and Strober (1996). Briefly, mouse serum was diluted 1:100 to 1:1600 (vol/vol) with 1% bovine serum albumin (BSA) in PBS. The alkaline phosphate-conjugated goat anti-mouse IgM (Jackson Immunoresearch; West Grove, PA) was diluted 1:1000, as determined by an optimization ELISA. After the addition of the substrate, *p*-nitrophenyl phosphate, the plates were read at 405 nm using a PerkinElmer Victor[3] at 15 min.

Splenic and thymic CD4/CD8 subpopulations. Spleen or thymus cells were labeled with fluorescent (PE or FITC) rat IgG_2 monoclonal antibodies specific for mouse CD4 or CD8 (rat-anti-mouse). In this procedure, single cell suspensions of thymocytes and splenocytes were washed and resuspended in PBS (pH 7.4; containing 0.1% sodium azide and 1% BSA). Monoclonal antibodies were incubated with cells for 30 min at 6°C in the dark. Red blood cells were lysed (0.83% NH_4Cl, 0.1% $NaHCO_3$, and 0.006% ethylenediaminetetraacetic acid in distilled water; pH 7.0) and removed by several washings with PBS. Lastly, the cells were fixed with 1% paraformaldehyde and stored at 6°C in the dark. Flow cytometric analysis was performed using a Becton Dickinson flow cytometer (FACSCalibur; San Jose, CA). Nonstained cells and isotypic antibody controls were used to establish gates for the CD4/CD8 subpopulations in thymic and splenic cells. Data are represented as absolute number of cells, determined by multiplying the percent gated cells by the total number of nucleated cells obtained by the Coulter Counter.

Serum PFOS determination. A stock prepared from PFOS potassium salt (Alfa Aesar, Ward Hill, MA) in methanol was used to create an 8-point calibration curve from 0.010 to 346 ng of the PFOS ion (not including the potassium salt) spiked into Milli-Q deionized water. Spiking of calibration and internal standards and weighing of serum extracted was performed gravimetrically to the nearest 0.00001 g. The calibration standards, nine aliquots of a National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 1957 (human serum), and nine procedural blanks consisting of Milli-Q water were extracted and processed identically alongside the serum samples and were used as quality control. Serum samples (0.00921–0.19633 g) were thawed, vortexed, and transferred to polypropylene tubes. All tubes were spiked with 0.100 ml of internal standard, containing [13]C_4-perfluorooctane sulfinate ([13]C_4-PFOSi) and/or [13]C_4-PFOS (both from Wellington Laboratories, Guelph, Ontario). [13]C_4-PFOSi was used mistakenly for a subset of samples, but both internal standards resulted in similar PFOS concentrations in subsequent sample batches. All tubes received 0.600 ml of 50% formic acid in water and were sonicated for 10 min. Extraction utilized 3 cm[3], 60 mg Oasis WAX solid-phase extraction columns (Waters, Milford, MA) on RapidTrace modules (Caliper, Hopkinton, MA). The columns were preconditioned with 2 ml of methanol and 2 ml of water. Samples were loaded onto columns, followed by a 1 ml of water rinse of the sample tubes. Biological compounds were rinsed from columns into waste with 1 ml of 2% formic acid, followed by 0.5 ml of methanol. Compounds were then eluted from columns into clean polypropylene tubes with 1.5 ml of methanol followed by 2.0 ml of 1% ammonium hydroxide in methanol. Extracts were evaporated under a stream of nitrogen until volumes ranged from 0.7 to 1.3 ml. Extracts were transferred to glass autosampler vials and analyzed with an API 4000 (Applied Biosystems, Foster City, CA) consisting of an Agilent 1100 liquid chromatograph interfaced to a negative electrospray ionization tandem mass spectrometer (LC/MS/MS). A precolumn (Thermo Betasil C8, 100 mm × 2.1 mm × 5 μm) was placed in between the LC pump and autosampler to capture and delay PFOS and other perfluorinated contaminants that might leach from the Teflon tubing and LC components upstream from the autosampler. Forty microliters of sample was injected onto an identical Betasil C8 analytical column. The solvent gradient started at 60% methanol and 40% 20 mmol/l ammonium acetate in water, and then increased to 75% methanol by 9 min, held for 5 min, and then increased to 95% methanol by 18 min, held for 2 min, before reverting back to original conditions by 20.5 min with a 10-min hold. The flow rate was 0.3 ml/min. The LC/MS/MS method included optimized parameters for PFOS (499 → 80 and 499 → 99), [13]C_4-PFOSi (487 → 83, 487 → 169, and 487 → 219), and [13]C_4-PFOS (503 → 80, 503 → 99). Branched and linear isomer peaks of PFOS were included when integrating peak areas, so the concentrations should be considered total PFOS concentrations. Amounts of PFOS were calculated using regressions of the calibration curve that used response and amount ratios to the available internal standard. Limits of detection were determined as the average ng measured in procedural blanks plus three times the standard deviation of these blanks, all divided by the grams of extracted sample, and most were between 0.1 and 2 ng PFOS/g of serum. Because the density of serum is approximately 1 g/ml, the values reported as ng/g here are approximately equivalent to ng/ml.

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 ($p < 0.05$), Dunnett's *t*-test was used to compare treatment groups to the control group. Unless specified all experiments were repeated twice.

The PFC dose–response was assessed by regression analysis. The log transformation of the endpoint (PFC/million cells) was plotted against the log

dose. The log dose needed to achieve 50% suppression of the immune response was determined from the regression equation and the inverse log calculated to determine the calculated ED_{50} (mg/kg TAD).

RESULTS

Serum PFOS Concentrations

PFOS concentrations measured in SRM 1957 here (mean ± SD = 22.4 ± 1.78 ng/g) agreed within 2% of the mean results from an interlaboratory comparison using this material (Lindström and van Leeuwen, 2006). This finding provides assurance of accuracy and precision of the PFOS measurements. Concentrations of PFOS in the mouse serum collected on day 29 just prior to euthanization (24 h after the last exposure) were determined for the control (0 mg/kg TAD), no observed effect level (NOEL), and low observed effect level (LOEL) dose groups for each gender (Table 2). Serum levels in males exposed to 0.05 mg/kg TAD, the LOEL, ranged from 64.3 to 118 ng/g. Serum levels in females exposed to 0.5 mg/kg TAD, the LOEL, ranged from 49 to 753 ng/g. The serum ranges for the NOEL in males and females respectively were 14.0–24.2 ng/g and 93.7–147 ng/g. Additionally, serum from both genders was analyzed in the 0.05 and 0.1 mg/kg TAD dose groups to provide a comparison between genders at the same dose levels (Table 2). Serum levels of males and females at matching doses were not significantly different. Concentrations for the high dose group were also assessed but fell above the calibration curve and are therefore not reported. When background levels in the control animals are subtracted from the respective treatment groups, the serum concentrations are on average 38% higher than the expected serum level as estimated by the TAD (Table 2).

Body and Organ Mass and Immune Organ Cellularity

There were no signs of overt toxicity in any of the animals as indicated by weight loss, lack of activity, lack of grooming, or cloudy eyes. In fact, mice in all groups appeared to gain weight equally over the course of the 28-day study, therefore, no differences were observed in final body mass or body mass change between the PFOS treated and control animals in either gender (see Supplementary Data, SD 1 and 2). Secondary immune organ mass (spleen and thymus), liver, kidney and gonad (uterus or testis) mass also exhibited no statistical change as compared with control groups in either gender (see Supplementary Data, SD 1 and 2). Cellularity and cell viability of the spleen and thymus were not altered following treatment with PFOS at any of the dose levels assessed in either gender (see Supplementary Data, SD 3).

### Lymphocyte Proliferation, NK-Cell Function, and Lysozyme Activity

Con A- and LPS-induced lymphocyte proliferation responses were not altered compared with control in either gender (see Supplementary Data, SD 4). NK-cell activity was not affected in females but it was significantly increased 2- to 2.5-fold in males at exposure levels of 0.5, 1, and 5 mg/kg TAD (Fig. 1). Plasma lysozyme activity was not altered by PFOS treatment in male mice, but in female mice it was significantly increased over control at the 0.1 and 5.0 mg/kg treatments (Fig. 2).

Antigen-Specific IgM Production

SRBC-specific IgM production (PFC response) was suppressed in both genders following PFOS exposure (Fig. 3). Males were more sensitive to this effect than females. Suppression of this response was dose-responsive beginning at exposures of 0.05 mg/kg TAD in males and at 0.5 mg/kg TAD in females. PFOS caused the male PFC response to decrease by 52–78%. In females, the PFC response was decreased by 50, 66, and 74% at the 0.5, 1, and 5 mg/kg treatments, respectively. The calculated ED_{50} for this response by gender is shown in Table 3.

Because suppression of IgM production by a T-dependent antigen such as SRBC can be the result of a functional deficit in either the T-cell or the B-cell, challenge with a TI antigen (TNP–LPS) was conducted to further elucidate this effect. An
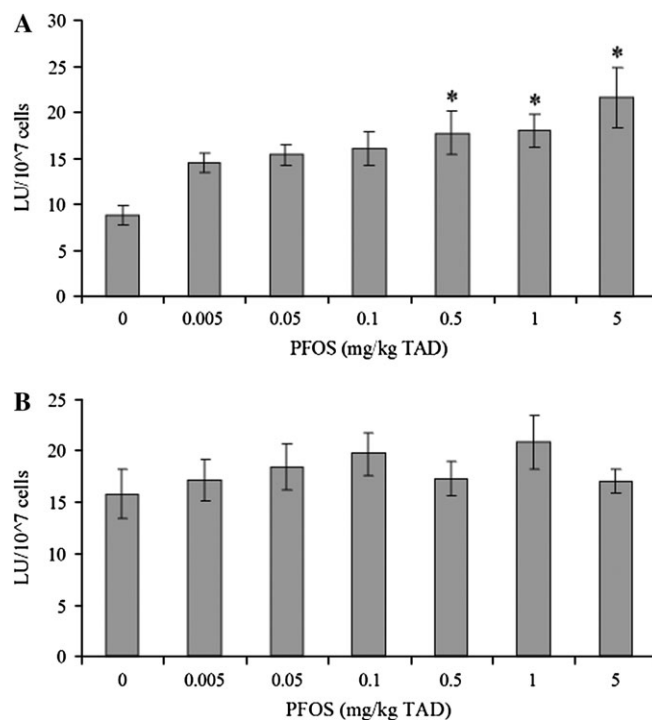
FIG. 1. Splenic NK-cell activity was measured using a standard chromium release method following oral exposure to PFOS for 28 days in adult (A) male or (B) female B6C3F1 mice. Data are presented as mean ± standard error. Sample size for all treatments in both genders is five. LU = lytic units. *Significantly different from control ($p \leq 0.05$). TAD = over the course of 28 days. This experiment was conducted twice. Data from a single experiment are shown as results were similar between both experiments.
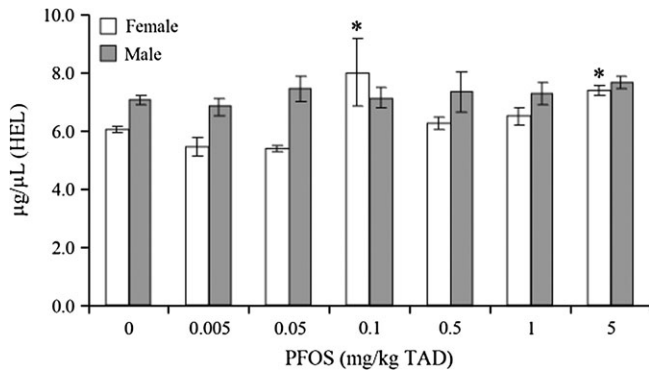
FIG. 2. Serum lysozyme activity measured in adult B6C3F1 mice following oral exposure to PFOS for 28 days using a standard turbidity assay and compared with a HEL standard curve. Data are presented as mean ± standard error. Sample size for all treatments in both genders is five. *Significantly different from control ($p \leq 0.05$). TAD = over the course of 28 days. This experiment was only conducted once.

additional group of female mice were used for this experiment and exposed orally for 21 days to 0.334 mg/kg/day PFOS (7 mg PFOS/kg TAD). Serum levels of TNP-specific IgM were significantly suppressed (62% decrease) compared with control following challenge with TNP–LPS (Fig. 4).

Lymphocyte Immunophenotypes

In males, thymic T-cell CD4/CD8 subpopulations were not affected by PFOS exposure (see Supplementary Data, SD 5), whereas numbers of all T-cell CD4/CD8 subpopulations in the spleen were significantly altered beginning at 0.1 mg/kg TAD. More specifically, splenic CD4−/CD8+ and CD4−/CD8− cells were increased, whereas numbers of CD4+/CD8− and CD4+/CD8+ cells were decreased (Table 4). In females, thymic T-cell populations were minimally affected by PFOS treatment. Numbers of thymic CD4−/CD8+ cells were increased compared with control at the 1 and 5 mg/kg treatment levels (see Supplementary Data, SD 5). Splenic CD4−/CD8+ cells were decreased in females at 0.1, 0.5, and 5 mg/kg as were numbers of CD4+/CD8− cells (0.1 and 0.5 mg/kg only) (Table 4). In general, the thymus cell populations in both genders were less sensitive to the effects of PFOS when compared with alterations observed in the spleen. Furthermore, the most clear dose-responsive effect observed in these data was increases in the CD4-/CD8− subpopulation in male spleen.

DISCUSSION

Immunomodulating effects of various xenobiotic classes including polycyclic aromatic hydrocarbons, halogenated aromatic hydrocarbons, organochlorine, and organophosphorous pesticides, and heavy metals have been relatively well characterized (Dean, 1994; Luebke *et al.*, 2007; Vial *et al.*,
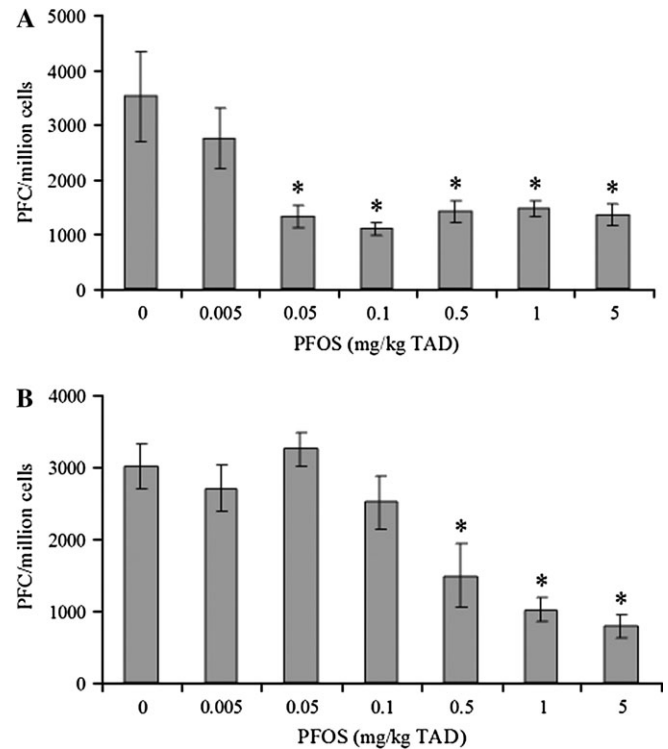
FIG. 3. SRBC-specific IgM PFC response was measured using the Cunningham modification of the Jerne plaque assay following oral exposure to PFOS for 28 days in adult (A) male or (B) female B6C3F1 mice. Data are presented as mean ± standard error. Sample size for all treatments in both genders is five. *Significantly different from control ($p \leq 0.05$). TAD = over the course of 28 days. This experiment was conducted twice. Data from a single experiment are shown as results were similar between both experiments.

1996), whereas the effects of perfluorinated compounds have not. A few studies have assessed immune modulation following exposure to PFOA, PFDA, PFOS, and the perfluorinated pesticide sulfluramid (DeWitt *et al.*, 2008; Keil *et al.*, 2008; Nelson *et al.*, 1992; Peden-Adams *et al.*, 2007, Yang *et al.*, 2000, 2001, 2002a, b). This, however, is the first study to examine the effects of PFOS using a suite of traditional endpoints as outlined by the National Toxicology Program's Tiered Immunotoxicity testing scheme (Luster *et al.*, 1988, 1992) and USEPA's Harmonized Test Guidelines for Immunotoxicity (OPPTS 870.7800) (CD4/CD8, PFC, and NK-cell activity).

Organ (spleen, thymus, and liver) and body mass were not altered in this study which is unlike other studies evaluating PFOS, PFOA, sulfluramid, and PFDA (Borges *et al.*, 1992; Kennedy, 1987; OECD, 2002; Nelson *et al.*, 1992; Peden-Adams *et al.*, 2007; Yang *et al.*, 2000, 2001). In all likelihood, this is attributed to the lower PFOS concentrations administered in this study. These observations, however, remind one that decreased immune organ mass is not always a sensitive indicator of immunotoxicity. This example clearly demonstrates the value of functional immunological assays and their contribution to the full scope of toxicological evaluations.

TABLE 3

ED_{50} values, NOELs, and LOELs for the PFC Response in Adult Male and Female B6C3F1 Mice following Daily Oral Exposure with the PFOS Ion for 28 Days

	Male			Female		
	mg/kg actual TAD	μg/kg/day[a]	Serum (ng/g[b])	mg/kg actual TAD	μg/kg/day	Serum (ng/g[b])
NOEL	0.00464	0.166	17.8 (4.24)[b]	0.0927	3.31	123 (18.7)[b]
LOEL	0.0464	1.66	91.5 (22.2)[b]	0.464	16.6	666 (108)[b]
ED_{50}	0.021[c]	0.69[d]	51.8[e]	0.59[f]	21.0[d]	781[e]

Note. TAD = over the course of 28 days.

[a] Daily dose = (Actual TAD using mass of PFOS ion only/28 days) × 1000.

[b] Mean measured serum concentration(standard deviation).

[c] Calculated from $y = -0.3095x + 2.7154$, $R^2 = 0.9996$ where actual TAD is on the x-axis and the PFC response is on the y-axis (value shown in table was calculated using the portion of the dose–response prior to the slope leveling out at 0.5 mg/kg. When the whole curve was utilized [$y = 0.087x^2 + 0.0651x + 3.0972$, $R^2 = 0.7703$], the ED_{50} was 0.019 mg/kg TAD and corresponding calculated serum concentration was 48.2 ng/g).

[d] Calculated using actual μg/kg/day on the x-axis with the PFC response on the y-axis. For males: $y = -0.3097x + 3.1962$, $R^2 = 0.9996$ (value shown in table was calculated using the portion of the dose–response prior to the slope leveling out at 0.5 mg/kg. When the whole curve was utilized [$y = 0.087x^2 + 0.1996x + 3.1995$, $R^2 = 0.77$], the ED_{50} in μg/kg/day was 0.62). For females: $y = -0.0806x^2 - 0.096x + 3.4457$, $R^2 = 0.9141$.

[e] Calculated serum concentrations corresponding to the calculated ED_{50}. These were determined by using the best fitting equation for TAD levels on x-axis and serum PFOS concentration means (from Table 2) on y-axis (each line had 4 points that spanned both sides of the calculated ED_{50} mg/kg TAD). The equation was solved for y using the calculated ED_{50} (0.021 for males or 0.59 for females) for x. The best fitting equations were $y = -9483.8x^2 + 2183.9x + 10.151$, $R^2 = 0.9991$ for males and $y = 1303.2x + 11.698$, $R^2 = 0.998$ for females [(ng/g ≈ ng/ml) = ppb].
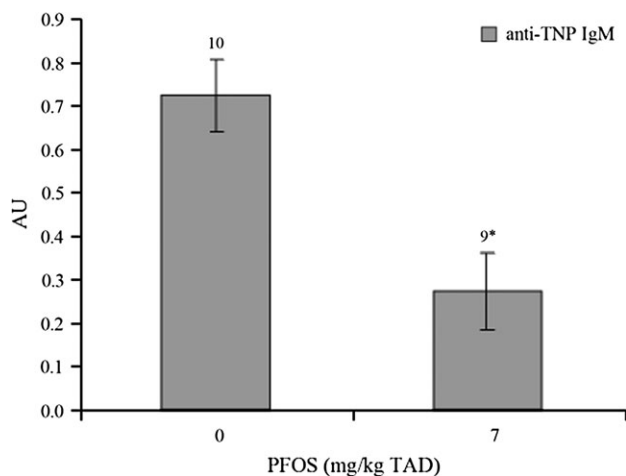
[f] Calculated from $y = -0.0806x^2 - 0.3464x + 3.1022$, $R^2 = 0.9143$ where actual TAD is on the x-axis and the PFC response is on the y-axis.

In the current study, NK-cell function was not altered by PFOS in female mice. This is consistent with our previous study with sulfluramid (0–57 μmol/kg/day) exposure (Peden-Adams *et al.*, 2007) and with reports by Nelson *et al.* (1992) following acute exposure to 20 and 50 mg PFDA/kg (39 and 97 μmol/kg, respectively). However, PFOS did cause increased NK-cell activity in male B6C3F1 mice. The reason for this

FIG. 4. Anti-TNP IgM serum levels as measured by ELISA in adult female B6C3F1 mice following oral exposure to PFOS for 21 days (0.334 mg/kg/day). Seven days prior to euthanization mice were injected with the TI antigen TNP–LPS. Data are presented as mean ± standard error. Numbers above error bars indicate sample size. One animal in the PFOS treatment group died. Cause of death could not be determined. *Significantly different from control ($p \leq 0.05$). This experiment was only conducted once. TAD = over the course of 21 days.

gender related difference is not clear but has been observed following gestational exposure to PFOS as well (Keil *et al.*, 2008). Due to known differences in gender elimination of perfluorinated compounds in some species, it could be speculated that differences in the half-life between males and females (males generally, but not always having a longer half-life) may have affected NK-cell function correspondingly (Lau *et al.*, 2007; OECD, 2002). However, analytical determination of serum levels reflected no difference in serum concentrations between genders as has been previously noted in mice (Lau *et al.*, 2007); therefore, differential accumulation of PFOS is not likely the basis for the gender differences observed in altered immune responses.

Although a dietary concentration of 0.02% PFOA for 10 days (reported as 20 mg/kg/day = 48 μmol/kg/day = 480 μmol/kg TAD) was shown to decrease T- and B-cell mitogen-induced lymphoproliferation (Yang *et al.*, 2002a), this was not observed in this study with PFOS in either gender. These results are similar to those reported with sulfluramid, a perfluorinated pesticide that is metabolized to PFOS (Peden-Adams *et al.*, 2007). This discrepancy could potentially be related to differences in effects between PFAA classes (i.e., carboxylates and sulfonates), but is more likely related to the dose administered (480 μmol PFOA/kg TAD vs. 9.3 μmol PFOS/kg TAD).

Suppression of the SRBC-specific IgM response (PFC response) was the most sensitive indicator of immune dysfunction following exposure to PFOS. In the males, the mean serum PFOS concentration at the LOEL (91.5 ng/g = 91.5 ppb) is approximately 14-fold lower than the reported

TABLE 4
Splenic CD4/CD8 Subpopulations in Adult B6C3F1 Mice Treated with PFOS Orally for 28 Days

PFOS (mg/kg TAD)	Gender	CD4−/CD8+ (cells × 10^7)	CD4+/CD8+ (cells × 10^6)	CD4−/CD8− (cells × 10^7)	CD4+/CD8− (cells × 10^7)
0	Male	0.10 ± 0.01	2.4 ± 11.0	2.1 ± 0.53	5.2 ± 1.2
0.005	Male	0.13 ± 0.01	1.0 ± 1.2	3.2 ± 0.31	6.3 ± 0.78
0.05	Male	0.32 ± 0.20	0.9 ± 2.1	4.2 ± 0.68	5.4 ± 0.77
0.1	Male	1.1 ± 0.10*	0.03 ± 0.00*	5.9 ± 0.38*	2.4 ± 0.17*
0.5	Male	1.1 ± 0.20*	0.04 ± 0.00*	6.8 ± 1.1*	2.3 ± 0.43*
1	Male	1.1 ± 0.30*	0.2 ± 1.7*	6.7 ± 1.5*	3.7 ± 0.74*
5	Male	1.2 ± 0.10*	0.04 ± 0.00*	7.3 ± 0.68*	2.7 ± 0.30*
0	Female	1.4 ± 0.05	4.8 ± 0.54[a]	6.4 ± 0.46	3.0 ± 0.19
0.005	Female	1.4 ± 0.15	7.6 ± 0.94[a]	8.4 ± 0.87	3.4 ± 0.39
0.05	Female	1.1 ± 0.10	4.8 ± 0.95[a]	5.5 ± 0.47	2.3 ± 0.18
0.1	Female	0.87 ± 0.08*	4.4 ± 0.84[a]	7.5 ± 2.6	2.1 ± 0.22*
0.5	Female	0.96 ± 0.07*	5.2 ± 1.1[a]	4.9 ± 0.27	2.0 ± 0.14*
1	Female	1.1 ± 0.06	4.2 ± 0.41[a]	6.2 ± 0.58	2.3 ± 0.18
5	Female	0.75 ± 0.20*	2.4 ± 0.72[a]	4.4 ± 1.2	2.2 ± 0.23

Note. Data are reported as the mean absolute number of cells ± SEM. Absolute values were determined by multiplying the percent gated cells by the total number nucleated cells counted in the spleen.

*Significantly different from control ($p \leq 0.05$). This experiment was only conducted once. Sample size is five per treatment per gender.

[a]Cells × 10^5. TAD = over the course of 28 days.

mean occupational exposure level of 1300 ppb (Olsen *et al.*, 2003a) and falls within the high end of ranges reported in several studies of serum from nonoccupationally exposed persons (i.e., reported maximum values of 145 ng/ml [145 ppb; Jin *et al.*, 2007]; 164 ng/ml [164 ppb; Kannan *et al.*, 2004]; and 1656 ppb [Olsen *et al.*, 2003b]). This suppression is similar to studies with PFOA, sulfluramid and PFDA challenged with T-dependent antigens (SRBCs or horse red blood cells or KLH) (Nelson *et al.*, 1992; Peden-Adams *et al.*, 2007; Yang *et al.*, 2002a). Taken together, these data suggest that humoral immunological function, T-dependent antibody responses specifically, may be a target for perfluorinated compounds. However, further studies using the TI antigen LPS conjugated with TNP showed that TI antibody production was also suppressed by PFOS exposure. This suggests that, for PFOS at least, the functional deficit may lie with the B-cell or with antigen presenting cells and not with the T-helper cell.

The mechanism disrupting antibody secretion is not known. Yang *et al.* (2001) demonstrated a 75% decrease in splenic CD19+ B-cells following exposure to PFOA; however, our studies do not demonstrate a selective decrease in the B-cell population. For example, numbers of B220+ B-cells, were not decreased following sulfluramid exposure suggesting the deficit in SRBC-specific IgM production was not due to a decreased total number of splenic B-cells (Peden-Adams *et al.*, 2007). Moreover, in a separate study, numbers of splenic CD19+, CD21+, or B220+ cells were not decreased in female B6C3F1 mice at the PFOS concentrations used in the current study (Driscoll, 2007).

The possible modes of action and molecular targets by which IgM production to both T-dependent and TI antigens is suppressed are numerous (i.e., CD27/CD70 signaling; PGE1; downregulation of Pax5, nuclear factor kappa B [NF-κβ], I kappa B, I kappa B alpha, or activator protein-1 [AP-1]; decreased B-cell interleukin-6 [IL-6] production) and discussion of most of these would be little more than highly speculative at this point. However, the role of peroxisome proliferator–activated receptors-alpha (PPAR-α) in mediating immunosuppression following PFOS exposure is worthy of comment. PFOS is a known agonist for PPAR-α, which is expressed in various tissues (Shipley *et al.*, 2004). PPAR-α is the primary PPAR isoform expressed in lymphocytes (Jones *et al.*, 2002) and is more highly expressed in B-cells than in T-cells (Cunard *et al.*, 2002). Several recent studies have suggested that PPAR-α-agonists including WY14,643 and PFOA have profound effects on murine immune responses including decreased IL-6, tumor necrosis factor alpha (TNF-α), and interferon gamma production, decreased spleen weight and splenocyte number and suppression of antibody responses following antigen challenge (Cunard *et al.*, 2002; Yang *et al.*, 2000, 2002a). Additionally, PPAR-α inhibits expression of inflammatory genes such as cyclooxygenase-2, IL-6, and endothelin-1 through negative regulation of NF-κβ and AP-1 signaling pathways. PPAR-α null mice have been used to determine that WY14,643-induced increases in plasma TNF-α levels and PFOA-induced suppression of Con A–induced lymphocyte proliferation, and thymic T-cell subpopulation depletion are PPAR-α dependent (Hill *et al.*, 1999; Yang *et al.*, 2002b). A plausible mode of action related to PPAR-α agonism is through antagonism of NF-κβ and c-Jun leading to inhibition of IL-6 production and consequently, suppression of IgM production (Baccam *et al.*, 2003; Delerive *et al.*, 1999). At this

time, it is not clear if PFOS agonism of PPAR-α through this pathway would account for the observed deficits in humoral immunity; however, studies are underway in our lab to explore this. Moreover, as PPAR-α is differentially expressed in species and effects mediated by PPAR-α vary by species, knowledge of the role of PPAR-α in the observed PFOS-induced IgM suppression is important to understanding human and wildlife health risks due to this compound.

In this study, the LOEL for females was conservatively established using the PFC data and not based on alterations observed in the CD4/CD8 lymphocytic subpopulation data. As the flow cytometric data demonstrates, splenic CD4+/CD8− and CD4−/CD8+ subpopulations were significantly altered in female mice beginning at the PFOS exposure level of 0.1 mg/kg (TAD). However, these changes were not clearly dose-responsive as observed with the PFC data and in follow up studies no change was seen in 3 experiments for female CD4+ cells at the 0.1 mg/kg TAD level (Driscol, 2007).

Differences in sensitivity between genders is most likely not related to gender differences in bioaccumulation or elimination of PFOS in these mice, as serum levels of PFOS in males and females at matching doses were not significantly different. This lack of gender difference in serum levels in mice has been previously noted (Lau *et al.*, 2007). These gender related immune effects could then be secondary to modulation of sex-steroids. Although not statistically significant, in this study PFOS decreased uterine wet weight at the higher doses. This is similar to results with sulfluramid (Peden-Adams *et al.*, 2007). Furthermore, uterine weight was decreased in rats following PFOS exposure (10 mg/kg/day) (Wetzel, 1983). Thus, it may be plausible that PFOS possesses antiestrogenic effects. Whether these observations are due to antagonism of the estrogen receptor (ER) or decreases in circulating estradiol is unclear. PFOS has been shown to decrease aromatase activity in fathead minnows causing increased plasma 11-ketotestosterone and testosterone (Ankley *et al.*, 2005). In studies with Cynomolgus monkeys exposed to PFOS, females did not exhibit a decrease in circulating plasma estradiol levels, yet males did without a corresponding increase in testosterone levels (Seacat *et al.*, 2002). The link to immunological function is demonstrated in that estradiol increases IgM production (Kanda and Tamaki, 1999), whereas testosterone inhibits IgM production (Kanda *et al.*, 1996). In respect to NK-cell activity, estradiol has been shown to increase NK-cell activity (Hao *et al.*, 2007) and *in vitro* studies suggest that this enhancement by estradiol is blocked by ER agonists; whereas testosterone has no effect on NK-cell function. Modulation of aromatase activity and the culminating changes in circulating testosterone and estradiol levels could, therefore, affect IgM production and potentially account for the differential responses between genders. This, however, would not explain the increase seen in NK-cell function in males. Studies are needed to address the possible link between endocrine modulation and alteration of immune function following PFOS exposure.

In conclusion, this is the first study to assess the immunotoxicological effects of PFOS following exposure in adult organisms. It is clear that PFOS targets antibody production at exposure levels less than those observed with overt toxicity (as indicated by decreases in body or organ weight) and that this humoral immune effect was the most sensitive immune endpoint. To our knowledge, this is currently the lowest reported PFOS concentration to cause differences as compared with control (0.05 mg/kg TAD = 1.66 µg/kg/day; serum mean = 91.5 ng PFOS/g) and these levels are environmentally relevant for current human and wildlife exposure. Males appeared more sensitive to the effects than females for three of the immune tests assessed: NK-cell activity, the PFC assay, and alteration in splenic T-cell subpopulations. The cause of this gender difference is unclear at present. More importantly, TI antibody production was also suppressed suggesting that the cellular or molecular target for the suppression of humoral immunity may be attributed to alterations in B-cells or antigen presenting cells, rather than T-helper cells. Further investigation is underway to clarify the mode and mechanism of action of PFOS-induced IgM suppression.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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