Gestational Exposure to Perfluorooctane Sulfonate Suppresses Immune Function in B6C3F1 Mice

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Perfluorinated alkyl acids (PFAAs) are used in a multitude of applications and are categorized as high-production volume chemicals produced in quantities exceeding 10,000 lbs/year. As a result, widespread exposure has been documented in adults, children, and infants. It is generally accepted that children are more sensitive to the effects of xenobiotic exposures during fetal and postnatal periods of development; therefore, considerable efforts are required to investigate the potential impact of a model PFAA, perfluorooctane sulfonate (PFOS) on children's immunological health. Using the pairing of female C57BL/6N mice with male C3H/HeJ, developmental immunotoxicity was evaluated in B6C3F1 pups following oral maternal exposure to PFOS on gestations days 1–17. Exposure levels included 0.1, 1, and 5 mg/kg/day PFOS. Natural killer (NK) cell activity, SRBC IgM plaque assay, CD4/8 lymphocytic subpopulations, nitrite production in peritoneal macrophages, and body/organ weights were evaluated at 4 and 8 weeks of age in F1 pups. No significant dose-responsive changes in maternal or pup body weights, flow cytometry, or macrophage function were observed, yet hepatomegaly was indicated in F1 male pups at 4 weeks of age. Functional deficits were not evident until 8 weeks of age when NK cell function and IgM production were significantly decreased. When compared with females, male pups were more sensitive to the effects of PFOS thereby establishing a no observed adverse effect level and low observed adverse effect level of 0.1 and 1.0 mg/kg/day (males only) following maternal PFOS exposure level, respectively. This study establishes that the developing immune system is sensitive to the effects of PFOS and results in functional deficits in innate and humoral immunity detectable at adulthood.

Key Words: developmental immunotoxicity; PFOS; perfluorinated compounds.

Perfluorinated alkyl acids (PFAAs) have been manufactured for over 50 years and are considered high-production volume (HPV) chemicals produced in quantities exceeding 10,000 lbs/year. Their applications are diverse including uses throughout the electronic industry as well as stain and water repellents, floor waxes, paper food wrappings, fire-fighting foams, denture cleaners, shampoos, carpet spot cleaners, pharmaceuticals, and as pesticides (Moody and Field, 2000). The two major classes of PFAAs include the perfluorinated carboxylates and the perfluorinated sulfonates. These compounds are simple carbon chains bound to fluorine with various substitution groups (R-groups) on the terminal end that impart their active properties. Because the carbon-fluorine bond is the strongest known covalent bond, some of these perfluorinated compounds are extremely resistant to environmental degradation processes and metabolism, thereby leading to persistence in the environment and an extended half-life in humans and other animals (Giesy and Kannan, 2001, 2002; Moody and Field, 2000).

Although the environmental sources, transportation, and fate of perfluorinated hydrocarbons have yet to be fully elucidated, an accumulating body of evidence demonstrates that exposure to wildlife and humans is widespread (Apelberg *et al.*, 2007; Butenhoff *et al.*, 2006; Calafat *et al.*, 2007; Giesy and Kannan, 2001, 2002; Inoue *et al.*, 2004; Kannan, *et al.*, 2001a, 2001b, 2002a, 2002b, 2002c; Keller *et al.*, 2005; Olsen *et al.*, 2001a, b, 2004; Table 1). Perfluorooctane sulfonate (PFOS) poses significant concern to the U.S. Environmental Protection Agency (USEPA) because it "combine(s) persistence, bioaccumulation, and toxicity properties to an extraordinary degree" (EPA, 2003). The USEPA has also identified "potential human health concerns from exposure to perfluorooctanoic acid (PFOA)" (EPA, 2003) and has nominated "PFOS, PFOA, and related fluorochemicals" to the Centers for Disease Control and Prevention for inclusion in the National Health and Nutrition Examination Survey (Calafat *et al.*, 2007; EPA, 2003). Cause for concern is driven not only by the environmental persistence of these compounds and their documented toxicity (Gratsy *et al.*, 2005; Hu *et al.*, 2002; Lau *et al.*, 2003; Luebker *et al.*, 2005a, b; Oakes *et al.*, 2005; Peden-Adams *et al.*, 2007; Thibodeaux *et al.*, 2003; Wolf *et al.*, 2006), but also by the uncertainty and data gaps regarding exposure routes, mechanisms of toxicity, and lack of understanding regarding sublethal developmental exposure effects.

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TABLE 1
Serum Levels (ppb) of Selected Perfluorinated Chemicals
Detected in Children's Serum Samples for a Clinical Trial and
Adult Serum Samples Obtained from the American Red Cross

		Mean serum concentration (ppb)	Range	Geometric mean	Cumulative 95th percentile
Adult	PFOS	43.7	LLOQ-1656	35.1	90.5
	PFOA	5.6	LLOQ-52.3	4.6	12.9
	PFHS	2.9	LLOQ-66.3	2.0	9.6
Children	PFOS	43.5	6.7-515	37.5	90.2
	PFOA	5.6	LLOQ-56.1	4.9	10.2
	PFHS	15.0	LLOQ-712	4.5	71.3

Note. LLOQ = lower limit of quantification. Children's ages ranged from 2 to 12 years old. Adult ages ranged from 20 to 69 years old. (Estimated dose is based on geometric mean. Derived from Olsen *et al.*, 2001a, b).

Recent reports indicate that perfluorinated compounds are widespread in children (ages 2–12) and levels are similar to those of adults (Table 1; Apelberg *et al.*, 2007; Butenhoff *et al.*, 2006; Olsen *et al.*, 2004). The primary PFAA detected in children's serum is PFOS, which has a human serum half-life of ~5 years (Olsen *et al.*, 2005). PFOS is 95% is absorbed via ingestion, not readily metabolized, and exhibits a unique toxicokinetic profile as it is not sequestered in adipose tissue but circulates primarily in the liver and blood (Johnson *et al.*, 1979a, 1984; Seacat *et al.*, 2002, 2003).

In humans, relationships between maternal and fetal exposure to PFOS have been demonstrated. Inoue *et al.* (2004) identified that maternal blood levels (4.9–17.6 ng/ml) highly correlated with blood levels measured in the fetus (1.6–5.3 ng/ml). A recent study evaluating infants born in Baltimore, MD, confirms the presence of PFOS (mean 4.9 ng/ml) and PFOA (1.6 ng/ml) in 99% and 100% of tested cord bloods, respectively (Apelberg *et al.*, 2007). Thus, PFOS is clearly distributed to the human fetus during gestation via placental transfer.

Maternal–fetal transfer of PFOS has also been demonstrated both *in utero* and via lactation in rodent models (Lau *et al.*, 2003; Luebker *et al.*, 2005a, b; Thibodeaux *et al.*, 2003; Wolf *et al.*, 2006). Previous studies in mice and rats (Grasty *et al.*, 2005; Lau *et al.*, 2003; Luebker *et al.*, 2005a, b; Thibodeaux *et al.*, 2003; Wolf *et al.*, 2006) identify that PFOS is a toxicologically significant exposure to the fetus. Adverse effects following gestational exposure include increased pup mortality, decreased birth weight; decreased serum levels of total T4 and T3, alterations in lung maturations, skeletal abnormalities, and altered motor skill development (Gratsy *et al.*, 2005; Lau *et al.*, 2003; Luebker *et al.*, 2005a; Thibodeaux *et al.*, 2003; Wolf *et al.*, 2006). Immunotoxicity following *in utero* exposure to PFOS, however, has not been previously examined. Therefore, this study was designed to investigate potential persistent immunological effects in pups following maternal exposure to PFOS during gestation.

METHODS

Animal dosing and exposures. The experimental design employed in this study was based on the developmental study conducted by Lau *et al.* (2003). PFOS (potassium salt; 91% pure) was obtained from Fluka Chemical (Sigma-Aldrich, Switzerland). It was prepared weekly in distilled water with 0.5% Tween 20 (Sigma-Aldrich, PA). Exposure levels applied in this study (0.1–5 mg/kg/day) were established based on pup LD_{50} levels (10 mg/kg/day) during gestation day (GD) 1–17 (Lau *et al.*, 2003).

Within our animal facilities, female C57BL/6N mice and male C3H/HeJ were paired to achieve a B6C3F1 inbred strain. Mice were obtained from Taconic Farms (Germantown, NY) or Charles River Laboratories (Wilmington, MA). Vaginal plugs were checked on a daily basis. All plug-positive females identified within a 5-day period (Mon–Fri) were moved to group housing with other plug-positive females. Plug-positive females that were ultimately not pregnant were eliminated from the study. PFOS was administered daily to plug-positive females (GD 1) by gavage in volumes of 100 μl. There were 10–12 plug-positive females assigned to each treatment group. Every 3–4 days, dams were weighed and the PFOS dosage was adjusted based on body weight. Dams were dosed until day 17 of gestation. Near delivery (i.e., GD 19), pregnant dams were single housed. Delivery of pups that occurred over a 5-day period (Mon–Fri) was selected for the study. If delivery exceeded this period, these litters were not included in the study as the age of the pups would exceed a 5-day window. Only litters with six to nine pups were maintained and were kept with their birth mother following the first 3 weeks of birth. For the immunotoxicity studies, groups were compromised of one female or male from each litter. This mating process was repeated twice to obtain data for two replicate studies.

Animal care. All mice were acclimated to the conditions of the treatment room (12-h light/dark cycle, 22 ± 2°C, 60–65% relative humidity) 1 week prior to pairing and dosing. Mice were maintained in an Association and Accreditation of Laboratory Animal Carc accredited animal facility and experiments were conducted under an approved National Institute for Occupational Health Animal Care and Use Committee protocol. Mice were housed in ventilated polycarbonate shoebox cages with hardwood bedding and were provided tap water and feed *ad libidum* with LM-485 (7912.15) purchased from Harlan Teklad (Madison, WI).

Body and organ mass and organ cellularity. Body mass was monitored weekly during the study. Upon dissection, spleen, thymus, liver, and uterus were collected and weighed following euthanization in a CO_2-saturated environment. All balances were calibrated prior to use with standardized weights. Organ mass, was normalized for body weight and reported as a somatic index {(organ weight/body wt) × 100}. Immunological organs (spleen and thymus) were aseptically processed into single-cell suspensions by gentle grinding with the use of sterile, frosted microscope slides. A 10-μl aliquot of spleen or thymus single-cell suspension was placed in 10 ml of Isoton with two drops of Zap-O-globin (Beckman Coulter, France). The total number of nucleated cells was counted using an electronic cell counter (Coulter Electronics, Inc., Hialeah, FL). Viability of splenic and thymic cells was verified to be greater than 95% based on flow cytometric gating to identify live cells.

Natural killer cell activity. An *in vitro* cytotoxicity assay using ^{51}Cr-labeled Yac-1 cells (ATCC, TIB 160, Manassas, VA) was used as described previously (Duke *et al.*, 1985; Holsapple *et al.*, 1988). Splenocytes were adjusted to 2×10^7 cells per ml in complete media (RPMI, 10% fetal calf serum, 50 IU penicillin and 50 μg streptomycin). Splenocyte and Yac-1 cells were prepared in ratios of 200:1, 100:1, 50:1, 25:1, 12.5:1, 6.25:1, and replicate wells plated for each concentration. After 4-h incubation at 37°C and 5% CO_2, 100 μl of supernatant was transferred to tubes and counted on a gamma counter

(Packard Cobra II Auto-Gamma, Packard Instrument Co., Downer's Grove, IL). Maximum release was determined by lysing ^{51}Cr-labeled Yac-1 cells with 0.1% Triton X in complete media. Spontaneous release was determined by incubating Yac-1 tumor cells in complete media. The results were expressed in lytic units (Bryant *et al.*, 1992) for each of the effector to target ratios. Positive control animals were administered 0.2 ml of a 1:10 dilution of anti-asialo-GM1 administered by intraperitoneal injection one day prior to assay day.

Antibody plaque-forming cell assay. The primary IgM response to sheep red blood cells (SRBC; Rockland, Gilbertsville, PA) was determined using a modified hemolytic plaque assay (Jerne and Nordin, 1963). Four days before sacrifice, mice were sensitized by intravenous injection of 7.5×10^7 SRBC in 0.2 ml of saline. On the day of sacrifice, mice were asphyxiated with carbon dioxide and spleens were aseptically removed. Single-cell suspensions in Hank's balanced salt solution (HBSS, Gibco, BRL, Grand Island, NY) were prepared from individual animals by pressing spleens between the frosted ends of microscopic slides. An aliquot of single cells (100 μl) at 1:30 or 1:120 dilutions of total spleen cells (in 2 ml of HBSS), was added to a test tube containing a 0.5 ml of warm agar/dextran mixture (0.5% Bacto-Agar, DIFCO Laboratories, Detroit, MI; and 0.05% diethylaminoethyl (DEAE) dextran; Sigma Chemical Co.), 25 μl of SRBC solution and 20 μl of 1:4 dilution guinea pig complement (Gibco BRL). The mixtures were poured into Petri dishes, covered with microscope coverslips, and incubated at 37°C for 3 h. The plaques (representing antibody forming cells) were viewed and quantified using a Bellco magnification viewer (Vineland, NJ). The results were expressed as specific activity (IgM plaque-forming units/10^6 splenocytes). In mice challenged with SRBC, only the plaque-forming cell (PFC) endpoint was evaluated. Mice used as positive controls for this response were administered 0.2 ml of 25 mg/kg cyclophosphamide (CYP) by intraperitoneal injection for 4 days prior to euthanization.

Splenic and thymic CD4/CD8 subpopulations. A 10-μl aliquot of spleen or thymus single-cell suspension was placed in 10 ml of Isoton with two drops of Zap-O-globin (Beckman Coulter, France), and the total number of nucleated cells was counted using an electronic cell counter (Coulter Electronics, Inc., Hialeah, FL). To enumerate B cells, T cells (CD3$^+$), and CD4$^+$ and CD8$^+$ T-cell subsets, spleen or thymus cells (cell density of 1.5×10^6/ml) were collected by centrifugation and suspended in phosphate-buffered saline (PBS) containing 1% bovine serum albumin and 0.1% sodium azide at a pH 7.4. The cells were incubated with Fc Block (clone 2.4G2 Pharmingen, San Diego, CA) for 5 min to prevent nonspecific binding and then labeled with an appropriate monoclonal antibody (mAb) conjugated to a fluorescent probe for visualization using flow cytometry. The antibodies were obtained from Pharmingen. B cells were enumerated using anti-mouse CD45R/B220 antibodies (clone RA3-6B2) conjugated to fluorescein isothiocyanate (FITC). Anti-mouse CD3 mAb (clone 145-2C11) conjugated to FITC was used to enumerate T cells. For the T-cell subsets, the cells were identified by using anti-mouse CD4$^+$ mAb (clone H129.19) conjugated to FITC and anti-mouse CD8$^+$ (clone 53-6.7) conjugated to phycoerythrin. An isotype specific negative control was used for each antibody. Cell suspensions were incubated with labeled antibodies on ice in the dark for at least 30 min, washed, and then resuspended in 0.1 ml of a 1 μg/ml solution of propidium iodide (PI). After 5-min incubation with PI, cells were washed, resuspended in PBS, and enumerated using a FacsVantage Flow Cytometer (Becton Dickinson, San Jose, CA). PI-stained cells (dead cells) were eliminated from the analysis. The forward scatter threshold was set to eliminate red blood cells. The percentage of gated live cells with the corresponding cell surface marker was converted to absolute numbers of cells per organ using the measured total numbers of splenic or thymic nucleated cells.

Nitrite production by peritoneal macrophages. Peritoneal macrophages were aseptically isolated by peritoneal lavage and incubated for 24 h with 10 μg/ml of lipopolysaccharide and 500 units/ml of interferon-gamma. These methods are described previously (Keil *et al.*, 1995). Nitric oxide was indirectly measured by spectrophotometrically quantitating nitrite, a stable end product of nitric oxide generation. A standard nitrite curve was established for each experiment and data were expressed in micromolar of nitrite production. Background control cells contained macrophages only without any stimulant

and background was less than 0.5μM. This endpoint was only assessed in control and high dose group adult pups only (8 weeks of age).

Statistics. Statistical analysis was performed using JMP 5.0 (SAS, Cary, NC). Data were tested for normality (Shapiro–Wilks) and homogeneity (Bartlett's) and if needed appropriate transformations were made. Because differences between genders could be expected and could skew mean values, data were tested for gender by treatment and gender within treatment interaction. If no gender dependent effect was observed, then male and female data were combined. If significant gender by treatment (gender*treatment) or gender within treatment (gender [treatment]) differences were observed ($p \leq 0.05$), data were separated by gender for analysis. Statistical significance was determined using a one-way analysis of variance (ANOVA) ($p \leq 0.05$). When significant differences were detected by ANOVA, Dunnett's Comparison was used to compare treatment groups and controls. Unless otherwise stated, all experiments were replicated using male and female pups obtained from two separate mating studies with virgin female C57BL/6N mice.

RESULTS

Body and Organ Mass and Immune Organ Cellularity

Body mass of male and female pups exposed to PFOS *in utero* was not significantly different from controls at either 4 or 8 weeks of age (Table 2). Furthermore, pregnant dams did not experience any significant weight loss due to PFOS exposure (data not shown). In female pups at 4 weeks of age, liver mass was significantly decreased at the 0.1 mg/kg/day treatment and kidney mass was increased at the 5 mg/kg/day treatment, whereas spleen and thymus mass were not altered (Table 2). In 4-week male pups spleen, thymus, and kidney mass were not altered, but liver mass was significantly increased compared with control at the 5 mg/kg/day treatment level (Table 2). In 8-week pups no alterations compared with control were noted in spleen, thymus, kidney, or liver mass regardless of gender (Table 2). Additionally, splenic and thymic cellularities were not altered compared with control at either 4 or 8 weeks of age in either gender (Table 2).

Natural Killer Cell Function and PFC Assessments

Natural killer (NK) cell activity was assessed at both 4 and 8 weeks of age. There was no statistical interaction related to gender in the 4-week-old pups; therefore, genders were combined for analysis. NK cell activity was not altered compared with control in the 4-week-old pups (Fig. 1A). At 8 weeks of age, however, differences in gender by treatment were noted and data were separated by gender for analysis. NK cell activity was suppressed in 8-week-old male offspring at the 1 and 5 mg/kg/day treatments (42.5% and 32.1% decrease, respectively) and was suppressed in female offspring at the 5 mg/kg/day treatment (35.1% decrease; Fig. 1B).

The PFC response for SRBC-specific IgM production by B cells was only assessed in the F1 adults (8 weeks of age). Differences in response by gender were observed, therefore, these data are presented with gender separated. In males, this

TABLE 2
Body Weight and Organ Parameters in B6C3F1 Pups after Gestational Exposure (GD 1–17) to PFOS

	Gender	PFOS gestational exposure level (mg/kg/day)			
		0.0	0.1	1.0	5.0
4 weeks of age					
Body weight (g)	Female	13.9 ± 0.44 (6)	14.2 ± 0.54 (6)	13.2 ± 0.25 (6)	13.1 ± 0.39 (6)
	Male	15.4 ± 0.52 (6)	16.2 ± 0.81 (6)	13.2 ± 0.61 (6)	15.2 ± 0.72 (6)
Liver[a]	Female	6.76 ± 0.07 (6)	6.16 ± 0.15 (6)*	6.61 ± 0.09 (6)	6.60 ± 0.17 (6)
	Male	6.84 ± 0.16 (6)	6.82 ± 0.15 (6)	6.91 ± 0.27 (6)	7.83 ± 0.32 (6)*
Kidney[a]	Female	1.75 ± 0.05 (6)	1.67 ± 0.03 (6)	1.70 ± 0.02 (6)	1.54 ± 0.04 (6)*
	Male	1.65 ± 0.03 (6)	1.71 ± 0.03 (6)	1.76 ± 0.05 (6)	1.68 ± 0.03 (6)
Spleen[a]	Female	0.47 ± 0.01 (6)	0.42 ± 0.02 (6)	0.42 ± 0.03 (6)	0.40 ± 0.02 (6)
	Male	0.50 ± 0.01 (6)	0.47 ± 0.03 (6)	0.44 ± 0.02 (6)	0.45 ± 0.02 (6)
Spleen cellularity ($\times 10^8$)	Female	0.85 ± 0.06 (6)	0.80 ± 0.05 (6)	0.79 ± 0.05 (6)	0.67 ± 0.06 (6)
	Male	1.28 ± 0.03 (6)	1.24 ± 0.09 (6)	1.18 ± 0.10 (6)	1.11 ± 0.05 (6)
Thymus[a]	Female	0.65 ± 0.02 (6)	0.62 ± 0.02 (6)	0.66 ± 0.03 (6)	0.62 ± 0.02 (6)
	Male	0.49 ± 0.01 (6)	0.48 ± 0.01 (6)	0.45 ± 0.02 (6)	0.49 ± 0.02 (6)
Thymus cellularity ($\times 10^8$)	Female	2.45 ± 0.06 (6)	2.25 ± 0.08 (6)	2.53 ± 0.11 (6)	2.35 ± 0.18 (6)
	Male	2.06 ± 0.05 (6)	2.08 ± 0.11 (6)	1.93 ± 0.06 (6)	2.07 ± 0.06 (6)
8 weeks of age					
Body weight (g)	Female	20.3 ± 0.88 (6)	21.0 ± 0.59 (6)	21.1 ± 0.49 (6)	19.9 ± 0.62 (6)
	Male	22.0 ± 0.38 (5)	25.1 ± 0.92 (6)	25.1 ± 0.27 (6)	22.4 ± 0.76 (5)
Liver[a]	Female	5.50 ± 0.19 (7)	5.69 ± 0.05 (7)	5.98 ± 0.06 (7)	5.99 ± 0.15 (6)
	Male	6.40 ± 0.13 (5)	6.24 ± 0.19 (6)	5.95 ± 0.09 (6)	6.12 ± 0.16 (5)
Kidney[a]	Female	1.60 ± 0.10 (7)	1.60 ± 0.02 (7)	1.62 ± 0.02 (7)	1.63 ± 0.04 (6)
	Male	1.82 ± 0.02 (5)	1.87 ± 0.03 (6)	1.96 ± 0.03 (6)	1.83 ± 0.03 (5)
Spleen[a]	Female	0.48 ± 0.04 (7)	0.45 ± 0.01 (7)	0.45 ± 0.02 (7)	0.45 ± 0.02 (6)
	Male	0.38 ± 0.01 (5)	0.43 ± 0.05 (5)	0.43 ± 0.02 (6)	0.33 ± 0.02 (5)
Spleen cellularity ($\times 10^8$)	Female	1.31 ± 0.13 (7)	1.44 ± 0.04 (7)	1.41 ± 0.06 (7)	1.47 ± 0.15 (6)
	Male	1.50 ± 0.14 (5)	1.78 ± 0.09 (5)	1.71 ± 0.10 (6)	1.36 ± 0.15 (5)
Thymus[a]	Female	0.35 ± 0.01 (6)	0.33 ± 0.02 (3)	0.31 ± 0.04 (6)	0.35 ± 0.02 (6)
	Male	0.26 ± 0.00 (6)	0.26 (2)	0.26 ± 0.01 (6)	0.27 ± 0.01 (6)
Thymus cellularity ($\times 10^8$)	Female	1.42 ± 0.05 (6)	1.45 ± 0.12 (3)	1.41 ± 0.05 (6)	1.40 ± 0.12 (6)
	Male	1.50 ± 0.07 (6)	1.68 (2)	1.57 ± 0.06 (6)	1.35 ± 0.04 (6)

Note. Data are reported as mean ± SEM. *Significantly different from respective control ($p \leq 0.05$). (*n*) = number of mice per group.
[a]Calculated as (organ mass/body mass) $\times$ 100.

response was significantly suppressed 53% in the 5 mg/kg/day exposure group, but it was not altered in the females (Fig. 2).

Lymphocyte Immunophenotypes

Splenic or thymic lymphocyte subpopulations (B220+, CD3+, CD4+, CD8+, CD4−CD8− [DN], CD4+CD8+ [DP]) were assessed in pups at 4 and 8 weeks of age. Due to statistical interactions, genders were analyzed separately. Absolute numbers of splenic B220+ cells were significantly decreased by 21% in 4-week-old female pups exposed to 5.0 mg/kg/day *in utero*, whereas male pups remain unaffected (Table 3). By the adult age of 8 weeks, however, no significant changes in B220 labeled splenocytes were detected in either female or males for any of the treatments (data not shown). Absolute numbers of splenic or thymic CD8+, DP, and DN cells were not significantly altered at either age or gender (Tables 3 and 4). In 4-week-old pups, CD3+ or CD4+ cells were not significantly affected in either males or females (Table 3).

In 8-week-old pups, the only significant differences detected were in male mice with a 25% decrease in CD3+ and 28% decrease in CD4+ thymocytes at the highest treatment level (Table 4). The thymocyte CD4:8 ratio was reduced from 4.2 in controls to 3.5 in the 5.0 mg/kg/day maternal dose group.

Nitrite Production by Peritoneal Macrophages

Peritoneal macrophage nitric oxide was assessed in F1 mice at 8 weeks of age only. It was not statistically altered in male or female offspring exposed to 5 mg/kg/day *in utero* (data not shown). Furthermore, no difference in peritoneal cellularity was noted (data not shown).
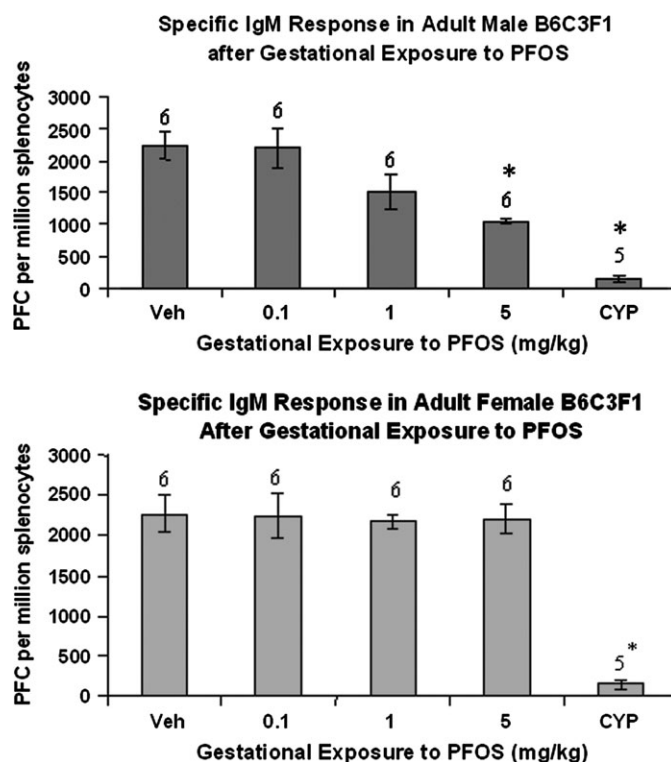
DISCUSSION

Few studies have assessed the immunotoxicity of PFOS and none to date have assessed potential immune effects of
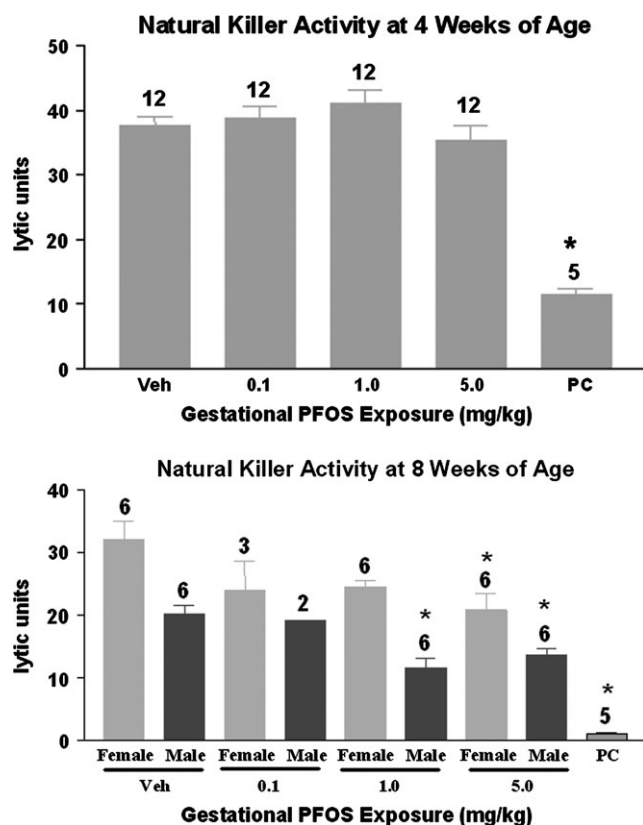
FIG. 1. NK activity in (A) 4- or (B) 8-week-old B6C3F1 pups after gestational exposure (GD 1–17) to PFOS. Data are presented as mean ± SEM. Numbers above the SEM bars indicate sample size. Veh = vehicle control (0.5% Tween in water). In the 4-week-old pups (A) there was no statistical interaction by gender; therefore, male ($n = 6$ per group) and female ($n = 6$ per group) data were combined. In the 8-week-old pups (B), data were separated by gender (M = male or F = female) due to statistical interactions. No significant difference was observed after gestational exposure to PFOS at 4 weeks (A), whereas significant suppression in activity was observed at 8 weeks of age (B). The positive control was 0.2 ml of a 1:10 dilution of anti-asialo-GM1 administered by intraperitoneal injection one day prior to assay day. *Significantly different from respective control ($p < 0.05$).

FIG. 2. Primary IgM antibody responses to SRBC immunization in (A) males or (B) females at 8 weeks of age following gestational exposure (GD 1–17) to PFOS. Data are presented as mean ± SEM. Numbers above the SEM bars indicate sample size. Veh = vehicle control (0.5% Tween in water). The positive control was 0.2 ml of 25 mg/kg CYP by intraperitoneal injection for 4 days prior to assay day. Exposure during gestation significantly and dose-responsively decreased PFC activity in adult males (A), but not females (B) *Significantly different from respective control ($p < 0.05$).

developmental exposure. As PFOS is a pervasive environmental contaminant, exposure during children's developmental periods can be expected and in fact has been documented (Apelberg *et al.*, 2007; Butenhoff *et al.*, 2006; Olsen *et al.*, 2004). This study sought to profile the effects of developmental exposure on immunity with the use of a rodent model.

Although little has been characterized regarding PFOS exposure and pregnancy, Lau *et al.* (2003) determined that serum PFOS concentrations measured in pups were comparable to the levels measured in maternal circulation. Thus, Lau *et al.* (2003) demonstrated that *in utero* exposure of neonates is proportional to maternal body burden which is proportional to cumulative doses of PFOS. Furthermore, cross-fostering studies and milk sampling by Luebker *et al.* (2005a) demonstrate that PFOS is bioavailable to neonates via lactation. Although serum levels for PFOS were not quantitated in this study, there is reason to believe that both placental and lactational transfer of PFOS provided considerable routes of exposure to the offspring.

Exposure to PFOS is typically associated with dose-responsive hepatomegaly and weight loss. In the study by Lau *et al.* (2003), no change in body weight with dose-responsive increases in liver weight were reported in mouse pups at the 5 and 10 mg/kg/day PFOS exposure level and this persisted until postnatal day 35. Thibodeaux *et al.* (2003) demonstrated no adverse effect on pup viability at exposure levels of 0.1, 1, and 5 mg/kg/day PFOS. Body weight observations in this study are comparable as no significant changes were observed until the high dose, 5 mg/kg/day PFOS. However, hepatomegaly was present only in male offspring. Except for differences in mouse strains between studies, it is not clear why female pups were less sensitive to the effects of PFOS.

In general, alterations in organ weights such as the aforementioned hepatomegaly in male pups were observed at the earlier time point of 4 weeks, whereas functional deficits in both innate and humoral immunity were noted at 8 weeks of

TABLE 3

Splenic Lymphocytic Subpopulations in 4-Week-Old B6C3F1 Pups Treated with PFOS during Gestation (GD 1–17)

PFOS and gender (mg/kg/day)	CD3+ (cells $\times 10^7$)	CD8+ (cells $\times 10^6$)	DP (cells $\times 10^5$)	DN (cells $\times 10^7$)	CD4+ (cells $\times 10^7$)	B220 (cells $\times 10^7$)
0 Female	2.141 ± 0.17	5.681 ± 0.55	3.90 ± 0.44	6.451 ± 0.41	1.416 ± 0.12	4.214 ± 0.23
0.1 Female	2.222 ± 0.22	5.244 ± 0.48	5.667 ± 7.2	6.031 ± 0.77	1.395 ± 0.13	4.387 ± 0.16
1.0 Female	2.357 ± 0.17	5.954 ± 0.37	5.650 ± 6.8	5.769 ± 0.44	1.496 ± 0.08	4.082 ± 0.29
5.0 Female	2.001 ± 0.22	5.081 ± 0.56	4.433 ± 0.6	4.87 ± 0.45	1.283 ± 0.15	3.317 ± 0.28*
0 Male	2.497 ± 0.11	6.121 ± 0.20	5.900 ± 3.7	10.56 ± 0.25	1.515 ± 0.10	6.158 ± 0.29
0.1 Male	2.515 ± 0.18	6.077 ± 0.35	5.567 ± 6.4	10.17 ± 0.86	1.527 ± 0.06	5.262 ± 0.23
1.0 Male	2.771 ± 0.21	6.458 ± 0.57	6.817 ± 3.1	9.66 ± 0.88	1.144 ± 0.13	5.243 ± 0.40
5.0 Male	2.386 ± 0.19	5.587 ± 0.33	6.983 ± 8.0	9.122 ± 0.45	1.374 ± 0.07	5.108 ± 0.29

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. Sample size is six for each group. These data correspond with organ and cellularity data compiled for Table 2. *Significantly different from control animals ($p \leq 0.05$). DP = CD4+/CD8+. DN = CD4−/CD8−. Thymic lymphocytic subpopulations in 4-week male and female pups were not significantly affected by gestational exposure to PFOS and thus, a table was not included.

age. In the current study, SRBC-specific IgM production was used to assess humoral immunological function. This function was decreased in males following gestational exposure to 5 mg/kg/day, but was not altered in female pups. This gender difference in humoral immunity has also been noted in adult B6C3F1 mice exposed to PFOS for 28 days. Peden-Adams *et al.* (2006a) have shown that male B6C3F1 mice appear, based on total administered dose concentrations, to be 10-fold more sensitive to the effects of PFOS on the PFC assay than are female mice.

Although the PFC assay was significantly affected, it was the deficit in NK cytolytic activity that established the LOAEL for male pups. The deficit in NK cytolytic activity was observed only at 8 but not 4 weeks of age. The reason for this unusual observation is not clear. NK cells may be vulnerable because NK cell numbers are significantly elevated during fetal life relative to the neonatal period (West, 2002). Perhaps alterations of the *in utero* environment lead to alterations in phenotypic expression of NK cells later in life. For instance, a recent study has

identified age-related changes in NK cell phenotype in children from birth to 5 years of age (Sundström *et al.*, 2007). LIR-1+ NK cells increased with age, whereas CD94+NKG2C− (NKG2A+) NK cells decreased. Furthermore, alterations in interleukin-15 production might also be considered as this is essential for NK cell maturation (Kennedy *et al.*, 2000). A recent study in chickens demonstrated that PFOS caused a greater fold increase in gene alterations such as those related to electron transport, metabolism of lipids and fatty acids, and gene regulation 4 weeks after exposure (Yeung *et al.*, 2007). Although it is not known if a link between delayed gene regulation and suppressed NK activity exists, it is notable that the effects were more dramatic several weeks post exposure.

Based on functional immunological assays, the no observed adverse effect level (NOAEL) and low observed adverse effect level (LOAEL) established in this study were 0.1 and 1.0 mg/kg/day maternal dose for male pups and 1.0 and 5.0 mg/kg/day maternal dose for female pups. These limits are consistent with previous reproductive and developmental studies using mice,

TABLE 4

Thymic Lymphocytic Subpopulations in 8-Week-Old B6C3F1 Pups Treated with PFOS during Gestation (GD 1–17)

PFOS and gender (mg/kg/day)	CD3+ (cells $\times 10^7$)	CD8+ (cells $\times 10^6$)	DP (cells $\times 10^7$)	DN (cells $\times 10^6$)	CD4+ (cells $\times 10^7$)
0 Female	1.879 ± 0.13	3.885 ± 0.23	11.88 ± 0.43	4.89 ± 0.29	1.382 ± 0.05
0.1 Female	1.984 ± 0.18	4.296 ± 0.34	11.97 ± 0.99	5.71 ± 0.78	1.539 ± 0.12
1.0 Female	2.040 ± 0.13	3.986 ± 0.25	11.84 ± 0.45	4.63 ± 0.30	1.419 ± 0.07
5.0 Female	2.131 ± 0.25	3.882 ± 0.40	11.66 ± 0.10	6.212 ± 0.90	1.290 ± 0.11
0 Male	2.419 ± 0.13	4.482 ± 0.21	11.88 ± 0.56	7.202 ± 0.71	1.833 ± 0.11
0.1 Male	2.500 ± 0.08	4.847 ± 0.16	13.50 ± 0.19	8.265 ± 0.53	1.860 ± 0.11
1.0 Male	2.111 ± 0.07	4.046 ± 0.26	12.99 ± 0.47	6.902 ± 0.67	1.550 ± 0.08
5.0 Male	1.861 ± 0.08*	3.761 ± 0.19	11.27 ± 0.39	5.417 ± 0.31	1.310 ± 0.03*

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. Sample size is six for each group. These data correspond with organ and cellularity data compiled for Table 2. *Significantly different from control animals ($p \leq 0.05$). DP = CD4+/CD8+. DN = CD4−/CD8−. Splenic lymphocytic subpopulations in 8-week male and female pups were not significantly affected by gestational exposure to PFOS and thus, a table was not included.

rats, and rabbits (Grasty *et al.*, 2003; Lau *et al.*, 2003; Luebker *et al.*, 2005a, b; Thibodeaux *et al.*, 2003). It was previously reported that the NOAEL for maternal toxicity was 0.1 mg/kg in the rabbit and 1.0 mg/kg for the rat, whereas the NOAEL for developmental toxicity was 1 mg/kg in both the species. In two-generation reproductive studies, no effect was observed in mating, fertility, and estrous cycle (Luebker *et al.*, 2005a). However, changes in fetal body weight were detected at 3.2 mg/kg, establishing a NOAEL for F0 and F1 generations of 1.6 mg/kg/day. Body weight changes, either maternal or neonatal, were often the primary effect that established NOAEL and LOAEL levels. Yet this was not the case in this study as functional changes in NK cell activity at adulthood (8 weeks of age) established the NOAEL and LOAEL for F1 pups.

Due to known differences in gender elimination of perfluorinated compounds (Kudo *et al.*, 2002; Lau *et al.* 2007; OECD, 2002), 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 the PFC and NK responses accordingly (Lau *et al.*, 2007; OECD, 2002). However, recent studies in mice have shown little difference in serum concentrations between genders (Lau *et al.*, 2007; Peden-Adams *et al.*, unpublished data); therefore, in this rodent model, differences in elimination rates between genders are not likely the basis for these altered responses.

The observed gender related differences in responses could be secondary to endocrine disruption. PFOS is not estrogenic in the MCF-7 E-screen assay (Ishibashi *et al.*, 2007; Maras *et al.*, 2006), whereas studies in *Xenopus*, rats, and mice suggest possible anti-estrogenic effects (Driscoll, 2007; Fort *et al.*, 2007; Peden-Adams *et al.*, 2007; Wetzel, 1983). In *Cynomolgus* monkeys, females did not exhibit a change in circulating plasma estradiol levels, whereas males had decreased estradiol levels (Seacat *et al.*, 2002). More recently, Ankley *et al.* (2005) showed decreased aromatase activity in fathead minnows following PFOS exposure leading to subsequent increases in plasma 11-ketotestosterone and testosterone. Similar results have been reported with Xenopus such that aromatase and 5α-reductase activity are decreased *in vitro*, whereas *in vivo* increases in plasma testosterone and dihydrotestosterone were observed with corresponding decreases in estradiol (Fort *et al.*, 2007). Moreover, decreased uterine weight, a marker of anti-estrogenic effects (Peden-Adams *et al.*, 2007), has been observed in rats and mice following PFOS exposure (Driscoll, 2007; Wetzel, 1983). Taken together, it seems plausible that PFOS may indeed alter endocrine function.

The importance of this to immunological function is that estrogen and androgen receptors are expressed in lymphocytes and primary lymphoid organs (Tanriverdi *et al.*, 2003) and alterations in levels of these hormones or antagonism or these receptors are known to modulate immune function. In regards to the observed effects in the PFC response, it has been demonstrated that estradiol increases IgM production (Kanda and Tamaki, 1999; Tanriverdi *et al.*, 2003), whereas testoster-

one 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 estrogen receptor (ER) agonists; although testosterone has no effect on NK cell function. Therefore, the IgM response and NK response observed in this study could be secondary to endocrine disruption by PFOS. Whether these observations are may be due to antagonism of the estrogen receptor or modulation of circulating hormones is unclear. It is apparent that additional studies are required to address the role of PFOS in modulation of endocrine function and to verify a possible link to immunotoxicity.

The specific role of peroxisome proliferator-activated receptor alpha (PPAR-α) in mediating immunosuppression following PFOS exposure has not been thoroughly investigated. PFOS is a known agonist for PPAR-α which is expressed in gestational tissues and integral to embryonic implantation, placental maturation, and regulation of hormones (Barak *et al.*, 1999; Berry *et al.*, 2003; Tarrade *et al.*, 2001). PPARα is also the primary PPAR isoform expressed on lymphocytes (Jones *et al.*, 2002) and is more highly expressed in B cells than in T cells (Cunard *et al.*, 2002). At this time, it is not clear if PFOS-mediated activation of PPARα would account for deficits in immunity; however, studies are underway to explore this mechanism.

In conclusion, this is the first study to assess the immunotoxicological effects of developmental exposure to PFOS. Our data demonstrate that the developing immune system is sensitive to PFOS. Male pups appeared more susceptible to the effects in three of the immunological tests assessed: NK cell activity, the PFC assay, and alteration in T-cell subpopulations (17% decrease in the CD 4:8 ratio). Based on Luster *et al.* (1992), changes in these three combined endpoints is highly predictive of immunotoxicity of a compound. Future studies exploring the effects of PFOS on the developing immune system should expand the range of concentrations assessed to include lower levels during a lifetime exposure model (Peden-Adams *et al.*, 2006b) and to explore specific developmental windows during *in utero* and postnatal growth.

declare. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

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