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Does developmental exposure to perflurooctanoic acid (PFOA) induce immunopathologies commonly observed in neurodevelopmental disorders?

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ABSTRACT

Immune comorbidities often are reported in subsets of patients with neurodevelopmental disorders, including autism spectrum disorders and attention-deficit hyperactivity disorder. A common immunopathology is an increase in serum autoantibodies against myelin basic protein (MBP) relative to control patients. Increases in autoantibodies suggest possible deficits in self-tolerance that may contribute to the formation of brain-specific autoantibodies and subsequent effects on the central nervous system (CNS). Oppositely, the formation of neuronal autoantibodies may be a reaction to neuronal injury or damage. Perfluorooctanoic acid (PFOA) is an environmental pollutant that induces multisystem toxicity in rodent models, including immunotoxicity and neurotoxicity. We hypothesized that developmental exposure to PFOA may induce immunotoxicity similar to that observed in subsets of patients with neurodevelopmental disorders. To test this hypothesis, we evaluated subsets of T cells from spleens, serum markers of autoreactivity, and levels of MBP and T cell infiltration in the cerebella of adult offspring exposed to 0.02, 0.2, or 2 mg/kg of PFOA given to dams from gestation through lactation. Litter weights of offspring from dams exposed to 2 mg/kg of PFOA were reduced by 32.6%, on average, from postnatal day one (PND1) through weaning (PND21). The percentage of splenic CD4+CD25+Foxp3+ T cells in male and female offspring from dams exposed to 2 mg/kg of PFOA was reduced by 22% relative to the control percentage. Ex vivo co-cultures of splenic CD4+CD25+ T cells and CD4+CD25− T cells from dosed male offspring produced less IL-10 relative to control cells. Anti-ssDNA, a serum marker of autoreactivity, was decreased by 26%, on average, in female offspring from dams exposed to 0.02 and 2 mg/kg PFOA. No other endpoints were statistically different by dose. These data suggest that developmental PFOA exposure may impact T cell responses and may be a possible route to downstream effects on other systems.

1. Introduction

The etiology of many neurodevelopmental disorders, including autism spectrum disorders and attention-deficit hyperactivity disorder, is largely unknown. Myriad hypotheses have suggested that exogenous agents, such as environmental pollutants, play a role in causing or triggering dysfunctional development that may culminate in a phenotypic expression of the disorder. Although genetic factors also have been suggested as causative or triggering stimuli, the phenotypic heterogeneity of neurodevelopmental disorders suggests a complex etiology that involves the interplay of a variety of events involving multiple systems and pre- and postnatal environmental factors. One specific set of initiating or triggering events may involve the immune system as alterations to the immune system have been reported in subsets of afflicted patients. In a recent review of immune dysfunctions described in patients with autism spectrum disorder, Onore et al. (2012) indicated that evidence was sufficient to implicate altered immune processes as a potential pathway. In addition, Dietert and Dietert (2008) made the case that early-life immune insults (ELII), including developmental immunotoxicity (DIT), share several features with autism spectrum disorders, suggesting an association between immune dysfunction and the disorder. Like several neurodevelopmental disorders, immune dysfunction is not fully heritable, which suggests that genetic background may be a shared susceptibility (Dietert and Dietert, 2008).

One type of immune dysfunction that has been reported in subsets of patients with neurodevelopmental disorders is an increase in the amount of serum autoantibodies against brain-specific proteins (Rout et al., 2012; Pessah et al., 2008; Silva et al.,

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2004; Wills et al., 2009). Increases in autoantibodies indicate that the immune system may be responding against self-proteins, an occurrence that is typically minimized by central and peripheral tolerance and the action of regulatory T cells (T_{regs} or CD4+CD25+Foxp3+ T cells). While autoreactive T cells are part of the normal immune cell pool, increases in serum autoantibodies reflect an increased risk of autoimmune disease. During tolerance, T cells that react against self proteins are isolated or eliminated; T_{regs} act peripherally to eliminate T cells that react to self proteins (Selgrade et al., 2008). However, the development of autoantibodies also may reflect an immune response against cellular products of neuronal damage. Autoimmune reactions are considered common features among neurological diseases and central nervous system (CNS) trauma (Zhang and Popovich, 2011).

Perfluorooctanoic acid (PFOA) is an environmentally pervasive pollutant that induces multisystem toxicity in rodent models, including immunotoxicity and neurotoxicity. The type of immunotoxicity most commonly observed after PFOA exposure is suppression of T cell dependent antibody responses (DeWitt et al., 2008, 2012). Although numerous cells and signals are involved in the mediation of T cell dependent antibody responses and suppression of this response may indicate effects on one or more cell populations (Plitnick and Herzyk, 2010), T cells may be one of the cell types affected when this response is suppressed. PFOA is capable of altering T cell populations, including the number of CD4+ and CD8+ cells (Son et al., 2009; Yang et al., 2000) and production of T cell-derived cytokines such as IL-4 and IL-10 (Corsini et al., 2011). Developmental exposure to PFOA also has been reported to induce neurotoxicity, including neurobehavioral deficits (Johansson et al., 2008; Pinkas et al., 2010; Onishchenko et al., 2011) and alterations in the levels of proteins necessary for normal brain development (Johansson et al., 2009). However, these neurotoxicological studies did not evaluate potential immune deficits associated with PFOA exposure.

We hypothesized that because PFOA has been reported to alter T cell populations and has been associated with neurotoxicity, developmental exposure to PFOA may induce immunotoxicity similar to that observed in subsets of patients with neurodevelopmental disorders. To test this hypothesis, we evaluated percentages of splenic CD4+CD25+FoxP3+ T cells from spleens, IL-10 production from ex vivo co-cultures of splenic CD4+CD25+ T cells co-cultured with CD4+CD25− T cells, serum markers of autoreactivity, and levels of MBP and T cell infiltration in the cerebella of adult offspring exposed to 0.02, 0.2, or 2 mg/kg of PFOA given to dams from gestation through lactation.

2. Materials and methods

2.1. Materials

PFOA, Tween-20, sterile water, and RPMI were obtained from Sigma-Aldrich Co. (St. Louis, MO). All antibodies and supplies for labeling splenic CD4+CD25+Foxp3+ T cells (T_{regs}) were obtained from eBioscience, Inc. (San Diego, CA). CD4+CD25+ T cell isolation kits were obtained from R&D Systems (Minneapolis, MN) and antibodies for the IL-10 production assay and for immunohistochemistry were obtained from Abcam (Cambridge, MA). Additional materials for immunohistochemistry were obtained from Vector Labs (Burlingame, CA) and all levels of serum autoantibodies were assessed with kits from Alpha Diagnostic International (San Antonio, TX).

2.2. Animals

Male and female C57BL/6N mice (6–7 weeks of age) were purchased from Charles River Laboratories (Raleigh, NC) and delivered to the East Carolina University (ECU) Brody School of Medicine (BSOM) animal facility (accredited by the Association for Assessment and Accreditation of Laboratory Animal Care). Animals were singly housed in polycarbonate cages with corn cob bedding, soft bedding material, and a hiding tube. They were provided a 12-h light:dark cycle (light, 0600–1800 h; dark, 1800–0600 h), maintained at 23 ± 3 °C and 30–70% relative humidity, and given ad libitum access to both food (5P00 Prolab RMH 3000) and water. All procedures employed in this study involving animal models were approved in advance by the Institutional Animal Care and Use Committee of ECU.

2.3. Experimental design

2.3.1. Dosing solution

Dosing solutions were prepared freshly once per week and were prepared so that 0.1 mL of dosing solution administered per 10 g of body weight resulted in the appropriate mg/kg concentration of PFOA. Stock solutions contained 0.002, 0.02, or 0.2 mg/L of PFOA in deionized water with 0.5% Tween. The vehicle solution contained 0.5% Tween in deionized water. Solutions were stored in plastics known to be free of PFOA.

2.3.2. Treatment

After a five-day acclimation period, all mice were weighed and randomly distributed to dose groups by weight so that each dose group contained 12 males and 12 females. Female mice were treated via gavage from the day they were paired with males through weaning of offspring at postnatal day 21 (PND21). On average, females were dosed for 12.9 days prior to becoming pregnant (±7.3; average range = 10.1–15.7 days; no statistical difference among dose in number of days to pregnancy). On the first day of dosing, females were weighed, gavaged, and then immediately placed into a male's home cage (one male:one female). Females were weighed and then gavaged each morning of the study until the day that pups were weaned at PND21. When females were obviously pregnant (body weight >24 g), males were removed and euthanized. Females that obviously were not pregnant by the end of the 5th week of the study were removed and euthanized and uteri were examined for implantation sites, embryos, or reabsorbing embryos. Females that lost >20% of body weight were removed from the study, euthanized, and necropsied for gross signs of pathology.

2.3.3. Offspring

On PND1, litters were weighed, sexed, and culled to three males and three females per dam. If possible, litters with an insufficient number of male or female pups were backfilled with extra pups of the same age from dams within the same dose group. Offspring were weighed once per week until weaning at PND21. At weaning, offspring were separated by sex and housed in sibling trios or pairs, depending on the number of offspring of each sex per litter. Once offspring reached adulthood (at least six weeks of age), they were euthanized by carbon dioxide intoxication and exsanguinated by neck vein transection. Brains and spleens were immediately harvested and prepared according to the endpoint to be evaluated.

Spleens of male and female offspring were divided into two subsets, one of which was used for immunophenotyping (Section 2.3.1) and one of which was used for IL-10 production (Section 2.3.2). Ideally, spleens were divided so that one male and one female offspring from each litter were used for IL-10 production (up to $N = 5$) and remaining spleens were used for immunophenotyping. Similarly, one male and one female offspring from each litter were evaluated for serum autoantibody concentrations and for brain endpoints. Figs. 2–6 indicate sample sizes per sex and per litter used for each endpoint.

2.4. Neuroimmune endpoints

2.4.1. Splenic T_{reg} percentage

The percentage of splenic CD4+CD25+Foxp3+ T cells (T_{regs}) was determined within a gated CD4+ T cell population from subsets of spleens of male and female offspring from each dose group. Freshly harvested spleens were prepared according to the manufacturer's protocol (eBioscience, Inc., San Diego, CA) and standardized to a concentration of 2×10^7 cells/mL in flow cytometry staining buffer. Two million (2×10^6) cells were removed from each spleen suspension and placed into a fresh tube. Cells were preincubated with anti-CD16/32 antibody and then stained with anti-mouse CD4-FITC and CD25-PE. For intracellular staining, cells were fixed and permeabilized and then stained with anti-mouse Foxp3-APC. Optimal concentrations of the antibodies and reagents were determined in previous experiments as were isotype controls to determine color compensation. Flow cytometric analysis was performed using an Accuri C6 flow cytometer and 25,000 events were collected from each sample.

2.4.2. IL-10 production

Ex vivo production of IL-10 from splenic CD4+CD25+ T cells was analyzed in subsets of freshly harvested spleens of male and female offspring from each dose group. Spleens were prepared according to the manufacturer's protocol (R&D Systems, Minneapolis, MN). Briefly, CD4+ T cells were selected from spleen single cell suspensions ($N = 5$ male or 5 female spleens/dose; if possible, offspring from the same dams were not pooled unless it was not possible due to low litter numbers for a particular dose) by negative selection and then from those CD4+ T cells, CD25+ T cells were selected by positive selection. CD4+CD25− T cells also were retained. Separate aliquots of CD4+CD25+ T cells and CD4+CD25− T cells were adjusted so that 100 μL of supplemented RPMI 1640 medium (S-RPMI; 10% fetal calf serum, 1% pen-strep, 20 mM Hepes, and 1 mM L-glutamine) contained 2×10^5 cells.

Culture was carried out as described by Ring et al. (2007). Briefly, purified splenic CD4+CD25- T cells were cultured in 96-well tissue culture plates in S-RPMI (2×10^5 cells/well in 100 μL of S-RPMI) and stimulated with anti-CD3 (0.5 μg/mL), anti-CD28 (0.5 μg/mL) and IL-2 (10 ng/mL). Splenic CD4+CD25+ T cells were added to the medium (2×10^5 cells/well in 100 μL of S-RPMI), and cells were co-cultured for 72 h (37 °C). After 72 h the supernatant was collected and stored at −80 °C. According to Ring et al. (2007), stimulation of splenic CD4+CD25− T cells with anti-CD3/anti-CD28 and IL-2 is an effective method for polyclonal expansion in vitro. In addition, stimulation of splenic CD4+CD25+ T cells by this means maximizes IL-10 production by these cells in the presence of CD4+CD25− T cells. Under these culture conditions, neither antigen presenting cells (APCs) nor dendritic cells (DCs) are required for expansion of splenic CD4+CD25+ T cells. Although the purity of both cell populations was not confirmed via flow cytometry due to the number of cells required for the IL-10 production assay, the cell isolation kit indicates a purity of 84–94% of both $CD25+^{low}$ and $CD25+^{high}$ T cells. Therefore, some of the IL-10 produced in our culture could be from CD4+ CD25− T cells. However, optimization studies in our laboratory prior to this experiment demonstrated that IL-10 production from cultures of splenic T cells was maximized in co-cultures (CD4+CD25− <CD4+CD25+ <CD4+CD25−/CD4+CD25+; data not shown). Thawed supernatant was assayed for IL-10 according to the manufacturer's protocol (R&D Systems, Minneapolis, MN).

2.4.3. Serum autoantibodies

Blood collected from all offspring at the time of euthanasia was held at room temperature for 30 min, centrifuged at 4 °C to separate serum, and then frozen at −80 °C until analysis of serum autoantibodies. Serum markers of autoreactivity were measured in subsets of male and female offspring and included levels of anti-dsDNA, anti-ssDNA, and anti-myelin basic protein (anti-MBP). All assays were performed according to the manufacturer's protocol (Alpha Diagnostic International, San Antonio, TX).

2.4.4. T cell infiltration and myelin basic protein levels in cerebella

Numbers of CD3+ T cells that had infiltrated brains were counted in cerebella of subsets of male and female offspring. Cerebella were chosen based on studies by Rout et al. (2012), Goines et al. (2011), and Wills et al. (2009), that suggested that cells in the cerebellum are targeted by autoantibodies in patients with autism spectrum disorders. Briefly, cerebella were immersion fixed in 10% neutral buffered formalin for 24 h, paraffin-embedded, and sliced at 6 μm with a rotary microtome. Six sections/offspring/sex/dose were stained immunohistochemically (Vectastain ABC Kit, Vector Labs, Burlingame, CA); two of the four sections were stained with anti-CD3+ antibody (Abcam, Cambridge, MA) and two of the four sections were stained with anti-MBP (Abcam, Cambridge, MA). The remaining two sections served as negative staining controls for either anti-CD3+ or anti-MBP. The number of cells stained with CD3+ were counted on every section stained for anti-CD3+. For sections stained with anti-MBP, the relative intensity of the stain was determined. Sections were photographed, desaturated of color and then a 500 by 500 pixel square was selected. Within selected squares, the average intensity of gray staining (where 0 = black and 255 = white) was calculated. Sections with lower intensity values therefore had stronger MBP staining than did sections with higher intensity values.

2.5. Replication

Steps 2.1–2.4 were independently replicated in two separate studies. Data therefore represent the combined results of two identical studies.

2.6. Statistics

All data are presented as mean ± standard deviation. Statistical analyses were performed with the SAS System (SAS Institute, Cary, NC). Dam and offspring body weights were analyzed by one-way repeated measures analysis of variance (ANOVA) by dose and time (repeated factor). Nested (hierarchical) ANOVA was used to determine if dams produced a statistically significant sub-group effect within offspring dose groups. Remaining data were analyzed by two-way ANOVA by dose and sex. When ANOVA results indicated a statistically significant dose, time, or sex effect within operation, individual post hoc comparisons were made using one-way ANOVA, Tukey's test, or an LSMeans *t*-test. Statistical significance was determined when *P*-values were less than 0.05. As all experimental procedures were performed twice, replicate endpoints were statistically compared before pooling of results to verify that results of each replicate did not differ statistically.

3. Results

3.1. General observations of dams and offspring

Dosing body weights of dams gavaged daily with PFOA did not differ statistically by dose over the course of the experiment. Similarly, terminal body weights of dams, number of pregnancies, litters delivered, and litters weaned did not differ statistically by dose (Table 1).

Number of offspring of dams/dose and sex ratio of offspring of dams/dose did not vary statistically by dose (Table 1). Mean body weight of offspring from the 2 mg/kg dose group was 32.6% lower,

Table 1
Terminal body weights (mean ± standard deviation) and reproductive outcome of dams dosed with PFOA via gavage from pairing with males through weaning of offspring. $N = 24$ dams/dose.

	0 mg/kg PFOA	0.02 mg/kg PFOA	0.2 mg/kg PFOA	2 mg/kg PFOA
Terminal BW (g)	28.6 ± 1.80	28.5 ± 0.97	28.1 ± 2.26	27.8 ± 2.04
Dams pregnant	16	11	13	14
Litters delivered	13	11	13	14
Litters weaned	7	9	11	9
Pup numbers [Total (male, female)]	6.3 (3.3, 3.0)	6.8 (3.6, 3.3)	7.4 (3.9, 3.5)	7.6 (3.8, 3.8)

Note. BW: body weight.

on average, relative to mean body weight of the control group from PND1 through weaning at PND21 (Fig. 1). At the time of sample collection, when offspring were at least six weeks of age, mean body weight of the 2 mg/kg dose group was statistically indistinguishable from the mean body weight of the control group (data not shown). Apart from body weight changes, no other differences were noted among offspring from the different dose groups.

3.2. Splenic T_{reg} percentage

The percentage of splenic CD4+CD25+Foxp3+ T_{regs} were assessed in subsets of male and female offspring from each dose (Fig. 2). Spleen cellularity did not vary between sexes or among dose groups within each sex (data not shown). Within each splenic CD4+ T cell population, the percentage of cells that also were positive for CD25+ and Foxp3+ was approximately 8% in control spleens, which was expected for this subset of T cells. Overall, spleens of offspring from dams treated with PFOA had a slightly lower percentage of T_{regs} relative to spleens of offspring from control dams. On average, male offspring of dams treated with PFOA had 16.2% less splenic T_{regs} relative to male offspring of control dams. Female offspring of dams treated with PFOA had 12.9% less splenic T_{regs}, on average, relative to female offspring of control dams; however, only spleens of males and females from the 2 mg/kg dose had a statistically lower percentage (20.1% and 23.1% lower, respectively) of T_{regs}.

3.3. IL-10 production

The ability of ex vivo splenic CD4+ T cells to produce IL-10 in culture was determined from subsets of male and female offspring from each dose (Fig. 3). In offspring from dams exposed to PFOA, IL-10 produced from cells obtained from male offspring was decreased statistically by 61%, 75%, and 75%, in the 0.02 mg/kg, 0.2 mg/kg, and 2 mg/kg dose groups, respectively. In female offspring from dams exposed to PFOA, ex vivo levels of IL-10 were increased statistically by 204% in the 0.02 mg/kg dose group relative to controls; no other differences were detectable in the female offspring.

3.4. Serum autoantibodies

Serum markers of autoreactivity were measured in subsets of male and female offspring from each dose (Fig. 4A–C). With the exception of a 26% decrease, on average, in anti-ssDNA in female offspring exposed to 0.02 mg/kg and 2 mg/kg of PFOA (C), no differences in autoreactivity markers were detected among dose groups or between sexes.

3.5. T cell infiltration and myelin basic protein levels in cerebella

Numbers of CD3+ T cells that had infiltrated brains were counted in cerebella of subsets of male and female of offspring from each dose. A representative section is displayed in Fig. 5. No

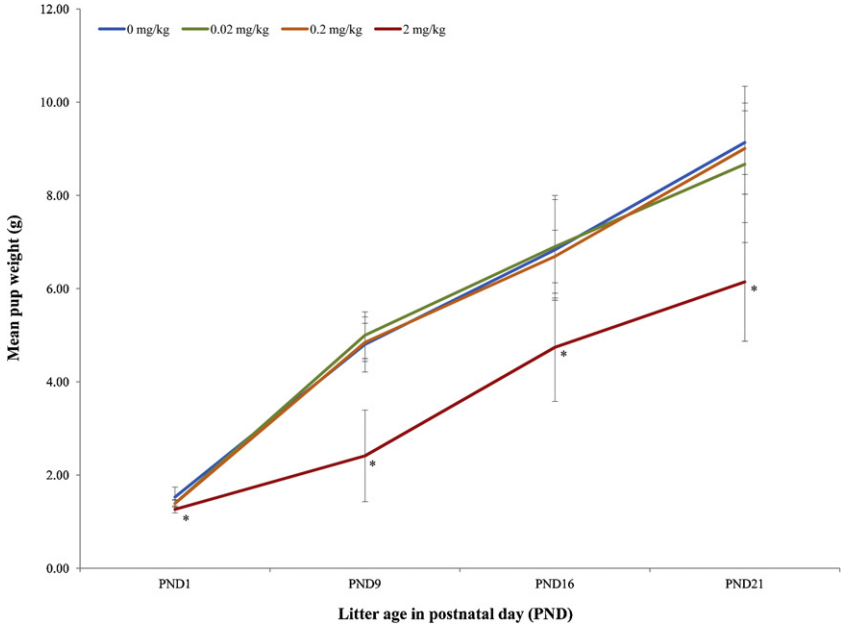

Fig. 1. Mean (±standard deviation) litter weights in offspring of dams dosed with PFOA via gavage from pairing with males through weaning of offspring. Offspring of dams exposed to 2 mg/kg of PFOA weighed 32.6% less, on average, relative to offspring of control dams from birth (PND1) through weaning (PND21). * Indicates a statistical ($P < 0.05$) difference from the 0 mg/kg group.

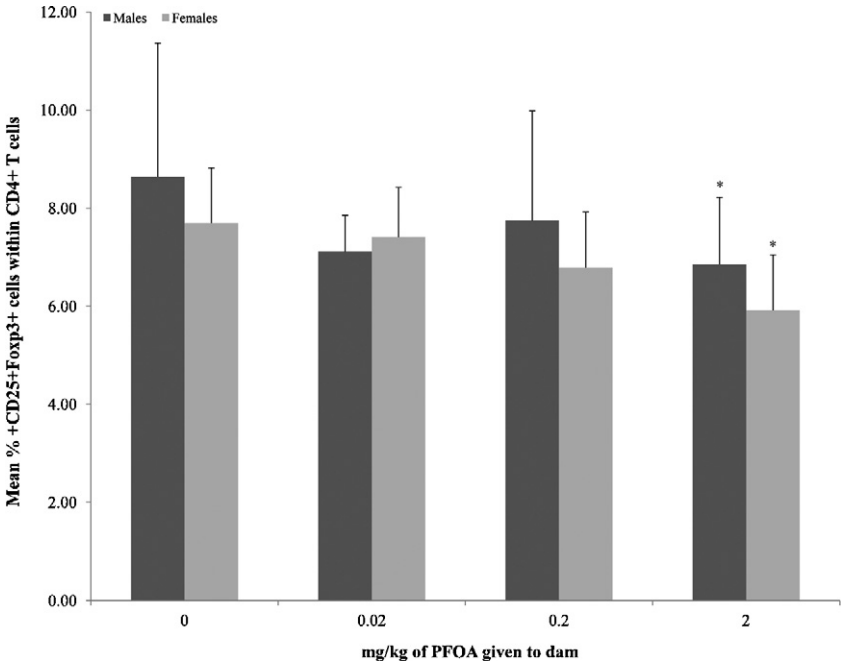

Fig. 2. Mean percent (±standard deviation) of lymphocytes from spleens that were positive for CD25+ and Foxp3+ of the 25,000 events that were collected within each CD4+ T cell population in offspring of dams dosed with PFOA via gavage from pairing with males through weaning of offspring. PFOA suppressed the percentage of T_{regs} in spleens from male and female offspring exposed to 2 mg/kg. * Indicates a statistical ($P < 0.05$) difference from the sex-matched 0 mg/kg group.

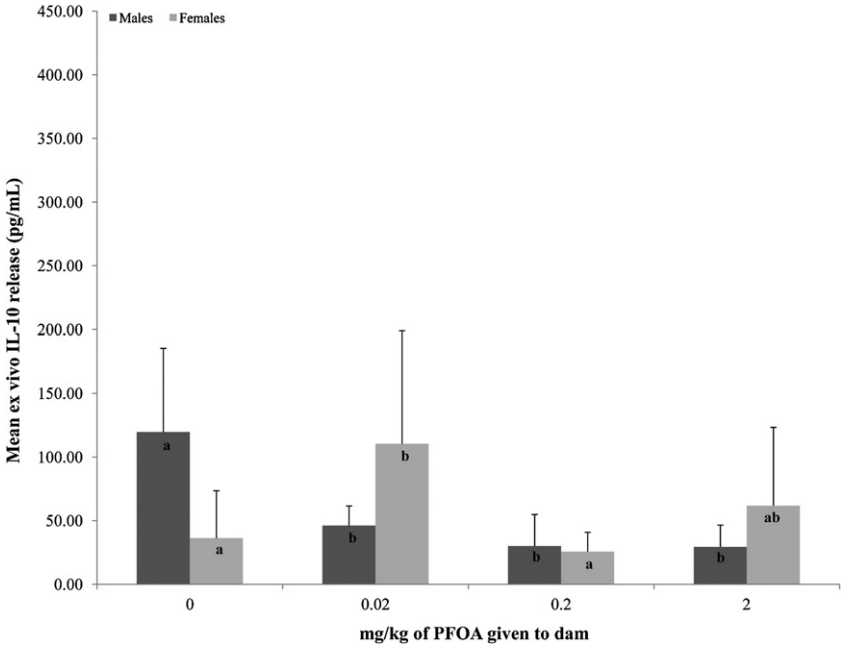

Fig. 3. Mean IL-10 (±standard deviation) produced (pg/mL) from CD4+CD25+ T cells isolated from spleens of offspring from dams dosed with PFOA via gavage from pairing with males through weaning of offspring. Splenic CD4+CD25+ T cells were co-cultured ex vivo for 72 h with splenic CD4+CD25− T cells stimulated with anti-CD3+, anti-CD28+, and IL-2 in RPMI culture medium. PFOA reduced the amount of IL-10 in the medium containing cells from spleens of male offspring at all doses and increased the amount of IL-10 from in the medium containing cells from spleens of female offspring exposed to 0.02 mg/kg. Within sex, means with the same letter do not differ statistically ($P < 0.05$).

CD3+ T cell infiltration was observed in any of the examined sections.

Relative MBP levels were measured in cerebella of subsets of male and female offspring from each dose. The average intensity of MBP staining did not differ by dose (Fig. 6A–D).

4. Discussion

Our study demonstrated that in C57BL/6 mice, gestational and lactational exposure to PFOA produced several effects on splenic CD4+ T cells. A reduction in the percentage of splenic CD4+CD25+Foxp3+ T cells (T_{regs}) was observed in male and female offspring exposed to 2 mg/kg. In female offspring exposed to 0.02 mg/kg and ex vivo IL-10 production was increased from splenic CD4+ T cells. In male offspring, all doses of PFOA reduced ex vivo IL-10 produced from splenic CD4+ T cells. These data suggest that developmental exposure to PFOA can have effects on CD4+ T cells and one of the regulatory cytokines that they produce. Additionally, although female offspring exposed to 0.02 and 2 mg/kg had a decrease in serum anti-ssDNA, no other markers of

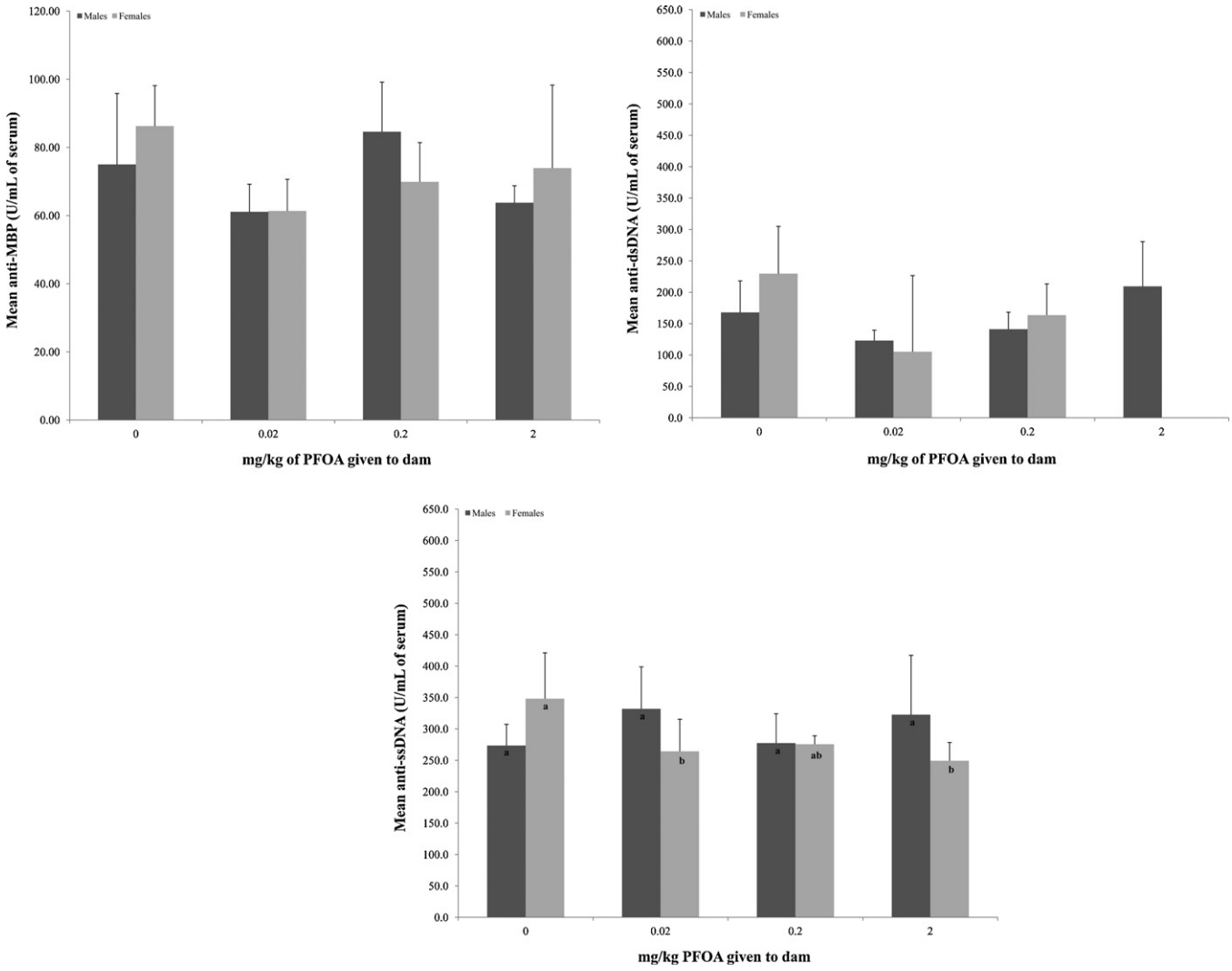

Fig. 4. Mean serum (±standard deviation) markers of autoreactivity in offspring of dams dosed with PFOA via gavage from pairing with males through weaning of offspring. With the exception of a slight decrease in anti-ssDNA in female offspring exposed to 0.02 and 2 mg/kg of PFOA (C), levels of serum markers for autoreactivity did not differ statistically by dose for either male or female offspring. (A) Anti-myelin basic protein of offspring exposed to PFOA; (B) anti-dsDNA of offspring exposed to PFOA; (C) anti-ssDNA of offspring exposed to PFOA. Within sex, means with the same letter do not differ statistically ($P < 0.05$).

autoreactivity were altered by developmental PFOA exposure. Overall, while developmental exposure to PFOA can affect T cell subpopulations and an associated cytokine, it is unclear whether PFOA exposure leads to increases in autoantibodies that would be indicative of immunotoxicity observed in certain neurodevelopmental disorders.

As a measure of autoimmunity, we evaluated serum levels of antibodies against dsDNA, ssDNA, and myelin basic protein (MBP). Increases in anti-MBP antibody levels have been reported in the serum of patients with neurodevelopmental disorders, including autism spectrum disorders (Mostafa and Al-Ayadhi, 2011; Singh et al., 1993, 2002). Additionally, increases in anti-ssDNA and anti-dsDNA antibodies can reveal autoimmune disease risk after toxicant exposures, although anti-dsDNA is typically most useful for diagnosing and evaluating the progression of lupus (Keil et al., 2009). The only change that we observed in any serum markers of autoimmunity was a decrease in anti-ssDNA in female offspring of dams exposed to 0.02 and 2 mg/kg of PFOA. In studies of the solvent trichloroethylene (TCE), levels of anti-ssDNA have been reported to increase after 24–30 weeks of exposure in adult animals (Keil et al., 2009); to our knowledge, no studies of toxicants and autoantibodies have reported decreases in autoantibodies after exposure. However, autoantibodies to ssDNA may be more commonly seen in chemically-induced autoimmune diseases; an increase in anti-ssDNA could indicate that PFOA is interacting with DNA to cause breakage or separation, which would generate anti-ssDNA autoantibodies (Keil et al., 2009). It is possible that the decrease in anti-ssDNA that we observed in female offspring from the 0.02 and 2 mg/kg dose groups reflects overall impacts on B or T cells, although additional studies are necessary to determine if this is the case. A related compound, perfluorooctane sulfonate (PFOS) has been reported to impact B cell populations (Peden-Adams et al., 2008); decreases in antibodies also may result from deficits in B cells. However, we did not assess antigen-specific antibody responses in this study.

To our knowledge, this is the first report of the effects of PFOA on of splenic CD4+CD25+Foxp3+ T cells. Two previous studies of the effects of PFOA on immune cell populations in adult animals reported that PFOA exposure alters the number of CD4+ T cells. Son et al. (2009) reported increases in the number of splenic CD4+CD8− T cells and decreases in the number of CD4+CD8+ T cells in adult male ICR mice exposed to PFOA for 21 days. Fewer days of PFOA treatment also impacts CD4+ T cells; seven or 10 days of PFOA treatment reduced numbers of splenic CD4+ T cells in male

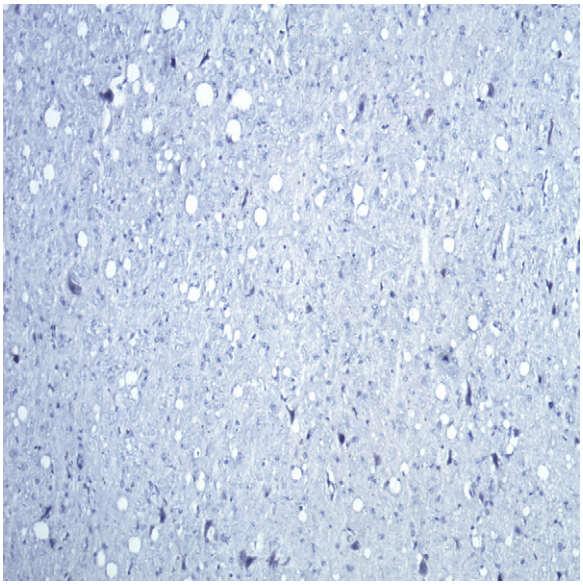

Fig. 5. A representative cerebellar section from a female offspring of a dam dosed with PFOA via gavage from pairing with males through weaning of pups. No CD3+ T cell infiltration was observed in any of the examined sections (magnification = 200× total magnification) of either male or female offspring.

C57BL/6 mice (Yang et al., 2000, 2001). These authors hypothesized that PFOA may interfere with the process of thymocyte proliferation. The reduction in splenic CD4+CD25+Foxp3+ T cells that we observed also could result from impacts of PFOA on the thymus or on peripheral expansion of splenic CD4+CD25+Foxp3+ T cells. As the developing fetuses were exposed to PFOA throughout all of gestation, all three critical windows of development for CD4+CD25+Foxp3+ T cells, including seeding of thymus with thymocytes, negative selection, and CD4+CD25+Foxp3+ T cell population generation, were potentially affected. Dietert and Dietert (2008) noted that targeted immunosuppression by environmental chemicals can impact thymus maturation and/or T_{regs} and increase the risk of postnatal autoimmunity. Sakaguchi et al. (1995) established that when T_{regs} are depleted from an animal's T cell repertoire, the afflicted animal will develop histologically and serologically evident autoimmune disease within about 90 days. However, as CD4+CD25+Foxp3+ T cells comprise only about 5–10% of CD4+ T cells in adult mice, a reduction in the percentage of splenic CD4+CD25+Foxp3+ T cells in an organism does not necessarily lead to a reduction in the functional capacity of the splenic CD4+CD25+Foxp3+ T cells to respond to a challenge. Therefore, although we observed a reduction in the number of splenic CD4+CD25+Foxp3+ T cells in both male and female offspring exposed to 2 mg/kg of PFOA, the remaining T_{regs}, including CD4+CD25+ T cells lacking Foxp3+, may still have retained adequate function to maintain peripheral tolerance.

Although constitutive expression of CD25 is generally a characteristic feature of T_{regs}, T cells also up-regulate CD25 when activated (Corsini et al., 2011). Natural T_{regs} are definitively identified by expression of the intracelluar transcription factor, Foxp3, and adaptive/inducible T_{regs} are defined, in part by their ability to secrete IL-10. T_{regs} suppress the proliferation of target cells by cell-to-cell contact and/or by production of inhibitory cytokines such as IL-10 (Oberg et al., 2011). Although we did not measure proliferation of either T_{regs} or target cell populations within the cultures, in vitro production of IL-10 by T_{regs} (Baecher-Allan et al., 2001) is a proportional measure of their suppressive ability. In vivo, suppression of target cells by IL-10 may lead to cell cycle arrest, prevention of maturation, and/or a reduction in antigen presentation (Peterson, 2012). If the activated target cell damages host tissue, suppression of that response by T_{regs} is desirable and helps to reduce the risk of an autoimmune, chronic inflammatory, or other undesirable immune response (Corsini et al., 2011). If the target cell is directed against neural tissue, neurological damage may occur. T_{regs} have been reported to be neuroprotective. Reynolds et al. (2007) found that when T_{regs} activated by anti-CD3 and IL-2 were adoptively transferred to a mouse model of Parkinson's disease, they protected dopaminergic neuronal bodies through a reduction in the number of immunoreactive microglia. Microglia are resident immune cells of the CNS and through their effector functions, can induce secondary damage to healthy tissue through inflammation and oxidative stress.

However, T_{regs} may not always play a neuroprotective role. Moalem et al. (1999) demonstrated that T cells directed against MBP may be neuroprotective. They reported that MBP-specific T cells protected CNS neurons from additional degeneration after an injury. This illustrates that the relative balance of cell populations is critical to disease management or formation. Additional research by Kipnis et al. (2004) verified that T_{regs} play a role in mediating the response of T cells to CNS injury. When microglia were pre-incubated with T_{regs} alone, activated T cells alone, or both, and were then added to organotypic hippocampal slice cultures, neuronal survival was reduced only in the cultures that contained both T_{regs} and T cells (Kipnis et al., 2004). Thus, by inhibiting activated T cells, T_{regs} apparently inhibited the ability of the T cells to reduce neuronal cell death, a phenomenon that they called protective autoimmunity. Additionally, because Kipnis et al. (2004) observed a difference in cell death associated with protective autoimmunity between two strains of mice (BALB/c versus C57BL/6), they hypothesized that a susceptible genotype (C57BL/6) is unable to manifest a sufficient autoimmune response to reduce neurological damage and/or to control the autoimmune response once it has turned on.

In our study, ex vivo production of IL-10 from splenic CD4+CD25+/CD4+CD25− T cells was statistically decreased in male, but not female offspring from all PFOA dose groups. This ex

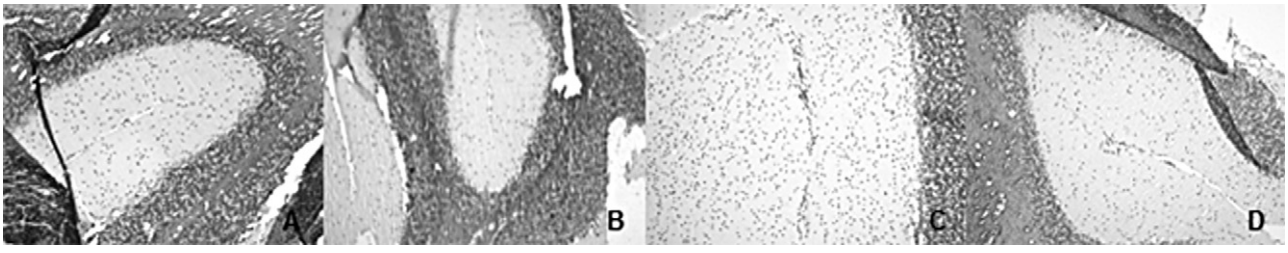

Fig. 6. Sample sections of cerebella from offspring exposed to PFOA used to determine relative intensity of myelin basic protein (MBP) staining. (A) 0 mg/kg; (B) 0.02 mg/kg; (C) 0.2 mg/kg; (D) 2 mg/kg. Magnification = 20×. No statistical difference in the intensity of MBP staining was detected among dose groups in male or female offspring.

vivo IL-10 production in cells isolated from the spleens of dosed animals was about 70% lower relative to IL-10 released from cells isolated from control animals. Corsini et al. (2011) demonstrated that in vitro exposure of human peripheral blood leukocytes to 10 μg/mL of PFOA decreased IL-10 release. In addition, levels of cerebellar MBP were consistent across all dose groups and no T cell infiltration into the cerebella was observed in any dose group. However, as our experiment did not induce a neuronal injury, we can only conclude that T cells did not enter the CNS or deplete MBP. Similarly, as serum markers of autoreactivity were not increased in any dose groups, we suspect that the decrease in T_{reg} number and ex vivo IL-10 production by CD4+ T cells, at least in male offspring, was not sufficient to affect the overall T cell pool. Although our data demonstrated that PFOA affects the percentage of splenic T_{regs} and the ability of isolated splenic CD4+T cells to produce IL-10, additional work is required to further elucidate this relationship and to determine if alterations to these cells impact neural development.

5. Conclusions

It is generally held that neurodevelopmental disorders arise from a combination of genetic and environmental factors and that multiple pathways may give rise to a diagnosis. Additionally, initiating or triggering events for neurodevelopmental disorders may involve the immune system as alterations to the immune system have been reported in subsets of afflicted patients. The results of our current model suggest that if exposure to PFOA occurs during development at levels below 2 mg/kg, no definitive changes indicative of the types of immunopathologies observed in neurodevelopmental disorders exist. Our results indicate that at an exposure level of 2 mg/kg, certain cells of the immune system can be altered by developmental exposure to PFOA. Therefore, in genetically susceptible individuals who experience the "right" combination of causes and triggers, developmental exposure to PFOA may tip the balance from health to disease. A recent report by Grandjean et al. (2012) concluded that elevated exposure to perfluorinated compounds was associated with a reduced response to routine childhood vaccinations in children aged five and seven years. If the ability of the immune system to respond to pathogens is altered by exposure to these compounds, it is possible that other functions of the immune system, i.e., neurological development, can be altered as well.

Conflict of interest

None of the authors have any conflict of interest of any kind.

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