

Studies in Experimental Autoimmune Encephalomyelitis Do Not Support Developmental Bisphenol A Exposure as an Environmental Factor in Increasing Multiple Sclerosis Risk

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Multiple sclerosis (MS), a demyelinating immune-mediated central nervous system disease characterized by increasing female penetrance, is the leading cause of disability in young adults in the developed world. Epidemiological data strongly implicate an environmental factor, acting at the population level during gestation, in the increasing incidence of female MS observed over the last 50 years, yet the identity of this factor remains unknown. Gestational exposure to bisphenol A (BPA), an endocrine disruptor used in the manufacture of polycarbonate plastics since the 1950s, has been reported to alter a variety of physiological processes in adulthood. BPA has estrogenic activity, and we hypothesized that increased gestational exposure to environmental BPA may therefore contribute to the increasing female MS risk. To test this hypothesis, we utilized two different mouse models of MS, experimental autoimmune encephalomyelitis (EAE) in C57BL/6J mice (chronic progressive) and in SJL/J mice (relapsing-remitting). Dams were exposed to physiologically relevant levels of BPA in drinking water starting 2 weeks prior to mating and continuing until weaning of offspring. EAE was induced in adult offspring. No significant changes in EAE incidence, progression, or severity were observed with BPA exposure, despite changes in cytokine production by autoreactive T cells. However, endocrine disruption was evidenced by changes in testes development, and transcriptomic profiling revealed that BPA exposure altered the expression of several genes important for testes development, including *Pdgfra*, which was downregulated. Overall, our results do not support gestational BPA exposure as a significant contributor to the increasing female MS risk.

Key Words: multiple sclerosis; experimental autoimmune encephalomyelitis; central nervous system; T cell; Bisphenol A; environmental exposure; gestational; developmental; autoimmunity; testes; endocrine disruption; PDGF.

Bisphenol A (BPA) is an organic compound used to manufacture polycarbonate plastics and epoxy resins, many of which

are used in food packaging (e.g., in can linings and polycarbonate plastic containers). BPA was first synthesized in 1891, and evidence of its estrogenicity came from experiments in the 1930s (Dodds and Lawson, 1938). In fact, BPA was intended for clinical use, but because it was less potent than diethylstilbestrol (DES, which was formulated around the same time), it was never used clinically. Subsequently, polymer chemists discovered that it could be polymerized to form polycarbonate plastic, and it has been in commercial use since 1957. Unfortunately, the ester bond that links BPA monomers to one another to form a polymer is not stable and hence the polymer decays with time, releasing BPA into materials with which it comes into contact, including food or water. Today, it can be consistently detected in the circulation and tissues of humans (Bushnik *et al.*, 2010). Multiple studies have shown that at low levels of exposure BPA can act as an endocrine disruptor, altering many physiological processes (Soto and Sonnenschein, 2010). BPA exposure during development (*in utero* or neonatal) can have particularly profound effects, many of which are manifested in adulthood. Importantly, maternal exposure to BPA has been shown to suppress DNA methylation in developing pups and change offspring phenotypes by stably altering the epigenome (Dolinoy *et al.*, 2007).

There is some evidence that developmental BPA exposure can alter the immune system in the adult state, although this has not been explored in detail. In general, BPA exposure in mice is associated with enhanced cytokine and antibody production, and decreased numbers of regulatory T cells (Alizadeh *et al.*, 2006; Ohshima *et al.*, 2007; Yan *et al.*, 2008; Yoshino *et al.*, 2004; Youn *et al.*, 2002; Yurino *et al.*, 2004), although many of these reports focused on adult, as opposed to gestational, exposure to BPA. Importantly, BPA exposure exacerbates the development of experimental asthma (Bauer

et al., 2012; Midoro-Horiuti *et al.*, 2010), and higher urinary levels of BPA were associated with increased asthma risk in humans (Donohue *et al.*, 2013; Spanier *et al.*, 2012; Vaidya and Kulkarni, 2012). Whether gestational BPA exposure can play a role in autoimmune disease remains almost completely unexplored.

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) characterized by demyelination, axonal damage, and progressive neurological dysfunction (Noseworthy *et al.*, 2000). MS is the most common disabling neurologic disease of young adults and adolescents, affecting ~400,000 individuals in the United States and ~2.5 million individuals worldwide (Courtney *et al.*, 2009). MS susceptibility is characterized by a maternal parent-of-origin (MPOO) effect and increased female penetrance. The MPOO effect has been well documented in studies of half-siblings (Ebers *et al.*, 2004), sibships including dizygotic twins (Willer *et al.*, 2003), a large extended Dutch pedigree (Hoppenbrouwers *et al.*, 2008), avuncular pairs (Herrera *et al.*, 2008), and Caucasian-North American Aboriginal admixture matings (Ramagopalan *et al.*, 2009). The incidence of female MS has been increasing over the last 50 years (Orton *et al.*, 2006) to the current female:male MS sex ratio of 3–4:1 in Caucasians and 6:1 in admixed African Americans (Weinstock-Guttman *et al.*, 2003). The temporal increase in female MS has been relatively rapid, suggesting that the increasing female MS risk may be due to environmental factors acting at the population level during gestation (MPOO effect) in an ethnic-specific manner, that is, gene-by-environment interactions occurring during gestation differentially affect MS risk more in female than in male offspring.

The increasing female MS incidence temporally coincides with increased exposure to BPA, and because gestational BPA exposure can have a profound impact that lasts through adulthood, it is a plausible environmental factor that could contribute to the MPOO effects in MS. Furthermore, susceptibility to MS and its primary animal model, experimental autoimmune encephalomyelitis (EAE) is strongly influenced by sex hormones, in particular estrogen (Ramagopalan *et al.*, 2009) and estrogen receptor signaling (Polanczyk *et al.*, 2003, 2004). Finally, the findings that BPA can enhance immune responses also support its potential role as an environmental factor influencing autoimmunity.

We hypothesized that developmental exposure to environmental endocrine disruptors such as BPA may contribute to the increasing female MS risk. Importantly, the increase in the female:male MS sex ratio has recently been shown to be due to an increase in relapsing-remitting (RR) MS (Ramagopalan *et al.*, 2010), which would be consistent with differential gene-by-BPA interactions specific to RR disease. In this regard, we and others have shown that responsiveness to estrogens and estrogen disruptors is genetically controlled (Griffith *et al.*, 1997; Nakai *et al.*, 1999; Roper *et al.*, 1999; Spearow *et al.*, 1999, 2001; Wall *et al.*, 2013; Zheng *et al.*, 1989). Hence, we investigated whether

gestational exposure to BPA differentially influenced the susceptibility of adult C57BL/6J (B6) mice to chronic progressive EAE and SJL/JCrHsd (SJL) mice to RR EAE, two different genetic models of MS. Although we observed evidence of mild endocrine disruption in BPA-exposed mice and alternations in immune responses, we found no significant effect on EAE susceptibility in either model, suggesting that BPA is not a good candidate for an environmental agent influencing MS susceptibility.

MATERIALS AND METHODS

Animals. C57BL/6J (B6) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). SJL/JCrHsd (SJL) mice were purchased from Harlan Laboratories. All animals were maintained under specific pathogen-free conditions on a 12:12 light-dark cycle and were fed Purina mouse pellets (Ralston-Purina, St Louis, MO) and water *ad libitum*, except where noted. The experimental procedures performed in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Vermont; IACUC protocol number 12-031, approved April 2012.

BPA exposure. Dams were purchased from the animal vendor at 6 weeks of age and rested for 2 weeks after arrival at the animal facility at the University of Vermont. Subsequently, the dams were switched to the appropriate diet, either standard mouse chow (Purina) or casein-based phytoestrogen-free (PF) diet (AIN-93G Rodent Diets; Research Diet, Inc.) for 1 week, followed by introduction of BPA at 10 µg/ml in 1% ethanol in the drinking water, or control 1% ethanol drinking water. The dose of BPA was chosen because it has been shown to result in BPA accumulation in mouse tissues at concentrations comparable with that in humans (Kabuto *et al.*, 2004). Stock BPA (Sigma-Aldrich, St Louis, MO) was dissolved at 1 mg/ml in 100% ethanol and diluted 1:100 in drinking water. Drinking water was purified using the Milli-Q Academic A10 system (Millipore) and was provided in glass water bottles with rubber stoppers. Water was replaced 2–3 times per week. Animals were housed in polysulfone cages, which do not leach significant amounts of BPA (Midoro-Horiuti *et al.*, 2010). Two weeks following the start of BPA exposure, breeder males were introduced for up to 4 weeks. BPA, control water, and special chow were maintained until the pups were weaned at 3 weeks of age, at which point the pups were switched to standard mouse chow and drinking water. Anogenital distance (AGD) and weight were measured at weaning. Weight-adjusted AGD was calculated by dividing AGD by the pup weight (which was normalized by the average pup weight for a given sex in the normal diet control group).

Induction and evaluation of EAE. EAE was induced and evaluated in B6 and SJL mice essentially as described previously (Noubade *et al.*, 2011). Briefly, 8- to 12-week-old mice were injected sc with an emulsion containing 100 µg of myelin oligodendrocyte glycoprotein 35–55 (MOG_{35–55}) peptide (B6) or 100 µg of proteolipid protein 139–151 (PLP_{139–151}) peptide (SJL) and complete Freund's adjuvant (CFA) (Sigma-Aldrich) supplemented with 200 µg of *Mycobacterium tuberculosis* H37RA (Difco Laboratories, Detroit, MI) in the posterior right and left flank; 1 week later, all mice were similarly injected at two sites on the right and left flank anterior of the initial injection sites (2× MOG_{35–55} or PLP_{139–151} + CFA). Mice were scored daily starting at day 10 postinjection for clinical signs of EAE according to the following scale: 0 = normal; 1 = limp tail; 2 = moderate hind limb weakness or mild ataxia; 3 = hind limb paralysis; 4 = hind limb paralysis with urinary or fecal incontinence; 5 = hind limb paralysis with front limb weakness or paralysis, or death. Clinical quantitative trait variables were generated as previously described (Noubade *et al.*, 2011).

Cytokine quantification by ELISA. Mice were immunized with 2× MOG_{35–55} (B6) or PLP_{139–151} (SJL) + CFA, and spleen and draining lymph node

(DLN) were harvested on day 30 postimmunization. Red blood cells were lysed by hypotonic lysis with Gey's solution. Single-cell suspensions were prepared at 1×10^6 cells/ml in RPMI containing 10% fetal bovine serum (Hyclone, Logan, UT) and stimulated with 50 $\mu\text{g/ml}$ of MOG₃₅₋₅₅ or PLP₁₃₅₋₁₅₁. Cell culture supernatants were collected at 72 h and cytokine levels were measured by ELISA as described below.

For the detection of cytokines in the cell culture supernatants, ELISAs were performed as described previously (Noubade *et al.*, 2011), using the capture mAbs: anti-IFN γ and anti-IL-17A and the corresponding biotinylated mAbs (BioLegend). Other ELISA reagents included: horseradish peroxidase-conjugated avidin D (Vector Laboratories, Burlingame, CA), TMB microwell peroxidase substrate and stop solution (Kirkegaard and Perry Laboratories, Gaithersburg, MD). rIFN γ and rIL-17A (BioLegend) were used as standards.

Proliferation assays. Mice were immunized with $2 \times$ MOG₃₅₋₅₅ (B6) or PLP₁₃₅₋₁₅₁ (SJL) + CFA, and spleen and DLN cells were collected on day 10 postimmunization. Single-cell suspensions of spleen and DLN were prepared at 2.5×10^5 cells/well in RPMI medium and stimulated in a 96-well plate with different concentrations of MOG₃₅₋₅₅ or PLP₁₃₅₋₁₅₁ for 72 h. During the last 18 h of culture, 1 μCi of ^3H -thymidine (PerkinElmer) was added. Cells were harvested onto glass fiber filters and thymidine uptake was determined with a liquid scintillation counter.

Tissue collection. For collection of uterus, testes, and ovaries, mice were terminally anesthetized with ketamine and intracardially perfused with PBS. For each animal, one of the testes or ovaries, and one of the uterine horns was snap frozen in liquid nitrogen for RNA extraction. The other uterine horn, ovary, or testis was fixed in 10% neutral buffered formalin for 24 h, then stored in 100% ethanol until further processing. The tissues were subsequently processed for paraffin embedding and hematoxylin and eosin (H&E) staining.

Microscopic evaluation of tissues. At least two 5- μm sections of testes, ovaries, or uterus were stained with H&E and examined by a board-certified veterinary pathologist (BD) under light microscopy. Tissues were examined without knowledge of treatment. Sections were excluded from evaluation if there were tissue or sectioning artifacts related to processing. Alterations observed were graded in severity representing minimal, mild, moderate, or severe.

RNA extraction and cDNA synthesis. RNA was extracted from frozen tissues using TRIzol reagent (Invitrogen) in accordance with manufacturer's instructions. Quantitative and qualitative analysis of RNA was performed using Nanodrop1000 spectrophotometer (ThermoScientific) and the Agilent 2100Bioanalyzer (Agilent Technologies, Santa Clara, CA), respectively. One microgram of the extracted total RNA was used for cDNA synthesis. First-strand cDNA was synthesized using SuperScript III and random hexamers (Life Technologies Corporation, Grand Island, NY) according to manufacturer's instructions.

Quantitative real-time PCR analysis of mRNA expression. Quantitative real-time PCR (qRT-PCR) was performed essentially as previously described (Williams *et al.*, 2008). Briefly, each reaction was performed using 10 ng cDNA, 1 pmol each of forward and reverse primers, and SYBR green PCR master mix (Life Technologies Corporation) in a 10 μl final reaction volume. Mouse 18S rRNA was used for normalization of mRNA expression. All runs were performed using the Applied Biosystems 7500 Fast Real-Time PCR System (Life Technologies Corporation), and all amplification products were checked with dissociation-curve analysis. Only amplifications with one defined melting curve were used for further analysis. Relative expression and SD were calculated using the comparative threshold cycle ($\Delta\Delta\text{Ct}$) method.

Microarray experiment and analysis and bioinformatics analysis. Two-color comparative microarray technique and Operon's 70-mer-oligonucleotide arrays (Microarray Inc., Huntsville, AL) were used, covering the mouse transcriptome of 37,000 genes and variants. Analysis was performed essentially as previously described (Williams *et al.*, 2008). RNA from 15 control mice was pooled and compared with a pool of RNA from 7 BPA-treated mice, and the comparison was replicated and dye swapped totaling four replicates. From

each pool, 15–20 μg of total RNA were used per cDNA synthesis and labeling reaction (eight in total). Labeled cDNA was hybridized to arrays for 35–40 h at 42°C, washed, and scanned at 10 μm resolution using the Axon GenePix 4400A (Molecular Devices, LLC, Sunnyvale, CA) scanner. The obtained Tiff images were analyzed using the GenePix Pro 6.1 software (Molecular Devices, LLC). All data analysis steps were performed in the R environment as previously described (Richter *et al.*, 2006). Differentially expressed genes were identified using the empirical Bayes moderated *t*-test within the Limma package, which generated a list of differentially expressed genes with *p* values and *B* scores. Genes were considered differentially expressed when *p* < 0.05 and log₂ fold change > 10.451. Microarray data are available on NCBI's GEO under accession GSE45917.

Gene ontology and subnetwork enrichment analysis were performed for differentially expressed genes using the Ariadne Pathway Studios program (Elsevier Inc., Rockville, MD). Because this type of analysis performs better with a large number of genes and tolerates false positives, a less stringent cutoff of *p* < 0.05 combined with log₂ fold change > 10.21 was used for the gene list of differentially expressed genes subjected to this analysis. *p* Values indicating overrepresentation using the Ariadne Pathway Studios Gene Ontology analysis were calculated with Fisher's exact test and considered significant when *p* < 0.05.

Statistical analyses. Statistical analyses were performed using GraphPad Prism 6 software (GraphPad software Inc.). Significance of differences was determined using one-way or two-way ANOVA or *t*-test, as described in the figure legends. For all analyses, *p* < 0.05 was considered significant. Numerical data are displayed as mean \pm SE of the mean throughout.

RESULTS

We utilized two different mouse models of MS: chronic progressive EAE in B6 mice and RR-EAE in SJL mice. In order to assess the effect of gestational BPA exposure, B6 and SJL dams were exposed to 10 $\mu\text{g/ml}$ BPA in drinking water beginning 2 weeks prior to mating and continuing until the offspring were weaned. This dose was chosen because it has been shown to result in BPA accumulation in mouse tissues (Kabuto *et al.*, 2004) at concentrations comparable with that in humans (summarized in Midoro-Horiuti *et al.*, 2010). Furthermore, this dose of BPA was sufficient to cause enhanced murine asthma (Midoro-Horiuti *et al.*, 2010). BPA-exposed mice were fed a casein-based diet free of soy protein, the major source of dietary phytoestrogens, such as genistein (Barnes, 2010). An extra control group receiving normal diet was included to test for effects of the PF diet. Another control group received normal diet and normal water to test for the effects of 1% ethanol in the drinking water of the other groups. This group did not show any significant difference from the normal diet + 1% ethanol drinking water group in any of the measured parameters (data not shown).

Effect of BPA Exposure on EAE

EAE was induced in adult (8–12 weeks) offspring from BPA-exposed and control B6 mothers by immunization with MOG₃₅₋₅₅ emulsified in CFA on D0 and D7 (Noubade *et al.*, 2011). This EAE induction protocol results in less severe EAE compared with the standard $1 \times$ MOG₃₅₋₅₅ + CFA + pertussis toxin protocol used in most EAE studies (Stromnes and Goverman, 2006), hence readily allowing for detection of

disease exacerbation, that is, onset, severity, and duration of clinical disease course. Immunized B6 mice showed typical chronic progressive EAE, which in males was modestly more severe in the normal diet group compared with PF diet (Fig. 1). However, the BPA-exposed group did not show a difference in EAE severity compared with the PF diet control group (Fig. 1). No differences in EAE were observed with either treatment in female B6 mice. EAE incidence in B6 mice was 100%.

Immunized SJL mice showed EAE remission after the first acute attack, followed by a second episode of lesser severity. The time of onset for the first and second EAE episodes was quite variable (Figs. 2A and 2B); hence, cumulative disease score (CDS) (Noubade *et al.*, 2011) was used as the primary quantitative measure of disease severity for each episode (Figs. 2C–F). None of the treatments affected CDS in either sex in SJL mice (Fig. 2). We also found that although the incidence of acute phase disease was high (90–100%), the incidence of relapse was ~60% (Figs. 2G and 2H). Most importantly, BPA treatment did not significantly affect incidence of relapse; therefore, the incidence of RR disease was not significantly different among treatment groups. Taken together, these results suggest that gestational BPA exposure does not affect the clinical course of EAE in the adult mouse.

Effect of BPA Exposure on Antigen-Specific Cytokine Responses

BPA has been reported to lead to dysregulated cytokine responses. To address this possibility in an organ-specific autoimmune disease setting, we measured MOG_{35–55}-specific responses in peripheral lymphoid organs by ELISA for IFN γ and IL-17, which are the signature cytokines produced by encephalitogenic Th1 and Th17 cells, respectively. No significant changes were observed in male B6 mice (Figs. 3A and 3C). In female B6 mice, the PF diet resulted in a significant upregulation of IL-17, an effect that was reversed by BPA exposure (Fig. 3D), suggesting that xeno- and phytoestrogens may suppress IL-17 production in a sex-specific fashion in B6 mice. These data were reanalyzed by comparing cytokine production by males versus females in each group to look for

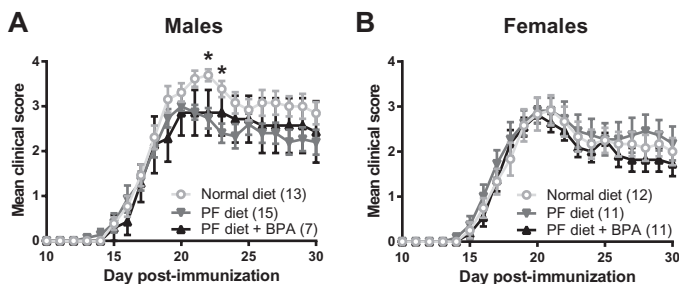


FIG. 1. Effect of gestational BPA exposure on EAE in B6 mice. EAE was induced in male (A) and female (B) B6 mice using the 2 \times MOG_{35–55} + CFA protocol. Data were analyzed two-way ANOVA and *post hoc* comparison. *indicates significant difference from PF diet, $p < 0.05$. The numbers in parentheses indicate the number of animals in each group.

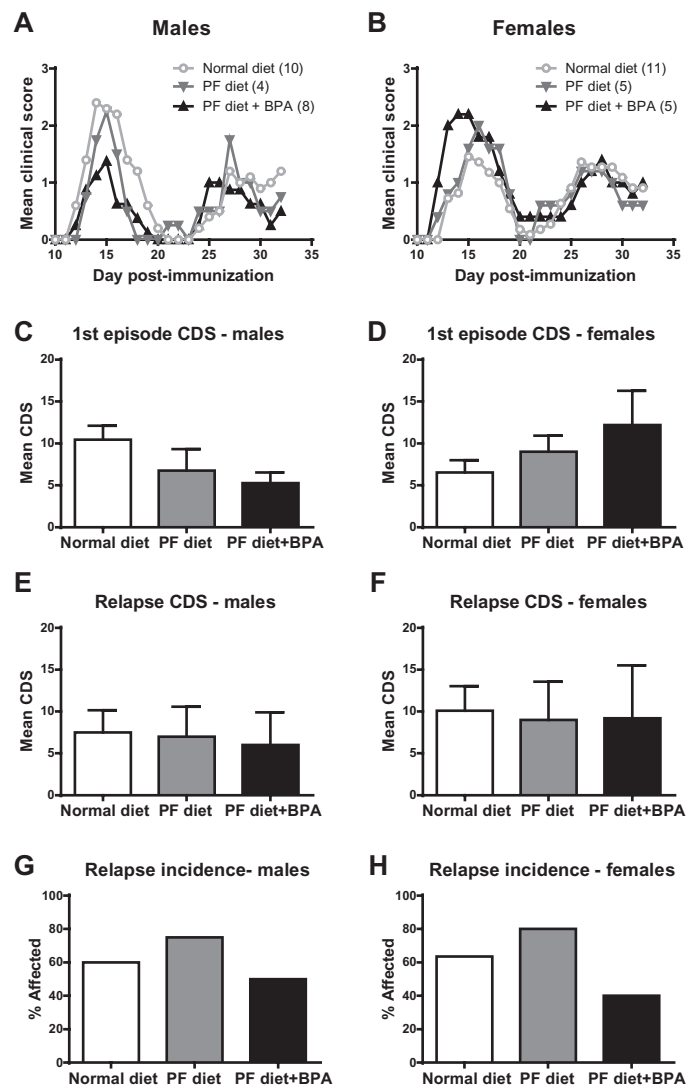


FIG. 2. Effect of gestational BPA exposure on EAE in SJL mice. EAE was induced in male (A, C, E, and G) and female (B, D, F, and H) B6 mice using the 2 \times or PLP_{139–151} + CFA protocol. CDS was calculated by adding the mean clinical scores for days 10–21 (first episode, C and D) or days 22–32 (second episode/relapse, E and F). Percentage of SJL mice exhibiting relapse is shown in (G and H). EAE course (A and B) was analyzed by two-way ANOVA with *post hoc* comparisons. CDS (C–F) was analyzed by one-way ANOVA with *post hoc* comparisons. Incidence of relapse (G and H) was analyzed by the chi-square and Fisher's exact tests. No significant differences were found among treatment groups. The numbers in parentheses indicate the number of animals in each group.

sexual dimorphisms and the effects of exposures on them. B6 males in the regular diet control group produced more IFN γ and IL-17 than females, consistent with our previous unpublished observations (Krementsov, Case, Saligrama, and Teuscher, unpublished data). However, this sexual dimorphism was reversed by BPA exposure for IFN γ and by PF diet for IL-17 (Figs. 3E and 3F).

In male SJL mice, the PF diet resulted in a significant decrease in IL-17 production, but this was not reversed by BPA

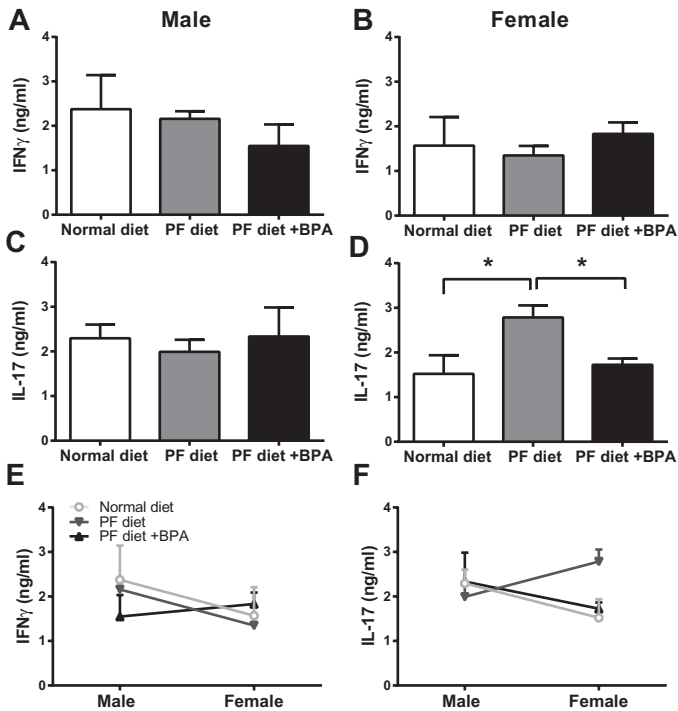


FIG. 3. Effect of gestational BPA exposure on antigen-specific cytokine production in B6 mice. Cells from spleen and LN were isolated from male (A, C, and E) and female (B, D, and F) B6 mice immunized with the 2 \times MOG₃₅₋₅₅ + CFA protocol and restimulated with 50 μ g/ml MOG₃₅₋₅₅ for 72 h. Cytokine production in the supernatants was measured by ELISA and analyzed by one-way ANOVA with *post hoc* comparisons. *indicates $p < 0.05$. $n = 5$ for all groups.

treatment (Fig. 4C). No significant changes were observed in female SJL mice with any treatment, although the pattern of IL-17 production (Fig. 4D) was remarkably similar to B6 mice (Fig. 3D), where xenoestrogens suppressed IL-17 production. In terms of sexual dimorphisms, IL-17 production was higher in males than in females, as in B6 mice. This dimorphism was eliminated by the PF diet, but only partially restored by BPA treatment (Fig. 4F). Antigen-specific proliferative responses were also measured, and these did not differ for any of the treatments in either B6 or SJL mice (Fig. 5). Taken together, our results show that gestational xenoestrogen exposure can modulate adult cytokine responses in a gene-by-sex-specific manner, but these effects are not sufficient to affect EAE severity.

Secondary Indicators of Endocrine Disruption

In order to confirm that our BPA exposure protocol produced the desired endocrine-disruptive effects, we tested multiple parameters associated with endocrine disruption, including body weight, AGD, adult reproductive organ weight and histology, and global gene expression in the gonad. Changes in AGD can serve as a marker of endocrine disruption, and potent estrogens such as DES can cause changes in AGD (LaRocca *et al.*, 2011). Pups born to control and BPA-exposed dams were weighed at weaning (3 weeks of age) and their AGD and weight were measured. We found no effect of either treatment

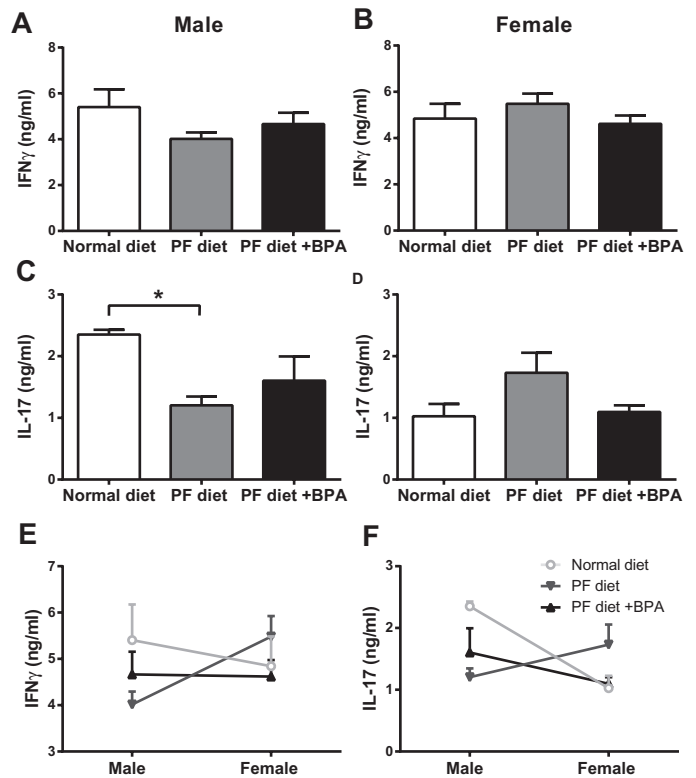


FIG. 4. Effect of gestational BPA exposure on antigen-specific cytokine production in SJL mice. Cells from spleen and LN were isolated from male (A, C, and E) and female (B, D, and F) SJL mice immunized with the 2 \times PLP₁₃₅₋₁₅₁ + CFA protocol and restimulated with 50 μ g/ml PLP₁₃₅₋₁₅₁ for 72 h. Cytokine production in the supernatants was measured by ELISA and analyzed by one-way ANOVA with *post hoc* comparisons. *indicates $p < 0.05$. $n = 5$ for all groups, except the male PF diet group, where $n = 4$.

on weight-corrected AGD in either strain (Figs. 6A–D). Pup weight was also not affected by either treatment in B6 mice (Figs. 6E and 6F); however, in female SJL mice, PF diet caused an increase in pup weight, an effect that was reversed by BPA exposure (Figs. 6G and 6H), a finding consistent with a previous report showing reduced body weight at postnatal day 22 due to gestational BPA exposure (Honma *et al.*, 2002).

Gestational BPA exposure has been reported to affect sexual differentiation in mice (Honma *et al.*, 2002; Xi *et al.*, 2011) and in humans (Miao *et al.*, 2011), particularly in males. Thus, we tested whether gestational BPA exposure was associated with changes in adult gonads in B6 and SJL mice. No significant changes in testis or uterus wet weight were found with either treatment (data not shown). At the histological level, uteri and ovaries from BPA-exposed mice had no remarkable lesions, although quantitative oocyte counts were not performed (data not shown). In testes, a basal low level of individual germ cell apoptosis was observed in both control and BPA-treated mice (Table 1). This observation was consistent with a background level of germ cell apoptosis typical in mouse testes in either mitotic or meiotic stages of spermatogenesis. A diagnosis of degeneration was used when there was greater level of germ

cell apoptosis compared with baseline, and a notable loss of germ cells or a general disruption of normal maturation observed in individual cross-sections of seminiferous tubules. Degeneration in the testes was diagnosed more often in BPA-exposed mice and with a slight increase in severity (Table 1) compared with other control groups. Degeneration was most common in late stages of spermatogenesis, VIII–XII.

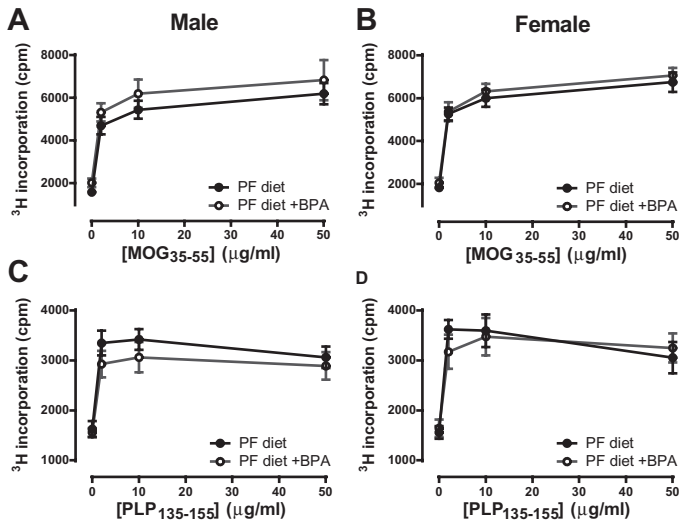


FIG. 5. Effect of gestational BPA exposure on antigen-specific proliferative responses. Cells from spleen and LN were isolated from male (A and C) and female (B and D) B6 mice (A and B) or SJL mice (C and D) immunized with the 2× MOG₃₅₋₅₅ or PLP₁₃₅₋₁₅₁ + CFA protocols, respectively. Cells were restimulated with the indicated dose of peptide for 72 h and ³H-thymidine incorporation during the last 18 h was measured. Data were analyzed by two-way ANOVA. No significant differences were found. $n = 5$ for all groups, except the SJL male PF diet group, where $n = 4$.

Based on these findings, we hypothesized that gestational BPA exposure resulted in changes in gene expression that persisted in the adult testes. To test this hypothesis, RNA was extracted from testes of B6 mice from the BPA-treated and PF diet control-treated groups, and subjected to microarray analysis. Results from the microarray experiments indicated that BPA exposure resulted in subtle, but detectable changes in gene expression (Table 2). Pathway analysis of differentially expressed genes suggested involvement of the innate immune response, as well as dysregulation of cell proliferation, spermatogenesis, and apoptosis, the latter being consistent with histopathological analyses (Table 3). Furthermore, in our microarray results, we observed downregulation of *Pdgfa* (platelet-derived growth factor, alpha), a gene well documented to be critical for proper testes development and function (Basciani *et al.*, 2010). This downregulation was confirmed by qRT-PCR in B6 mice, yet was absent in SJL mice (Fig. 7), suggesting a genetic component in the response to BPA, consistent with other's observations (Spearow *et al.*, 1999, 2001) and our reports on genetic control of responsiveness to estrogen (Griffith *et al.*, 1997; Wall *et al.*, 2013). Taken together, these results indicate that BPA exposure caused subtle, but detectable degeneration in the adult testes in our study.

DISCUSSION

Based on several lines of evidence, we hypothesized that gestational BPA exposure may be responsible for the increased MS risk observed in females over the past decades. This hypothesis was tested using the EAE model in mice exposed to BPA during gestation and early development. Although BPA and dietary phytoestrogens affected antigen-specific production of some of

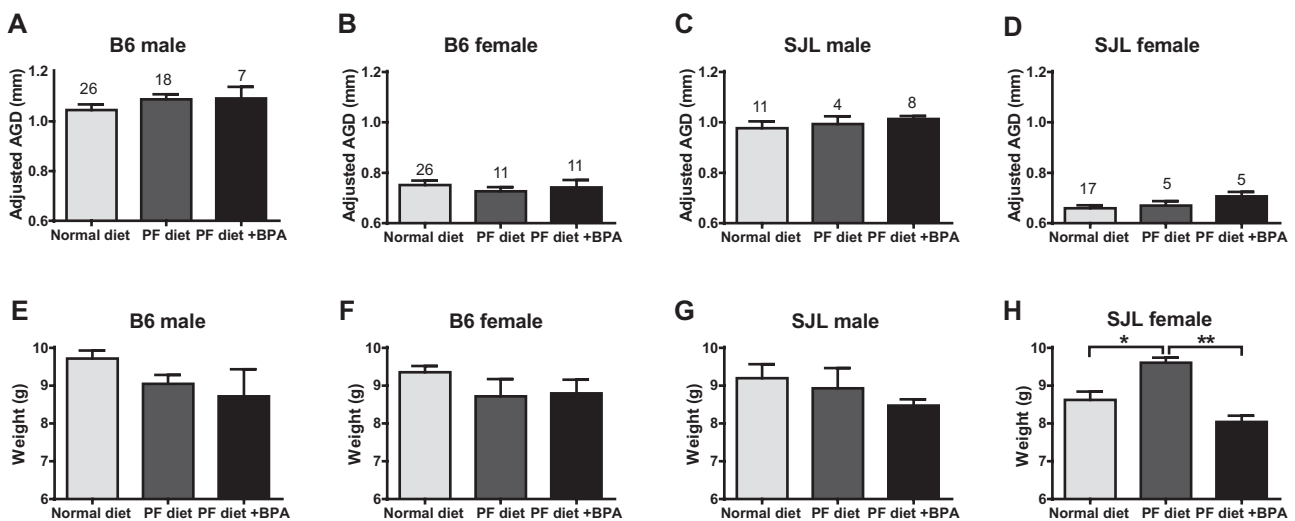


FIG. 6. Effect of gestational BPA on weight and AGD. AGD (A–D) and weight (E and F) were measured at weaning (3 weeks of age) in B6 mice (A, B, E, and F) or SJL mice (C, D, G, and H). AGD was adjusted by weight as described in the Materials and Methods section. Data were analyzed by one-way ANOVA. *indicates $p < 0.05$, **indicates $p < 0.01$. Numbers above the bars indicate the number of animals in each group. The number of animals for weight measurements was the same as what is listed for AGD measurements.

TABLE 1
Effect of BPA Exposure on Testes Histology

	B6			SJL		
	Normal diet	PF diet	PF diet + BPA	Normal diet	PF diet	PF diet + BPA
Mice/group	13	15	7	10	4	8
No. of testes examined ^a	7	10	5	9	3	8
Diagnosis						
Degeneration ^b	1	0	2	0	0	5
Severity grade ^c						
1	1		2			3
2						2
3						
4						
Apoptosis	0	3	0	2	3	3
Severity grade ^c						
1		3		2	3	3
2						
3						
4						
Tubular atrophy ^d	0	1	1	0	0	0

Note. At least two 5- μ m sections of testes, ovaries, or uterus were stained with H&E and examined by a board-certified veterinary pathologist under light microscopy.

^aTestes from some mice were not examined due to sectioning artifacts, etc.

^bDegeneration was used when there was greater than individual cell apoptosis and notable loss of germ cells or a general disruption of normal maturation process in individual cross-sections of seminiferous tubules.

^cSeverity grades 1–4 correlate to minimal, mild, moderate, or severe, respectively. The grade represents the extent or distribution (focal, multifocal, locally extensive, diffuse).

^dAtrophy is defined as complete loss of spermatogenesis, this could be in one to several tubules, and tubules were lined by Sertoli cells only.

the cytokines associated with EAE, these effects did not translate into significant changes in EAE severity. Given the fact that the efficacy of our BPA exposure protocol was confirmed by alterations in several indicators of endocrine disruption, including pup weight and reproductive organ development, our results do not support BPA as a modifier of CNS autoimmunity.

Multiple reports examined immune function and cytokine production by T lymphocytes after gestational BPA exposure. [Holladay et al. \(2010\)](#) reported an increase in concanavalin A–induced IL-17, granulocyte-macrophage colony-stimulating factor, granulocyte colony-stimulating factor, and IL-4 production in male C57B6/129svj mice exposed to BPA *in utero*, although this study was statistically underpowered and has not been reproduced. Other reports observed enhanced Th1 and Th2 responses in DBA/1 and BALB/c mice ([Yan et al., 2008](#); [Yoshino et al., 2004](#)). Additional reports showed diminished T regulatory cell (Treg) numbers ([Ohshima et al., 2007](#); [Yan et al., 2008](#)), in one case together with enhanced Th2 but diminished Th1 responses ([Ohshima et al., 2007](#)). Consistent with enhanced Th2 responses and diminished Treg numbers, gestational BPA exposure resulted in exacerbated experimental asthma in mice ([Bauer et al., 2012](#); [Midoro-Horiuti et al., 2010](#); [Nakajima et al., 2012](#)). Importantly, the asthma findings in mice are also supported by several recent human studies that show associations between BPA levels in urine and asthma incidence ([Donohue et al., 2013](#); [Spanier et al., 2012](#); [Vaidya and Kulkarni, 2012](#)). In contrast, with regard to autoimmune

disease, there is one recent report that shows a lack of effect of developmental or adult BPA exposure in an experimental colitis model, a model of tissue-specific autoimmunity driven by Th1/17 cells ([Roy et al., 2013](#)), and another report examining the role of adult BPA exposure in a lupus model, where BPA actually dampened Th1 responses and delayed autoimmunity ([Sawai et al., 2003](#)). Lastly, in full agreement with our results, the aforementioned study also reported a lack of an effect of gestational BPA exposure on EAE, although experimental data pertaining to this observation or information regarding the EAE model used were not shown ([Roy et al., 2013](#)). Taken together with our results demonstrating no effect of gestational BPA exposure in a Th1/17-driven EAE model, these findings suggest a more prominent role for BPA in allergic responses, with no effect on Th1/17-mediated autoimmunity. Similarly, gestational BPA exposure did not affect adaptive antiviral responses or virus clearance in an influenza infection model in mice ([Roy et al., 2012](#)).

Our findings that gestational BPA exposure influenced gene expression in the adult testes are in line with previous studies demonstrating various effects of similar exposure on male gonad development ([Honma et al., 2002](#); [Miao et al., 2011](#); [vom Saal et al., 1998](#); [Xi et al., 2011](#)), although to our knowledge, a genome-wide transcriptomic analysis has never been carried out. Thus, our analysis not only generates a useful database for comparisons with effects of other endocrine disruptors on testes but also generates insights into the mechanism of

TABLE 2
List of Differentially Expressed Genes in BPA-Treated Mice Versus PF Diet Control

Genes downregulated by BPA			Genes upregulated by BPA		
Gene	LogFC	p Value	Gene	LogFC	p Value
<i>Lars2</i>	-1.47	0.000	<i>Ikbip</i>	1.14	0.035
<i>Nap11l</i>	-1.29	0.002	<i>Fam82a1</i>	0.95	0.041
<i>Vrk3</i>	-1.08	0.001	<i>Eno2</i>	0.83	0.042
<i>Ropn1l</i>	-0.91	0.009	<i>1700025E21Rik</i>	0.83	0.011
<i>Irgc1</i>	-0.85	0.000	<i>Serpina3n</i>	0.82	0.010
<i>Dpcd</i>	-0.84	0.005	<i>Tmx3</i>	0.81	0.004
<i>Eif2b1</i>	-0.77	0.022	<i>Slit1</i>	0.77	0.046
<i>Mocos</i>	-0.75	0.006	<i>Msln</i>	0.75	0.024
<i>Efcab8</i>	-0.74	0.003	<i>C3</i>	0.70	0.002
<i>Gm5617</i>	-0.70	0.010	<i>Tmem50a</i>	0.68	0.015
<i>Git1</i>	-0.67	0.008	<i>Dcum1d4</i>	0.65	0.027
<i>Tnp2</i>	-0.64	0.027	<i>Ssxb2</i>	0.62	0.016
<i>Rap1gds1</i>	-0.64	0.022	<i>Gpx3</i>	0.61	0.002
<i>Eprs</i>	-0.64	0.038	<i>Sdcbp</i>	0.61	0.012
<i>Hapln1</i>	-0.63	0.048	<i>Txndc9</i>	0.60	0.014
<i>Prm1</i>	-0.63	0.025	<i>Pdhb</i>	0.60	0.028
<i>Prss55</i>	-0.62	0.006	<i>Txndc9</i>	0.59	0.020
<i>Odf2</i>	-0.61	0.004	<i>Olfir706</i>	0.58	0.016
<i>Lrch4</i>	-0.61	0.012	<i>Gnpda2</i>	0.55	0.003
<i>Tbk1</i>	-0.61	0.014	<i>Gramd4</i>	0.54	0.028
<i>2610318N02Rik</i>	-0.59	0.002	<i>4930522H14Rik</i>	0.53	0.011
<i>Ubc</i>	-0.57	0.007	<i>Cenpp</i>	0.52	0.021
<i>Iars2</i>	-0.57	0.044	<i>Prpf40a</i>	0.52	0.008
<i>Mpg</i>	-0.56	0.049	<i>Sall4</i>	0.50	0.043
<i>Cisd2</i>	-0.55	0.021	<i>C1qc</i>	0.50	0.015
<i>Cdc42ep3</i>	-0.53	0.009	<i>Smoc2</i>	0.49	0.012
<i>Col15a1</i>	-0.52	0.048	<i>Serping1</i>	0.47	0.002
<i>Fam108a</i>	-0.52	0.002			
<i>Cdk2</i>	-0.52	0.003			
<i>Gapdhs</i>	-0.52	0.016			
<i>Rnf181</i>	-0.51	0.033			
<i>Zfp219</i>	-0.51	0.003			
<i>Cypt4</i>	-0.51	0.031			
<i>Pdgfa</i>	-0.49	0.047			
<i>Clpb</i>	-0.49	0.006			
<i>Amhr2</i>	-0.49	0.012			
<i>Trim36</i>	-0.46	0.005			
<i>1810013L24Rik</i>	-0.46	0.005			
<i>Larp1</i>	-0.45	0.010			

Note. RNA from testes from mice gestationally exposed to BPA or PF diet controls was subjected to microarray analysis as detailed in the Materials and Methods section. To generate this list, a cutoff of $p < 0.05$ and log₂ fold change (LogFC) $> |0.45|$ was used.

action of BPA. Gene pathway analysis suggests dysregulated proliferation and apoptosis, and this is supported by histological observations. We also observed strain-specific BPA-induced downregulation of *Pdgfa*, suggesting that the endocrine-disrupting effects of BPA on the testes in B6 and SJL mice may involve different molecular pathways. PDGF signaling is well documented to be critical for testes development and function. Previous reports show that BPA and other estrogens can stimulate testicular gonocyte proliferation in a PDGF-dependent manner *in vitro* (Thuillier *et al.*, 2010). It is likely that in our *in vivo* experiments, this process is dysregulated by continuous gestational BPA exposure, which in the end leads to downregulation of PDGF and germ cell apoptosis.

The finding that BPA exposure can alter the expression of receptors for PDGF in neonatal testes (Thuillier *et al.*, 2003) further supports dysregulation of the PDGF signaling pathway as a general theme in BPA-mediated endocrine disruption in the testes. Other differentially expressed genes of interest included cell cycle/apoptosis-related genes: *Vrk3*, *c3*, *Cdk2*, *Cdc42ep3*, *Ikbip*, *Nap11l*, *Smoc2* (Cerione, 2004; Kang and Kim, 2008; Lee *et al.*, 2007; Liu *et al.*, 2008; Park and Luger, 2006; Ricklin *et al.*, 2010); or genes with documented or hypothetical function in the testes or sperm development/motility: *Ropn1l/ASP*, *Dpcd*, *Ssxb2*, *Tnp2*, *Prm1*, *Odf2*, *Smoc2* (Chen *et al.*, 2003; Fiedler *et al.*, 2013; Haueter *et al.*, 2010; Hoyer-Fender *et al.*, 1998; Pazin and Albrecht, 2009; Zariwala

TABLE 3
Pathway Analysis of Differentially Expressed Genes in BPA-Treated Mice

Category	<i>p</i> Value
Gene set enrichment analysis	
Innate immune response	0.0003
Translation	0.0009
Complement activation	0.006
Carbohydrate metabolic process	0.021
Antiapoptosis	0.033
Negative regulation of cell proliferation	0.039
Spermatid development	0.049
Pathway	
<i>p</i> Value	
Subnetworks enrichment analysis	
Expression targets of IL1 family	0.010
Expression targets of TGFA	0.011
Expression targets of IL1A	0.021
Expression targets of CEBPA	0.041
Expression targets of HIF1A	0.043

Note. Gene ontology and subnetwork enrichment analysis were performed for differentially expressed genes using the Ariadne Pathway Studios program as detailed in the Materials and Methods section. *p* Values < 0.05 were considered significant.

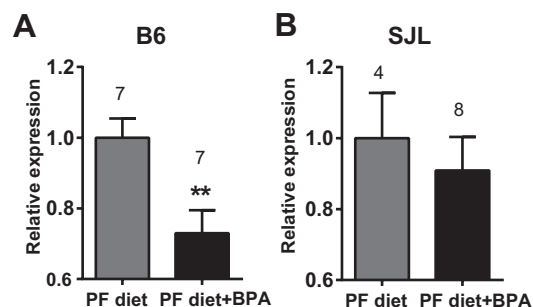


FIG. 7. Effect of gestational BPA exposure on *Pdgfra* expression in the adult testis. RNA was isolated from B6 (A) and SJL (B) testes and subjected to qRT-PCR analysis for *Pdgfra* expression. 18S RNA served as an endogenous control. Data were analyzed using unpaired *t*-test. **indicates *p* < 0.01. Numbers above the bars indicate the number of animals in each group.

et al., 2004; Zhao *et al.*, 2004). These results are also consistent with dysregulated apoptosis in the testes.

The observation that BPA-enhanced degeneration in the testes was most often associated with late stages of spermatogenesis suggests potential developmentally induced alterations in Sertoli cell-dependent germ cell maturation. The results from the testes microarray studies are consistent with the histopathologic observations and provide further support that this BPA exposure paradigm appears to affect Sertoli cells. A limitation of this analysis was the few testes available from diet-matched controls and exclusion of testes due to substantial artifact from fixation or tearing of tissue from sectioning (Table 1). Our results are consistent with multiple previous

reports showing effects of BPA on male gonad development and sperm production (Honma *et al.*, 2002; Miao *et al.*, 2011; vom Saal *et al.*, 1998; Xi *et al.*, 2011) but are at odds with a different study showing a lack of effect of BPA on Sertoli cells (LaRocca *et al.*, 2011). However, the latter report used a much shorter BPA exposure protocol in C57BL/6N mice (gestational days 10–16), whereas in our study exposure began prior to pregnancy. Moreover, C57BL/6N mice, a substrain of C57BL/6J mice that have been separated since 1951, have extensive genetic differences (<http://phenome.jax.org>) that most certainly contribute to phenotypic differences between the substrains (Bryant, 2011). Repeated studies and follow-up studies focusing specifically on Sertoli cell function will be necessary to confirm our observations.

Many recent reports have implicated BPA exposure in a wide variety of physiological abnormalities, and, as a result of this, many countries have banned the use of BPA in food packaging (Erler and Novak, 2010). Yet, compared with other xenoestrogens, for example, DES, there is often a lack of consensus on the effects of BPA. This may be in part due to the fact that many different exposure protocols and animal models are used and the doses of BPA vary widely from study to study (Richter *et al.*, 2007). It is now recognized that some physiological responses to BPA and other environmental agents do not always show classical dose dependence (Fagin, 2012). This is also a limitation in the interpretation of our study, as only one dose of BPA was used, although this exact dose and exposure protocol was sufficient to exacerbate experimental asthma in mice in several studies (Midoro-Horiuti *et al.*, 2010; Nakajima *et al.*, 2012) and affected brain and testes development in another study (Kabuto *et al.*, 2004). Another potential limitation of our study is that animals were maintained on a normal diet (containing phytoestrogens) after developmental BPA exposure; hence, it is possible that this adulthood exposure to phytoestrogens may have negated any effects of developmental BPA exposure on EAE. However, this is not supported by our data because maintaining the animals on normal chow after weaning did not mask the effects of BPA on cytokine responses (Figs. 3 and 4). Although BPA binds to and activates estrogen receptors 1000- to 10,000-fold less efficiently than estradiol (Kuiper *et al.*, 1998), BPA can pass the placenta and result in unparalleled exposure to fetuses (Balakrishnan *et al.*, 2010; Nishikawa *et al.*, 2010). In addition, many reports suggest that it may activate nonclassical estrogen signaling pathways, or affect other nuclear receptors, such as the arylhydrocarbon receptor and peroxisome proliferator-activated receptors (Richter *et al.*, 2007; Rogers *et al.*, 2013). Although this may be another explanation for the lack of consistent results with BPA studies, it is also possible that BPA exposure alone may not be sufficient to affect the complex physiological processes that culminate in autoimmune disease. In combination with exposure to other environmental agents, whether estrogenic (e.g., equol) (Shor *et al.*, 2012) or not (e.g., vitamin D)

(Handunnetthi *et al.*, 2010), gestational BPA exposure may indeed influence the incidence of adult onset disease. Further studies will be needed to address these possibilities. At present, our data suggest that gestational BPA exposure does not affect EAE development, and thus do not support gestational BPA exposure as a significant contributor to the increasing female MS risk.

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