

The Effects of Maternal Exposure to Bisphenol A on Allergic Lung Inflammation into Adulthood

Stephen M. Bauer,* Anirban Roy,* Jason Emo,† Timothy J. Chapman,† Steve N. Georas,*‡ and B. Paige Lawrence*‡¹

*Department of Environmental Medicine, †Division of Pulmonary and Critical Care Medicine, and ‡Department of Microbiology and Immunology, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

¹To whom correspondence should be addressed at Department of Environmental Medicine, University of Rochester School of Medicine and Dentistry, 601 Elmwood Avenue, Box EHSC, Rochester, NY 14642. Fax: (585) 276-0239. E-mail: Paige_Lawrence@urmc.rochester.edu.

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Bisphenol A (BPA) is a high-production volume chemical classified as an environmental estrogen and used primarily in the plastics industry. BPA's increased usage correlates with rising BPA levels in people and a corresponding increase in the incidence of asthma. Due to limited studies, the contribution of maternal BPA exposure to allergic asthma pathogenesis is unclear. Using two established mouse models of allergic asthma, we examined whether developmental exposure to BPA alters hallmarks of allergic lung inflammation in adult offspring. Pregnant C57BL/6 dams were gavaged with 0, 0.5, 5, 50, or 500 μg BPA/kg/day from gestational day 6 until postnatal day 21. To induce allergic inflammation, adult offspring were mucosally sensitized with inhaled ovalbumin containing low-dose lipopolysaccharide or ip sensitized using ovalbumin with alum followed by ovalbumin aerosol challenge. In the mucosal sensitization model, female offspring that were maternally exposed to ≥ 50 μg BPA/kg/day displayed enhanced airway lymphocytic and lung inflammation, compared with offspring of control dams. Peritoneally sensitized, female offspring exposed to ≤ 50 μg BPA/kg/day presented dampened lung eosinophilia, compared with vehicle controls. Male offspring did not exhibit these differences in either sensitization model. Our data demonstrate that maternal exposure to BPA has subtle and qualitatively different effects on allergic inflammation, which are critically dependent upon route of allergen sensitization and sex. However, these subtle, yet persistent changes due to developmental exposure to BPA did not lead to significant differences in overall airway responsiveness, suggesting that early life exposure to BPA does not exacerbate allergic inflammation into adulthood.

Key Words: asthma; plastics; developmental exposure; developmental basis of adult disease.

Asthma is a global disease with no definitive cause or cure. This complex, chronic condition is described by variable symptoms, including reversible airway obstruction, wheezing, chest tightness, and shortness of breath. In a 2010 assessment, asthma affected over 300 million people (GINA, 2011), and in 2009, it claimed 250,000 lives worldwide (GINA, 2009), making

its study a global priority. Allergic asthma is the most common form of asthma and describes about 60% of all asthmatics (Milgrom, 2003). Allergic asthma is triggered by the response to allergens, such as pollens or house dust mites. Asthma prevalence has increased since the 1970s, a period that also saw an increased use of numerous chemicals in manufacturing processes, with increased exposure of the general population (Kwak *et al.*, 2009; Vollmer *et al.*, 1998). This association has raised concern about the potential for a cause-and-effect relationship between the widespread use of some chemicals, particularly those that are not only used in manufacturing and industrial processes but are detected in a large proportion of the population, with the increased incidence or severity of diseases such as asthma. One chemical that has garnered considerable attention of late is bisphenol A (4,4'-(propane-2,2-diyl) diphenol or BPA) (Braun and Hauser, 2011; Hengstler *et al.*, 2011; Vandenberg *et al.*, 2007).

BPA is composed of two functional phenol groups, giving it properties that are useful in many manufacturing processes and commonly used consumer products. BPA's primary use is to make polycarbonate plastics. Other applications include epoxy resins lining food and beverage containers, dental sealants, carbonless copy and thermal papers, and as a precursor in flame retardants (Debenest *et al.*, 2010; Liao and Kannan, 2011; Lopez-Cervantes and Paseiro-Losada, 2003; Suzuki *et al.*, 2000). Concerns about BPA stem from several factors, including the knowledge that it is one of the highest production volume chemicals in the world, and products that contain BPA often break down, releasing monomers (Cannon *et al.*, 2000). Biomonitoring surveys by the U.S. Centers for Disease Control and Prevention have found BPA in blood and urine samples from 95% of the U.S. population (Calafat *et al.*, 2005). Additional concern arises because BPA is an endocrine disruptor. Specifically, BPA binds to estrogen receptor- α (ER- α) and ER- β . Although BPA's affinity for ER- α and ER- β is $\geq 10,000$ -fold lower than 17 β -estradiol (Kuiper *et al.*, 1998),

studies suggest that BPA may initiate biological effects through both ER-dependent and ER-independent signaling pathways (Miyakoshi *et al.*, 2009; Singleton *et al.*, 2004). Multiple reports, in a variety of animal models, paint a concerning, albeit inconsistent, picture of the potential for BPA to contribute to negative health effects, including chromosomal and reproductive system abnormalities, impaired brain, neurological, and cardiovascular functions, as well as possibly contributing to diabetes and obesity (Allard and Colaiacovo, 2010; Larocca *et al.*, 2011; Leranthe *et al.*, 2008; Melzer *et al.*, 2010; Sakurai *et al.*, 2004). The differential effects of estrogen and the modification of ER signaling on allergic asthma have also been reported although the mechanisms by which estrogens influence this disease remain to be determined (de Oliveira *et al.*, 2010; Shah *et al.*, 2010). These reports highlight the potential for BPA to perturb human health and contribute to diseases, such as allergic lung inflammation and asthma, although the underlying mechanisms by which BPA could cause these effects remain an area of active research.

Few epidemiological studies have explicitly examined the relationship between BPA exposure and the developing human immune system although contact dermatitis and asthma have been reported in industrial workers exposed to BPA and BPA conjugates, suggesting a possible link between chronic BPA exposure and immune-mediated diseases (Hannu *et al.*, 2009; Jensen and Andersen, 2003). Several *in vitro* and animal studies have examined the consequences of BPA exposure on mast cell degranulation, lymphocyte proliferation, antibody responses, and regulatory T cells, with data suggesting that the developing immune system may be a particularly sensitive target of BPA (Midoro-Horiuti *et al.*, 2010; Nakajima *et al.*, 2012; Shin-ichiro *et al.*, 2007; Yan *et al.*, 2008; Yoshino *et al.*, 2004). However, there are still gaps in our knowledge concerning BPA's effects in the context of allergic asthma. This study examines, for the first time, whether maternal exposure permanently affects metrics of allergic inflammation development/severity into adulthood and whether gender influences the potential proallergic effects of BPA (Melgert *et al.*, 2005; Shah *et al.*, 2010). Furthermore, we examined whether the route of allergen sensitization affects any BPA-related differences.

MATERIALS AND METHODS

Animals. C57BL/6 mice (6–8 weeks; NCI, Bethesda, MD) were housed in prewashed polysulfone microisolator cages under pathogen-free conditions. Mice received soy-free, AIN76-semi-PD1RR chow (Test Diet, Richmond, IN) to avoid phytoestrogenic effects that might mimic possible estrogenic effects of BPA. Glass water bottles and reverse osmosis-purified water were used. Paired mice were checked daily for vaginal plugs, indicating pregnancy. Pregnant females were housed singly and initially treated by oral gavage with 50 or 500 μg BPA/kg/day (d) (Sigma-Aldrich, St Louis, MO) or peanut oil vehicle control, beginning on gestational day 6 and continuing daily through postnatal day (PND) 21. An estrogen control was not used as BPA may act via other receptors in addition to ERs; thus it is not clear that BPA and natural estrogens will have the same effects (Chung *et al.*, 2011). The results of these initial

experiments and new information in the literature prompted the subsequent examination of maternal exposure to lower BPA doses (0.5 and 5 μg BPA/kg/day). Dosing to the dam was initiated on the 6th day of pregnancy to eliminate any possible effects of BPA on implantation, which occurs in the mouse between the 4th and 5th day of gestation, and all pups were weaned on PND 21, at which time all maternal transfer of BPA ceased. Offspring were not directly given any BPA. The selected dose range spans the current tolerable daily intake of 50 μg BPA/kg/day (USEPA, 2010). Maternal treatment with these doses of BPA did not affect offspring bodyweight, which was monitored over the life span of the offspring, pregnancy duration, litter size, or the sex ratio of litters (Roy *et al.*, 2012).

Using a subset of pregnant dams, we determined the amount of circulating BPA detected following this oral dose of BPA. BPA was measured at the Laboratory of Analytical Chemistry, New York State Department of Health, using high performance liquid chromatography coupled with electrospray triple-quadrupole mass spectrometry (Padmanabhan *et al.*, 2008). Four hours after gavage of pregnant mice, free and total BPA were measured (7 mice per treatment group). The mean circulating levels of total BPA in pregnant mice given 50 μg /kg/day were 2.941 ng/ml, whereas mean circulating BPA levels in vehicle control-treated dams was 0.434 ng/ml ($p = 0.0173$). Interestingly, in the BPA-treated group, none of the samples tested were below the limit of quantitation (LOQ), whereas in the samples from the group dosed with peanut oil vehicle, 4 out of 7 samples were below the LOQ.

On PND 21, offspring were weaned and housed in same-sex groups under the BPA-controlled conditions described above until maturity (6–10 weeks). Over the course of this research, several separate cohorts of maternally exposed mice were generated over a period of many months. Vehicle-treated dams were included in all pregnancy cohorts, so that BPA exposed offspring and vehicle-exposed offspring were age matched and derived from a single shipment of nulliparous mice (i.e., each batch of mice that we used as dams). Our experimental strategy utilized the available offspring in the various exposure groups as they were available. For all presented data, the offspring of vehicle- and BPA-treated dams were impregnated and treated in the same time frame. For experiments, age and sex-matched, maternally exposed, mature offspring were derived from separate vehicle- or BPA-treated dams. For example, a group with an $N = 5$ contained five mice, each from a different exposed dam of the same vehicle or BPA dose. Animal work was done in accordance with protocols approved by the University of Rochester Animal Care and Use Committee.

Mucosal sensitization model. Mice were anesthetized with avertin ((2,2,2-tribromoethanol); Aldrich, Milwaukee, WI) and intratracheally (*i.t.*) sensitized on days 0, 1, and 2 with 100 μg of endotoxin-depleted ovalbumin (OVA), with, or without, 100 ng of *Escherichia coli* lipopolysaccharide (LPS) (O55:B5; Sigma-Aldrich) in PBS (50 μl /mouse). OVA (Grade V, Sigma-Aldrich) was endotoxin-depleted using Endotoxin Detoxi-Gel (Pierce, Rockford, IL). This protocol results in T_H2 -type allergic airway inflammation following aerosol OVA challenge (Eisenbarth *et al.*, 2002). Mice were challenged twice per day on days 14, 15, and 16 with aerosolized 1% OVA in PBS (1 h), using a jet nebulizer (DeVilbiss, Somerset, PA) and constant airflow rate. Forty-eight hours after the final aerosol challenge, mice were sacrificed by avertin overdose. We chose the 48-h time point based on prior studies and to better compare outcomes with mice in the peritoneal sensitization groups (Hogaboam *et al.*, 2008; Lawrence *et al.*, 2008).

Peritoneal sensitization model. Mice were ip sensitized on days 0 and 4 with PBS or 100 μg OVA in PBS with 100 μl Imject Alum (Pierce; 200 μl /mouse). Although this is an artificial route of allergen encounter, this widely used model relies on the robust T_H2 -promoting properties of injected alum. Mice were challenged once (1 h), on day 11, with aerosolized 1% OVA in PBS, using a challenge chamber, jet nebulizer, and constant airflow rate. Forty-eight hours post-aerosol challenge, mice were sacrificed using avertin overdose. In previous studies, inflammatory cells, cytokines, and OVA-specific IgE were detected 48 h after single aerosol challenge (Hogaboam *et al.*, 2008; Lawrence *et al.*, 2008). Based on this work, we elected to use the time point for the current study.

Histological analysis. Lungs were fixed *in situ* with 10% neutral-buffered formalin, embedded in paraffin, and 5- μ m sections were stained with hematoxylin and eosin (H&E). Coded samples were scored for tissue inflammation by at least two different scientists using the following scale: 0, no airway involvement, few/no inflammatory cells in tissue; 1, limited inflammation, small number of airways with inflammatory cell clusters, most airways have no inflammation; 2, modest inflammation, several airways with small inflammatory cell clusters, no areas of intense infiltrate; 3, moderate-to-intense inflammation, many airways with inflammatory cells and occasional areas of intense infiltrate; 4, significant airway inflammation with multiple intense foci, massive perivascular/periairway inflammation, most/all airways involved. The combined scores of five lobes resulted in an overall number. The maximum possible inflammation score for each whole lung was 20.

Bronchoalveolar lavage. Lungs were lavaged as previously described (Warren *et al.*, 2000). The supernatant from the first 1 ml wash was frozen at -80°C for cytokine analysis, whereas recovered cells from all washes were pooled. A TC-10 (Bio-Rad, Hercules, CA) was used to calculate the total number of cells recovered. Bronchoalveolar lavage (BAL) cells (5×10^4) were spun onto glass microscope slides using a cytospin (Shandon Scientific, Waltham, MA), fixed, and stained with H&E.

Flow cytometric analysis of lung-derived immune cells. The pulmonary vasculature was perfused (PBS) via the right ventricle, and immune cells were obtained from collagenase-digested whole lung as described previously (Neff-LaFord *et al.*, 2007). Leukocytes were filtered, washed, and counted, and nonspecific binding was blocked using anti-CD16/32 and rat IgG. Subsequently, cells were stained with fluorochrome-conjugated antibodies against cell surface CD4, CD8, CD11b, CD11c, CD25, CD45.2, MHCII, Siglec-F, or the intracellular protein, FoxP3 (eBioscience, San Diego, CA; BD Pharmingen, San Jose, CA). For the latter, cells were permeabilized with saponin postfixation and incubated with an anti-FoxP3 antibody. A live/dead stain (Invitrogen, Carlsbad, CA) was used to selectively gate on live cells during analyses. Events were acquired using an LSR II cytometer (BD Biosciences, Franklin Lakes, NJ) with subsequent analysis using FlowJo 8.8.7 (Tree Star, Ashland, OR).

OVA-specific antibody measurements. Circulating OVA-specific IgE levels were analyzed by stacking ELISA (Hogaboam *et al.*, 2008). Serial dilutions of OVA-specific IgE (GeneTex, Irvine, CA) and plasma from unexposed/unsensitized/unchallenged C57BL/6 mice were used as positive and negative controls, respectively. Plasma from exposed mice was serially diluted, and biotinylated anti-mouse IgE subclass antibody (eBioscience) was used, in combination with avidin-peroxidase and ABTS substrate (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid, Sigma-Aldrich) to detect OVA-specific IgE in the samples.

Measurement of airway hyperresponsiveness. Airway responsiveness to methacholine was assessed using the FlexiVent, invasive, airway plethysmography system (Scireq, Chandler, AZ) and analyzed using Lab Chart Version 6 software (ADInstruments, Inc., Colorado Springs, CO). Mice were paralyzed with succinylcholine, anesthetized with avertin, tracheostomized, and intubated with a cannula connected to a ventilator. The system was calibrated for airflow and pressure. Pressure measurements, postbaseline, were obtained in response to airway challenge with saline and increasing concentrations of aerosolized methacholine in saline (0.1, 0.3, 1, 3, 10, 30, and 100 mg/ml) using a jet nebulizer. Resistance and elastance measurements to describe overall airway responsiveness were derived from the pressure measurements. Resistance is a measurement describing a reduction in airflow due to factors such as bronchoconstriction, whereas elastance describes the "stretching/recoil" potential of the airway.

Statistical analysis. Given that the dams, not offspring, were directly exposed, all offspring from a litter are considered as a single statistical unit. Statistical analyses were performed on all measured endpoints using JMP software (Version 9, SAS, Cary, NC). Mean differences between independent variables were compared among treatment groups using two-way ANOVA, followed by a *post hoc* test (Tukey's-Kramer HSD [Honestly Significant Difference]).

Also, nonparametric analyses of data were performed using a Kruskal-Wallis test. Differences between two groups at a single point in time were evaluated using a Student's *t*-test or Tukey's-Kramer HSD test. Differences were considered significant when *p* values were less than 0.05. All statistical analyses were conducted within animals of the same sex.

RESULTS

In light of the disparity among allergic inflammation mouse models currently employed in research and our aim to better understand the potential long-term impact of maternal exposure to BPA on allergic inflammation, we utilized two established mouse models of allergic inflammation. These models differ in the route of antigen sensitization, but share a similar model allergen (OVA) and strategy for allergen challenge (OVA aerosolization). In one model, sensitization is via the airway, and in the other model, OVA is administered into the peritoneum. Airway sensitization is a physiological route of mucosal allergen exposure, and low-dose LPS is thought to act as a T_H2 -promoting adjuvant, activating airway epithelial cells and lung-resident dendritic cells (Eisenbarth *et al.*, 2002; Tan *et al.*, 2010). In contrast, inhalation of endotoxin-depleted OVA alone usually results in tolerance to the antigen. In the more widely used peritoneal sensitization model, OVA is suspended in the adjuvant alum. The precise mechanisms by which alum and LPS act as adjuvants in these models remain to be fully determined; however, it is thought that they cue innate and inflammatory responses differently and may even influence aspects of antigen processing and presentation (Eisenbarth *et al.*, 2002; Kool *et al.*, 2008a,b). In both models, OVA sensitization and challenge were performed in immunologically mature offspring of both sexes.

Maternal BPA Exposure Enhances Airway and Lung Inflammation

In the mucosal sensitization model, mice inhaled either endotoxin-depleted OVA alone, or with low-dose LPS as adjuvant, followed 2 weeks later by OVA aerosol challenge. We observed that adult female offspring, but not adult male offspring, that were maternally exposed to 50 and 500 μg BPA/kg/day developed slightly enhanced lymphocyte airway infiltration when compared with offspring of vehicle control-treated dams (Fig. 1). Furthermore, when we examined lung histopathology, only adult female offspring demonstrated consistently enhanced whole-lung inflammation compared with adult offspring of vehicle control-treated dams (Fig. 2). This enhanced airway and whole-lung inflammation that occurred in adult female offspring sensitized using OVA alone suggests that the threshold for mounting a response to inhaled adjuvant-free OVA, as a model allergen, was lower in these mice. In contrast, the effects of maternal exposure to BPA on lung inflammation in the male offspring were less pronounced, with some statistically significant differences in mean score of the OVA-alone groups. However, the overall level of lung

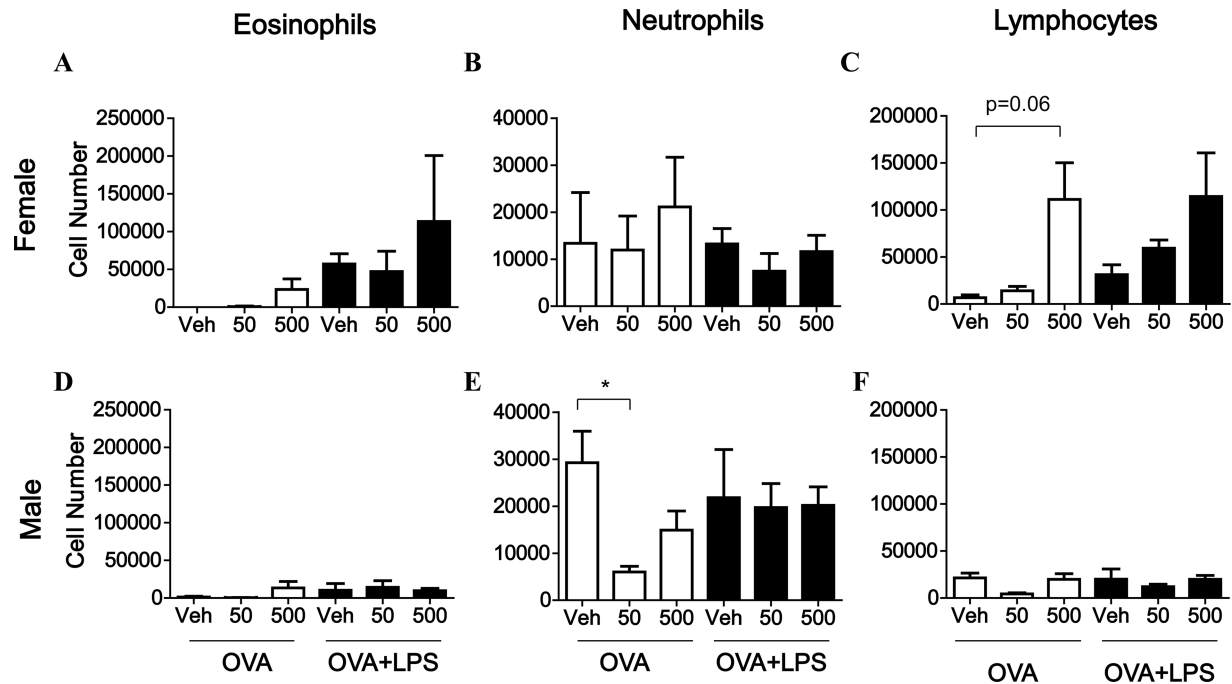


FIG. 1. Airway cell influx in the mucosal sensitization model. Adult female ($N = 4-6$) or male ($N = 5-8$) offspring of dams treated daily with BPA (50 or 500 $\mu\text{g}/\text{kg}$) or the vehicle control were sensitized (*i.t.*) with OVA alone or OVA + LPS prior to aerosol challenge. Differential cell counts of H&E stained BAL cells (200 cells counted/slide) were acquired using coded slides. Graphs depict the mean percentage ($\pm$ SEM) of eosinophils (A, D), neutrophils (B, E), and lymphocytes (C, F) in developmentally exposed adult female (A–C) and male (D–F) offspring. Please note that in order to make comparison of sex differences easier, the same y-axis scaling for each cell type is used to present data from female and male offspring. An asterisk (*) indicates $p \leq 0.05$, for comparisons of means between the same sensitization/challenge group, but different BPA exposure groups. Data are representative of findings from two independently performed experiments.

inflammation was similar among the groups of male offspring, regardless of maternal exposure and whether sensitization was with OVA or OVA + LPS. Regardless of BPA dose, neither female nor male adult offspring presented differences in airway eosinophilia or neutrophilia when sensitized with OVA + LPS. Of note, the statistically significant reduction in airway neutrophilia observed in adult male offspring (Fig. 1E) is unexplained, but it was consistent among experiments.

We next further characterized the lymphocytic lung inflammation. Given that maternal BPA exposure has been shown to influence helper (Yoshino *et al.*, 2004) and regulatory CD4^+ T cell populations (Yan *et al.*, 2008), we examined common lung T-cell populations by flow cytometry. There were no differences in the number (or percentage) of CD4^+ , CD8^+ , or $\text{CD4}^+\text{CD25}^+\text{FoxP3}^+$ T lymphocyte subpopulations in adult offspring that were maternally exposed to 50 or 500 μg BPA/kg/day, when compared with the adult offspring of corresponding vehicle-treated dams (Fig. 3). Furthermore, although we noted differences in some metrics of allergic inflammation that could be attributed to maternal BPA exposure, we did not observe such differences in disease-relevant lavage fluid cytokines, such as IL-5 and IL-17, or circulating OVA-specific IgE levels (data not shown).

U.S. Federal guidelines currently put the daily upper limit of safe human exposure to BPA at 50 $\mu\text{g}/\text{kg}/\text{day}$ (USEPA, 2010).

Hence, we selected this daily dose and an order of magnitude higher for our aforementioned initial investigations. However, several animal studies have demonstrated that acute or maternal exposure to BPA at levels < 50 $\mu\text{g}/\text{kg}/\text{day}$ can have deleterious effects in various disease models (Al-Hiyasat *et al.* 2002; Chitra *et al.* 2003; Maffini *et al.* 2006; Sakaue *et al.* 2001). In light of these studies, we investigated the consequences of maternal exposure to 10 and 100 times lower levels of BPA (0.5 or 5 μg BPA/kg/day) in adult offspring, using the mucosal sensitization model described above. When comparing like-sensitization/challenge groups, there were no significant differences in total airway eosinophils, neutrophils, or lymphocytes in adult offspring (Fig. 4). Furthermore, the overall magnitude of the response, as measured by the number of airway eosinophils, neutrophils, and lymphocytes, generally appeared to be less in adult male than in adult female offspring. As previously observed with higher BPA doses, an investigation of the CD4^+ , CD8^+ , and $\text{CD4}^+\text{CD25}^+\text{FoxP3}^+$ lymphocyte populations in the lung revealed few differences between like-sensitization/challenge groups in adult offspring that were previously maternally exposed to 0, 0.5, or 5 μg BPA/kg/day (Fig. 5). Moreover, similar to the higher maternal BPA doses tested, although we noted subtle, insignificant differences in some metrics of allergic inflammation that could be attributed to maternal BPA exposure, we found no differences in cytokines,

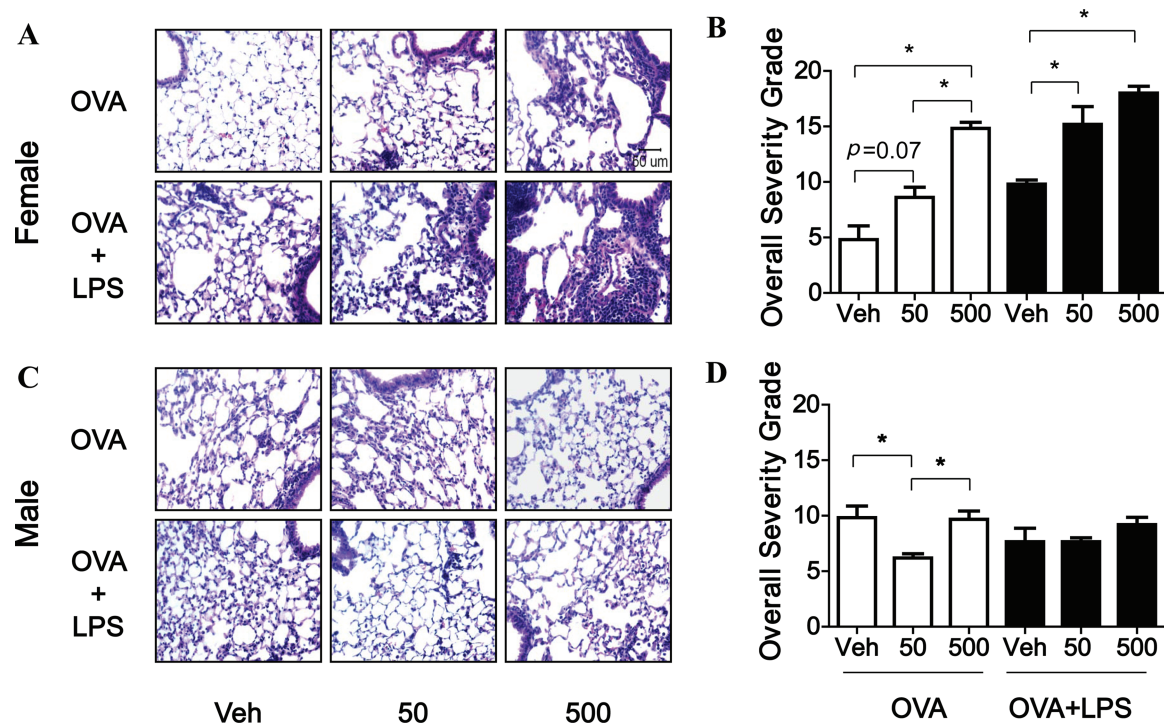


FIG. 2. Developmental exposure to BPA alters overall lung inflammation. Adult female ($N = 5-6$) or male offspring ($N = 3-6$) of BPA exposed or vehicle control dams were sensitized (*i.t.*) with OVA alone or OVA + LPS prior to aerosol challenge. Scoring was performed by three independent scientists, using coded slides. Representative images are from H&E-stained lung tissue at 40 $\times$ magnification (A, C) and depict the cumulative histopathological assessment. Graphs (B, D) present the mean inflammation scores for each group (error bars = SEM). An asterisk (*) indicates $p \leq 0.05$ when mean values from the same sensitization/challenge group, but different BPA exposure groups, were compared.

such as IL-5 and IL-17, in lung lavage fluid, or circulating OVA-specific IgE levels (data not shown). Previous allergic asthma-modeling studies in mice (Melgert *et al.*, 2005) have shown that female mice are more prone to display characteristics of asthma than male mice. Despite the proposed link between estrogens and asthma, these data show that maternal BPA exposure, at the doses used, does not alter this sex-specific characteristic and that maternal exposure to BPA does not promote allergic inflammation in adult male offspring.

Maternal BPA Exposure Dampens Airway and Lung Inflammation

In the commonly used peritoneal sensitization model, mice were ip sensitized with OVA + alum followed by OVA aerosol challenge. This leads to OVA uptake by spleen antigen-presenting cells and is believed to promote robust T_H2 immune responses in both inflammasome-dependent and -independent manners (Eisenbarth *et al.*, 2008; Kool *et al.*, 2008a). Interestingly, and in contrast to developmentally exposed offspring that were sensitized with inhaled OVA plus low-dose LPS, we found that adult female offspring that were maternally exposed to 0.5, 5, or 50 μg BPA/kg/day displayed significantly dampened airway eosinophilia, compared with adult female offspring exposed to either vehicle control or 500 μg BPA/kg/day (Fig. 6A). There were no significant, BPA-related

differences in airway neutrophil or lymphocyte frequency in these sensitized/challenged mice (Figs. 6B & C). The attenuated airway inflammation observed was similarly reflected in the total number of lung-derived eosinophils, wherein adult female offspring that were maternally exposed to 0.5–50 μg BPA/kg/day had significantly fewer CD11b $^+$ CD11c $^+$ Siglec-F $^+$ cells (eosinophils) compared with adult female offspring of dams that received vehicle or 500 μg BPA/kg/day (Fig. 6D and Supplementary fig. 1). Although mean differences did not reach statistical significance for any comparison, the total number of CD4 $^+$ T cells in the lung displayed a similar reduced trend for the same maternal BPA exposure groups (Fig. 6E). Furthermore, circulating OVA-specific IgE levels were significantly lower in adult female offspring that were maternally exposed to 0.5, 5, or 50 μg BPA/kg/day (Fig. 6F), compared with vehicle-exposed offspring.

Given that we observed statistically significant differences in multiple endpoints of allergic inflammation using the peritoneal sensitization model, we wanted to determine whether these differences affected overall airway mechanics. Airway hyper-responsiveness was measured using invasive plethysmography. This measurement relates to a clinically relevant human endpoint, and because multiple pathways contribute to changes in this measurement, the results may reveal differences that are not apparent in the examination of individual phenotypic or

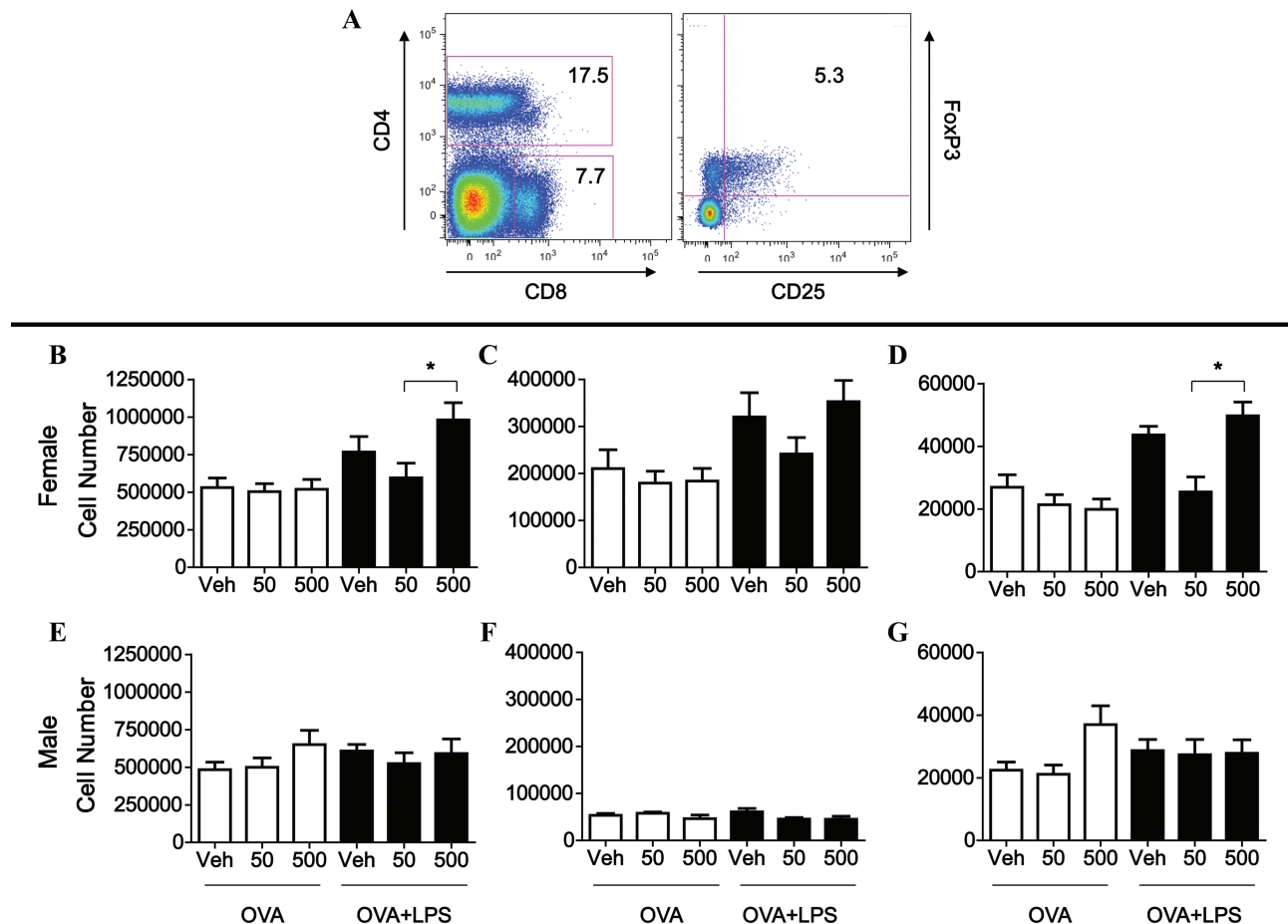


FIG. 3. Effects of developmental exposure to BPA on T-lymphocyte subpopulations in the lung. Lung tissues were examined from adult female ($N = 5-7$) or male offspring ($N = 5-8$) from dams exposed to BPA or vehicle control. Immune cells were obtained from intact lung, stained with fluorochrome-conjugated monoclonal antibodies and analyzed by flow cytometry, as described in the Materials and Methods section. (A) Representative dot plots show the gating strategy for lung lymphocyte populations. After gating on CD45⁺ cells (all leukocytes), fluorescence minus one (FMO) controls were used to identify CD4⁺ and CD8⁺ cells, or CD4⁺ cells that are CD25⁺FoxP3⁺ (T_{reg}). The number in each region of the dot plot indicates the percentage of cells in that gate. (B-G) The graphs depict the average number ($\pm$ SEM) of CD4⁺ (B, E), CD8⁺ (C, F), and CD4⁺CD25⁺FoxP3⁺ (D, G) lymphocytes after mucosal allergic sensitization and challenge. Asterisks (*) indicate $p \leq 0.05$ when mean values from the same sensitization/challenge group, but different BPA exposure groups, were compared. The same y-axis scaling for each cell type is used to simplify comparisons across sex.

cellular endpoints. As shown in Figure 7, there were no significant alterations in airway hyperresponsiveness in sensitized/challenged adult female offspring that were maternally exposed to BPA, compared with vehicle control. This suggests that although maternal exposure to BPA caused subtle changes in some metrics of allergic airway inflammation, the net effect of developmental exposure to BPA on overall airway mechanics during antigen challenge remain unaffected.

DISCUSSION

In light of increasing concern about human exposure to BPA from a variety of sources and the reported correlation of this exposure with increasing rates of allergic asthma, we investigated the hypothesis that maternal exposure to BPA affects the development and severity of allergic inflammation

into adulthood using two distinct mouse models of allergic asthma. In addition to using the conventional and commonly used OVA + alum, peritoneal sensitization model, we felt it was critical to investigate the consequences of maternal BPA exposure on allergic inflammation in the context of a model that uses mucosal sensitization, a more physiological route of antigen encounter. Importantly, although antigen sensitization differs in the models, both models used aerosol challenges with antigen to induce allergic inflammation. Furthermore, we wanted to determine whether any impact that gestational exposure to BPA may have on allergic inflammation would persist into immunologically mature, adult animals.

The major findings of this study were that the effects of maternal exposure to a range of doses of BPA on allergic inflammation in adult offspring were subtle. Many immune endpoints were unaffected by developmental exposure. For

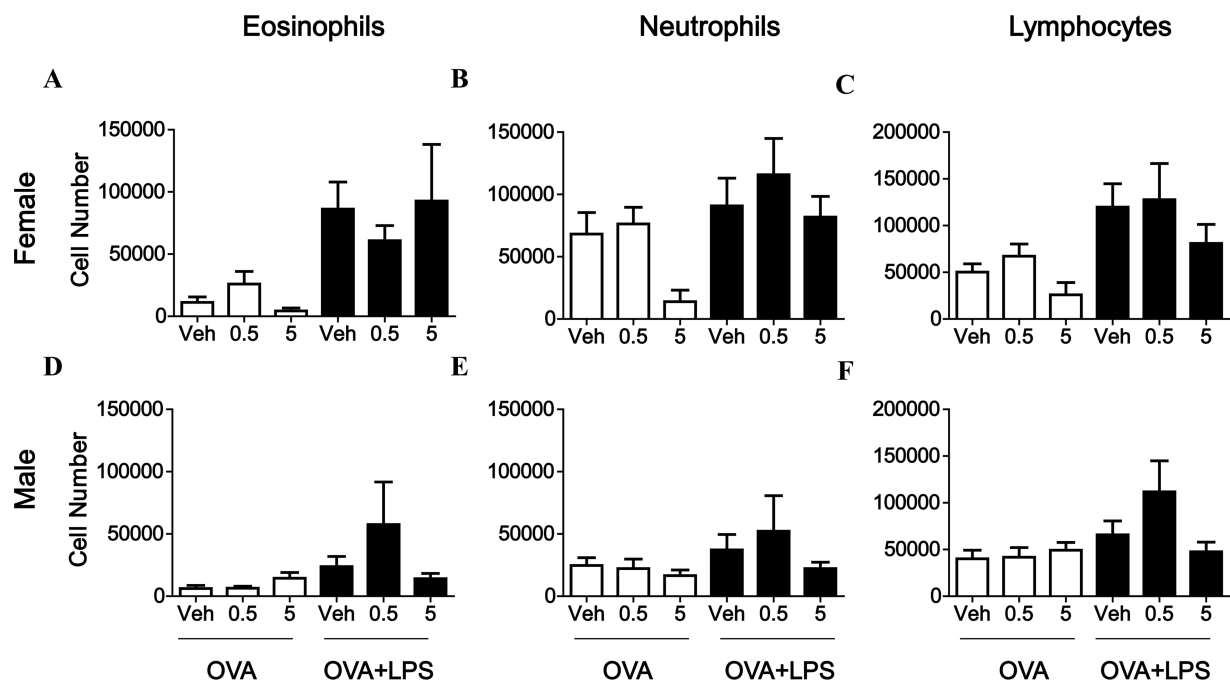


FIG. 4. Exposure to lower maternal doses of BPA does not affect airway cell populations in a mucosal sensitization model. Airway cells from adult female (A–C) or male (D–F) offspring (6–8 mice/group) maternally exposed to 0, 0.5, or 5 µg BPA/kg/day were assessed. The total number of cells collected per mouse was determined, and differential cell counts were performed to enumerate leukocytes in BAL cells (200 BAL cells counted/mouse). The graphs depict the average number ($\pm$ SEM) of eosinophils (A, D), neutrophils (B, E), and lymphocytes (C, F). The same y-axis scaling is used for each cell type to simplify comparison of data from female and male offspring; however, the scaling is different from the data presented in Figure 1. An * indicates $p \leq 0.05$ between same sensitization/challenge group, but different maternal BPA dose.

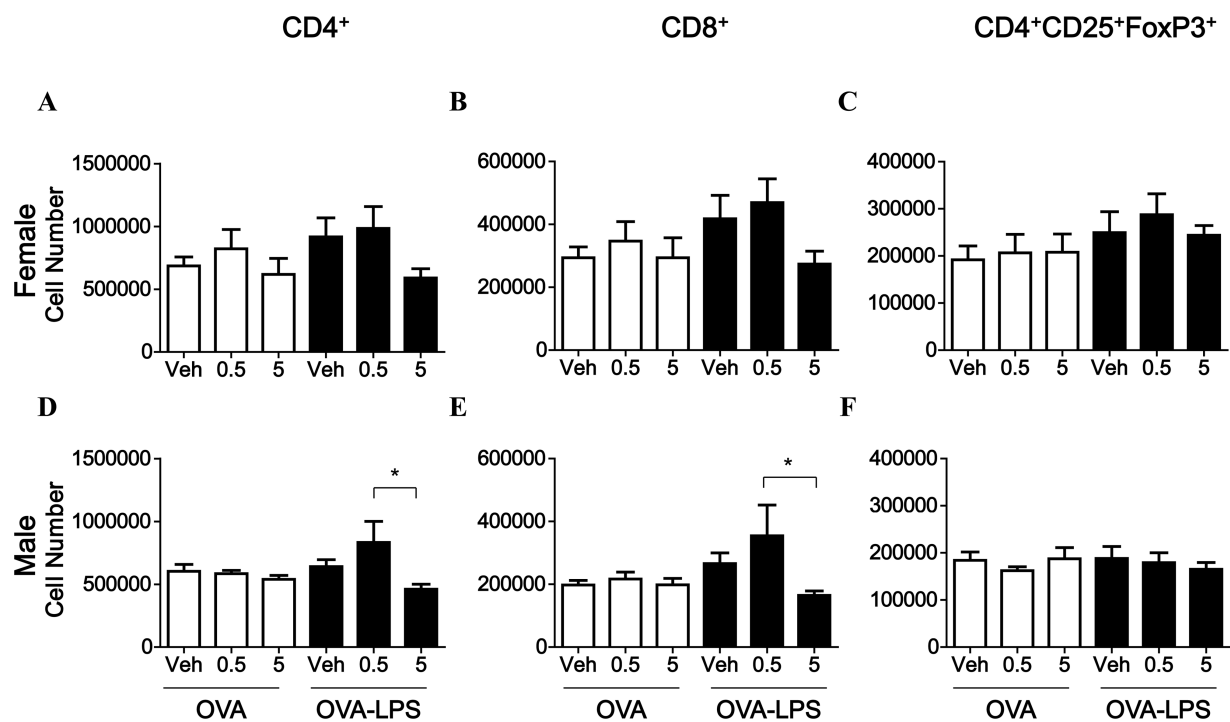


FIG. 5. Lung lymphocyte populations in offspring maternally exposed to < 50 µg BPA/kg/day. Adult female (A–C), or male offspring (D–F) from dams exposed daily to BPA or vehicle control were compared after mucosal sensitization modeling. Immune cells from the lung were obtained, processed, and analyzed as in Figure 3. Data are expressed as mean number ($\pm$ SEM) of each lymphocyte subset from 6–8 mice/group. Asterisks (*) indicate $p \leq 0.05$ between same sensitization/challenge group, but different maternal BPA doses. The same y-axis scaling for each cell type is used to simplify sex comparisons.

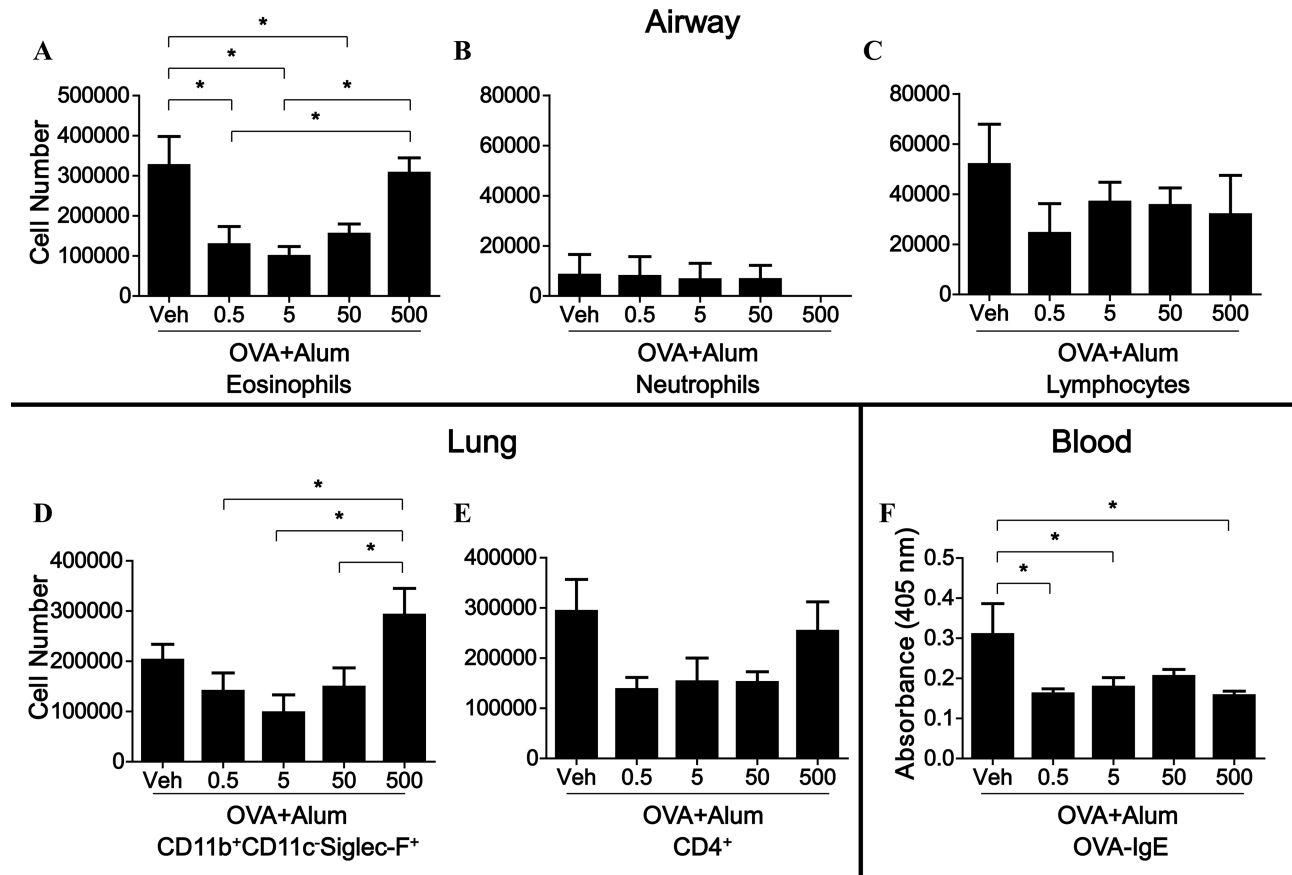


FIG. 6. Maternal BPA exposure alters several endpoints in offspring using a peritoneal sensitization model. Dams were treated with BPA doses ranging from 0.5 to 500 µg/kg/day from day of pregnancy 6 through postpartum day 21. Adult female offspring were sensitized by *i.p.* injection of OVA in alum and challenged with OVA aerosol. Airway cells, lung-derived immune cells, and blood were obtained from mice in each group. Total cellular recovery was determined and differential cell counts of H&E stained airway cells (200 cells/slide) were assessed by morphology to determine the percentage and number of eosinophils (A), neutrophils (B), and lymphocytes (C). Lung-derived (interstitial) immune cells were collected by collagenase digestion and stained for flow cytometry. Graphs show the average number of lung eosinophil (D) and CD4⁺ lymphocyte (E) populations in each group. OVA-specific IgE was accessed by stacking ELISA (F). All data are expressed as mean ± SEM for 4–5 mice/group; **p* ≤ 0.05 between same sensitization/challenge group, but different BPA exposure dose.

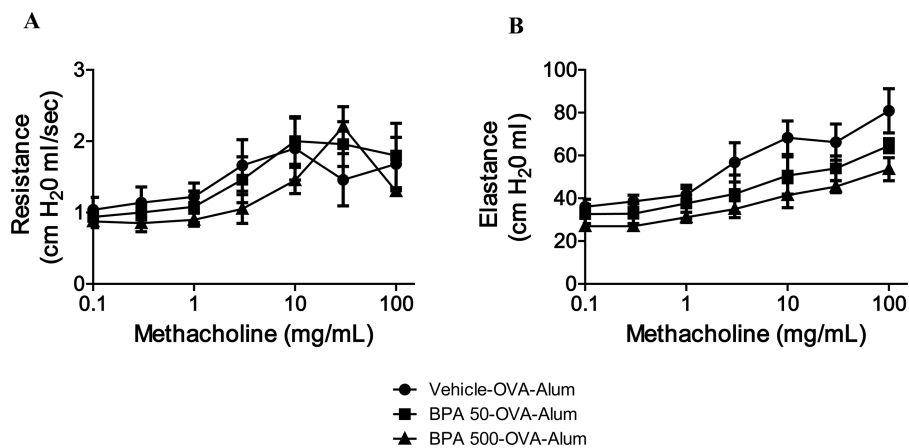


FIG. 7. Maternal exposure to BPA did not adversely affect airway hyperresponsiveness measurements of lung resistance (A) or elastance (B) in adult female offspring. Pressure measurements, postbaseline, were obtained in response to airway challenge with saline and increasing concentrations of aerosolized methacholine using a jet nebulizer. Resistance and elastance measurements to describe overall airway function were derived from the pressure measurements. Data are expressed as mean value for each group + SEM from 4–5 mice per group. **p* ≤ 0.05 between mice of the same sensitization/challenge group, but different maternal BPA exposure dose.

the endpoints that were altered by developmental exposure to BPA, the effects observed were qualitatively different and critically dependent on the route of antigen sensitization and the sex of the offspring. Furthermore, when tested, the subtle effects on allergic inflammation caused by maternal BPA exposure did not have a significant impact on airway function, compared with vehicle control-exposed mice. Interestingly, in the more widely used peritoneal sensitization model of allergen immunization, maternal exposure to 0.5, 5, and 50 μg BPA/kg/day attenuated lung eosinophilia in adult female offspring following OVA challenge. In contrast, when antigen sensitization occurred via the mucosal route, maternal exposure to BPA resulted in enhanced airway lymphocytic inflammation in adult female offspring, which occurred even in the absence of inhaled low-dose LPS adjuvant. The differences observed between these two mouse models of allergic inflammation suggest that maternal BPA exposure differentially influences immune responses into adulthood, but the nature of this effect depends on the site and context of initial antigen encounter. Additionally, in mice that were maternally exposed to BPA, we have shown that these influences did not impact the airway response in mice that underwent the peritoneal model of allergic inflammation.

The mucosal sensitization model involves toll-like receptor (TLR)-4, CD14, and LPS-binding protein in response to LPS, thereby facilitating the OVA-specific response. It has been shown that the amount of LPS influences the type of inflammatory response generated (pro- T_H1 or pro- T_H2), thus influencing the development and severity of allergic asthma (Eisenbarth *et al.*, 2002; Tan *et al.*, 2010). In contrast, insoluble aluminum salts, like those used in the peritoneal sensitization model, are the principle adjuvants used in clinical vaccine preparations and have been shown to elicit T_H2 -type-specific immune responses to allergens (Grun and Maurer, 1989). The mechanism by which alum acts as an adjuvant remains controversial. However, multiple studies indicate that aluminum salts elicit their actions through TLRs. One such study showed that MyD88-deficient mice produced normal amounts of IgG1 but excessive amounts of IgE in response to antigen immunization in aluminum salts (Schnare *et al.*, 2001). Another study showed enhanced antibody responses in MyD88/TRIF double-deficient mice, where no TLR signaling could occur (Gavin *et al.*, 2006). There is controversy, some showing dependence (Kool *et al.*, 2008b) and others showing independence (McKee *et al.*, 2009) of the inflammasome in this process. Thus, it is possible that maternal BPA exposure influences these different mechanisms of allergen uptake and presentation, which offers an explanation for differential effects observed when we used these distinct models for antigen sensitization.

An unexpected and important finding of our studies using the peritoneal model was that maternal BPA exposure dampened multiple features of adult allergic inflammation. Other recent studies (Midoro-Horiuti *et al.*, 2010; Nakajima *et al.*, 2012) utilized a similar OVA + alum peritoneal sensitization model

but reported enhanced airway hyperresponsiveness, eosinophilia, and circulating OVA-specific IgE in neonatal offspring that were maternally exposed to 10 $\mu\text{g}/\text{ml}$ BPA in the dams' drinking water. Several distinctions in study design, such as the maternal dose of BPA, mouse strain, and no segregation of male and female offspring for neonatal sensitization, may contribute to the differences in our findings and those reported by this other group. However, the age difference of the mice at the time of antigen sensitization and challenge offers a likely and very important distinction between these studies and ours. The development of the fetal immune system is under the integrated control of multiple systems, including those involving hormones, and one possibility is that chemical exposure is more detrimental during this period. In fact, there is evidence that the developing immune system is considerably more sensitive to perturbation than the fully mature immune system (Hogaboam *et al.*, 2008; Luebke *et al.*, 2004). It would appear that although all studies of maternal exposure to BPA demonstrated modulatory effects on allergic inflammation in the offspring, the exacerbation of overall airway responsiveness observed in neonatally sensitized mice appears to result in a different outcome than when sensitization occurs later in life. When taken together, it may be that gestational exposure to BPA leads to transient early life changes in allergic lung inflammation, but that this effect wanes with age and may not be permanent. Furthermore, the metabolic capacity of the exposed dam may also determine exposure to the fetus (Doerge *et al.*, 2011).

Several other pieces of evidence support the idea that there may be a developmental window in which BPA exposure affects allergic sensitization. A recent study showed that *Ugt2b1* expression, an isoform of UDP glucuronosyltransferase that is related to clearance of BPA in rats, is undetectable in mouse fetuses and newborn pups but approaches adult levels by PND 25 (Nakajima *et al.*, 2012). This suggests that BPA conjugation mechanisms change with developmental state, which may influence the potential for BPA to modulate biological systems during different windows of development. Further support for a developmental window for BPA to affect the immune system comes from separate studies, in which we examined whether daily, acute, oral exposure of adult mice to BPA alters allergic lung inflammation in either the mucosal and peritoneal sensitization models. Our data revealed no effect of BPA treatment on allergic inflammation using either model of allergic inflammation (unpublished observations). Consistent with these findings, a recent study found no correlation between urinary BPA levels and allergy/hay fever (Rees Clayton *et al.*, 2011). Taking all of the above into consideration suggests that the BPA-related subtle effects we have observed in maternally exposed adult mice occurred during development; however, these changes did not confer a permanent detrimental impact on allergic inflammation, nor was airway responsiveness adversely affected.

Allergic lung inflammation is just one of many facets of immune system responsiveness. Interestingly, in parallel

animal studies, we recently reported that maternal exposure to BPA exposure did not compromise virus-specific adaptive immunity against primary and recall influenza A virus infection in adult offspring. However, we observed that infected adult mice that were developmentally exposed to BPA had a transient reduction in the extent of infection-associated pulmonary inflammation and anti-viral gene expression in lung tissue (Roy *et al.*, 2012). These observations are consistent with the idea that early life exposure to BPA affects aspects of tissue inflammation, even in the absence of modulating adaptive immune responses, and suggest that early life exposure to BPA may affect epithelial cells resident in peripheral tissues, such as the lung.

In summary, we present here a novel evaluation of the effects of maternal exposure to four different doses of BPA and report the effect of this exposure on several measures of allergic lung inflammation in adult offspring. In contrast to reports that maternal, and even gestational only, exposure to BPA alters aspects of lung inflammation and airway responsiveness when antigen sensitization and challenge occur shortly after birth, using multiple doses of BPA to the dam, our data reveal that maternal exposure to BPA has only subtle effects on allergic inflammation in adult offspring, with some endpoints unaffected at any dose. Moreover, when observed, the direction of change and magnitude of the effect of developmental exposure to BPA are critically dependent upon route of antigen sensitization and sex of the adult offspring. Finally, it is important to consider that these subtle effects, although persistent, did not lead to significant differences in overall airway responsiveness, suggesting that early life exposure to BPA may not exacerbate allergic inflammation into adulthood.

SUPPLEMENTARY DATA

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

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