



Prior oral exposure to environmental immunosuppressive chemicals methoxychlor, parathion, or piperonyl butoxide aggravates allergic airway inflammation in NC/Nga mice

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ABSTRACT

Background: Immunosuppressive environmental chemicals may increase the potency of allergens and thereby play a role in the development of respiratory tract allergies, such as allergic rhinitis and asthma. **Objectives:** We investigated the association between environmental immunosuppressive chemicals and the allergic airway inflammation development.

Methods: We used a mouse model of ovalbumin (OVA)-induced allergic airway inflammation. NC/Nga mice were exposed orally to pesticides parathion (an organophosphate compound) or methoxychlor (an organochlorine compound), or to an insecticide synergist piperonyl butoxide, prior to OVA intraperitoneal sensitization and inhalation challenge. We assessed serum IgE levels, B-cell counts, cytokine production, IgE production in hilar lymph nodes, eosinophil counts, chemokine levels in bronchoalveolar lavage fluid, and cytokine gene expression in the lung.

Results: Exposure to environmental immunosuppressive chemicals markedly increased serum IgE – IgE-positive B-cells, IgE and cytokines in lymph nodes – eosinophils and chemokines in BALF – *IL-10a* and *IL-17* in the lung.

Conclusions: Allergic airway inflammation can be aggravated by prior exposure to immunosuppressive environmental chemicals.

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1. Introduction

Recent reports suggest that prior exposure to environmental immunosuppressive chemicals induces autoimmune reactions and allergic diseases in immature rodents (Bakker et al., 2000; Casillas et al., 1999; Mustafa et al., 2009; Sobel et al., 2005; Yanagisawa et al., 2008). We have previously demonstrated that prior exposure to immunosuppressive environmental chemicals aggravates T cell-mediated allergic reactions (Fukuyama et al., 2010), as well as mite and chemical allergen-induced atopic dermatitis-like immunoreaction in immature mice (Fukuyama et al., 2011a,b, 2012). These data indicate that immunosuppression by environmental chemicals is closely related to the aggravation of allergic reactions. However, the reasons for the increasing prevalence of asthma are unclear, although environmental factors such as air pollutants have been implicated (Pearce et al., 2007).

Many environmental allergens, viral infections, and environmental irritants are known contributors to the development of asthma. Asthma is a chronic inflammatory disease of the airways

characterized by reversible airway obstruction, airway hyperreactivity, and remodeling of the airways. Infiltration of eosinophils in the lungs is a fundamental trait of the inflammatory response in asthma and may be important in the pathogenesis of this disease (Lambrecht et al., 2000; Maestrelli et al., 2009; De Monchy et al., 1985; Whitehead et al., 2003).

OVA-sensitized and challenged mouse model is one of the most popular allergic airway inflammation models, in particular in the BALB/c strain (Lin et al., 2006). However, different susceptibilities to OVA may exist in different laboratory strains and with different antigen doses (Morokata et al., 1999, 2000). In the previous studies, more massive and prolonged allergic responses, such as eosinophilic infiltration and IgE production, were observed in NC/Nga strain mice relative to BALB/c strain mice. Therefore, it is suggested that NC/Nga strain is highly sensitive to several types of allergic diseases/reactions, such as atopic dermatitis and allergic airway inflammation (Iwasaki et al., 2001; Yamamoto et al., 2007). In addition, Iwasaki et al. (2001) demonstrated that both OVA-sensitized and challenged mice revealed severer airway inflammation than only OVA-challenged mice (there were almost no effect in only OVA-challenged group). Similarly, according to our unpublished data on strain-dependent differences in susceptibility to OVA-induced allergic airway inflammation, both OVA-sensitized

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and challenged protocol in NC/Nga mice are the most sensitive condition (only OVA-sensitized or -challenged group showed much the same patterns as intact group in allergic airway inflammation). Therefore, to detect the aggravation of allergic airway inflammation by environmental immunosuppressive chemicals, we selected the OVA-sensitized and challenged NC/Nga strain mice as a model for allergic airway inflammation. Moreover, to explore the mechanisms of allergic airway inflammation development, we analyzed leukocyte numbers, chemokine production, immunoglobulin (Ig) E levels in serum and B-cells, cytokine production in hilar lymph node (LN) cells, and cytokine gene expression in the lung.

In this paper, we investigate the association between environmental immunosuppressive chemicals and allergic airway inflammation development following exposure to several types of environmental immunosuppressive chemicals: organochlorine pesticides (OC) such as methoxychlor, organophosphorus pesticides (OP) such as parathion, and an agricultural insecticide synergist used mainly with OP or pyrethroids such as piperonyl butoxide. These three chemicals were chosen on the basis of previous studies. Methoxychlor exposure has been demonstrated to result in atrophy of CD4+CD8+ double-positive T-cells in the thymus (Takeuchi et al., 2002a,b). Parathion markedly inhibits antigen-specific IgM production (Casale et al., 1984), and piperonyl butoxide administration depletes T-cells in the spleen and thymus, induces hypoplasia of the bone marrow, and inhibits T-cells proliferation in lymphoid tissues (Mitsumori et al., 1996; Diel et al., 1999; Battaglia et al., 2010). In addition, we have previously shown that methoxychlor, parathion and piperonyl butoxide exposure results in an increase in thymocyte apoptosis *in vitro*, and markedly inhibits sheep red blood cells (SRBC)-specific IgM production in mice (Fukuyama et al., 2012). However, although these chemicals contribute to suppressing immune functions, we have obtained contradictory results from studies in which T-lymphocyte-mediated allergic reactions and atopic dermatitis were exacerbated by prior oral exposure to methoxychlor, parathion, or piperonyl butoxide (Fukuyama et al., 2010, 2011a,b, 2012). To establish these paradoxes, we undertook the current study using a typical animal model of allergic airway inflammation.

2. Materials and methods

2.1. Animals

Female inbred NC/Nga mice (3-week old) were purchased from Charles River Japan (Atsugi, Kanagawa, Japan) and acclimatized for 6 days before the start of the experiment. Mice were housed individually under controlled lighting (lights on from 7:00 to 19:00 h), temperature ($22 \pm 3^\circ\text{C}$), humidity ($50 \pm 20\%$), and ventilation (at least 10 complete fresh-air changes hourly). Food (Certified Pellet Diet MF, Oriental Yeast, Tokyo, Japan) and water were available *ad libitum*. The current study was conducted in accordance with the Animal Care and Use Program of the Institute of Environmental Toxicology (IET IACUC Approval No. AC12008).

2.2. Chemicals

Methoxychlor standard ($\text{C}_{16}\text{H}_{15}\text{Cl}_3\text{O}_2$, >97% pure), parathion standard ($\text{C}_{10}\text{H}_{14}\text{NO}_5\text{PS}$, 99.5% pure), piperonyl butoxide ($\text{C}_{19}\text{H}_{30}\text{O}_5$, >98% pure) standard, and 0.5% methylcellulose solution were purchased from Wako Pure Chemical Industries (Osaka, Japan). Corn oil was purchased from Hayashi Chemicals (Tokyo, Japan). Albumin from chicken egg white (ovalbumin, OVA) was purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan) and Sigma–Aldrich Japan K.K. (Tokyo, Japan). Aluminum hydroxide hydrate gel suspension (ALUM) was purchased from Cosmo Bio Co., Ltd. Methoxychlor, parathion and piperonyl butoxide were diluted in corn oil to concentrations (w/v) described below. For the intraperitoneal (i.p.) sensitization, OVA (10 μg) was dissolved in PBS (0.2 mL/animal) containing ALUM (2 mg). For the inhalation challenge, OVA was dissolved in PBS to 0.1% (w/v). To confirm the effect of the methoxychlor, parathion or piperonyl butoxide, we adopted OVA concentrations that were no adverse effect to allergic airway inflammation only sensitization/challenge.

2.3. Experimental protocol

The experimental protocol used in this study is depicted in Fig. 1. Following a 6 days acclimatization period, NC/Nga mice (4-week old) were allocated randomly to groups ($n=8$ mice per group) for dosing, vehicle control, or no treatment (intact) group. According to our preliminary study, only OVA sensitization or challenge group showed much the same patterns as intact group in allergic airway inflammation. Therefore, data of only OVA sensitization or challenge are not shown in this issue. Doses of the chemicals were as follows: methoxychlor, 30 or 300 mg/kg day; parathion, 0.15 or 1.5 mg/kg day; piperonyl butoxide, 30 or 300 mg/kg day. Dosing groups and vehicle control group were given an oral dose of the test solution or vehicle (corn oil) only on days 1–5, respectively. Based on the EPA Immunotoxicity Guidelines established in 1998, the highest dose level used in a host should “not produce significant stress, malnutrition, or fatalities”. Accordingly, in this study, the maximum doses used were selected to be $<1/3$ of the LD50 (dose at which $\geq 50\%$ of animals would be expected to die) and concurrently to avoid the induction of clear systemic toxicity (i.e., changes in appearance, posture, behavior, respiration, consciousness, neurologic status, body temperature and excretion) (Fukuyama et al., 2012, *in press*). Actually, there were no abnormal signs during examination period. In body weight measurement, treated groups were comparable with vehicle control and intact groups (data not shown). In addition, the objective in this study was to investigate the relationship between immunosuppressive chemicals and allergic responses using confirmed environmental immunosuppressive chemicals using a mice model. The examination of relation to human exposures is now underway. For sensitization, OVA/ALUM was injected intraperitoneally into each mouse on days 29, 36, and 42. For challenge, OVA/PBS was administered by inhalation on days 43, 45, 47, 50, and 52. One day after the last challenge (day 53), all animals were anesthetized and sacrificed by injecting pentobarbital sodium (75 mg/kg, i.p.). Blood samples were taken from the inferior vena cava, and serum samples were assayed for IgE levels. Bronchoalveolar lavage fluid (BALF) was collected by cannulating the trachea and lavaging the lungs 3 times with 1 mL PBS supplemented with 1% heat-inactivated fetal calf serum (FCS; Life Technologies Co., Ltd., Tokyo, Japan). The first BALF fraction from each animal was centrifuged at $350 \times g$ for 5 min, then supernatants were pooled, and chemokine levels were measured. The cell pellets of all three fractions were resuspended, pooled and centrifuged at $350 \times g$ for 5 min. The supernatants were removed, and the cell pellets were used for differential cell counts. Hilar lymph nodes (LN) removed from each mouse were pooled in RPMI 1640 medium (Life Technologies Co., Ltd., USA). Single-cell suspensions were prepared from LNs by passage through a sterile 70- μm nylon cell strainer in 1 mL RPMI 1640 supplemented with 5% FCS. Single-cell suspensions were used to analyze the IgE-positive B-cells and cytokine production. Each animal's accessory lobe was removed, submerged in RNAlater Solution (Life Technologies Co., Ltd., Tokyo, Japan) overnight, and then used to extract total RNA.

2.4. Inhalation exposure

For the inhalation challenge, animals were exposed to 0.1% OVA/PBS mist continuously for 30 min/day. The animals were individually held in animal holders (Tokiwa Kagakukikai Co., Ltd., Tokyo, Japan) attached to a nose-only exposure chamber (total volume 31.2 L, Tokiwa Kagakukikai Co., Ltd.) so that only their noses were exposed to the chemical mist. The mist was generated by an atomizer (Ikeuchi Co., Ltd., Tokyo, Japan) with compressed air (ES4AD-5, Kobelco, Tokyo, Japan), supplied to the exposure chamber through an air filter (F3000-10-Y, CKD Corporation, Aichi, Japan). Airflow to the chamber was controlled by an area flowmeter (NSPO-4, Nippon Flow Cell, Tokyo, Japan) at a rate of 20 L/min. The chamber air was exhausted through an air filter system consisting of a glass wool filter, a mist trap, and an activated charcoal filter (Tokiwa Kagakukikai Co., Ltd.), and was emitted to the atmosphere by using a blower (TFO-K4P, Hitachi, Ltd., Tokyo, Japan). The actual concentration, mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) were monitored by gravimetric analysis by using an air sampler (MF-200, Oct Science Co., Ltd., Osaka, Japan) and an Andersen type personal sampler (Model 1312S, Kanomax Japan, Inc., Osaka, Japan). The mean values of actual concentration of active ingredient, MMAD and GSD were kept at approximately 5 mg/m³, 4.0 μm and 2.0, respectively, throughout the inhalation exposure.

2.5. Assay for total IgE

Total IgE levels in serum were measured by using enzyme-linked immunosorbent assay (BD OptEIA Mouse IgE ELISA Set, BD Pharmingen, Tokyo, Japan) according to the manufacturer's protocol.

2.6. Flow cytometry of BALF and hilar LN cells

The following antibodies used for flow cytometric analysis were purchased from BD Pharmingen: fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgE (clone R35-72), phycoerythrin (PE)-conjugated hamster anti-mouse CD11c (HL3), PE-conjugated anti-mouse Gr-1 (RB6-8C5), peridinin chlorophyll protein-conjugated anti-mouse CD3 (145-2C11), PE-cyanin-7-conjugated anti-mouse CD4 (RM-4-5), allo-phycoerythrin-conjugated anti-mouse CD45R/B220 (RA3-6B2), and allo-phycoerythrin-cyanin-7-conjugated anti-mouse CD8 (53-6.7). To avoid

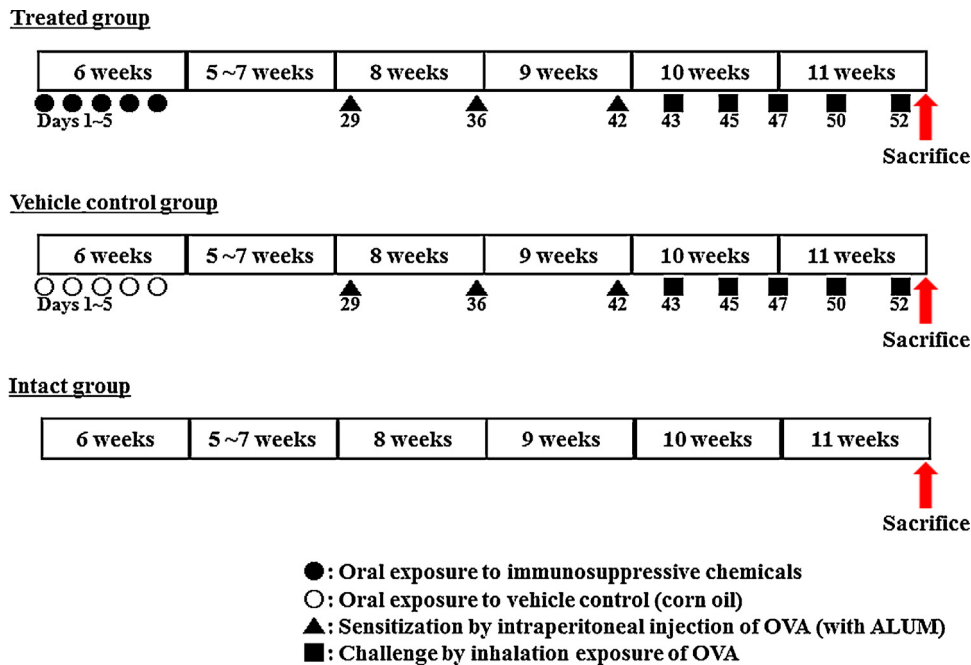


Fig. 1. Experimental protocol. See Section 2 for a detailed description. OVA, Ovalbumin; ALUM, aluminum hydroxide hydrate gel suspension.

nonspecific binding, 1×10^6 cells were incubated with $1 \mu\text{g}$ Mouse BD Fc Block™ (BD Pharmingen) for 5 min at 4°C , followed by incubation with monoclonal antibodies for 30 min at 4°C in the dark. Cells were washed twice with 5% FCS in PBS, resuspended at 1×10^6 cells per tube in $500 \mu\text{L}$ PBS, and analyzed on a FACSVerse flow cytometer (BD Pharmingen) by using FACSuite (BD Pharmingen). For each sample, 20,000 events were collected and analyzed for expression of antigens.

2.7. Cytokine production in LN cells

To stimulate T-cell receptor signaling, we cultured single-cell suspensions recovered from LNs (1×10^6 cells/well) for 24 or 96 h with anti-CD3 ($2 \mu\text{g}/\text{mL}$) and anti-CD28 ($2 \mu\text{g}/\text{mL}$) antibodies (BD Pharmingen) in 24-well plates at 37°C in a 5% CO_2 atmosphere. The levels of cytokines (interleukin (IL)-4, IL-5, IL-6, IL-10, IL-13, IL-17, IFN γ and TNF) in supernatants (cell culture medium) were measured by using the cytometric bead array (BD™ CBA Mouse Flex Set, BD Pharmingen) according to the manufacturer's protocol.

2.8. IgE production by B-cells in LN cells

B-cells were isolated from single-cell suspensions with autoMACS (Miltenyi Biotec K.K., Tokyo, Japan) and B220 micro beads (Miltenyi Biotec K.K.). To stimulate IgE production, B-cells were cultured (1×10^6 cells/well) for 9 days with $1 \text{ mg}/\text{mL}$ OVA in 24-well plates at 37°C in a 5% CO_2 atmosphere. Total IgE concentrations in supernatants were measured by ELISA as described above.

2.9. Chemokine levels in BALF

The levels of chemokines (KC, MIP-1 β , RANTES) were measured by the cytometric bead array (BD™ CBA Mouse Flex Set, BD Pharmingen) according to the manufacturer's protocol.

2.10. IL-10 and IL-17 gene expression in the lung

Total RNA was extracted from accessory lobes by use of NucleoSpin RNA II (Takara Bio, Tokyo, Japan) according to the manufacturer's protocol. RNA was reverse transcribed with the PrimeScript RT Reagent Kit with gDNA Eraser (Takara Bio) according to the manufacturer's protocol. cDNAs obtained were amplified by quantitative real-time polymerase chain reaction (QRT-PCR) by using the Thermal Cycler Dice system (Takara Bio). The data acquired for each sample were normalized to the expression levels of *Actb* (β -actin; a housekeeping protein). PCR primers (Takara Bio) for *Il10*, *Il17a* and *Actb* are listed in Table 1.

2.11. Statistical analysis

The data were transformed logarithmically to equalize the variance, and analysis of variance (ANOVA) was used to evaluate the results. For statistically significant ANOVA, the differences between groups were assessed by using Dunnett's multiple comparison test.

3. Results

3.1. Total IgE levels in serum

All groups given the test substances showed dose-dependent increases in total IgE levels in serum (Fig. 2). In the methoxychlor (300 mg/kg) treatment group, total IgE level was approximately 1.3-times that of the vehicle control groups, respectively. The total IgE in the parathion (1.5 mg/kg) treatment group ($3836 \pm 1893 \text{ ng}/\text{mL}$; mean $\pm$ SD) was significantly higher ($P < 0.05$) than that in the vehicle control group; this total IgE level is 11- and 2-times the levels in the intact and vehicle control groups, respectively. In the piperonyl butoxide (300 mg/kg) treatment group, total IgE level was approximately 8.5- and 1.5-times those in the intact and vehicle control groups, respectively.

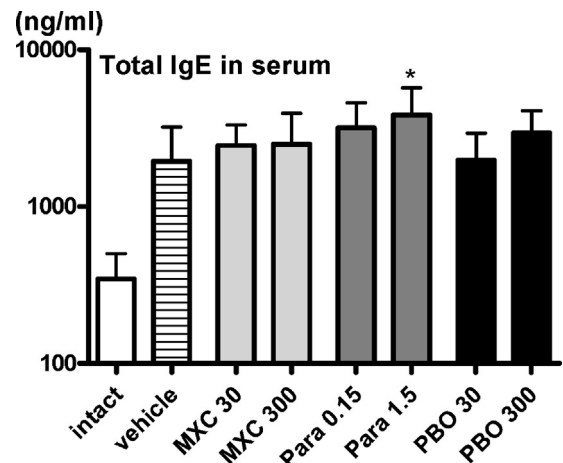


Fig. 2. Total serum IgE levels in mice with no treatment (intact), or with prior exposure to vehicle, or to the low or high doses (see Section 2) of methoxychlor (MXC), parathion (PARA) or piperonyl butoxide (PBO). Total IgE levels are expressed as mean $\pm$ SD (ng/mL; $n = 8$ per group). Statistical significance of the differences from the vehicle control group is indicated by asterisks (* $P < 0.05$; Dunnett's multiple comparison test).

Table 1
Primer sets used.

Gene symbol	Gene bank accession no.	Primers	Product (bp)
<i>Il10</i>	NM.010548.2	F: 5'-GCCAGAGCCACATGCTCCTA-3' R: 5'-GATAAGGCTTGGCAACCCAAGTAA-3'	145
<i>Il17a</i>	NM.010552.3	F: 5'-ACGCGCAAACATGAGTCCAG-3' R: 5'-AGGCTCAGCAGCAGCAACAG-3'	66
<i>Actb</i>	NM.007393.3	F: 5'-CATCCGTAAAGACCTCTATGCCAAC-3' R: 5'-ATGGAGCCACCGATCCACA-3'	171

F: forward; R: reverse.

3.2. BALF analysis

To assess the allergic airway inflammation in the lung, we counted eosinophils, macrophages and neutrophils (Fig. 3) and measured the levels of chemokines (KC, MIP-1 β , and RANTES) (Fig. 4) in BALF.

Methoxychlor and piperonyl butoxide treated groups given test substances showed dose-dependent increases in each parameter. Groups that received high doses of test substances showed greatly increased numbers of eosinophils and chemokine levels. The number of eosinophils was significantly higher in the groups treated with methoxychlor (both doses) and with the high doses of parathion and piperonyl butoxide as compared to the vehicle control group. In the groups treated with the high doses of methoxychlor, parathion and piperonyl butoxide, the eosinophil numbers were approximately 10.1-, 9.8- and 4.2-times, respectively, those in the vehicle control group. The numbers of macrophages and neutrophils were significantly higher in the groups treated with the high doses of methoxychlor and piperonyl butoxide than in the vehicle control group.

The levels of KC were significantly higher in the groups that received the high doses of methoxychlor, parathion and piperonyl butoxide than in the vehicle control group (approximately 2.2-, 3.1- and 2.4-times, respectively, that in the vehicle control). The levels of RANTES significantly increased in the groups that received high doses of methoxychlor, parathion and piperonyl butoxide (approximately 4.3-, 7.2- and 2.9-times, respectively, the level in the vehicle control group). The levels of MIP-1 β were significantly higher in the groups treated with the high doses of methoxychlor, parathion and piperonyl butoxide (approximately 2.1-, 3.1- and 2.4-times, respectively, that in the vehicle control group).

3.3. Analysis in hilar LN cells

We measured the number of IgE-positive B-cells (Fig. 5a). The numbers of IgE-positive B-cells were significantly higher in the groups treated with either dose of methoxychlor or parathion, and with the high dose of piperonyl than in the vehicle control group; for the high dose treatments with methoxychlor, parathion, and piperonyl, the numbers of IgE-positive B-cells were approximately 5.7-, 5.5- and 3.1-times, respectively, that of the vehicle control.

We also examined the total IgE production by B-cells derived from LN cells (Fig. 5b). Total IgE production in the test substance treatment groups showed a dose-dependent increase, and statistically significant differences from the vehicle control were observed in the groups exposed to the high doses of methoxychlor, parathion and piperonyl butoxide. These effects paralleled the increases in IgE-positive B-cells. In the groups that received the high doses of methoxychlor, parathion and piperonyl butoxide, total IgE production was approximately 3.1-, 3.1- and 1.6-times, respectively, the level in the vehicle control group.

Finally, we examined the cytokine production (Fig. 6). Significant increases in production of Th1 cytokines (IFN γ , TNF) were

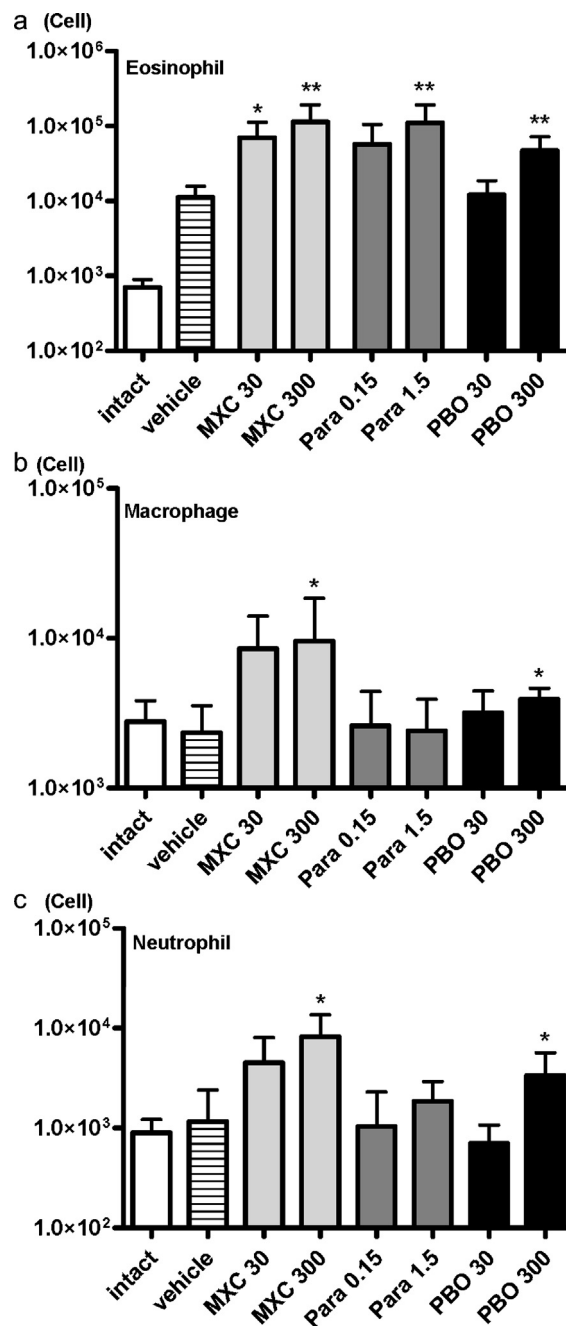


Fig. 3. Eosinophil (a), macrophage (b) and neutrophil (c) counts in BALF. Designations of treatments are as in Fig. 2. Cell counts are expressed as mean $\pm$ SD ($n = 8$ per group). Statistical significance of the differences from the vehicle control group is indicated by asterisks (* $P < 0.05$ and ** $P < 0.01$; Dunnett's multiple comparison test).

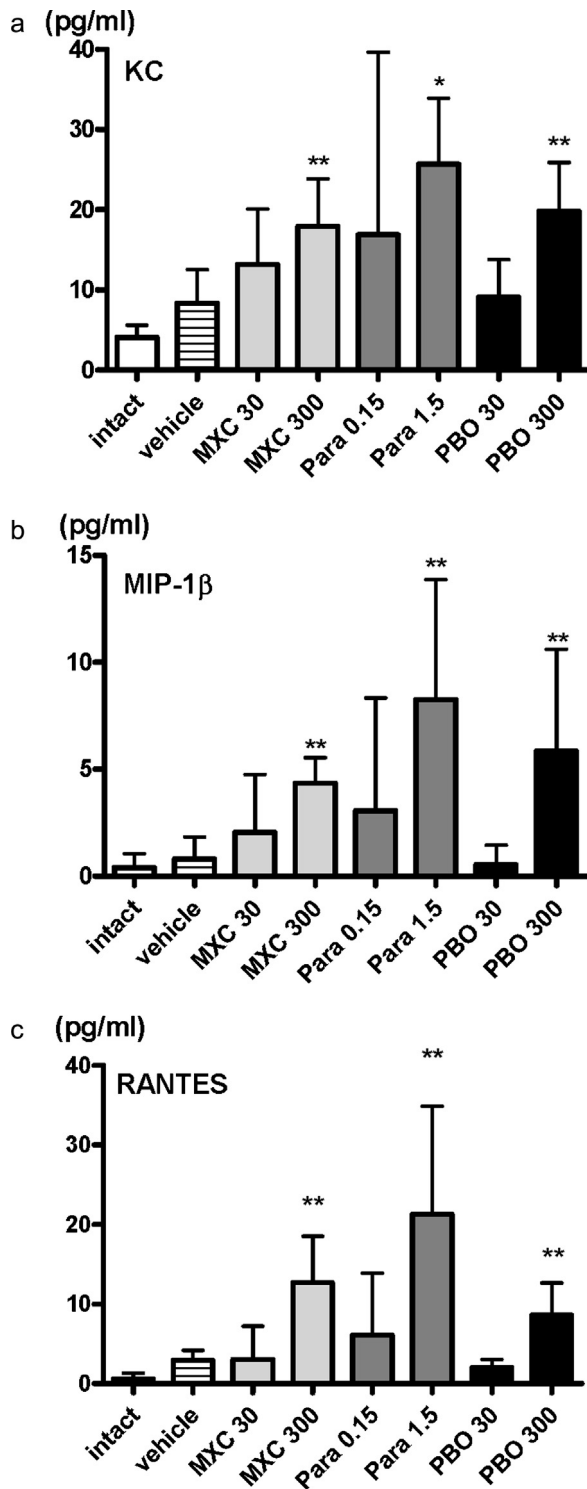


Fig. 4. Chemokine levels in BALF. The levels of (a) KC, (b) MIP-1 β , and (c) RANTES are expressed as mean $\pm$ SD (pg/mL; $n = 8$ per group). Designations of treatments are as in Fig. 2. Statistical significance of the differences from the vehicle control group is indicated by asterisks (* $P < 0.05$ and ** $P < 0.01$; Dunnett's multiple comparison test).

observed upon treatment with the high doses of methoxychlor and piperonyl butoxide, and both doses of parathion. The Th2 (IL-4, -5, -6, -13), Th9 (IL-10) and Th17 (IL-17A) cytokine production induced by the high doses of methoxychlor and parathion was significantly higher than in the control groups. In particular, IL-6 and IL-13 were markedly induced by the high doses of methoxychlor, parathion

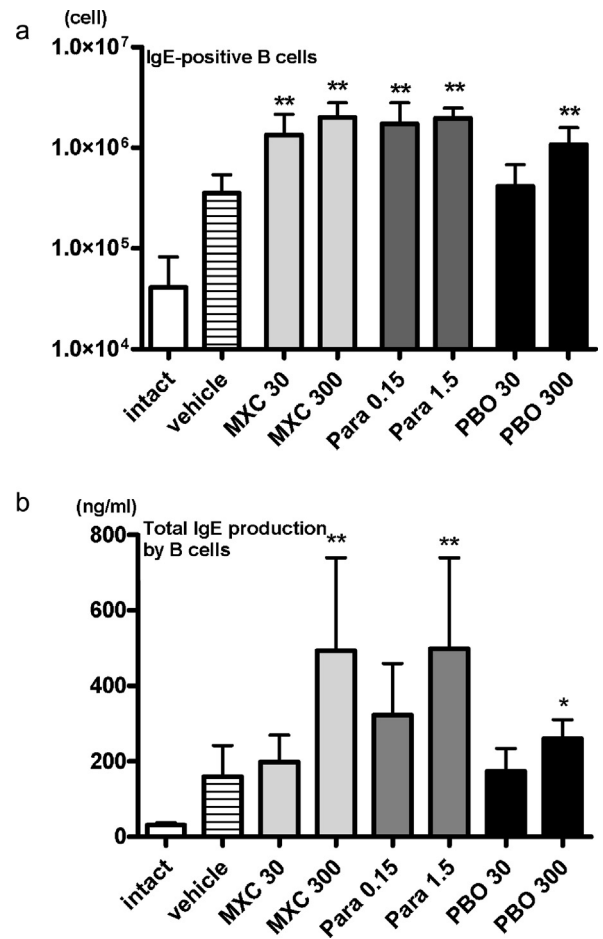


Fig. 5. IgE-positive B-cell counts (a) and total IgE production by B-cells (b) in LN cells. Designations of treatments are as in Fig. 2. Cell counts and total IgE levels (ng/mL) are expressed as mean $\pm$ SD ($n = 8$ per group). Statistical significance of the differences from the vehicle control group is indicated by asterisks (* $P < 0.05$ and ** $P < 0.01$; Dunnett's multiple comparison test).

and piperonyl butoxide. IL-6 concentrations in the groups treated with the high doses of methoxychlor, parathion and piperonyl butoxide were approximately 14-, 11- and 4-times, respectively, the level in the vehicle control group. IL-13 concentrations in the same groups were approximately 4-, 17- and 4-times, respectively, that in the vehicle control group.

3.4. Cytokine gene expression in the lung

To further explore the effects of test substances on allergic airway inflammation induced by OVA, we examined the expression of *Il10* and *Il17a* (Fig. 7). *Il10* expression in the test substance treatment groups showed a dose-dependent increase, and statistically significant differences were observed in the groups exposed to both doses of methoxychlor, and to the high doses of parathion and piperonyl butoxide compared with the vehicle control group. The values for *Il10* expression with high doses of methoxychlor, parathion and piperonyl butoxide were approximately 2.2-, 5.1- and 3.6-times, respectively, the level in the vehicle control group. *Il17a* expression in the methoxychlor or parathion treatment groups also showed a dose-dependent increase, but the high doses of piperonyl butoxide were lower than in the vehicle control group. In the groups treated with the high doses of methoxychlor, parathion and piperonyl butoxide, *Il17a* expression was approximately 1.3-, 2.0- and 1.3-times, respectively, the level in the vehicle control group.

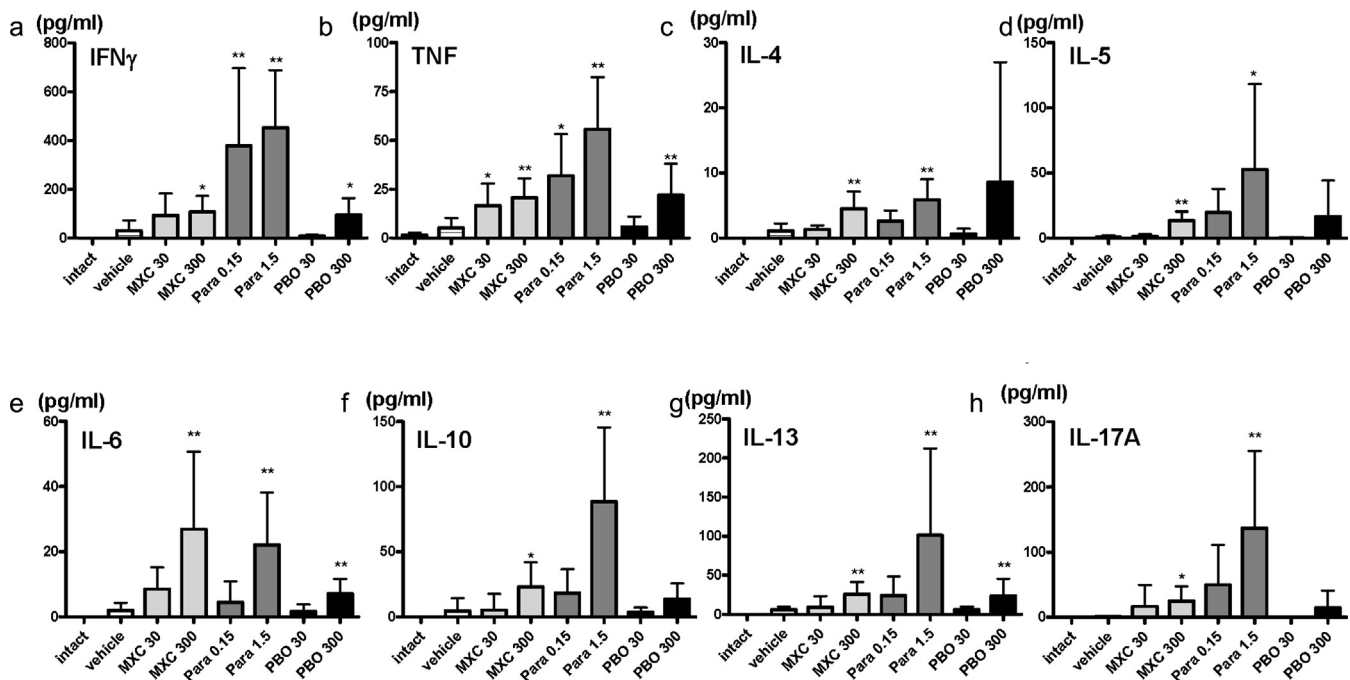


Fig. 6. Cytokine production in LN cells. The levels of (a) IFN γ , (b) TNF, (c) IL-4, (d) IL-5, (e) IL-6, (f) IL-10, (g) IL-13 and (h) IL-17A are expressed as mean $\pm$ SD (pg/mL; $n = 8$ per group). Designations of treatments are as in Fig. 2. Statistical significance of the differences from the vehicle control group is indicated by asterisks (* $P < 0.05$ and ** $P < 0.01$; Dunnett's multiple comparison test).

4. Discussion

Our objective in this study was to reveal the association between environmental immunosuppressive chemicals and the mechanisms of asthma development. To that end, we used NC/Nga mice exposed orally to 3 commonly used environmental immunosuppressive chemicals prior to intraperitoneal sensitization and inhalation challenge with OVA.

OVA is one of the most widely used antigens to evoke allergic airway inflammation and NC/Nga mouse strain is one of highly sensitive allergic reactions (Lin et al., 2006; Iwasaki et al., 2001). Therefore, in this study, we used NC/Nga mice and OVA to develop an animal model of allergic airway inflammation. In general, intra-tracheal and intranasal administration is used for sensitization and challenge with OVA in mice (Wills-Karp et al., 1998; Hammad et al., 2003; Akbari et al., 2001). However, these methods are different from exposure in actual environments and sometimes induce excessive inflammatory responses. To more accurately mimic the environmental exposure, we used an inhalation exposure system for the OVA challenge.

IgE antibody plays an important role in the development of allergic asthma, and can be used as a marker of OVA-induced allergic asthma model (Eum et al., 1995). In addition, in the development of an allergic response, activated B-cells can act as antigen-presenting cells for helper T-cells, resulting in the hyper-production of IgE (Goutet et al., 2005; Janeway et al., 2004). To evaluate the IgE production following the exposure to environmental immunosuppressive chemicals and OVA, we measured total IgE in serum, the number of IgE-positive B-cells, and IgE production by B-cells in the LNs. The exposure of mice to environmental immunosuppressive chemicals before treatment with OVA led to a marked increase in serum IgE levels. Consistent with these effects, we also noted significant increases in the number of IgE-expressing B-cells and in the production of IgE by the LN cells of mice exposed to the environmental chemicals.

In general, cellular inflammation in the airways is a clinical sign of allergic airway inflammation. Degranulation and release of inflammatory mediators such as KC, RANTES and MIP-1 β by activated eosinophils may cause extensive tissue damage and propagate airway obstruction (Janeway et al., 2004; Whitehead et al., 2003; L  tvall et al., 1998). In the current study, all groups given a test substance had a dose-dependent increase in the number of eosinophils in BALF. Furthermore, we analyzed the levels of these chemokines, and found that they were increased in the test substance-treated groups. These data suggest that modulation of eosinophil numbers and related chemokine levels by the exposure to environmental chemicals may be of clinical importance for the development and severity of allergic airway inflammation.

T-lymphocytes also play a pivotal role in the development of asthma. CD4 $^{+}$ T-cells infiltrate the lung lumen and express a T-helper type 2 (Th2) pattern of cytokines (Andersson et al., 2000; Whitehead et al., 2003). Cytokines secreted by these cells, including IL-4, -5, -6 and -13, appear to function in concert with chemokines and other mediators to recruit and activate the eosinophils of the allergic inflammatory response (Janeway et al., 2004). In our study, prior exposure to immunosuppressive chemicals induced a dose-dependent increase in the production of Th2 cytokines (IL-4, -5, -6, -13) by LN cells. Production of Th1 cytokines (IFN γ and TNF) also increased in the test substance treatment groups. These Th1 cytokines are associated with allergic contact dermatitis (Kimber et al., 2011), and involved in part in allergic airway inflammation (Brightling et al., 2008). Thus, our results showed that the test substance treatment groups increased allergic airway inflammation in lungs.

Recently, cytokines secreted by Th9 and Th17 cells have been implicated in the development of asthma. Th9 cells produce IL-9 and IL-10, associated with tissue inflammation in asthma (Veldhoen et al., 2008; Soroosh and Doherty, 2009). Th17 cells produce IL-17A, considered as a pivotal proinflammatory molecule in asthma (Song et al., 2008). In our study, high doses of the test substances increased the production of IL-10 and IL-17A by LN cells and

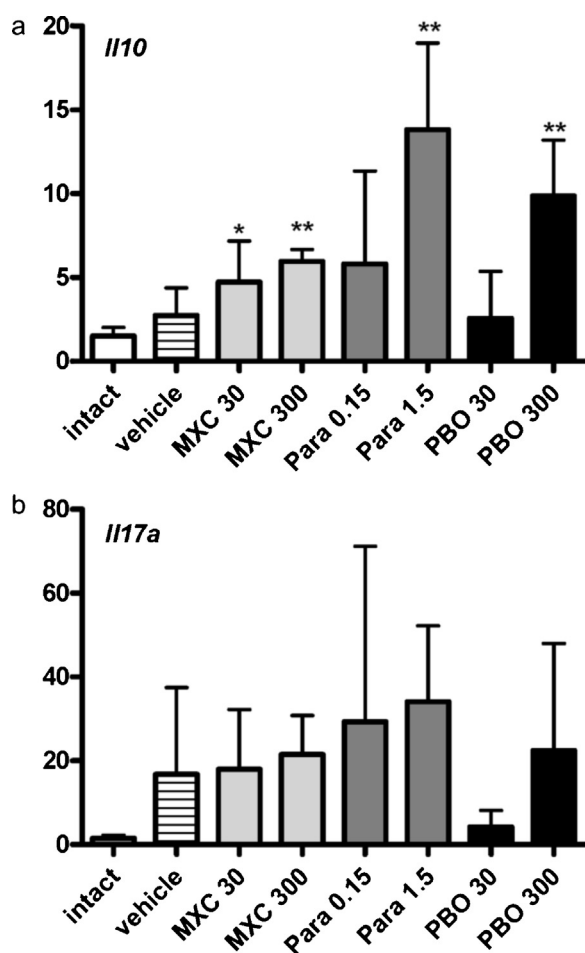


Fig. 7. Cytokine gene expression in the lung. The levels of expression of (a) *Il10* and (b) *Il17a* in the accessory lobes (normalized to the expression of *Actb*) are expressed as mean $\pm$ SD ($n = 8$ per group). Designations of treatments are as in Fig. 2. Statistical significance of the differences from the vehicle control group is indicated by asterisks (* $P < 0.05$ and ** $P < 0.01$; Dunnett's multiple comparison test).

expression of *Il10* and *Il17a* genes increased in the test substance treated groups. Our data suggest that the immune responses by various T-helper cells are enhanced in the test substance-treated groups.

Overall, the results reported here demonstrate that prior exposure to immunosuppressive environmental chemicals such as methoxychlor, parathion, and piperonyl butoxide can modulate immune functions and increase the severity of allergic airway inflammation in mice. This is in line with our previous study, which found that coinstantaneous exposure to an allergen and to immunosuppressive environmental chemicals has no effect, but prior exposure to these chemicals can aggravate allergen-induced atopic dermatitis in mice (Fukuyama et al., 2011b). In addition, according to our previous report, prevention of the central tolerance of autoreactive T or B cells is implicated because mice exposed to immunosuppressive environmental chemicals showed significant decreases in Treg cell populations (Fukuyama et al., 2012). Thus, after the withdrawal of the immunosuppressive compounds, a recovery from immunosuppression occurs, and an immune disorder develops such as increasing production antibodies and autoreactive T- or B-cells in mice. Then, if some antigens are encountered, abnormal hypersensitive reactions are induced. These reactions may have different mechanisms and varying degrees of hypersensitivity depending on the immunosuppressive chemicals. This model may be helpful in elucidating the etiology and the mechanisms of allergic diseases.

Although several studies have shown that immunosuppression leads to allergic diseases (Akdis et al., 2004), the relationship between these diseases and the breakdown of immune regulation induced by the exposure to environmental chemicals is not fully understood. Our report helps to elucidate the cause of the increase in the incidence of allergies, and suggests possible directions for further investigation of the mechanisms of allergic diseases caused by environmental chemicals.

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Conflict of interest

The authors state that they have no conflicts of interest.

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