

Article title

Oral administration of bisphenol A directly exacerbates allergic airway inflammation but not allergic skin inflammation in mice

Running title: Bisphenol A exacerbates airway inflammation

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Abstract: Bisphenol A (BPA) is used in various areas of daily life as a major component of plastic products. However, it is also known as a strong endocrine disruptor that affects the human immune system. Studies have indicated that BPA possibly exacerbates allergic diseases such as atopic dermatitis and asthma. The main aim of this study was to elucidate whether BPA is directly involved in the exacerbation of allergic inflammation. Initially, *in vivo* experiments with mouse models of allergic inflammation induced by Th2 type hapten toluene-2,4-diisocyanate (TDI) was performed. Mice were subjected to oral administration of BPA 48 h, 24 h, and 4 h before challenge with TDI. Dermal challenge of TDI onto the ear auricle was performed for the allergic dermatitis model, and intratracheal challenge of TDI was performed for the allergic airway inflammation model. In the allergic dermatitis model, ear-swelling response was significantly downregulated by high doses of BPA. The opposite reaction was observed in the allergic airway inflammation model, including significant exacerbation of red coloration in the lung, local cytokine levels, and total IgE levels in serum by BPA administration. To confirm the *in vivo* results, *in vitro* experiments with human epidermal keratinocytes (HEKs) and bronchial epithelial (BEAS-2B) cells were carried out. Significant enhancement of cytokine release from BEAS-2B cells but not HEKs in the BPA-treated group supported the *in vivo* observations. Our results imply that exposure to BPA directly exacerbates allergic airway inflammation but not allergic dermatitis.

Keywords: Bisphenol A; allergic dermatitis; allergic airway inflammation; keratinocyte;

bronchial epithelial cell, thymic stromal lymphopoietin

Introduction

Bisphenol A (BPA) is used in various areas of daily life as a major component of plastic products including epoxy resin containers, dental sealants, carbonless copy, and as a precursor in flame retardants (Liao *et al.*, 2011; Lopez-Cervantes *et al.*, 2003; Suzuki *et al.*, 2000). However, persistent organic pollutant contamination of BPA has become an important public concern recently as BPA has serious toxic effects on human health and wildlife (Huang *et al.*, 2012). In particular, BPA is recognized as a representative endocrine-disrupting substance that alters the reproductive system (Karnam *et al.*, 2015; Peretz *et al.*, 2014). Studies have indicated that BPA induces immunosuppression with consequences such as apoptosis in thymocytes (Rochester, 2013). It has also been shown that BPA could possibly exacerbate allergic diseases such as allergic dermatitis, rhinitis, and allergic asthma. Bauer *et al.* (2012) reported that when pregnant mice were exposed to BPA, enhanced airway lymphocytic and lung inflammation was observed in adult offspring. In humans, Gascon *et al.* (2015) showed that increasing concentrations of maternal urinary BPA are correlated with an increasing risk of asthma symptoms and respiratory tract infection in children. Zhou *et al.* (2017) also focused on the human data and found the evidence which mothers of infants with allergic diseases had significantly higher urinary BPA levels than those of infants without

allergic diseases. This finding indicates that, increased risk of infant allergic diseases is possibly associated with creatinine-adjusted maternal urinary BPA concentrations. However, above mentioned studies have only focused the indirect effects of BPA on inflammatory diseases by prenatal exposure, and the events between administration and outcome remain quite uncertain. He *et al.* (2016) demonstrated that simultaneous exposure to BPA and ovalbumin enhanced lung eosinophilia by promoting Th2-type immune response in adult male mice and Kim *et al.* (2017) suggested that exposure to phthalates and concentration of bisphenol A in urine sample is associated with aggravation of AD symptoms in children. However, little is known about the detail mechanisms.

Interestingly, there are also opponents to the abovementioned pre-clinical and clinical reports. Nygaard *et al.* (2015) disclaimed the relationship between BPA administration and development of allergy at exposure levels relevant for humans. They showed that early-life exposure to BPA modestly augmented allergic responses in a mouse model of airway allergy only at high BPA dose (100 µg/mL/day), and not in mouse models for food-allergy and tolerance. Thus, it remains unclear whether exposure to BPA definitely exacerbates allergic reaction, and the detailed mechanisms of action are unexplored.

Therefore, the main aim of this study was to elucidate whether BPA is directly involved in the exacerbation of allergic inflammation. In order to confirm this, *in vivo* experiments with mouse models of allergic dermatitis and allergic airway inflammation were

initially conducted. *In vitro* experiments using a human keratinocyte cell line, a human bronchial epithelial cell line, and mouse bone marrow-derived dendritic cells, as well as mixed leukocyte reaction assay were additionally performed to confirm the detail mechanisms of action. Epithelial cells including keratinocytes and bronchial epithelial cells play important roles in the development of local allergic inflammation by secretion of cytokines such as thymic stromal lymphopoietin (Mitchell *et al.*, 2017; Wilson *et al.*, 2013). Antigen-presenting cells including dendritic cells (DCs) are also contributors to allergic inflammation by recognizing exogenous allergens and transferring information to helper T-cells (Guermonprez *et al.*, 2002; Polte *et al.*, 2015).

Materials and Methods

Experimental animals

Seven-week-old both sexes of BALB/cAnN and female C57BL/6J mice were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan) and housed in groups of fewer than six mice per cage under controlled lighting (a 12-h light-dark cycle), temperature ($22 \pm 3^{\circ}\text{C}$), humidity ($55 \pm 15\%$), and ventilation (at least ten complete fresh-air changes per hour). Standard rodent diet (Certified Pellet Diet MF; Oriental Yeast Co., Tokyo, Japan) and local tap water were provided *ad libitum*. All aspects of this study were conducted in accordance with the Animal Care and Use Program of the Institute of Environmental

Toxicology, Japan (IET IACUC Approval No. AC17063), which was fully accredited by Association for Assessment and Accreditation of Laboratory Animal Care International.

Reagents

Acetone, BPA, mycophenolic acid, RPMI 1640 medium, fetal bovine serum (FBS), 5% trichloroacetic acid, and Tween 20 were acquired from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Lipopolysaccharide (LPS), Pefabloc® SC, polyinosinic-polycytidylic acid sodium salt (Poly (I:C)), and toluene-2,4-diisocyanate (TDI) were obtained from Sigma-Aldrich Co. LLC. (Tokyo, Japan). 3H-methyl thymidine and Pico-Fluor Plus were obtained from PerkinElmer Japan Co., Ltd. (Kanagawa, Japan). DC protein assay kit was purchased from Bio-Rad Laboratories, Inc. (Tokyo, Japan). Mouse IgE Ready-SET-Go, phosphate-buffered saline (PBS), 1-Step™ Ultra TMB-ELISA Substrate Solution, Blocker™ BSA (10×) in PBS, and Dynabeads mouse T-Activator CD3/CD28 were ordered from Thermo Fisher Scientific Inc. (Kanagawa, Japan). BEGM™ Bronchial Epithelial Cell Growth Medium was acquired from Lonza Japan Ltd. (Tokyo, Japan). EpiVita basal medium with hydrocortisone was obtained from Cell Applications Inc. (San Diego, CA). Recombinant mouse granulocyte-macrophage colony-stimulating factor was obtained from PeproTech, Inc. (Rocky Hill, NJ). Enzyme-linked immunosorbent assay (ELISA) kits for mouse and/or human interleukin (IL)-1β, -4, -8, -12, -13, -33, interferon gamma (IFNγ), tumor necrosis

factor (TNF) α , and thymic stromal lymphopoietin (TSLP) were purchased from R&D Systems (Minneapolis, MN). Mouse BD Fc Block, PE-conjugated anti-mouse CD3 (hamster IgG1, clone 145-2C11), PE-Cy7-conjugated anti-mouse CD4 (rat IgG2a, clone RM4-5), FITC-conjugated anti-mouse CD40 (Rat IgG2a, clone 3/23), PerCP-Cy5.5-conjugated anti-mouse Gr-1 (rat IgG2b, clone RB6-8C5), APC-conjugated anti-mouse CD49b (hamster IgG1, clone HMA2), and Mouse T Lymphocyte Enrichment Set-DM were purchased from BD Pharmingen (Tokyo, Japan). APC-conjugated anti-mouse CD11c (hamster IgG, clone N418) was purchased from Miltenyi Biotec K.K. (Tokyo, Japan). NucleoSpin RNA II, a PrimeScript RT Reagent Kit with gDNA Eraser, and primers were purchased from TaKaRa Bio (Tokyo, Japan).

TDI-induced allergic inflammation model

For the *in vivo* model of inflammation, male BALB/c mice were sensitized and challenged with TDI, which is a type 2 helper T-cells (TH2 cells)-dominated hapten, according to a previous study (Fukuyama *et al.*, 2015). To exclude the effects of the estrus cycle and endogenous estrogen, male mice were used to generate the model in this study. The abdominal skin of the mice was depilated with a commercially available depilating cream one day before the first sensitization. Immediately before the first sensitization, the horny layers of the depilated skin were stripped off ten times with adhesive tape. Subsequently, 50 μ L of

5% TDI solution in acetone was applied to the stripped epidermis (day 1). The same procedure was performed at the same site without tape stripping on days 2 and 3. Three weeks after the first sensitization, the allergic reaction was boosted by the application of 50 μ L of 0.5% TDI onto the depilated abdominal skin.

BPA administration in TDI-induced allergic contact dermatitis model

Five days after the final sensitization of TDI, TDI-sensitized male BALB/c mice were subjected to oral administration of the vehicle at a concentration of 0.06 or 0.2 mg/kg BPA 48 h, 24 h, and 4 h before topical challenge of TDI (0.5%) onto the shaved rostral part of the neck (30 μ L) and left ear auricle (20 μ L). The doses of BPA used in this study were selected based on previous reports to avoid systemic toxicity or excessive immune suppression (Bauer *et al.*, 2012). To measure the itching behavior, the mice were video-monitored for 1 h and scratching bouts (directed against the neck and ear regions) were determined directly after TDI challenge. For the ear swelling test used to evaluate the dermal inflammation potential, the thickness of the left ear was measured before and 24 h after TDI challenge. Swelling was calculated by comparing the ear thickness (cutimeter, Mitutoyo Corporation., Tokyo, Japan) before and 24 h after challenge. Right after measuring the 24-h post-TDI ear thickness, mice were euthanized by exsanguination from the abdominal aorta and posterior vena cava under anesthesia by isoflurane inhalation. Blood samples were taken

from the inferior vena cava, and serum samples were assayed for IgE levels. The left ear auricle was removed from each mouse and stored until use for cytokine evaluation. Single-cell suspensions were prepared from the auricular lymph node (LN) removed from each mouse by passage through a sterile 70- μ m nylon cell strainer in 1 mL of RPMI 1640 supplemented with 5% FBS. Single-cell suspensions were used in fluorescence-activated cell sorting (FACS) and cytokine evaluation.

Chemical administration in TDI-induced allergic airway inflammation model

Five days after the final sensitization of TDI, oral administration of BPA to TDI-sensitized male BALB/c mice was performed with the same doses of BPA as those in the contact dermatitis model 48 h, 24 h, and 4 h before intratracheal challenge of TDI (0.001%, 50 μ L). Twenty-four hours after TDI challenge, mice were euthanized by exsanguination from the abdominal aorta and posterior vena cava under isoflurane anesthesia. The red coloration in the lung was evaluated by a semi-quantitative score based on the criteria described in previous study (Nishino *et al.*, 2016) (0 = no visible changes, 1 = diffuse or patchy red coloration, 2 = moderate dispersed red coloration, 3 = severe red coloration). Blood samples, the vice lobe of the lung, bronchoalveolar lavage fluid (BALF), and hilar LNs were isolated from each mouse for total IgE level measurement, cytokine evaluation, and FACS analysis. BALF was collected by cannulating the trachea and lavaging the lungs three

times with 1 mL of PBS supplemented with 1% FBS. The supernatants from the first BALF fraction were stored for cytokine evaluation. The cell pellets of all three fractions were resuspended, pooled, and used for differential cell counts by FACS.

FACS analysis for BALF and LNs

To avoid non-specific binding, 1×10^6 cells were first incubated with 1 μ g of Mouse BD Fc Block, followed by incubation with monoclonal antibodies. Cells were washed and analyzed on a FACSVerse flow cytometer (BD Pharmingen) using the FACSuite program (BD Pharmingen). To analyze antigen expression, 10,000 events were collected from BALF and LN samples.

Cytokine evaluation in LNs

Single-cell suspensions of LNs (5×10^5 cells/well) were incubated with Dynabeads mouse T-Activator CD3/CD28 for 24 h. IL-4 and IFN γ productions in the supernatant were evaluated by ELISA.

IgE measurements in serum

Total IgE levels in the serum were measured by ELISA using Mouse IgE Ready-SET-Go according to the manufacturer's protocol. Briefly, each flat-bottomed

microplate well was coated with capture antibody in PBS and incubated overnight at 4 °C. The content of each well was removed and the plate was washed with wash buffer. Non-specific binding was blocked by incubation with 1% bovine serum albumin in PBS for 2 h at room temperature. The diluted serum was added to each well and incubated for 2 h at room temperature. Detection antibody (mixture of biotin-conjugated rat anti-mouse IgE monoclonal antibody and streptavidin–horseradish peroxidase conjugate) was added to each well and incubated for another 1 h at room temperature. The plate was developed with tetramethylbenzidine (TMB) in the dark, at room temperature, for 30 min. Optical density (OD) was read at a wavelength of 450 nm with a microplate reader.

Evaluation of cytokines in ear and lung tissues

Ear and lung tissues were frozen in dry ice and stored at –80°C until use. Cytokines in tissues were evaluated according to Fukuyama, *et al.* (2015). The protein content was determined with the DC protein assay kit. Levels of IL-1 β , -4, -33, IFN γ , TNF α , and TSLP were measured by ELISA. Recently investigated reports (Cruse *et al.*, 2016; Fukuyama, *et al.*, 2015; Stover *et al.*, 2016) demonstrated that secretions of IL-1 β , IL-6, KC, TARC and TNF α were detected in the same TDI-induced allergic inflammation model, although they were not TH2 dominated cytokines. In addition, Nishino, *et al.* (2016) showed that similar TH2 dominated hapten, trimellitic anhydride, also upregulated the secretions of TH1, TH9 and

TH17 type cytokines apart from TH2 type cytokines. Therefore, in this study, we measured not only the TH2 type cytokines such as IL-4 but also other types of cytokines including IL-1 β , -33, IFN γ , TNF α and TSLP which play an important role to maintain the allergic response.

Human keratinocyte and bronchial epithelial cell line culture

Human epidermal keratinocytes (HEKs) and bronchial epithelial (BEAS-2B) cells were cultured following the standard protocol with EpiVita basal medium and BEGM™ Bronchial Epithelial Cell Growth media, respectively, and incubated at 37°C in 5% CO₂ and 100% humidity. Cells (1×10^4 cells/200 μ L) at 70% confluency were seeded into 96-well culture plates and pre-exposed for 24 h to BPA at 1, 10, or 100 nmol/L followed by exposure to the toll-like receptor 3 ligand Poly (I:C) (2 μ g/mL) for another 24 h. BPA at high concentrations (100 nmol/L) did not induce any cell toxicity. After exposure, the production of TNF α and IL-8 in supernatants was evaluated by ELISA. In another setting, TSLP gene expression in cell pellets was semi-quantified by reverse transcriptase polymerase chain reaction. Three independent experiments were performed in different settings.

Preparation of bone marrow-derived dendritic cells and cytokine assay

Bone marrow-derived dendritic cells (BMDCs) were generated from bone marrow

cells of female BALB/c mice, as described previously (Fukuyama, et al., 2015). At day 8, non-adherent and loosely adherent cells were isolated and seeded at 5×10^4 cells/200 μ L into 96-well plates. The cells were pre-exposed for 24 h to BPA at 1, 10, or 100 nmol/L followed by exposure to the toll-like receptor 2 ligand LPS (25 ng/mL) for another 24 h. The high concentrations of each chemical (100 nmol/L) did not induce any cell toxicity. After exposure, cytokine production (IL-12) in BMDCs was evaluated by ELISA. Three independent experiments were performed in DCs from different animals.

Mixed leukocyte reaction assay

Mixed leukocyte reaction assay was conducted according to previous studies (Beyer *et al.*, 2004; Stover, *et al.*, 2016) with slight modifications. High-purity BMDCs from female BALB/c mice were generated and treated with the same doses of BPA as mentioned in the BMDC cytokine assay. A mycophenolic acid solution at 10 μ mol/L was used as a positive control. To initiate the mixed leukocyte reaction, T-cells were isolated from the spleen of female C57BL/6 mice. Single-cell suspensions were prepared from the spleen by passage through a sterile 70- μ m nylon cell strainer in 1 mL of culture medium (RPMI 1640 with L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 10% heat-inactivated FBS). The spleen cells were then incubated in an erythrocyte lysis buffer for 5 min, followed by an enrichment of T-lymphocytes achieved using Mouse T Lymphocyte Enrichment Set-DM.

This leads to a T-cell purity of higher than 90%. Enriched T-cells (1×10^6 cells/tube) and BPA-treated BMDCs (1×10^5 cells/tube) were seeded in a 2-mL tube and incubated for five additional days. ^3H -thymidine was added for the final 16 h (5 μCi /tube). After five days, the mixed cells were washed twice with PBS, re-suspended with 5% trichloroacetic acid, and stored at 4°C for 18 h. Cell pellets were re-suspended in 1 mL of trichloroacetic acid and transferred to scintillation vials containing 9 mL of scintillation fluid. T-cell proliferation (DPM, disintegrations per minute) was determined using a liquid scintillation counter.

Statistical analysis

Statistical significance of the difference between the vehicle control and treated groups was estimated at 5% and 1% levels of probability. Data from the vehicle-only control and the BPA-treated groups were evaluated by Bartlett's test for equality of variance. When group variances were homogeneous, a parametric one-way analysis of variance was conducted to determine statistical differences among groups. When the analysis of variance was significant, Dunnett's multiple comparison test was applied. When group variances were heterogeneous, data were evaluated by Kruskal-Wallis non-parametric analysis of variance. When differences were significant, Dunnett's mean rank-sum test was applied. Data are expressed as mean $\pm$ standard error of the mean. The data were analyzed using Prism 4 (GraphPad Software, San Diego, CA).

Result

Administration of BPA slightly reduced inflammatory and itch responses in mouse model of allergic dermatitis

When mice with allergic dermatitis were exposed to BPA before the final TDI challenge, their skin inflammation (ear swelling response) was dose-dependently reduced when compared to the mice in the vehicle control group, and significant change was observed in highest concentration group (Figure 1a). Corresponding reduction of scratching bouts to ear swelling response was seen in the BPA-administered group (Figure 1b). The reduced ear swelling and scratching behavior were corroborated by local cytokine levels in ear samples, particularly with the significant reduction in IL-1 β , IL-4, TNF α , and TSLP secretion (Table 1). IFN γ secretion was not altered by BPA administration (Table 1). The number of helper T-cells (TH cells) in auricle LNs, and IL-4 and IFN γ production in auricle LNs were also downregulated by exposure to BPA, but the change in TH cell number and IFN γ production was not significant (Figure 1c-e). There was no impact of BPA administration on total IgE level in serum (Figure 1f).

Respiratory allergic inflammation was significantly exacerbated by BPA administration

While allergic skin inflammation was suppressed by exposure to BPA, allergic airway inflammation was upregulated by BPA administration. The score of red coloration in

the lung was significantly increased by exposure to BPA compared to that in the control group (Figure 2a), and this was corroborated by the significant induction of eosinophils into the BALF (Figure 2b). The cytokine levels in the lung were also increased by BPA administration, and significant increases in IL-1 β , IL-4, IL-33, TNF α , and TSLP were observed in the BPA-treated groups (Table 2). Typical TH1 type cytokine IFN γ was not influenced by BPA administration (Table 2). TH cell number and IL-4 production in hilar LNs were simultaneously induced by exposure to BPA compared to those in the vehicle controls (Figure 2c and 2d). BPA administration also enhanced IFN γ production, but the change was not significant (Figure 1e). Interestingly, total IgE level in serum was also altered by BPA administration, although the change was not significant (Figure 2f).

Pre-exposure to BPA significantly induced cytokine production from human bronchial epithelial cells, but not from human epidermal keratinocytes

Anti- or pro-inflammatory responses observed in the *in vivo* studies were confirmed by *in vitro* experiments with HEKs and BEAS-2B cells. While TNF α production from HEKs was not influenced by exposure to BPA, IL-8 production from BEAS-2B cells was significantly induced by exposure to BPA (Figure 3a and 3c). Comparable upregulation of TSLP gene expression in BEAS-2B cells was found in the BPA-treated groups (Figure 3d). On the other hand, TSLP gene expression in HEKs was significantly downregulated by BPA

exposure (Figure 3b).

No impact of BPA exposure on cytokine release from DCs and proliferation of T-cells

Murine BMDCs and T-cells, which play important roles in the development of allergic inflammation, were analyzed *in vitro* as the next step. When BMDCs were exposed to BPA for 24 h followed by LPS stimulation, the release of IL-12 was comparable with that of the vehicle control group (Figure 4a). T-cell proliferation was measured by a mixed leukocyte reaction assay after cells were exposed to BPA. As observed in the BMDC assay, BPA exposure had no impact on T-cell proliferation (Figure 4b).

Discussion

The current study demonstrated three crucial aspects: (1) oral administration of BPA directly exacerbated TDI-induced respiratory allergic inflammation in mice, whereas skin inflammatory potential in the TDI-induced allergic dermatitis model was slightly interfered by oral administration of BPA; (2) *in vitro* experiments with HEKs and BEAS-2B cells supported the pro- and anti-inflammatory responses observed *in vivo*; and (3) DCs and T-cells might not be involved in pro- and anti-inflammatory responses by oral administration of BPA.

Initially, we performed *in vivo* experiments using established TDI-induced allergic

models to identify whether oral BPA administration alters allergic reaction. As all previous studies focused on the off-target effects of BPA, the present study investigated the direct impact of BPA administration on allergic inflammation and itch behavior. Mice were subjected to oral administration of BPA right before the final TDI challenge. Thus, the direct relationship between allergic response and BPA exposure could be elucidated. To compare the different types of allergies, the allergic airway inflammation model and allergic dermatitis model were taken into consideration in this study. As a result, allergic airway inflammation was significantly upregulated by BPA exposure, with signs such as red coloration of the lung, increased number of eosinophils in the BALF, and increased secretion of pro-inflammatory cytokines (IL-1 β , IL-4, IL-33, TSLP, and TNF α) in lung tissues (Figure 2 and Table 2). These responses were corroborated by significant induction of TH cells and cytokine release from hilar LNs (Figure 2). However, the opposite results were found in the TDI-induced allergic dermatitis model, such as decreased ear swelling responses and pro-inflammatory cytokine levels in the skin tissues (IL-1 β , IL-4, TSLP, and TNF α) by exposure to BPA (Figure 1 and Table 1). Interestingly, itch behavior was also reduced by administration of BPA, although the response was not significant. Wilson, et al. (2013) and Liu *et al.* (2016) recently demonstrated that TSLP and IL-33 evoked itch behavior via activation of pruritic receptors on peripheral neurons in dorsal root ganglia. Thus, the reduction of TSLP and IL-33 secretion in skin tissue possibly influenced the itch behavior. Comparable results were observed in TH

cell number and cytokine secretion in auricular LNs (Figure 1). Several previous reports (Kim, et al., 2017; Wang *et al.*, 2016) have implied that BPA exposure and development of skin allergy were correlated with each other. However, the correlation might be attributed to the off-target effects of BPA exposure based on the above results.

In the next step, we performed *in vitro* studies to see whether activated keratinocytes, bronchial epithelial cells, and immune cells produced lower levels of cytokines or exhibited changes in proliferation in response to incubation with BPA. Recent studies clearly pointed to the active participation of keratinocytes and the airway epithelial barrier in cutaneous and airway immune responses (Gon *et al.*, 2018; Steinhoff *et al.*, 2001). In addition, immune cells such as DCs and T-cells play important roles in both the initiation and maintenance phases of allergic inflammatory diseases. Baumer *et al.* (2007) used toll-like receptor activated canine, murine and human keratinocyte to demonstrate the anti-allergic effect of cyclosporin A and cilomilast. Reines *et al.* (2009) used toll-like receptor activated BMDCs to reinforce the anti-inflammatory effect of Sphingosine-1-Phosphate and FTY720 seen in TDI-induced allergic model in mice. Baumer *et al.* (2010) reported that the immunomodulatory action in T-cell of miltefosine using TDI-induced allergic dermatitis model and mixed leukocyte reaction assay. Thus, we can at least partly predict the effect of immunomodulatory substances by inhibition or activation of keratinocytes, epithelial cells, and DCs as well as T-cell functions.

Activation of HEKs with the toll-like receptor agonist Poly (I:C) induced TNF α production and *TSLP* gene expression, which are related to itch behavior (Wilson, *et al.*, 2013). We observed a slight dose-dependent inhibition for both TNF α and TSLP (Figure 3). On the other hand, activation of BEAS-2B cells with the toll-like receptor agonist LPS induced IL-8 production and *TSLP* gene expression, which are associated with airway inflammation and coughing (Gon, *et al.*, 2018; Russo *et al.*, 2014; Shi *et al.*, 2017; Tang *et al.*, 2011). We observed an upregulation of both IL-8 and TSLP that corresponded to the *in vivo* evidence (Figure 3). LPS also induced cytokine production in BMDCs, but interestingly we did not see any changes in IL-12 with BPA exposure (Figure 4). In addition, we evaluated the proliferation of T-cells in the mixed leukocyte reaction (Figure 4) and found that BPA exposure had almost no impact on T-cell proliferation.

Our findings implied that oral administration of BPA is directly associated with allergic inflammatory diseases. However, the profiles of allergic response to BPA administration are different depending on the type of allergy. Our results demonstrate the ability of BPA to significantly reduce cytokine response from keratinocytes *in vitro* (Figure 3) and reduce skin allergic inflammatory response in a TH2 allergen model (Figure 1). Interestingly, an opposite response to the skin allergic model was observed in IL1- β , IL-4, IL-33, TNF α , and TSLP, a group of cytokines involved in inflammation processes in the respiratory tract (Table 1 and 2). It is not clear why such strong upregulation of these

cytokines was observed only in the *in vivo* model of allergic airway inflammation. However, it is possible that bronchial epithelial cells and TSLP, which have the potential to activate antigen-presenting cells and TH cells, and promote itch behavior by themselves, could play important roles based on our *in vivo* and *in vitro* results.

This study focuses on the acute and direct effect of BPA subjecting to oral administration of BPA at 48 h, 24 h, and 4 h before challenge with TDI, then it becomes important to determine if the effect is reversible. According to the pharmacokinetics data of BPA in orally administered adult CD-1 mice by Doerge *et al.* (2011), oral administration of BPA gave rise to a rapid absorption phase (Tmax: 30 min) with similar distribution kinetics for unconjugated and total BPA. A rapid absorption phase was followed by the distribution phase (2 to 6 h post-oral dosing) and a slower elimination phases for both unconjugated and total BPA until probably 48h post-dosing. These evidences indicate that; (1) our schedule to detect the direct effect of BPA on allergic inflammation was appropriate, (2) “direct” effect of BPA on inflammatory response may not prolong for a long time and it should be reversible. However, accurate evidence is needed to be exhibited in future studies.

In future studies, it would be beneficial to continue characterizing the inflammatory and pruritic potentials of BPA and other types of endocrine disruptors. Evaluating both parameters in chronic models of allergy such as NC/Nga mice would be useful to determine the inflammatory and pruritogenic mechanisms further.

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Conflict of Interest

The authors state that there is no conflict of interest.

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Figure legends

Figure 1. Oral administration of BPA inhibits skin inflammation and scratching behavior in a mouse model of allergic dermatitis. (a) The ear swelling response (μm) was significantly inhibited by oral exposure to BPA. (b) Scratching behavior was also decreased by administration of BPA. (c) Helper T-cell infiltration in auricular LNs was correspondingly reduced by BPA administration. (d) Significant increase in IL-4 production (pg/mL) in auricle LNs supported the pro-inflammatory response to BPA exposure. (e) $\text{IFN}\gamma$ production (pg/mL) in auricle LNs was also increased by BPA exposure but the change was not significant. (f) Total IgE levels (pg/mL) in serum were not altered by BPA exposure. All results are represented as mean $\pm$ SEM, $n = 5$ in each group. * $p < 0.05$, ** $p < 0.01$ (Dunnett's multiple comparison test) vs. vehicle-only control group.

Figure 2. Oral administration of BPA exacerbates allergic airway inflammation in a mouse model of allergic airway inflammation. (a) Dose-dependent significant increase was observed in scoring of red color in the lung. Grade was determined by semi-quantitative examination (0 = no visible changes, 1 = diffuse or patchy red coloration, 2 = moderate dispersed red coloration, 3 = severe red coloration). (b) Eosinophil infiltration in BALF was also induced by oral administration of BPA in a dose-dependent manner. (c) Comparable induction of helper T-cell infiltration was observed in hilar LNs. (d) Significant increase in

IL-4 production (pg/mL) in hilar LNs supported the pro-inflammatory response to BPA exposure. **(e)** IFN γ production (pg/mL) in hilar LNs was also decreased by BPA exposure but the change was not significant. **(f)** Total IgE levels (pg/mL) in serum were exacerbated by BPA exposure, although the response was not significant. All results are represented as mean $\pm$ SEM, n = 5 in each group. *p < 0.05, **p < 0.01 (Dunnett's multiple comparison test) vs. vehicle-only control group.

Figure 3. BPA exposure contributes to cytokine secretion from activated HEKs and BEAS-2B cells. **(a)** Pre-exposure to BPA did not influence TNF α secretion (pg/mL) from activated HEKs. **(b)** TSLP gene expression in activated HEKs was significantly downregulated by pre-exposure to BPA. **(c)** IL-8 (pg/mL) secretion from activated BEAS-2B cells was enhanced by pre-exposure to BPA. **(d)** TSLP gene expression in activated BEAS-2B cells was also upregulated by pre-exposure to BPA. All results are represented as mean $\pm$ SEM, n = 7 in each group. *p < 0.05, **p < 0.01 (Dunnett's multiple comparison test) vs. vehicle-only control group.

Figure 4. Pre-exposure to BPA did not contribute to DC and T-cell function. **(a)** IL-12 production (pg/mL) from activated BMDCs was slightly inhibited by pre-exposure to BPA. **(b)** T-cell proliferation (DPM) was not altered by pre-exposure to BPA. The group treated with 10 μ mol/L mycophenolic acid (Positive) was selected as a positive control. All results are represented as mean $\pm$ SEM, n = 6–7 in each group. *p < 0.05, **p < 0.01 (Dunnett's

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TABLE 1

Response of ear auricle to oral administration of BPA in a mouse model of allergic dermatitis

Ear sample was collected 24 hours after TDI challenge. Results are expressed as mean $\pm$ SEM, n = 5 per group.

*p < 0.05 and **p < 0.01 (Dunnett's multiple comparison test) vs. TDI + vehicle control group.

Group	Untreated	TDI	TDI	TDI
		+	+	+
		Vehicle	BPA 0.06 mg/kg	BPA 0.2 mg/kg
IL-1 β (pg/mg protein)	136 $\pm$ 6	276 $\pm$ 21	198 $\pm$ 15 *	204 $\pm$ 16 *
IL-4 (pg/mg protein)	111 $\pm$ 18	184 $\pm$ 15	141 $\pm$ 10 *	146 $\pm$ 11
IL-33 (pg/mg protein)	100 $\pm$ 11	329 $\pm$ 55	276 $\pm$ 21	291 $\pm$ 23
TNF α (pg/mg protein)	158 $\pm$ 21	246 $\pm$ 20	184 $\pm$ 10 *	189 $\pm$ 15 *
TSLP (pg/mg protein)	20 $\pm$ 3	62 $\pm$ 2	46 $\pm$ 3 **	50 $\pm$ 4 *
IFN- γ (pg/mg protein)	34 $\pm$ 4	30 $\pm$ 1	28 $\pm$ 3	29 $\pm$ 3

TABLE 2

Response of lung to oral administration of BPA in a mouse model of allergic airway inflammation

Lung sample was isolated 24 hours after TDI challenge. Results are expressed as mean $\pm$ SEM, n = 5 per group.

*p < 0.05 and **p < 0.01 (Dunnett's multiple comparison test) vs. TDI + vehicle control group.

Group	Untreated	TDI	TDI	TDI
		+	+	+
		Vehicle	BPA 0.06 mg/kg	BPA 0.2 mg/kg
IL-1 β (pg/mg protein)	27 $\pm$ 3	71 $\pm$ 8	88 $\pm$ 13 *	158 $\pm$ 9 **
IL-4 (pg/mg protein)	45 $\pm$ 5	138 $\pm$ 14	183 $\pm$ 17	261 $\pm$ 10 **
IL-33 (pg/mg protein)	102 $\pm$ 14	612 $\pm$ 81	1009 $\pm$ 123 *	1148 $\pm$ 53 **
TNF α (pg/mg protein)	48 $\pm$ 7	397 $\pm$ 38	518 $\pm$ 115	1070 $\pm$ 86 **
TSLP (pg/mg protein)	13 $\pm$ 2	49 $\pm$ 4	60 $\pm$ 8	111 $\pm$ 5 **
IFN- γ (pg/mg protein)	12 $\pm$ 1	27 $\pm$ 4	33 $\pm$ 3	28 $\pm$ 4

Figure 1

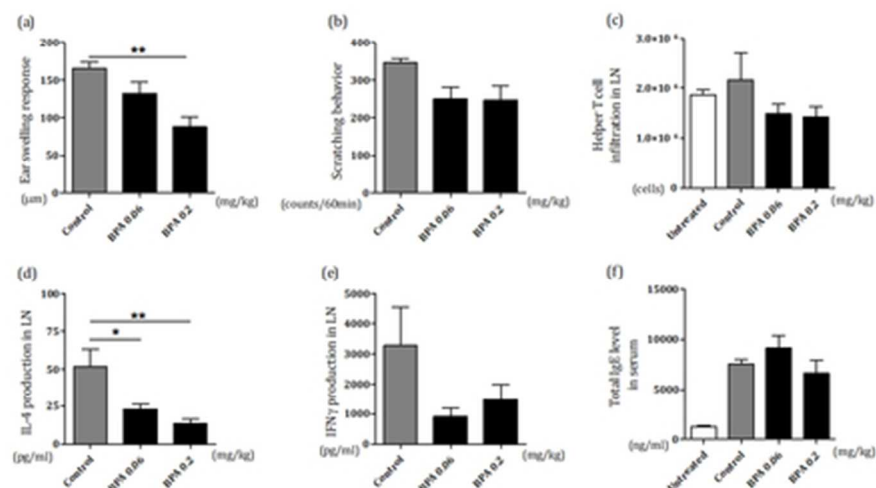


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Figure 2

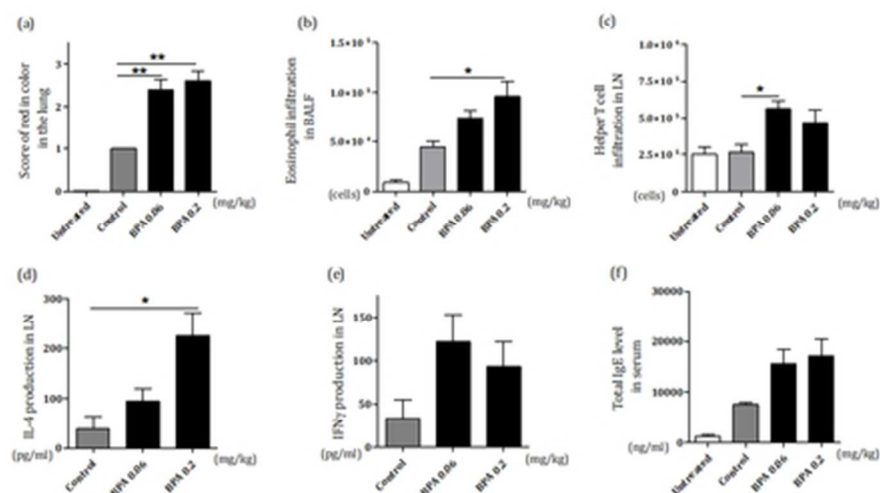


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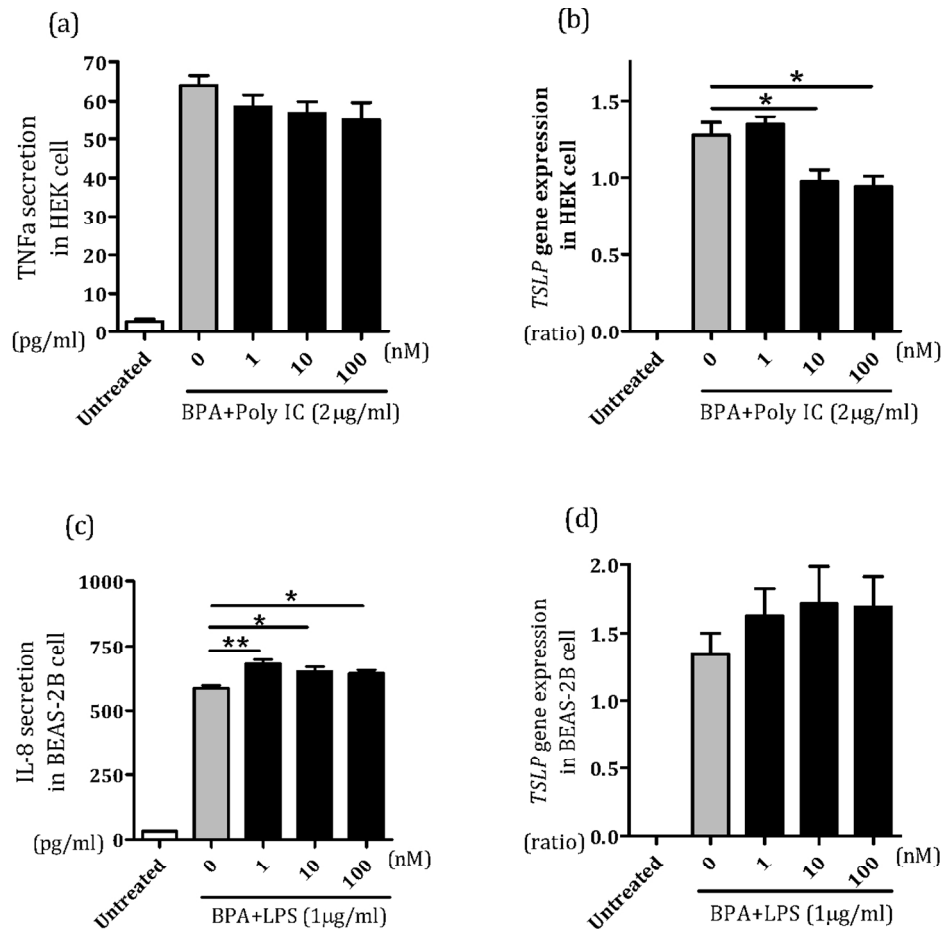


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Figure 4

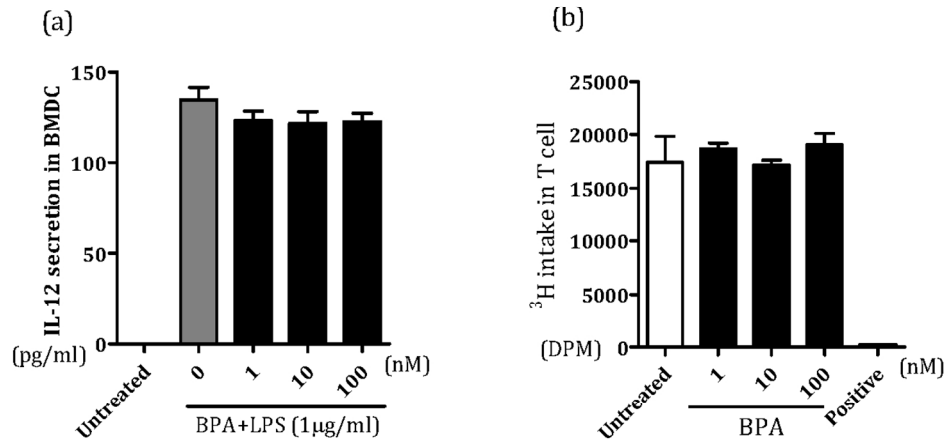


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