



Role of estrogen receptors α and β in the development of allergic airway inflammation in mice: A possible involvement of interleukin 33 and eosinophils

Yuko Watanabe^a, Risako Tajiki-Nishino^a, Hitoshi Tajima^a, Tomoki Fukuyama^{a,b,*}

^a The Institute of Environmental Toxicology, 4321, Uchimoriya-machi, Joso-shi, Ibaraki, 303-0043, Japan

^b Laboratory of Veterinary Pharmacology, School of Veterinary Medicine, Azabu University, 1-17-71, Fuchinobe, Chuo-ku, Sagami-hara, Kanagawa, 252-5201, Japan



ARTICLE INFO

Keywords:

Oestrogen receptor α
Oestrogen receptor β
Allergic airway inflammation
Bronchial epithelial cell
Interleukin 33
Eosinophil

ABSTRACT

Recent studies have shown that the estrogen receptor α (ER α), but not ER β , is involved in the proinflammatory and pruritic responses in cutaneous allergy. In addition, results from our recent study showed that while oral administration of the rather ER β -selective agonist bisphenol A exacerbated the respiratory allergic inflammation, the potential inflammatory reaction in the skin was decreased after administration of bisphenol A. This study aimed to elucidate whether ER α and ER β are involved in the progression of an allergic airway inflammation. We performed an *in vivo* experiment using an animal model of allergic airway inflammation using male BALB/c mice to confirm an increase in the proinflammatory response induced by propylpyrazoletriol (PPT), an ER α agonist, and diarylpropionitrile (DPN), an ER β agonist. Oral administration of PPT or DPN showed a significant increase in the inflammation of the lung and infiltration of eosinophils. While the expression of Th2 cytokines such as interleukin 4 (IL-4) and IL-13 was not affected by exposure to PPT or DPN, administration of these agonists significantly increased the expression of IL-33. The mechanism underlying the development of such allergic inflammatory responses was determined by an *in vitro* study using the human bronchial epithelial cell line (BEAS-2B) and the human eosinophilic leukemia cell line (EoL-1). Activated cells were exposed to PPT or DPN for 24 h, and the cytokine levels were measured. The IL-33 levels in BEAS-2B cells increased significantly after exposure to PPT or DPN. In addition, pretreatment with PPT or DPN increased the expression of IL-8 in activated EoL-1 cells. Our findings indicate that ER α and ER β are involved in the proinflammatory response in respiratory allergy, and their effects may be mediated by an increase in the expression of IL-33 and infiltration of eosinophils.

1. Introduction

Many chemicals released into the environment are hazardous to humans and to the wildlife populations worldwide. Results from studies in the past few decades indicate that among the various toxic chemicals, endocrine disruptors pose a considerable health risk to humans. Many studies to date have showed that endogenous and exogenous estrogens affect the mammalian reproductive function via several types of estrogen receptors (ERs). In addition, the effect of these estrogens and ERs on the immune system has been well established. Previous studies show immunosuppression, including atrophy of the thymus and a decrease in the specific antibody titer (Bernardi et al., 2015; Fukuyama et al., 2011a, 2010).

We examined the estrogen-induced exacerbation of immune

disorders, and our results showed that transient immunosuppression induced by exposure to exogenous estrogens such as the organochlorine agent methoxychlor and the organophosphorus agent parathion at early stages in life may exacerbate immune disorders, including atopic dermatitis and asthma, in the later stages of life (Fukuyama et al., 2012, 2011b; Fukuyama et al., 2011c; Nishino et al., 2013). However, the studies performed to date have described the indirect effects of endocrine disruptors on allergic diseases, and the events between administration and outcome remain to be established. Petzold et al. (2014) reported that the endocrine disruptor bisphenol A has an age-dependent effect on the risk of asthma; thus, the relationship between exposure to estrogen and the development of allergic symptoms should be investigated to understand the effects of such agents. Therefore, we recently performed a study to investigate the direct involvement of ERs in

* Corresponding author at: Laboratory of Veterinary Pharmacology, School of Veterinary Medicine, Azabu University, 1-17-71, Fuchinobe, Chuo-ku, Sagami-hara, Kanagawa, 252-5201, Japan.

E-mail addresses: fukuyama@iet.or.jp (Y. Watanabe), tajiki@iet.or.jp (R. Tajiki-Nishino), tajima@iet.or.jp (H. Tajima), t-fukuyama@azabu-u.ac.jp (T. Fukuyama).

<https://doi.org/10.1016/j.tox.2018.11.002>

Received 7 September 2018; Received in revised form 31 October 2018; Accepted 12 November 2018

Available online 13 November 2018

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proinflammatory and pruritic responses in a model of cutaneous allergy (Watanabe et al., 2018). Our results showed that oral administration of the ER α -selective agonist propylpyrazoletriol (PPT) significantly increased the inflammatory response and scratching behavior, whereas the ER β -selective agonist diarylpropionitrile (DPN) showed no such effects in a mouse model of allergic dermatitis; results of *in vitro* experiments using keratinocytes were consistent with those of our *in vivo* experiment, in that ER α agonists induced production of cytokines in the keratinocytes, whereas an ER β agonist did not affect the cytokine production in keratinocytes; moreover, cytokine secretion by murine bone marrow-derived dendritic cells (mBMDs) and T-cell proliferation were not significantly altered by treatment with ER α or ER β agonists. Our findings indicated that ER α , but not ER β , is involved in the proinflammatory and pruritic responses in cutaneous allergy through the activation of keratinocytes.

On the basis of the findings from previous studies, we hypothesized that exacerbation of allergic airway inflammation by ER α occurs in the same manner as that in a model of allergic dermatitis, but the ER β does not alter the proinflammatory response. Further, a previous study (Tajiki-Nishino et al., 2018) indicated that oral administration of an exogenous rather ER β -selective agonist bisphenol A directly exacerbated toluene-2,4-diisocyanate (TDI)-induced respiratory allergic inflammation in mice, whereas the inflammatory reaction on the skin was not altered after administration of bisphenol A in an animal model of TDI-induced allergic dermatitis. Moreover, the results of *in vitro* experiments on pro- and anti-inflammatory responses using human epidermal keratinocytes and human bronchial epithelial cells supported those observed *in vivo*. Our findings indicate that ER β has opposite effects on the inflammatory responses in skin allergy and respiratory allergy. To date, a limited number of studies have investigated these effects and further studies are required to substantiate our findings.

This study aimed to investigate whether ER α and ER β are directly involved in the exacerbation of allergic airway inflammation similar to that observed previously in an animal model of allergic dermatitis. We performed *in vivo* experiments using a mouse model of allergic airway inflammation. In addition, we performed *in vitro* experiments using a human bronchial epithelial cell line (BEAS-2B) and a human eosinophilic leukemia cell line (EoL-1) to determine the detailed mechanisms of action. The epithelial cells of the airway not only serve as a barrier to pathogens and allergens but also regulate immune responses and play a crucial role in the pathogenesis of allergic airway inflammation (Penberthy et al., 2014). When exposed to allergens, bronchial epithelial cells produce the inflammatory cytokines thymic stromal lymphopoietin (TSLP), interleukin 25 (IL-25), and IL-33, which contribute to the innate inflammatory responses (Allakhverdi et al., 2007; Angkasekwinai et al., 2007; Willart et al., 2012).

2. Materials and methods

2.1. Mice

We purchased 7-week-old male BALB/c mice were purchased from Charles River Japan Laboratories (Kanagawa, Japan), and they were used for developing a model of allergic airway inflammation. A previous study (Watanabe et al., 2018) showed that proinflammatory responses after oral administration of estrogen were similar between ovariectomized female mice and male mice although males can convert testosterone to only small amounts of estradiol *via* aromatase. Therefore, we used male mice to develop the experimental model in this study to exclude the effects of the estrus cycle and endogenous estrogen. After the acclimatization period, healthy animals were allocated to the study. On the day of initiation of estrogens treatment, animals were weighed and allocated to each dose group through a stratified random sampling method based on body weights. After allocation, it was confirmed that there are no statistically significant differences in the mean body weights among groups and that all individual body

weights are within a range of 80% to 120% of the mean value. During the study, the animals were maintained in a controlled environment with 12 h of light per day at $22 \pm 2^\circ\text{C}$ and $50 \pm 20\%$ humidity. The mice received a certified pellet diet and water *ad libitum*. The study protocol was approved by the Institute of Environmental Toxicology Animal Care and Use Committee, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International (IACUC Protocol No. AC17149).

2.2. Responses to oral administration of E2 in a TDI-induced model of allergic airway inflammation in male mice

We induced allergic airway inflammation in mice by repeated topical sensitization with Th2 hapten toluene-2,4-diisocyanate (TDI, Sigma-Aldrich Co., LLC, Tokyo, Japan) according to the method described by Tajiki-Nishino et al. (Tajiki-Nishino et al., 2018). TDI-sensitized mice were orally administered β -Estradiol (E2, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan; 0.03 and 0.1 mg/kg) 48, 24, and 1 h before intratracheal challenge of TDI (0.001%, 50 μL). E2 showed no systemic toxicity or immunosuppression at the doses used in this study, which was similar to the findings reported in a previous study (Watanabe et al., 2018). The mice were killed under isoflurane anesthesia 24 h after TDI challenge. Red coloration in the lung was evaluated in a blinded manner using a semi-quantitative score based on the criteria described in a previous study (Nishino et al., 2016) (0 = no visible changes, 1 = diffuse or patchy red coloration, 2 = moderate dispersed red coloration, and 3 = severe red coloration). We collected the bronchoalveolar lavage fluid (BALF) and resected the hilar lymph nodes (LNs) from each mouse for evaluation of the cell number. The BALF was collected by cannulating the trachea and lavaging the lungs three times with 1 mL of phosphate-buffered saline (PBS).

2.3. Responses to oral administration of ER α or ER β agonists in a TDI-induced model of allergic airway inflammation in male mice

In the second experiment, a similar protocol with 2.2. was used, with the addition of oral administration of selective agonists of PPT (a selective agonist of ER α , Sigma-Aldrich Co., LLC; 2.5 and 5 mg/kg) and DPN (a selective agonist of ER β , Sigma-Aldrich Co., LLC; 2.5 and 5 mg/kg). PPT and DPN showed no systemic toxicity or immunosuppression at the doses used in this study, which was similar to the findings reported in a previous study (Watanabe et al., 2018). The mice were killed under isoflurane anesthesia 24 h after TDI challenge. Red coloration in the lung was evaluated in a blinded manner. We isolated the affected lobe of the lung, collected the BALF, and resected the hilar LNs from each mouse for evaluation of the cytokines and fluorescence-activated cell sorting.

2.4. Analysis of hilar LNs

We prepared single-cell suspensions of LNs using a 70- μm cell strainer and performed fluorescence-activated cell sorter (FACS) analyses and determined the levels of IL-4 produced. FACS analysis for infiltrations of helper T cells and DCs were performed by staining 5×10^5 cells with monoclonal antibodies specific to helper T cells (anti-CD3 and anti-CD4) or DCs (anti-CD11c and anti-CD40) (antibodies were ordered from Miltenyi Biotec K.K., Tokyo, Japan and BD Pharmingen, Tokyo, Japan). We analyzed 10,000 cells in a FACSVerse flow cytometer using the FACSuite software (BD Pharmingen). The levels of cytokines were determined by culturing 5×10^5 single-cell suspensions of LNs with Dynabeads Mouse T-Activator CD3/CD28 reagent (Thermo Fisher Scientific, Inc., Kanagawa, Japan) for 24 or 96 h to stimulate the T-cell receptor. Then, the levels of IL-4 in the culture supernatants were measured using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (R&D Systems, Minneapolis, MN). Optical density was measured at a wavelength of

450 nm using a microplate reader (SPECTRA MAX 190; Molecular Devices, LLC., Tokyo, Japan).

2.5. Analysis of the BALF

The supernatants from the first fraction of the BALF were used for measurement of the cytokine levels. The cell pellets of all three fractions were used for determination the eosinophil counts using the FACS. We measured the levels of IL-4, IL-13, IL-33, TSLP, eotaxin, and RANTES using ELISA according to the manufacturer's protocol (R&D Systems). For FACS analysis, 1×10^6 cells were incubated with monoclonal antibodies (anti-Gr-1, anti-CD49b and anti-CD11c; BD Pharmingen). Ten thousand cells were washed and analyzed on a FACSVerse flow cytometer using the FACSuite program.

2.6. Cytokine levels in the lung tissue

The frozen lung tissue was homogenized in RPMI-1640 medium (FUJIFILM Wako Pure Chemical Corporation) containing 1 mmol/L Pefabloc (Sigma-Aldrich Co., LLC) with an electric homogenizer. The protein content in the isolated supernatant was determined using a DC protein assay kit (Bio-Rad Laboratories, Inc., Tokyo, Japan) according to the manufacturer's protocol. The levels of IL-4, IL-13, IL-17, IL-33, TSLP, eotaxin, and RANTES were measured using ELISA according to the manufacturer's protocol (R&D Systems).

2.7. BEAS-2B cell line culture

The BEAS-2B cell line was obtained from the European Collection of Cell Culture (ECACC) and cultured according to the standard protocol in the bronchial epithelial cell growth media (BEGM™; Lonza Japan Ltd., Tokyo, Japan). The cells (1×10^4 cells/200 μ L) at 70% confluency were seeded in a 96-well culture plate and were exposed to PPT or DPN at 1, 10, or 100 nmol/L for 24 h before being stimulated with the Toll-like receptor (TLR) ligand Lipopolysaccharide (LPS, Sigma-Aldrich Co., LLC) at 1 μ g/mL for an additional 4 (for reverse transcription polymerase chain reaction (RT-PCR)) or 24 h (for ELISA). TLR ligands such as lipopolysaccharide (LPS) and Poly (IC) may mimic the conditions of the skin, and we used LPS and Poly (IC) because they are well-known (and characterized) stimuli for BEAS-2B cells (Tajiki-Nishino et al., 2018). The concentrations of PPT and DPN used in *in vitro* studies were determined on the basis of the results of the preliminary cytotoxicity test and reporter gene assay for ER α and ER β (Tajiki-Nishino et al., 2018; Watanabe et al., 2018). None of the test substances induced any signs of cytotoxicity even at the highest concentrations. Our previous results showed that the binding affinity of PPT and DPN to ER α and ER β ranged from 1 to 1000 nmol/L. The levels of IL-8 in the supernatants were measured using ELISA, and the expression levels of the genes encoding IL-4 and IL-33 were determined in a semi-quantitative manner using RT-PCR.

2.8. RT-PCR

BEAS-2B cells were washed twice in ice-cold PBS, and total RNA was isolated using the NucleoSpin RNA II kit according to the manufacturer's instructions (TaKaRa Bio, Inc., Tokyo, Japan). PrimeScript RT reagent Kit with gDNA Eraser (Takara Bio, Inc.) was used to reverse transcribe RNA into cDNA, in accordance with the manufacturer's protocol. The corresponding cDNAs were amplified using quantitative real-time RT-PCR using the Thermal Cycler Dice system (Takara Bio, Inc.). The data acquired for each sample were normalized to expression levels of the housekeeping gene β -actin (*ACTB*). The following primers were used for human *IL-4* and *IL-33* mRNA: *IL-4* forward, 5'-TCTCGAATGTACCAGGAGCCATATC-3'; *IL-4* reverse, 5'-AGCACCTTGAAGCCCTACAGA-3'; *IL-33* forward, 5'-CACATTGGGCAAAGTTGCTTCTAA-3'; and *IL-33* reverse, 5'-GCATCCAGAGGTCAGGTGATACAG-3'.

2.9. EoL-1 cell line culture

The EoL-1 cell line was obtained from the ECACC and cultured according to the standard protocol in RPMI-1640 medium containing 10% fetal calf serum. It has been recognized that phenol red indicates the estrogen-like activity, therefore, phenol red-free RPMI-1640 was used in this experiment to exclude the endogenous estrogen effects. The cells (5×10^4 cells/200 μ L) were seeded in a 96-well culture plate and were exposed to PPT or DPN at 1, 10, or 100 nmol/L for 24 h before being stimulated with the TLR2 ligand LPS (Sigma-Aldrich Co., LLC) at 25 ng/mL for a further 24 h. The levels of IL-8 in the supernatants were measured using ELISA.

2.10. Statistical analyses

Data are expressed as the mean $\pm$ 1 standard error of mean (SEM). Analysis of variance was used to evaluate the results. For significant results, differences between the vehicle control and treatment groups were assessed using Dunnett's multiple comparison test. The statistical significance of differences between the vehicle control and treated groups was estimated at the 5% and 1% probability levels.

3. Results

3.1. Oral exposure to E2 significantly exacerbated inflammatory responses in a TDI-induced mouse model of allergic airway inflammation

To elucidate the direct contribution of estrogens to development of the allergic airway inflammation, we first analyzed the pro-inflammatory potential of E2 as an endogenous estrogen, *in vivo* using a TDI-induced mouse model of allergic airway inflammation. Compared to the vehicle-only control, the E2 groups showed a significant increase in the score of red coloration of the lung (Supplemental Fig. 1a). Analysis of the local immune response in BALF and hilar LNs revealed that total cell influxes were elevated following oral treatment with E2 compared to the vehicle control group (Supplemental Fig. 1b and c).

3.2. Oral exposure to PPT or DPN significantly increased the inflammatory response in a TDI-induced mouse model of airway inflammation

To elucidate the direct effects of ER α and ER β in the development of allergic airway inflammation, we determined the proinflammatory potential of the ER α agonist PPT and ER β agonist DPN *in vivo* using a mouse model of TDI-induced allergic airway inflammation. Compared to the vehicle-only control, the PPT or DPN groups showed an increase in the score of red coloration of the lung (Fig. 1a). In particular, oral administration of DPN significantly increased the red coloration in the lung. Results similar to those mentioned above were observed with infiltration of eosinophils into the BALF. We observed a significant increase in the infiltration of eosinophils in the group receiving 5 mg/kg PPT and 2.5 or 5 mg/kg of DPN (Fig. 1b). The group receiving DPN showed a higher extent of eosinophil infiltration than the group treated with PPT. Treatment with PPT or DPN had no effect on levels of Th2 cytokines such as IL-4 and IL-13 (Table 1). We observed only a slight increase in the expression of eosinophil-activating cytokines, including eotaxin and RANTES, in the PPT or DPN treatment groups. The production of IL-33 significantly increased in a dose-dependent manner after oral administration of PPT or DPN (Table 1). The expression of cytokines in the lung samples increased in a dose-dependent manner. While we observed no significant changes in the levels of Th2, Th17, and eosinophil-activating cytokines (IL-4, -13, -17, TSLP, eotaxin and RANTES), the levels of IL-33 were significantly increased in the PPT or DPN groups (Table 2). Analysis of the local immune responses in hilar LNs showed that compared to the vehicle control group, the group treated with DPN 5 mg/kg showed a significant increase in the infiltration by DCs (Fig. 1d), whereas the helper T cells were not

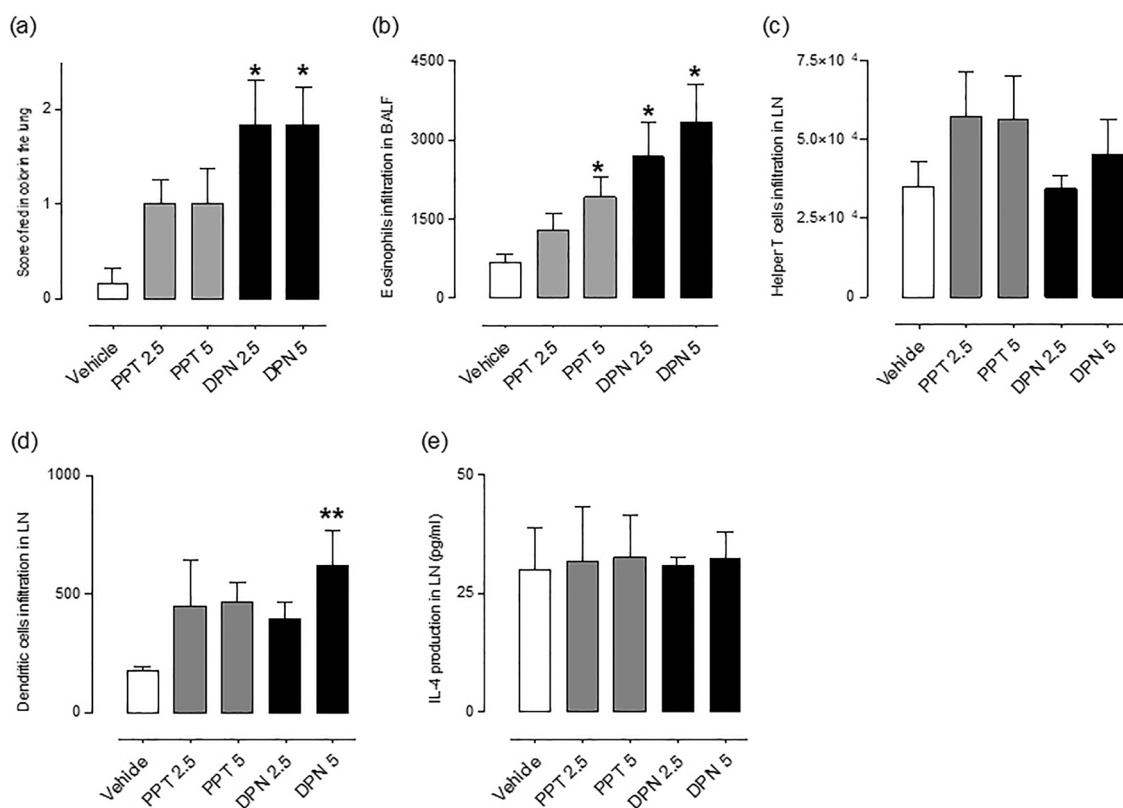


Fig. 1. Oral administration of propylpyrazoletriol (PPT) or diethylpropionitrile (DPN) exacerbates inflammation in a toluene-2,4-diisocyanate (TDI)-induced allergic airway inflammation in male mice.

The score of red color in the lung was significantly increased after exposure to propylpyrazoletriol (PPT) or diethylpropionitrile (DPN) (a). An increase in the infiltration of eosinophils into the bronchoalveolar lavage fluid (BALF) was observed 24 h after the toluene-2,4-diisocyanate (TDI) challenge (b). After the administration of PPT or DPN, we observed only a slight effect on the presence of CD3⁺CD4⁺ T cells (c), but the number of CD11c⁺CD40⁺ dendritic cells (d) significantly increased after exposure to DPN 5 mg/kg. The levels of interleukin 4 (IL-4) (pg/mL) were not changed after exposure to PPT or DPN (e). Each value is presented as the mean $\pm$ 1 standard error of mean (SEM). n = 6 per group. *p < 0.05, **p < 0.01 (Dunnett's multiple comparison test) vs. the vehicle-only control group.

influenced by treatment with PPT or DPN (Fig. 1c). In addition, IL-4 production was not observed in the hilar LNs in the PPT- or DPN-treated groups (Fig. 1e).

3.3. Involvement of the bronchial epithelium in ER α - and ER β -mediated cytokine production *in vitro*

Results of the *in vivo* study showed a significant increase in the production of IL-33 after oral administration of PPT or DPN; thus, we examined the role of ERs in the bronchial epithelium *in vitro* using BEAS-2B cells, which express the ER α and ER β (Tajiki-Nishino et al., 2018). Compared to the vehicle control group, the group of TLR ligand-activated BEAS-2B cells treated with PPT or DPN showed a significant dose-dependent increase in the secretion of IL-8 (Fig. 2). The activation

of BEAS-2B cells by PPT or DPN was corroborated by a significant increase in the expression of IL-33 (Fig. 3), which indicated a possible relationship between airway inflammation and an increase in the IL-33 levels.

3.4. Role of eosinophils in the exacerbation of airway inflammation by PPT or DPN

Results of the *in vivo* study showed a significant increase in the number of eosinophils in the BALF; thus, we examined the role of eosinophils using the EoL-1 cells. Pretreatment with PPT or DPN significantly increased the secretion of IL-8 and tumor necrosis factor α (TNF α) (Fig. 4), which corroborated the significant increase in eosinophil infiltration into the BALF observed *in vivo*.

Table 1

Responses in the BALF to oral administration of PPT or DPN in a TDI-induced male mouse model of allergic airway inflammation.

	TDI + vehicle control	TDI + 2.5 mg/kg PPT	TDI + 5 mg/kg PPT	TDI + 2.5 mg/kg DPN	TDI + 5 mg/kg DPN
IL-4 (pg/mg protein)	68.2 $\pm$ 12.8	70.4 $\pm$ 9.5	57.2 $\pm$ 5.9	79.9 $\pm$ 16.2	54.7 $\pm$ 10.8
IL-13 (pg/mg protein)	17.7 $\pm$ 4.6	15.7 $\pm$ 4.0	15.9 $\pm$ 1.7	19.8 $\pm$ 7.7	14.2 $\pm$ 4.6
IL-33 (pg/mg protein)	72 $\pm$ 12	113 $\pm$ 15	125 $\pm$ 15*	95 $\pm$ 12	163 $\pm$ 26**
TSLP (pg/mg protein)	3.91 $\pm$ 1.52	6.55 $\pm$ 1.78	8.81 $\pm$ 2.76	5.46 $\pm$ 1.93	7.63 $\pm$ 2.93
EOTAXIN(pg/mg protein)	18.3 $\pm$ 4.2	28.0 $\pm$ 5.7	36.3 $\pm$ 8.9	27.9 $\pm$ 7.3	39.5 $\pm$ 14.5
RANTES (pg/mg protein)	8.2 $\pm$ 2.1	10.1 $\pm$ 2.1	12.4 $\pm$ 2.7	16.4 $\pm$ 7.2	14 $\pm$ 5

BALF samples were collected 24 h after TDI challenge. Results are expressed as means $\pm$ SEM. n = 6 per group. *p < 0.05, **p < 0.01 (Dunnett's multiple comparison test) vs. the TDI + vehicle control group.

Table 2

Responses in the lung tissue to oral administration of PPT or DPN in a TDI-induced male mouse model of allergic airway inflammation.

	TDI + vehicle control	TDI + 2.5 mg/kg PPT	TDI + 5 mg/kg PPT	TDI + 2.5 mg/kg DPN	TDI + 5 mg/kg DPN
IL-4 (pg/mg protein)	182 ± 2	165 ± 11	162 ± 6	175 ± 14	162 ± 18
IL-13 (pg/mg protein)	58.5 ± 1.4	50.1 ± 5.3	52.2 ± 2.2	55.5 ± 5.3	56.2 ± 7.0
IL-17 (pg/mg protein)	29.4 ± 0.4	27.4 ± 2.1	29.2 ± 1.7	29.4 ± 2.9	28.0 ± 2.9
IL-33 (pg/mg protein)	823 ± 49	1322 ± 83 [*]	1402 ± 177 ^{**}	1748 ± 347 ^{**}	1797 ± 145 ^{**}
TSLP (pg/mg protein)	31.6 ± 0.9	26.9 ± 2.5	26.6 ± 1.5	30.4 ± 3.5	25.9 ± 3.2
EOTAXIN (pg/mg protein)	129 ± 5	110 ± 7	134 ± 6	128 ± 15	137 ± 10
RANTES (pg/mg protein)	88.8 ± 6.8	70.0 ± 4.0	82.5 ± 8.1	92.8 ± 19.2	82.5 ± 4.5

Lung samples were collected 24 h after TDI challenge. Results are expressed as means ± SEM. n = 6 per group. *p < 0.05, **p < 0.01 (Dunnett's multiple comparison test) vs. the TDI + vehicle control group.

4. Discussion

Allergic diseases, including asthma, atopic dermatitis, and food allergy are more common in women than in men (Melgert et al., 2007; Schatz and Camargo, 2003), and the symptoms are exacerbated during the menstrual cycle and pregnancy (Farha et al., 2009; Vrieze et al., 2003). Although the role of gender differences in the severity of allergic diseases has been established, the mechanisms by which sex steroids such as estrogen influence the target organs/tissues have not been clarified thus far. Therefore, we performed several studies that focus on the relationship between exposure to sex steroids and development of allergies. Results of our previous study showed that transient exposure to endocrine disrupting agents such as methoxychlor or parathion (4 weeks after transient exposure to an endocrine disruptor) during the early stages of life increase the risk of development of atopic dermatitis and allergic airway inflammation in the later stages of life (Fukuyama et al., 2012, 2011b; Fukuyama et al., 2011c; Nishino et al., 2013). Previous studies have only described the indirect effects of endocrine disruptors on inflammatory diseases, and the mechanism underlying these effects remains to be established. Therefore, this study aimed to elucidate the direct link between the activation of ERs and allergic airway inflammation.

We investigated the involvement of ERα and ERβ, which are of the greatest importance and have an opposite impact on the immune system. Previous studies have shown that ERβ-selective agonists are used for the treatment of autoimmune diseases and several other inflammatory diseases (Ponnusamy et al., 2017; Wu et al., 2013), and the activation of ERα is involved in most of the undesirable proliferative effects (Patemi et al., 2014). In addition, the results of our recent study

showed that while the activation of ERα after oral administration of ERα agonists was directly associated with proinflammatory and pruritic responses in a mouse model of allergic dermatitis, these effects were not observed after administration of ERβ-selective agonists. The secondary objective of this study was to identify the roles of these ERs in increasing or decreasing the allergenicity in our mouse model of allergic airway inflammation using ERα- and ERβ-specific agonists.

Initially, we analyzed the effects of E2 as a representative endogenous estrogen *in vivo* using a mouse model of the Th2-type hapten TDI-induced allergic airway inflammation. The mice received oral administration of E2 immediately before (48, 24, and 1 h) the intratracheal challenge of TDI in an attempt to establish a direct relationship between the activation of ERs and development of an allergy. The pro-inflammatory effects of E2 were evidenced by an enhanced the score of red coloration in the lung (Supplemental Fig. 1a), which was corroborated by total cell influx in BALF and hilar LNs (Supplemental Fig. 1b and c). These findings demonstrated a direct relationship between estrogen administration and the development of allergic airway inflammation. However, as E2 is a dual agonist of both ERα and ERβ, the contribution of ERα or ERβ alone to allergic airway inflammation remained uncertain.

Therefore, we secondary investigated the effects of ERα agonist (PPT) or ERβ agonist (DPN) *in vivo* using a mouse model of allergic airway inflammation. The mice received oral administration of PPT or DPN immediately before (48, 24, and 1 h) the intratracheal challenge of TDI. Our results showed the proinflammatory effects of not only PPT but also DPN by an increase in the score of red coloration in the lung (Fig. 1a) and eosinophil infiltration into the BALF (Fig. 1b). The number of eosinophils is significantly increased in patients with asthma (Bossley

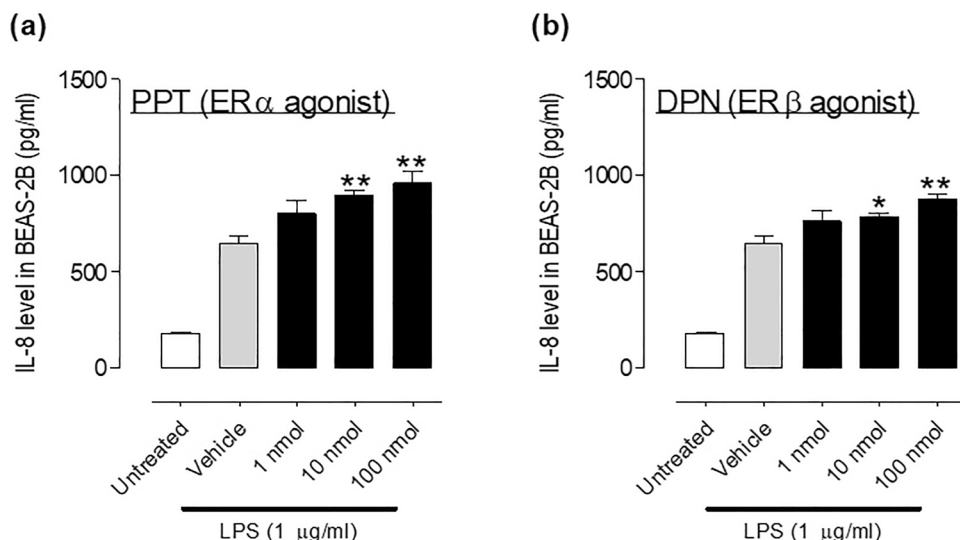


Fig. 2. Both estrogen receptor α (ERα) and ERβ play a role in lipopolysaccharide (LPS)-stimulated secretion of interleukin 8 (IL-8) by the human bronchial epithelial cells. Pretreatment with propylpyrazoletriol (PPT) or diarylpropionitrile (DPN), significantly increased the activation of the bronchial epithelium as determined by lipopolysaccharide (LPS)-stimulated secretion of interleukin 8 (IL-8) (a and b). Each value is the mean (pg/mL) ± 1 standard error of mean (SEM). n = 7 per group. *p < 0.05, **p < 0.01 (Dunnett's multiple comparison test) vs. the vehicle-only control group. BEAS-2B = human bronchial epithelial cell line.

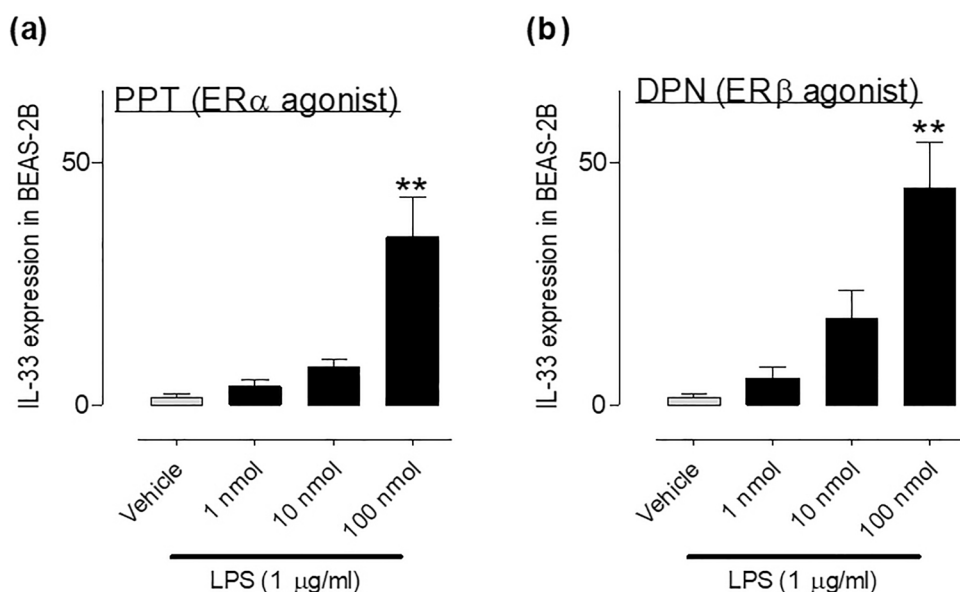


Fig. 3. Expression of the interleukin 33 (IL-33) gene in the human bronchial epithelium was significantly upregulated after exposure to estrogen receptor α (ER α) and ER β agonists. Pretreatment with propylpyrazoletriol (PPT) or diarylpropionitrile (DPN) significantly upregulated the lipopolysaccharide (LPS)-induced expression of interleukin 33 (IL-33) gene in the bronchial epithelium (a and b). Each value is the mean $\pm$ 1 standard error of mean (SEM). $n = 7$ per group. * $p < 0.05$, ** $p < 0.01$ (Dunnett's multiple comparison test) vs. the vehicle-only control group.

et al., 2012), and eosinophil-deficient mice show a decrease in airway hyperresponsiveness, which results in a decrease in airway regeneration and mucus production (Lee et al., 2004). Eosinophils are considered to play an essential role in the activation of allergic airway inflammation; thus, we concluded that oral administration of PPT or DPN aggravated the allergic airway inflammation through infiltration of eosinophils. A significant increase in the infiltration of DCs into the hilar LNs (Fig. 1d) indicated that oral administration of PPT or DPN affects the inflammatory response in the lung because DCs orchestrate immune responses, which presents antigens to T lymphocytes and drives their activation and differentiation to effector T cells (Diebold, 2008). The infiltration of helper T cells into the LNs (Fig. 1c) and production of IL-4 (Fig. 1e) were not entirely consistent with an enhanced score of red coloration in the lung and eosinophil infiltration into the BALF. In addition, oral administration of PPT or DPN did not affect the levels of Th2-type cytokines such as IL-4 and IL-13 and the Th17-type cytokine IL-17 in the BALF and lung tissue (Tables 1 and 2). Thus, our results showed that activation of ER α or ER β induced proinflammatory responses in the respiratory system, but Th2- or Th17-mediated immune responses do not play a role in this inflammatory response. Our mouse model of allergic airway inflammation was driven by Th2 cells, and thus, we expected an association between inflammation and Th2 response. Although an increased infiltration of eosinophils was observed in mice treated with PPT or DPN, the levels of eosinophil-activating cytokines, including eotaxin and RANTES, did not increase in these groups (Tables 1 and 2). Oral administration of PPT or DPN significantly increased the production of IL-33 in the BALF and lung tissue (Tables 1 and 2). IL-33 is mainly secreted by the bronchial epithelial cells and plays a pivotal role in the pathogenesis of allergic airway diseases (Penberthy et al., 2014; Willart et al., 2012; Yao et al., 2018); thus, we hypothesized that IL-33 and bronchial epithelial cells play an important role in the aggravation of allergic airway inflammation by activation of ER α or ER β .

Results of our *in vivo* study showed that bronchial epithelial cells play an important role in the aggravation of allergic airway inflammation; thus, we performed an *in vitro* experiment using BEAS-2B cells to confirm the results obtained in the *in vivo* experiment. We compared the difference in the levels of IL-8 secreted by BEAS-2B cells stimulated by the TLR-ligand LPS; our results showed that compared to the cells treated with the vehicle control, the BEAS-2B cells pretreated with PPT or DPN showed a dose-dependent and significant increase in the levels of IL-8 (Fig. 2a and b). IL-8 is secreted by any cells upon activation of the TLRs that are involved in the innate immune response,

and previous studies indicate that the levels of IL-8 are increased in patients with allergic airway diseases and atopic diseases (Hatano et al., 1999; Kaburagi et al., 2001; Kimata and Lindley, 1994; Reynolds et al., 2018). Although an upregulation in the secretion of IL-8 indicates an involvement of ER α or ER β in the activation of epithelial cells, a direct link between expression of IL-33 and airway inflammation has not been established thus far. Therefore, we examined the expression of IL-33 in LPS-stimulated BEAS-2B cells pretreated with PPT or DPN. Our results showed a significant upregulation in the expression of IL-33 after pretreatment with PPT or DPN, which confirmed the involvement of ER α and ER β in the activation of epithelial cells and production of IL-33 (Fig. 3). Schmitz et al. (2005) showed that the secretion of IL-33 induced by IL-1 β and TNF α plays an important role in Th2-associated immune responses; the results of their *in vitro* study showed that polarized Th2 cells upon re-stimulation respond to IL-33 by significantly increasing the secretion of IL-4 and IL-13. Lee et al. (2014) showed that blockade of the IL-33/ST2 pathway ameliorates airway inflammation in a murine model of allergic asthma; they showed that the production of Th2-type cytokines, including IL-4, IL-5, and IL-13, was inhibited by anti-IL-33 antibody or soluble ST2. Inhibition of airway hyperresponsiveness upon exposure methacholine was observed after blockade of IL-33/ST2 pathway. In addition, Poulsen et al. (2018) showed that the expression of IL-33 in airway inflammation is associated with an increase in the expression of Th2-type cytokines observed in adult patients with an exacerbation of asthma, which provides *in vivo* evidence supporting that IL-33 may be responsible in driving type-2 inflammation in acute asthma. Although we observed no significant increase in the levels of Th2-type cytokines in mice in our study, the activation of ER α or ER β increased the production of IL-33 in the airway followed by the production of specific Th2-type cytokines.

Moreover, the results from our *in vivo* experiment showed that the significant increase in the infiltration of eosinophils in the BALF after administration of PPT or DPN may be directly associated with the exacerbation of airway inflammation. A recent study shows that IL-33 precedes IL-5 in regulating eosinophil commitment, and IL-33 is required for eosinophil homeostasis (Johnston et al., 2016). In addition, IL-33 is associated with eosinophilic airway inflammation (Tworek et al., 2018). On the basis of the results from previous studies, we hypothesized that the significant increase in eosinophil infiltration observed in our mouse model was not directly induced by the administration of PPT or DPN, but by the PPT- or DPN-induced upregulation of IL-33. We confirmed these findings by performing an *in vitro* study using the EoL-1 cell line. The results of the assay to determine TLR-stimulated

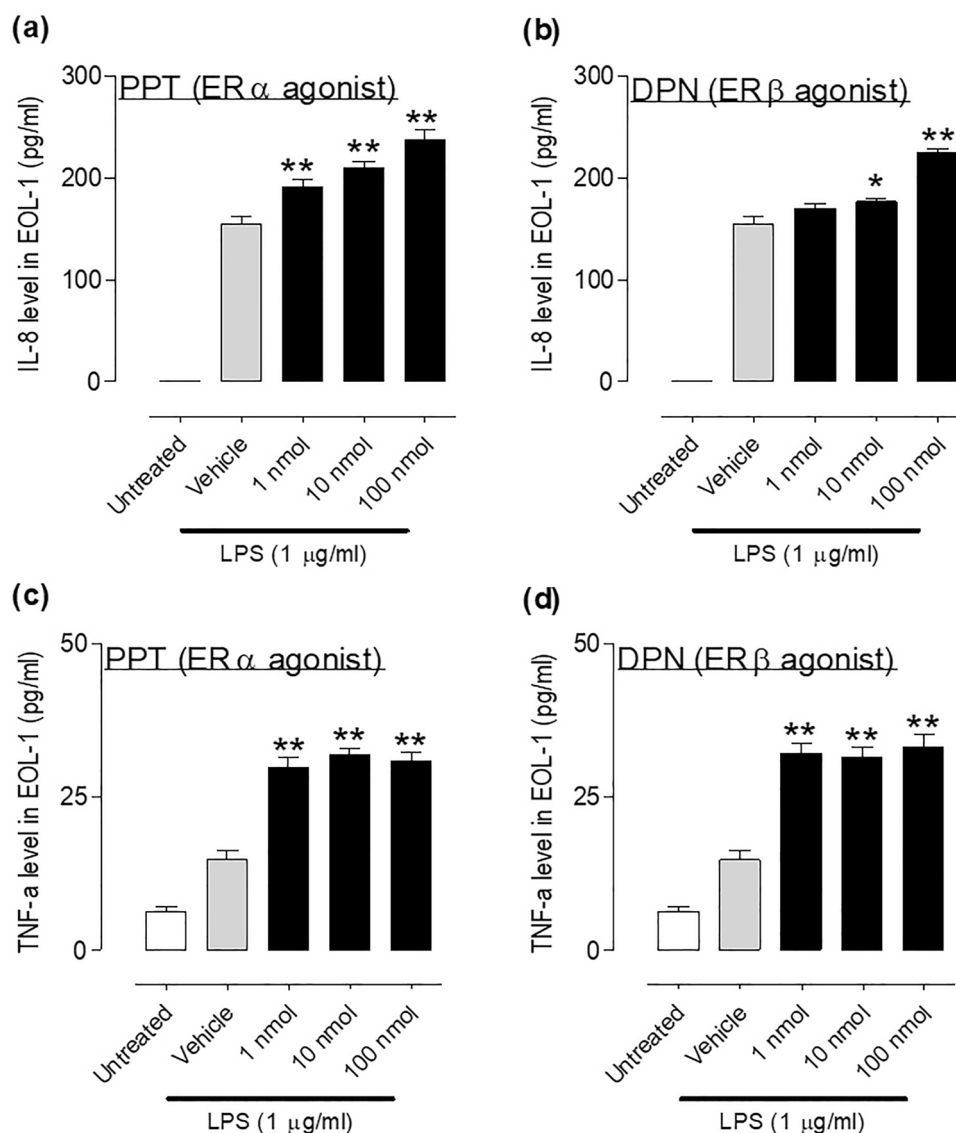


Fig. 4. Estrogen receptor α (ER α) and ER β agonists significantly inhibit the lipopolysaccharide (LPS)-stimulated secretion of interleukin 8 (IL-8) and tumor necrosis factor α (TNF α) by the eosinophils.

Pretreatment with propylpyrazoletriol (PPT) or diarylpropionitrile (DPN) significantly increased the lipopolysaccharide (LPS)-mediated secretion of interleukin 8 (IL-8) (a and b) and tumor necrosis factor α (TNF α) (c and d). Each value is the mean (pg/mL) $\pm$ 1 standard error of mean (SEM). $n = 7$ per group. * $p < 0.05$, ** $p < 0.01$ (Dunnett's multiple comparison test) vs. the vehicle-only control group. EoL-1 = human eosinophilic leukemia cell line.

production of cytokines showed that the activation of ER α or ER β had a significant effect on the production of IL-8 and TNF α (Fig. 4). Results from our *in vitro* study showed that several pathways are involved in the ER α - or ER β -mediated development of allergic airway inflammation, and overexpression of IL-33 induced by modulation of the function of the airway epithelium indicate a direct association with the eosinophil function.

Taken together, our findings show that the activation of ER α or ER β by oral administration of their respective agonists is directly associated with proinflammatory responses in a mouse model of allergic airway inflammation mainly via the production of IL-33 and infiltration of eosinophils, whereas the administration of these drugs has no effect on the Th2-type immune responses. Results from our *in vitro* experiment corroborated those obtained *in vivo* in that both ER α and ER β significantly induced cytokine responses, including the expression of IL-8, IL-33, in BEAS-2B and EoL-1 cell lines. On the basis of our results, we can conclude that the airway epithelium and IL-33, which have the potential to activate immunocytes such as antigen-presenting cells, eosinophils and mast cells and polarize Th2 cells, play an

important role in respiratory allergy exacerbated by ER α or ER β agonists. Moreover, the results from our recent study showed that ER α , but not ER β , was involved in the exacerbation of inflammatory and pruritic responses in the development of cutaneous allergy, which suggested that response to ERs might be organ dependent. Further studies using models of food allergy or ER α /ER β -deficient mice should be performed to further characterize the mechanism underlying the effects of estrogens in the induction of proinflammatory responses.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interest

The authors state no conflict of interest.

Transparency document

The [Transparency document](#) associated with this article can be found in the online version.

Acknowledgements

We are grateful to Dr. Tadashi Kosaka, Dr. Hiroaki Aoyama, and Dr. Takanori Harada for their valuable suggestions. We would like to thank Editage (www.editage.jp) for English language editing.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.tox.2018.11.002>.

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Supplemental Fig. 1. Oral administration of E2 exacerbates allergic airway inflammation in a TDI-induced male mouse model of allergic airway inflammation.

The score of red color in the lung was significantly increased after exposure to β -Estradiol (E2) (a). An increase in the infiltration of total cells into the bronchoalveolar lavage fluid (BALF) was observed 24 h after the toluene-2,4-diisocyanate (TDI) challenge (b). After the administration of E2, we observed only a slight effect on the cell influx into hilar lymph node (LN) (c). Each value is presented as the mean $\pm$ 1 standard error of mean (SEM). n = 5 per group. *p < 0.05, **p < 0.01 (Dunnett's multiple comparison test) vs. the vehicle-only control group.