

Involvement of estrogen receptor α in pro-pruritic and pro-inflammatory responses in a mouse model of allergic dermatitis

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ABSTRACT

It has been reported that endogenous or exogenous estrogens can affect the immune system, resulting in immune disorders; however, their direct involvement in such conditions remains to be demonstrated. The purpose of this study was to investigate whether estrogen receptors (ER) are directly implicated in pro-pruritic and pro-inflammatory reactions in cutaneous allergy. Initially, enhancement of the pro-inflammatory response by several ER agonists [methoxychlor (MXC), β -estradiol (E2), propylpyrazoletriol (PPT; an ER α agonist), and diarylpropionitrile (DPN; an ER β agonist)] was examined *in vivo* using a male BALB/c mouse model of allergic dermatitis induced by toluene-2,4-diisocyanate administration. The ear swelling response, itch response, and local cytokine secretion were measured. Subsequently, the mechanism underlying the development of such allergic reactions was analyzed *in vitro* using human epidermal keratinocytes, murine bone marrow-derived dendritic cells (mBMDCs), and the mixed leucocyte reaction assay. Activated cells were exposed to each ER agonist for 24 h, and cytokine secretion and cell proliferation were measured. Our *in vivo* experiments indicated significant up-regulation of pro-inflammatory and pro-pruritic responses in the E2-, MXC-, and PPT-treated groups compared to the control group; however, no change was observed in the DPN-treated group. Levels of cytokines expressed by keratinocytes, such as TSLP and IL-33, were particularly increased by exposure to E2, MXC, or PPT. These *in vivo* results were confirmed *in vitro* in keratinocytes, but not mBMDCs or T cells. Our findings imply that ER α is involved in pro-inflammatory and pro-pruritic responses in cutaneous allergy through activation of keratinocytes.

1. Introduction

The effect of endogenous or exogenous estrogens on the mammalian immune system, particularly in immunosuppression involving thymus atrophy, is well documented (Sakazaki et al. 2007; Fukuyama et al. 2010, 2011a; Bernardi et al. 2015). Interestingly, some reports have also indicated that transient immunosuppression induced by estrogens early in life may exacerbate immune disorders, including atopic dermatitis, asthma, rheumatoid arthritis, and food allergy, in later life stages. In order to examine this phenomenon, our group recently conducted several studies using mouse models of atopic dermatitis and allergic airway inflammation involving oral exposure to the endocrine disruptors methoxychlor (MXC) and parathion during immaturity (4 weeks of age) (Fukuyama et al., 2011a, b, 2012; Nishino et al. 2013).

Analysis of allergic responses in maturity (4 weeks after exposure) revealed skin and airway allergic reactions to be significantly increased by pre-exposure to MXC or parathion, suggesting that endocrine disruptors aggravate such reactions in mice. Sobel et al. (2005) presented similar findings with respect to the progression of systemic lupus erythematosus due to administration of an estrogenic substance. Moreover, Sakazaki et al. (2006) demonstrated that 17 β -estradiol (E2) enhances inflamed skin contact hypersensitivity in BALB/c mice. However, previous studies have only described the indirect effects of endocrine disruptors on inflammatory diseases, and the events between administration and outcome remain quite uncertain. Indeed, Petzold et al. (2014) have implied that the impact of the endocrine disruptor bisphenol A on asthma risk is strongly age-dependent, and demonstration of a direct relationship between estrogen exposure and the

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development of allergic symptoms is necessary for a proper understanding of the situation. Therefore, the primary aim of this study was to elucidate whether estrogen exposure directly exacerbates allergic responses using a mouse model of allergic dermatitis.

Endogenous or exogenous estrogens exert their effects by binding to estrogen receptors (ERs). Of such receptors, ER α and ER β are generally of greatest importance and have a considerable impact on the immune system. From a clinical perspective, ER β -selective agonists might be therapeutically useful for some types of cancer and several other inflammatory diseases. Since most of the undesirable proliferative effects of estrogens involve activation of ER α , fewer efforts have been devoted to the development of pharmaceuticals that ER α -selective agonists than for ER β -selective agonists (Paterni et al. 2014). However, only very limited data concerning their effects on allergic diseases are currently available. Therefore, the secondary objective of this study was to identify the roles of these ERs in enhancing or diminishing allergenicity in our mouse model of allergic dermatitis using several types of ER agonists selective to ER α and/or ER β . In addition, the detailed mode of action was confirmed *in vitro*. In particular, type 2 helper T (Th2) cells, dendritic cells and keratinocytes play a central role to develop and promote the cutaneous inflammation and pruritus *via* cytokine release including IL-4, IL-5, IL-8, IL-12, IL-13, TNF α and thymic stromal lymphopoietin (TSLP) (Danso et al. 2014; Mu et al. 2014). Previous reports have shown that Th2 cytokines such as IL-4 and IL-13 promotes T cell activation and differentiation into the Th2 subtype and can switch the antibody isotype from IgM to IgE (Kaplan et al. 1996; Oettgen 2000). TSLP is also a possible candidate protein involved in the initiation, development and progression of atopy and atopic diseases both in mice and in humans (Indra 2013). We particularly focused on the cytokine release to elucidate the functions of ERs.

2. Material and methods

2.1. Materials

Dinitrochlorobenzene (DNCB), MXC, RPMI-1640 medium, and β -Estradiol (E2) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Lipopolysaccharide (LPS), Pefabloc® SC, polyinosinic-polycytidylic acid sodium salt [poly(I:C)], propylpyrazoletriol (PPT; a selective ER α agonist), diarylpropionitrile (DPN; a selective ER β agonist), hydroxychlor (HPTE; a major metabolite of MXC), toluene-2,4-diisocyanate (TDI), and TMB Liquid substrate system for membranes were obtained from Sigma-Aldrich Co., LLC. (Tokyo, Japan). Methylpiperidinopyrazole dihydrochloride (MPP; a selective ER α antagonist) was supplied by Tocris Bioscience (Minneapolis, MN). Methyl-³H-thymidine was provided by PerkinElmer Japan Co., Ltd. (Kanagawa, Japan). A DC protein assay kit, TGX precast gels (10%) was purchased from Bio-Rad Laboratories, Inc. (Tokyo, Japan). Dynabeads Mouse T-Activator CD3/CD28 reagents were obtained from Thermo Fisher Scientific, Inc. (Kanagawa, Japan). EpiVita basal medium with hydrocortisone was supplied by Cell Applications, Inc. (San Diego, CA). Recombinant mouse GM-CSF was provided by PeproTech, Inc. (Rocky Hill, NJ). ELISAs for mouse and/or human IL-4, -5, -8, -12, -13 and -33, TNF α and TSLP; antibodies for human GAPDH, ER α /NR3A1 and ER β /NR3A2; and mouse IgG HRP conjugated antibody were purchased from R&D Systems (Minneapolis, MN). Mouse BD Fc Block PE-conjugated anti-mouse CD3, PE-Cy7-conjugated anti-mouse CD4, and FITC-conjugated anti-mouse CD40 antibodies and a Mouse T Lymphocyte Enrichment Set-DM were obtained from BD Pharmingen (Tokyo, Japan). An APC-conjugated anti-mouse CD11c antibody was supplied by Miltenyi Biotec K.K. (Tokyo, Japan). NucleoSpin RNA II, SYBR® Premix Ex Taq™ GC, primers and Western BLoT Blocking Buffer were provided by TaKaRa Bio (Tokyo, Japan). Pro-Prep Protein extraction solution was ordered from iNtRON Biotechnology, Inc. (Seongnam, South Korea). Human ER α and ER β Reporter Assay Systems were purchased from Indigo Biosciences, Inc. (State College, PA).

2.2. Mice

Seven-week-old male, female and ovariectomized BALB/c mice and female C57BL/6 mice were purchased from Charles River Japan Laboratories (Kanagawa, Japan). C57BL/6 mice were only used for isolating the T cells for mix leucocyte reaction assay. The animals were kept in a controlled environment with 12 h of light per day at 22 $\pm$ 2 °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 AAALAC International (IACUC Protocol No. AC17030).

2.3. Responses to oral or topical administration of E2 or MXC in a TDI-induced male mouse model of allergic dermatitis

The mouse model of allergic dermatitis was induced by repeated topical sensitization with Th2 hapten TDI according to the method described by 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. In the first experiment, TDI-sensitized mice were orally or topically administered E2 (0.01% for topical application and 0.1 mg/kg for oral administration) or MXC (1% for topical application and 10 mg/kg for oral administration) 48, 24, and 4 h before the final TDI treatment. In this study, E2 and MXC are used as representative estrogens which can bind to the estrogen receptors. E2 or MXC was topically applied onto both rostral neck skin and ear auricle (30 μ l for rostral neck skin and 25 μ l for ear auricle). Both substances were diluted in corn oil and acetone for oral administration and topical application, respectively. E2 and MXC concentrations used in the topical and oral dosing were decided according to the previous studies as well as preliminary dose-finding test (data not shown), which did not induce the systemic toxicity and immunosuppression (Fukuyama et al. 2010; Fukuyama et al. 2011a; Fukuyama et al. 2011b; Fukuyama et al. 2011c; Fukuyama et al. 2012). Immediately after TDI challenge, scratching behavior was monitored by video for 60 min. The ear swelling response was determined by subtracting ear thickness before TDI administration from that 24 h afterwards. Animals were euthanized 24 h after TDI challenge under isoflurane inhalation anesthesia. The ear auricle and auricular lymph node (LN) were isolated for FACS analysis, cytokine measurements and histological evaluation.

2.4. Responses to oral administration of selective agonists of ER α (PPT) and ER β (DPN) in a TDI-induced mouse model of allergic dermatitis

In the second experiment, a similar protocol with 2.3. was used, with the addition of oral administration of selective agonists of ER α (PPT; 2.5 and 5 mg/kg) and ER β (DPN; 2.5 and 5 mg/kg). Since males can convert testosterone to estradiol *via* aromatase; similar experiment with ovariectomized female mice instead of male mice was also performed with the oral administration of 5 mg/kg of each PPT and DPN. Both substances were suspended in olive oil by ultrasonication for 5 min just before each administration. At the doses for PPT and DPN used, none of the test substances employed in this study resulted in any signs of systemic toxicity or immunosuppression, according to our preliminary experiments (data not shown).

2.5. Responses to oral administration of PPT and DPN in a DNCB-induced male mouse model of allergic dermatitis

Th1 hapten DNCB-induced mouse model of allergic dermatitis was generated according to the method described by Yu et al. (2015) with slight modifications. In short, the abdominal skin of the BALB/c mice was depilated with a commercially available depilating cream. On the day after depilation, the abdominal skin was stripped 10 times with adhesive tapes. Just after the tape stripping, a 50 μ l of 1% DNCB in

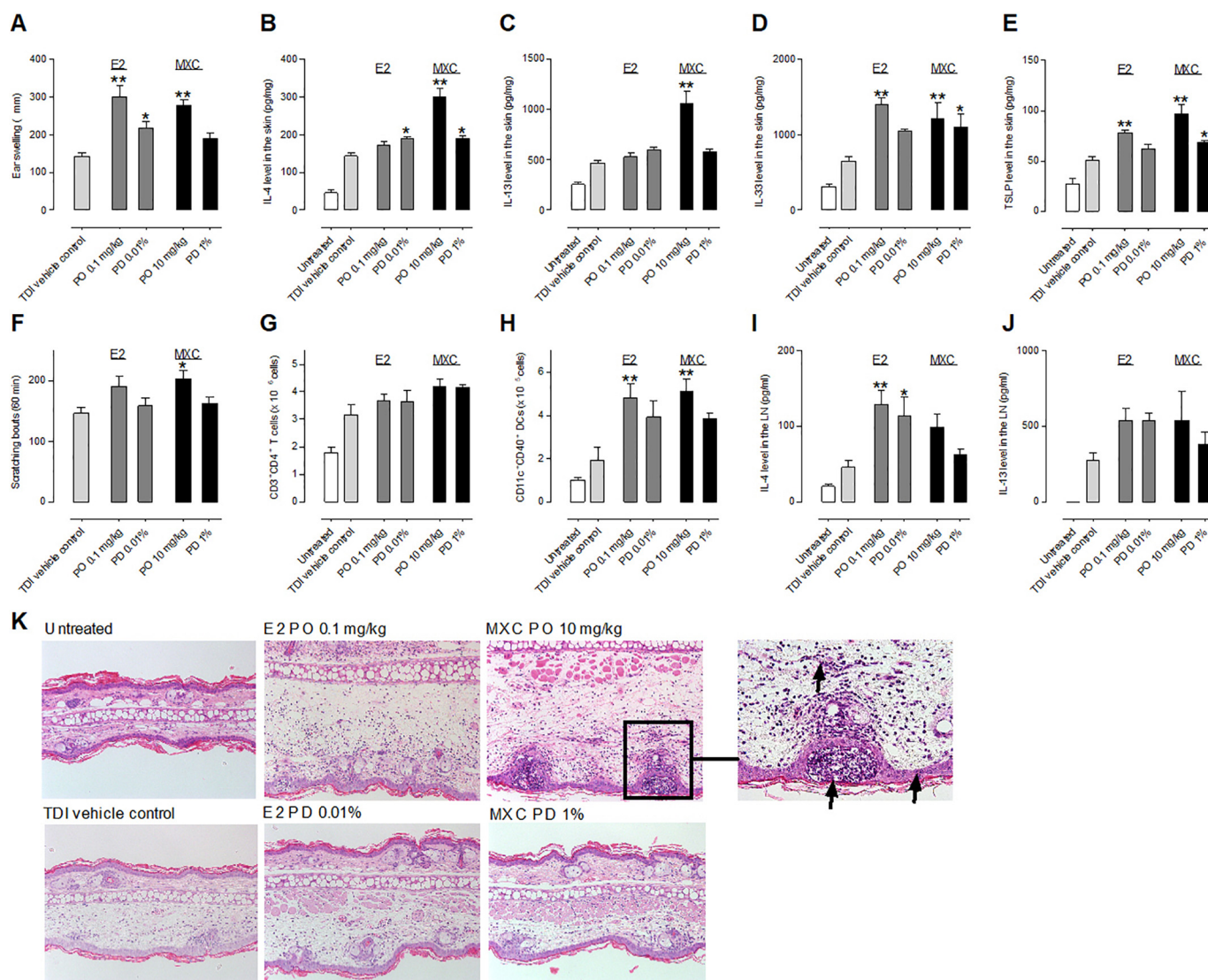


Fig. 1. Oral or topical administration of E2 or MXC exacerbates skin inflammation and scratching behavior in a TDI-induced male mouse model of allergic dermatitis. The ear swelling response (μm) was significantly increased by exposure to E2 or MXC (A). Comparable changes in pro-inflammatory cytokine levels in ear skin were seen 24 h following TDI challenge (B–E). Scratching behavior also increased following administration of E2 or MXC (F). After administration of E2 or MXC, only a slight impact on the presence of CD3⁺CD4⁺ T cells (G) was noted, but the number of CD11c⁺CD40⁺ dendritic cells (H) was significantly increased. IL-4 (I) and IL-13 (J) levels (pg/ml) were also increased by exposure to E2 or MXC. Representative histological images of ear skin (K). Arrows indicated typical examples of the cellular infiltrate, folliculitis and epidermal hyperplasia. Each result is presented as the mean $\pm$ 1 SEM. $n = 5$ to 8 per group. * $p < .05$, ** $p < .01$ (Dunnett's multiple comparison test) vs. the vehicle-only control group. E2 = β -Estradiol, MXC = methoxychlor, TDI = toluene-2,4-diisocyanate, PO = perioral administration, PD = peridermal application, LN = lymph node.

Table 1

Histological evaluation of the ear auricle to oral or topical administration of E2 or MXC in a TDI-induced mouse model of allergic dermatitis.

Group	Untreated	TDI vehicle	E2 PO 0.1 mg/kg	E2 PD 0.01%	MXC PO 10 mg/kg	MXC PD 1%
Dermal edema	0.00 $\pm$ 0.00	1.60 $\pm$ 0.24	2.00 $\pm$ 0.00	1.60 $\pm$ 0.24	2.20 $\pm$ 0.37	1.40 $\pm$ 0.24
Cellular infiltration	0.00 $\pm$ 0.00	1.20 $\pm$ 0.20	2.00 $\pm$ 0.45	1.60 $\pm$ 0.24	2.40 $\pm$ 0.24	1.20 $\pm$ 0.20
Folliculitis	0.00 $\pm$ 0.00	1.00 $\pm$ 0.32	2.00 $\pm$ 0.45	1.20 $\pm$ 0.37	2.20 $\pm$ 0.37	1.80 $\pm$ 0.20
Hyperkeratosis	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.20 $\pm$ 0.20	0.20 $\pm$ 0.20	0.00 $\pm$ 0.00
Epidermal hyperplasia	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.20 $\pm$ 0.20	0.00 $\pm$ 0.00
Total score	0.00 $\pm$ 0.00	3.80 $\pm$ 0.49	6.00 $\pm$ 0.89	4.60 $\pm$ 0.75	7.20 $\pm$ 1.24*	4.40 $\pm$ 0.51

Histological score (0, within normal limits; 1, mild; 2, moderate; and 3, severe) was given for each finding. Results are expressed as the mean $\pm$ SEM. $n = 5$ to 8 per group. * $p < .05$, ** $p < .01$ (Dunnett's multiple comparison test) vs. the TDI + vehicle control group. E2 = β -Estradiol, MXC = methoxychlor, TDI = toluene-2,4-diisocyanate, PO = perioral administration, PD = peridermal application.

Table 2

Responses in the ear auricle to oral administration of PPT or DPN in a TDI-induced male mouse model of allergic dermatitis.

	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
Ear swelling (μm)	107 $\pm$ 3	110 $\pm$ 12	187 $\pm$ 15**	110 $\pm$ 13	78 $\pm$ 14
Scratching bouts	87 $\pm$ 15	97 $\pm$ 18	175 $\pm$ 14**	78 $\pm$ 12	97 $\pm$ 9
IL-4 (pg/mg protein)	77 $\pm$ 2	84 $\pm$ 5	87 $\pm$ 4	77 $\pm$ 6	72 $\pm$ 8
IL-5 (pg/mg protein)	172 $\pm$ 9	206 $\pm$ 13	221 $\pm$ 18*	208 $\pm$ 19	172 $\pm$ 24
IL-13 (pg/mg protein)	569 $\pm$ 18	557 $\pm$ 15	584 $\pm$ 24	574 $\pm$ 20	560 $\pm$ 26
IL-33 (pg/mg protein)	335 $\pm$ 10	410 $\pm$ 25**	478 $\pm$ 6**	332 $\pm$ 19	302 $\pm$ 19
TNF α (pg/mg protein)	148 $\pm$ 5	163 $\pm$ 7	165 $\pm$ 9	152 $\pm$ 10	147 $\pm$ 4
TSLP (pg/mg protein)	22 $\pm$ 1	27 $\pm$ 2	39 $\pm$ 2**	23 $\pm$ 1	19 $\pm$ 2

Ear samples were collected 24 h after TDI challenge. Results are expressed as means $\pm$ SEM. n = 6 per group. *p < .05, **p < .01 (Dunnett's multiple comparison test) vs. the TDI + vehicle control group.

Table 3

Responses in the auricular LN to oral administration of PPT or DPN in a TDI-induced male mouse model of allergic dermatitis.

	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
CD3 ⁺ CD4 ⁺ cells ($\times 10^6$)	1.71 $\pm$ 0.13	2.21 $\pm$ 0.15	2.56 $\pm$ 0.35*	1.37 $\pm$ 0.16	1.32 $\pm$ 0.13
CD11c ⁺ CD40 ⁺ cells ($\times 10^5$)	0.92 $\pm$ 0.14	1.89 $\pm$ 0.08	2.39 $\pm$ 0.47**	0.87 $\pm$ 0.15	0.61 $\pm$ 0.10
IL-4 (pg/ml)	15 $\pm$ 3	24 $\pm$ 4	42 $\pm$ 6**	13 $\pm$ 4	7 $\pm$ 3
IL-5 (pg/ml)	74 $\pm$ 26	250 $\pm$ 61*	238 $\pm$ 32*	75 $\pm$ 28	56 $\pm$ 28
IL-13 (pg/ml)	219 $\pm$ 94	598 $\pm$ 142	943 $\pm$ 181**	207 $\pm$ 115	134 $\pm$ 59
TNF α (pg/ml)	137 $\pm$ 11	139 $\pm$ 10	176 $\pm$ 16	125 $\pm$ 18	110 $\pm$ 10

Cytokine production was measured in response to anti-CD3 and -CD28 antibody stimulation. Each result is the mean $\pm$ 1 SEM. (n = 6 per group). *p < .05, **p < .01 (Dunnett's multiple comparison test) vs. the TDI + vehicle control group.

acetone/olive oil (4:1) was applied to the stripped epidermis. On the next day, again 50 μl of 1% DNCB in acetone/olive oil were applied. A week after the last sensitization, the challenge with topically applied DNCB (0.1%, 10 μl) onto the ear auricle was performed 48 h, 24 h and 4 h after oral administrations of 5 mg/kg of each PPT and DPN. The ear swelling response, LN analysis and cytokine determination were carried out in the same manner as described in 2.3.

2.6. Ear skin histology

Samples of ear skin were fixed in 10% formalin solution, sectioned, and stained with hematoxylin and eosin. Semi-quantitative histological evaluation (0, nothing of note; 1, mild; 2, moderate; and 3, severe) of dermal edema, cellular infiltration, folliculitis, hyperkeratosis, and epidermal hyperplasia was then performed. The summed score of above 5 categories was used for further statistical evaluation.

2.7. Ear skin tissue cytokine levels

Frozen ear tissue was homogenized in 500 μl RPMI-1640 medium containing 1 mmol/l Pefabloc with an electric homogenizer. The protein content of the isolated supernatant was determined with a DC protein assay kit according to the manufacturer's protocol. IL-4, -5, -13, and -33, IFN γ , TNF α , and/or TSLP levels were measured by ELISA according to the manufacturer's protocol. The plate was developed with tetramethylbenzidine in the dark at room temperature for 20 min. Optical density was read at a wavelength of 450 nm with a microplate reader (SPECTRA MAX 190, Molecular Devices, LLC., Tokyo, Japan.).

2.8. Analysis of auricular LNs

Single-cell suspensions prepared from LNs were subjected to cytokine production and FACS analyses. Cell survival rate was confirmed by trypan blue staining as > 85% for all samples. To stimulate the T-cell receptor, these suspensions were cultured with anti-CD3 and -CD28 antibodies for 24 or 96 h, and levels of cytokines (IL-4, -5, and -13 and TNF α) in the culture supernatants were then measured by ELISA. For FACS, 5×10^5 cells were stained with monoclonal antibodies specific to helper T cells (anti-CD3 and -CD4) or dendritic cells (anti-CD11c and

-CD40), and analyzed (10,000 events) in a FACSVerse flow cytometer using FACSsuite software (BD Pharmingen).

2.9. Reporter assay for human ER α and ER β

The agonistic effect of each test substance was confirmed using human ER α and ER β reporter assay systems according to the manufacturer's instructions. In brief, reporter cells were seeded in a 96-well white culture plate and pre-exposed for 24 h to each test substance (E2, HPTE, PPT, or DPN) at 0.1 to 1000 nmol/l. Luminescence was developed by luciferase detection reagent and quantified with luminometer (Spectra Max L, Molecular Devices, LLC.). Concentrations for E2, PPT, or DPN were based on the manufacturer's protocol as the representative agonists for each ER. Concentrations for HPTE were determined by cytotoxicity assay. Even at the highest concentration for each test substance, none of the test substances induced cytotoxicity (data not shown). Seven concentrations for each test substance were selected based on the abovementioned criteria. HPTE (the major metabolite of MXC) was used for *in vitro* studies instead of MXC, as it mainly exhibits estrogenic activity according to a previous report (Liu et al. 2016b).

2.10. Human epidermal keratinocyte line

Human epidermal keratinocyte cell line was cultured following the standard protocol in EpiVita basal medium. The cells (1×10^4 cells/200 μl) were seeded in a 96-well culture plate and pre-exposed for 24 h to each test substance (E2, HPTE, PPT, or DPN) at 1, 10, or 100 nmol/l, before being stimulated with the Toll-like receptor (TLR) 3 ligand poly (I:C) at 2 $\mu\text{g}/\text{ml}$ for a further 4 h (for RT-PCR) or 24 h (for ELISA). TLR ligands such as LPS and Poly (IC) might mimic what is happening in/on skin but we used LPS and Poly (IC) as they are well known (and characterized) stimulus for keratinocytes and BMDCs (Lee et al. 2011; Fukuyama et al. 2015). Concentrations for each test substance used *in vitro* studies were determined based on the preliminary cytotoxicity test and reporter gene assay for ER α and ER β . Even at the highest concentration, none of the test substances induced any signs of cytotoxicity (data not shown). Our results from reporter gene assay showed that binding affinity of all test substances to ER α and/or ER β was ranged 1 to 1000 nmol/l (see Supplemental Fig. 1). Levels of IL-1 β , IL-8 and

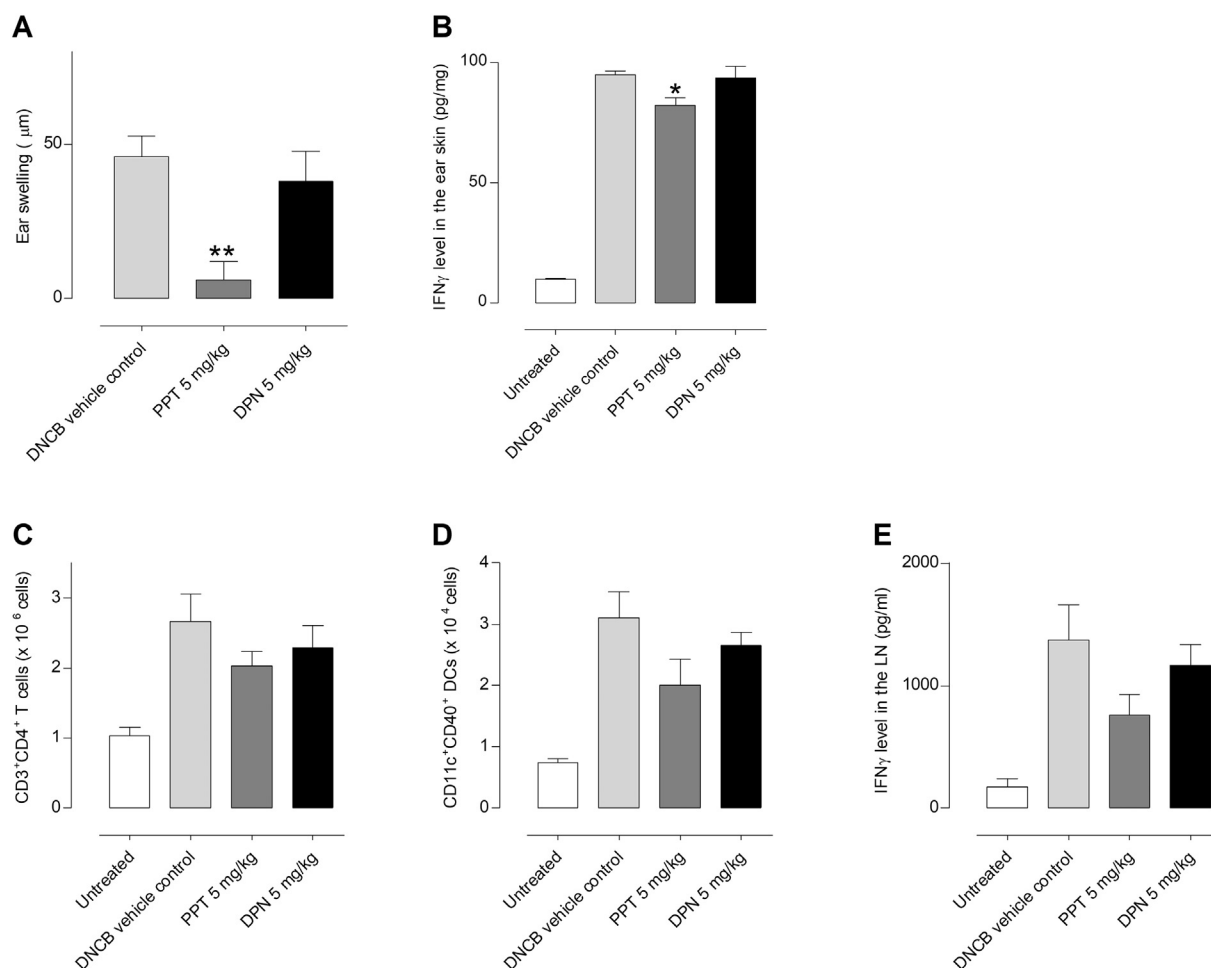


Fig. 2. Responses in the ear auricle and auricular LN to oral administration of PPT or DPN in a DNCB-induced male mouse model of allergic dermatitis. The ear swelling response (μm) was significantly suppressed by exposure to PPT (A). Comparable change in IFN γ levels in ear skin were seen 24 h following DNCB challenge (B). A slight downwards trend on the presence of CD3 $^{+}$ CD4 $^{+}$ T cells (C), CD11c $^{+}$ CD40 $^{+}$ dendritic cells (D) and IFN γ levels (pg/ml) (E) in auricular LN were observed after administration of PPT. Each result is presented as the mean $\pm$ 1 SEM. $n = 5$ per group. * $p < .05$ (Dunnett's multiple comparison test) vs. the vehicle-only control group. DNCB = dinitrochlorbenzene.

TNF α in supernatants were measured using ELISA, and expression of the genes encoding IL-4, IL-33 and TSLP was tested in a semi-quantitative manner by subjecting cell pellets to RT-PCR. In addition, the contribution of ER α to IL-8 secretion was confirmed with the ER α antagonist MPP and agonist E2. Three independent experiments were performed for each treatment.

2.11. Western blot

Keratinocytes were washed twice in ice-cold PBS, and total protein was extracted using a Pro-Prep Protein extraction solution according to the manufacturer's instructions. A total protein was loaded on Mini-Protean TGX precast gels. Blotting was performed using the semi dry electro blotter. The membranes were blocked with Western BLoT Blocking Buffer. Antibodies were used as 1:3000 dilution in the Western BLoT Blocking Buffer. Incubation time for primary antibodies was 2 h and 1 h for secondary antibodies (in the Western BLoT Blocking Buffer). Signals were developed by incubating with TMB Liquid substrate system for membranes and imaged with calibrated densitometer (GS-800, Bio-Rad Laboratories, Inc.).

2.12. RT-PCR

Keratinocytes were washed twice in ice-cold PBS, and total RNA was isolated using a NucleoSpin RNA II kit according to the manufacturer's

instructions. 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 by quantitative real-time polymerase chain reaction (PCR) with 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*, *IL-33* and *TSLP* mRNA: *IL-4* forward, 5'- TCTCGAATGTACCAGGAGCC ATATC -3'; *IL-4* reverse, 5'- AGCACCTTGAAGCCCTACAGA -3'; *IL-33* forward, 5'- CACATTGGGCAAAGTTGCTTCTAA -3'; *IL-33* reverse, 5'- GCATCCAGAGGTCAGGTGATACAG -3'; *TSLP* forward, 5'- CGCCTATG AGCAGCCACATT -3'; *TSLP* reverse, 5'- TCTTCTTCATTGCCTGAGTAG CATT -3'.

2.13. Preparation of murine bone marrow-derived dendritic cells (mBMDCs) and cytokine assay

mBMDCs were generated from the bone marrow cells of female BALB/c mice, as described previously by Fukuyama et al. (2015). Eight days after initiation of mBMDCs culture, dendritic cells were isolated, purified, seeded at 5×10^4 cells/200 μl in a 96-well plate, and pre-exposed for 24 h to each test substance (E2, HPTE, PPT, or DPN) at 1, 10, or 100 nmol/l, before being exposed to the TLR2 ligand LPS at 25 ng/ml for a further 24 h. Concentrations for each test substance in this assay were determined under the similar criteria with the

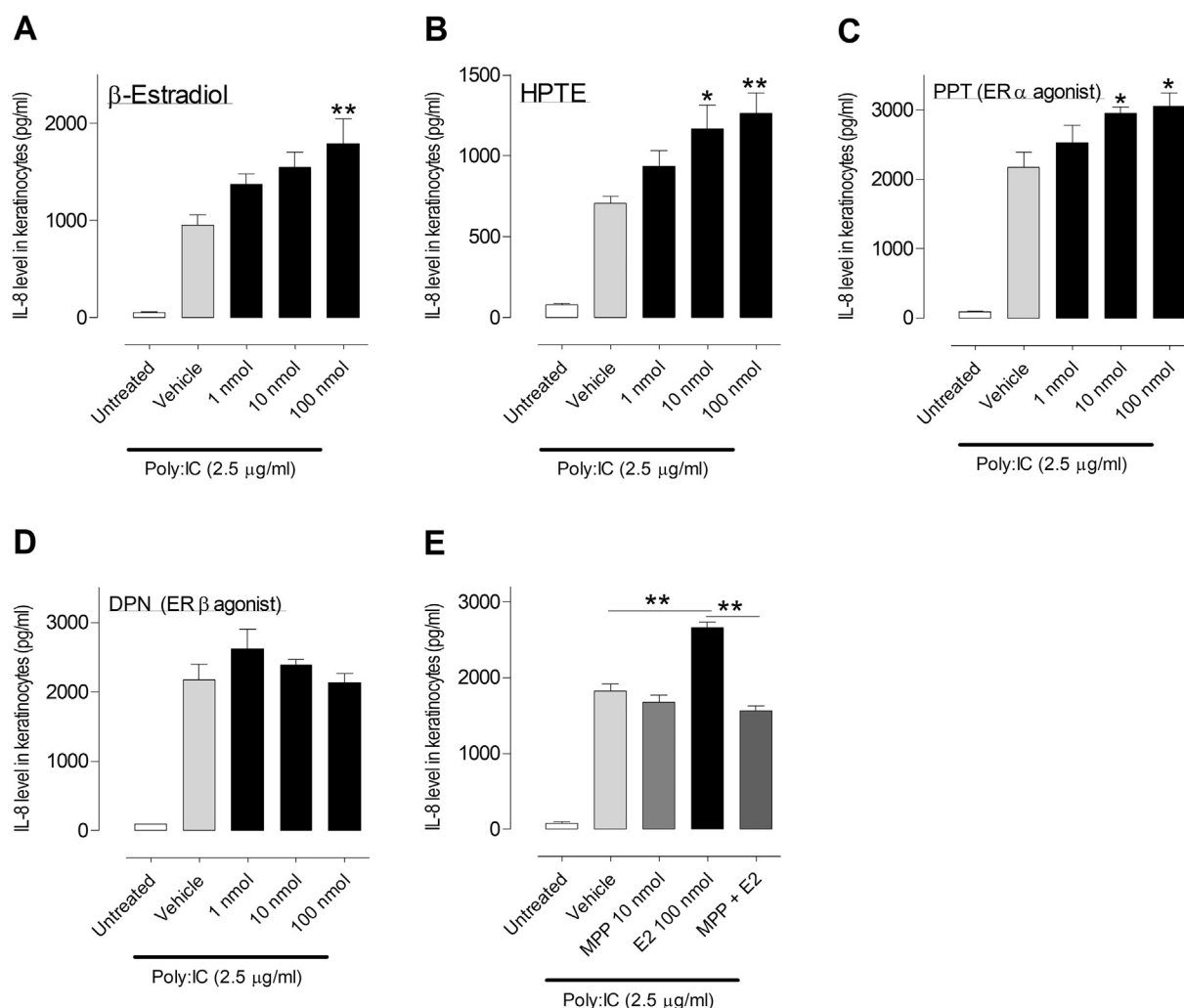


Fig. 3. ER α contributes to Poly (I:C)-stimulated IL-8 secretion by human epidermal keratinocytes.

Pre-exposure to E2, HPTE, or PPT, but not DPN, significantly enhanced keratinocytes activation, as determined by Poly (I:C)-stimulated IL-8 secretion (A–D). A direct link between keratinocyte IL-8 secretion and ER α was confirmed by administration of the selective ER α antagonist MPP, which significantly interfered with the effect of E2 on IL-8 secretion (E). Each result is the mean (pg/ml) $\pm$ 1 SEM. n = 7 per group. * p < .05, ** p < .01 (Dunnett's multiple comparison test) vs. the vehicle-only control group. HPTE = hydroxychlor.

keratinocytes assay. Levels of IL-12 and TNF α in the supernatants were measured using ELISA. Three independent experiments were performed using dendritic cells from different animals.

2.14. Mixed leukocyte reaction assay

The mixed leukocyte reaction assay was performed according to the protocol described by Beyer et al. (2004), with slight modification. In short, highly purified mBMDCs (from BALB/c mice) were pre-treated with PPT or DPN at 1, 10, or 100 nmol/l for 24 h. To initiate the mixed leukocyte reaction, T cells were isolated from the spleens of female C57BL/6 mice and enriched using a Mouse T Lymphocyte Enrichment Set-DM (use of this system results in a T-cell purity > 90%). Enriched T cells (1×10^6 cells/tube) and test substance-treated mBMDCs (1×10^5 cells/tube) were seeded in a 2 mL tube, and incubated for an additional 5 days. Methyl- ^{3} H-thymidine was added for the final 16 h (5 μ Ci/tube). After this period, the mixed cells were washed twice with PBS, resuspended in 5% trichloroacetic acid, and stored at 4 $^{\circ}$ C for 18 h. The cell pellets were resuspended in scintillation fluid and transferred to scintillation vials for determination of T-cell proliferation [in disintegrations per minute (DPM)] using a liquid scintillation counter.

2.15. Statistical analyses

Data are expressed as the mean $\pm$ 1 SEM. Analysis of variance was used to evaluate the results. For significant results, differences between the vehicle control and treatment groups were then assessed using Dunnett's multiple comparison test except for histological evaluation. For histological evaluation, Mann-Whitney U test was performed to analyze differences between vehicle control and treatment groups. 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 or topical exposure to E2 or MXC significantly exacerbated inflammatory and itch responses in a TDI-induced mouse model of allergic dermatitis

To elucidate the direct contribution of estrogens to development of the skin allergy, we first analyzed the pro-inflammatory and pruritic potential of E2 and MXC, endogenous and exogenous estrogens, respectively, *in vivo* using a TDI-induced mouse model of allergic dermatitis. The ear swelling response was significantly increased by oral or

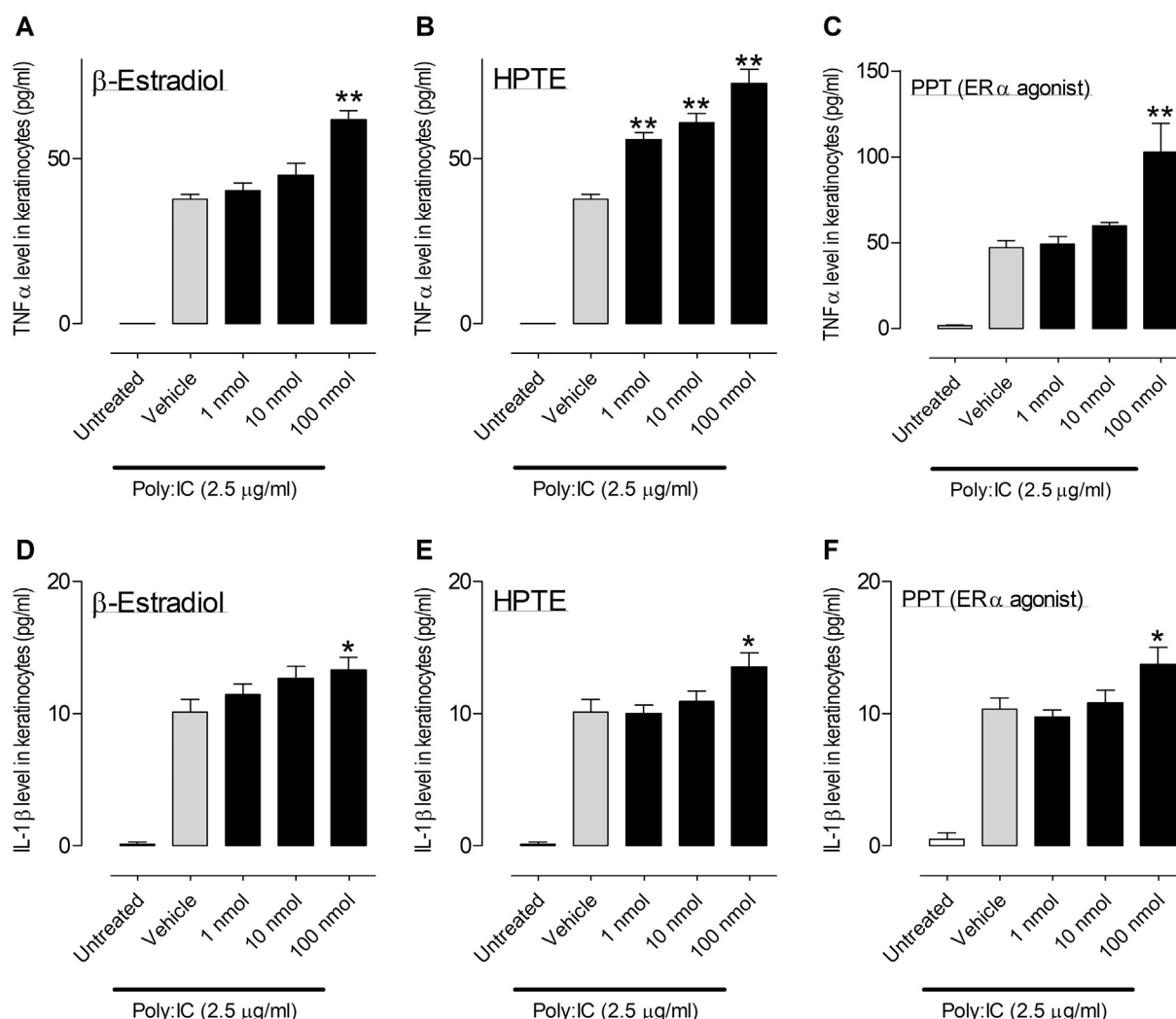


Fig. 4. Significant contribution of ERα to Poly (I:C)-stimulated TNFα and IL-1β secretion by human epidermal keratinocytes.

Pre-exposure to E2, HPTE, or PPT, significantly induced Poly (I:C)-stimulated TNFα (A-C) and IL-1β (D-F) secretion. Each result is the mean (pg/ml) ± 1 SEM. n = 7 per group. *p < .05, **p < .01 (Dunnett's multiple comparison test) vs. the vehicle-only control group.

topical exposure to E2 or oral exposure to MXC compared with the vehicle-only control (Fig. 1A). Scratching behavior was also significantly enhanced by oral administration of MXC (Fig. 1F). Comparable increases were noted in the histological scores of ear skin specimens from mice treated orally with MXC (Table 1 and Fig. 1K). Significantly increased IL-4, -13, and -33 and TSLP levels were observed in the ear skin tissue of E2- or MXC-treated mice, consistent with the ear swelling response, scratching behavior, and histological scores recorded (Fig. 1B–E). Analysis of the local immune response in auricular LNs revealed that infiltration by dendritic cells was significantly elevated following oral treatment with E2 or MXC compared to the vehicle control group (Fig. 1H), whereas helper T cells were not influenced by estrogens exposure (Fig. 1H). Significant changes in LN cell IL-4 production were also evident in the E2 treatment groups (Fig. 1I).

3.2. Inflammatory and itch responses were significantly heightened by an ERα agonist, but not an ERβ agonist, in a TDI-induced mouse model of allergic dermatitis

At this point in our study, the type of estrogen receptor predominantly responsible for the observed exacerbation of cutaneous allergy remained uncertain, as E2 is a dual agonist and HPTE (the major metabolite of MXC) is principally an ERα agonist (Supplemental Fig. 1). Thus, we next investigated the contributions of ERα and ERβ to pro-

inflammatory and pro-pruritic responses in the TDI-induced male and ovariectomized female BALB/c mouse model of allergic dermatitis by pre-treatment with an ERα or ERβ agonist (PPT and DPN, respectively). The ear swelling response in the PPT treatment group was significantly increased compared to that in the vehicle control group; however, DPN treatment had no impact in this respect (Table 2). Scratching behavior was also heightened due to treatment with PPT, but not DPN (Table 2). Corresponding results were obtained with respect to cytokine secretion in ear skin tissue. Levels of IL-5 and -33 and TSLP were significantly increased in the PPT but not the DPN treatment group (Table 2). Consistent with the enhanced inflammatory and itch responses seen, increased numbers of helper T cells and dendritic cells and elevated secretion of the cytokines IL-4, -5, and -13 and TNFα were observed in auricular LNs from mice in the PPT treatment group (Table 3). In addition, similar experiment with ovariectomized female mice instead of male mice was performed with the oral administration of 5 mg/kg PPT and 5 mg/kg DPN. Corresponding results were obtained with respect to ear swelling response, itch behavior, cytokine secretion in ear skin tissue, and LN activation (Supplemental Tables 1 and 2).

3.3. Th1 responses were conversely suppressed by an ERα agonist in a DNCB-induced mouse model of allergic dermatitis

The results from the TDI-induced allergic model indicated that ERα

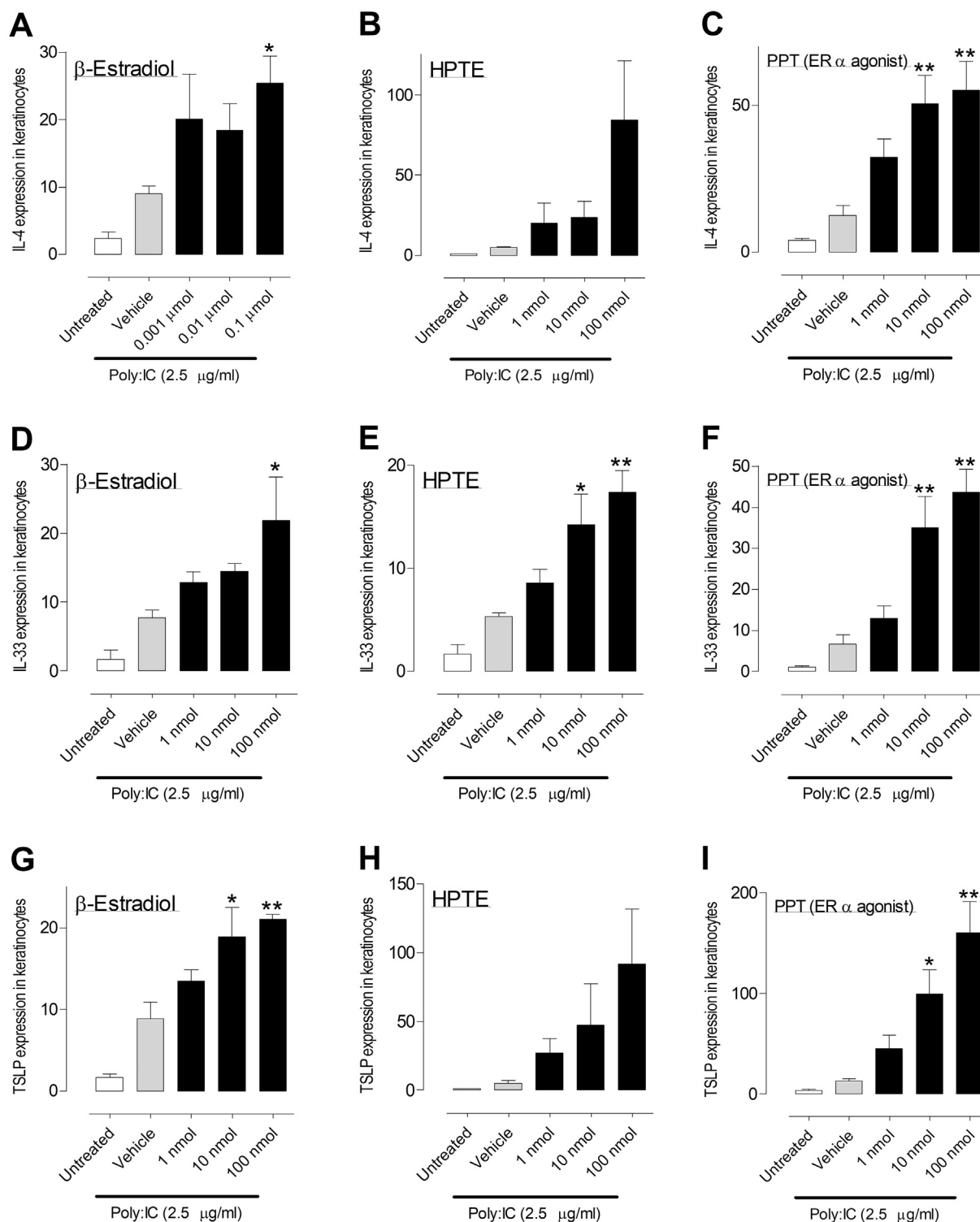


Fig. 5. Expression of the genes *IL-4*, *IL-33* and *TSLP* in keratinocytes was significantly upregulated by exposure to ERα agonists.

Pre-exposure to E2, HPTE, or PPT significantly upregulated Poly (I:C)-stimulated *IL-4* expression in keratinocytes (A–C). Increases in the expression of *TSLP* comparable to those seen in that of *IL-33* were evident following E2, HPTE, or PPT pre-exposure (D–F). Each result is the mean $\pm$ 1 SEM. n = 7 per group. *p < .05, **p < .01 (Dunnett's multiple comparison test) vs. the vehicle-only control group. TSLP = thymic stromal lymphopoietin.

agonist significantly exacerbated the Th2-driven inflammatory and itch responses. However, at this point in our study, it was still uncertain how the ERα and ERβ agonists act for the Th1 pathway. Thus, we next investigated the contributions of ERα and ERβ to pro-inflammatory responses in the DNCB-induced male BALB/c mouse model of allergic dermatitis by pre-treatment with PPT and DPN. The ear swelling

response in the PPT treatment group was significantly suppressed compared to that in the vehicle control group; however, DPN treatment had no impact in this respect (Fig. 2A). Corresponding results were obtained with respect to Th1 cytokine IFNγ secretion in ear skin tissue (Fig. 2B). Although there were no statistical significant changes, decreased numbers of helper T cells and dendritic cells and reduced

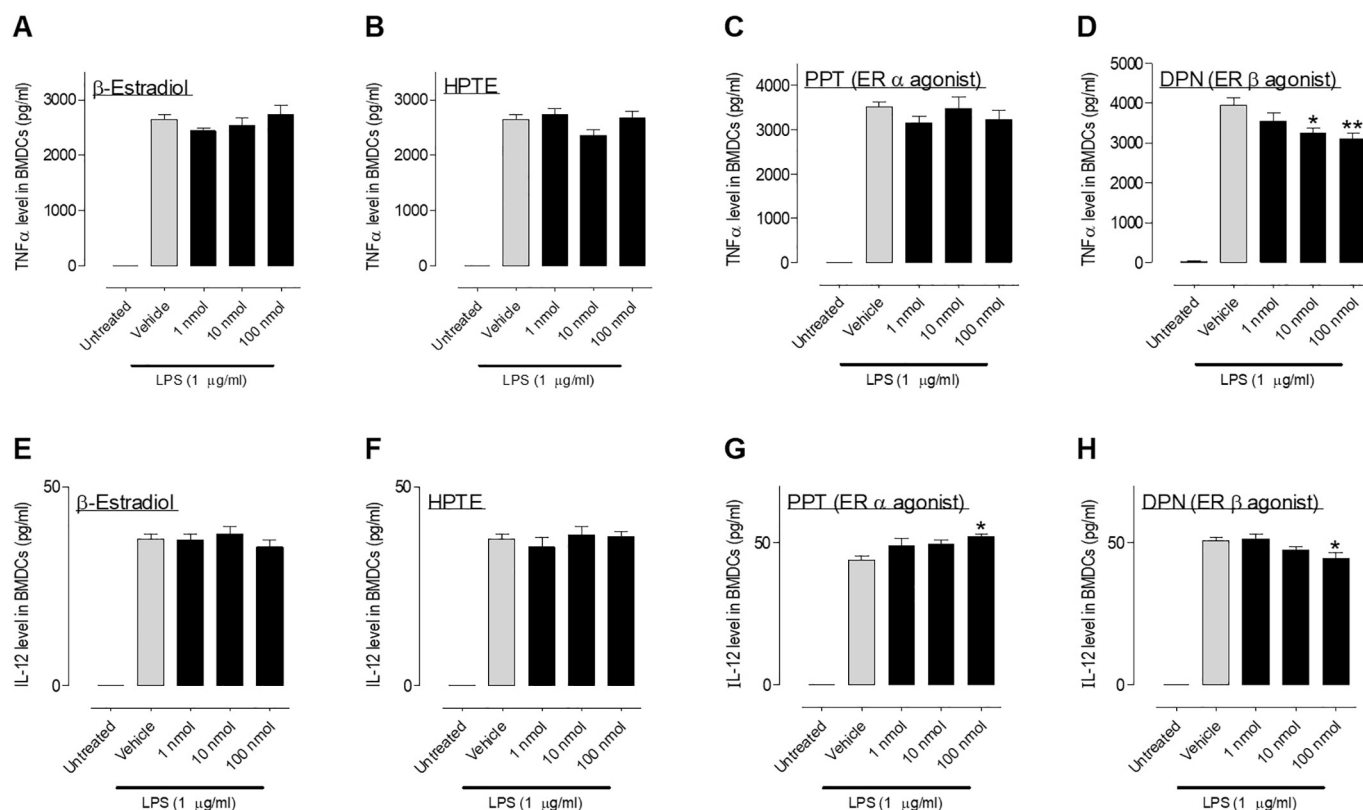


Fig. 6. ERα agonists do not counteract LPS-stimulated TNFα and IL-12 secretion by mBMDs.

TNFα secretion was not affected by E2, HPTE, or PPT treatment (A–C), whereas a slightly significant reduction was observed after DPN administration (D). Only a slightly significant increase in IL-12 secretion was noted following exposure to the highest concentration of PPT (E–G). A reduction in IL-12 corresponding to that in TNFα was seen in the DPN group (H). Each result is the mean (pg/ml) ± 1 SEM. n = 7 per group. *p < .05, **p < .01 (Dunnett's multiple comparison test) vs. the vehicle-only control group.

secretion of IFNγ were observed in auricular LNs from mice in the PPT treatment group (Fig. 2C–E).

3.4. Keratinocytes were activated by pre-exposure to ERα agonists, but not an ERβ agonist, *in vitro*

In order to examine the possible contribution of ERs to keratinocytes activation, *in vitro* experiments with human epidermal keratinocytes, which express the ERα and ERβ (Supplemental Fig. 2), were conducted. Compared to those in the vehicle control group, activated keratinocytes pre-exposed to E2, HPTE (a major metabolite of MXC), or PPT demonstrated significantly increased IL-8, TNFα and IL-1β secretion (Fig. 3A–C, Fig. 4). In addition, the E2-induced upregulation of IL-8 secretion was significantly inhibited by the ERα antagonist MPP (Fig. 3E). On the other hand, pre-exposure to DPN did not alter IL-8 secretion (Fig. 3D). This activation of keratinocytes by ERα agonists was corroborated by observations of significantly upregulated IL-4, IL-33 and TSLP expression (Fig. 5).

3.5. Dendritic cells and T cells may not contribute to the exacerbation of skin allergy by ERα agonists

Possible contributions of dendritic cells and T cells were also examined using BMDs and mix leucocyte reaction assay. Whereas ERα agonists significantly increased cytokines secretion by activated keratinocytes, they did not influence TNFα and IL-12 secretion by mBMDs (Fig. 6A–C, E–G). In contrast, a slightly significant reduction in the secretion of these cytokines was seen in the DPN group (Fig. 6D, H). In the mixed leucocyte reaction assay, slight reduction of T-cell proliferation was noted in DPN pre-treatment groups although differences

were not significant (Fig. 7).

4. Discussion

Several key findings resulted from the present study: (1) in a mouse model of allergic dermatitis, oral or topical treatment with E2 or MXC significantly heightened the inflammatory response and scratching behavior; (2) in this same model, the selective ERα agonist PPT also significantly induced both scratching behavior and skin inflammation, whereas the selective ERβ agonist DPN had no such impact; (3) *in vitro* experiments using keratinocytes yielded results consistent with those of our *in vivo* investigation, in that ERα agonists induced keratinocyte cytokine production, whereas an ERβ agonist did not affect keratinocyte activation; (4) cytokine secretion by mBMDs was slightly influenced by ERα or ERβ agonist treatment, but reaction was not consistent those of our *in vivo* investigation; and (5) T-cell proliferation was not significantly altered by ERα or ERβ agonist treatment.

Although the contribution of estrogenic agents to the development of allergic diseases has been extensively investigated (Sobel et al. 2005; Sakazaki et al. 2007; Fukuyama et al. 2011a, 2011b, 2012; Bernardi et al. 2015), there is only limited evidence of the direct involvement of ERs in these conditions. Furthermore, the relative importance of each ER type to the alteration of allergic responses has not been clarified. Thus, we investigated here whether ER agonists directly affect allergic inflammatory and itch responses using a TDI-induced mouse model of allergic dermatitis, and attempted to characterize the active participation of ERs in pro-inflammatory and pro-pruritic responses with several *in vitro* assays relevant to cutaneous allergy.

Initially, we analyzed the effects of E2 and MXC, representative endogenous and exogenous estrogens, respectively. The organochlorine

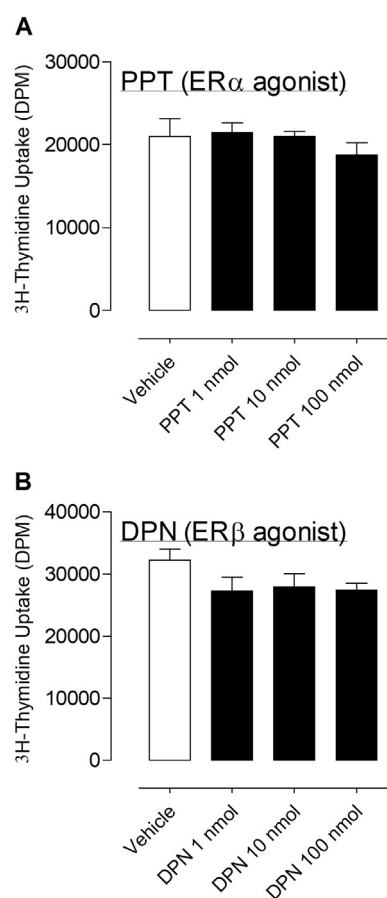


Fig. 7. Agonists of ER α and ER β do not affect T-cell proliferation. T-cell proliferation was not altered by PPT pre-treatment (A). DPN also had no effect on T-cell proliferation *in vitro* (B). Each result is the mean (DPM) $\pm$ 1 SEM. $n = 6$ per group. MA, mycophenolic acid.

pesticide MXC was developed as an alternative to dichlorodiphenyltri-chloroethane, owing to its shorter half-life, lower toxicity in mammals, and greater biodegradability (Kapoor et al. 1970; Bulger et al. 1978; Bal 1984). Some previous studies have reported that MXC affects the immune and reproductive systems as an endocrine disruptor (Chapin et al. 1997; Guo et al. 2002; Fukuyama et al. 2011a). In the current work, we orally and topically administered estrogens immediately prior (48, 24, and 4 h) to the final TDI treatment in an attempt to establish a direct relationship between estrogen exposure and allergy development. The pro-inflammatory effects of E2 and MXC were evidenced by an enhanced ear swelling response (Fig. 1A), which was corroborated by histological evaluation (Table 1 and Fig. 1K). Interestingly, for both E2 and MXC, oral administration had a greater impact than topical application. Toxicokinetic study of topical application of E2 or MXC will be performed in our laboratories to clear up this question. Although levels of Th2-type cytokines, such as IL-4 and IL-13, in ear skin tissue were not entirely consistent with the ear swelling response and histological results obtained (Fig. 1B and C), the secretion of keratinocytes, fibroblasts, epithelial cells and dendritic cells cytokines, including TSLP and IL-33, corresponded well to these observations (Fig. 1D and E). Recent studies have particularly indicated that keratinocytes actively participate in the cutaneous immune response (Steinhoff et al. 2001); thus, we hypothesized that these cells play an important role in the aggravation of cutaneous allergy by estrogen exposure. In addition, we could find an upwards trend of scratching behavior by administration of E2 or MXC, and mice orally given MXC showed a significant change compared to vehicle control mice (Fig. 1F), suggesting the possible contribution of estrogens to pruritogenic response.

The findings of our first experiment demonstrated a direct relationship between estrogen administration and the development of allergic dermatitis. However, as MXC is predominantly an ER α agonist, and E2 is a dual agonist of both ER α and ER β (Supplemental Fig. 1), the contribution of ER β to cutaneous allergy remained uncertain. In the next set of experiments, male mice with TDI-induced allergic dermatitis were orally pre-administered an agonist of ER α (PPT) or ER β (DPN), and the effects on inflammatory and itch responses were compared. The pro-inflammatory response was significantly increased by pre-treatment with PPT (only at 5 mg/kg) but not DPN, and this was corroborated by measurements of the ear swelling response and ear tissue cytokine secretion such as IL-5, IL-33 and TSLP (Table 2). Helper T-cell and dendritic cell infiltration and activation in local LNs were also only enhanced by PPT (only at 5 mg/kg) pre-treatment (Table 3). Since males can convert testosterone to estradiol *via* aromatase; similar experiment with ovariectomized female mice instead of male mice was additionally performed with the oral administration of PPT and DPN. Corresponding results were obtained with respect to inflammatory and itch responses (Supplemental Tables 1 and 2), which demonstrates that endogenous estrogens did not influence our *in vivo* result. As seen in the first experiment, secretion of keratinocyte cytokines, including IL-33 and TSLP, in ear skin tissue was particularly up-regulated compared to that of Th2-cell-related cytokines, such as IL-4 and IL-13. Interestingly, scratching behavior was also induced by administration of PPT. Recent work by Liu et al. (2016a) and Wilson et al. (2013) has demonstrated that IL-33 and TSLP evoke scratching behavior *via* activation of pruritic receptors on peripheral neurons in the dorsal root ganglion. Thus, induction of TSLP and IL-33 secretion in skin tissue may have influenced the scratching behavior observed. The findings of our second experiment clearly indicate that ER α actively contributes to the development of cutaneous allergy.

As TDI is the Th2-type hapten, we only saw the contribution of ER α to Th2 polarizing cells and related-cytokines including IL-4, IL-5 and IL-13 in the first and second experiments. Therefore, we next attempt to see the involvement of ER α in Th1-dominated contact dermatitis using DNCB-induced allergic dermatitis model. PPT exerted the opposite effects including significant reduction of ear swelling response and IFN γ secretion, which is a typical Th1-derived cytokine (Fig. 2). This was suggestive that the process of ER α activation in skin allergy is Th2 cytokine-driven.

Finally, our *in vivo* results were confirmed *in vitro* using keratinocytes, mBMDCs, and T cells. Activation of keratinocytes with poly(I:C) induced production of the cytokine IL-1 β , IL-8, TNF α and expression of the genes encoding IL-4, IL-33 and TSLP. IL-8 can be secreted by any cells with TLR activation that are involved in the innate immune response, and previous studies demonstrated that IL-8 are elevated in human patients with AD (Kimata and Lindley 1994; Hatano et al. 1999; Kaburagi et al. 2001). We observed significant dose-dependent upregulation of IL-8 production following pre-treatment with ER α agonists, including E2, HPTE (a major metabolite of MXC), and PPT, whereas pre-treatment with the ER β agonist DPN had no effect in this respect (Fig. 3). The active participation of ER α was further confirmed by the inhibition of the IL-8 production to E2 by the ER α antagonist MPP. Moreover, elevation of IL-8 secretion was corroborated by significant increases of typical pro-inflammatory cytokines IL-1 β and TNF α secretion, which are also produced by keratinocyte *via* TLR-activation (Fig. 4). Schmitz et al. (2005) indicated that IL-1 β and TNF α promotes the secretion of IL-33 which plays an important role in Th2-associated immunology. They showed that *in vitro* polarized Th2 cells upon re-stimulation respond to IL-33 by significantly increasing secretion of IL-4 and IL-13. Also, keratinocytes produce TSLP which is a central player involved in the initiation, development and progression of atopy and atopic diseases both in mice and in humans (Indra 2013). Therefore, we next evaluated the IL-4, IL-33 and TSLP gene expression in keratinocytes. Significant upregulation of IL-4, IL-33 and TSLP expression due to ER α agonist pre-treatment verified the contribution of ER α to

keratinocyte activation (Fig. 5). In particular, significant enhancement of IL-4 expression in keratinocytes was very supportive of the concept that ER α is acting to enhance Th2 pathway. On the other hand, immune cells such as dendritic cells and T cells, which also play an important role in both the initiation and maintenance of allergic inflammatory diseases, might not be influenced by ER α . LPS induced IL-12 and TNF α production by mBMDs, but exposure to ER α agonists had only a moderate effect on the levels of these cytokines secreted by these cells (Fig. 6). In addition, the mixed leukocyte reaction assay revealed that activation of ER α had almost no impact on T-cell proliferation (Fig. 7). Our *in vitro* findings indicated that the DCs and T cells might not directly be related to allergy development but definitely give a first hint, that the ER α dependent allergic development is mainly *via* modulation of keratinocyte function.

Taken together, our findings demonstrate that ER α activation by oral administration of ER α agonists is directly associated with pro-inflammatory and pro-pruritic responses in a mouse model of allergic dermatitis. Our results also demonstrate the ability of ER α to significantly induce a cytokine response in keratinocytes *in vitro*, while having only a slight impact on dendritic cell and T-cell function. Based on the above results, we can conclude that keratinocytes and cytokines such as IL-33 and TSLP, which have the potential to activate antigen-presenting cells and polarize Th2 cells and promote scratching behavior, play an important role in cutaneous allergy exacerbated by ER α agonists.

However, there are also opponents to the abovementioned pro-inflammatory responses by estrogens exposure. Previous studies reported that E2 inhibits the production of MCP-1 and RANTES in human keratinocytes, and PPT and DPN administration were equally effective in normalizing the inflammatory response of keratinocytes (Kanda and Watanabe 2003a; Kanda and Watanabe 2003b; Moenipour et al. 2008). When we additionally measured the RANTES and MCP-1 levels in the ear skin samples of PPT- or DPN-pretreated TDI-induced mice, no significant changes were observed among the groups (data not shown), suggesting that RANTES and MCP-1 are not typical players to enhance the inflammatory responses by estrogens in our *in vivo* model. However, significant elevations of IL-1 β , IL-8 and TNF α secretions and IL-4, IL-33 and TSLP gene expressions in our *in vitro* keratinocytes experiment clearly demonstrated that the direct contribution of ER α to pro-inflammatory responses. Exacerbation of *in vivo* allergic dermatitis was also very supportive. These phenomena might closely depend on the background of the experiment such as chemical concentrations, length of exposure and types of animal model used. In addition, our recently investigated report indicated that, rather ER β dependent agonist bisphenol A elicits anti-inflammatory responses in TDI-induced allergic dermatitis, but pro-inflammatory responses in TDI-induced respiratory allergic inflammation (Tajiki-Nishino et al. 2018), thus these reactions might be organ specific. In order to clear up these questions, future studies using models of human chronic atopic dermatitis, such as NC/Nga mice, would be beneficial to further characterize the mechanism of action of estrogens in the induction of pro-inflammatory and pro-pruritic responses.

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Conflict of interest

The authors state no conflict of interest.

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References

- Bal, H.S., 1984. Effect of methoxychlor on reproductive systems of the rat. *Proc. Soc. Exp. Biol. Med.* 176, 187–196.
- Bernardi, A.I., Andersson, A., Stubelius, A., Grahnmö, L., Carlsten, H., Islander, U., 2015. Selective estrogen receptor modulators in T cell development and T cell dependent inflammation. *Immunobiology* 220, 1122–1128.
- Beyer, M., Bartz, H., Horner, K., Doths, S., Koerner-Rettberg, C., Schwarze, J., 2004. Sustained increases in numbers of pulmonary dendritic cells after respiratory syncytial virus infection. *J. Allergy Clin. Immunol.* 113, 127–133.
- Bulger, W.H., Muccitelli, R.M., Kupfer, D., 1978. Interactions of methoxychlor, methoxychlor base-soluble contaminant, and 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane with rat uterine estrogen receptor. *J. Toxicol. Environ. Health* 4, 881–893.
- Chapin, R.E., Harris, M.W., Davis, B.J., Ward, S.M., Wilson, R.E., Mauney, M.A., Lockhart, A.C., Smalowicz, R.J., Moser, V.C., Burka, L.T., Collins, B.J., 1997. The effects of perinatal/juvenile methoxychlor exposure on adult rat nervous, immune, and reproductive system function. *Fundam. Appl. Toxicol.* 40, 138–157.
- Danso, M.O., van Drongelen, V., Mulder, A., van Esch, J., Scott, H., van Smeden, J., El Ghalbzouri, A., Bouwstra, J.A., 2014. TNF-alpha and Th2 cytokines induce atopic dermatitis-like features on epidermal differentiation proteins and stratum corneum lipids in human skin equivalents. *J. Invest. Dermatol.* 134, 1941–1950.
- Fukuyama, T., Tajima, Y., Ueda, H., Hayashi, K., Shutoh, Y., Harada, T., Kosaka, T., 2010. Apoptosis in immunocytes induced by several types of pesticides. *J. Immunotoxicol.* 7, 39–56.
- Fukuyama, T., Kosaka, T., Tajima, Y., Hayashi, K., Shutoh, Y., Harada, T., 2011a. Detection of thymocytes apoptosis in mice induced by organochlorine pesticides methoxychlor. *Immunopharmacol. Immunotoxicol.* 33, 193–200.
- Fukuyama, T., Tajima, Y., Hayashi, K., Ueda, H., Kosaka, T., 2011b. Prior or co-instantaneous oral exposure to environmental immunosuppressive agents aggravates mite allergen-induced atopic dermatitis-like immunoreaction in NC/Nga mice. *Toxicology* 289, 132–140.
- Fukuyama, T., Tajima, Y., Ueda, H., Hayashi, K., Kosaka, T., 2011c. Prior exposure to immunosuppressive organophosphorus or organochlorine compounds aggravates the TH1- and TH2-type allergy caused by topical sensitization to 2,4-dinitro-chlorobenzene and trimellitic anhydride. *J. Immunotoxicol.* 8, 170–182.
- Fukuyama, T., Kosaka, T., Miyashita, L., Nishino, R., Wada, K., Hayashi, K., Ueda, H., Harada, T., 2012. Role of regulatory T cells in the induction of atopic dermatitis by immunosuppressive chemicals. *Toxicol. Lett.* 213, 392–401.
- Fukuyama, T., Ehling, S., Cook, E., Baumer, W., 2015. Topically administered Janus-kinase inhibitors tofacitinib and oclacitinib display impressive antipruritic and anti-inflammatory responses in a model of allergic dermatitis. *J. Pharmacol. Exp. Ther.* 354, 394–405.
- Guo, T.L., Zhang, X.L., Bartolucci, E., McCay, J.A., White Jr., K.L., You, L., 2002. Genistein and methoxychlor modulate the activity of natural killer cells and the expression of phenotypic markers by thymocytes and splenocytes in F0 and F1 generations of Sprague-Dawley rats. *Toxicology* 172, 205–215.
- Hatano, Y., Katagiri, K., Takayasu, S., 1999. Increased levels in vivo of mRNAs for IL-8 and macrophage inflammatory protein-1 alpha (MIP-1 alpha), but not of RANTES mRNA in peripheral blood mononuclear cells of patients with atopic dermatitis (AD). *Clin. Exp. Immunol.* 117, 237–243.
- Indra, A.K., 2013. Epidermal TSLP: a trigger factor for pathogenesis of atopic dermatitis. *Expert Rev. Proteomics* 10, 309–311.
- Kaburagi, Y., Shimada, Y., Nagaoka, T., Hasegawa, M., Takehara, K., Sato, S., 2001. Enhanced production of CC-chemokines (RANTES, MCP-1, MIP-1alpha, MIP-1beta, and eotaxin) in patients with atopic dermatitis. *Arch. Dermatol. Res.* 293, 350–355.
- Kanda, N., Watanabe, S., 2003a. 17Beta-estradiol inhibits MCP-1 production in human keratinocytes. *J. Invest. Dermatol.* 120, 1058–1066.
- Kanda, N., Watanabe, S., 2003b. 17beta-estradiol inhibits the production of RANTES in human keratinocytes. *J. Invest. Dermatol.* 120, 420–427.
- Kaplan, M.H., Schindler, U., Smiley, S.T., Grusby, M.J., 1996. Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. *Immunity* 4, 313–319.
- Kapoor, I.P., Metcalf, R.L., Nystrom, R.F., Sangha, G.K., 1970. Comparative metabolism of methoxychlor, methiochlor, and DDT in mouse, insects, and in a model ecosystem. *J. Agric. Food Chem.* 18, 1145–1152.
- Kimata, H., Lindley, I., 1994. Detection of plasma interleukin-8 in atopic dermatitis. *Arch. Dis. Child.* 70, 119–122.
- Lee, K.H., Cho, K.A., Kim, J.Y., Kim, J.Y., Baek, J.H., Woo, S.Y., Kim, J.W., 2011. Filaggrin knockdown and Toll-like receptor 3 (TLR3) stimulation enhanced the production of thymic stromal lymphopoietin (TSLP) from epidermal layers. *Exp. Dermatol.* 20, 149–151.
- Liu, B., Tai, Y., Achanta, S., Kaelberer, M.M., Caceres, A.I., Shao, X., Fang, J., Jordt, S.E., 2016a. IL-33/ST2 signaling excites sensory neurons and mediates itch response in a mouse model of poison ivy contact allergy. *Proc. Natl. Acad. Sci. U. S. A.* 113, E7572–E7579.
- Liu, S., Mao, B., Bai, Y., Liu, J., Li, H., Li, X., Lian, Q., Ge, R.S., 2016b. Effects of methoxychlor and its metabolite hydroxychlor on human placental 3beta-hydroxysteroid

- dehydrogenase 1 and aromatase in JEG-3 cells. *Pharmacology* 97, 126–133.
- Moeinpour, F., Choudhry, M.A., de Figueiredo, L.F., Bland, K.I., Chaudry, I.H., 2008. Estradiol's salutary effects on keratinocytes following trauma-hemorrhage are mediated by estrogen receptor (ER)-alpha and ER-beta. *Mol. Med.* 14, 689–696.
- Mu, Z., Zhao, Y., Liu, X., Chang, C., Zhang, J., 2014. Molecular biology of atopic dermatitis. *Clin. Rev. Allergy Immunol.* 47, 193–218.
- Nishino, R., Fukuyama, T., Tajima, Y., Miyashita, L., Watanabe, Y., Ueda, H., Kosaka, T., 2013. Prior oral exposure to environmental immunosuppressive chemicals methoxychlor, parathion, or piperonyl butoxide aggravates allergic airway inflammation in NC/Nga mice. *Toxicology* 309, 1–8.
- Oettgen, H.C., 2000. Regulation of the IgE isotype switch: new insights on cytokine signals and the functions of epsilon germline transcripts. *Curr. Opin. Immunol.* 12, 618–623.
- Paterni, I., Granchi, C., Katzenellenbogen, J.A., Minutolo, F., 2014. Estrogen receptors alpha (ERalpha) and beta (ERbeta): subtype-selective ligands and clinical potential. *Steroids* 90, 13–29.
- Petzold, S., Averbek, M., Simon, J.C., Lehmann, I., Polte, T., 2014. Lifetime-dependent effects of bisphenol A on asthma development in an experimental mouse model. *PLoS One* 9, e100468.
- Sakazaki, F., Ueno, H., Nakamuro, K., 2006. 17beta-estradiol enhances contact hypersensitivity and IFN-gamma expression in inflamed skin of BALB/c mice. *Toxicol. Lett.* 166, 60–66.
- Sakazaki, F., Ueno, H., Nakamuro, K., 2007. 17beta-estradiol enhances contact hypersensitivity in a hair cycle-dependent manner and retards thymic atrophy with age. *Immunopharmacol. Immunotoxicol.* 29, 597–609.
- Schmitz, J., Owyang, A., Oldham, E., Song, Y., Murphy, E., McClanahan, T.K., Zurawski, G., Moshrefi, M., Qin, J., Li, X., Gorman, D.M., Bazan, J.F., Kastelein, R.A., 2005. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* 23, 479–490.
- Sobel, E.S., Gianini, J., Butfiloski, E.J., Croker, B.P., Schiffenbauer, J., Roberts, S.M., 2005. Acceleration of autoimmunity by organochlorine pesticides in (NZB x NZW)F1 mice. *Environ. Health Perspect.* 113, 323–328.
- Steinhoff, M., Brzoska, T., Luger, T.A., 2001. Keratinocytes in epidermal immune responses. *Curr. Opin. Allergy Clin. Immunol.* 1, 469–476.
- Tajiki-Nishino, R., Makino, E., Watanabe, Y., Tajima, H., Ishimota, M., Fukuyama, T., 2018. Oral administration of bisphenol A directly exacerbates allergic airway inflammation but not allergic skin inflammation in mice. *Toxicol. Sci.* (28 May).
- Wilson, S.R., The, L., Batia, L.M., Beattie, K., Katibah, G.E., McClain, S.P., Pellegrino, M., Estandian, D.M., Bautista, D.M., 2013. The epithelial cell-derived atopic dermatitis cytokine TSLP activates neurons to induce itch. *Cell* 155, 285–295.
- Yu, X., Niu, Y., Zheng, J., Liu, H., Jiang, G., Chen, J., Hong, M., 2015. Radix Saposhnikovia extract suppresses mouse allergic contact dermatitis by regulating dendritic-cell-activated Th1 cells. *Phytomedicine* 22, 1150–1158.

Supplemental TABLE 1

Responses in the ear auricle to oral administration of PPT or DPN in a TDI-induced ovariectomized female mouse model of allergic dermatitis

Ear samples were collected 24 h after TDI challenge. Results are expressed as means ± SEM. n = 5 per group. *p < 0.05, **p < 0.01 (Dunnett’s multiple comparison test) vs. the TDI + vehicle control group.

	TDI + vehicle control	TDI + 5 mg/kg PPT	TDI + 5 mg/kg DPN
Ear swelling (µm)	120 ± 15	164 ± 12 [*]	130 ± 8
Scratching bouts	103 ± 6	165 ± 10 ^{**}	133 ± 9
IL-4 (pg/mg protein)	136 ± 14	269 ± 26 ^{**}	132 ± 7
IL-5 (pg/mg protein)	186 ± 16	273 ± 18 ^{**}	189 ± 7
IL-13 (pg/mg protein)	272 ± 34	369 ± 19	248 ± 28
IL-33 (pg/mg protein)	985 ± 113	1559 ± 80 ^{**}	565 ± 34 ^{**}
TNFα (pg/mg protein)	120 ± 11	153 ± 3 [*]	121 ± 8
TSLP (pg/mg protein)	44 ± 3	76 ± 7 ^{**}	38 ± 4

Supplemental TABLE 2

Responses in the auricular LN to oral administration of PPT or DPN in a TDI-induced ovariectomized female mouse model of allergic dermatitis

Cytokine production was measured in response to anti-CD3 and -CD28 antibody stimulation. Each result is the mean $\pm$ 1 SEM. (n = 5 per group). *p < 0.05, **p < 0.01 (Dunnett's multiple comparison test) vs. the TDI + vehicle control group.

	TDI + vehicle control	TDI + 5 mg/kg PPT	TDI + 5 mg/kg DPN
CD3 ⁺ CD4 ⁺ cells ($\times 10^6$)	2.11 $\pm$ 0.35	3.32 $\pm$ 0.42	2.37 $\pm$ 0.25
CD11c ⁺ CD40 ⁺ cells ($\times 10^5$)	0.79 $\pm$ 0.13	1.18 $\pm$ 0.19	0.88 $\pm$ 0.12
IL-4 (pg/ml)	97 $\pm$ 15	186 $\pm$ 26*	117 $\pm$ 16
IL-5 (pg/ml)	24 $\pm$ 3	46 $\pm$ 10	31 $\pm$ 6
IL-13 (pg/ml)	64 $\pm$ 16	202 $\pm$ 55*	83 $\pm$ 23
TNF α (pg/ml)	113 $\pm$ 8	170 $\pm$ 16*	122 $\pm$ 15