

## Phthalate ester-induced thymic stromal lymphopoietin mediates allergic dermatitis in mice

Tomomi Shigeno,\* Mayako Katakuse,\* Tomoyuki Fujita, Yohei Mukoyama and Hideki Watanabe\*

*Drug Discovery Research, Kyoto R&D Centre, Maruho Co., Ltd, Chudoji, Kyoto, Japan*

### Summary

Recently air pollutants and irritants have been labelled as possible exogenous risk factors for allergic disorders. Although the underlying causes of allergic disorders such as atopic dermatitis and asthma remain unclear, the T helper type 2 (Th2) cell-mediated allergic inflammatory cascade may contribute to their pathogenesis. In the last decade, it has been documented that one of the candidates for triggering Th2 commitment is thymic stromal lymphopoietin (TSLP), the expression of which is up-regulated in the lesions of allergic patients. Here, we describe TSLP function in a fluorescein isothiocyanate (FITC) -induced contact hypersensitivity (CHS) model. A cytokine profile indicated that the model was dominantly mediated by the Th2 milieu. Interestingly, TSLP was increased in the skin during the sensitization phase when stimulated by a solvent, dibutyl phthalate (DBP), but not by FITC hapten or another solvent, acetone. Ear swelling in FITC-induced CHS was totally abrogated by removing DBP from the sensitization or elicitation phase, and was restored by complementary injection of TSLP. Inversely, the ear swelling was suppressed by injection of small interfering RNA against TSLP during the sensitization phase, which was concomitant with decreasing expression of interleukin-4 at the swollen skin site. Taken together, DBP-induced TSLP during the sensitization phase plays a role in establishing FITC-induced CHS and may be one of the causes of Th2 commitment in the model, suggesting that certain environmental toxins, such as DBP, may endow pro-allergic and atopic predisposition in humans or animals.

**Keywords:** adjuvants; allergic contact dermatitis/contact sensitivity; cytokines; T cells

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\*These authors contributed equally to this work.

Correspondence: H. Watanabe, Kyoto R&D Centre, Maruho Co., Ltd, 93 Awata-cho, Chudoji, Kyoto 600-8815, Japan. Email: watanabe\_cfb@mii.maruho.co.jp  
Senior author: H. Watanabe

### Introduction

Contact hypersensitivity (CHS) is one of the common allergic skin diseases mediated by T cells. Pathological T cells have been educated by antigen-presenting cells (APCs), such as dermal dendritic cells (DCs) or Langerhans cells migrated from the skin, which have been subjected to and are activated by a specific allergen. This pathological reaction is easily mimicked in experimental

animals by painting a chemical hapten, such as trinitrochlorobenzene, oxazolone, picryl chloride or fluorescein isothiocyanate (FITC), on the skin.

Most types of CHS are mediated by T helper type 1 (Th1) cells producing interferon- $\gamma$  and interleukin-12 (IL-12). Repeated-application of hapten results in the development of site-restricted and antigen-specific immediate-type hypersensitivity, which is widely regarded as chronic CHS. It is believed that the chronic CHS is

Abbreviations: AD, atopic dermatitis; ADBP, acetone : DBP (1 : 1); AOO, acetone : olive oil; CHS, contact hypersensitivity; DBP, dibutyl phthalate; Elicit, elicitation; FITC, fluorescein isothiocyanate; LNCs, lymph node cells; NT, non-treated; PC, picryl chloride; rmTSLP, recombinant mouse TSLP; Sens, sensitization; siRNA, small interfering RNA; Th2 cells, T helper type II cells; TSLP, thymic stromal lymphopoietin.

associated with a change in the local cytokine profile from Th1 to Th2 with elevated serum immunoglobulin E (IgE) levels.<sup>1–4</sup> Therefore Th2 cells are also potentially able to mediate CHS in the chronic phase. Interestingly, FITC-induced CHS is dominantly mediated by Th2 rather than Th1 cells, although the model is an acute model. However, it is unclear how the type of T-cell-mediated immunity develops in each model.

In animal models of CHS, DC activation by an immunoreactive antigen is an initiation step where the T cells memorize the characteristics of the antigen. Therefore, the activation of DCs could be critical for determining the pathway of the downstream responses mediated by the T cells.

One of the candidates for the local activation of skin DCs during an allergic inflammatory reaction is the IL-7-like cytokine thymic stromal lymphopoietin (TSLP). Over the last few years, it has been determined that TSLP works to skew the immune system toward Th2-dominance by activating DCs that promote differentiation of naïve T cells to Th2 cells by an OX40 ligand–OX40 interaction<sup>5,6</sup> which then locally produces Th2 chemokines, such as thymus and activation regulated chemokine and macrophage-derived chemokine.<sup>5</sup>

Patients with atopic dermatitis (AD) or asthma, which are clinically regarded as Th2-dominant diseases,<sup>7</sup> express elevated TSLP in the epidermis<sup>5</sup> or airway epithelia,<sup>8</sup> respectively, correlating to their disease severity.<sup>8</sup> Interestingly, skin-specific over-expression of TSLP in mice resulted in spontaneous AD-like symptoms accompanied by severe eczematous skin.<sup>9</sup> These facts suggest that TSLP plays an important role in the pathogenesis of AD.

Interestingly, dibutyl phthalate (DBP), which is an airborne pollutant derived from industrial activity,<sup>10</sup> is used as a solvent for FITC and is required for the sensitization and the elicitation as an immunological adjuvant under the experimental conditions of FITC-induced CHS.<sup>11</sup> Moreover, the morbidity rate of AD has dramatically increased in the past decades, which corresponds to an increase in industrial pollution.<sup>12</sup> It is tempting to speculate that environmental toxins, such as DBP, cause Th2-dominant immune responses.

We hypothesized that the use of DBP as a solvent for FITC, characteristically required for FITC-induced CHS, played an important role in skewing the immune system toward a Th2 response. In the present study, we found that DBP potently modified the mouse immune system by locally produced TSLP, resulting in Th2 cell-mediated allergic skin inflammation. The FITC alone could not be detected as an immunoactive hapten. These results suggested that long-lasting exposure to environmental toxins, such as DBP, might affect human immunity by promoting an atopic disposition that senses non-allergenic substances as allergens.

## Materials and methods

### *Animals*

Female BALB/c mice were obtained from Charles River (Yokohama, Japan). The mice were acclimated for at least 5 days before the experiments and were used at 7–8 weeks old. *In vivo* procedures underwent veterinary review and approval by the Animal Care and Use Committee.

### *Chemicals and reagents*

The FITC, acetone, DBP, olive oil and penicillin–streptomycin mixed solution (final 100 U/ml per 100 µg/ml) were purchased from Nacalai Tesque (Kyoto, Japan). Picryl chloride (PC) was purchased from Tokyo Chemical Industry (Tokyo, Japan). The PC was dissolved in acetone : olive oil (AOO; 3 : 1) and FITC was dissolved in acetone : DBP (ADBP; 1 : 1) or acetone or AOO (1 : 1). Recombinant mouse TSLP (rmTSLP), enzyme-linked immunosorbent assay (ELISA) kits for mouse IL-4 and mouse TSLP were purchased from R&D Systems (Minneapolis, MN). The ELISA kit for mouse IL-12p40 was purchased from BD Biosciences (Franklin Lakes, NJ), the TaqMan Gene Expression Assay kit [IL-4, IL-12p40, tumour necrosis factor (TNF)], TaqMan Rodent GAPDH Control Reagents (4308313) and mouse TSLP primers and probe set were obtained from Applied Biosystems (Foster City, CA). Small interfering (si) RNAs were purchased from Ambion (Austin, TX) and 2-mercaptoethanol, fetal bovine serum, and RPMI-1640 Glutamax<sup>®</sup> medium were purchased from Invitrogen (Carlsbad, CA).

### *Induction of contact hypersensitivity*

The FITC-induced CHS was generated as described previously.<sup>13</sup> Briefly, mice were topically sensitized on the shaved abdomens or left ears with 0.5% FITC/ADBP at a volume of 400 µl or 20 µl, respectively, on day 0 and 1. Then the right ears of the sensitized mice were elicited by 20 µl 0.5% FITC/ADBP more than 5 days after the first sensitization. Similarly, PC-induced CHS was performed by sensitizing the shaved abdomens with 100 µl 3% PC/AOO on day 0 followed by elicitation with 20 µl 1% PC/AOO on the right ears. Ear thickness was measured using a calibrated thickness gauge (Misutoyo, Tokyo, Japan) before and 24 hr after the elicitation.

### *Lymph node cells culture*

Lymph node cells (LNCs) were obtained 24 hr after the elicitation and cultured in RPMI-1640 supplemented with 10% fetal bovine serum, penicillin–streptomycin, 2-mer-

captoethanol (50  $\mu$ M) and 2  $\mu$ g/ml concanavalin A (Seikagaku, Tokyo, Japan) for 24 hr for the production of IL-4 or were cultured in the same medium without concanavalin A for 5 days for the production of IL-12p40.

#### *Protein extraction and total RNA purification from ear tissue*

Central parts of the ears were punched out to the size of a circle 5 mm in diameter, were soaked in 200  $\mu$ l phosphate-buffered saline containing proteinase inhibitors (Calbiochem, Darmstadt, Germany) and 0.1% Tween-20, and then homogenized to extract protein. The homogenates were centrifuged (15 000 g at 4° for 5 min) and then the supernatants were supplied as protein samples for ELISA. For total RNA preparation, the ear discs were soaked in RNAlater®, and then total RNA was extracted and purified using the RNeasy® kit (QIAGEN, Germantown, MD).

#### *Measurement of cytokine concentration*

The cytokine levels in the supernatants from the culture media or from the ear homogenates were determined using commercially available ELISA kits (IL-4, IL-12p40 and TSLP).

#### *Measurement of plasma IgE concentration*

Blood samples were collected using heparinized tubes 24 hr after the elicitation and centrifuged (300 g, at room temperature for 5 min). The supernatants were supplied as plasma samples for the determination of IgE concentrations by ELISA kit (Bethyl, Montgomery, TX).

#### *Real-time polymerase chain reaction*

First-strand complementary DNA was synthesized from 1–5  $\mu$ g total RNA with random hexamers (GE Healthcare, Buckinghamshire, UK). Real-time polymerase chain reaction (PCR) was conducted with the ABI PRISM 7700 Sequence Detector (Applied Biosystems), according to the manufacturer's instructions. The PCR consisted of one cycle at 50° for 2 min and then at 95° for 10 min, followed by 40 cycles at 95° for 15 seconds and at 60° for 1 min. The TSLP primer pair was used for amplification: forward, 5'-GCTTGTCTCCTGAAAATCGAGTATT-3' and reverse, 5'-CTCCGGGCAAATGTTTTGTC-3'. For the other cytokines or glyceraldehyde 3-phosphate dehydrogenase (GAPDH), primer pairs, in the TaqMan gene expression assay kit [IL-4 (Mm00445259\_m1), IL-12p40 (Mm00434170\_m1), TNF (Mm00443258\_m1)] or in the TaqMan Rodent GAPDH control reagents kit, were used. During amplification, fluorescence measured the relative amount of amplicon produced in each cycle using specific

probes [TSLP (5'-FAM-TACTCTCAATCCTATCCCTGGCTGCCCT-TAMRA-3'), IL-4, IL-12p40, TNF, and GAPDH included in each kit]. These data were used to make a relative determination of gene expression under each experimental condition.

#### *In vivo DBP stimulation*

Ears were topically treated with several stimuli on days 0 and 1 (for acetone, DBP, ADBP, FITC/ADBP, FITC/acetone) or on only day 0 (for PC/AOO). On day 2, the expression of TSLP at the messenger RNA (mRNA) and protein levels were determined in the ear.

#### *In vivo rmTSLP treatment*

Recombinant mouse TSLP (500 ng) was injected into the dermis of the ears 30 min before each sensitization and elicitation with 0.5% FITC/AOO. In the case of sensitization, the injection was also performed 24 hr after the second sensitization.

#### *In vivo siRNA treatment*

The siRNAs were used in FITC-induced CHS. Forty micrograms siRNA against TSLP (5'-GGCAAGGAUUU-UUCUACCUTT-3') or negative control siRNA (AM4636) was intradermally injected into the sensitized ears on days 0, 1 and 2. Eighty micrograms of each siRNA was injected into the elicited ears 30 min before the elicitation.

#### *Skin histopathology*

Two days after the first sensitization, draining lymph nodes were acquired and were prepared for frozen sections (30  $\mu$ m). The cell nucleus was stained with 0.125  $\mu$ g/ml DAPI for 5 min. The lymph nodes were analysed under a confocal microscope (Carl Zeiss, Jena, Germany).

#### *Flow cytometric analysis*

Two days after the first sensitization, draining lymph nodes were acquired and were ground to prepare single-cell suspensions. The cells labelled with FITC were quantified as cells migrated from skin using a FACSCalibur flow cytometer and CELLQUEST software (BD Biosciences).

#### *Statistical analysis*

All data are reported as the mean  $\pm$  SD. Differences between groups were analysed using the Student's *t*-test, or Dunnett's multiple comparisons test with EXSAS

software (Arm Systex Co., Ltd., Osaka, Japan). Statistical significance was indicated by *P* values < 0.05.

## Results

### FITC-induced CHS elevates Th2 cytokines and serum IgE levels

Fluorescein isothiocyanate is known as a hapten with potential to induce CHS.<sup>13</sup> The ear swelling in FITC-induced CHS was increased 24 hr after the elicitation, but not after 6 hr. Meanwhile, the ear swelling in PC-induced CHS was increased at both 6 and 24 hr (Fig. 1a). To determine the properties of the CHS responses, we investigated the production of cytokines from LNCs and plasma IgE levels. The production of IL-4 was increased in LNCs derived from the mice elicited with FITC/ADBP by stimulation with 2 µg/ml of concanavalin A for 24 hr *in vitro*, but not in LNCs derived from the mice elicited with PC/AOO (PC-LNCs). Inversely, the production of IL-12p40 was increased only in PC-LNCs after 5 days of culture (Fig. 1b). The plasma IgE level was increased in FITC-induced CHS, not in PC-induced CHS, correlating with the production of IL-4 in LNCs (Fig. 1b,c). The mRNA expression in elicited ears was investigated. The level of IL-4 and TNF was increased in the swollen ears elicited with FITC/ADBP in sensitized mice compared with in the elicited ears without sensitization or in non-treated ears. Meanwhile, the expression of IL-12p40 mRNA was not changed by FITC/ADBP elicitation (Fig. 1d). These results indicate that FITC-induced CHS is a Th2-dominant model and is also associated with the induction of the pro-inflammatory cytokine TNF.

### TSLP expression is induced by DBP during the sensitization phase in FITC-induced CHS

Thymic stromal lymphopoietin is one of the potent determinants of Th2 commitment in T-cell immunity by local activation of skin DCs. To investigate the contribution of TSLP to the Th2 commitment during the sensitization phase in FITC-induced CHS, ears were stimulated on days 0 and 1, and then mice were killed on day 2 to determine the expression of TSLP in the sensitized ears. The sensitization with FITC/ADBP, but not with PC/AOO, clearly induced TSLP expression at the mRNA level. Unexpectedly, application of DBP-free FITC/acetone failed to induce TSLP expression. Meanwhile, DBP and ADBP, but not acetone alone, potently induced TSLP expression at the mRNA level. Interestingly, FITC and ADBP (FITC/ADBP) synergistically increased the expression level of TSLP (Fig. 2a). The induction of TSLP expression at the protein level showed a similar trend to that at the mRNA level. Exceptional induction of TSLP

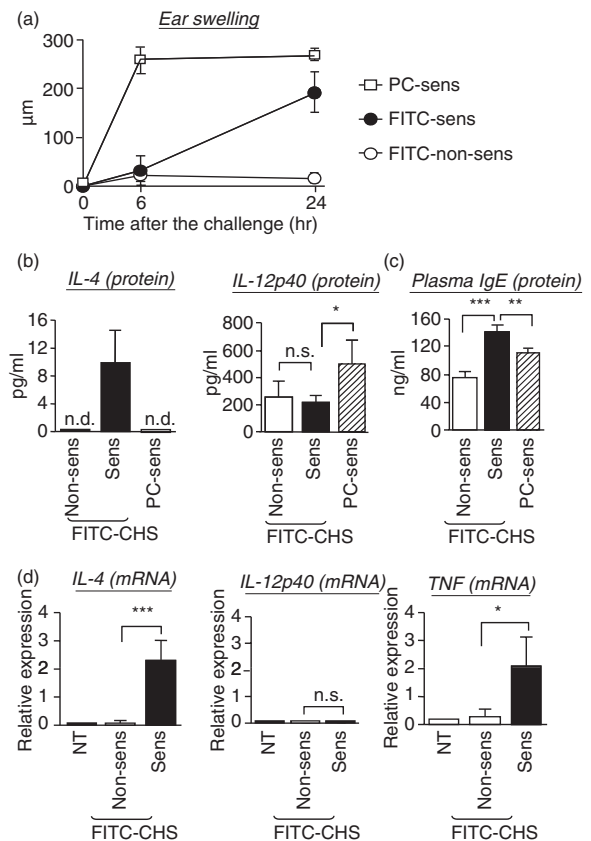


**Figure 1.** Fluorescein isothiocyanate (FITC)-induced contact hypersensitivity (CHS) elevates T helper type 2 cytokines and serum immunoglobulin E (IgE) level. Mice were sensitized and elicited with FITC/acetone : dibutyl phthalate (ADBP) or picryl chloride/acetone : olive oil (PC/AOO). (a) Ear swelling was measured 0, 6 and 24 hr after the elicitation. (b) Lymph node cells (LNCs) were harvested 24 hr after the elicitation and cultured with (for interleukin-4) and without (for interleukin-12p40) concanavalin A. Cytokine levels in the culture media were determined by enzyme-linked immunosorbent assay (ELISA; n.d. = not-detected). (c) Plasma was collected 24 hr after the elicitation, and plasma IgE level was determined by ELISA. (d) The messenger RNA expression levels of cytokines in elicited ears were determined as relative gene expression normalized to housekeeping gene, *GAPDH*. Data are mean  $\pm$  SD of six or seven animals (a), mean  $\pm$  SD of three or four animals (b, c), and mean  $\pm$  SD of two to four animals. Student's *t*-test was used for statistical evaluation of the results. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, and n.s. = not significant (*P* > 0.05).

was observed in the case of PC/AOO stimulation (Fig. 2b).

### DBP is indispensable for FITC sensitization and elicitation

The TSLP was probably induced by DBP in FITC-induced CHS, suggesting the importance of DBP in the model. Therefore, we attempted to confirm the importance of DBP for establishment of the model. Removing DBP

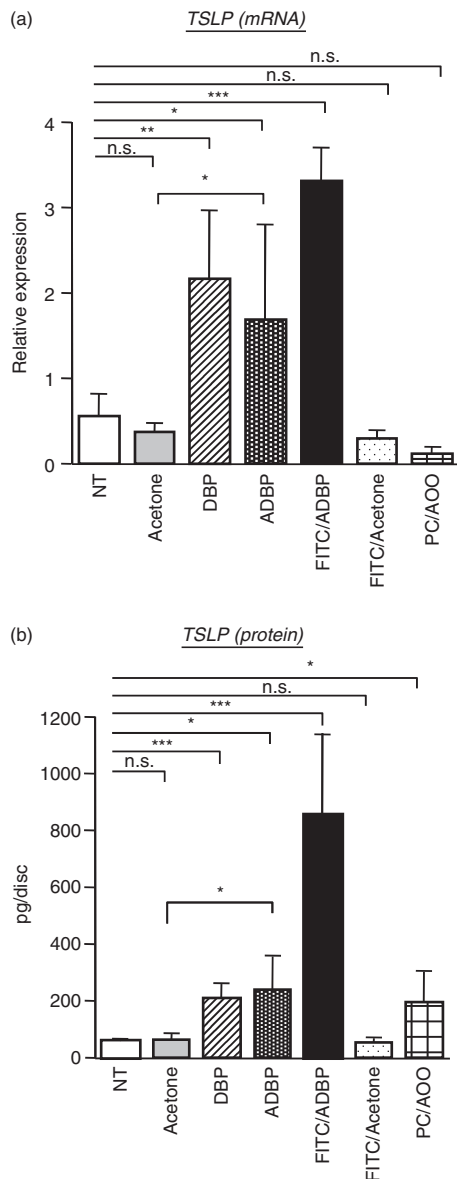


**Figure 2.** Thymic stromal lymphopoietin (TSLP) expression is induced by dibutyl phthalate (DBP) during the sensitization phase in fluorescein isothiocyanate (FITC)-induced contact hypersensitivity (CHS). Mice were topically stimulated once daily on days 0 and 1 [acetone, DBP, acetone : DBP (ADBP), FITC/ADBP, FITC/acetone] or on day 0 [picryl chloride/acetone : olive oil (PC/AOO)]. On day 2, the skin punches were taken from the ears and the following experiments were undertaken. (a) The messenger RNA expressions of TSLP in the ears were determined. The relative gene expression was normalized to *GAPDH*. (b) The protein expression of TSLP in punched samples was detected by enzyme-linked immunosorbent assay. Data are mean + SD of three or four animals. Student's *t*-test or Dunnett's test was used for statistical evaluation of the results. \**P* < 0.05, \*\**P* < 0.01 and \*\*\**P* < 0.001.

from the solvent of FITC in the sensitization or the elicitation phase completely abolished the induction of ear swelling (Fig. 3).

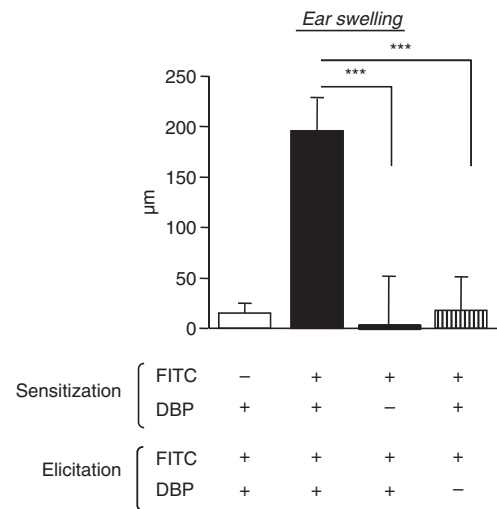


**Figure 3.** Dibutyl phthalate (DBP) is indispensable for fluorescein isothiocyanate (FITC) sensitization and elicitation. Mice were sensitized and elicited with FITC/acetone : DBP (ADBP) or FITC/acetone. The ear swelling was measured 24 hr after the elicitation. Data are mean + SD of four to eight animals. Dunnett's test was used for statistical evaluation of the results. \*\*\**P* < 0.001.

#### Recombinant mouse TSLP complements the absence of DBP in the sensitization phase

As shown in Fig. 3 the absence of DBP in the sensitization and elicitation phases resulted in failed FITC-induced CHS. We next determined whether exogenous TSLP was able to complement the failures. Recombinant mTSLP or saline, as a vehicle control, was injected into the ears where DBP-free FITC/acetone would be applied during sensitization and/or elicitation phase. These injections were performed 30 min before each sensitization and/or elicitation and in the case of the sensitization phase, it was also performed 24 hr after the second sensitization. Ear swelling was abolished by removing DBP from the sensitization and/or the elicitation, which was significantly restored to the level of that of FITC/ADBP-sensitized and -elicited mice when rmTSLP was injected during the sensitization phase. However, rmTSLP injection during the elicitation phase or both the sensitization and the elicitation phases only partially restored the ear swelling. The intradermal injection of saline vehicle established pseudo-sensitization with DBP-free FITC/acetone, resulting in increased ear swelling, which might be the result of the physical activation of cells participating in the sensitization (Fig. 4a). The expression of IL-4 in the ears was increased in the FITC/ADBP-sensitized and -elicited mice. As expected, its level in the ears of mice that were sensitized and/or elicited without DBP was not increased. Interestingly, the unchanged IL-4 level was enhanced by the complementary injection of rmTSLP during the sensitization phase, but there was no effect during the elicita-

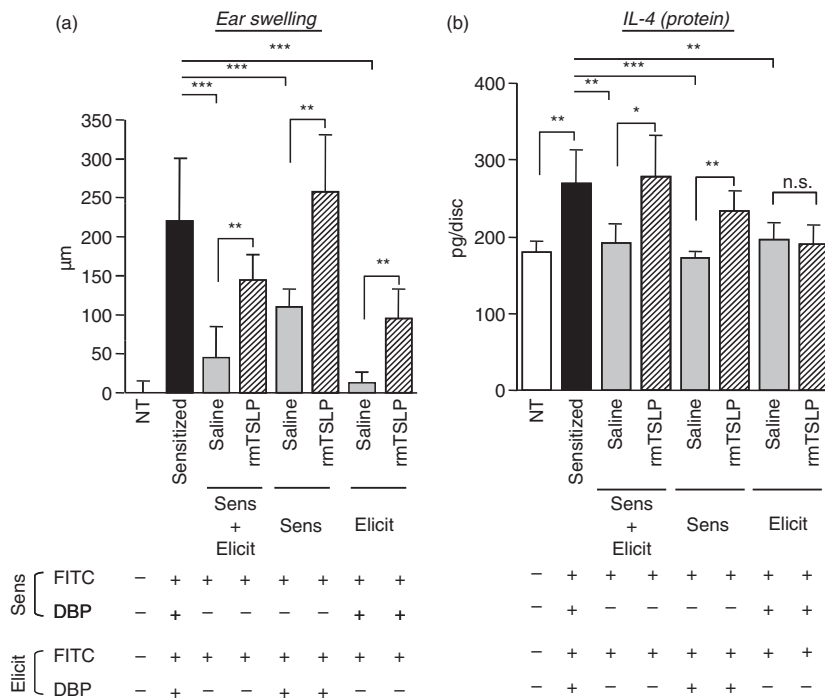


**Figure 4.** Recombinant mouse thymic stromal lymphopoietin (TSLP) complements the absence of dibutyl phthalate (DBP) in the sensitization phase. Recombinant mouse TSLP or saline was intradermally injected into the ear sensitized or elicited with DBP-free fluorescein isothiocyanate (FITC)/acetone 30 min earlier. (a) Ear swelling was determined 24 hr after the elicitation. (b) Interleukin-4 (IL-4) levels in the ear were determined by enzyme-linked immunosorbent assay 24 hr after the elicitation. Data are mean + SD of three or four animals. Student's *t*-test or Dunnett's test was used for statistical evaluation of the results. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001 and n.s. = not significant (*P* > 0.05).

tion phase (Fig. 4b). These data suggest that the exogenously injected TSLP plays the surrogate role of DBP during the sensitization phase committing to Th2 milieu, and it may do so only partially during the elicitation phase.

#### Small interfering RNA against TSLP suppresses Th2-dominant FITC-induced CHS

The expression of TSLP in the sensitization phase might contribute to the establishment of FITC-induced CHS. We next investigated the contribution of TSLP in FITC-induced CHS using siRNA against TSLP, which was injected into the dermis at the sensitized site. The ear swelling in FITC-induced CHS was significantly suppressed by the treatment with siRNA against TSLP (Fig. 5a). To investigate the effect of the siRNA, we investigated the level of cytokines in the ear. The siRNA against TSLP decreased the IL-4 level in the elicited ear (Fig. 5b), but did not affect the IL-12p40 at the protein level (data not shown). The negative control of siRNA did not show any significant effects on ear swelling (Fig. 5a), or on the levels of IL-4 and IL-12p40 (Fig. 5b and data not shown) during elicitation. These data indicated that TSLP played a role during the sensitization phase in FITC-induced CHS at least in part through IL-4 production.

#### DBP induces APC migration

The action of DBP as an adjuvant is believed to occur via the migration of DCs,<sup>11,14</sup> so we evaluated whether DBP-

induced TSLP could evoke the migration of APCs. Ears were stimulated on days 0 and 1. Recombinant mTSLP, saline, siRNA against TSLP or negative control siRNA was intradermally injected into the ear 30 min before stimulation. The FITC-positive cells were observed histochemically in the draining lymph node of mouse stimulated with FITC/ADBP on day 2 (Fig. 6a). The LNCs were subjected to flow cytometric analysis. The presence of DBP markedly increased the number of FITC-positive cells in the draining lymph nodes and all the FITC-positive cells were CD11c-positive, which meant that the FITC-bearing APCs were DCs. Furthermore, the FITC-positive DCs were activated, highly expressing CD40 and CD80 (Fig. S1). Recombinant mouse TSLP did not evoke the migration of FITC-positive DCs and siRNA against TSLP did not inhibit the DBP-induced migration (Figs 6b and S2).

#### Discussion

The FITC-induced CHS in mouse has been characterized as an animal model mediated by a Th2-dominant immune system.<sup>13,15</sup> Similar to previous reports, we showed here that FITC-induced CHS was dominantly committed to the Th2 lineage, expressing high IL-4 at the protein level in LNCs and at the mRNA level in the inflamed ear, and increasing plasma IgE levels, as compared with those in the PC-induced CHS model in which IL-12p40 was increased at the protein level in LNCs. Consequently, FITC-induced CHS had a different component from PC-induced CHS, although the experimental methodologies were similar. The sole and characteristic differ-

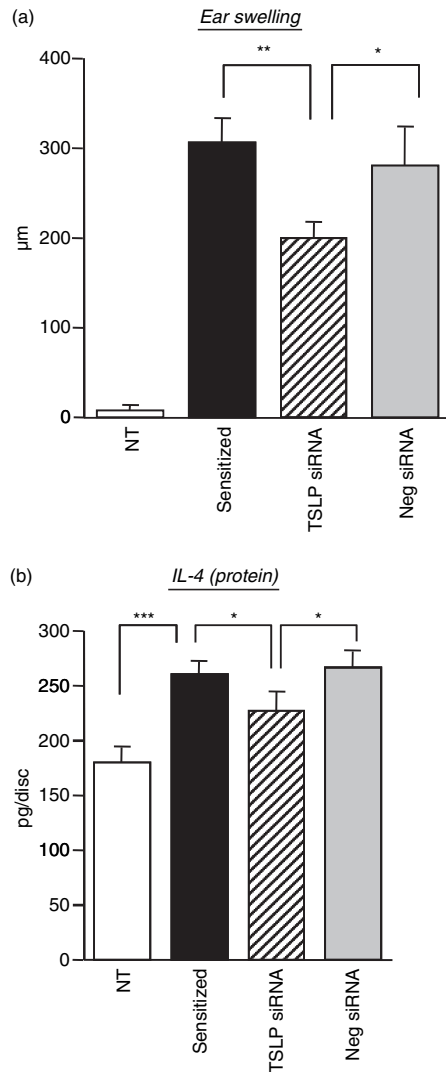


**Figure 5.** Small interfering (si) RNA against thymic stromal lymphopoietin (TSLP) suppresses T helper type 2 (Th2)-dominant fluorescein isothiocyanate (FITC)-induced contact hypersensitivity (CHS). The siRNA against TSLP or negative control siRNA was intradermally injected into the sensitized ears. (a) Ear swelling was determined 24 hr after the elicitation. (b) Interleukin-4 (IL-4) levels in the ear were determined by enzyme-linked immunosorbent assay 24 hr after the elicitation. Data are mean + SD of three animals. Student's *t*-test was used for statistical evaluation of the results. \**P* < 0.05 and \*\**P* < 0.01 and \*\*\**P* < 0.001.

ence was the DBP component, which had been reported to act as an indispensable adjuvant for FITC-induced CHS.<sup>11</sup> Therefore, we hypothesized that DBP might contribute to Th2 commitment in FITC-induced CHS.

A newly identified cytokine, TSLP, is believed to be one of the factors involved in the pathogenesis of allergic disorders such as AD or asthma<sup>5,9,16</sup> and locally activates DCs in humans, which in turn promotes differentiation of naïve T cells to Th2 cells.<sup>5,6</sup> It has been reported that the Th2 cells differentiated by TSLP-activated DCs produced not only

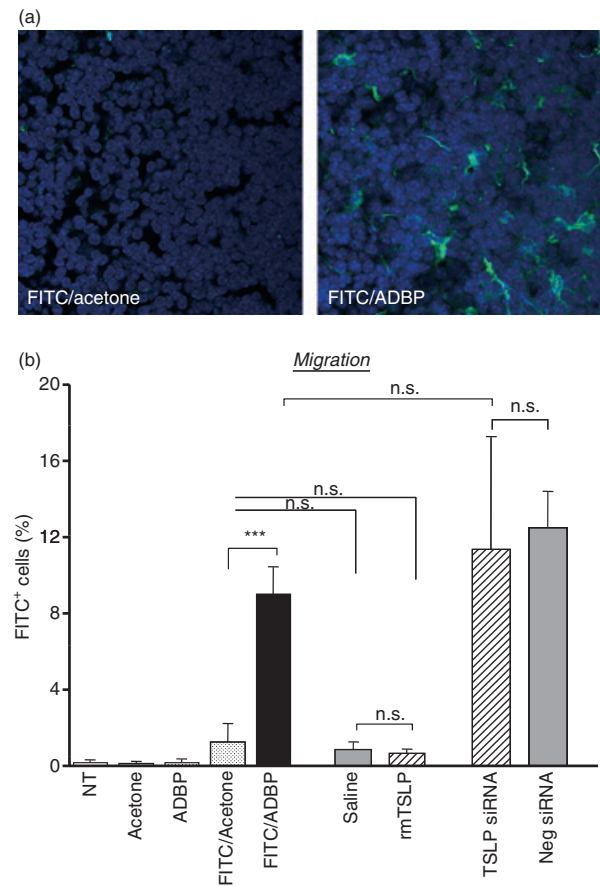


**Figure 6.** Dibutyl phthalate (DBP) induces migration of antigen-presenting cells. Ears were topically stimulated once daily on days 0 and 1 [acetone, acetone : DBP (ADBP), fluorescein isothiocyanate (FITC)/acetone, FITC/ADBP]. Recombinant mouse thymic stromal lymphopoietin (TSLP) or saline was intradermally injected into the ear stimulated with FITC/acetone 30 min earlier. Small interfering (si) RNA against TSLP or negative control siRNA was intradermally injected into the ear stimulated with FITC/ADBP 30 min earlier. On day 2, FITC-positive cells in draining lymph nodes were determined by (a) confocal microscopy or (b) FACSCalibur. The percentages of FITC-positive cells are indicated as the mean + SD of four or five animals. Student's *t*-test or Dunnett's test was used for statistical evaluation of the results. \*\*\**P* < 0.001 and n.s. = not significant (*P* > 0.05).

Th2 cytokines but also TNF- $\alpha$  and consequently induced allergic and inflammatory reactions. Interestingly, the mRNA expression of TNF in the inflamed ear tissues in FITC-induced CHS was also increased as well as IL-4, suggesting that TSLP was implicated in the model. As we expected, the TSLP level was elevated at the mRNA and protein levels in the sensitized ears, which was clearly dependent on DBP application, but not FITC or acetone.

Since a DBP-free FITC/acetone mixture completely failed to sensitize or elicit mice, DBP seemed to play a crucial role in the model. DBP and some of its relatives, phthalates, have been regarded as one of the adjuvants of

the reaction. Their adjuvanticity is believed to be the result of the migration of APCs, such as dermal DCs and Langerhans cells, to skin-draining lymph nodes bearing antigen.<sup>11,14</sup> Although APC migration and activation are essential to provoke innate and adaptive immunity, the precise functions of DBP for the adjuvanticity remain to be defined. Since TSLP could uniquely activate DCs<sup>5,6</sup> and was inducing inflammation in mice,<sup>17</sup> we hypothesized that TSLP played a substantial role in DBP adjuvanticity. Indeed, the epicutaneous application of DBP during the sensitization phase markedly increased the migration of FITC-positive DCs to the draining lymph nodes. Though rmTSLP did not induce the migration, it restored the loss of adjuvanticity of DBP-free sensitization in FITC-induced CHS. Inversely, siRNA against TSLP did not inhibit the DBP-induced migration of DCs, but it suppressed the oedematous inflammation and rescued the immune response from Th2 commitment, suppressing IL-4 expression. Additionally our preliminary data showed that intradermal injection of rmTNF- $\alpha$  in the sensitization phase did not play any surrogating role for DBP (data not shown), although it had been reported as enough to provoke APC migration.<sup>18</sup> These facts suggested that the induction of APC migration was not critical to exerting the adjuvanticity of DBP for the sensitization in FITC-induced CHS. The surrogate role of TSLP for DBP might be independent of the induction of migration. It was reported that TSLP might not be important for the migration of DCs to skin-draining lymph nodes, although TSLP was essential for Th2 immunity.<sup>19</sup> Consistent with the plausible adjuvanticity of DBP, these data therefore suggested that the TSLP pathway was one of the ways, but crucial, to exert the adjuvanticity of DBP, which might be because of the activation of APCs and/or undetermined effects but not the migration of APCs.

Although clinically nickel could be an antigen in humans, the prevalence of nickel-contact allergy was only 8.6% of the general population,<sup>20</sup> meaning that only a portion of people could sense nickel as the antigen and so some other factors contribute to the sensitivity of these people or the antigenicity of the pre-antigen. In addition, only a portion of the population has a pollen allergy that depends on their genetic background and also on exogenous factors such as air pollution as suggested by a report indicating that the morbidity caused by pollen allergy was higher in urban than rural areas.<sup>21</sup> This suggests that environmental factors predispose humans to allergic diseases. Like nickel-contact allergy and pollen allergy, AD is also one of the allergic diseases that is well documented as strongly implicated by genetic background. However, evidence from a range of researchers has shown a rapid increase in prevalence and in the social and geographical variation, suggesting that secondary environmental factors, such as irritant allergens, infection

with micro-organisms, breastfeeding, foods and environmental pollutants, play a crucial role in the skin pathogenesis in AD.<sup>12,22</sup> As shown in the present study, DBP-free FITC completely failed at sensitization or elicitation in CHS. These results suggest that FITC itself is insufficient to sensitize or elicit immune systems to produce a response. These facts led us to speculate that exogenous factors such as phthalates endowed a sensitive predisposition in humans or animals, or are necessary in models to add the antigenicity to the 'pre-antigen' such as FITC. Our life has many opportunities to be exposed to phthalates because they are often used as a plasticizer for vinyl flooring, toys for children, etc., and they can migrate out of these things and be absorbed in dust.<sup>23</sup> The causal association between phthalates in dust and allergic wheezing in humans has been reported<sup>24</sup> and intraperitoneal injection of phthalate augmented mite-allergen-induced dermatitis in mice,<sup>25</sup> suggesting that phthalates might contribute to atopic pathogenesis. Here we showed that the elevated-expression of TSLP in mice by epicutaneous exposure to phthalates impaired AD. This result supports the evidence that indicates the relation between phthalates and allergic responses.<sup>10</sup>

The present study is the first report to show the important effects of DBP in eliciting Th2 immunity via TSLP expression in mice. This provides new explanations of the acquirement of atopic predisposition and the expansion of atopic prevalence in modern society, i.e. long-lasting exposure to types of environmental toxins, such as phthalates, may endow atopic predisposition in humans or animals. However, it remains unclear why DBP induces TSLP, how DBP is recognized and which cells sense DBP. Our preliminary data showed that an intracellular danger sensor inflammasome might not contribute to sensing DBP and so some other molecule(s) may recognize DBP. The resolutions of these questions promise to give us a better understanding of the relationship between atopic predisposition and environmental materials and also may help to provide new effective treatments and prevention strategies against atopic diseases like AD.

## Disclosures

The authors have no potential conflicts of interest.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Dibutyl phthalate (DBP) induces migration and activation of dendritic cells (DCs). The lymph node cells (LNCs) were isolated as described in Fig. 6(b) and were stained with antibodies [Alexa Fluor 647-conjugated anti-mouse CD11c (clone N418; AbD Serotec, Oxford, UK), phycoerythrin-conjugated anti-mouse CD40 (clone 1C10; eBioscience, San Diego, CA), or phycoerythrin-conjugated anti-mouse CD80 antibody (clone 16-10A1; eBioscience)]. Fluorescein isothiocyanate (FITC)-positive (R2) or negative (R3) cells were analysed for CD11c (x-axis) together with CD40 or CD80 (y-axis). Results are representative data of five individuals.

**Figure S2.** Thymic stromal lymphopoietin (TSLP) did not contribute to the migration of antigen-presenting cells to draining lymph nodes. The lymph node cells (LNCs) isolated as described in Fig. 6(b) were analysed by FACSCalibur to determine fluorescein isothiocyanate (FITC)-positive cells. Results are representative data of four or five individuals.

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