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**Langerhans cells are critical in epicutaneous sensitization with protein antigen via  
TSLP receptor signaling**

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## Abstract

**Background:** Clarification of cutaneous dendritic cell (DC) subset and the role of thymic stromal lymphopoietin (TSLP) signaling in epicutaneous sensitization with protein antigens, as in the development of atopic dermatitis (AD), is a crucial issue.

**Objectives:** Since TSLP is highly expressed in the vicinity of Langerhans cells (LCs), we sought to clarify our hypothesis that LCs play an essential role in epicutaneous sensitization with protein antigens through TSLP signaling.

**Methods:** Using Langerin-diphtheria toxin receptor knockin mice and human Langerin-diphtheria toxin A transgenic mice, we prepared mice deficient in LC. We also prepared mice deficient in TSLP receptor in LCs using TSLP receptor deficient mice with bone marrow chimeric technique. We applied these mice to an ovalbumin-induced epicutaneous sensitization model.

**Results:** Upon the epicutaneous application of OVA, conditional LC-depletion attenuated the development of clinical manifestations as well as serum OVA-specific IgE increase, OVA-specific T cell proliferation, and IL-4 mRNA expression in the draining lymph nodes. Consistently, even in the steady state, permanent LC depletion resulted in decreased serum IgE levels, suggesting that LCs mediate Th2 local environment. In addition, mice deficient in TSLP receptor on LCs abrogated the induction of OVA-specific IgE levels upon epicutaneous OVA sensitization.

**Conclusion:** LCs initiate epicutaneous sensitization with protein antigens and induce Th2-type immune responses via TSLP signaling.

## Clinical implications

TSLP receptors on LCs can be a therapeutic target of skin inflammatory reactions

induced by epicutaneous sensitization with protein antigens, such as in the development of atopic dermatitis.

### **Capsule summary**

LCs initiate epicutaneous sensitization with protein antigens and induce Th2-type immune responses via TSLP-TSLP receptor signaling.

**Key words:** Langerhans cell, TSLP, TSLP receptor, epicutaneous sensitization, protein antigen

### **Abbreviations used**

AD, atopic dermatitis

BM, bone marrow

BMC, bone marrow chimera

CCR, CC chemokine receptor

DCs, dendritic cells

DTA, diphtheria toxin subunit A

DTR, diphtheria toxin receptor

EGFP, enhanced green fluorescent protein

LCs, Langerhans cells

LN, lymph node

MDC, macrophage-derived chemokine

MFI, mean fluorescence intensity

OVA, ovalbumin

90 TARC, thymus and activation-regulated chemokine

91 TSLP, thymic stromal lymphopoietin

92 TSLPR, TSLP receptor

93 TJ, tight junction

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## INTRODUCTION

Skin plays an important immunological role by eliciting a wide variety of immune responses to foreign antigens (1). Atopic dermatitis (AD) is a pruritic chronic retractable inflammatory skin disease that is induced by the complex interaction between susceptibility genes encoding skin barrier components and stimulation by protein antigens (2, 3). Patients with AD exhibit compromised barrier function that leads to the activation of keratinocytes and immune cells, which favors a Th2 bias. A wide array of cytokines and chemokines interact to yield symptoms that are characteristic of AD. For example, thymus and activation-regulated chemokine (TARC/CCL17) and macrophage-derived chemokine (MDC/CCL22) both attract Th2 cells through CC chemokine receptor 4 (CCR4) (4), levels of which correlate well with the severity of AD (5). Elevation of serum IgE levels is also frequently found in patients with AD, sometimes concomitant with food allergy, allergic rhinitis, and asthma (3). Yet it remains unknown how elevation of serum IgE levels to protein antigens is induced in the pathogenesis of AD.

Upon protein antigen exposure, dendritic cells (DCs) acquire antigens and stimulate the proliferation of T cells to induce distinct T helper cell responses to external pathogens (6). Therefore, it has been suggested that DCs initiate AD in humans (7), however, it remains unclarified which cutaneous DC subset initiates epicutaneous sensitization to protein antigens. In the mouse skin, there are at least three subsets of DCs: LCs in the epidermis, and Langerin-positive and Langerin-negative DCs in the dermis (Langerin<sup>+</sup> dermal DCs and Langerin<sup>-</sup> dermal DCs, respectively) (8-10). It has been reported that application of large molecules are localized above the size-selective barrier, tight junction (TJ), and that activated LCs extend their dendrites through the TJ

to take up antigens (11). Therefore, it can be hypothesized that not dermal DCs but rather LCs initiate epicutaneous sensitization with protein antigens, as in the development of AD.

In human, polymorphisms in the gene encoding the cytokine thymic stromal lymphopoietin (TSLP) are associated with the development of multiple allergic disorders through TSLP receptor (TSLPR), which is expressed in several cell types, such as DCs, T cells, B cells, basophils, and eosinophils (12, 13). Thus, TSLP seems to be a critical regulator of Th2 cytokine-associated inflammatory diseases.

Recently, it has been reported that basophils induce Th2 through TSLPR (13). On the other hand, it is also known that skin DCs elicit a Th2 response in the presence of mechanical injury by inducing cutaneous TSLP (14), and that LCs are critical in the development of skin lesions induced by the topical application of vitamin D3 analogues through TSLP signaling (15). However, these skin inflammation models are induced in an antigen-independent manner; therefore, it is important to address the degree to how TSLP is essential in Th2 shifting and to identify the cells that are essential for TSLP signaling transduction upon epicutaneous sensitization, which is relevant to inflammatory skin diseases, such as AD. This will lead to the understanding of the underlying mechanism and to develop new therapeutic targets for inflammatory skin diseases.

It is known that TSLP activates human epidermal LCs and DCs *in vitro* (16-18) and that TSLP is highly expressed in the epidermis of the lesional skin of AD patients. Since LCs are localized in the epidermis, we hypothesized that LCs initiate epicutaneous sensitization through TSLP signaling. By applying an LC ablation system, we found that LCs are crucial for Th2 induction and IgE production upon epicutaneous protein



exposure through TSLP signaling.

## MATERIALS AND METHODS

### Animals and bone marrow chimera

C57BL6 (B6) and BALB/c mice were purchased from Japan SLC (Shizuoka, Japan).

OT-II TCR transgenic mice were purchased from the Jackson Laboratory (Bar Harbor,

ME, USA). Langerin-DTA mice were generated by Dr. Daniel Kaplan (19), and

Langerin-eGFP-DTR knock-in mice were kindly provided by Dr. Bernard Mallissen

(CIML, Institut National de la Santé et de la Recherche Médicale, Marseille, France).

TSLPR<sup>-/-</sup> mice (BALB/c or B6 background) were generated by Dr. Steven Ziegler

(20). Seven- to twelve-week-old female mice bred in specific pathogen-free facilities at

Kyoto University were used for all experiments.

For LC depletion specifically, Langerin-eGFP-DTR mice were used. Intraperitoneal

injection of 1 µg DT (Sigma-Aldrich, St. Louis, MO, USA, in 500 µl of PBS) depleted

Langerin<sup>+</sup> DC subsets, including LCs and Langerin<sup>+</sup> dermal DCs. Langerin<sup>+</sup> dermal DCs

in the dermis recover one week after DT injection, but LCs remain undetectable for four

weeks after depletion (21). Since only LCs are depleted between one and three weeks

after DT injection, we can evaluate the role of LCs in epicutaneous sensitization by

applying OVA between one and three weeks after DT injection. Therefore, we injected

DT seven days before epicutaneous sensitization. Control mice were intraperitoneally

injected with 500 µl of PBS on the same day.

To generate bone marrow chimeric mice, 6-week-old mice were irradiated (9 Gy) and

transplanted with bone marrow cells ( $1 \times 10^7$  cells/recipient). All experimental

procedures were approved by the institutional animal care and use committee of Kyoto University Graduate School of Medicine.

### **Epicutaneous sensitization**

Mice were anesthetized with diethylether (Nacalai Tesque, Kyoto, Japan), and then shaved with an electric razor (THRIVE Co. Ltd., Osaka, Japan). A single skin site on each mouse was tape-stripped at least five times with adhesive cellophane tape (Nichiban, Tokyo, Japan). One hundred  $\mu\text{g}$  of OVA in 100  $\mu\text{l}$  of normal saline or placebo (100  $\mu\text{l}$  of normal saline) was placed on patch-test tape (Torii Pharmaceutical Co., Ltd., Tokyo, Japan). Each mouse had a total of three two-day exposures to the patch, separated by one-day intervals. Mice were euthanized at the end of the third cycle of sensitization (day 9).

### **Antigen-specific T cell proliferation**

To assess the OVA-specific T cell priming capacity of cutaneous LCs, 100  $\mu\text{l}$  of normal saline with or without 100  $\mu\text{g}$  of OVA was placed on the shaved and tape-stripped mouse back skin. CD4 T cells were isolated from OT-II mice using magnetic bead separation (Miltenyi Biotec, Bergisch Gladbach, Germany) and labeled with 8  $\mu\text{M}$  CFSE. Forty-eight hours after epicutaneous sensitization,  $5 \times 10^6$  CFSE labeled OT-II T cells were transferred to naïve mice via the tail vein. An additional 48 hours later, skin draining brachial lymph nodes (LNs) were collected and analyzed by means of flow cytometry.

### **Statistical analysis**

Unless otherwise indicated, data are presented as means  $\pm$  standard deviations (SD), and each data point is representative of three independent experiments. *P* values were calculated according to the two-tailed Student's *t*-test.

A complete description of the materials and methods, and any associated references are available in the Online Repository.

## RESULTS

### **LC depletion impaired the development of OVA-induced allergic skin dermatitis model**

To assess the role of LCs in epicutaneous sensitization with protein antigens and induction of IgE, we applied OVA to mice epicutaneously (22). In this model, we observed a rise in OVA-specific serum IgE and IgG1, both of which are induced in a Th2-dependent manner, as well as the development of dermatitis characterized by the infiltration of CD3<sup>+</sup> T cells, eosinophils, and neutrophils and local expression of mRNA for the cytokines interleukin (IL)-4, IL-5, and interferon (IFN)- $\gamma$  (22). These findings exhibited characteristics of allergic skin inflammation such as AD. To evaluate the roles of LCs, we used knock-in mice expressing enhanced green fluorescent protein (EGFP) and diphtheria toxin receptor (DTR) under the control of the Langerin gene, called Langerin-eGFP-DTR mice (23).

In the OVA-induced allergic skin dermatitis model, LC-depleted mice showed milder clinical manifestations than LC-non-depleted mice did (Fig. 1A, left panel). Histology of the patched skin area showed pronounced lymphocyte infiltration and edema in the dermis of sensitized LC-non-depleted mice, which was less apparent in sensitized

LC-depleted mice (Fig. S1A, B). The histological score of LC-depleted mice was also lower than that of LC-non-depleted mice (Fig. 1A, right panel). In addition, serum OVA-specific IgE and IgG1 levels in LC-depleted mice were significantly lower than those in wild-type (WT) mice (Fig. 1B). On the other hand, the Th1-dependent immunoglobulin IgG2a was not induced by application of OVA (Fig. 1B). These data suggest that LCs are involved in the development of OVA-induced AD-like skin inflammation and induction of IgE.

### **Impaired T cell proliferation and Th2 induction by LC depletion**

Priming of antigen-specific Th2 cells and proliferation is an important step in the development of this model. To assess the T cell priming capacity of cutaneous LCs upon protein allergen exposure, LC-depleted and non-depleted mice were sensitized with OVA percutaneously on the back and transferred with carboxyfluorescein succinimidyl ester (CFSE)-labeled OT-II T cells which express an OVA-specific T cell antigen receptor. Next, single-cell suspensions prepared from the skin-draining brachial lymph nodes (LNs) were analyzed by means of flow cytometry to evaluate T cell division by LCs in the draining LNs. LC-depleted mice showed impaired T cell division after OVA sensitization compared with LC non-depleted mice, suggesting that LCs stimulate T cell proliferation, at least to some degree, in this model (Fig. 2A and B).

To evaluate the role of LCs in T cell priming, we examined the mRNA expression of Th2 cytokine IL-4 and Th1 cytokine IFN- $\gamma$  in draining LNs after OVA sensitization. The IL-4 mRNA expression level of draining LNs was significantly decreased in LC-depleted mice, while the IFN- $\gamma$  mRNA expression level was significantly higher in LC-depleted mice than in LC-non-depleted mice (Fig. 2C). These results suggest that

LCs are crucial for stimulating T cell proliferation to a certain extent and Th2 induction pronouncedly in skin-draining LNs in this model.

### **LCs are responsible for initiating epicutaneous sensitization to protein antigens**

It has been reported that LCs are dispensable for initiating contact hypersensitivity to haptens, which may cast a discrepancy to our findings on the necessity of LCs to protein antigen sensitization (21, 24). To evaluate the extent of skin penetration by protein antigens and haptens, we patched fluorescein isothiocyanate (FITC)-conjugated OVA or painted FITC on the back skin of B6 mice, and performed immunohistochemical analysis. FITC-conjugated OVA retained above the TJ was indicated by staining with anti-claudin-1 antibody (Fig. S2, left panel). On the other hand, when we painted FITC on the skin of the mouse back skin, it readily penetrated into the dermis where dermal DCs locate (Fig. S2, right panel).

### **LCs are critical for IgE production**

To further assess the role of LCs in IgE production, we used gene-targeted Langerin-diphtheria toxin subunit A (DTA) mice (named Langerin-DTA mice), which constitutively lack LCs throughout life (19). WT and Langerin-DTA mice were bred under SPF conditions for six to ten weeks, and serum IgE levels were measured by means of ELISA. On the FVB background, the serum IgE level was lower in Langerin-DTA mice than in WT controls (Fig. 3A, left panel), while no significant difference was seen on the C57BL/6 (B6) background (Fig. 3A, right panel). We also found that the expression level of IgE on peritoneal mast cells was decreased in LC-deficient mice in both the FVB and B6 backgrounds (Fig. 3B). Pre-incubation of

mast cells with IgE *in vitro* did not change the data arguing that surface expression of FcεRI on mast cells was decreased in LC deficient mice, which is an indicator of lower serum IgE. Therefore, the above data strongly suggest that LCs are crucial for IgE production, which is consistent with the findings in the OVA-induced skin inflammation model (Fig. 1, Fig. 2).

### **TSLP receptor on LCs is upregulated by protein antigen exposure**

It has been reported that TSLP is involved in exacerbation of mouse Th2-mediated allergic inflammation through direct stimulation of Th2 effector cells (25). However, it remains unknown which cells initiate Th2 induction via TSLP signaling under epicutaneous sensitization of protein antigens. TSLP is highly expressed in the skin lesions of human AD (17, 18, 26, 27), and the major cells in proximity to keratinocytes are LCs; therefore, we evaluated the effect of TSLPR expression on LCs. We found that LCs expressed TSLPR, but the expression level was low under the steady state. On the other hand, the expression level of TSLPR on LCs was pronouncedly enhanced by topical application of OVA (Fig. 4).

### **Establishment of BMC mice deficient in TSLPR on LC**

Next we sought to clarify the significance of TSLP in epicutaneous sensitization with protein antigens and to identify responsible cells mediating TSLP signaling. Since cells ensuring epidermal LC renewal are radioresistant, LCs and their derivatives found in skin-draining LNs are of host origin (28). We irradiated B6 mice and B6 background TSLPR-deficient (TSLPR<sup>-/-</sup>) mice, and then transferred bone marrow cells from B6 mice into the irradiated mice. TSLPR is expressed on not only LCs, but also T cells, B

cells, basophils, eosinophils, and dermal DCs. Of note LCs are radioresistant while T cells, B cells, basophils, eosinophils, and dermal DCs are radiosensitive. When mice were irradiated and transplanted with bone marrow cells, more than 95% of the blood cells in the recipient mice had been replaced with donor-derived cells within two months after the transfer, whereas almost 100% of LCs were derived from the host, unlike the vast majority of dermal DCs that were donor-derived at this point (Fig. 5A). Therefore, given that TSLPR<sup>-/-</sup> mice were reconstituted with bone marrow cells from B6 mice, these mice were deficient in TSLPR on LCs, but other bone marrow-derived cells expressing TSLPR were present. Accordingly, using a hematopoietic bone marrow chimeric (BMC) system, we generated mice in which TSLPRs were lacking in LCs (LC-TSLPR<sup>-/-</sup> BMC mice) (Fig. S3).

#### **Essential target of TSLP is TSLPR on LCs in OVA-induced allergic skin dermatitis model**

In the context of OVA-induced AD-like skin inflammation, LC-TSLPR<sup>-/-</sup> BMC mice showed milder clinical and histological findings than TSLPR<sup>+/+</sup> BMC mice did, but these findings were nearly comparable with those of TSLPR<sup>-/-</sup> BMC mice (Fig. 5B, Fig. S4). Consistently, OVA-specific IgE levels in the serum after OVA challenge were significantly lower in LC-TSLPR<sup>-/-</sup> BMC mice than in TSLPR<sup>+/+</sup> BMC mice (Fig. 5C). These data indicate LCs play an important role in epicutaneous sensitization upon protein antigens in accord with IgE induction through TSLP-TSLPR signaling.

#### **TSLPR on LCs are dispensable for antigen-specific T cell proliferation, but vital for Th2 induction**

The above results suggest that LCs stimulate T cells to differentiate into Th2, resulting in IgE induction. To clarify this issue, we assessed the T cell proliferation and differentiation capacity of LCs in the presence or absence of TSLPR. We transferred CFSE-labeled OT-II T cells into mice topically treated with OVA, and dividing cells in the draining LNs were measured by means of flow cytometry (Fig. 6A). The ratio of dividing OT-II CD4<sup>+</sup> T cells to undivided OT-II CD4<sup>+</sup> T cells was comparable among LC-TSLPR<sup>-/-</sup> BMC, TSLPR<sup>+/+</sup> BMC, and TSLPR<sup>-/-</sup> BMC mice (Fig. 6B). In addition, IFN- $\gamma$  mRNA level in the draining LNs 96 hours after OVA application was similar among these three groups (Fig. 6C). On the other hand, the IL-4 mRNA expression level in skin-draining LNs was significantly lower in LC-TSLPR<sup>-/-</sup> BMC mice than in the other two groups (Fig. 6C). These results indicate that TSLPR on LCs are dispensable for antigen-specific T cell proliferation but vital for inducing Th2 differentiation.

### **TSLP promotes expression of OX40L and production of Th2 chemokines by DCs**

We next sought to elucidate the mechanism underlying Th2 induction of LCs via TSLP-TSLPR signaling. Modulation of costimulatory molecule expression was among the candidates, as it has been demonstrated that the interaction between membrane OX40L on DCs and OX40 on naive T cells results in the induction of IL-4 production by T cells in humans (26), and that treating mice with OX40L-blocking antibodies substantially inhibited Th2 immune responses induced by TSLP in the lung and skin (29).

Therefore, it is important to evaluate the expression levels of costimulatory molecules on LCs in OVA-sensitized skin by means of flow cytometry. TSLPR<sup>-/-</sup> (BALB/c



background) and WT control BALB/c mice were sensitized with OVA percutaneously. Seventy-two hours later, epidermal cell suspensions were prepared and stained with anti-OX40L, CD80, and CD40 antibodies. The MFI of OX40L expressed by LCs from OVA-sensitized TSLPR<sup>-/-</sup> mice was significantly lower than that in WT control mice. On the other hand, expression levels of CD40 and CD80 on LCs were comparable between WT control and TSLPR<sup>-/-</sup> mice (Fig. S5A).

It is known that serum levels of CCL17 and CCL22 correlate with the severity of AD (5). We incubated bone marrow-derived DCs (BMDCs) from BALB/c mice with recombinant mouse TSLP, and found that TSLP induced DCs to express CCL17 and CCL22 mRNA (Fig. S5B), while the expression level of the Th1 chemokine CXCL10 was suppressed by TSLP (Fig. S5C). These results suggest that TSLP instructs cutaneous DCs to create a Th2-permissive microenvironment by modulating the expression levels of chemokines.

## DISCUSSION

In this study, we have demonstrated that LCs are the essential cutaneous DC subset in the induction of IgE upon epicutaneous sensitization with protein antigens. We also found that TSLPR expression on LCs is enhanced upon protein antigen exposure to the skin and that LCs plays an important role in this process through TSLP-TSLPR signaling. In addition, we have demonstrated that TSLP stimulation causes LCs to express OX40L as shown previously in human studies, and that BMDCs induce Th2 chemokines while suppressing Th1 chemokines, which may shift the immune environment to a Th2 milieu.

While a previous report suggests the significance of LCs in the induction of Th2

immune responses in humans (30), other studies have reported that dermal DCs, but not LCs, are essential for murine epicutaneous sensitization with hapten, as in contact hypersensitivity that is mediated by Th1 (19, 21, 31, 32). In our study, we have demonstrated that LCs seem to be indispensable for Th2 induction upon protein antigen sensitization. Therefore, dermal DCs and LCs may play an important role for Th1 and Th2 type immune reactions, respectively.

While protein antigens remain above the TJ, haptens can readily penetrate into the dermis as shown in Fig. S2; therefore, LCs may not be essential for sensitization to hapten as reported previously (21, 24). Upon protein antigen exposure to the skin, on the other hand, LCs are vital in the induction of antigen-specific IgE. It is still an intriguing issue how clinical and histological scores, T cell proliferation, and IL-4 production were only partially suppressed by deficiency of LCs. These results suggest that other antigen presenting cells, such as dermal DCs, might be able to induce antigen-specific T cell proliferation in the draining LNs and that other Th2 inducing cells, such as basophils and mast cells, may contribute to produce IL-4 in the draining LNs. These issues need to be answered in the future.

It has been reported that basophils induce Th2 through TSLPR and that LCs are essential in the vitamin D3 induced-skin lesions through TSLP signaling (13, 15). In this study, we have demonstrated the significance of TSLP-TSLPR signaling on LCs under epicutaneous sensitization with protein antigens, which is clinically relevant to AD. Our findings will lead to the understanding of underlying mechanism and developing new therapeutic targets for inflammatory skin diseases.

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**FIGURE LEGENDS****FIG 1. LCs are crucial for epicutaneous sensitization with OVA.**

(A) Total clinical severity scores (left panel) and total histology scores (right panel) of LC-non-depleted (LC+) and LC-depleted (LC-) mice (n = 5 mice per group). (B) Serum OVA-specific antibodies as determined by ELISA. Optical density value for IgE, IgG1, and IgG2a levels were measured at a wavelength of 450 nm. \*,  $P < 0.05$

**FIG 2. LCs are critical for antigen-specific T cell proliferation.**

Mice in the presence or absence of LCs (LC+ and LC-, respectively) were treated with OVA and transplanted with CFSE-labeled OT-II T cells (n = 5 mice per group). Skin-draining LNs were analyzed for OVA-specific T cell proliferation (A and B) and mRNA expression levels for IFN- $\gamma$  and IL-4 (C). Boxes in (A) demarcate divided cells (left) and undivided cells (right) \*,  $P < 0.05$ . N.D., not detected.

**FIG 3. LCs are essential for IgE production.**

(A) The serum IgE levels and (B) IgE expression levels on peritoneal mast cells (indicated by MFI) of WT and Langerin-DTA mice on FVB (left panel) and B6 (right panel) backgrounds. Mast cells were also pre-incubated with IgE (labeled with pre IgE) *in vitro* before measurement of IgE expression (B). Each symbol represents an individual animal. \*,  $P < 0.05$ .

**FIG 4. TSLPR on LCs is a responsible target of TSLP upon epicutaneous OVA sensitization.**

Epidermal cell suspensions from B6 (WT) mice with (sensitized) or without (non-sensitized) epidermal application of OVA were stained with TSLPR antibody. TSLPR expressions of MHC class II<sup>+</sup> CD11c<sup>+</sup> LCs was analyzed by flow cytometry (left, histogram; right, average  $\pm$  SD of MFI). n = 3 per group. \*,  $P < 0.05$ .

**FIG 5. An essential target of TSLP for IgE induction is TSLPR on LCs.**

(A) B6 (Ly45.2) mice were irradiated and transplanted with BM cells from B6 (Ly45.1) mice. The epidermis and dermis of BMC mice separated, and single-cell suspensions were stained and analyzed by flow cytometry. (B) Total clinical severity scores (left panel) and histology scores (right panel) of TSLPR<sup>+/+</sup> BMC, LC-TSLPR<sup>-/-</sup> BMC, and TSLPR<sup>-/-</sup> BMC mice (n=5 mice per group). (C) Serum OVA-specific antibodies as determined by ELISA. Optical density value for IgE, IgG1, and IgG2a levels were measured at a wavelength of 450 nm. \*,  $P < 0.05$ .

**FIG 6. TSLPR on LCs are vital for Th2 induction**

TSLPR<sup>+/+</sup> BMC, LC-TSLPR<sup>-/-</sup> BMC, and TSLPR<sup>-/-</sup> BMC mice were treated with OVA or saline and transplanted with CFSE-labeled OT-II T cells. Skin-draining LNs were analyzed for OVA-specific T cell proliferation (A and B) and cytokine mRNA expression levels for IFN- $\gamma$  and IL-4 (C). Boxes in (A) demarcate divided cells (left) and undivided cells (right). n = 5 mice per group. \* $P < 0.05$ . N.D., not detected.

**Figure 1**

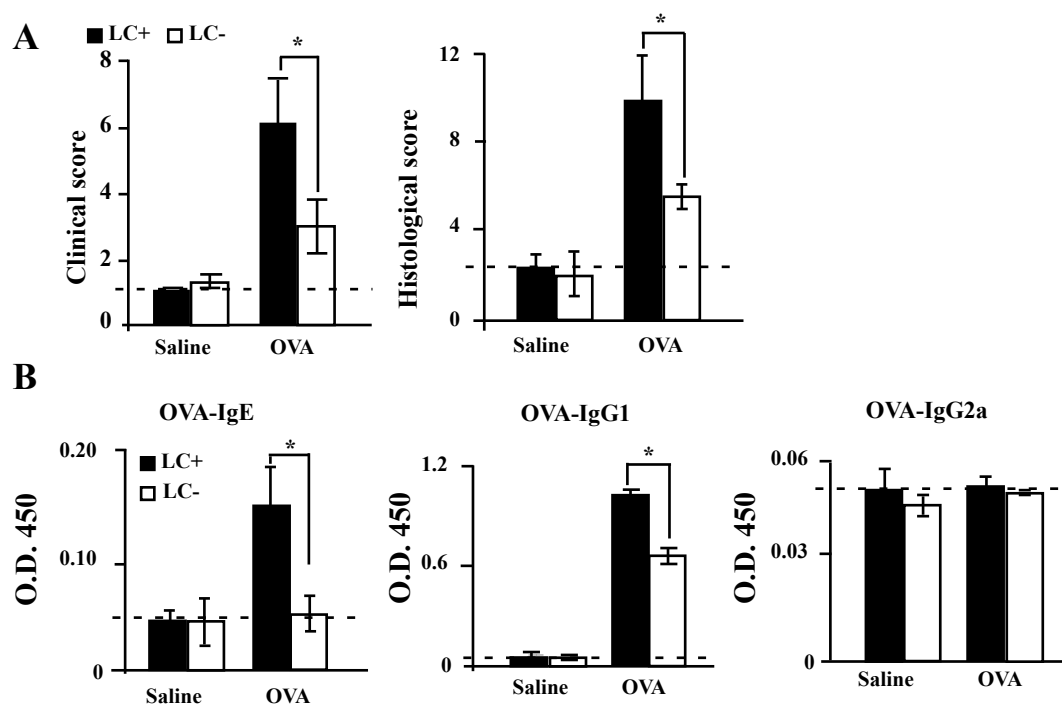




Figure 2

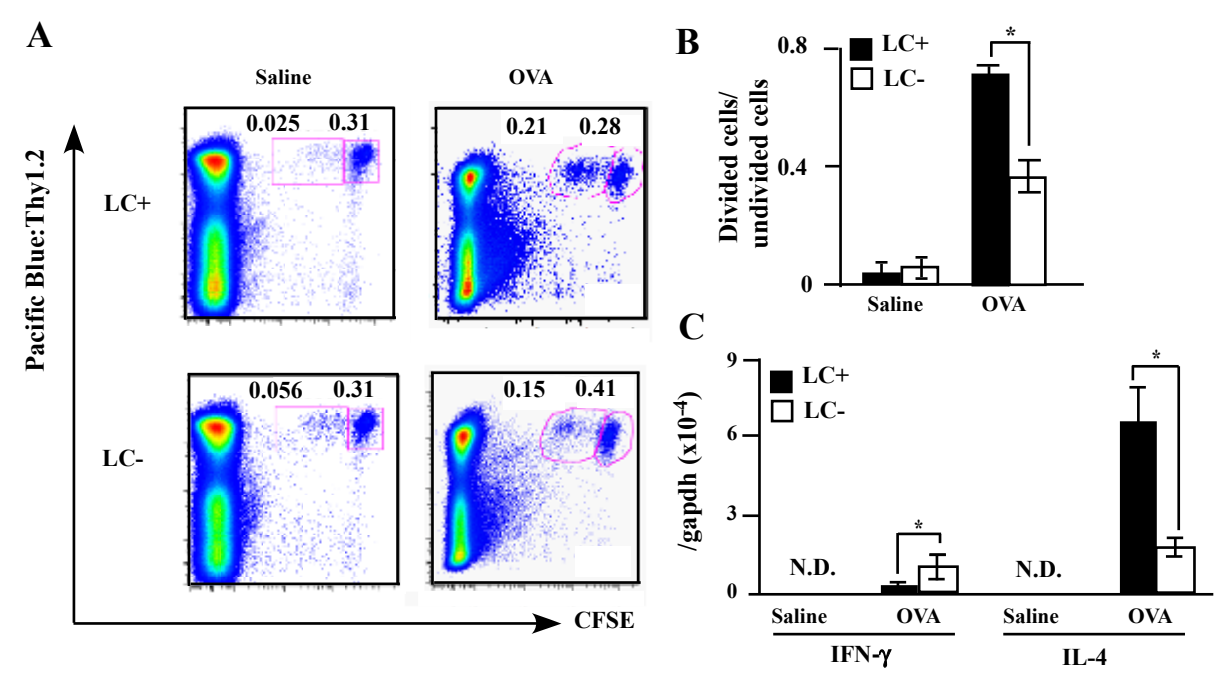
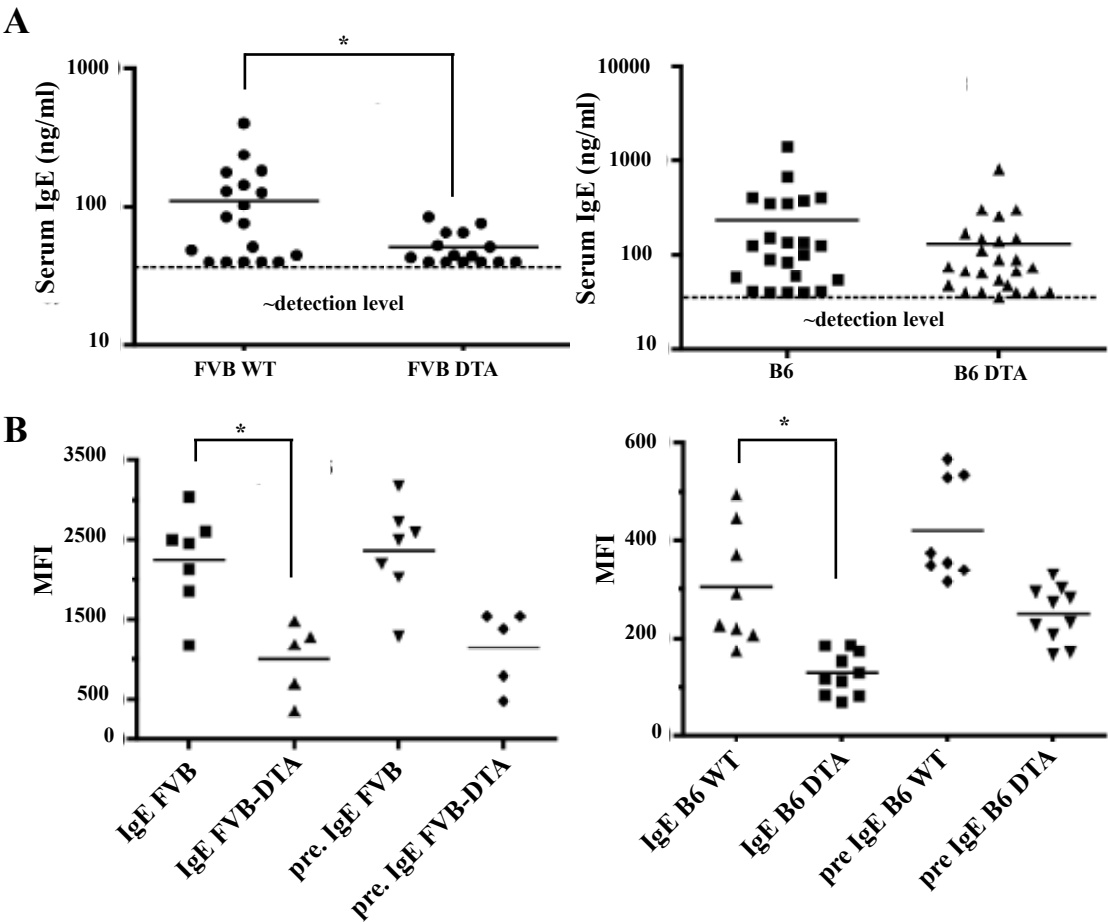


Figure 3



**Figure 4**

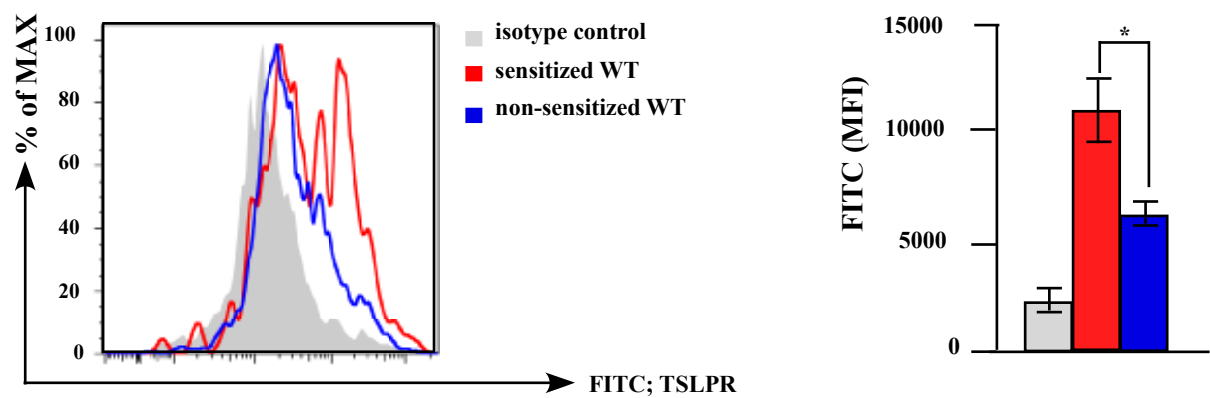


Figure 5

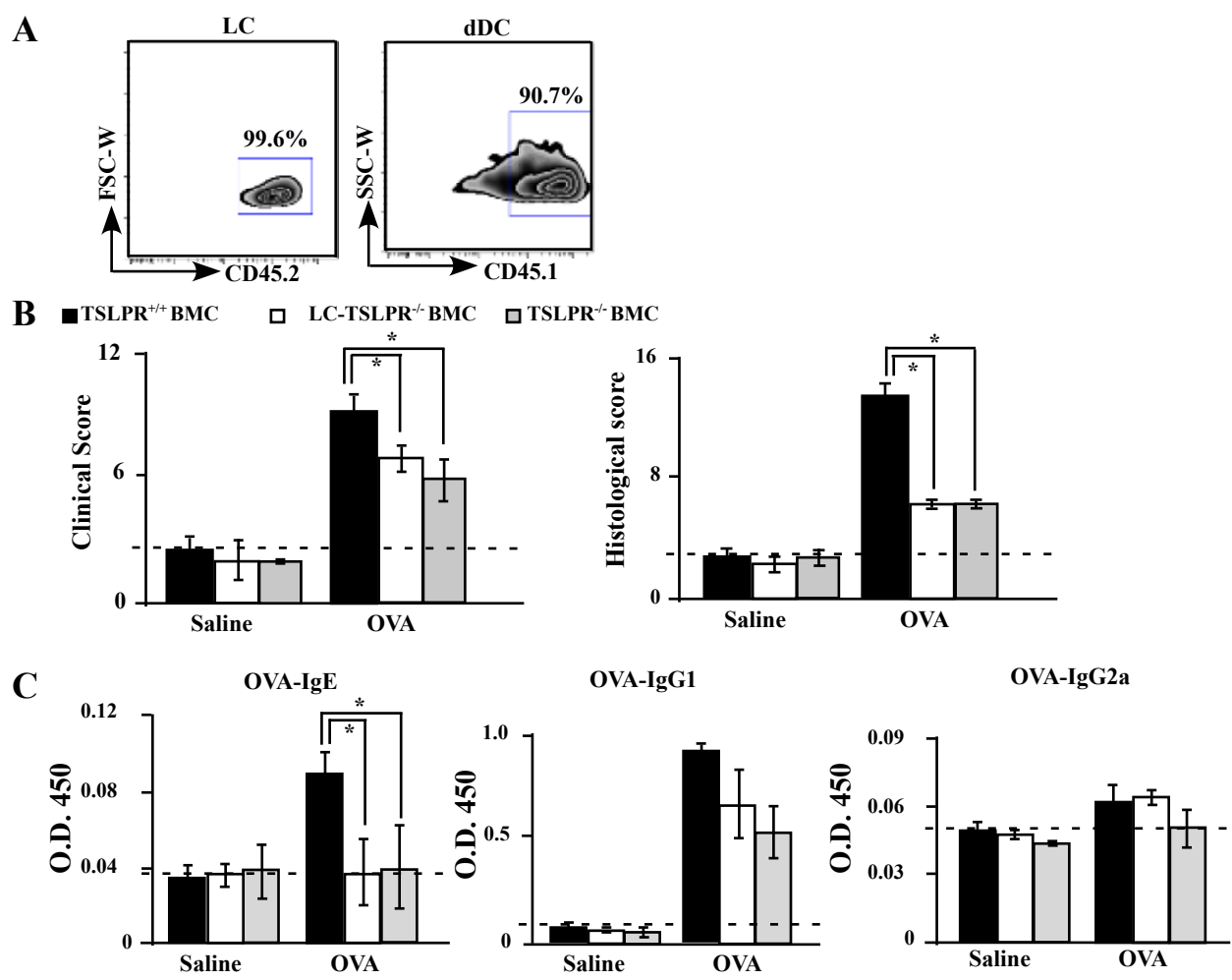
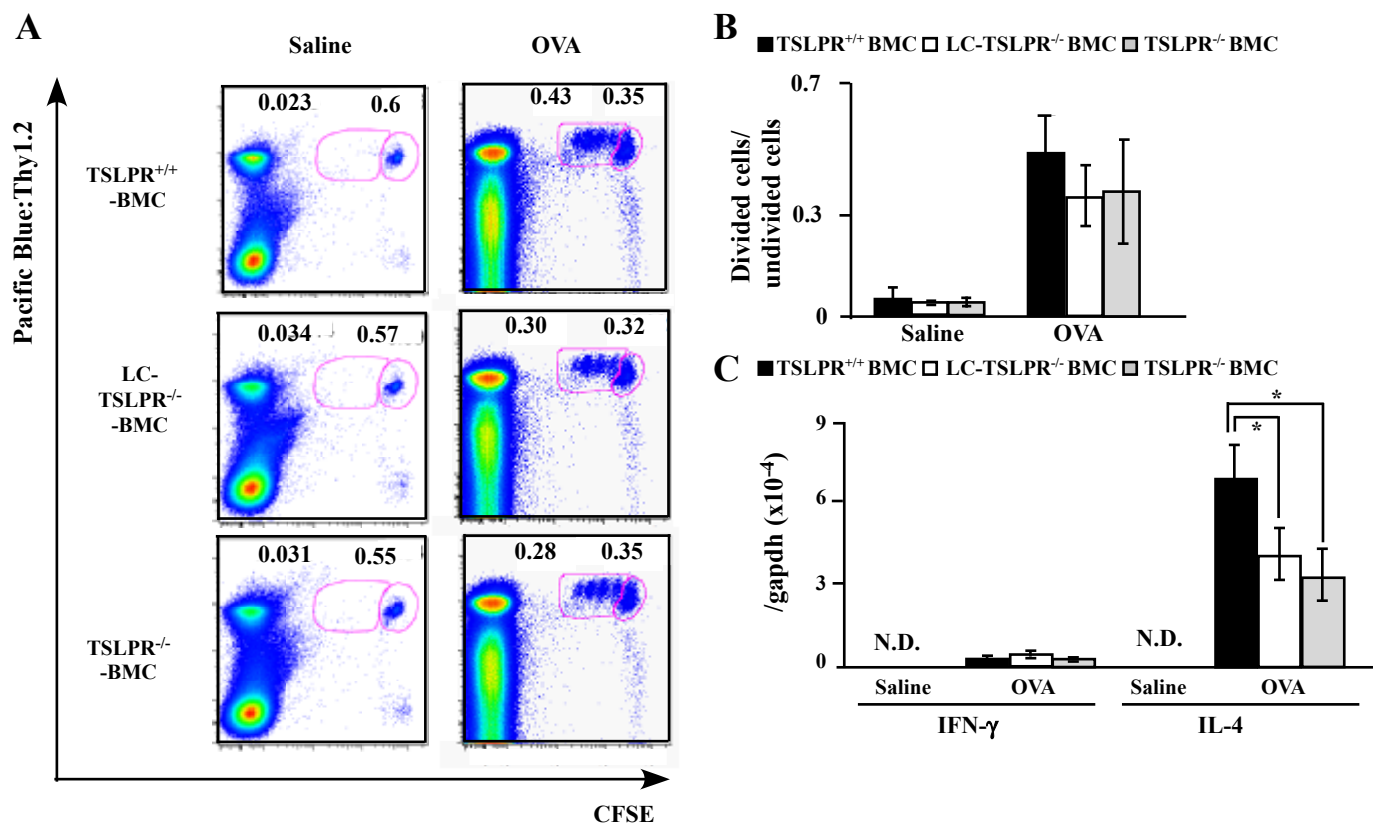


Figure 6



## Online Repository

### Langerhans cells are critical in epicutaneous sensitization with protein antigen via TSLP receptor signaling

Saeko Nakajima, MD, Botond Igyarto, PhD, Tetsuya Honda, MD, PhD, Gyohei Egawa, MD, PhD, Atsushi Otsuka, MD, PhD, Mariko Hara-Chikuma, PhD, Norihiko Watanabe, MD, PhD, Steven F Ziegler, PhD, Michio Tomura, PhD, Kayo Inaba, PhD, Yoshiki Miyachi, MD, PhD, Daniel H Kaplan, MD, PhD, and Kenji Kabashima, MD, PhD

## SUPPLEMENTAL MATERIALS AND METHODS

### Cell culture, reagents, antibodies, and flow cytometry

The complete RPMI (cRPMI) culture medium consisting of RPMI 1640 (Invitrogen, Carlsbad, CA, USA) containing 10% heat-inactivated fetal calf serum,  $5 \times 10^{-5}$  M 2-mercaptoethanol, 2 mM L-glutamine, 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 units/mL penicillin, and 100  $\mu$ g/mL streptomycin, was used, unless otherwise indicated.

For bone marrow-derived DC (BMDC) culture,  $5 \times 10^6$  BM cells generated from WT and TSLPR<sup>-/-</sup> mice were cultured in 10 mL of cRPMI supplemented with 3 ng/mL recombinant murine granulocyte-macrophage colony-stimulating factor (PeproTech,

Rocky Hill, NJ, USA) for 5 to 7 days. Then,  $5 \times 10^5$  cells were seeded in a 24-well culture dish (Nunc, Rochester, NY, USA) in 500  $\mu$ l cRPMI and stimulated with 100 ng/ml recombinant mouse TSLP (R&D Systems, Minneapolis, MN, USA) for six hours.

For epidermal cell suspensions, dorsal skin sheets were floated on dispase II (GODO SHUSEI CO., LTD, Aomori, Japan) diluted to 5 mg/ml in cRPMI for one hour at 37°C and 5% CO<sub>2</sub>. The epidermis was separated from the dermis with forceps in RPMI medium supplemented with 2% fetal calf serum. The isolated epidermis was cut finely with scissors and floated in 0.25% trypsin-EDTA for 10 min at 37°C and 5% CO<sub>2</sub>, and filtered through a 40- $\mu$ m cell strainer (BD Bioscience, San Diego, CA, USA).

We purchased OVA from Sigma-Aldrich, and carboxyfluorescein succinimidyl ester (CFSE) was acquired from Invitrogen. Fluorochrome-conjugated antibodies to CD4, CD11c, CD90.1, MHC class II, OX40L, CD40, and CD80 were purchased from eBioscience Inc. (San Diego, CA, USA). Anti-mouse TSLPR and isotype control were purchased from R&D systems. Cells were analyzed using the FACS LSR Fortessa flow cytometric system (BD Bioscience) and FlowJo software (Tree Star, Ashland, OR, USA).

## **Histology, and allergen penetration in the skin**

The clinical severity of skin lesions was scored according to the macroscopic diagnostic criteria that were used for the NC/Nga mouse (4). In brief, the total clinical score for skin lesions was designated as the sum of individual scores, graded as 0 (none), 1 (mild), 2 (moderate), and 3 (severe), for the symptoms of pruritus, erythema, edema, erosion, and scaling. Pruritus was observed clinically for more than two minutes.

For histological examination, tissues were fixed with 10% formalin in phosphate buffer saline, and then embedded in paraffin. Sections with a thickness of 5  $\mu$ m were prepared and subjected to staining with hematoxylin and eosin. The histological findings were evaluated as reported previously (5).

For immunohistochemical analysis, OVA-sensitized skin samples were directly frozen at -80°C in Tissue-Tek O.C.T. (Sakura Finetek, Tokyo, Japan). Skin cryosections were fixed with 4% paraformaldehyde (Nacalai Tesque) and permeabilized with 0.1% Triton-X (Sigma-Aldrich) in PBS for 10 minutes at room temperature. Next, slides were incubated with anti-claudin-1 polyclonal antibody (Abcam, Cambridge, UK). Immunodetection was performed using Alexa Fluor 594-coupled secondary antibody (Invitrogen). The slides were mounted in ProLong Gold Antifade reagent (Invitrogen), and fluorescence images were obtained using a BIOREVO BZ-9000 system (Keyence, Osaka, Japan).

For assessing penetration of allergen, mice were percutaneously sensitized with 100  $\mu$ g of fluorescein isothiocyanat (FITC)-conjugated OVA (Molecular Probes, Inc., Eugene, OR, USA) diluted in 100  $\mu$ l normal saline onto the shaved and tape-stripped



back skin. Seventy-two hours later, immunohistochemical analysis of the skin to assess allergen penetration was performed. Similarly, 100  $\mu$ l of 1% FITC (Sigma-Aldrich) in acetone/dibutyl phthalate (1/1) was applied to shaved dorsal skin of B6 mice; 72 hours later, immunohistochemical analysis was performed to assess hapten penetration into the skin.

#### **ELISA for OVA-specific serum IgE**

Total serum IgE levels were measured using a Bio-Rad (Hercules, CA, USA) Luminex kit according to the manufacturer's instructions. To measure OVA-specific IgE/IgG1/IgG2a levels, the appropriate mouse IgE/IgG1/IgG2a ELISA kit (Bethyl Laboratories, Montgomery, TX, USA) was used with slight modifications. Specifically, plates were coated and incubated with 10  $\mu$ g/ml OVA diluted with coating buffer for 2 hours. After a blocking period of 30 minutes, 100  $\mu$ l of 5 x diluted serum was added into each well and incubated for 2 hours. Anti-mouse IgE/IgG1/IgG2a-horseradish peroxidase conjugate (1:15,000; 100  $\mu$ L) was used to conjugate the antigen-antibody complex for 60 minutes at room temperature; from this point on the ELISA kit was used according to the manufacturer's instructions. Absorbance was measured at 450 nm. The difference between the sample absorbance and the mean of negative control absorbance was taken as the result.

To measure IgE levels on peritoneal mast cells, the peritoneal cavity was rinsed with 10 ml of ice-cold, sterile PBS. The collected cell suspension was incubated with

Fc-block antibody (BD Biosciences; 2-4G2), washed and split in half. Half of the cells were kept untreated while the other half were incubated with 10 µg/ml of anti-DNP-IgE (mouse monoclonal IgE, Sigma-Aldrich) for 40 minutes on ice. After being washed with staining media, the cells were further incubated with an anti-c-kit and anti-mouse IgE and analyzed using a flow cytometer.

### **Quantitative reverse-transcribed PCR analysis**

Total RNAs were isolated with RNeasy kits and digested with DNase I (Qiagen, Hilden, Germany). cDNA was reverse transcribed from total RNA samples using the Prime Script RT reagent kit (Takara Bio, Otsu, Japan). Quantitative RT-PCR was performed by monitoring the synthesis of double-stranded DNA during the various PCR cycles, using SYBR Green I (Roche, Basel, Switzerland) and the Light Cycler real time PCR apparatus (Roche) according to the manufacturer's instructions. All primers were obtained from Greiner Japan (Tokyo, Japan). The primer sequences were IFN- $\gamma$ , 5'-GAA CTG GCA AAA GGA TGG TGA -3' (forward), 5'- TGT GGG TTG TTG ACC TCA AAC -3' (reverse); IL-4, 5'- GGT CTC AAC CCC CAG CTA GT -3' (forward), 5'- GCC GAT GAT CTC TCT CAA GTG AT -3' (reverse); CCL17, 5'- CAG GGA TGC CAT CGT GTT TCT -3' (forward), 5'- GGT CAC AGG CCG TTT TAT GTT -3' (reverse); CCL22, 5'- TCT TGC TGT GGC AAT TCA GA -3' (forward), 5'- GAG GGT GAC GGA TGT AGT CC -3' (reverse); CXCL10, 5'- CCA AGT GCT GCC GTC ATT TTC-3' (forward), 5'- GGC TCG CAG GGA TGA TTT CAA-3' (reverse). The cycling

conditions were as follows: initial enzyme activation at 95°C for 10 min, followed by 40 cycles at 95°C for 10 seconds, and 60°C for 20 seconds. All cycling reactions were performed in the presence of 3.5 mM MgCl<sub>2</sub>. Gene-specific fluorescence was measured at 60°C. For each sample, triplicate test reactions and a control reaction lacking reverse transcriptase were analyzed for expression of the genes, and results were normalized to those of the 'housekeeping' glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

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E3. Honda T, Nakajima S, Egawa G, Ogasawara K, Malissen B, Miyachi Y, et al. Compensatory role of Langerhans cells and langerin-positive dermal dendritic cells in the sensitization phase of murine contact hypersensitivity. *J Allergy Clin Immunol*. 2010 May;125(5):1154-6 e2.

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## SUPPLEMENTAL FIGURE LEGENDS

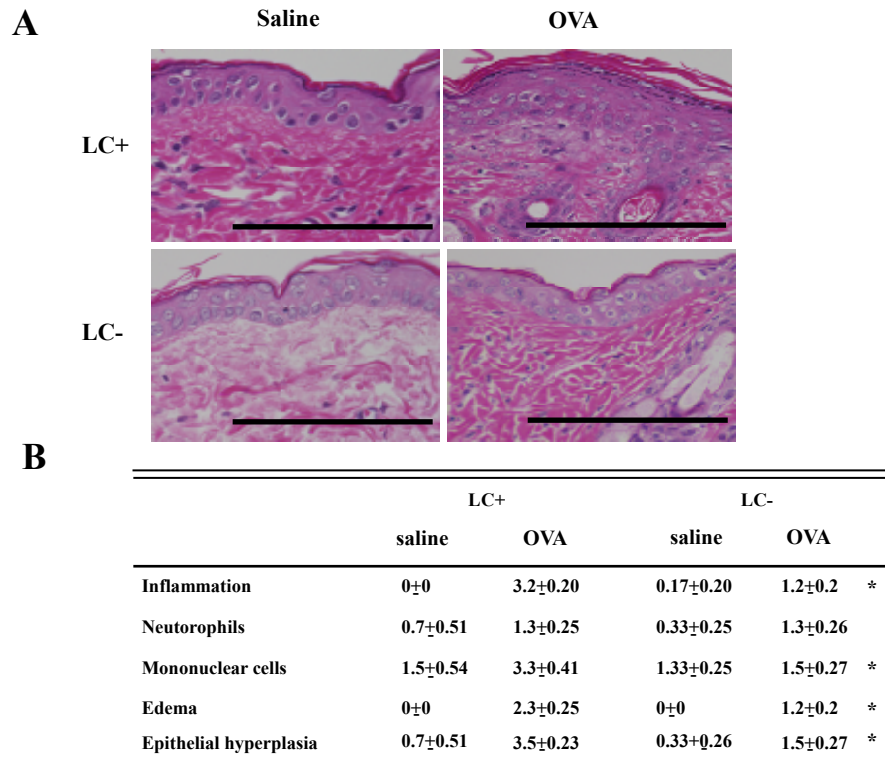
**Figure S1.** (A) H&E staining of the back skin of LC-non-depleted or LC depleted mice after OVA application for three times (H&E, original magnification x400). Scale bar, 100  $\mu$ m. (B) The histological findings were scored by inflammation, neutrophil infiltration, mononuclear cell infiltration, edema and epithelial hyperplasia. Data are presented as means  $\pm$  SD (n = 5).

**Figure S2. Impaired penetration of protein antigen into the dermis.** B6 mice were patched with FITC-conjugated OVA on the back skin; 72 hours later, patched skin area was analyzed by immunohistochemistry. FITC-conjugated OVA (green) retained above the TJ was indicated by staining with anti-claudin-1 antibody (red) (left panel). FITC (green) readily penetrated into the dermis (right panel). Blue staining (DAPI) indicates nuclei. Dashed white lines represent the border between dermis and epidermis. Scale bars, 100  $\mu$ m.

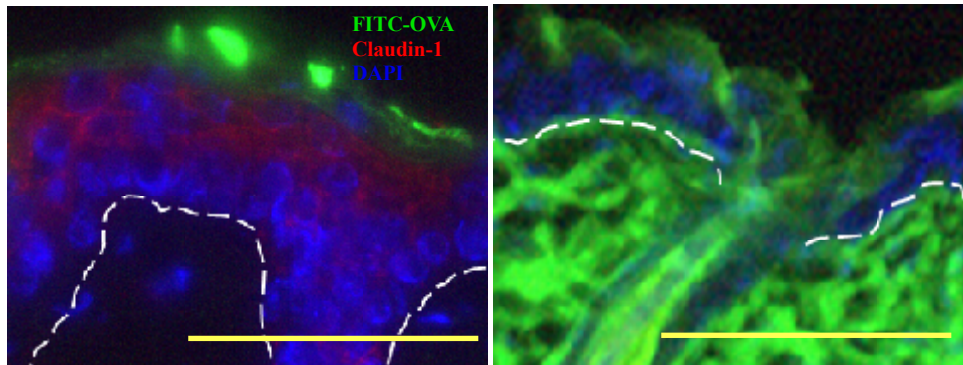
**Figure S3. Establishment of bone marrow chimeric mice deficient in TSLPR on LC (LC-TSLPR<sup>-/-</sup> BMC).** B6 mice and B6-background TSLPR<sup>-/-</sup> mice were irradiated (IR) and transplanted with BM cells (BMT) from B6 mice or TSLPR<sup>-/-</sup> mice. Since LCs were radioresistant, when TSLPR<sup>-/-</sup> mice were reconstituted with BM cells from B6 mice, they were deficient in TSLPR on LCs (LC-TSLPR<sup>-/-</sup> BMC mice).

**Figure S4.** (A) H&E staining of the back skin of TSLPR<sup>+/+</sup>, LC-TSLPR<sup>-/-</sup>, and TSLPR<sup>-/-</sup> mice after OVA application for three times (H&E, original magnification x400). Scale bar, 100  $\mu$ m. (B) The histological findings were scored by inflammation, neutrophil infiltration, mononuclear cell infiltration, edema and epithelial hyperplasia. Data are presented as means  $\pm$  SD (n = 5).

154 **Figure S5. TSLP promotes expression of OX40L and production of Th2**  
155 **chemokines by DCs.** (A) The expression levels of OX40L, CD80 and CD40 of LCs  
156 with (sen+) or without (sen-) OVA sensitization in TSLPR<sup>+/+</sup> and TSLPR<sup>-/-</sup> mice (n = 5  
157 mice per group). Cells were pregated on MHC class II<sup>+</sup> CD11c<sup>+</sup> LC cells. (B, C)  
158 BMDCs were incubated with or without recombinant TSLP (rTSLP), and mRNA levels  
159 of chemokines, CCL17, CCL22, and CXCL10, were measured by real-time qPCR. \*P  
160 <0 .05.



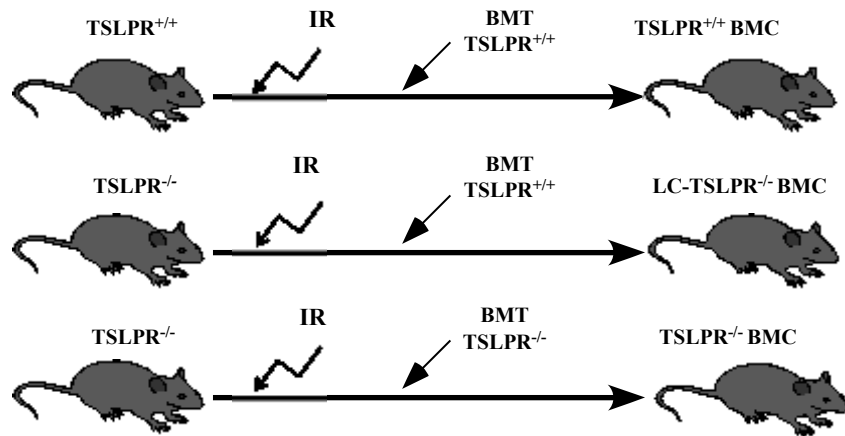
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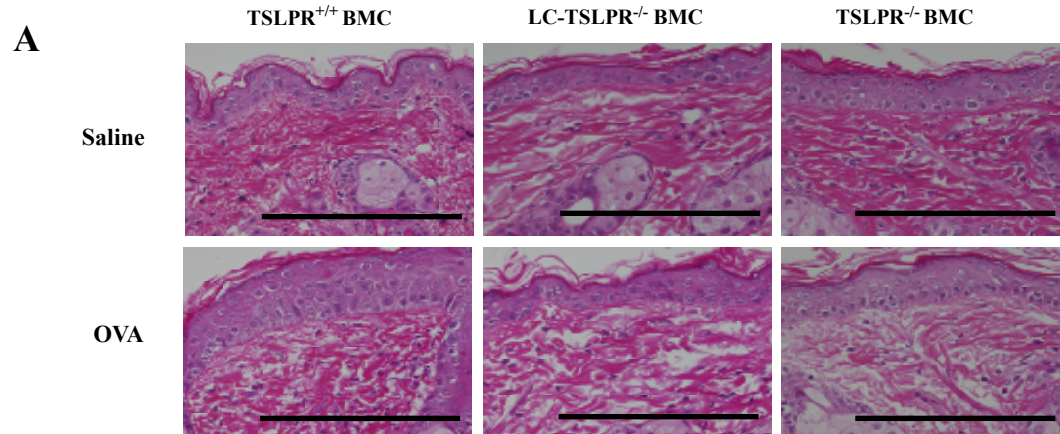
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B6 mice and B6-background TSLPR<sup>-/-</sup> mice were irradiated (IR) and transplanted with BM cells (BMT) from B6 mice or TSLPR<sup>-/-</sup> mice. Since LCs were radioresistant, when TSLPR<sup>-/-</sup> mice were reconstituted with BM cells from B6 mice, they were deficient in TSLPR on LCs (LC-TSLPR<sup>-/-</sup> BMC mice).



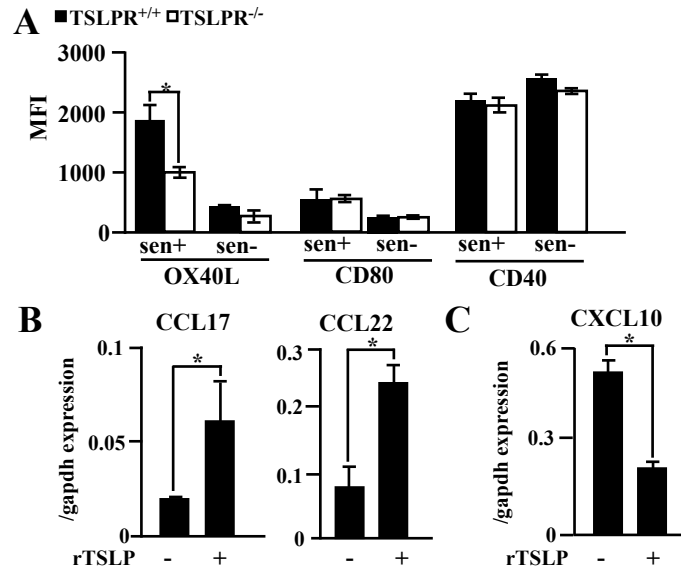


**B**

	TSLPR <sup>+/+</sup> BMC		LC-TSLPR <sup>-/-</sup> BMC			TSLPR <sup>-/-</sup> BMC	
	saline	OVA	saline	OVA		saline	OVA
Inflammation	0±0	3.2±0.2	0.4±0.24	1.2±0.2	*	0.4±0.24	1.4±0.24
Neutrophils	0.8±0.2	1.8±0.37	0.4±0.24	1.2±0.2	*	0.6±0.24	1.4±0.24
Mononuclear cells	1.6±0.24	3.2±0.37	1.2±0.2	1.6±0.24	*	1.0±0.32	1.2±0.2
Edema	0.2±0.2	2.4±0.24	0±0	1.4±0.24	*	0.2±0.2	1.4±0.24
Epithelial hyperplasia	0.8±0.2	3.6±0.24	0.4±0.24	1.2±0.2	*	0.6±0.24	1.2±0.24

**Figure S4.** (A) H&E staining of the back skin of TSLPR<sup>+/+</sup>, LC-TSLPR<sup>-/-</sup>, and TSLPR<sup>-/-</sup> mice after OVA application for three times (H&E, original magnification x400). Scale bar, 100  $\mu$ m.

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**Figure S5. TSLP promotes expression of OX40L and production of Th2 chemokines by DCs.**

(A) The expression levels of OX40L, CD80 and CD40 of LCs with (sen+) or without (sen-) OVA sensitization in TSLPR<sup>+/+</sup> and TSLPR<sup>-/-</sup> mice (n = 5 mice per group). Cells were pregated on MHC class II<sup>+</sup> CD11c<sup>+</sup> LC cells. (B, C) BMDCs were incubated with or without recombinant TSLP (rTSLP), and mRNA levels of chemokines, CCL17, CCL22, and CXCL10, were measured by real-time qPCR. \*P < 0.05.