

TSLP Produced by Keratinocytes Promotes Allergen Sensitization through Skin and Thereby Triggers Atopic March in Mice

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Atopic dermatitis often precedes the development of asthma, a phenomenon known as “atopic march”. An important role of allergen sensitization developed through barrier-defective skin has been recognized in the onset of atopic march; however, the underlying mechanism remains poorly understood. In this study, we use an experimental atopic march mouse model, in which the sensitization to allergen is achieved through barrier-impaired skin, followed by allergen challenge in the airway. By using thymic stromal lymphopoietin (TSLP)^{iep-/-} mice in which the cytokine TSLP is selectively and inducibly ablated in epidermal keratinocytes, we demonstrate that keratinocytic TSLP, the expression of which is induced by skin barrier impairment, is essential for generating skin allergic inflammation and allergen-induced T helper type 2 response, for developing sensitization to allergen, and for triggering a subsequent allergic asthma. Furthermore, using TSLP^{over} mice in which overexpression of keratinocytic TSLP is induced by skin topical application of MC903 (a vitamin D3 analog) in a dose-dependent manner, we show that keratinocytic TSLP levels are correlated with skin sensitization strength and asthma severity. Taken together, our study uncovers a crucial role of keratinocytic TSLP in the “atopic march” by promoting allergen sensitization occurring in barrier-impaired skin, which ultimately leads to allergic asthma.

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INTRODUCTION

Atopic dermatitis (AD, eczema) frequently starts in early infancy, and is characterized by skin inflammation and impaired skin barrier function (Bieber, 2008; Boguniewicz and Leung, 2011). More than 50% of AD patients with moderate to severe AD develop asthma and/or allergic rhinitis at a later stage, and the severity of AD appears to influence the course of respiratory allergy. Although this phenomenon is well known as the “atopic march” (Spergel and Paller, 2003; Hahn and Bacharier, 2005; Boguniewicz and Leung, 2011), how asthma progresses from AD still remains obscure, and an effective prevention of the atopic march is still lacking.

It has been recognized that, often during the course of AD, a majority of patients develop sensitization to common food and/or environmental allergens (Bieber, 2008; Cork *et al.*, 2009; Boguniewicz and Leung, 2011), and moreover that the severity of AD correlates with the degree of sensitization and with the risk of developing asthma (Schafer *et al.*, 1999; Oettgen and Geha, 2001). Indeed, allergen sensitization developed through barrier-defective skin during AD could be a critical event leading to subsequent allergic asthma (Bieber, 2008; Cork *et al.*, 2009; Benedetto *et al.*, 2012); however, the mechanism underlying skin sensitization is still poorly understood.

Thymic stromal lymphopoietin (TSLP) has emerged as a key epithelium-derived cytokine in the AD pathogenesis (Liu, 2006; Ziegler and Artis, 2010). Increased expression of TSLP has been revealed in skin keratinocytes from AD patients (Soumelis *et al.*, 2002), and elevated serum TSLP level was reported in AD children (Lee *et al.*, 2010a). Our previous study (Li *et al.*, 2005) and that of others (Yoo *et al.*, 2005) have demonstrated that transgenic mice overexpressing TSLP in keratinocytes developed a spontaneous AD-like dermatitis. We have further established an experimental TSLP^{over} protocol through which a skin topical application of MC903 (calcipotriol; a low-calcemic analog of vitamin D3) induces keratinocytic TSLP expression and triggers an AD-like syndrome (Li *et al.*, 2006, 2009). More recently, we reported that MC903 skin treatment aggravated experimental allergic asthma induced by intraperitoneal (i.p.) sensitization to ovalbumin

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Abbreviations: AD, atopic dermatitis; CT, wild-type control; DC, dendritic cell; e.c., epicutaneous; i.n., intranasal; i.p., intraperitoneal; LN, lymph node; MT, mutant; NT, nontreated; OVA, ovalbumin; PBS, phosphate-buffered saline; Tam, tamoxifen; Th2, T helper type 2; TSLP, thymic stromal lymphopoietin

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(OVA) and airway OVA challenge, which was mediated by TSLP produced in keratinocytes (Zhang *et al.*, 2009). Interestingly, we also found that overexpressed keratinocytic TSLP during the OVA sensitization phase was able to aggravate OVA-induced asthma (Zhang *et al.*, 2009). This study led us to posit that TSLP overproduced in the skin of AD patients could be a risk factor for the development of allergic airway inflammation. A similar suggestion was made by Demehri *et al.* (2009). However, in these previous experiments, allergen sensitization was “artificially” achieved by i.p. injection of OVA complexed with exogenous adjuvant (Takeda and Gelfand, 2009; Holgate, 2011), which does not mimic the sensitization occurring in epithelium sites such as skin (Bieber, 2008). In this study, by using an experimental “atopic march” mouse model, in which allergen sensitization is achieved through barrier-impaired skin, followed by airway challenge, our study demonstrates that keratinocyte-produced TSLP has a crucial role in promoting allergen sensitization occurring in skin, which ultimately triggers the “atopic march” leading to allergic asthma.

RESULTS

An experimental “atopic march” model induced by OVA epicutaneous (e.c.) sensitization through barrier-impaired skin and intranasal (i.n.) challenge

We established a mouse model representing the atopic march features (outlined in Figure 1a), which is initiated by an e.c.

sensitization phase by OVA treatment on barrier-impaired skin through tape stripping (mimicking scratching in AD patients) (Strid *et al.*, 2004; Cork *et al.*, 2009; Jin *et al.*, 2009), and followed by an OVA i.n. challenge phase. In this model, e.c. OVA-treated wild-type Balb/c mice exhibited an AD-like skin allergic inflammation (see Figure 2), and developed an allergen sensitization evidenced by systemic immune responses, including the production of OVA-specific IgS (indicating an allergen-specific B-cell response), and cytokine production of splenocyte cells upon *in vitro* OVA stimulation (indicating an allergen-specific T-cell response; see Figure 3). Upon i.n. challenge, these e.c. OVA-sensitized mice exhibited an asthmatic phenotype (see Figure 4).

Barrier impairment induces TSLP production in keratinocytes

In skin extracts of nontreated (NT) wild-type Balb/c mice, TSLP protein levels were undetectable by ELISA. In contrast, upon phosphate-buffered saline (PBS) or OVA treatment on barrier-impaired skin (e.c. PBS or e.c. OVA), TSLP protein levels were upregulated after 3 hours, and further increased 9, 24, and 48 hours later (Figure 1b). These levels were not significantly different between e.c. OVA and e.c. PBS treatment, suggesting that TSLP induction was mainly due to skin barrier impairment but not due to OVA application.

To examine whether epidermal keratinocytes were the source of stripping-induced TSLP, we used TSLP^{iep-/-} mutant (MT) mice, in which selective ablation of TSLP was induced

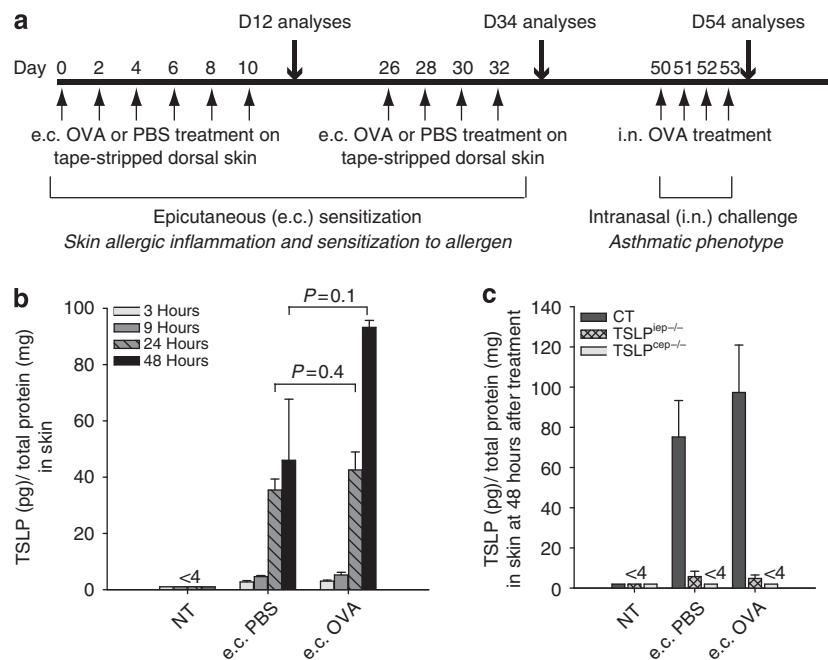


Figure 1. Thymic stromal lymphopoietin (TSLP) production is induced in keratinocytes by tape stripping during epicutaneous (e.c.) sensitization in an experimental mouse asthma model. (a) Experimental protocol. Dorsal skin of 10–12-week-old female mice was shaved and tape stripped (until TEWL (transepidermal water loss) value reached 15–20 gm⁻² per hour) and treated with ovalbumin (OVA) every other day (D) from D0 to D10. Similar e.c. treatment was repeated from D26 to D32. Nontreated (NT) mice or mice treated with phosphate-buffered saline (PBS) on tape-stripped skin (e.c. PBS) were used as controls. Three weeks later, mice were intranasally (i.n.) challenged with OVA for 4 consecutive days (D50–D53). (b) TSLP protein levels in the skin of wild-type Balb/c mice measured by ELISA at 3, 9, 24, and 48 hours after one e.c. PBS or e.c. OVA treatment. (c) TSLP protein levels measured by ELISA in the skin of wild-type control (CT), TSLP^{iep-/-} (inducible ablation of TSLP in epidermal keratinocytes), and TSLP^{cep-/-} (constitutive ablation of TSLP in epidermal keratinocytes) mice 48 hours after one e.c. treatment. Values are mean ± SEM (n ≥ 4 mice per group).

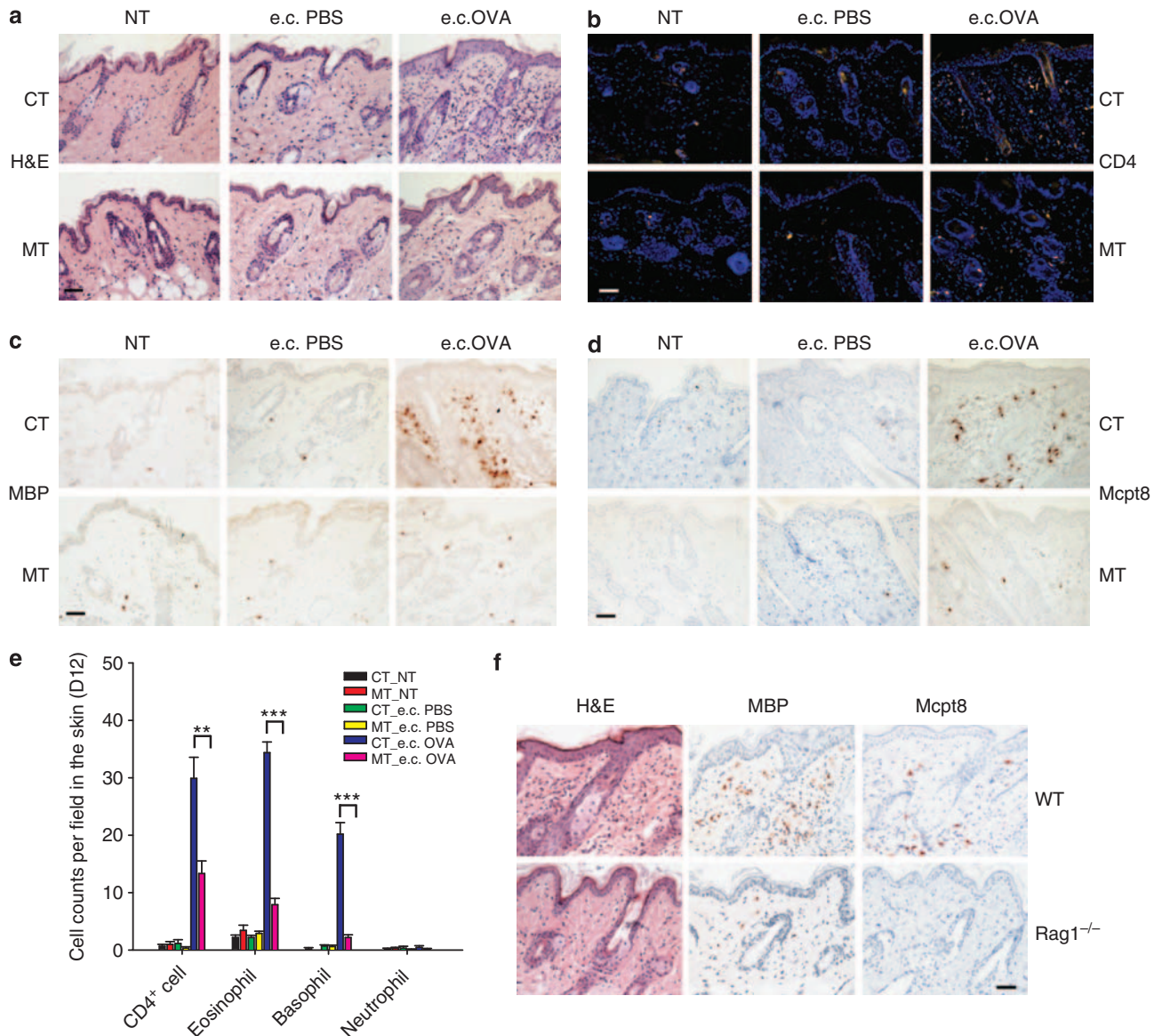


Figure 2. Reduced allergic inflammatory infiltrate in skin of epicutaneous (e.c.) ovalbumin (OVA)-treated thymic stromal lymphopoietin (TSLP)^{iep-/-} mutant (MT) mice. (a) Hematoxylin and eosin (H&E) staining of skin paraffin sections from day (D)12, showing reduced epidermal thickening and dermal infiltration in MT mice compared with wild-type control (CT) mice, upon e.c. OVA treatment. (b-d) Immunohistochemical (IHC) staining performed on skin sections at D12, (b) with antibodies against CD4 (yellow corresponds to CD4⁺ staining and blue for DAPI staining of nuclei), (c) MBP (specific for eosinophils; in dark red), or (d) Mcpt8 (specific for basophils; in dark red). (e) A summary of cell counts for skin-infiltrating immune cells at D12. Results were obtained by calculating the average number of positively stained cells per microscopic field (at $\times 200$ magnification; $n \geq 12$) ** $P < 0.01$; *** $P < 0.001$. Values are mean $\pm$ SEM. (f) Comparison of H&E and IHC staining with antibodies against MBP or Mcpt8 in e.c.OVA-treated wild-type (WT) and Rag1^{-/-} skin at D12. NT, nontreated; e.c. phosphate-buffered saline (PBS), treatment of PBS on tape-stripped skin; e.c. OVA, treatment of OVA on tape-stripped skin. Bar = 50 μ m.

at the adult stage in epidermal keratinocytes by tamoxifen (Tam) treatment of K14-CreER^{T2}/TSLP^{L2/L2} mice (Li *et al.*, 2009). The TSLP increase was nearly abolished in both e.c. PBS- and e.c. OVA-treated TSLP^{iep-/-} mice (Figure 1c). The residual induction of TSLP seen in e.c.-treated TSLP^{iep-/-} mice was most likely due to a $<100\%$ efficiency in Tam-induced TSLP gene ablation in keratinocytes, as the induction of TSLP was fully abolished in TSLP^{cep-/-} MT mice (K14-Cre/TSLP^{L2/L2}) (Li *et al.*, 2009) in which the TSLP gene was

“constitutively” ablated in keratinocytes at the embryonic stage (Figure 1c). Therefore, barrier impairment by tape stripping induces a production of TSLP in keratinocytes.

Ablation of TSLP in mouse skin keratinocytes results in a reduced allergic inflammation upon e.c. OVA treatment

Wild-type control (CT; Tam-injected TSLP^{L2/L2} mice) and TSLP^{iep-/-} MT mice were treated as described in Figure 1a. At D12, e.c. OVA-treated CT mice exhibited epidermal

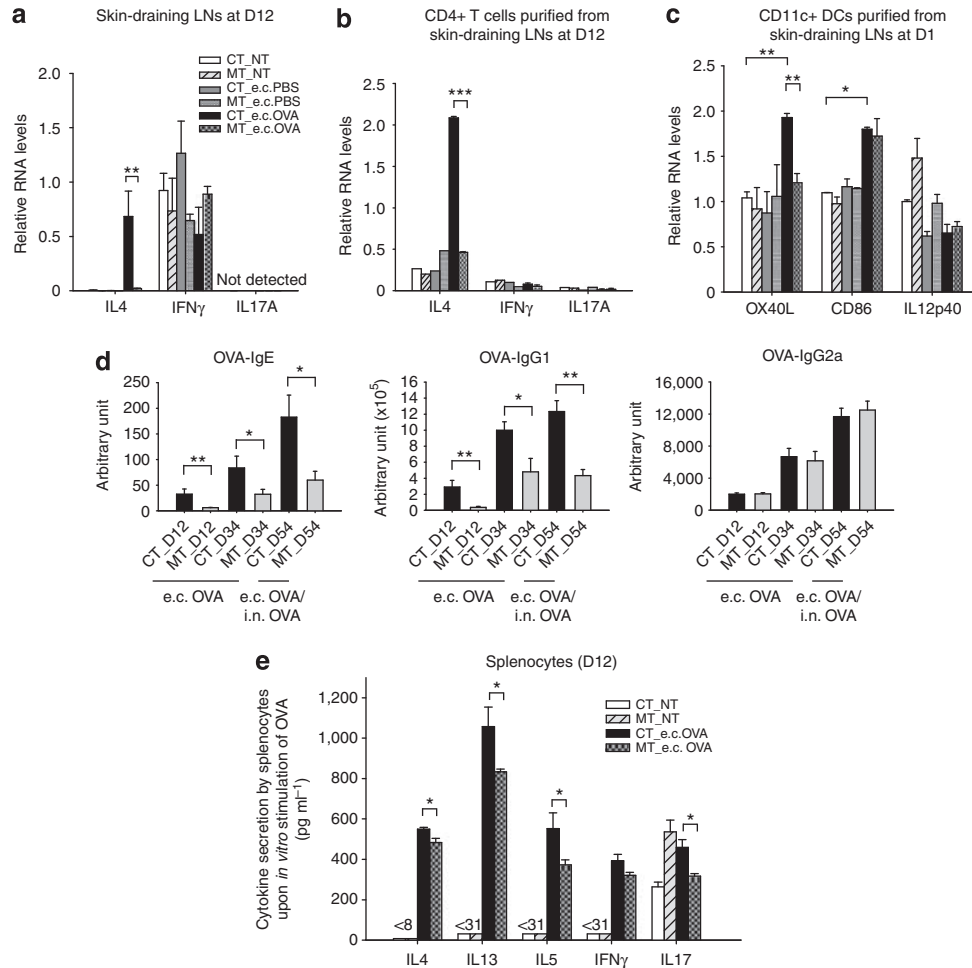


Figure 3. Impaired T helper type 2 (Th2) cell response and defective allergen sensitization in epicutaneous (e.c.) ovalbumin (OVA)-treated thymic stromal lymphopoietin (TSLP)^{iepi-/-} mutant (MT) mice. (a, b) Quantitative reverse-transcriptase-PCR (RT-PCR) analyses of (a) skin-draining lymph nodes (LN) and (b) purified CD4 $^{+}$ T cells from skin-draining LNs at day (D)12. (c) Quantitative RT-PCR analyses of purified CD11c $^{+}$ dendritic cells (DCs) from skin-draining LNs at D1. (d) Serum OVA-specific IgE, IgG1, and IgG2a levels at various time points (D12, D34, and D54). (e) Cytokine secretion by splenocytes at D12, in response to *in vitro* OVA stimulation. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ ($n \geq 5$ mice per group). Values are mean $\pm$ SEM. CT, wild-type control mice; NT, nontreated.

hyperplasia and dermal cell infiltration (Figure 2a). The infiltrated cells comprised CD4 $^{+}$ cells (Figure 2b), eosinophils (Figure 2c), and basophils (Figure 2d), all of which are characteristic cells in allergic skin inflammation (Leung *et al.*, 2004; Ito *et al.*, 2011). This local skin infiltrate was not seen in NT or e.c. PBS-treated CT skin (Figure 2a–e), indicating that it was an immune response upon allergen treatment on barrier-impaired skin, but was not induced by barrier disruption alone. Notably, the infiltrate of eosinophils and basophils was abolished in e.c. OVA-treated Rag1 $^{-/-}$ mice (Figure 2f), indicating that it was a T- and B-cell-dependent, but not an innate inflammatory, response. Very few CD8 $^{+}$ T cells or neutrophils were detected in e.c.-treated CT skin (Figure 2e and data not shown). At D34, e.c. OVA-treated CT mice showed an immune infiltrate similar to that seen on D12 (data not shown).

In contrast, e.c. OVA-treated MT mice displayed a drastically reduced skin allergic inflammation, exhibiting lesser

epidermal hyperplasia and dermal infiltrate of CD4 $^{+}$ cells, eosinophils, and basophils at D12 (Figure 2a–e), as well as at D34 (data not shown). Altogether, these results indicate that keratinocytic TSLP is essential for generating skin allergic inflammation upon OVA treatment on barrier-impaired skin.

The T helper type 2 (Th2) response induced by e.c. OVA treatment is impaired in mice lacking TSLP in keratinocytes
When examined at D12, skin-draining lymph nodes (LN) from the e.c. OVA-treated CT mice showed a significant induction of IL-4, compared with NT or e.c. PBS-treated CT mice (Figure 3a). This increase was abrogated in e.c. OVA-treated TSLP^{iepi-/-} MT mice (Figure 3a). In contrast, the expression of Th1 cytokine (IFN- γ) was not significantly affected, whereas that of Th17 cytokine (IL-17A) was below detectable levels (Figure 3a). Furthermore, we found that IL-4 expression was induced in purified CD4 $^{+}$ T cells from skin-draining LNs of e.c. OVA-treated CT (Figure 3b), but not in

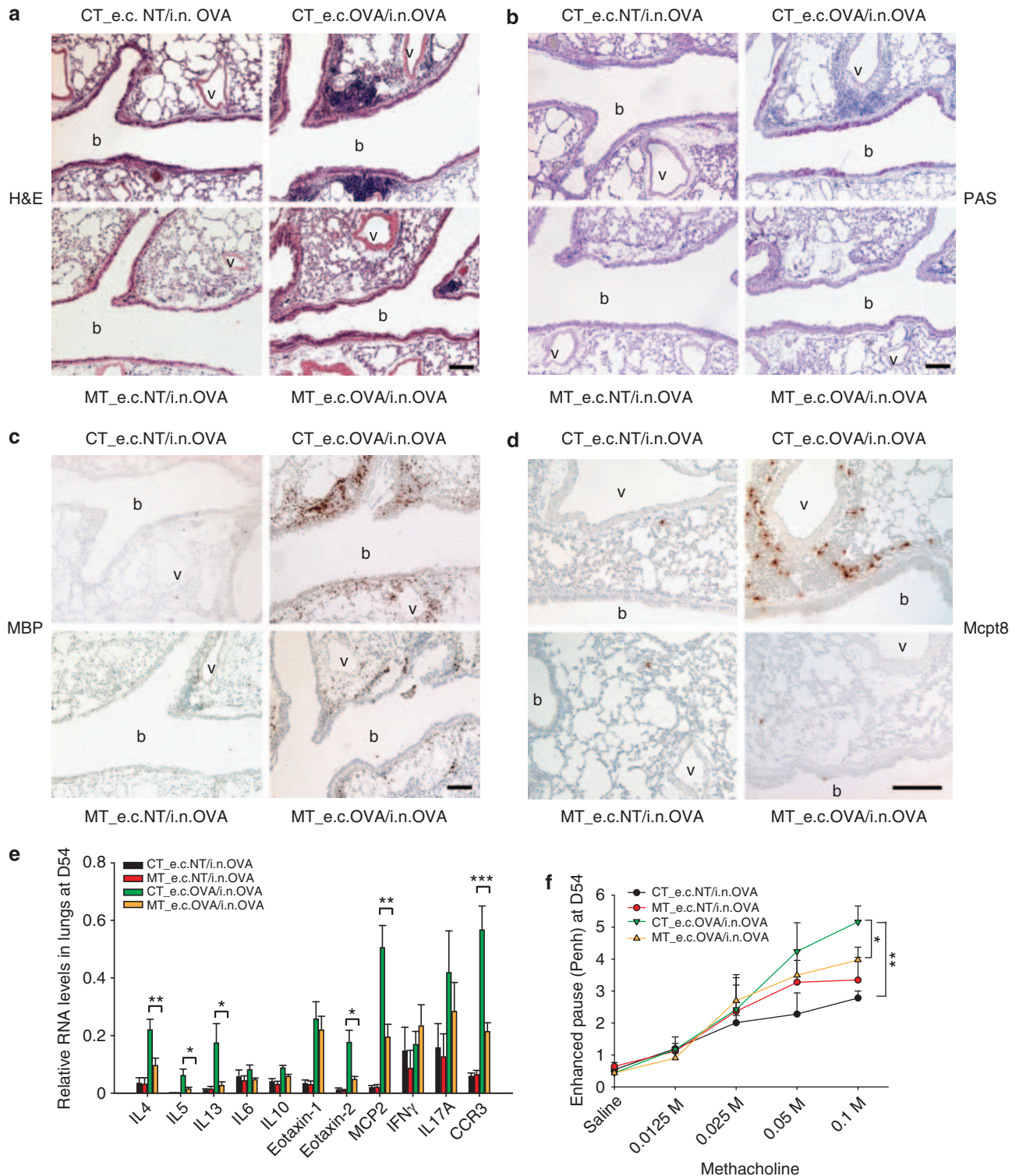


Figure 4. An attenuated allergic asthma phenotype in epicutaneous (e.c.)-sensitized thymic stromal lymphopoietin (TSLP)^{ep-/-} mutant (MT) mice upon intranasal (i.n.) challenge. (a) Hematoxylin and eosin (H&E) staining of lung section at day (D)54. “e.c. ovalbumin (OVA)/i.n.OVA”, mice with e.c. OVA sensitization and i.n. OVA challenge; “e.c. nontreated (NT)/i.n.OVA”, mice without any e.c. treatment but with i.n. challenge of OVA. (b) Periodic acid Schiff (PAS) staining of lung sections at D54. Mucus-secreting goblet cells are stained purple in bronchioles. (c, d) Immunohistochemical (IHC) with antibodies against (c) MBP (for eosinophils) and (d) Mcpt8 (for basophils) of lung sections at D54. Dark red color stands for stained cells. (e) Quantitative reverse-transcriptase-PCR (RT-PCR) analyses of cytokines and chemokines in lungs at D54. (f) Airway hyperresponsiveness (AHR) evaluated by plethysmography after exposure to increasing doses of methacholine. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ ($n \geq 6$ mice per group). Values are mean $\pm$ SEM. b, bronchiole; CT, wild-type control mice; v, vessel. Bar = 200 μ m.

non-CD4⁺ T cells (data not shown), indicating that CD4⁺ T cells were the major source of IL-4. This IL-4 induction was clearly abolished in CD4⁺ T cells from e.c. OVA-treated MT mice (Figure 3b). Notably, even though tape stripping on its own induced TSLP expression in keratinocytes of CT mice, it did not lead to IL-4 induction in CD4⁺ T cells in the absence of OVA (compare CT_e.c.PBS with CT_e.c.OVA, Figure 3b).

It has been reported that dendritic cells (DCs) targeted *in vitro* with TSLP express OX40L, which has been implicated in the initiation of the Th2 cell response (Ito *et al.*, 2005). The expression of OX40L was therefore examined in purified DCs from skin-draining LNs of CT and MT mice at D1 (i.e., 24 hours after the first e.c. treatment), a time at which skin DCs migrate to draining LNs after encountering an antigen (Inoue *et al.*, 2005). OX40L was induced in DCs from CT mice upon e.c. OVA (but not upon e.c. PBS); however, this induction was largely reduced in DCs from e.c. OVA-treated MT mice (Figure 3c), indicating that keratinocytic TSLP was required for OVA-induced OX40L expression in DCs. In contrast, other costimulatory factors including CD40, CD86, and CD80 were similarly increased in DCs from CT and MT mice upon OVA treatment (Figure 3c and data not shown). The expression of Th1-polarizing cytokine IL-12 was not different between OVA-treated CT and MT (Figure 3c). Taken together, these results indicate that keratinocytic TSLP has an essential role in promoting e.c. OVA-induced Th2 response.

Allergen sensitization through skin is defective in mice lacking TSLP in keratinocytes

To examine the sensitization to allergen, we first analyzed the production of OVA-specific IgE, IgG1, and IgG2a. TSLP^{iep-/-} MT mice exhibited lower levels of OVA-specific IgE and IgG1, whereas OVA-IgG2a levels were comparable in CT and MT mice, after either e.c. sensitization (at D12 and D34) or i.n. challenge (at D54; Figure 3d). We then analyzed cytokine production by splenocytes upon *in vitro* OVA stimulation. Th2 cytokine (IL-4, IL-13, and IL-5) levels were all significantly reduced in MT mice at D12 (Figure 3e). A decrease of IL-17 was also observed, whereas IFN- γ levels were comparable (Figure 3e). Similar results were obtained at D34 (data not shown). Taken together, these data indicate that keratinocytic TSLP is required for an optimal Th2 allergen sensitization through barrier-defective skin.

Allergic asthma induced by e.c. sensitization and airway challenge is attenuated in mice lacking TSLP in keratinocytes

Upon i.n. OVA challenge, e.c. OVA-sensitized CT mice (CT_e.c.OVA/i.n.OVA) exhibited an allergic airway inflammation with peribronchiolar and perivascular infiltrates (compared with CT_e.c.NT/i.n.OVA, Figure 4a), comprising eosinophils (Figure 4c), basophils (Figure 4d), and CD4⁺ T cells (data not shown). Very few CD8⁺ T cells or neutrophils were observed (data not shown). These mice also exhibited hyperplasia of mucus-secreting goblet cells (Figure 4b). Reverse-transcriptase-PCR analyses of lungs showed an upregulated expression of Th2 cytokines (IL-4, IL-5, and IL-13), Th2 chemokines (eotaxin-1, eotaxin-2, and MCP-2),

and CCR3 (mainly expressed by eosinophils and basophils), whereas expression levels of IFN- γ and IL-17A were not significantly affected (Figure 4e). Whole-body plethysmography analyses showed that these mice exhibited an enhanced airway hyperresponsiveness (Figure 4f).

In contrast to CT mice, TSLP^{iep-/-} MT mice developed an attenuated asthmatic phenotype upon e.c.OVA/i.n.OVA treatment, as shown by much lesser infiltrates of eosinophils, basophils, and CD4⁺ cells (Figure 4a, c and d, and data not shown), reduced goblet cell hyperplasia (Figure 4b), decreased expression of Th2 cytokines and chemokines (Figure 4e), and diminished airway hyperresponsiveness (Figure 4f).

This attenuated asthmatic phenotype in MT mice was due to defects in skin sensitization, but not due to airway challenge, because TSLP was selectively ablated in skin keratinocytes and not in airways (Li *et al.*, 2009; data not shown). Importantly, when sensitization was achieved through i.p. route (by injection of OVA/alum), and followed by i.n. OVA challenge, TSLP^{iep-/-} MT and CT mice developed a similar sensitization and asthmatic phenotype (Supplementary Figure S1 online). Altogether, these data demonstrate that keratinocytic TSLP is crucial for promoting sensitization to allergen through skin, which triggers the “atopic march” and leads to allergic asthma.

Topical MC903 treatment induces keratinocytic TSLP expression, enhances e.c. sensitization, and promotes allergic asthma in a dose-dependent manner

It has been shown that TSLP is overexpressed in keratinocytes in lesioned skin of AD patients (Soumelis *et al.*, 2002). To examine whether overproduction of TSLP could boost skin sensitization, thus triggering the atopic march, keratinocytic TSLP was induced by MC903 application during the first phase of e.c. sensitization (Figure 5a). Note that upon MC903 treatment of wild-type Balb/c mice, TSLP expression was rapidly induced in a dose-dependent manner (Figure 5b), whereas ending the MC903 treatment normalized TSLP levels within 3 days (Zhang *et al.*, 2009; data not shown).

In e.c. OVA-treated TSLP^{over} Balb/c mice, Th2 and Th17 (but not Th1) cytokines secreted by splenocytes at D34 were upregulated (Figure 5c), and serum OVA-specific IgE and IgG1 were concomitantly increased (Figure 5d). It is interesting to note that these enhancing effects of MC903 were clearly dose dependent.

Upon i.n. OVA challenge, MC903-treated e.c. OVA-sensitized wild-type mice developed an aggravated asthmatic phenotype. They exhibited enhanced mucus secretion and increased airway inflammatory infiltrate comprising eosinophils, basophils, and CD3⁺ T lymphocytes (consisting mainly of CD4⁺ but not CD8⁺ T cells; Figure 5e and f). The aggravation of these asthmatic phenotypes was again correlated with TSLP expression levels (Figure 5f). Accordingly, mRNA levels of Th2 cytokines (e.g., IL-4, IL-5, and IL-13), eosinophil-attractant chemokine (eotaxin-2) and receptor (CCR3) in lungs, as well as in bronchoalveolar lavage cells, were all dose-dependently increased (Figure 5g). Importantly, in the absence of e.c. OVA sensitization, MC903-induced

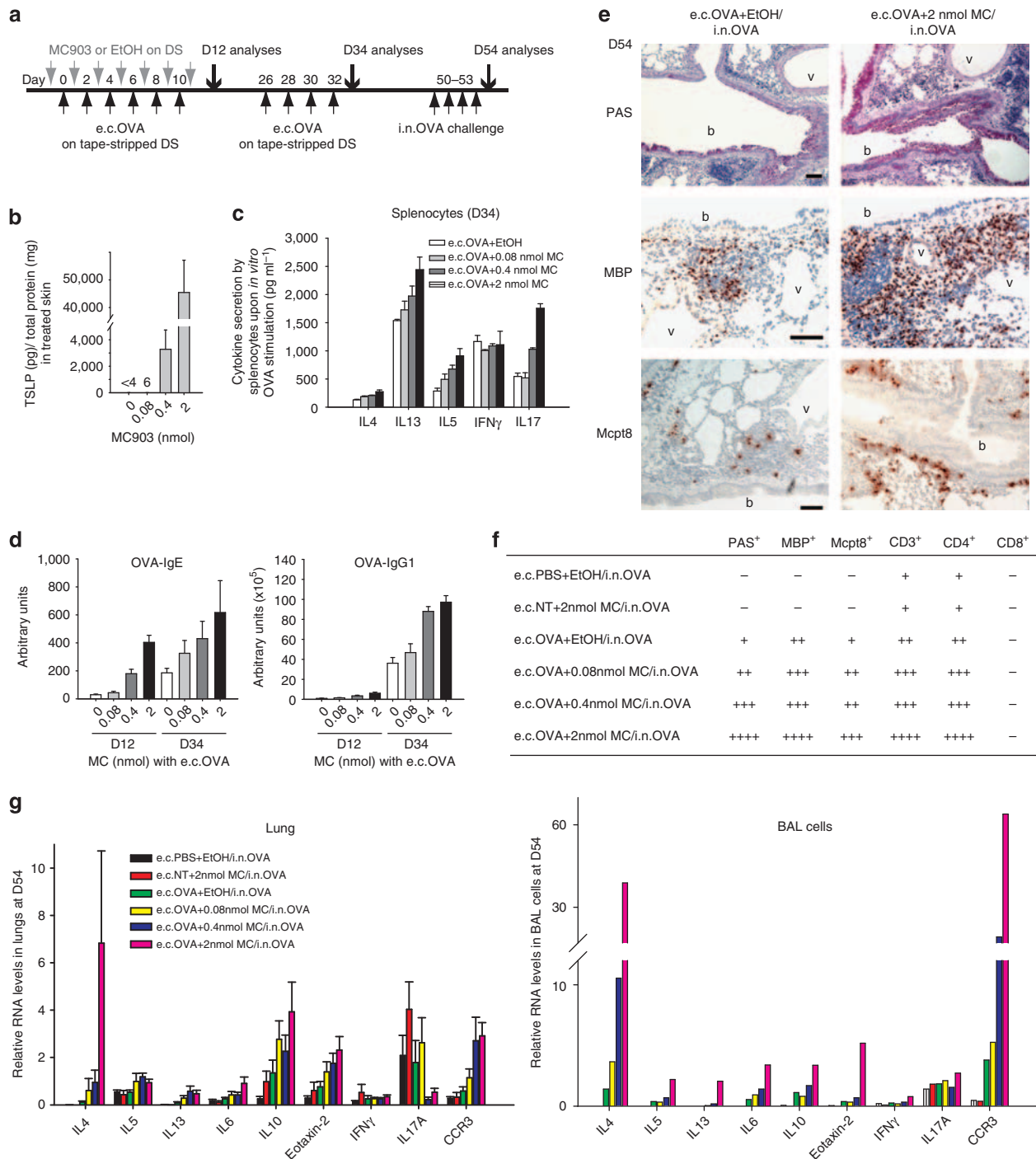


Figure 5. Skin topical application of MC903 induces keratinocytic thymic stromal lymphopoietin (TSLP) expression, enhances epicutaneous (e.c.) sensitization, and promotes allergic asthma in a dose-dependent manner. (a) Experimental protocol. Eight to twelve-week-old female wild-type Balb/c mice were subjected to the mouse model as described in Figure 1a, and concomitantly treated with MC903 or ethanol (EtOH) (its vehicle control) on the same area of dorsal skin (DS), during the first phase of e.c. sensitization. (b) TSLP protein levels measured by ELISA at day (D)2 in dorsal skin upon topical application of 0.08, 0.4, or 2 nmol MC903 at D –1 and D1. (c) Cytokine secretion by *in vitro* ovalbumin (OVA)-stimulated splenocytes from D34. (d) Serum OVA-specific IgE, IgG1, and IgG2a levels at D12 and D34. Values are mean $\pm$ SEM ($n \geq 6$ per group). (e) Mucus production (periodic acid Schiff (PAS) staining; in purple) and infiltrate of eosinophils (immunohistochemical (IHC) with MBP antibody) and basophils (IHC with Mcpt8 antibody) in lungs at D54. (f) A summary of comparison of mucus production (PAS staining) and inflammatory infiltrates of various cells (by IHC) in lungs of e.c.-sensitized and intranasal (i.n.)-challenged mice combined with different doses (0.08, 0.4, or 2 nmol) of MC903. Abundance of positive cells detected in lung sections is presented with – (not detected) to ++++ (the most abundant). (g) Quantitative reverse-transcriptase-PCR (RT-PCR) analyses in mouse lungs (left panel; values are mean $\pm$ SEM; $n = 6$ mice per group) and bronchoalveolar lavage (BAL) cells (right panel; pool of six mice per group) at D54. Results are representative of three independent experiments. b, bronchiole; NT, nontreated; v, vessel. Bar = 100 μ m.

production of skin TSLP did not induce any asthmatic phenotype upon OVA airway challenge (see e.c.NT + 2 nmol MC/i.n.OVA; Figure 5f and g), indicating that the over-produced skin TSLP on its own (without allergen) does not promote asthma. Altogether, these data indicate that keratinocytic TSLP expression level correlates with the strength of skin sensitization and the severity of asthma.

DISCUSSION

An understanding of the mechanism of sensitization to allergen through skin is crucial for elucidating the mechanism underlying the “atopic march”, and for possibly preventing its development. In the present study, we demonstrate that TSLP produced by keratinocytes is crucial for developing allergen sensitization through barrier-impaired skin, and for subsequent generation of allergic asthma.

The epidermis of AD patients is characterized by barrier impairment, and the majority of the patients develop sensitization to common allergens (Bieber, 2008; Cork *et al.*, 2009; Boguniewicz and Leung, 2011). We show that impairing the mouse epidermal barrier through tape stripping induces TSLP production in keratinocytes, in agreement with a recent report (Oyoshi *et al.*, 2010). Interestingly, impairment of human skin barrier by tape stripping or by application of sodium lauryl sulfate was also found to induce epidermal TSLP expression (Angelova-Fischer *et al.*, 2010). Actually, various extrinsic or intrinsic factors have been shown to impair the epidermal barrier, accompanied by TSLP production in keratinocytes (Demehri *et al.*, 2008; Briot *et al.*, 2009; Angelova-Fischer *et al.*, 2010; Takai and Ikeda, 2011). How these factors lead to TSLP expression remains to be elucidated. The induction of TSLP by tape stripping does not appear to involve the majority of TLR pathways, as TSLP expression was similarly observed in tape-stripped MyD88^{-/-} mouse skin (Supplementary Figure S2 online). Whether it could be mediated via the proteinase-activated receptor 2 (Briot *et al.*, 2009; Lee *et al.*, 2010b) needs to be determined.

Our results demonstrate that keratinocytic TSLP induced by barrier impairment is essential for generating a Th2 allergic immune response, as ablation of TSLP in keratinocytes leads to the abrogation of allergic skin inflammation and Th2 response upon allergen treatment on barrier-impaired skin (Figures 2 and 3). However, TSLP induced by tape stripping does not on its own, in the absence of allergen, lead to the skin recruitment of CD4⁺ cell, eosinophils, and basophils (see Figure 2a–e). Similarly, it does not induce IL-4 (indicative of Th2 differentiation) in CD4⁺ T cells (Figure 3b), nor does it induce OX40L (a key Th2 costimulatory factor) in DCs (Figure 3c). These observations clearly suggest that TSLP acts as a crucial Th2 “adjuvant” contributing to the allergic immune response upon allergen treatment on barrier-impaired skin. This conclusion differs from that drawn from our own previous reports and that of others, showing that transgenic TSLP (Li *et al.*, 2005; Yoo *et al.*, 2005) or MC903-induced TSLP (Li *et al.*, 2006, 2009) overexpression in mice leads to a “spontaneous” AD, in the absence of allergen treatment on skin. Indeed, in those previous mouse models,

TSLP expression was much higher: e.g., TSLP was detected in a high level in serum (Li *et al.*, 2005, 2006), whereas serum TSLP was below detectable level (<7.8 pg ml⁻¹) in the present case. One possible explanation would be that above a certain threshold level TSLP could trigger an immune cascade even in the absence of penetration of any exogenous allergens into the skin. Whether such a high TSLP level exists in human AD patients is actually uncertain, but in any event our present data demonstrate that a low level of TSLP production is essential and sufficient for exerting its Th2 “adjuvant” effect in promoting allergen sensitization through barrier-impaired skin.

How mouse keratinocytic TSLP promotes the allergic Th2 response remains to be elucidated. Human TSLP has been shown to target DCs *in vitro* to induce a Th2 response (Soumelis *et al.*, 2002; Ito *et al.*, 2005; Liu *et al.*, 2007), but whether mouse TSLP exerts similar function was unclear (Soumelis *et al.*, 2002; Al-Shami *et al.*, 2005; He *et al.*, 2008). We found that keratinocytic TSLP was essential for OX40L expression in DCs in response to OVA, as early as 24 hours after OVA encountering in barrier-defective mouse skin (Figure 3c), suggesting that a TSLP (in keratinocytes)-OX40L (in DCs) pathway could be critically involved in the initiation of allergen-dependent Th2 response. In contrast to *in vitro* studies (Al-Shami *et al.*, 2005; Ito *et al.*, 2005), we found that e.c. OVA-induced expression of CD40, CD80, and CD86 in DCs did not require TSLP (Figure 3c and data not shown). Interestingly, a recent study has shown that the complete Th2 induction upon i.p. immunization of OVA plus adjuvant (Nod1 or Nod2 agonists) was dependent on both TSLP production in stromal cells and upregulation of OX40L on DCs (Magalhaes *et al.*, 2011). Therefore, a TSLP-OX40L axis could be one common mechanism underlying Th2 priming either through e.c. or i.p. route. In addition, we observed that antigen presentation was impaired in TSLP^{iep-/-} MT mice (Supplementary Figure S3 online). Which subset(s) of DCs in the skin (Henri *et al.*, 2010) is (are) possibly responsible for the TSLP-promoted Th2 response to allergens and whether TSLP may act on other antigen-presenting cells (e.g., basophils) (Maddur *et al.*, 2010) to initiate the skin allergic response remain to be investigated.

The impaired Th2 response in TSLP^{iep-/-} MT mice correlates with defective skin sensitization and subsequent asthma generation. Whereas OVA-induced IL-4 expression is abolished in skin-draining LNs in these MT mice (Figure 3a), OVA-specific IgE and IgG1, as well as OVA-stimulated Th2 cytokine production in splenocytes, were not fully abrogated (Figure 3d and e), suggesting that factors (Cork *et al.*, 2009) other than TSLP also contribute to the complete Th2 sensitization to allergen in barrier-impaired skin. In contrast to its effect on Th2 response, TSLP appears to have no or a much less effect on the Th1 or Th17 response in skin (Figure 3a). Accordingly, the expression of IFN- γ or IL-17A was not different in the lung upon OVA i.n. challenge (Figure 4e). Moreover, neutrophils were barely detected in the skin upon e.c. sensitization (Figure 2e), or in the lung upon i.n. challenge (data not shown), in either CT or TSLP^{iep-/-} MT mice.

In contrast to TSLP^{iepe/-} MT mice in which skin sensitization is defective, TSLP^{over} mice in which TSLP expression in keratinocytes is induced by MC903 topical application develop an enhanced e.c. sensitization and subsequently an aggravated allergic asthma (Figure 5). It is interesting to note that our data establish a significant correlation of skin TSLP levels with sensitization strength, and with asthma severity, further supporting the fact that keratinocytic TSLP represents a risk factor for sensitization developed in AD patients and for triggering the onset of the “atopic march”.

In conclusion, our study has revealed that keratinocyte-derived cytokine TSLP is crucial for promoting allergen sensitization that occurs in barrier-impaired skin, which accounts for the “atopic march” leading ultimately to allergic asthma. It suggests that blocking TSLP production in skin could be therapeutically useful in preventing or limiting allergen sensitization that is commonly developed in AD patients, and halting the progress of the “atopic march”.

MATERIALS AND METHODS

Mice

Wild-type Balb/c and Rag1^{-/-} mice (in Balb/c background) were purchased from Charles River Laboratories (Lyon, France). Eight to twelve-week-old K14-Cre-ER^{T2(tg/0)}/TSLP^{L2/L2} female mice (in a Balb/c background) were injected i.p. with Tam (0.1 mg in 100 µl sunflower oil, for 5 consecutive days) (Li *et al.*, 2000) to generate TSLP^{iepe/-} mice. Tam-injected age- and sex-matched littermates (K14-Cre-ER^{T2(0/0)}/TSLP^{L2/L2}) were used as CT. TSLP^{iepe/-} and CT mice were subjected to experiments 4 weeks after the first Tam injection. Breeding and maintenance of mice were performed under institutional guidelines, and all of the experimental protocols were approved by the Animal Care and Use Committee of the Institut de Génétique et de Biologie Moléculaire et Cellulaire.

OVA e.c. sensitization and i.n. challenge

An area of approximately 2 cm² of the dorsal skin of mice was shaved and tape stripped until TEWL (transepidermal water loss) measurement reached between 15 and 20 g m⁻² per hour (DermaLab, Cortex Technology, Hadsund, Denmark), and then treated with 200 µg of OVA (Sigma, St Louis, MO) in 50 µl PBS with a cotton swab, every other day from D0 to D10. Similar e.c. treatment was repeated for 4 times 2 weeks later from D26 to D32. NT mice or mice treated with PBS on tape-stripped skin (e.c. PBS) were used as controls. To induce an experimental asthma, mice were i.n. challenged with OVA (50 µg in 25 µl saline) 3 weeks later, on 4 consecutive days (D50 to D53). Airway reactivity was measured with whole-body plethysmography (Emka Technologie, Paris, France) 1 day after the last i.n. treatment before mice were killed for other analyses.

MC903 topical application

MC903 (calcipotriol; Leo Pharma, Ballerup, Denmark) was dissolved in ethanol (Zhang *et al.*, 2009) and topically applied on mouse dorsal skin with the quantity indicated. Ethanol was used as vehicle control.

Skin TSLP protein levels

Mouse skin was chopped and homogenized using Mixer Mill MM301 (Retsch, Haan, Germany) in lysis buffer (25 mM Tris, pH 7.8,

2 mM EDTA, 1 mM DTT, 10% glycerol, and 1% Triton X-100) supplemented with protease inhibitor cocktail (Roche). Protein concentration of skin extract was quantified using the Bio-Rad Protein Assay (Bio-Rad, Marnes-la-Coquette, France). TSLP levels in skin extracts were determined with the DuoSet TSLP ELISA development kit (R&D, Minneapolis, MN).

Antibodies

The following antibodies were used in the immunohistochemical staining: rat anti-mouse MBP (provided by Dr James J Lee, Mayo Clinic, Scottsdale, AZ), rat anti-mouse MCP-8 (clone TUG-8, Biolegend, San Diego, CA), rat anti-mouse NIMP-R14 (Abcam, Cambridge, MA), rat anti-mouse CD3 (clone 17A2, BD, Heidelberg, Germany), rat anti-mouse CD4 (clone RM4-5, or clone GK1.5, BD) and rat anti-mouse CD8 (clone 53-6.7, BD).

Splenocyte culture

Single-cell suspensions of spleen were cultured in medium (RPMI 1640 without HEPES supplemented by 10% FCS, 2 mM glutamine, 100 U ml⁻¹ penicillin, and 100 µg ml⁻¹ streptomycin) at a density of 2.5 × 10⁶ ml⁻¹ in the presence of 500 µg ml⁻¹ OVA in anti-CD3 (10 µg ml⁻¹; clone 145-2C11 (e-Bioscience, Paris, France))-coated 96-well plates for 3 days. Cytokine levels of IL-4, IL-5, IL-13, IL-17, and IFN-γ in culture supernatants were measured using the DuoSet ELISA Development system (R&D).

Isolation of CD4⁺ T cells and DCs from mouse LNs

LN CD4⁺ T cells were purified using the mouse CD4⁺ T-cell Isolation Kit II (Miltenyi Biotec, Paris, France; purity was confirmed to >94%). LN CD11c⁺ DCs were first enriched using CD11c Microbeads (Miltenyi Biotec) and then sorted with FACS Aria II (BD; purity was confirmed to >95%).

Statistical analysis

Data were analyzed using SigmaStat (Systat Software, Richmond, CA) by the Student's *t*-test or the Mann-Whitney rank-sum nonparametric test depending on results from the Kolmogorov-Smirnov test (with Lilliefors' correction) for normality and Levene Median test for equal variance. Two-way repeated-measures ANOVA was used to compare airway hyperresponsiveness between groups responding to various doses of methacholine.

Other methods

Histopathology, immunohistochemical staining, serum Ig determination, quantitative reverse-transcriptase-PCR, and airway reactivity were performed as previously described (Zhang *et al.*, 2009; Hener *et al.*, 2011). Details of these methods, as well as *in vivo* proliferation of the adoptively transferred T cell, can be found in the Supplementary Materials and Methods online.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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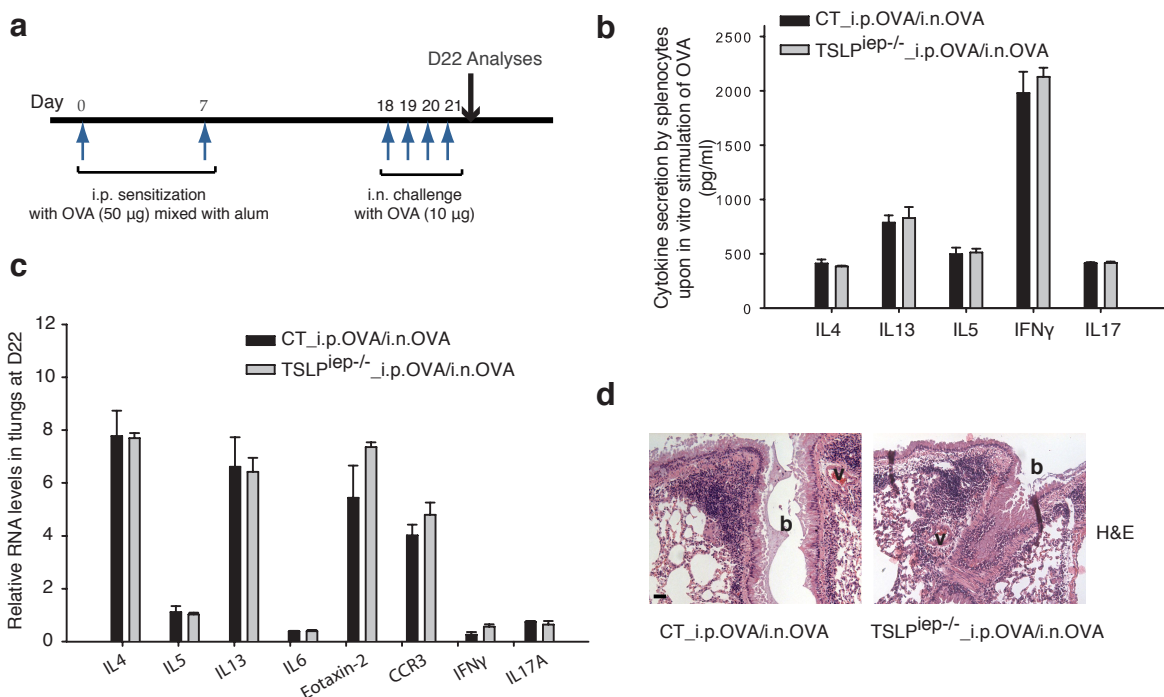
Frossard, and Susan Chan for helpful discussions, and Pierre Chambon for critical reading of the manuscript. We acknowledge James J Lee (Mayo Clinic, USA) for rat anti-mouse MBP monoclonal antibody, and LEO Pharma (Denmark) for MC903. This work was supported by funds from l'Agence Nationale de la Recherche (Grant ANR 2010 JCJC-1106-01 to M Li), the Association pour la Recherche à l'IGBMC (ARI for a predoctoral fellowship to JM Leyva-Castillo and a postdoctoral fellowship to H Jiang), the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale, and the Université de Strasbourg.

SUPPLEMENTARY MATERIAL

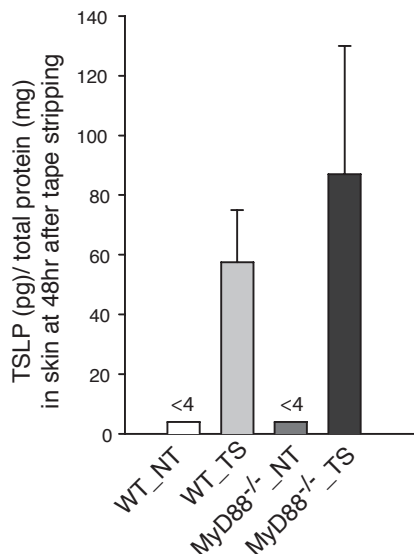
Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

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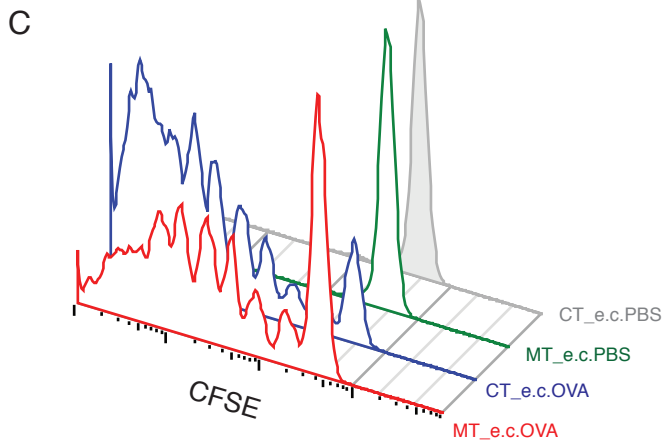
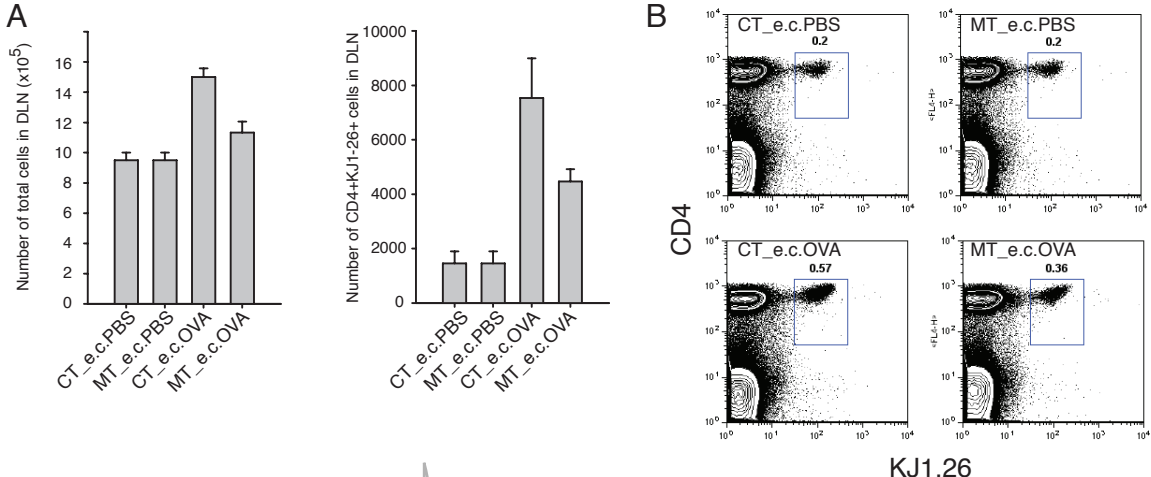
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Supplementary Figure S1. Keratinocytic TSLP is not required for intraperitoneal (i.p.) sensitization with OVA/Alum, and for the generation of asthmatic phenotype upon intranasal (i.n.) OVA challenge. (a) An experimental asthma protocol employing i.p. sensitization of OVA mixed with alum (aluminum hydroxide) (at D0 and D7) and i.n. challenge of OVA (at D18, 19, 20 and 21). (b) Cytokine secretion by splenocytes at D22 upon in vitro stimulation of OVA, showing comparable levels between TSLP^{ie⁻/i.p.OVA/i.n.OVA and wildtype control (CT) mice. (c) Quantitative RT-PCR analyses of lungs at D22 from TSLP^{ie⁻/i.p.OVA/i.n.OVA and CT mice. Values are mean $\pm$ SEM ($n \geq 5$ mice per group). (d) H&E staining of lung paraffin sections from D22, showing comparable inflammatory infiltrates between TSLP^{ie⁻/i.p.OVA/i.n.OVA and CT mice. b, bronchiole; v, vessel. Scale bar, 50 μ m.}}}



Supplementary Figure S2 MyD88 is not required for tape-stripping-induced TSLP expression in keratinocytes. Dorsal skin of twelve weeks old MyD88^{-/-} (in a mixed background of 75% C57BL/6 and 25% Balb/c) mice and their wildtype (WT) littermates was shaved and tape-stripped, and was analyzed 48 hrs later. TSLP protein levels in skin were measured by ELISA. NT, non-treated. TS, tape-stripped. Values are mean $\pm$ SEM (n=3 mice per group).



Supplementary Figure S3. Defective expansion of adoptively transferred TCR-OVA transgenic T cells in skin-draining lymph nodes (LNs) of e.c. OVA-treated TSLP^{ie}^{-/-} mutant (MT) mice. Naïve CD4⁺ DO11.10 cells were adoptively transferred into wildtype control (CT) or MT recipients, and followed by e.c. treatment with OVA (200 μ g) or saline on the tape-stripped dorsal skin at 48 hours later. Skin-draining LNs were analyzed 4 days later for the accumulation of CD4⁺KJ1.26⁺ cells. **(a)** Numbers of total cells and CD4⁺KJ1.26⁺ cells in skin-draining LNs. **(b)** Representative FACS plots of CD4⁺KJ1.26⁺ cells of skin-draining LNs of recipients of CD4⁺ DO11.10 cells. **(c)** Less cell division of CFSE-labeled CD4⁺ DO11.10 cells in skin-draining LNs of e.c. OVA-treated MT, compared to those of CT mice. No cell division was seen in e.c. PBS-treated CT or MT mice.

SUPPLEMENTARY MATERIALS AND METHODS

Histopathology. Skin or lung tissues were fixed overnight at 4 °C in 4% paraformaldehyde (PFA), and embedded in paraffin. Sections (3µm) were stained with hematoxylin/eosin (H&E), or Periodic acid-Schiff (for goblet cells in lung).

Immunohistochemical (IHC) staining. For IHC staining of MBP (for eosinophils) or NIMP-R14 (for neutrophils), 3 µm paraffin sections were treated with 0.6% H₂O₂ to block the endogenous peroxidase activity, followed by digestion with Pepsin solution (Invitrogen) in order to retrieve antigen. For IHC staining of MCP-8 (for basophils), paraffin sections were treated with citric buffer (10mM citric acid, pH6) to retrieve antigen, followed by blocking the endogenous peroxidase activity with 0.6% H₂O₂. Slides were then blocked with rabbit serum (Vector Laboratories), and incubated with rat-anti-mouse MBP (provided by Dr James J Lee, Mayo Clinic, Rochester, USA), NIMP-R14 (Abcam) or rat-anti-mouse MCP-8 (clone TUG-8, Biolegend) antibodies respectively. After incubation with biotinylated rabbit anti-rat IgG, followed by AB complex (Vector Laboratories), staining was finally visualized with AEC+ high sensitivity substrate chromogen solution (Dako) and counterstained with hematoxylin.

For CD3, CD4 and CD8 labeling, 10 µm frozen sections were fixed in 4% paraformaldehyde, permeabilized with cold acetone, and blocked with normal goat serum (Vector laboratories). Slides were then incubated with primary antibody [rat monoclonal anti-CD3 (clone 17A2); rat monoclonal anti-CD4 (clone RM4-5, or clone GK1.5); or rat monoclonal anti-CD8 (clone 53-6.7)]. After washing, sections were incubated with CY3-conjugated goat anti-rat IgG antibody (Jackson ImmunoResearch) and mounted with vectashield medium (Vector laboratories) containing DAPI (invitrogen).

Serum immunoglobulin determination. For OVA-specific Igs, microtiter plates (R&D Systems) were first coated with OVA (20 µg/ml in 0.1M sodium bicarbonate buffer). After blocking with 1% BSA (Sigma), the coated plates were then incubated with serum samples, followed by incubation with a biotinylated rat anti-mouse IgE (clone R35–118, BD Biosciences PharMingen), rat anti-mouse IgG1 (clone A85-1, BD Biosciences PharMingen), or rat anti-mouse IgG2a (clone R19-15, BD Biosciences PharMingen). ExtrAvidin peroxidase (Sigma) and TMB (tetramethylbenzidine) Substrate Reagent Set (BD Biosciences PharMingen) were used for detection. Serum levels of OVA-specific Igs were related to a pooled serum from OVA-sensitized and challenged BALB/c mice (internal standard) and expressed as arbitrary units.

Quantitative RT-PCR. RNA was reverse-transcribed using random oligonucleotide hexamers and amplified by quantitative PCR with a Ligthcycler 480 (Roche Diagnostics) and the QuantiTect SYBR Green kit (Qiagen), according to the manufacturer's instructions. Relative RNA levels were calculated using hypoxanthine phosphoribosyltransferase (HPRT) an internal control. Sequences of PCR primers: HPRT, TSLP, IL4, IL5, IL13, IFN γ , IL10, IL6, Eotaxin-1, Eotaxin-2, MCP2 and CCR3 were as previously reported (Hener *et al.*, 2011); IL17A (239bp) (5'-CCAGGGAGAGCTTCATCTGT-3'; 5'-ACGTGGAACGGTTGAGGTAG-3'); OX40L (204 bp) (5'-GCTAAGGCTGGTGGTCTCTG-3'; 5'-ACCGAATTGTTCTGCACCTC-3'); CCR7 (165 bp) (5'-AAAGCACAGCCTTCCTGTGT-3'; 5'-AGTCCACCGTG GTATTCTCG-3') ; CD86 (246 bp) (5'-CACGAGCTTTGACAGGAACA-3'; 5'-TTAGGTTTCGGGTGACCTTG-3').

Airway Reactivity. Airway reactivity was measured by noninvasive whole-body plethysmography (Emka Technologies, France) in response to inhaled methacholine (Sigma), using enhanced pause (Penh) as an index of airway responsiveness.

In vivo proliferation of adoptively transferred T cell. OVA-specific CD4⁺ T cells were purified from DO11.10 mice (The Jackson Laboratory) using mouse CD4⁺ T cell Isolation Kit II (Miltenyi), stained with carboxyfluorescein diacetate succinimidyl ester (CFSE; Sigma), and were *i.v.* injected (1x10⁶ cells) to TSLP^{+/+} or TSLP^{iep-/-} recipient mice. At 48h after, mice were *e.c.* treated with 200 µg OVA or PBS on tape-stripped dorsal skin. Four days after, skin-draining LNs were harvested and *in vivo* proliferation of the adoptively transferred DO11.10 cells was analyzed by CFSE dilution in CD4⁺KJ1-26⁺ cells.

Hener P, Friedmann L, Metzger D, *et al* (2011). Aggravated TSLP-Induced Atopic Dermatitis in Mice Lacking Dicer in Adult Skin Keratinocytes. *J Invest Dermatol* 131:2324-7.