Human epithelial cells trigger dendritic cell–mediated allergic inflammation by producing TSLP

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Whether epithelial cells play a role in triggering the immune cascade leading to T helper 2 (T_H2)-type allergic inflammation is not known. We show here that human thymic stromal lymphopoietin (TSLP) potently activated CD11c$^+$ dendritic cells (DCs) and induced production of the T_H2-attracting chemokines TARC (thymus and activation-regulated chemokine; also known as CCL17) and MDC (macrophage-derived chemokine; CCL22). TSLP-activated DCs primed naïve T_H cells to produce the proallergic cytokines interleukin 4 (IL-4), IL-5, IL-13 and tumor necrosis factor-α, while down-regulating IL-10 and interferon-γ. TSLP was highly expressed by epithelial cells, especially keratinocytes from patients with atopic dermatitis. TSLP expression was associated with Langerhans cell migration and activation *in situ*. These findings shed new light on the function of human TSLP and the role played by epithelial cells and DCs in initiating allergic inflammation.

About 20% of the population in Western countries suffers from allergic diseases, which include asthma, allergic rhinitis, atopic dermatitis and food allergy[1]. Allergic inflammation is the result of a complex immunological cascade that leads to dysregulated production of T helper type 2 (T_H2)-derived cytokines such as interleukin 4 (IL-4), IL-5 and IL-13[2–4], which trigger immunoglobulin E (IgE) production, eosinophilia and mucus production[5–7]. Dendritic cells (DCs), which are professional antigen-presenting cells[8], play an important role in the pathogenesis of allergic diseases[9–11]. However, the initial signal that primes DCs to induce T cells to produce proallergic T_H2 cytokines is unknown. Epithelial cells are located at the sites of allergen entry into the body and interact closely with DCs *in situ*. However, it is not known whether DCs play a role in triggering the allergic immune cascade. Although skin keratinocytes and mucosal epithelial cells produce proinflammatory cytokines such as IL-1, IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor-α (TNF-α) after activation[12], none of these cytokines explain the mechanism that underlies the induction of allergic inflammation.

Thymic stromal lymphopoietin (TSLP) is an IL-7–like cytokine, cloned from a murine thymic stromal cell line[13]. The TLSP receptor is a heterodimer that consists of the IL-7 receptor α chain (IL-7Rα) and a common γ-like receptor chain called TSLP receptor (TSLPR)[14–17]. Mouse TSLP supports murine early B and T cell developments[18,19] and does not appear to have any biological effects on murine DCs (unpublished data). In contrast, human TSLP activates CD11c$^+$ DCs, but does not appear to have any direct biological effects on B cells, T cells, NK cells, neutrophils or mast cells[17]. This is in accordance with the coexpression of IL-7Rα chain and TSLPR mRNA in CD11c$^+$ DCs, but not in other cell types. We show here that human TSLP potently activated human CD11c$^+$ DCs, which subsequently primed naïve T_H cells to produce high concentrations of IL-13, IL-5 and TNF-α, moderate amounts of IL-4 and down-regulate IL-10 and interferon-γ (IFN-γ). TSLP is highly expressed by epithelial cells of inflamed tonsils and keratinocytes of atopic dermatitis. Thus, TSLP represents a key epithelial cell or keratinocyte-derived cytokine that directly triggers DC-mediated allergic inflammation.

Results

TSLP potently activates human CD11c$^+$ DCs

We compared the effects of TSLP, IL-7, CD40 ligand (CD40L)and lipopolysaccharide (LPS) on human CD11c$^+$ DC activation. TSLP, IL-7, CD40L and LPS all up-regulated surface HLA-DR, CD40, CD80, CD86 and CD83 on DCs when compared with medium alone (**Fig. 1a**). Whereas TSLP induced the most CD40 and CD80 expression on DCs, CD40L induced more HLA-DR and CD83. Like CD40L, TSLP not only activated DCs, but also maintained the survival of DCs in 24-h cultures, as shown by annexin V staining (**Fig. 1a**) and cell counts (data not shown).

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The ability of TSLP to up-regulate HLA-DR and costimulatory molecules was blocked by two neutralizing monoclonal antibodies (mAbs)—5E5 and 12F3—that were specific for human TSLP (**Fig. 1b**). The two TSLP mAbs did not block the up-regulation of HLA-DR and costimulatory molecules on DCs induced by CD40L, IL-7 or LPS (data not shown). These data indicated that the observed effects of TSLP on CD11c$^+$ DCs were TSLP-specific. Morphologically, both TSLP-activated DCs (referred to hereafter as TSLP-DCs) and CD40L-activated DCs (referred to hereafter as CD40L-DCs) showed long dendrites and expressed more HLA-DR and DC–lysosome-associated membrane protein (DC-LAMP, which is a DC activation marker) compared to DCs in medium alone or IL-7–activated DCs (referred to hereafter as IL-7–DCs) (**Fig. 1c**).

TSLP-DCs induce CD4$^+$ T cell expansion

Compared to CD40L-DCs, LPS-activated DCs (referred to hereafter as LPS-DCs) or IL-7–DCs, TSLP-DCs induced stronger naïve CD4$^+$ T cell proliferation in an allogeneic mixed lymphocyte reaction (**Fig. 2a**). At a 1:150 ratio of DCs:T cells, TSLP-DCs still induced allogeneic naïve CD4$^+$ T cell proliferation that was about three-times stronger than that induced by CD40L-DCs (**Fig. 2a**). After 6 days of culture, TSLP-DCs induced a 7.5- to 9-fold increase in total T cell numbers, which was more than that induced by CD40L-DCs, LPS-DCs or IL-7–DCs (**Fig. 2b**). Therefore, human TSLP represents one of the most potent DC activation factors, and TSLP-DCs induce the most marked allogeneic naïve CD4$^+$ T cell proliferation and expansion.

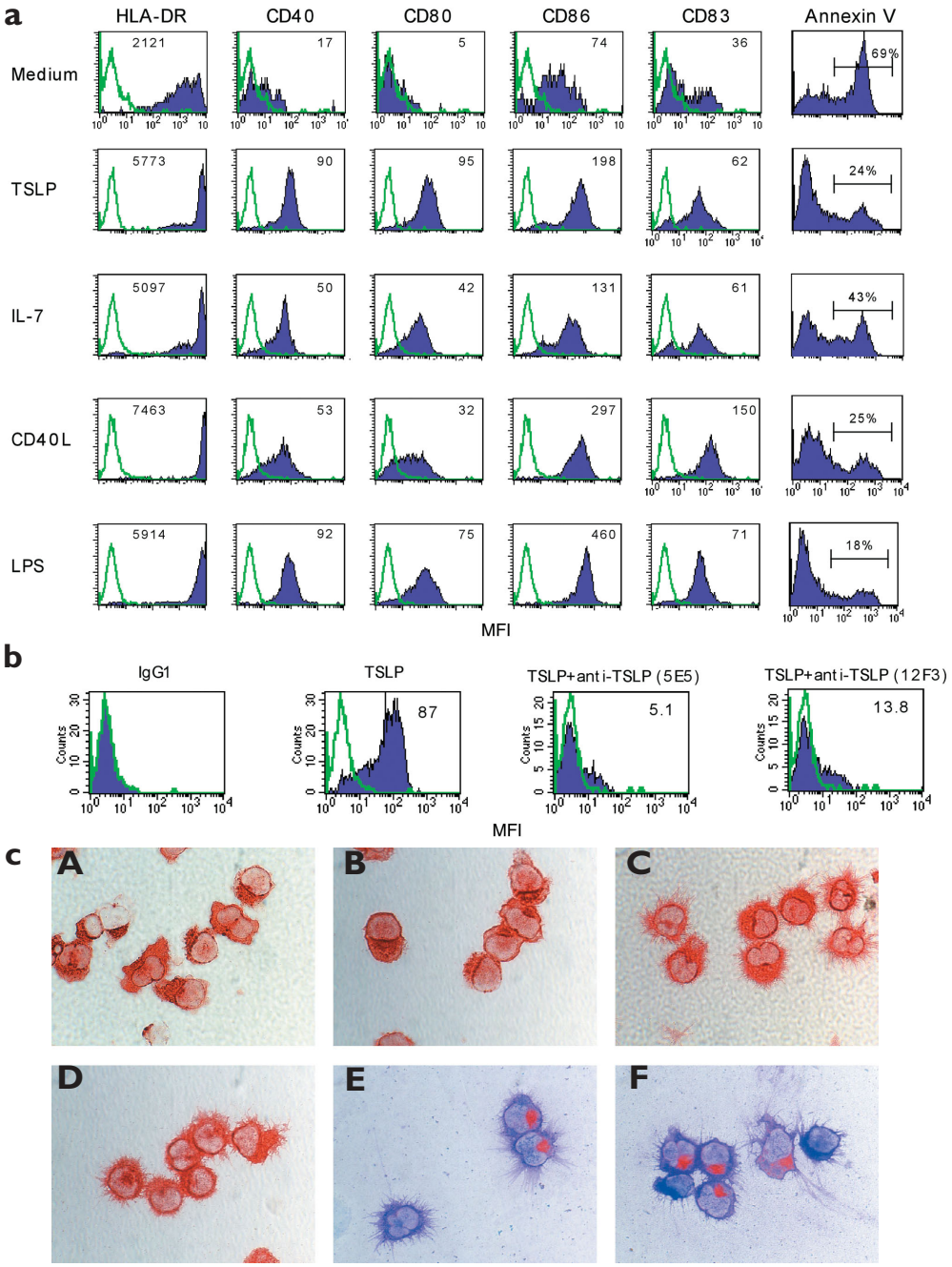

Figure 1. TSLP potently activates CD11c$^+$ DCs and maintains their survival. (**a**) TSLP strongly up-regulates HLA-DR, CD40, CD80, CD86 and CD83 compared to medium alone, and it potently up-regulates CD40 and CD80 expression compared to other DC activators (CD40L, IL-7 and LPS). Filled histograms represent staining of DC activation markers; open histograms represent the isotype control. Numbers indicate the mean fluorescence intensity (MFI). The percentage of annexin V$^+$ apoptotic DCs was also greatly reduced after 24 h of culture with TSLP (24%), CD40L (25%) and LPS (18%) compared with those cultured with medium (69%) or IL-7 (43%). Data represent one of six independent experiments. (**b**) Surface CD80 expression by DCs is strongly induced by TSLP, which is specifically blocked by rat anti-TSLP (mAbs 5E5 and 12F3). Numbers indicate the MFI for CD80 expression. Data represent one of three independent experiments. (**c**) Morphological analysis of DCs on cytospins. Major histocompatibility complex (MHC) class II staining of CD11c$^+$ DCs cultured with (**A**) medium (**B**) IL-7 (**C**) CD40L or (**D**) TSLP. MHC class II (blue) and DC-LAMP (red) double-staining of DCs cultured with (**E**) CD40L or (**F**) TSLP.

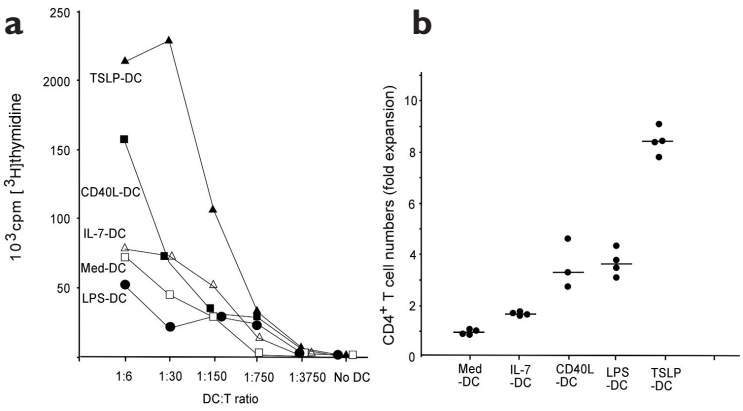

Figure 2. Naïve CD4$^+$ T cell proliferation and expansion with DCs activated by TSLP and other activators. (**a**) TSLP-DCs induced the strongest CD4$^+$ T cell proliferation after 5 days of culture, as assessed by [^{3}H]thymidine incorporation both at high (1:6) and low (1:150) DC:T ratios. (**b**) TSLP-DCs induced the highest CD4$^+$ T cell expansion after 6 days of culture. Results are expressed as fold expansion compared to the initial T cell number (50,000 cells). Data represent five independent experiments; horizontal bars indicate the median.

TSLP-DCs induce T_H2 development

Most DC activation signals, such as CD40L and LPS, induce DCs to produce the proinflammatory cytokines IL-1α/β, IL-6 and IL-12[20,21] and prime naïve CD4$^+$ T cell differentiation towards T_H1[22–24]. We performed a global quantitative mRNA screening of 11 different cytokines—IL-1α, IL-1β, IL-4, IL-6, IL-10, IL-12p35, IL-12p40, IL-13, IL-18, IL-23p19 and TNF-α—and 12 different chemokines— thymus and activation-regulated chemokine (TARC also known as CCL17), DCCK1, macrophage-derived chemokine (MDC or CCL22), monocyte chemoattractant protein 1 (MCP-1 or CCL2), MCP-2 (or CCL8), MCP-3 (or CCL7), MCP-4 (or CCL13), eotaxin (or CCL11), macrophage inflammatory protein (MIP-3β or CCL19), monokine induced by γ interferon (MIG or CXCL9), RANTES (or CCL5) and IL-8 (or CXCL8)—which have potential effects either on naïve CD4$^+$ T cell polarization or the migration of T_H1 or T_H2 cells. Unlike CD40L-DCs and LPS-DCs, TSLP-DCs did not produce mRNA for all the proinflammatory cytokines tested, but did produce high levels of mRNA for the chemokines TARC and MDC (data not shown). Enzyme-linked immunosorbent assay (ELISA) analyses confirmed at the protein level that TLSP-DCs did not produce detectable amounts of the proinflammatory cytokines IL-1β, IL-6, IL-12p70 and TNF-α, but did produce high amounts of the chemokines TARC and MDC (**Fig. 3**). TARC and MDC preferentially attract CCR4-expressing T_H2 cells[25].

The capacity of TSLP-DCs to polarize naïve CD4$^+$ T cells was compared to DCs cultured with medium, IL-7, CD40L or LPS. Naïve human CD4$^+$CD45RA$^+$ T cells purified from adult peripheral blood were cultured with DCs at a 1:5 ratio for 6 days; they were then washed to remove all cytokines, restimulated for 24 h with anti-CD3 and anti-CD28 and then cytokine production was measured in the culture supernatant by ELISA. TSLP-DCs induce naïve CD4$^+$ T cells to produce large amounts of IL-13, IL-5 and TNF-α and a moderate amount of IL-4 (**Fig. 4a**). Compared to DCs cultured with medium alone or other activators, TSLP-DCs induced naïve CD4$^+$ T cells to produce the lowest amounts of the anti-inflammatory cytokine IL-10 and the T_H1 cytokine IFN-γ (**Fig. 4a**). The ability of TSLP-DCs to induce naïve CD4$^+$ T cells to produce high IL-13 and TNF-α, moderate IL-4 and low IFN-γ and IL-10 was confirmed by intracellular cytokine staining (**Fig. 4b**). Therefore, TSLP-DCs induced naïve CD4$^+$ T cells to produce a unique set of cytokines that was distinct from a T_H1 profile (IFN-γ) or a classical T_H2 profile (IL-4, IL-5 and IL-10). Compared with CD4$^+$ T cells activated by DCs cultured in medium, IL-7–DCs, CD40L-DCs or LPS-DCs, CD4$^+$ T cells primed with TSLP-DCs produced the highest amounts of TNF-α, one of the most potent proinflammatory cytokines. In contrast, TSLP-DCs inhibited IL-10 as well as IFN-γ production by CD4$^+$ T cells.

Therefore, TSLP-DCs may induce robust T_H2 allergic inflammation by inducing naïve CD4$^+$ T cells to produce large amounts of IL-13 and IL-5 and a moderate amount of IL-4 in the presence of TNF-α and in the absence of two physiologic inhibitors of T_H2 inflammation, IL-10 and IFN-γ[26,27]. In addition, TSLP-DCs may further enhance T_H2-mediated inflammation by producing chemokines such as TARC and MDC, which may preferentially recruit T_H2 cells into the original inflamed tissues[28–30].

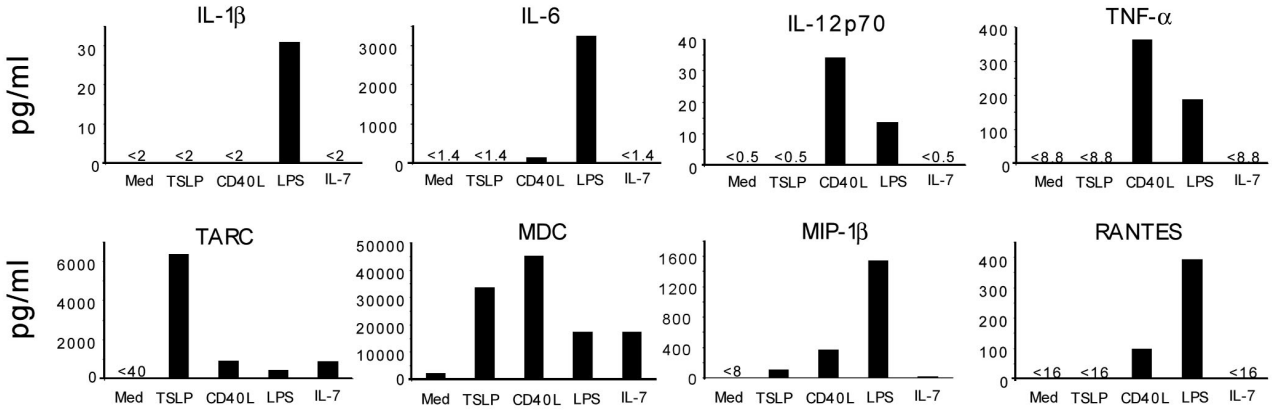

Figure 3. Cytokine and chemokine production by DCs activated with TSLP. TSLP-DCs did not produce inflammatory cytokines compared to CD40L-DCs or LPS-DCs, but produce high amounts of the T_H2-attracting chemokines TARC and MDC. Data represent one of five experiments.

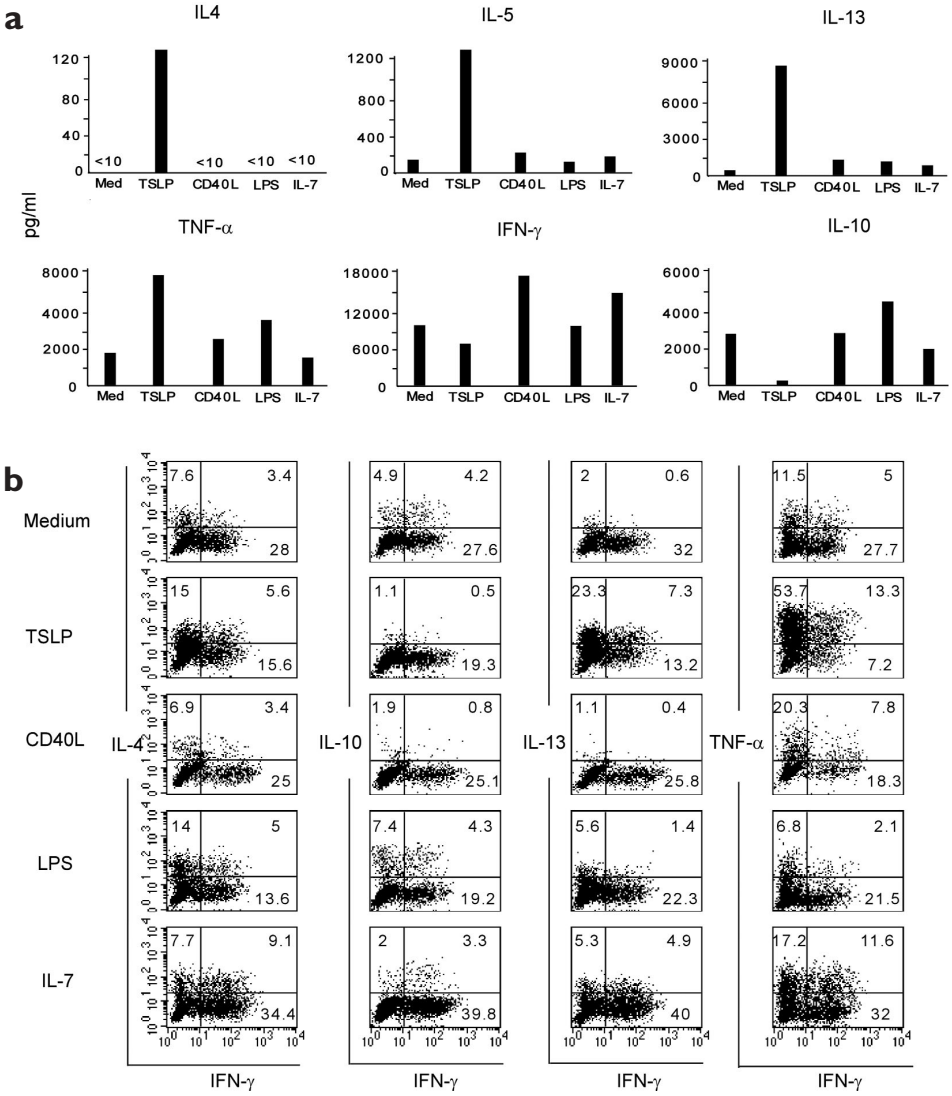

Figure 4. Cytokine production by naïve CD4⁺ T cells primed for 6 days with TSLP-DCs. (**a**) TSLP-DCs prime CD4⁺ T cells to produce the highest amounts of IL-4, IL-5, IL-13 and TNF-α, but lower amounts of IFN-γ and IL-10, compared to medium-activated DCs, CD40L-DCs, LPS-DCs or IL-7-DCs. Data represent one of six independent experiments. (**b**) Intracellular cytokine staining of T cells after 5-h restimulation with PMA and ionomycin shows similar results. Data represent one of three independent experiments.

TSLP protein expression by epithelial cells

Human tonsils contain two types of epithelium: crypt epithelium, which frequently harbors viruses and bacteria and represents the site of antigen-entry and constitutive inflammation, and squamous epithelium. Using the mAb 12F3, we showed that TSLP is expressed by crypt epithelial cells, which were in close contact with cells expressing DC-LAMP in five different tonsillar samples tested[31] (**Fig. 6**). In all tonsil samples tested, only a few small foci of TSLP expression were found within the apical part of the squamous epithelium (**Fig. 7**). The expression of TSLP was associated with the infiltration of DC-LAMP⁺-activated DCs (**Fig. 7a,b**) and the concurrent loss of langerin⁺ Langerhans cells within the squamous epithelium (**Fig. 7c,d**). Staining with 12F3 was specific for TSLP because recombinant TSLP, but not IL-7, completely blocked the staining and rat Ig isotype control antibody did not give any positive staining (data not shown). These results suggested that TSLP may contribute to constitutive inflammation within the crypt epithelium and sporadic inflammation within the squamous epithelium.

TSLP mRNA expression

To further understand the biology and pathophysiology of TSLP, expression of TSLP mRNA was analyzed by real-time quantitative polymerase chain reaction (PCR) in a panel of cDNA libraries from different cells or cell lines and a panel of purified primary cells (cell purity >99%) (**Fig. 5**). TSLP expression was not found in most hematopoietic cell types, including B cells, T cells, NK cells, granulocytes, macrophages, monocyte subsets and DC subsets; the exception was mast cells. Mast cells activated by mAbs that cross-link high-affinity IgE receptors express high amounts of TSLP. Human primary non-hematopoietic cells, such as skin keratinocytes, epithelial cells, smooth muscle cells and lung fibroblasts, cultured in growth medium, also expressed TSLP at high amounts, suggesting that these cells have the ability to produce TSLP (**Fig. 5**). Compared to cells cultured in medium, bronchial smooth muscle cells and skin keratinocytes activated by IL-4, IL-13 and TNF-α or TNF-α and IL-1β expressed higher TSLP. TSLP expression was not found in endothelial cells. Therefore, TSLP mRNA was mainly expressed by stromal cells, epithelial cells and mast cells, but not other hematopoietic cell types or endothelial cells.

High TSLP expression in atopic dermatitis

To investigate whether TSLP expression is associated with T_H2-type allergic inflammation *in vivo*, TSLP protein expression was analyzed in skin lesions, including atopic dermatitis (a T_H2-mediated allergic disease), nickel-induced contact dermatitis (an IFN-γ–producing T cell–mediated allergic disease) and cutaneous lupus erythematosus samples. Although TSLP was undetectable in normal skin (11 separate samples) (**Fig. 8a**), high expression of TSLP was found in the keratinocytes of acute (ten patients, **Fig. 8b,c**) and chronic atopic dermatitis (five patients, **Fig. 8d–f**). Isotype control–staining of an adjacent section (as shown in **Fig. 8d**) gave a negative result. Expression of TSLP was found mainly in keratinocytes of the apical layers of the epidermis, which ranged from small foci to the whole apical areas in both acute and chronic atopic dermatitis. The characteristics of these patients with atopic dermatitis are summarized (**Table 1**).

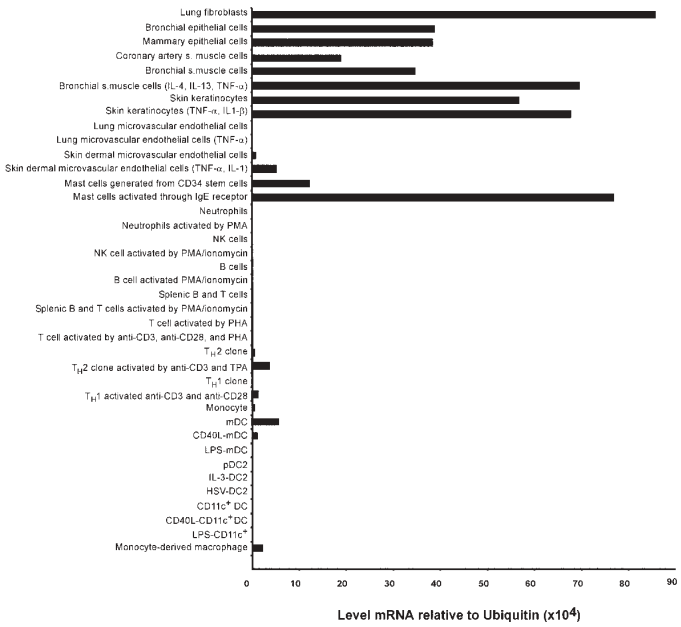

Figure 5. Quantification of TSLP mRNA levels in different human hematopoietic and stromal cell types. High levels of TSLP mRNA were detected in epithelial cells, different types of stromal cells and mast cells, but not in other hematopoietic cells.

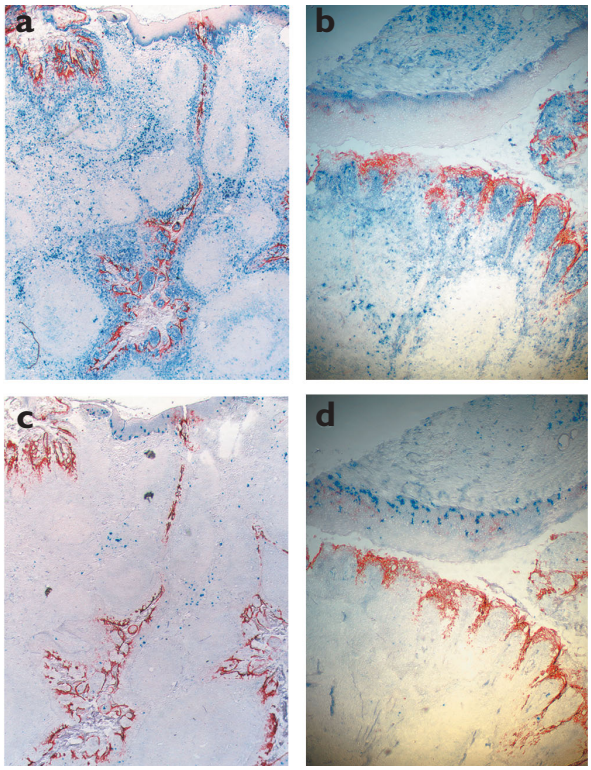

Figure 6. Expression of TSLP by crypt epithelial cells of human inflamed tonsils. (**a**,**b**) Double staining of TSLP (red) and DC-LAMP (blue). Expression of TSLP by crypt epithelial cells (red), which were in close association with DC-LAMP$^+$ lymphocytes and DCs (blue). (**a**) Magnification: ×100 (**b**) magnification: ×200. (**c**,**d**) Double staining of TSLP (red) and langerin (blue). TSLP expression (red) by crypt epithelial cells, but not by squamous epithelial cells characterized by the presence of langerin$^+$ Langerhans cells (blue). Langerin$^+$ Langerhans cells within the epidermis do not express DC-LAMP. (**c**) Magnification: ×100; (**d**) magnification: ×200.

Nonlesional skin samples were available for 9 of 15 atopic dermatitis patients. None of these samples stained positive for TSLP, which confirmed the specificity of TSLP for atopic dermatitis lesions and its absence from normal skin. Using Fisher's exact test, we were able to show that the difference between the normal skin group and atopic dermatitis group was statistically significant ($P < 0.001$). TSLP was not found in skin lesions from nickel-induced allergy contact dermatitis (**Fig. 8h**) or cutaneous lupus erythematosus (**Fig. 8i**) patients.

TSLP associates with Langerhans cell activation

To investigate whether TSLP expression in atopic dermatitis is associated with DC activation, TSLP was stained together with either langerin (the Langerhans cell marker) or DC-LAMP (the DC activation marker) in double immunohistology experiments (**Fig. 9**). In normal skin, or nonlesional skin of from atopic dermatitis samples, many langerin$^+$ Langerhans cells were found within the epidermis, but not within the dermis (**Fig. 9a**); and no DC-LAMP$^+$ activated DCs were found in either the epidermis or the dermis (**Fig. 9b**). Strong TSLP expression in atopic dermatitis was associated with the disappearance of langerin$^+$

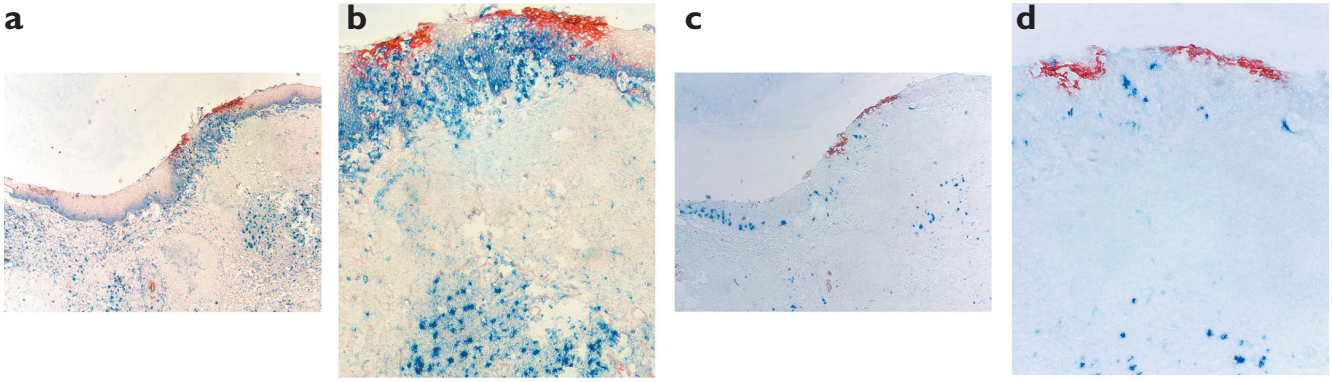

Figure 7. Sporadic expression of TSLP by squamous epithelial cells of inflamed tonsils is associated with the presence of activated DC-LAMP$^+$ DCs. (**a**,**b**) Double staining with TSLP (red) and DC-LAMP (blue), showing DC-LAMP$^+$ DCs within the epithelium area, which is positive for TSLP. (**c**,**d**) Double staining of TSLP (red) and langerin (blue), showing a decreased number of langerin$^+$ Langerhans cells within the TSLP-expressing epithelium.

Langerhans cells within the epidermis (**Fig. 9c**) and the concurrent appearance of many DC-LAMP$^+$ activated DCs within the dermis (**Fig. 9d**). Many of the DC-LAMP$^+$ activated DCs within the dermis expressed langerin, suggesting that epidermal Langerhans cells may be activated and migrate into the dermis (**Fig. 9d**). These results suggested that TSLP expression by keratinocytes in atopic dermatitis lesions may contribute directly to the activation of Langerhans cells, which may then migrate into the draining lymph nodes and prime allergen-specific T_H2 responses.

Table 1. Characteristics of patients with atopic dermatitis

Patient	TSLP[a]	Age	Sex	Surface area[b]	Other allergies	Prick test[c]	IgE[d]
			Acute atopic dermatitis[e]				
1	+++	23	M	50–75%	Rhinitis	+	1250
2	++	20	F	50–75%	Asthma	+	5350
3	++	40	M	75%	Rhinitis	+	45260
4	+++	34	M	NA[f]	NA[f]	NA[f]	NA[f]
5	+++	30	F	>75%	Asthma, rhinitis	+	795
6	++	66	F	25%	Rhinitis	+	2230
7	+++	53	F	35%	No[g]	+	99
8	++	25	M	40%	No[g]	+	NA[f]
9	+	40	F	3%	No[g]	+	983
10	+	21	F	85%	Rhinitis	+	NA[f]
			Chronic atopic dermatitis[h]				
11	+++	26	M	30%	No[g]	–	NA[f]
12	+++	49	M	5%	No[g]	–	210
13	+++	35	F	10%	No[g]	+	1207
14	+	24	M	15%	No[g]	+	NA[f]
15	+	45	F	15%	No[g]	+	4572

[a]Quantification of the TSLP staining on skin sections: weak and focal (+), intermediate (++), strong and diffused (+++). [b]Skin surface area involved at the time of biopsy. [c]For the prick test, + indicates a positive test to at least one allergen. [d]Total blood IgE concentrations (IU/ml): normal values are 0–200 IU/ml or 0–110 IU/ml, depending on the center. [e]Skin lesions were <1-week-old. [f]Not available. [g]Absence of other allergies. [h]Skin lesions were >1-week-old.

Discussion

We have shown here that the epithelial cells of skin and mucosa may directly interact with DCs during allergic inflammation by producing TSLP. Human TSLP is a DC activator that displays several unique features compared with other DC activation factors, such as CD40L, LPS or IL-7. It induced more CD40 and CD80 expression on DCs compared to other activators, activated DCs to induce strong naïve CD4$^+$ T cell proliferation and expansion, did not induce DCs to produce proinflammatory cytokines (instead it produced the T_H2-attracting chemokines TARC and MDC) and it endowed DCs with the ability to prime naïve CD4$^+$ T cells to produce large amounts of IL-13, IL-5 and TNF-α and moderate amounts of IL-4. However, expression of the anti-inflammatory cytokine IL-10 and the T_H1 cytokine IFN-γ were inhibited. These features suggested that TSLP represents a critical mediator in uncontrolled allergic inflammation.

Activation of DCs appears to be a critical step in the pathogenesis of T_H2-mediated allergic inflammation. Although DCs from allergic individuals preferentially induce a T_H2-type response[32–36], the molecular mechanism underlying the signaling of DCs to induce T_H2 allergic diseases is not understood. Our findings that TSLP is highly expressed by

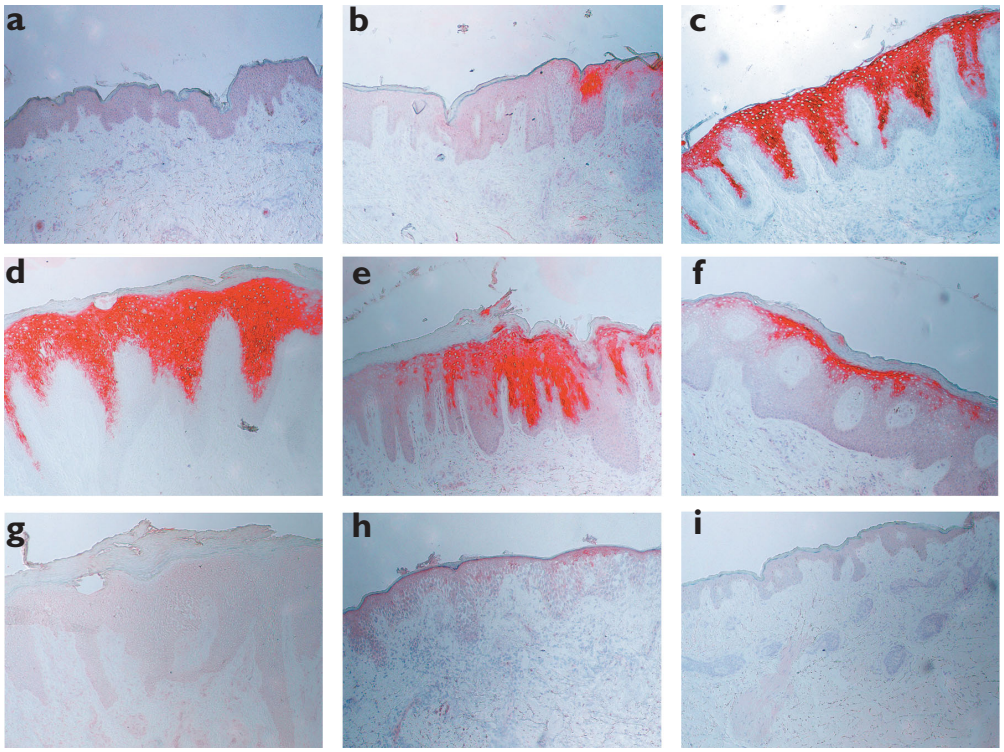

Figure 8. Expression of TSLP in atopic dermatitis. (**a**) Nonlesional skin from an atopic dermatitis patient. (**b**,**c**) Samples from acute atopic dermatitis patients. (**d–f**) Samples from chronic atopic dermatitis patients. (**g**) An adjacent section of **d**, showing negative staining with isotype control. (**h**) Lesion from a nickel-induced contact allergic dermatitis patient. (**i**) Lesion from a cutaneous lupus erythematosus patient. TSLP stained red.

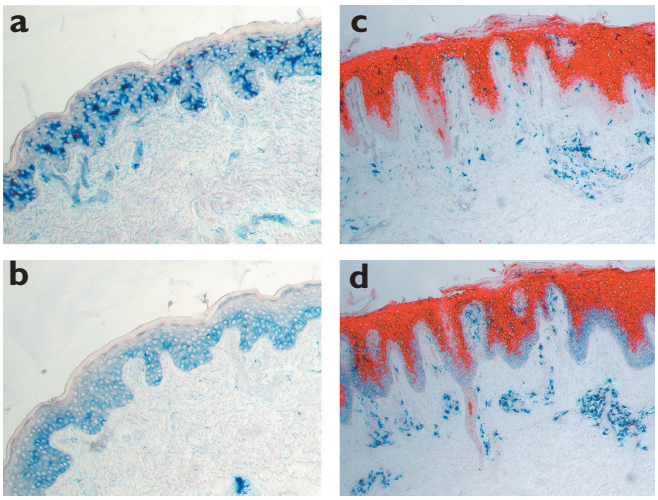

Figure 9. TSLP expression in atopic dermatitis associates with Langerhans cell migration and activation. (**a**) Double staining of TSLP (red) and DC-LAMP (blue) with a normal skin sample, showing no TSLP expression and no DC-LAMP$^+$ activated DCs within the epidermis or dermis. (**b**) Double staining of TSLP (red) and langerin (blue), showing the presence of many Langerhans cells within the epidermis. (**c**) High TSLP expression in atopic dermatitis skin lesion samples, which is associated with the presence of many activated DC-LAMP$^+$ DCs in epidermis and dermis. (**d**) Many of the DC-LAMP$^+$ DCs within the dermis in **c** express langerin.

keratinocytes of atopic dermatitis and TSLP-DCs strongly prime naïve CD4$^+$ T cells to produce IL-13, IL-5 and TNF-α suggest that TSLP likely represents the missing critical factor, which is key to understanding the pathogenesis of allergic diseases. TSLP produced by epithelial cells, or other stromal cells at the site of antigen entry, may activate DCs and stimulate them to produce T_H2-attracting chemokines such as TARC and MDC. Migration of TSLP-DCs to the draining lymph nodes would induce allergen-specific T cell proliferation and differentiation into T_H2 cells. These allergen-specific T_H2 T cells may then migrate back towards TARC and MDC, within the original site of inflammation, to trigger allergic inflammation. Indeed, T cell infiltration was found in skin samples from all the atopic deremititis patients tested in another study[37]. In addition, skin-homing T cells from atopic dermatitis produce T_H2 cytokines[38]. Therefore, our work provides a direct functional link between epithelial cells, DCs and T cell–mediated immune responses.

Unlike classical T_H2 cells, which produce IL-4, IL-5, IL-10 and IL-13, human CD4$^+$ T cells primed with TSLP-DCs produce high IL-13 and IL-5, moderate IL-4 but little IL-10. Although, historically, IL-10 has been included as a T_H2 cytokine[27], its contribution to the T_H2-mediated allergic inflammation has been controversial. Some studies showed that IL-10 mRNA levels in the lung, gut and skin were increased in patients with allergic asthma or atopic dermatitis[39]. However, direct measurement of IL-10 protein by ELISA showed markedly lower amounts of IL-10 in the bronchealveolar lavage or in the culture supernatants of activated peripheral blood mononuclear cells from atopic patients compared to normal control subjects[40]. Studies in mouse models confirm a role for IL-10 in suppressing airway inflammation and cytokine production[41,42]. Therefore, high concentrations of IL-13, IL-5 and TNF-α, moderate amounts of IL-4 and decreased production of IL-10 and IFN-γ by TSLP-DCs activated T cells and may represent the real allergic inflammatory cytokines that underlie the pathophysiology of atopic dermatitis or asthma.

Historically, the study of allergic diseases has been focused on the effector cells, such as mast cells and eosinophils. T_H2 cells and DCs are implicated as playing key roles in the upstream steps of allergic inflammation. Our study indicates that epithelial cells may well provide the initial trigger of the allergic immune cascade. Epithelial cell–derived TSLP not only potently activates DCs, but also endows DCs with the ability to polarize naïve T cells to produce proallergic T_H2 cytokines. TSLP may represent a new target to block inflammatory diseases, in particular allergic diseases.

Methods

DC purification and culture. CD11c$^+$ DCs were purified from the adult blood buffy coats of healthy volunteer blood donors (Stanford Medical School Blood Center, Stanford, CA) after separation of peripheral blood mononuclear cells (PBMC) by Ficoll centrifugation and negative depletion of cells that were expressing CD3, CD14, CD19, CD56 and glycophorin A with magnetic beads (Dynal, Oslo, Norway). Depleted cells were further stained with tricolor(TC)-conjugated anti-CD4 (Caltag, Burlingame, CA), phycoerythrin (PE)–anti-CD11c, anti-CD3, anti-CD14 and fluorescein isothiocyanate (FITC)–anti-CD16 (Becton Dickinson, Franklin Lakes, NJ). FITC-CD11c$^+$ cells were isolated with a Vantage FACsorter (Becton Dickinson) to reach >99% purity. CD11c$^+$ DCs were cultured immediately after sorting in RPMI containing 10% fetal calf serum (FCS), 1% pyruvate, 1% HEPES and penicillin-streptomycin. Cells were seeded at 0.5×10^6/ml in flat-bottomed 96-well plates in the presence of TSLP (15 ng/ml), IL-7 (50 ng/ml), LPS (1 μg/ml), CD40L-transfected L-fibroblasts (2.5×10^4/well) or culture medium alone.

DC activation and viability. After 24 h of culture, DCs were collected and resuspended in an EDTA-containing medium to dissociate the clusters. Viable DCs were first counted with trypan blue exclusion of dead cells. Remaining cells were stained with FITC-conjugated mouse anti-human mAbs that included anti–HLA-DR (Becton Dickinson), anti-CD40, anti-CD80 and anti-CD86 (all from Pharmingen, San Diego, CA) and an IgG1 isotype control (Becton Dickinson); they were analyzed with a FACScan flow cytometer (Becton Dickinson). Dead cells were excluded based on side- and forward-scatter characteristics. For apoptosis detection, cells were stained for 5–10 min with FITC–annexin V (Promega, Madison, WI) and analyzed on a FACScan flow cytometer (Becton Dickinson) without dead-cell exclusion.

DC cytokine production. DC culture supernatants were collected at 24 h, frozen at –80 °C and analyzed within 3 months with protein ELISA kits for IL-1β, IL-6, IL-12p70, TNF-α, TARC, MDC, MIP-1β and RANTES (all from R&D Systems, Minneapolis, MN).

DC–T cell cocultures. CD11c$^+$ DCs were collected after 24 h of culture under different conditions, washed twice to remove any cytokine and cocultured with 5×10^4 freshly purified allogeneic naïve CD4$^+$ T cells in round-bottomed 96-well culture plates. Cultures were done in triplicate at increasing DC:T cell ratios. DCs and T cells alone were used as controls. After 5 days, cells were pulsed with 1 mCi [^{3}H]thymidine (Amersham Biosciences, Piscataway, NJ) for 16 h before collecting and counting radioactivity.

T cell cytokine production. After 6 days of coculture, DC-primed CD4$^+$ T cells were restimulated for 24 h with plate-bound anti-CD3 (10 μg/ml) and soluble anti-CD28 (2 ng/ml). Cytokine production was assessed in the culture supernatant by protein ELISA for IL-4, IL-5, IL-10, IL-13, TNF-α and IFN-γ (all from R&D Systems). For intracellular cytokine production, T cells were collected on day 6 of the culture, washed twice and restimulated with PMA + ionomycin in flat-bottomed 96- or 48-well plates at a concentration of 1×10^6/ml. After 2.5 h, brefeldin A was added at 10 mg/ml. After 5 h, cells were collected, fixed with 2% formaldehyde, permeabilized with 10% saponin and stained with PE-conjugated mAbs to IL-4, IL-5, IL-10, IL-13 and TNF-α and FITC-conjugated anti–IFN-γ (all from Pharmingen). Stained cells were analyzed on a FACScan flow cytometer (Becton Dickinson).

Quantification of TSLP mRNA in human primary cells. Cryopreserved primary human fibroblasts, epithelial cells, smooth muscle cells, endothelial cells were from Clonetics (Biowhittaker, San Diego, CA), and seeded at 2500–3500 cells/cm^2 in T75 flasks with the appropriate fully supplemented culture medium (Biowhittaker). After two or three passages,

cells were collected by light trypsinization and reseeded in 12 or 6-well plates (5×10^5 cells/well) under different culture conditions, with or without addition of cytokines. After 12–15 h, culture supernatants were collected, adherent cells were lysed, mRNA was extracted with a Qiagen kit (Qiagen, Valencia, CA) and analyzed by real-time quantitative PCR. Mast cells were generated from human $CD34^+$ stem cells cultured with stem cell factor. Final culture contained >95% $CD117^+CD14^-CD16^-$ mast cells. Activation was initiated by cross-linking of the high-affinity IgE receptor with the DX55 mAb (J. Philips, DNAX, Palo Alto, CA). DCs and DC precursors (>99% purity) were isolated by cell-sorting peripheral blood cells as described[43]. The following freshly sorted peripheral blood cell subsets, before and after culture, were used for the Taqman-PCR analyses: monoctyes; monocyte-derived immature DCs (imDCs, monocytes cultured for 6 days with GM-CSF and IL-4); monocyte-derived mature DCs activated by CD40L (CD40L-mDC) or LPS (LPS-mDCs); $CD4^+Lin^-CD11c^-$ plasmacytoid DC precursors (pDC2); pDC2-derived DCs induced by IL-3 (IL-3-DC2) or by HSV-1 (HSV-DC2); $CD11c^+$ DCs ($CD11c^+$ DCs); $CD11c^+$ DCs activated by CD40L (CD40L-$CD11c^+$ DCs) or by LPS (LPS-$CD11c^+$ DCs); monocyte-derived macrophages (monocyte cultured for 6 d with M-CSF); and freshly isolated $CD68^+CD16^+$ neutrophils, $CD16^+CD56^+$ NK cells, $CD19^+$ B cells and $CD3^+$ T cells. For other cell types, cDNA libraries were prepared and used as templates for Taqman-PCR analyses as described[17]. Results are expressed as mRNA levels relative to ubiquitin.

Skin biopsy samples. After obtaining informed consent from patients, 3–6 mm punch biopsies were taken from either lesional or nonlesional skin from atopic dermatitis (n = 15) or disseminated lupus erythematosus patients (n = 5) or from normal healthy individuals (n = 11). Patients with a history of allergic contact dermatitis against nickel (n = 8) underwent diagnostic nickel patch tests, and skin biopsies (three per patient) were taken before and 6, 24 or 48 h after allergen exposure. Skin samples were immediately frozen in liquid nitrogen and stored at –80 °C. The study was approved by the local ethics committees of the Department of Medicine of the Helsinki-Uusimaa Hospital District, Finland and the Heinrich-Heine University, Dusseldorf, Germany.

Immunohistology. Frozen sections (8 μm) of human tonsil or skin were incubated with rat anti–human TSLP (mAb 12F3, DNAX, Palo Alto, CA) at room temperature for 1 h in PBS. The slides were washed with PBS twice and incubated with biotinylated secondary antibody for 30 min (PK-4004, Vector Laboratories, Burlingame, CA). The slides were washed and incubated with avidin-peroxidase complex reagents for 30 min (PK-4004, Vector Laboratories). The slides were washed and incubated with substrate SK-4200, which stained red (Vector Laboratories). For double staining of human TSLP with DC-LAMP or langerin, the slides were incubated with mouse anti–human DC-LAMP (IM3448) or mouse anti–human langerin (IM3449, Immunotech, Marseille, France) for 1 h; this was followed by red anti-TSLP staining. The slides were washed and incubated with biotinylated anti–mouse Ig (AK-5002, Vector Laboratories), then the avidin-peroxidase complex reagents (AK-5002, Vector Laboratories) for 30 min each. The slides were washed and the incubated with the substrate SK-5300, which stained blue (Vector Laboratories).

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Competing interests statement

The authors declare competing financial interests: see the *Nature Immunology* website (http://immunology.nature.com) for details.

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