

Flow cytometry imaging identifies rare T_H2 cells expressing thymic stromal lymphopoietin receptor in a “proallergic” milieu

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Background: Thymic stromal lymphopoietin (TSLP) is expressed at sites of allergic inflammation, including eczematous skin. This cytokine has been reported to exert its T_H2-inducing properties through dendritic cells. Expression of TSLP receptor on the surface of activated T_H2 cells could amplify T_H2 responses at inflamed sites through the direct actions of TSLP.

Objective: To test rigorously whether T_H2 cells induced by “proallergic” factors express TSLP receptor and characterize these cells using an experimental platform that combines flow cytometry with microscopic capabilities.

Methods: CD4⁺ T cells isolated from patients with atopic dermatitis or normal healthy controls were cocultured with autologous dendritic cells in the presence of T_H2-promoting stimuli (TSLP ± allergen and staphylococcal enterotoxin B ± TSLP). Surface expression of TSLP receptor was analyzed by image-based flow cytometry, and responsiveness of purified T cells to TSLP was assessed by phosphorylation of signal transducer and activator of transcription-5 and cytokine secretion.

Results: T_H2-promoting stimuli induced a robust population of activated T_H2 cells (CD25⁺IL-4⁺). Regardless of the nature of the stimulus, flow cytometry imaging confirmed that T cells expressing TSLP receptor were rare, constituting a minor fraction of the IL-4⁺ T cell pool; however, TSLP responsiveness was nonetheless detectable. Analysis of cell size and nuclear morphology revealed preferential expression of TSLP receptor on IL-4-expressing cells undergoing mitosis. Analysis of lesional skin in atopic dermatitis supported the view that rare IL-4⁺ T cells expressing TSLP receptor are present at inflamed sites.

Conclusion: In a “proallergic” milieu, TSLP receptor is preferentially expressed on rare actively dividing T_H2 cells. The

direct action of TSLP on T cells could amplify T_H2 responses at sites of allergic inflammation. (J Allergy Clin Immunol 2010;■■■:■■■-■■■.)

Key words: TSLP, TSLP receptor, atopic dermatitis, T_H2 cells, flow cytometry imaging

The cytokine thymic stromal lymphopoietin (TSLP) plays a key role in the pathogenesis of a variety of allergic diseases, including asthma and atopic dermatitis (AD).¹⁻⁵ TSLP exhibits potent T_H2-promoting properties based on its ability to differentiate T_H2 cells from naive CD4⁺ T-cell precursors in human beings.^{6,7} High expression of TSLP is a feature of keratinocytes in AD skin lesions,⁶ and TSLP-priming of dendritic cells (DCs) *in situ* may serve to induce or enhance T_H2 responses within the skin, as well as systemically. Consistent with this viewpoint, TSLP was originally reported to exert its T_H2-promoting properties through a DC-mediated pathway in human beings that involved induction of OX40 ligand on DCs.⁷

Disruption of the skin barrier in AD provides a portal of entry for antigens that drive T_H2 responses associated with this disease.^{8,9} Skin DCs also likely play a pivotal role in this process by sampling antigens at the subepithelial interface.¹⁰ We recently reported that co-priming DCs with TSLP and an allergen variant targeted to DCs enhanced T_H2 responses *in vitro* compared with either stimulus alone in cultures from atopic subjects.¹¹ This suggested that TSLP and allergen provide a “proallergic” milieu that favors the induction of a robust T_H2 response.

TSLP receptor is a heterodimer made up of the IL-7 receptor α chain and the TSLP receptor (TSLPr) chain.^{12,13} Although TSLPr was originally thought to be a key feature of CD11c⁺ myeloid DCs, this receptor was recently reported on activated human T cells, and these cells had the capacity to respond directly to TSLP.¹⁴ This observation made us question whether a T-cell-mediated TSLP pathway could contribute to T_H2 responses in an inflammatory milieu that includes TSLP. In accordance with this theory, TSLP has been shown to enhance T_H2 cytokine secretion by skin-infiltrating CD4⁺ T cells in a mouse model of allergic skin inflammation in a manner dependent on TSLPr expression on T cells, but not DCs.¹⁵

It is not known whether TSLPr is expressed on human T_H2 cells. The current study was designed to test rigorously whether TSLPr is expressed on these cells in the presence of a “proallergic” milieu. Using flow cytometry imaging, we found that TSLPr is preferentially expressed on rare actively dividing T_H2 cells. The functional relevance and implications for allergic inflammation are addressed.

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Abbreviations used

AD: Atopic dermatitis
 DC: Dendritic cell
 HDM: House dust mite allergen
 SEB: Staphylococcal enterotoxin B
 STAT: Signal transducer and activator of transcription
 TSLP: Thymic stromal lymphopoietin
 TSLPr: Thymic stromal lymphopoietin receptor

METHODS**Human subjects**

Subjects were recruited from the University of Virginia Allergic Diseases Clinic or by advertisement. Patients with AD reported physician-diagnosed eczema and fulfilled the diagnostic criteria of Hanifin and Rajka¹⁶ for AD. All patients had moderate-to-severe disease based on the SCORAD index,¹⁷ markedly elevated serum titers of total IgE (>400 IU/mL), and high titer IgE Ab to dust mite allergen (CAP ≥ 0.7 IU/mL). Subjects with dust mite allergy without AD had high titer IgE Ab to dust mite allergen (CAP ≥ 0.7 IU/mL). Healthy nonatopic controls had no measurable serum IgG or IgE antibodies to common allergens. All studies were approved by the University of Virginia Human Investigations Committee.

Cells and reagents

See this article's Methods section in the Online Repository at www.jacionline.org.

Cell cultures for flow cytometry imaging studies

Blood DCs (1×10^5) were cocultured in 24-well plates with autologous CD4⁺ T cells (4×10^5 cells per well) in complete medium containing 10% autologous serum.¹⁸ Cultures were stimulated with staphylococcal enterotoxin B (SEB; 20 ng/mL) $\pm$ TSLP (50 ng/mL) for 3 days, or with TSLP (50 ng/mL) $\pm$ house dust mite allergen (HDM; 20 μ g/mL house dust mite extract containing 10 μ g/mL Der p 1) for 7 days. In some experiments, THP-1 cells were pulsed for 24 hours with H22-Fel d 1 (10 μ g/mL) in medium containing 10% autologous serum.

Flow cytometry imaging

Immunostaining of cells. After culture, T cells or THP-1 cells were stained for surface markers and washed to remove excess antibody. Cells were then fixed and permeabilized using Caltag Fix and Perm kit (Invitrogen, Carlsbad, Calif) followed by staining for intracellular IL-4 (T cells only) and DNA (T cells and THP-1 cells). Before staining for IL-4, T cells were restimulated with phorbol 12-myristate 13-acetate (50 ng/mL; Fisher Scientific, Pittsburgh, Pa) and ionomycin (2 μ g/mL; Invitrogen) on the final day of culture for 5 hours, followed by addition of brefeldin A (BD Biosciences, San Jose, Calif) for 4 hours. The purity of CD4⁺ T cells harvested from coculture assays was determined to be 96% to 99% on the basis of forward and side scatter gating in conjunction with CD3 staining using standard flow cytometry.

Analysis. Image-based flow cytometry was performed on an Imagestream100 (Amnis Corp, Seattle, Wash) using the manufacturer's collection program, INSPIRE.¹⁹ Fluorometric compensation was digitally calculated by analysis software (IDEAS; Amnis Corp) on the basis of single-stain controls. The data were analyzed according to the manufacturer's instructions by use of IDEAS software. Briefly, debris and cell aggregates were excluded and single cells were identified after compensation by gating on cell area and aspect ratio followed by visual inspection. After identifying single cells, cell integrity was confirmed by morphology and DNA staining. Identification of TSLPr⁺ cells was based on selection of distinct cell populations above background fluorescence and confirmed visually by the appearance of a circumferential fluorescence signal.

Analysis of intracellular and surface markers by standard flow cytometry

See the Methods in the Online Repository.

Analysis of the direct effects of TSLP on T cells

See the Methods in the Online Repository.

Assay for secreted cytokines

See the Methods in the Online Repository.

Statistical analysis

See the Methods in the Online Repository.

RESULTS**Validation of anti-TSLPr mAbs by image-based flow cytometry**

Initially, we compared the surface staining properties of 2 commercially available anti-TSLPr monoclonal antibodies. To do this, THP-1 monocytes were activated using an allergen variant that upregulates TSLPr on DCs.¹¹ Cells were stained with different anti-TSLPr clones and analyzed by image-based flow cytometry. In dot plots, activated THP-1 cells staining for TSLPr were readily detectable (30.9% for clone 1A6 and 36.5% for clone 1B4; Fig 1, A). For both clones, visual inspection of cells within the TSLPr⁺ gate confirmed circumferential staining for TSLPr similar to that for HLA-DR (Fig 1, B). Overlaying digital images for surface and nuclear markers revealed a circumferential band of TSLPr staining consistent with surface staining (Fig 1, C). The specificity of each antibody for TSLPr staining as judged microscopically was equivalent. Visual inspection of all THP-1 cells within the TSLPr⁺ gate revealed 82% and 88% true positives for cells stained with clones 1A6 and 1B4, respectively. These experiments validated surface staining using anti-TSLPr antibodies and demonstrated the ability to enumerate TSLPr⁺ cells accurately by using image-based flow cytometry. All subsequent experiments were performed with anti-TSLPr clone 1A6 because this antibody, combined with other available fluorochrome conjugates, yielded an optimal quality image.

T_H2-promoting factors induce activated T_H2 cells

Initial experiments using standard flow cytometry showed that costimulating T cells from atopic subjects with TSLP and HDM selectively amplified the induction of IL-4⁺ T cells compared with either stimulus alone. By contrast, there was no effect on IFN- γ ⁺ or IL-10⁺ T cells. Moreover, only a small fraction of IL-4⁺ T cells coexpressed IFN- γ (see this article's Fig E1, A, in the Online Repository at www.jacionline.org). This confirmed our previous findings indicating that costimulating T cells with TSLP and allergen provides a potent T_H2-promoting milieu.¹¹ Next, we tested the capacity for TSLP and HDM to induce T_H2 cells by using image-based flow cytometry. Subjects selected for study were highly atopic patients with AD and healthy nonatopic controls (see this article's Table E1 in the Online Repository at www.jacionline.org). Stimulation of T cells with TSLP alone induced a robust population of cells in all subjects that expressed IL-2R α chain (CD25), a molecule expressed on activated T cells (Fig 2, A). Costimulation with TSLP and allergen amplified the response, although this effect was variable and not related to atopic status (Fig 2, A). On visual inspection, the majority of CD25⁺ T cells induced under T_H2-promoting conditions expressed IL-4;

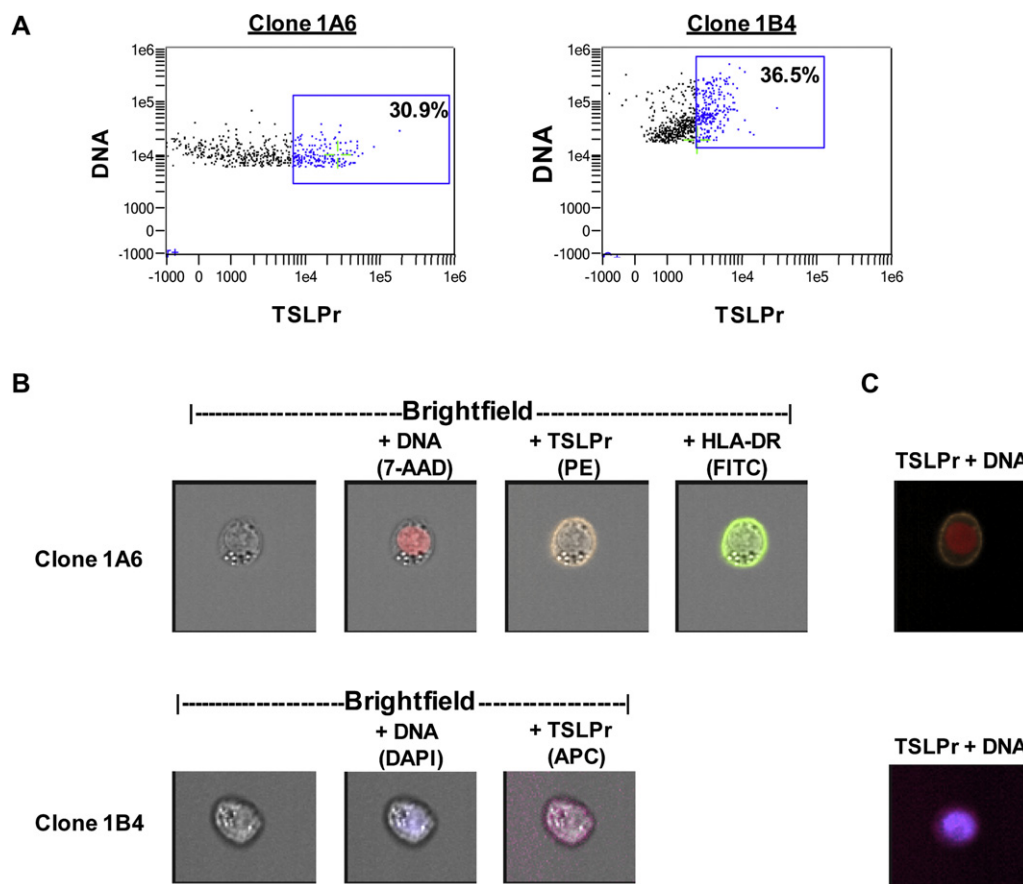


FIG 1. Validation of anti-TSLP receptor mAbs by image-based flow cytometry. Activated THP-1 monocytes were stained for TSLPr, HLA-DR, and DNA. **A**, Dot plots represent gated focused single cells with DNA negative cells excluded. Boxes denote TSLPr⁺ gates determined by visual inspection of single cells inside the gate versus cells outside the gate. Green crosshairs denote single cells shown in **B** and **C**. **B**, Single-cell images of TSLPr⁺ cells using different anti-TSLPr clones. Images for each stain are overlaid on the brightfield image. **C**, Composite images corresponding to cells shown in **B**. FITC, Fluorescein isothiocyanate; PE, phycoerythrin.

however, the intensity of cytoplasmic staining for IL-4 varied considerably within the CD25⁺IL-4⁺ gate, as evidenced by the presence of clearly distinguishable IL-4^{hi} and IL-4^{lo} cells (Fig 2, B). Analysis of secreted cytokines confirmed a T_H2-dominated cytokine profile in cultures stimulated with TSLP + allergen as judged by 6 to >100-fold levels of IL-5 or IL-13 compared with IFN- γ (Fig E1, B). Interestingly, IL-10 was also induced; however, intracellular staining of T cells coupled with production of peak levels at day 3 indicated that DCs were the source of this cytokine (Fig E1, B, and data not shown).

TSLPr⁺ T cells are rare under T_H2-promoting conditions

Next, TSLPr expression was analyzed on T cells. The strategy involved setting a TSLPr⁺ gate for total CD4⁺ T cells by identifying a threshold of staining intensity based on visual confirmation of bona fide TSLPr⁺ cells with a circumferential pattern of staining (Fig 3, A and B). Each cell within this gate was then visually inspected to confirm whether surface TSLPr was expressed to enumerate rare TSLPr⁺ T cells accurately. By this approach, analysis of unstimulated cells on day 7 revealed a very low frequency of TSLPr⁺ cells (mean = 0.02 $\pm$ 0.02%). Stimulation

with TSLP or TSLP + allergen enhanced the frequency of these cells in all subjects tested (mean = 0.21 $\pm$ 0.11%, P = .015, and 0.30 $\pm$ 0.25%, P = .061, respectively; Fig 3, C). Nonetheless, TSLPr⁺ cells accounted for <0.7% of total T cells. Notably, TSLPr⁺ cells constituted only a small fraction of cells within the TSLPr⁺ gate (<15%), with false-positive events accounting for the remainder (see this article's Fig E2 in the Online Repository at www.jacionline.org). These observations confirmed that TSLPr⁺ T cells are rare under T_H2-promoting conditions.

To recapitulate the *in vivo* microenvironment, TSLP was present for the duration of culture *in vitro*. Because cytokine receptor expression may be downregulated by the presence of its ligand, it was important to exclude receptor modulation in our system. No TSLPr was detected on CD4⁺ T cells isolated directly *ex vivo* from subjects with or without AD (data not shown). Time course studies performed by using standard flow cytometry showed that costimulation with TSLP + allergen induced TSLPr expression as early as 24 hours. Notably, TSLPr⁺ cells were predominantly CD25^{neg} or CD25^{lo} early in culture with a trend toward increased expression of CD25 at day 7 (see this article's Fig E3 in the Online Repository at www.jacionline.org). These findings indicate that TSLPr expression precedes upregulation of CD25 on activated T cells and persists in the presence of its ligand.

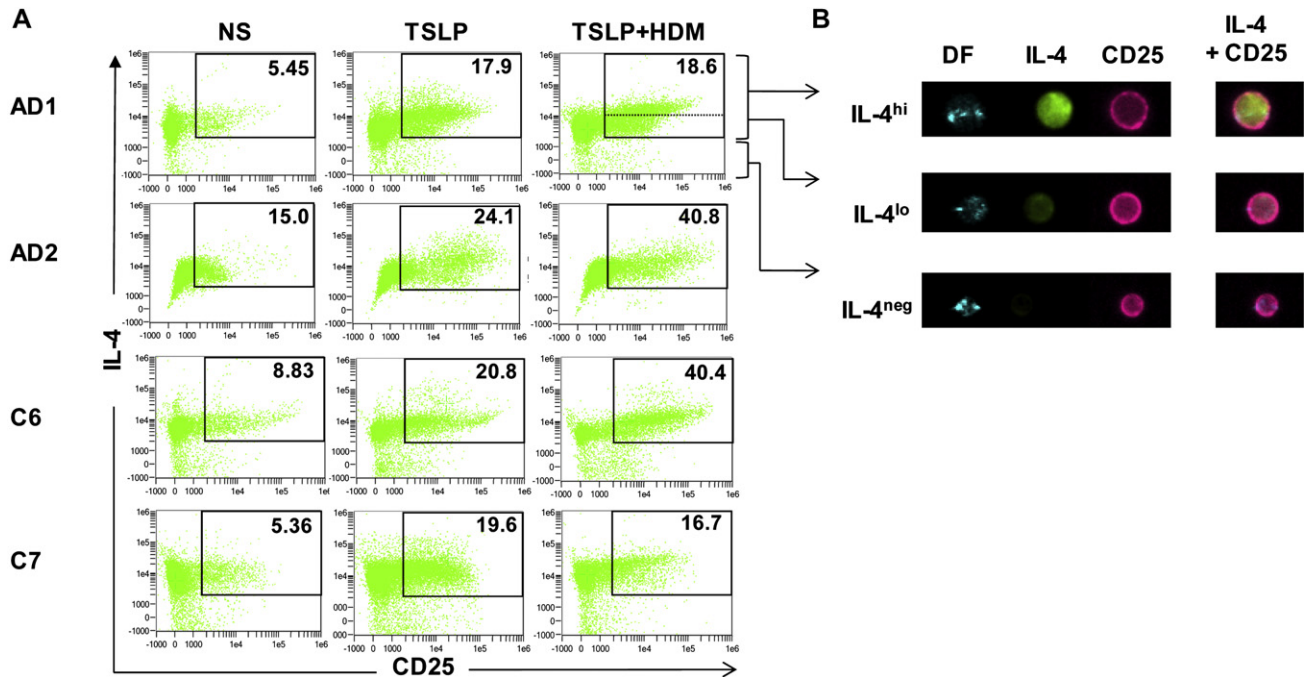


FIG 2. T_H2-promoting factors induce activated T_H2 cells. **A**, CD4⁺ T cells from DC/T-cell cocultures analyzed for expression of CD25 and IL-4 by image-based flow cytometry. Values denote percentages of CD25⁺IL-4⁺ T cells for each condition. Dotted line indicates the gate used to delineate IL-4^{hi} from IL-4^{lo} cells. NS, Not stimulated. **B**, Images of 3 representative T cells showing different staining intensity for IL-4 (green). Right, Composite images. DF, Darkfield. Data are representative of 5 subjects.

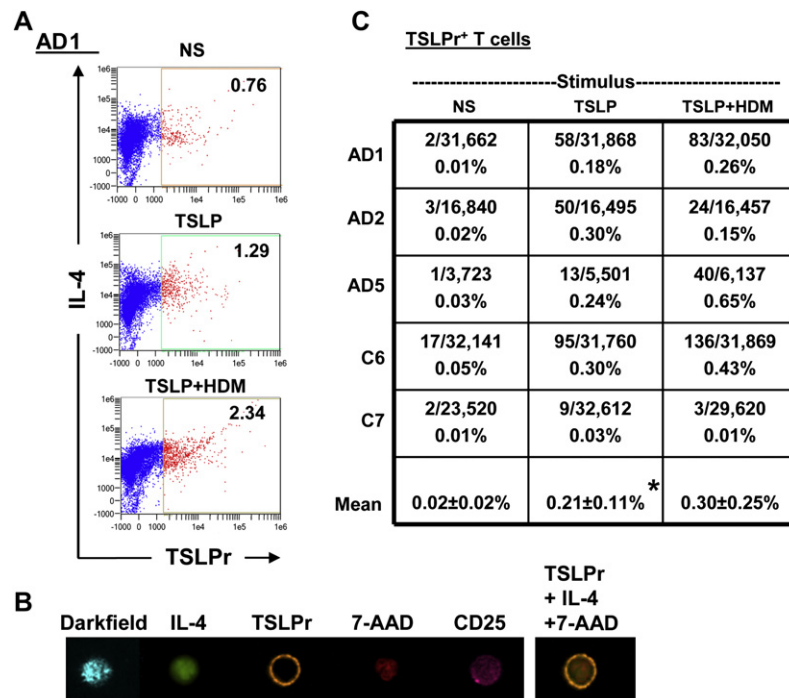


FIG 3. TSLPr⁺ T cells are rare under T_H2-promoting conditions. **A**, CD4⁺ T cells were stimulated and analyzed for TSLPr expression by image-based flow cytometry. Values denote the percentage of cells within a gate that captured all TSLPr⁺ events by visual inspection. NS, Not stimulated. Data are representative of 5 subjects. **B**, Image of a single representative TSLPr⁺ T cell with corresponding composite image. **C**, Table of actual frequencies of TSLPr⁺ T cells confirmed by inspection of cells (data from 5 subjects; **P* = .015).

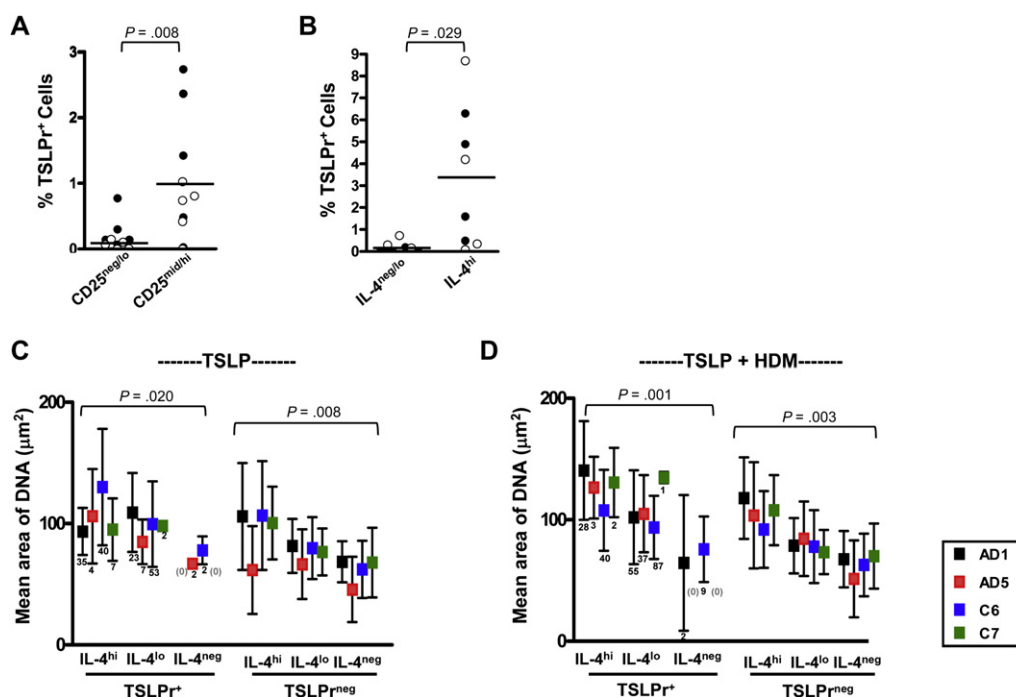


FIG 4. TSLPr⁺ T cells are enlarged and express high levels of CD25 and IL-4. **A and B,** Percentage of TSLPr⁺ T cells within CD25^{mid/hi} and CD25^{neg/lo} subsets (**A**) and IL-4^{hi} and IL-4^{neg/lo} subsets (**B**) after stimulation with TSLP (*closed symbols*) or TSLP + HDM (*open symbols*). Bars represent the mean ($n = 5$ subjects). **C and D,** Area of nuclear staining for IL-4^{hi}, IL-4^{lo}, and IL-4^{neg} subsets induced by TSLP (**C**) or TSLP+HDM (**D**) (mean $\pm$ SD). Numbers of TSLPr⁺ cells for each observation are shown. Data for TSLPr-negative subsets were compiled by using 200 cells randomly selected from each subset ($n = 4$ subjects).

TSLPr is a feature of actively dividing T_H2 cells

Surface TSLPr was further analyzed in the context of CD25 and IL-4 to determine whether this molecule was preferentially expressed on activated T_H2 cells. Under T_H2-promoting conditions, visual inspection confirmed that TSLPr⁺ cells were enriched within T-cell subsets expressing increased levels of CD25 ($P = .008$; Fig 4, A; see this article's Fig E4 in the Online Repository at www.jacionline.org). In addition, the majority of TSLPr⁺ cells induced under T_H2-promoting conditions expressed IL-4 (>93%), and these cells were enriched within the IL-4^{hi} subset, accounting for up to 8.7% of these cells ($P = .029$; Figs 4, B, and E4).

Because dividing T cells will stain more brightly for IL-4 as a result of increased cytoplasmic content, we postulated that IL-4^{hi} cells induced by T_H2-promoting factors represented dividing cells. Analysis of the area of nuclear staining confirmed a trend toward larger cell size with higher expression of IL-4 ($P = .008$ for TSLP alone and $P = .003$ for TSLP + allergen). A similar trend was observed for TSLPr⁺ cells (Figs 4, C and D). Collectively, these findings indicate that a large proportion of TSLPr⁺ cells is blasting T cells.

The relationship between IL-4 content, TSLPr expression, and cell division was further analyzed. A major advantage of flow cytometry imaging is that dividing T cells can be identified by the characteristic morphology of their mitotic nuclei. To avoid exclusion of dividing cells and thus capture all TSLPr⁺ dividing T-cell events, total cells were analyzed without first adjusting for cell area or nuclear aspect ratio. This strategy was adopted because elongated dividing cells with a lower nuclear aspect ratio

than nondividing spherical cells, would be excluded by preferentially selecting single cells based on a high nuclear aspect ratio. To avoid inclusion of doublets, which can result in artifactual single cells with high DNA content on scatter plot, cells with a low darkfield aspect ratio were excluded (≤ 0.4). This strategy excluded all doublets while retaining dividing cells. Two discrete populations were identified by dot plot based on DNA staining intensity (Fig 5, A, *gray spheres*). In the experiment shown (stimulus: TSLP + HDM), approximately one third of total TSLPr⁺ T cells (28/80) resided within a DNA^{hi}IL-4⁺ subset, and visual inspection indicated that these cells were blasting or actively dividing, as evidenced by their large size and the presence of mitotic nuclei characteristic of cells in early or late anaphase (Fig 5, A, *gate B*). Consistent with this, cells with high DNA staining intensity generally exhibited a lower nuclear aspect ratio (Fig 5, B, *gates A and B*). The majority of remaining TSLPr⁺ T cells showed lower DNA staining intensity; however, a disproportionately high number of these cells stained brightly for IL-4 and exhibited a lower nuclear aspect ratio indicating a mitotic phenotype (Fig 5, A and B, *gate E*). DNA imaging of these cells revealed intact nuclei, suggesting that the high IL-4 content masked DNA staining (see representative images for *gate E*, Fig 5, A). The increased cell size of the majority of TSLPr⁺ T cells was readily evident on microscopic examination compared with resting effector cells identified by low DNA content and high expression of CD127 (Fig 5, A, *gate C*).^{20,21} Collectively, these observations indicate that TSLPr is expressed on T_H2 cells, and a large proportion of these cells are actively dividing.

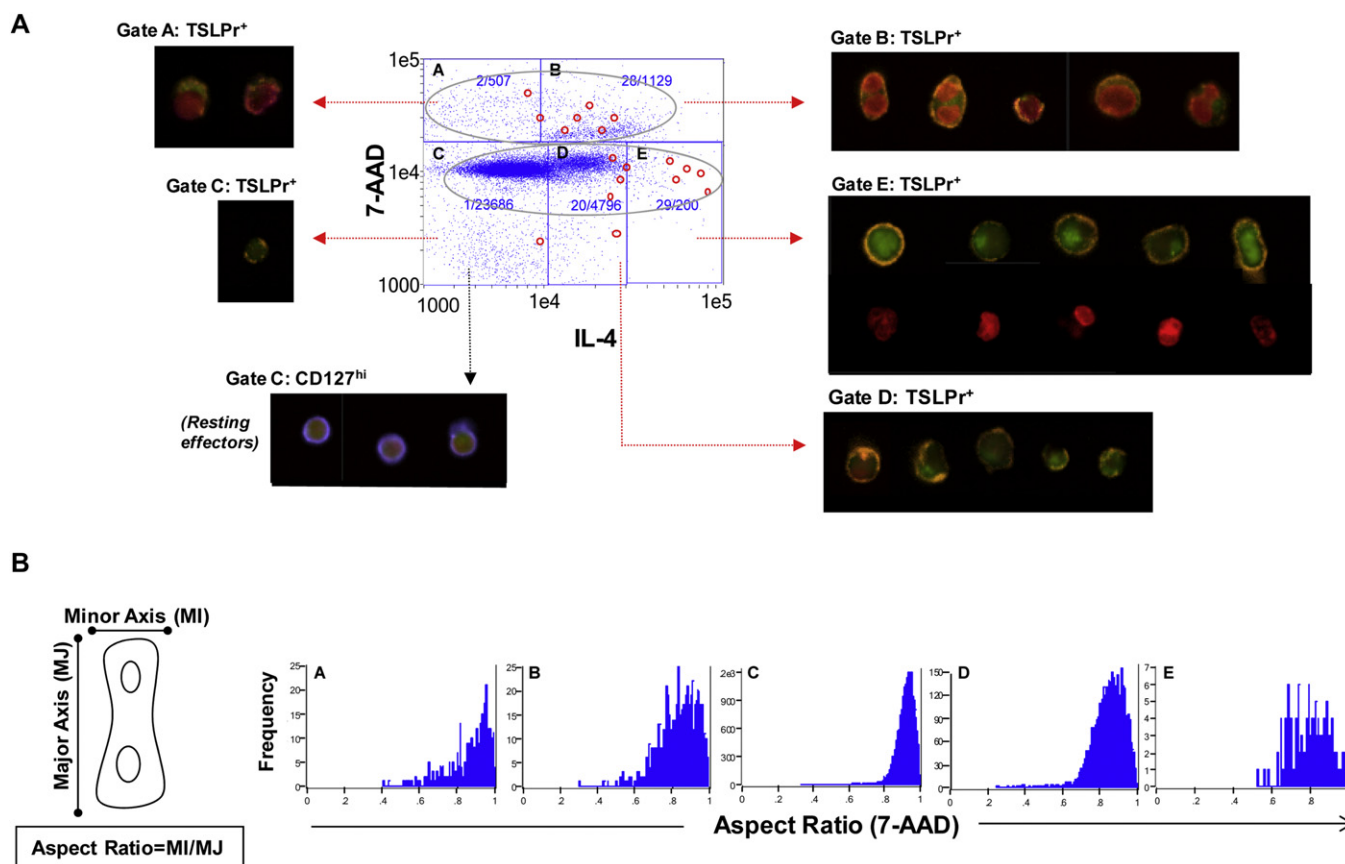


FIG 5. TSLPr⁺ T cells are actively dividing. **A**, Scatter plot showing T cells stimulated with TSLP + allergen analyzed for DNA (7-AAD) and IL-4. Values denote the frequency of TSLPr⁺ T cells within each gate, and red circles denote TSLPr⁺ cells shown in corresponding composite images. DNA staining for cells in gate E is shown in bottom row of cell panel. Composite images of representative resting effector cells in gate C are shown for comparison (CD127 [blue] and DNA [red]). **B**, Nuclear aspect ratios for gates in A. Representative data are shown (AD 1).

Bacterial superantigen induces rare T_H2 cells that express TSLPr

Many patients with AD are colonized with *Staphylococcus aureus*, and IgE antibodies to staphylococcal enterotoxin B (SEB) are common.^{22,23} We examined whether SEB had the capacity to induce TSLPr⁺ T cells. Interestingly, only 1 of 3 SEB-sensitized patients with AD responded to SEB (Table E1, AD 3), as judged by increased CD25 (Fig 6, A). A similar frequency of TSLPr⁺ T cells was observed as compared with other T_H2-promoting stimuli (64/9850; 0.65%), and cells expressing TSLPr were not amplified by costimulating with SEB + TSLP (32/12,326; 0.26%). Stimulation with SEB induced a robust population of IL-4^{hi} T cells that were predominantly CD25⁺ (Fig 6, B). On imaging, all TSLPr⁺ T cells were CD25⁺, and most cells (34/64) were IL-4^{hi}. TSLPr was also visible on T cells expressing lower levels of IL-4, including IL-4^{neg} cells (Figs 6, B and C). TSLPr⁺ cells exhibited a morphology similar to that induced by other T_H2-promoting factors (Fig 6, C). Analysis of secreted cytokines showed that SEB induced a mixed T_H1/T_H2 profile in the responder subject (AD 3), in contrast with a T_H2-dominated profile in a nonsensitized subject (AD1) (see this article's Fig E5, A, in the Online Repository at www.jacionline.org). Consistent with this, IL-4⁺ T cells from the responder subject coexpressed other cytokines including IFN- γ and IL-10, and to a lesser extent, IL-17

(Fig E5, B). These findings confirmed the capacity for SEB to induce TSLPr on rare IL-4-expressing T cells.

T cells induced in a "proallergic" milieu respond directly to TSLP

Formation of a functional TSLP receptor requires heterodimerization of the TSLPr chain with IL-7 receptor α chain (CD127).^{12,13} Interestingly, CD127 expression was visible on only a minority of total TSLPr⁺ T cells induced by TSLP + allergen (34.1% $\pm$ 23.3%) and was generally absent on those induced by TSLP alone (see this article's Table E2 and Fig E6 in the Online Repository at www.jacionline.org). By contrast, CD127 was readily detected on both unstimulated and stimulated T cells, including those expressing CD25, consistent with its expression on resting memory and activated T cells (Table E2; see this article's Fig E7 in the Online Repository at www.jacionline.org).²⁴

It was previously reported that T cells preactivated with anti-CD3 and anti-CD28 respond directly to TSLP by activation of signal transducer and activator of transcription (STAT)-5.¹⁴ To test whether T cells that had been preactivated with TSLP + allergen could respond directly to TSLP, T cells activated with TSLP + allergen were isolated, rested for 1 day, and then restimulated with TSLP. As expected, restimulation with IL-2

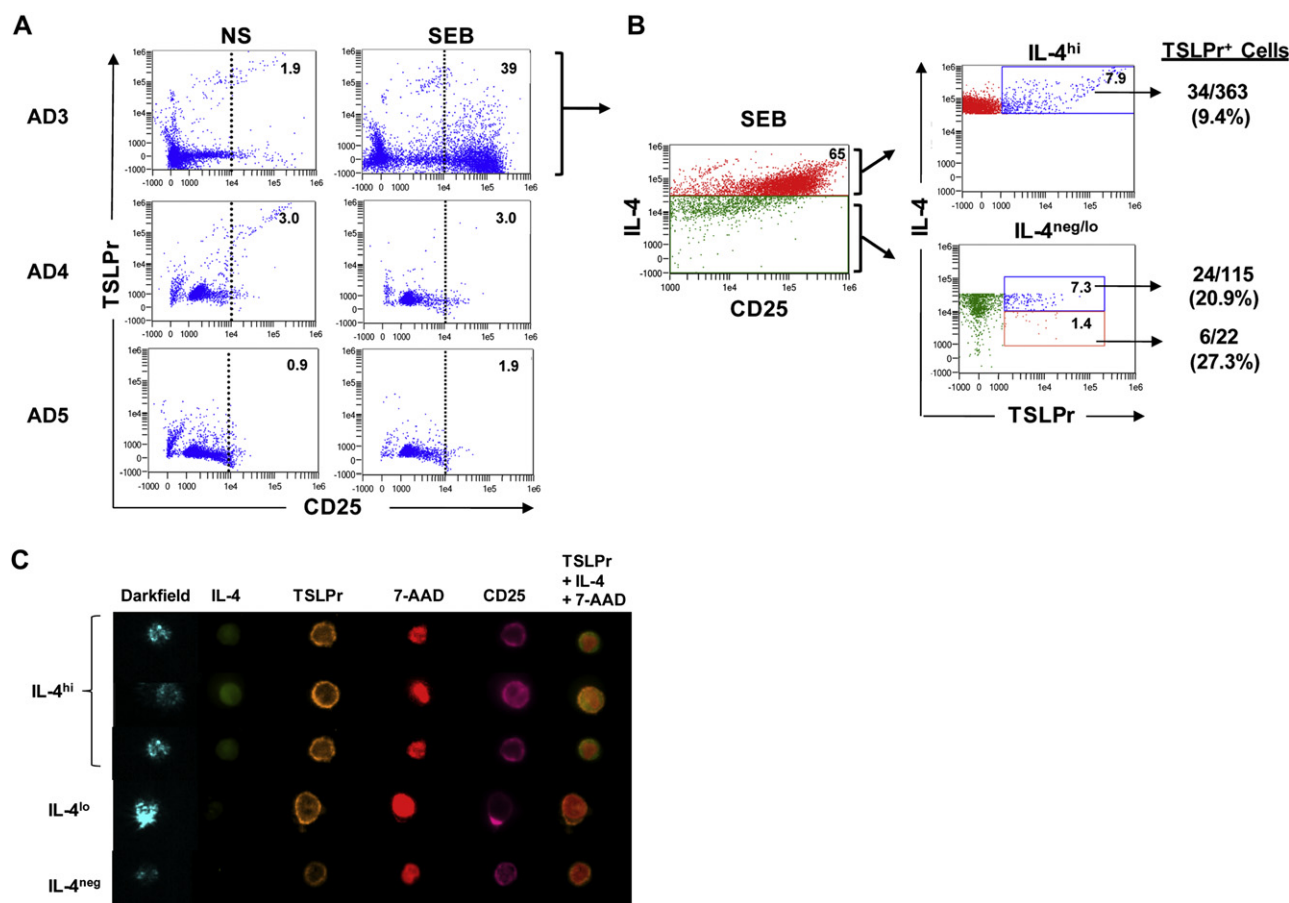


FIG 6. Bacterial superantigen induces rare TSLPr⁺ T_H2 cells. **A**, Analysis of CD25 and TSLPr on CD4⁺ T cells stimulated with SEB (day 3). Percentage of T cells within the CD25⁺ gate (dotted line) is denoted. NS, Not stimulated. **B**, Frequency of TSLPr⁺ T cells within IL-4^{hi}, IL-4^{lo} and IL-4^{neg} subsets in SEB-stimulated cultures from a responder patient. Left scatter plot shows gating strategy for IL-4^{hi} (red) and IL-4^{neg/lo} cells (green). **C**, Images of representative TSLPr⁺ cells.

resulted in phosphorylation of STAT5, reflecting signaling through the IL-2 receptor pathway. By contrast, only a weak signal for phosphorylated STAT5 was detected on restimulation with TSLP (Fig 7, A). Moreover, no secreted cytokines were detected for T cells preactivated with TSLP + allergen followed by TSLP restimulation. However, T_H2 cytokines were selectively induced on restimulation with TSLP or IL-2 when T cells were sequentially preactivated with TSLP + allergen and anti-CD3 + anti-CD28 (Fig 7, B). Collectively, these findings suggest that T_H2 cells induced by TSLP + allergen are weakly responsive to TSLP, and reactivation by T-cell receptor stimulation enhances this responsiveness.

IL-4⁺ T cells expressing TSLP receptor are detectable in AD skin

To determine the *in vivo* relevance of our observations, we examined whether IL-4⁺ T cells expressing TSLPr were detectable in T cells isolated from the skin. Stimulation of T cells from normal skin with TSLP + allergen induced T_H2-like (IL-4⁺IFN- γ) T cells; however, no TSLPr⁺ T cells were detected by standard flow cytometry (Fig 8, A). By contrast, direct *ex vivo* analysis of T cells from AD skin identified a low frequency of IL-4⁺TSLPr⁺ T cells in lesional but not in nonlesional skin

(Fig 8, B). These findings supported our *in vitro* observations obtained using image-based flow cytometry.

DISCUSSION

Our findings confirm that TSLPr is preferentially expressed on rare actively dividing T_H2 cells under T_H2-promoting conditions. Although a large proportion of cells expressing TSLPr did not co-express CD127, T cells that were preactivated with TSLP + allergen alone did respond weakly to TSLP as judged by phosphorylation of STAT5. Alternate STATs implicated in TSLP signaling in human DCs and T cells, including STAT6, were not examined in our study.^{24,25} Interestingly, sequential preactivation of T cells with TSLP + allergen followed by anti-CD3 + anti-CD28 resulted in secretion of TSLP-induced T_H2 cytokines, whereas preactivation with TSLP + allergen alone did not. Collectively, these findings suggest that T_H2 cells induced in a “proallergic” milieu require reactivation by a potent TCR stimulus to respond optimally to TSLP. In terms of the biological relevance, it is tempting to speculate that T_H2 cells respond to the direct actions of TSLP during the effector phase of the response, after T_H2 differentiation has occurred. Such a scenario would equip T_H2 cells primed in regional nodes with the capacity to respond to TSLP in inflamed tissues. Consistent with this theory,

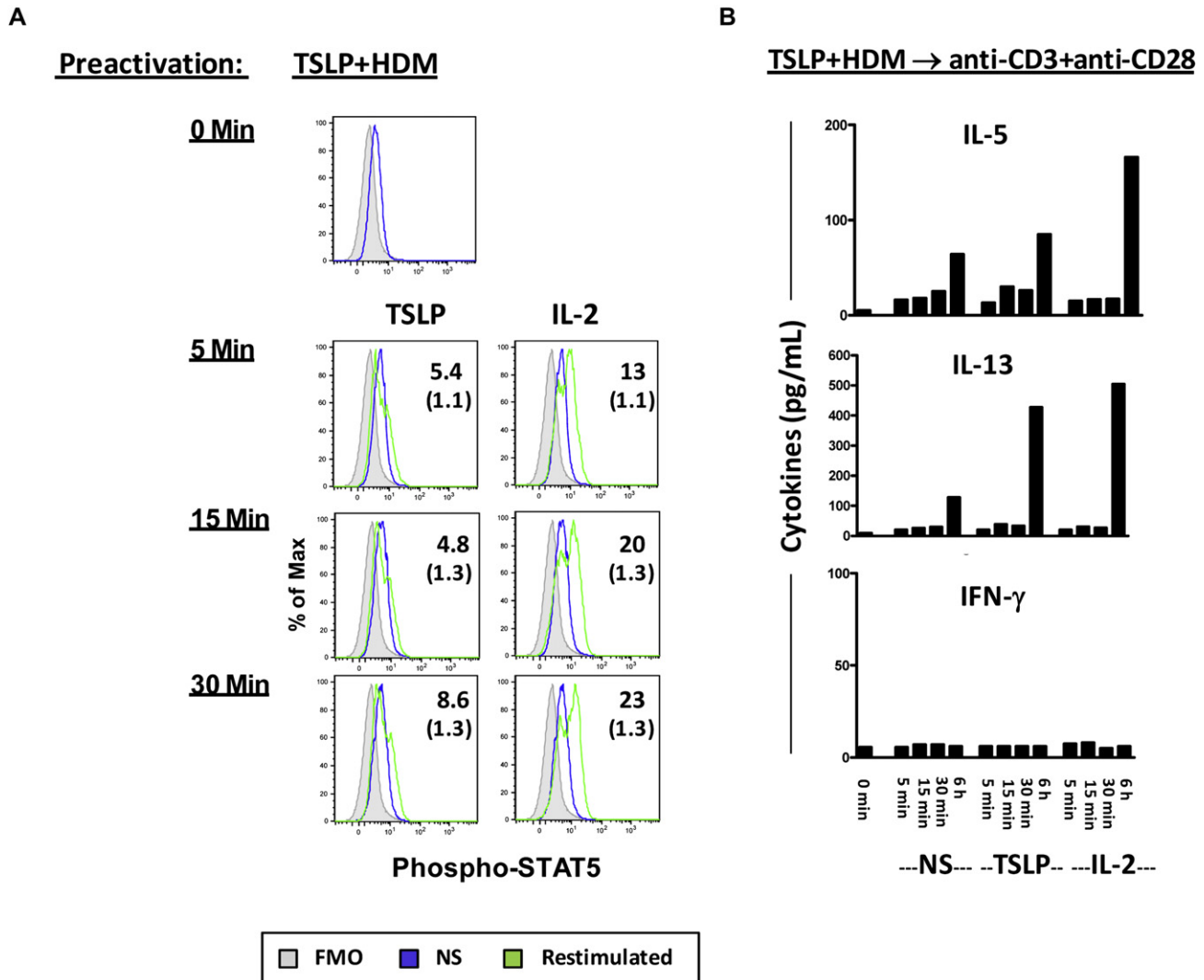


FIG 7. Direct effects of TSLP on purified T cells induced in a “proallergic” milieu. **A**, Purified T cells that had been preactivated with TSLP + allergen were not stimulated or restimulated with TSLP or IL-2 for 0 to 30 minutes and analyzed for phosphorylated STAT5. Values represent the percentage of cells positive for phospho-STAT5 after restimulation (values for nonstimulated cells are shown in parentheses for each corresponding time point). Data are representative of 3 experiments. **B**, Culture supernatants from purified T cells that had been preactivated sequentially with TSLP + allergen followed by anti-CD3 + anti-CD28 were assayed for cytokines after restimulation with TSLP or IL-2 for up to 6 hours. NS, Not stimulated; FMO, fluorescence-minus-one control.

TSLPr expression on T cells, but not DCs, was required for enhanced T_H2 cytokine secretion by skin-infiltrating $CD4^+$ T cells in a mouse model of allergic skin inflammation.¹⁵ Analysis of cells isolated from AD skin supported the presence of T cells expressing TSLPr at inflamed sites, and these cells constituted ~10% of $IL-4^+$ T cells. Although low expression of this receptor could reflect a transient event associated with T_H2 differentiation or cell division *in situ*, its presence in lesional but not nonlesional skin suggests a functional role at the site of allergic inflammation.

It has been reported that $IL-4$ -expressing T cells infiltrating AD skin, a site where TSLP is present, represent a heterogeneous population.²⁶ Our findings indicate that expression of TSLPr on T cells may not be unique to classic T_H2 cells because $IL-4$ -expressing cells induced by SEB coexpressed a variety of other cytokines. Thus, further studies are warranted to elucidate whether

TSLPr expression is exclusive to T cells with an $IL-4$ -restricted cytokine profile.

The lack of a functional TSLPr complex on some TSLPr⁺ T cells was a novel observation. The absence of CD127 on TSLPr⁺ T cells that were stimulated specifically with TSLP alone did not appear to be explained by downregulation of the TSLPr complex on binding of TSLP because the ligand binding component (TSLPr chain) of the TSLPr complex¹² was readily detectable on the cell surface. T cells were maintained in culture with TSLP in our system to reproduce a “proallergic” milieu. Several lines of evidence argue against receptor downregulation in the presence of TSLP. First, time course studies indicated a consistent signal for TSLPr after stimulation with TSLP. Second, TSLPr⁺ T cells were detected at low frequencies in SEB-stimulated cultures in the absence of TSLP. Third, work performed by other groups

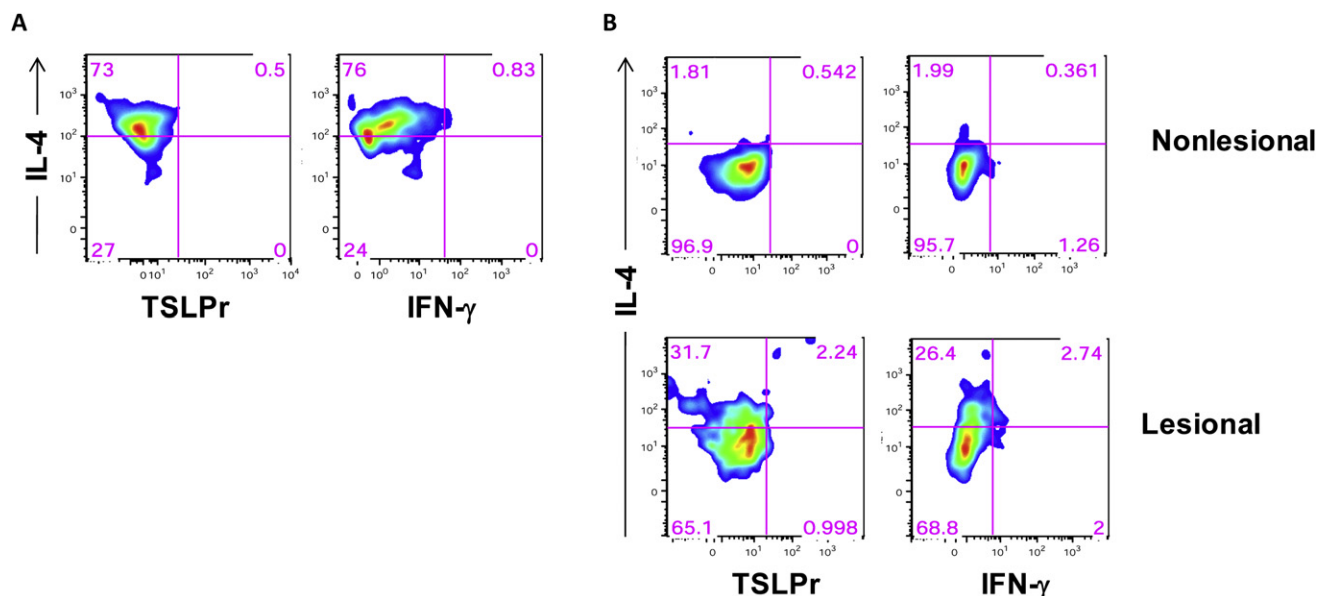


FIG 8. Analysis of TSLPr on skin T cells. **A**, Cells isolated from normal skin obtained from a nonatopic donor were cultured for 7 days with TSLP + allergen and analyzed by standard flow cytometry. **B**, Cells isolated directly *ex vivo* from lesional or nonlesional skin from an AD patient (AD1) were analyzed for TSLPr. Both panels show density plots for cells in the CD3⁺ T lymphocyte gate.

has demonstrated only low surface TSLPr and a modest increase in TSLPr mRNA in human T cells activated in the absence of TSLP.^{14,27}

In the current study, identification of TSLPr⁺ T cells by using image-based technology was a labor intensive process requiring visual inspection of thousands of cells. This approach facilitated the enumeration of bona fide TSLPr⁺ T cells. The microscopy component of this platform also allowed us to elucidate the presence of a functional receptor by confirming whether CD127 was expressed on rare TSLPr⁺ cells at the single-cell level. Another advantage of the approach described was the ability to visualize mitotic cells on the basis of a distinctive nuclear and surface morphology, thereby linking TSLPr to actively dividing T cells. The technology we describe allowed the simultaneous assessment of up to 5 cellular markers (CD127, CD25, IL-4, DNA, and TSLPr). To analyze the maximal number of markers related to TSLPr cells, the T-cell marker CD4 was not included. Thus, it could be argued that the rare TSLPr⁺ T cells identified in our system constituted a contaminating DC population. There are several key immunobiologic features of DCs that argue against this. Notably, expression of IL-4 is not an attribute of human DCs.²⁸ To the contrary, secretion of the Th1-promoting cytokine IL-12 is a notable characteristic of DCs under Th2-promoting conditions.^{29,30} Furthermore, DCs primed specifically with TSLP do not produce detectable amounts of IL-4.¹ In addition, studies performed using standard flow cytometry detected a TSLPr signal on CD4⁺ T cells under Th2-promoting conditions at a frequency similar to that obtained by image-based technology, thereby providing further evidence that TSLPr expression was a feature of T cells in our system. Finally, when considered collectively, the morphologic attributes and staining properties of TSLPr⁺ cells were characteristic of a T-cell phenotype.

In summary, our findings support the view that in a “proallergic” milieu, TSLPr is preferentially expressed on rare actively dividing Th2 cells. The capacity for these cells to respond directly

to TSLP may contribute to amplification of Th2 responses at sites of allergic inflammation.

We thank Holly Carper, MS, and Deborah Murphy, RN, (University of Virginia) for assistance with blood draws.

Key messages

- TSLP receptor is expressed on rare actively dividing Th2 effectors.
- T cells induced in a “proallergic” milieu can respond directly to TSLP.

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METHODS

Cells and reagents

Antigen preparations. House dust mite extract, natural Der p 1, and recombinant H22–Fel d 1 were obtained from Indoor Biotechnologies, Inc, Charlottesville, Va. H22–Fel d 1 contains the major cat allergen (Fel d 1) linked to a single chain fragment of the variable region (sFv) of the anti-CD64 humanized mAb, H22, that targets the high-affinity IgG receptor, FcγRI.^{E1,E2} SEB was obtained from Sigma, St. Louis, Mo.

Cells. Blood DCs were isolated from PBMCs by magnetic-activated cell sorting according to the manufacturer's instructions (Blood Dendritic Cell Isolation Kit II; Miltenyi Biotec, Auburn, Calif). CD4⁺ T cells were purified from fresh PBMCs by negative selection using magnetic-activated cell sorting (Miltenyi Biotec), and purity was assessed by flow cytometry (>95%). Cells were isolated from 4-mm skin biopsy specimens by tissue dissociation according to the manufacturer's instructions (GentleMACS Dissociator; Miltenyi Biotec). The THP-1 cell line was generously provided by K. Ravichandran (University of Virginia, Charlottesville, VA). Monocyte-derived DCs were generated as previously described.^{E1}

Antibodies for flow cytometry. mAbs used in flow cytometry imaging experiments were as follows: APC-anti-TSLPr (1B4; BioLegend), PE-anti-TSLPr (1A6), and Pacific Blue-anti-CD127 (eBioRDR5) (eBioscience), FITC-anti-HLA-DR (L243), PE-Cy5-anti-CD25 (M-A251), and (FITC-anti-IL-4 (8D4-8) (BD Biosciences). Antibodies used in experiments analyzed by standard flow cytometry were as follows: Pacific Orange-anti-CD3 (UCHT1; Invitrogen), APC-H7-anti-CD3 (SK7; BD Biosciences), PE-anti-IL-4 (8D4-8; BioLegend), FITC-IL-4 (MP4-25D2, BD Biosciences), Alexa Fluor 488-anti-IL-10 (JES3-9D7; BioLegend), Pacific Blue-anti-IL-17 (BL168; BioLegend), PE-Cy7-anti-IFN-γ (4S.B3; BD Biosciences), V450-anti-IFN-γ (B27; BD Biosciences), PE-anti-TSLPr (1B4; BioLegend), and phycoerythrin-Cy5-anti-CD25 (M-A251; BD Biosciences).

Other reagents. TSLP was purchased from R&D Systems, Minneapolis, Minn, and IL-2 was obtained from Invitrogen. DNA binding dyes used were 7-AAD (BD Biosciences) and DAPI (Invitrogen).

Analysis of intracellular and surface markers by standard flow cytometry

T cells stimulated in DC/T-cell cocultures were analyzed for intracellular expression of cytokines. Before staining for intracellular cytokines, cells were restimulated with phorbol 12-myristate 13-acetate (50 ng/mL; Fisher Scientific) and ionomycin (2 μg/mL; Invitrogen) on the final day of culture for 5 hours, followed by addition of brefeldin A (BD Biosciences) for 4 hours. For some experiments, intracellular cytokines were assessed in T cells cocultured for 10 days with monocyte-derived DCs primed for 48 hours with TSLP alone or TSLP + HDM as previously described.^{E1} For kinetic studies, TSLP receptor expression was analyzed on T cells harvested from DC/T cell cocultures stimulated with TSLP + allergen at different time points. For skin biopsy specimens, cells were analyzed immediately or else cultured with TSLP + allergen for 7 days before analysis of intracellular cytokines and surface TSLPr.

Cells were analyzed on a Cyan ADP LX 9 color Dako Cytomation, and data were analyzed by using FlowJo version 8.8.3 (Tree Star Inc, Ashland, Ore). Compensation controls (single stains, 1 for each fluorochrome) and gating controls (cells stained with all reagents minus 1 (fluorescence-minus-one [FMO])) were used to identify positive cell populations.

Analysis of the direct effects of TSLP on T cells

T cells that had been activated in DC/T cell cocultures by stimulation with TSLP + allergen (7 days) were isolated by magnetic activated cell sorting (CD4⁺ T Cell Isolation Kit II Kit; Miltenyi Biotec). Initial studies using fresh PBMCs confirmed high purity of CD4⁺ T cells (>99.5%) on the basis of counterstaining for CD11c (B-ly6; BD Biosciences), B220 (RA3-6B2; BD Biosciences), and CD14 (MoP9; BD Biosciences) and analysis by standard flow cytometry. For some experiments, cells were then reactivated with anti-CD3 (BD BioCoat Anti-Human CD3 plate) and anti-CD28 (10 μg/mL, R&D Systems) for 24 hours. Isolated T cells preactivated with TSLP + allergen alone or TSLP + allergen followed by anti-CD3 and anti-CD28 were then rested for 24 hours. After resting, cells were either not stimulated or restimulated for 5, 15, or 30 minutes or 6 hours with TSLP (50 ng/mL) or IL-2 (100 U/mL). T cells were stained with PE-anti-phospho-STAT5 mAb (pY694; BD Biosciences), FITC-anti-CD4, and PE-Cy5-CD11c and analyzed for phospho-STAT5 by standard flow cytometry after excluding CD11c⁺ cells. Secreted cytokines were measured by cytometric bead assay.

Assay for secreted cytokines

Cytokines were measured in culture supernatants by cytometric bead assay (T_H1/T_H2 panel; Bio-Rad Laboratories, Hercules, Calif).

Statistical analysis

A paired Student *t* test was used to compare the frequency of TSLPr⁺ T cells induced under different conditions, the percentage of TSLPr⁺ T cells within T-cell subsets expressing different levels of CD25 or IL-4, and the frequency of different T-cell subsets expressing CD127 under different conditions (SPSS 14.0; SPSS Inc, Chicago, Ill). Trends in area of DNA staining within discrete TSLPr⁺ and TSLPr-negative cell subsets were examined by using multivariate random coefficient regression. Model parameters were estimated by using the PROC MIXED procedure of SAS version 9.1.3 EAS (SAS Institute Inc, Cary, NC).

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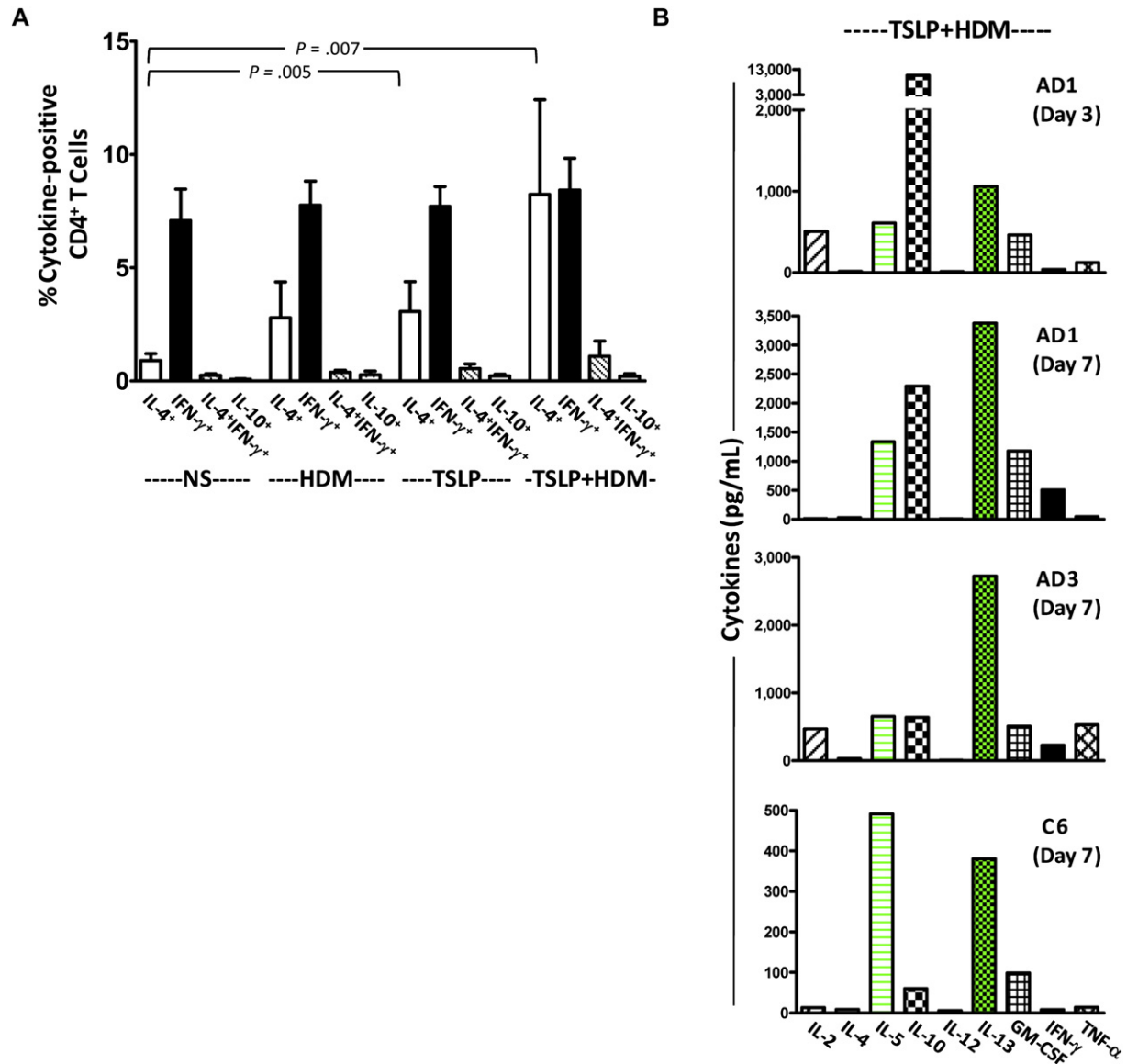


FIG E1. Induction of T_H2 cytokines by TSLP and HDM. **A**, $CD4^+$ T cells were cocultured with monocyte-derived DCs that had been primed for 48 hours with allergen alone, TSLP alone, or both stimuli. Intracellular cytokines were measured on day 10. Values represent the means $\pm$ SDs for 10 subjects with dust mite allergy (5 AD and 5 non-AD). *NS*, not stimulated. **B**, Cytokines in supernatants harvested from DC/T-cell cocultures that were analyzed by image-based flow cytometry.

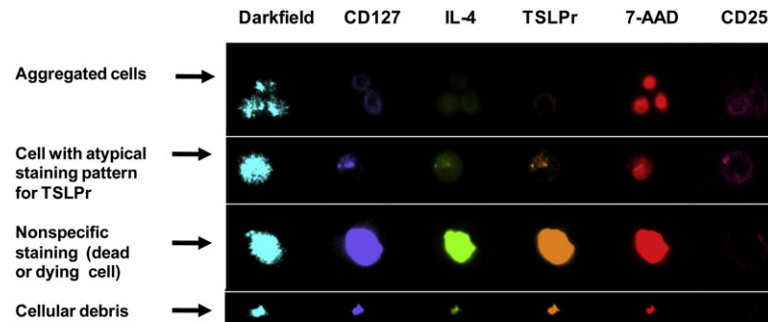


FIG E2. False-positive' TSLPr⁺ T cells. Images of 4 representative cells within the TSLPr⁺ gate that were confirmed as false-positives on visual inspection.

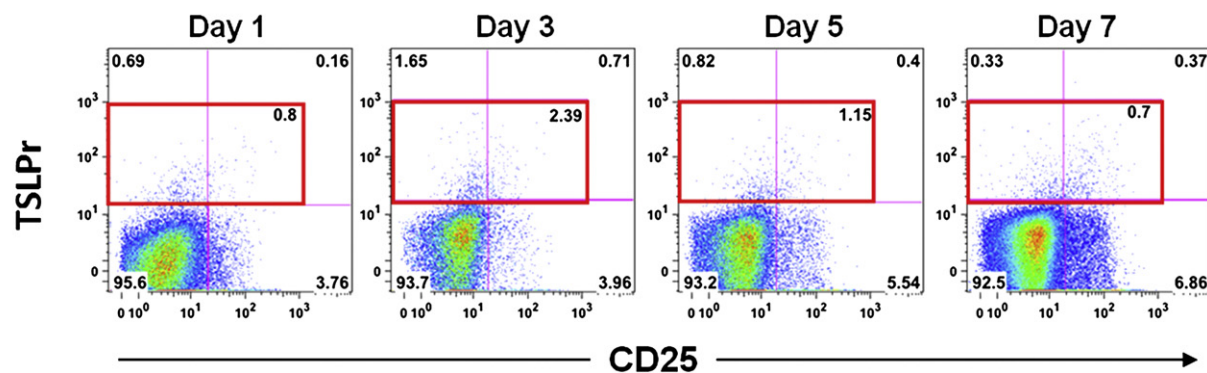
AD5

FIG E3. Kinetics of TSLP receptor expression. CD4⁺ T cells were cocultured with autologous DCs in the presence of TSLP + allergen and analyzed for expression of TSLPr and CD25 at different time points by standard flow cytometry.

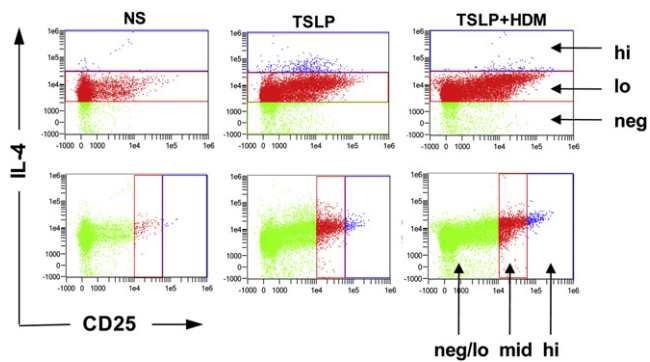


FIG E4. Gating strategies for defining T-cell subsets that express different levels of CD25 and IL-4. Differences in staining intensity for CD25 and IL-4 were confirmed by visual inspection. The same gating strategy was applied to data from 5 subjects. *NS*, not stimulated.

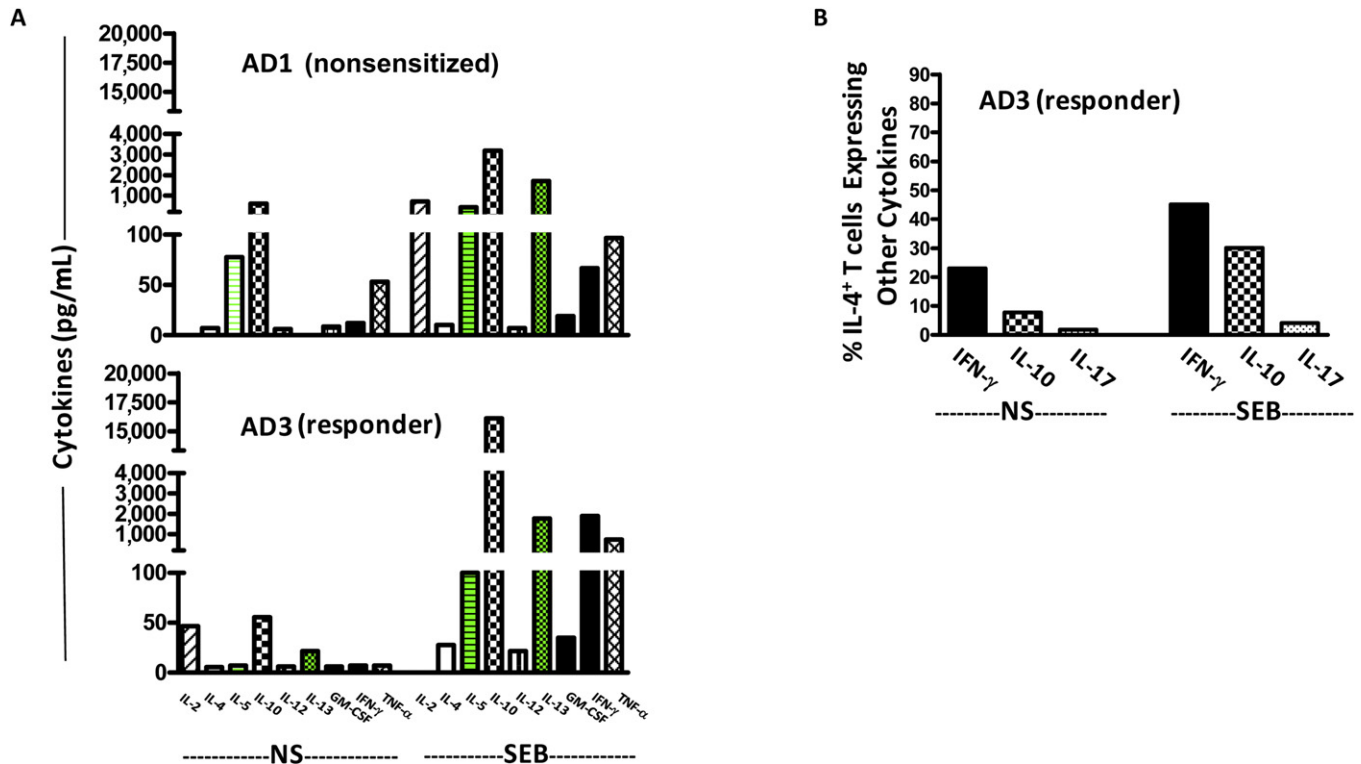


FIG E5. Cytokines induced by SEB. **A**, Secreted cytokines detected at 3 days after stimulation with SEB in patients with AD. **B**, Frequency of IL-4⁺ T cells expressing other cytokines after stimulation with SEB. NS, not stimulated.

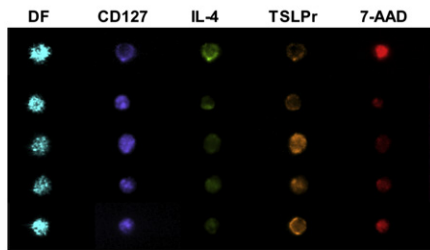
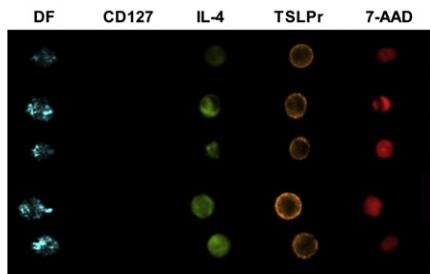
TSLPr⁺CD127⁺TSLPr⁺CD127^{neg}

FIG E6. Images of CD127⁺ and CD127^{neg} T cells that express TSLPr. Data are shown for representative cells from a single subject after stimulation with TSLP + HDM and are representative of 4 subjects. *DK*, darkfield.

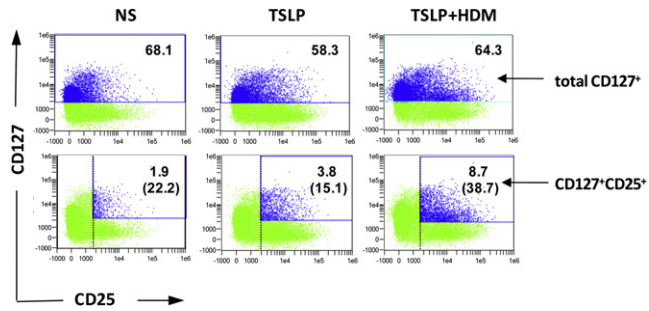


FIG E7. Gating strategy for identifying CD127⁺ T cells. *Upper panel* shows gated CD127⁺ T cells (*blue*) for different stimuli. *Lower panel* shows the gate for CD25⁺CD127⁺ T cells (*blue*). Values denote the percentage of cells within the CD127⁺ (*upper panel*) or CD25⁺CD127⁺ (*lower panel*) gates. Values in parentheses denote the percentage of CD25⁺ T cells that are CD127⁺. *Dotted line* denotes the CD25⁺ gate. The same gating strategy was applied to data from 4 different subjects. *NS*, not stimulated.

TABLE E1. Serum IgE antibody profiles in patients with AD and nonatopic controls

Subject	Total IgE (IU/mL)	IgE Ab (IU/mL)	
		House dust mite	SEB
AD 1	583	9.6	<0.35
AD 2	441	14.1	<0.35
AD 3	423	27.6	0.9
AD 4	3966	35.4	0.4
AD 5	6878	414	10.0
Control 6	28.3	<0.35	<0.35
Control 7	4.0	<0.35	<0.35

TABLE E2. Percentage of cells within discrete T-cell subsets that express CD127

Stimulus	NS			TSLP			TSLP + HDM			SEB	SEB + TSLP
T subset	CD4 ⁺	CD25 ⁺	TSLPr ⁺	CD4 ⁺	CD25 ⁺	TSLPr ⁺	CD4 ⁺	CD25 ⁺	TSLPr ⁺	TSLPr ⁺	TSLPr ⁺
AD 1	68.1 (21,549/31,662)	22.2 (591/2661)	0 (0/2)	58.3 (18,571/31,868)	15.1 (1222/8091)	0 (0/58)	64.3 (20,564/31,993)	38.7 (2797/7224)	25.3 (21/83)	ND	ND
AD 2	68.5 (11,528/16,840)	61.7 (8439/13,768)	0 (0/3)	26.6 (3493/13,113)	15.6 (1290/8247)	0 (0/50)	52.9 (9978/18,877)	43.8 (8269/13,513)	50.0 (12/24)	ND	ND
AD 3	ND	ND	ND	ND	ND	ND	ND	ND	ND	46.9 (30/64)	37.5 (12/32)
AD 5	28.2 (1050/3723)	84.1 (74/88)	100 (1/1)	22.3 (1226/5501)	24.3 (206/849)	15.4 (2/13)	29.4 (1806/6137)	25.7 (265/1030)	15.0 (6/40)	ND	ND
C 6	56.7 (18,227/32,141)	46.3 (2499/5396)	23.5 (4/17)	41.6 (13,382/32,186)	35.6 (4219/11,851)	0 (0/95)	46.4 (14,788/31,869)	37.4 (6729/18,011)	14.0 (19/136)	ND	ND
C 7	15.2 (3582/23,520)	53.4 (1557/2916)	0 (0/2)	13.7 (4467/32,613)	64.0 (2288/3575)	0 (0/9)	35.6 (10,884/30,545)	74.1 (3917/5286)	66.7 (2/3)	ND	ND
Mean	47.3 ± 24.3	53.5 ± 22.6	24.7 ± 43.3	32.5 ± 17.6	30.9 ± 20.3	3.1 ± 6.9*	45.7 ± 13.8	43.9 ± 18.1	34.1 ± 23.3	ND	ND

ND, not done; NS, not stimulated.

Values in parentheses correspond to the frequency of positive cells among total number of observations collected.

* $P = .05$ and $P = .036$ for TSLPr⁺ T cells vs CD4⁺ T cells and CD25⁺ T cells, respectively (stimulus: TSLP).