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Cutting Edge: Proinflammatory and Th2 Cytokines Synergize to Induce Thymic Stromal Lymphopoietin Production by Human Skin Keratinocytes¹

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Thymic stromal lymphopoietin (TSLP) is an epithelial cell-derived cytokine that strongly activates dendritic cells (DC) and can initiate allergic inflammation. The factors inducing the production of human TSLP are not known. In this study, we show that proinflammatory (TNF- α or IL-1 α) and Th2 (IL-4 or IL-13) cytokines synergized to induce the production of TSLP in human skin explants. TSLP production in situ was restricted to epidermal keratinocytes of the suprabasal layer. TSLP production could not be inhibited by factors regulating Th2 inflammation, such as IL-10, TGF- β , or IFN- γ . Cytokine-treated skin culture supernatants induced the maturation of blood CD11c⁺ DC in a TSLP-dependent manner. Our data provide the first evidence of TSLP induction and subsequent DC activation in human skin. Blocking TSLP-inducing cytokines could represent a novel strategy for the treatment of allergic diseases. The Journal of Immunology, 2007, 178: 3373–3377.

Thymic stromal lymphopoietin (TSLP)³ is an epithelial cell-derived cytokine that strongly activates myeloid dendritic cells (DC) to induce proallergic CD4⁺ and CD8⁺ T cell responses in vitro (1–3) and in vivo (4–6). TSLP is produced by human keratinocytes in atopic dermatitis (AD) (1) and could induce an AD phenotype when transgenically expressed in mouse keratinocytes (7). TSLP mRNA was detected in bronchial epithelial cells and lung fibroblasts in vitro (1) as well as in the asthmatic lung (8). When selectively expressed in the mouse lung, TSLP induced airway inflammation with characteristics of asthma (4). TSLP expression was also found in intestinal epithelial cells and could participate in the tolerance to the commensal flora through the modulation of DC function (9). Importantly, TSLP is constitutively expressed in the human thymus and instructs thymic DC to induce the differenti-

ation of CD4⁺CD25⁺Foxp3⁺ regulatory T cells (10). These studies suggest that TSLP plays an important role in the cross-talk between various epithelial cells and DC to induce or modulate physiological and pathological immune responses. However, the factors inducing the production of human TSLP are not known, and the molecular pathways regulating this production are still poorly defined. The retinoid X receptor was recently shown to be involved in the induction of TSLP in mouse epidermis but the physiopathological relevance of this finding is still unclear (11, 12).

Materials and Methods

Tissue samples

Skin samples considered as surgical wastes were obtained from healthy donors undertaking esthetic or reconstructive surgery and processed within 6 h of resection. This discarded human surgical material was obtained anonymously according to the institutional regulations, in compliance with French legislation. Donors were Caucasian females, aged 24–67 years, with no reported history of allergy. This study was approved by the Internal Review Board and Clinical Research Committee of the Institut Curie.

Cell and tissue culture

After removing s.c. fatty tissue, 5-mm skin punch-biopsies were cultured in RPMI 1640 containing 10% FCS, 1% pyruvate, 1% HEPES, and penicillin/streptomycin (referred to as medium) as described (13, 14), with combinations of the following soluble factors: 20 ng/ml TNF- α , 10 ng/ml IL-1 α , 100 ng/ml IL-4, 100 ng/ml IL-5, 100 ng/ml IL-13, 1000 U/ml IFN- γ , 5 ng/ml TGF- β , 100 ng/ml IL-10, and 10 μ g/ml anti-TGF- β blocking mAb (all obtained from R&D Systems) or 2 μ g/ml ALK5 inhibitor I (Calbiochem). After 48 h, culture supernatants and skin samples were collected and frozen at -80°C until assayed. Similar experiments were performed using epidermal explants, obtained from the punch-biopsies after 3 h of digestion at 37°C in the presence of 4 mg/ml Dispase II (Roche Diagnostics), or primary normal human epidermal keratinocytes (Cambrex/BioWhittaker).

Immunohistology

Frozen skin sections were stained with a sheep anti-human TSLP mAb (R&D Systems), followed by a donkey anti-sheep biotinylated polyclonal Ab (R&D Systems) and revealed using a Vectastain ABC peroxidase system (Vector Laboratories).

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³ Abbreviations used in this paper: TSLP, thymic stromal lymphopoietin; AD, atopic dermatitis; MFI, mean fluorescence intensity; DC, dendritic cell.

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Cytokine production

TSLP and IL-8 were quantified using protein ELISA kits (R&D Systems) in culture supernatants within 1 mo of collection.

Blood dendritic cell purification and culture

CD11c⁺ DC were purified to 99% by FacSorting from buffy coats of healthy adult volunteer blood donors (Crozatier blood bank, Paris, France) as previously described (1). Freshly sorted CD11c⁺ DC were cultured in complete medium. Cells were seeded at 0.5×10^6 /ml in flat-bottom 96-well plates in the presence of TSLP (R&D Systems), MRC-5 cell line (American Type Culture Collection), or cytokine-treated skin culture supernatants, with or without sheep anti-human anti-TSLP (10 μ g/ml), mouse anti-human anti-TNF- α (10 μ g/ml) blocking mAb (R&D Systems) or isotype-matched IgG1 (10 μ g/ml) (Sigma-Aldrich).

Dendritic cell phenotype

Blood DC were harvested after 24 h of culture and resuspended in an EDTA-containing medium. Cells were stained with mouse anti-human CD80- or CD40-FITC or an IgG1 isotype (BD Bioscience) and were analyzed with a FACScan flow cytometer (BD Bioscience). Dead cells were excluded based on side and forward scatter characteristics.

Statistical analysis

TSLP production was compared for each skin culture condition using a two-tailed Student *t* test. Statistical significance was retained for *p* values <0.05.

Results and Discussion

Proinflammatory and Th2 cytokines synergize to induce TSLP production in human skin

We previously showed that TSLP is highly produced in human AD (1). To identify TSLP-inducing cytokines, we used a cytokine combination that mimics the atopic microenvironment. This included TNF- α , together with the Th2 cytokines IL-4, IL-5, and IL-13 (15, 16). In cultured primary normal human skin keratinocytes, we detected baseline levels of TSLP mRNA (Ref. 1 and data not shown) that could not be up-regulated by

the addition of exogenous pro-allergic cytokines and no TSLP protein could be detected in culture supernatants (data not shown). A limitation of this system could be the relatively undifferentiated state of proliferating keratinocytes. Since TSLP production in situ is absent from the undifferentiated basal layer (1), we switched to whole skin explants, a model that preserves the differentiation of keratinocytes. After 48 h of culture in the presence of a pro-allergic cytokine combination (IL-4, IL-5, IL-13, and TNF- α), TSLP was consistently detected at levels ranging from 90 to 285 pg/ml (Fig. 1A). Removing TNF- α or IL-4 from the cytokine cocktail suppressed 90% and 40% of the TSLP production, respectively (Fig. 1A). Removing IL-13 and IL-5 had no major effect (Fig. 1A). Contrary to TSLP, IL-8, an NF- κ B-dependent cytokine, was highly produced at baseline (Fig. 1B). TNF- α was required for IL-8 up-regulation but removing IL-4 had no effect on IL-8 levels (Fig. 1B).

Next, we asked whether TNF- α was sufficient to induce TSLP. TNF- α alone induced low TSLP levels in only 1 out of 4 donors, similar to IL-4 and IL-5, whereas IL-13 did not induce detectable TSLP (Fig. 1D). However, when TNF- α was combined to IL-4, and to a lesser extent IL-13, we detected TSLP levels comparable to those induced by the pro-allergic cytokine combination (Fig. 1D). Among other pro-inflammatory cytokines, IL-1 α , but not IL-6, could replace TNF- α for TSLP induction (Fig. 1C). Interestingly, IL-8 production was differentially regulated, since TNF- α alone could enhance the baseline levels of IL-8, but IL-4 inhibited IL-8 production (Fig. 1E), as was shown by others for keratinocyte-derived chemokines (17, 18).

To exclude a role of dermal cells, such as dermal fibroblasts, in the induction of TSLP, we repeated these experiments using

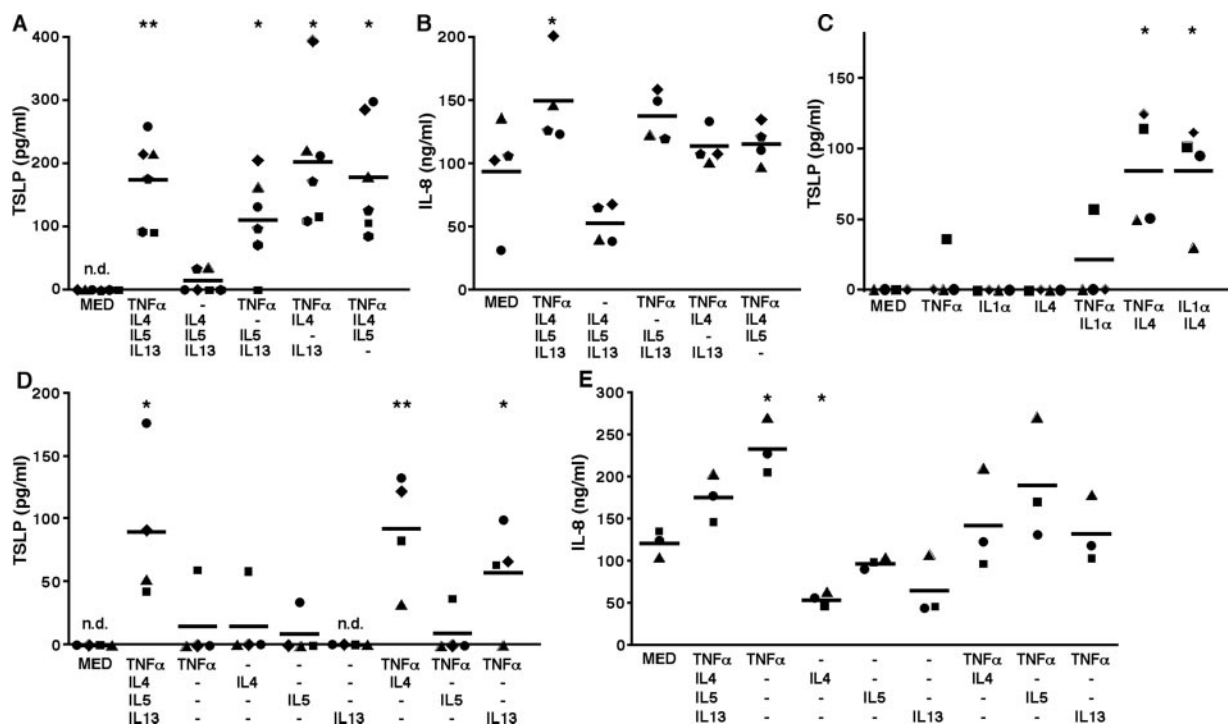


FIGURE 1. TNF- α or IL-1 α and Th2 cytokines synergize to induce TSLP production in human skin explants. TSLP (A, C, and D) and IL-8 (B and E) were measured by protein ELISA in 48-h skin explant culture supernatants. Each symbol represents an independent experiment performed with a sample from a different donor. Horizontal bars indicate the mean. Statistical significance is indicated for pairwise comparisons of culture condition groups. *, *p* < 0.05; **, *p* < 0.01.

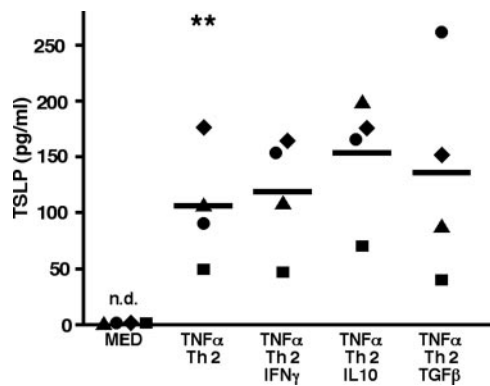


FIGURE 2. TSLP production is not sensitive to factors regulating Th2 inflammation. IFN- γ , IL-10, or TGF- β were added to the TNF- α and Th2 cytokine combination to modulate TSLP production. Each symbol represents an independent experiment performed with a sample from a different donor. Horizontal bars indicate the mean. Differences observed were not statistically significant ($p > 0.05$).

epidermal explants. TSLP induction by TNF- α plus IL-4 was comparable to full thickness skin explants (data not shown), indicating that the dermis is not involved in this process.

IL-4 and IL-13 were both described as major pro-allergic cytokines, mostly by promoting B cell growth and differentiation into IgE producing cells (19, 20). Our data suggest a novel mechanism by which these cytokines induce and sustain allergic inflammation. Interestingly, TNF- α , IL-4, IL-5, and IL-13 were shown to be produced by CD4⁺ T cells primed by TSLP-activated DC (1, 21). Combined with our current data, this suggests a potentially pathogenic amplification loop where TSLP-activated DC would prime T cells to produce pro-allergic cytokines, which would in turn sustain or increase the production of TSLP by keratinocytes.

TSLP production is not sensitive to factors regulating Th2 inflammation

It was previously suggested that TSLP could account for the dysregulated Th2 inflammation observed in allergy (22). We thus asked whether TSLP could be inhibited by factors implicated in the physiological regulation of Th2 inflammation. IFN- γ , a Th1 cytokine that can inhibit Th2 responses (23), did not have any effect on TSLP production (Fig. 2). IL-10 was shown to negatively regulate allergic inflammation (24, 25) but did not affect TSLP levels in the skin cultures (Fig. 2). TGF- β , another anti-inflammatory factor mediating the suppressive effect of regulatory T cells (26–28), did not significantly inhibit TSLP production ($p > 0.05$) (Fig. 2). Inhibition of endogenous TGF- β using a blocking mAb and a TGF- β receptor kinase inhibitor (ALK5 inhibitor I) did not increase the levels of TSLP

production by the skin explant (data not shown) excluding a saturating effect of endogenous TGF- β . These results suggest that physiological regulators of Th2 inflammation are not efficient in inhibiting TSLP.

Production of TSLP in situ is restricted to keratinocytes

Although TSLP production was mostly attributed to epithelial cells, fibroblasts, or other stromal cells can express TSLP mRNA in vitro (1). We assessed the cellular source of TSLP in our system by immunohistochemistry. Skin sections derived from explants cultured with medium alone did not show any TSLP-specific staining (Fig. 3*B*), whereas the TNF- α and Th2 cytokine combinations induced a TSLP-specific staining that was restricted to differentiated keratinocytes (Fig. 3, *C* and *D*). Removing TNF- α suppressed most of the TSLP-specific staining (Fig. 3*E*). Importantly, we did not observe any TSLP-specific staining in the dermis (Fig. 3, *C* and *D*), consistent with our previous description of TSLP in atopic dermatitis (1).

Cytokine-stimulated skin induces DC maturation in a TSLP-dependent manner

To assess the role of skin-derived TSLP in DC maturation, we used skin culture supernatants to stimulate purified blood CD11c⁺ DC in the presence or absence of a blocking anti-TSLP mAb (Fig. 4*A*). We selected CD80 as a maturation marker that is more rapidly and specifically induced by TSLP (1, 29). Supernatants of medium-, TNF- α -, or IL-4-stimulated skin cultures slightly up-regulated CD80 expression on DC, and this effect was inhibited by anti-TSLP mAb (Fig. 4*A*). Supernatants of TNF- α plus IL-4-containing conditions induced a stronger CD80 up-regulation (mean fluorescence intensity (MFI): 51 or 57), which was almost completely inhibited by anti-TSLP mAb, reaching back levels obtained with medium-cultured skin supernatants (MFI: 16 or 18) (Fig. 4*A*). Interestingly, medium-cultured skin supernatant induced a TSLP-independent up-regulation of CD80 (MFI: 17), indicating that skin explants might produce DC activating factors other than TSLP (Fig. 4*A*). These factors, as well as exogenous cytokines, may act in synergy with keratinocyte-derived TSLP, which could explain the higher CD80 levels observed with skin supernatants of TNF- α plus IL-4-containing conditions (MFI: 51 or 57) as compared with exogenous TSLP at similar concentration (100 pg/ml) (MFI: 23) (Fig. 4*A*). As a control, exogenous TNF- α and Th2 cytokines induced a TSLP-independent up-regulation of CD80, ruling out a production of TSLP by DC (Fig. 4*A*). MRC-5, a lung carcinoma cell line producing TSLP (27), induced a TSLP-dependent CD80 up-regulation (Fig. 4*A*).

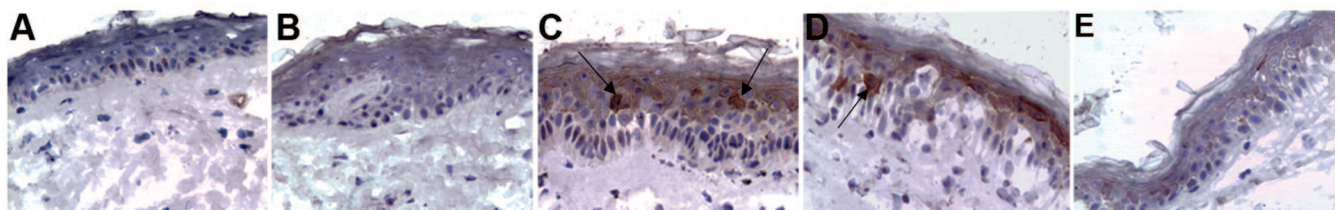


FIGURE 3. Production of TSLP in situ is restricted to keratinocytes. Frozen sections of skin explants cultured in medium (*B*) or with TNF- α plus IL-4 plus IL-13 plus IL-13 (*A* and *C*), TNF- α plus IL-4 plus IL-13 (*D*), or IL-4 plus IL-5 plus IL-13 (*E*) were stained with a TSLP-specific mAb or with sheep IgG isotype (*A*). The brown staining indicates TSLP production by epidermal keratinocytes. Results are from one representative of three independent experiments.

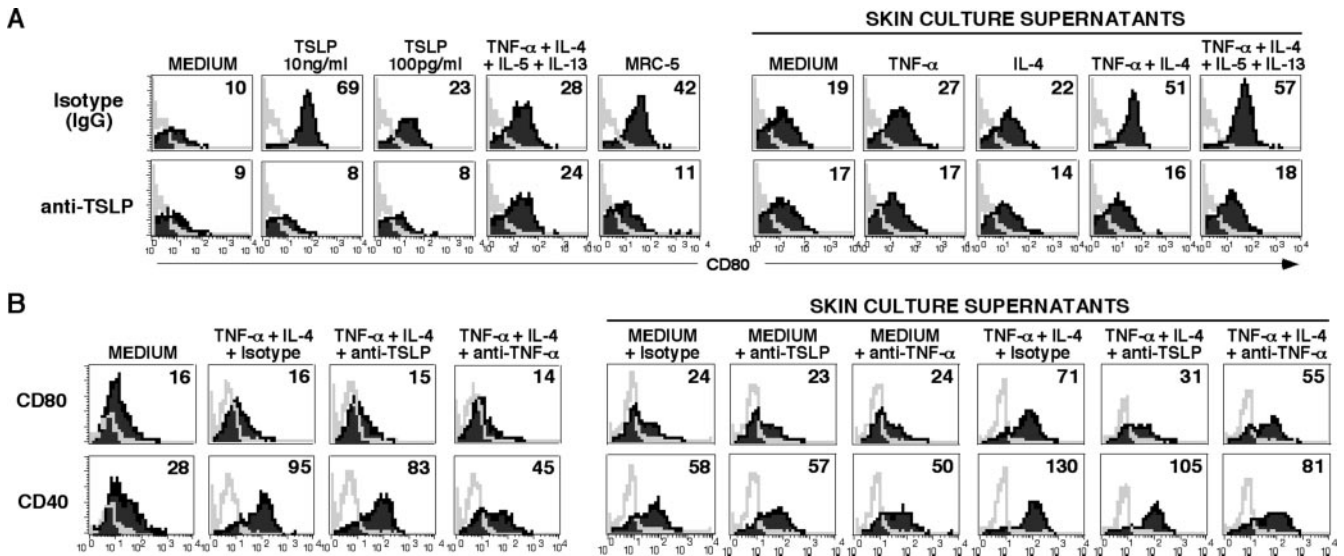


FIGURE 4. Cytokine-stimulated skin cultures induce DC maturation in a TSLP-dependent manner. *A*, Blood CD11c⁺ DCs were cultured for 24 h with exogenous cytokines, MRC-5 cell line, or skin explant culture supernatants, in the presence of a blocking anti-TSLP or matched nonspecific IgG isotype. DC maturation was assessed based on surface CD80 expression. *Top row*, Isotype control; *bottom row*, blocking anti-TSLP mAb. *B*, DC maturation was assessed based on surface CD80 and CD40 expression. Exogenous cytokines or skin supernatants were used in the presence of an IgG isotype, anti-TSLP, or anti-TNF- α blocking mAb. For clarity of the figure, only the mouse IgG isotype is shown. A sheep IgG was used in all experiments to match with the sheep anti-human anti-TSLP mAb and gave similar results (data not shown). Results are from one representative of three independent experiments for each figure panel.

Next, we studied the relative role of TSLP and TNF- α on DC maturation, as assessed by surface CD80, which is more sensitive to TSLP, and CD40, which is more sensitive to TNF- α (our unpublished observation). In the presence of TNF- α plus IL-4-stimulated skin culture supernatant, blocking TSLP down-regulated CD80 expression by approximately 60%, as compared with only 25% when blocking TNF- α (Fig. 4*B*). However, CD40 expression was only 20% TSLP-dependent and 40% TNF- α -dependent (Fig. 4*B*). This suggests that the role of TSLP could be dominant for some aspects of DC activation, such as CD80 induction, but not for others.

Although previous studies have shown that exogenous (1, 2, 29) or transgenic (4, 5) TSLP could drive a strong DC activation, the role of TSLP in DC activation within a complex tissue microenvironment was never assessed. In our system, the skin explant microenvironment contains: 1) exogenous cytokines that mimic the atopic skin cytokine milieu; and 2) keratinocyte-derived factors, which include, but are not restricted to, TSLP (1, 30). Although exogenous TNF- α was present at much higher doses (20 ng/ml) than endogenously produced TSLP (50–100 pg/ml), our data strongly support that TSLP is an important, but not exclusive, DC maturation factor within this complex tissue environment.

In this study, we have provided the first evidence of TSLP induction in human skin. We identified pro-inflammatory and Th2 cytokines as potential early triggers of a TSLP-mediated immune cascade, or as factors that could sustain allergic inflammation in the chronic phase. Keratinocyte-derived TSLP was efficient in mediating DC maturation. We propose that blocking TSLP-inducing cytokines could represent a novel strategy for the treatment of allergic diseases or other TSLP-related pathologies.

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Disclosures

The authors have no financial conflict of interest.

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