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Induction of IL-4 Expression in CD4⁺ T Cells by Thymic Stromal Lymphopoietin¹

Miyuki Omori* and Steven Ziegler²*†

The cytokine thymic stromal lymphopoietin (TSLP) has been implicated in the development and progression of allergic inflammation in both humans and mice. Although the underlying mechanism is not known, TSLP-stimulated dendritic cells have been shown to prime human CD4⁺ T cells into Th2 cytokine-producing cells. However, its direct effect on CD4⁺ T cells has not been extensively investigated. In this study, we show that TSLP can drive Th2 differentiation in the absence of exogenous IL-4 and APCs. IL-4 blockade inhibited TSLP-mediated Th2 differentiation, demonstrating that IL-4 is involved in this process. Further analysis has shown that TSLP-induced Th2 differentiation is dependent on Stat6 and independent of IL-2 and that TSLP treatment leads to immediate, direct IL-4 gene transcription. Taken together, these data demonstrate that TSLP is directly involved in Th2-mediated responses via the induction of IL-4 production. The Journal of Immunology, 2007, 178: 1396–1404.

Allergic diseases including atopic dermatitis and asthma share common pathogenic features as a result of an inflammatory cascade that is caused by the release of soluble factors including the Th2-derived cytokines IL-4, IL-5, and IL-13, chemokines, and elevated IgE in serum (1–5). In particular, IL-4 plays a critical role in priming naive CD4⁺ T cells into Th2 cells (6, 7). Although the signals that drive the Th2 differentiation of naive CD4⁺ T cells during an immune response have extensively been studied (8–11), the initiating mechanisms of Th2 polarization for the development of allergic diseases still remain unclear.

A candidate for a cytokine that can initiate Th2 differentiation in allergic diseases is the thymic stromal lymphopoietin (TSLP). TSLP was first identified as a biological activity in conditioned medium from a thymic stromal cell line and has been shown to support the growth of immature B220⁺IgM⁺ B cells from bone marrow and fetal liver in mice (12–15). TSLP expression has been found in epithelial cells at barrier surfaces (lung, skin, and gut), activated bronchial smooth muscle cells, and activated mast cells (15, 16). A link to allergic inflammation comes from the finding that TSLP was highly expressed by keratinocytes in the lesional skin of atopic dermatitis patients (16) and in bronchial biopsies from asthmatic patients (17).

The TSLP receptor (TSLPR) complex consists of a heterodimer of the TSLPR and IL-7Rα with expression found on a variety of cell types, including dendritic cells, monocytes, and T cells (18). TSLP signaling appears to be dispensable for immune system development, as mice lacking TSLPR are normal (19). However, TSLPR-deficient mice are resistant to Ag-induced airway inflammation, demonstrating a critical role of TSLP in the development of allergic diseases (20).

A role for TSLP in Th2-mediated inflammation comes from studies using organ-specific TSLP transgenes. Mice expressing TSLP transgenes specifically in the skin or lung developed inflammatory diseases indistinguishable from human atopic dermatitis and asthma, respectively (20–22). Concomitant with disease development was the induction of a robust Th2 response. To further investigate whether TSLP plays a role in priming Th2 differentiation, we used an in vitro culture system to determine the effect of TSLP treatment of naive CD4⁺ T cells. We show here for the first time that TSLP can prime Th2 differentiation in the absence of APCs and that the treatment of naive CD4⁺ T cells with TSLP in the presence of TCR stimulation led to IL-4 production. Further analysis demonstrated that TSLP treatment resulted in transcription of the Il-4 gene and that further Th2 differentiation in these cultures was IL-4 dependent. Consistent with this finding, initial Il-4 gene transcription was partially Stat6 independent, while IL-4 production and Th2 differentiation was completely Stat6-dependent. These data demonstrate a unique and important role of TSLP in CD4⁺ T cell differentiation into effector Th2 cells, suggesting that TSLP orchestrates Th2-mediated allergic diseases including atopic dermatitis and asthma.

Materials and Methods

Mice

Young adult BALB/c (8–12 wk old), Stat6-deficient (Stat6⁻/⁻), and Il-4-deficient (Il-4⁻/⁻) mice were purchased from Taconic Farms and The Jackson Laboratory. Tslpr-deficient (Tslpr⁻/⁻) mice (19) were backcrossed to BALB/c for seven generations. All mice for this study were maintained under pathogen-free conditions. The animal care and use at Benaroya Research Institute at Virginia Mason (Seattle, WA; accredited by American Association for the Accreditation of Laboratory Animal Care) is in accordance with National Institutes of Health guidelines.

Immunoﬂuorescent staining and ﬂow cytometry analysis

In general, a half million cells originally prepared from the spleen were stained with Abs. For surface staining, the Abs, FITC- and PE-conjugated anti-CD4 mAb (clone GK1.5; eBioscience), and FITC-conjugated

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3 Abbreviations used in this paper: TSLP, thymic stromal lymphopoietin; TSLPR, TSLP receptor; phospho-Stat5, phosphorylated Stat5.

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anti-CD62L mAb (clone MEL-14; eBioscience) were used. For the cytokine intracellular staining, FITC-conjugated anti-IFN-γ mAb (clone XMG1.2; eBioscience), PE- and allophycocyanin-conjugated anti-IL-4 mAb (clone 11B11; eBioscience) were used. Cells were cross-linked with 4% paraformaldehyde in PBS for 10 min at 37°C, permeabilized in permeabilizing buffer (50 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, and 0.02% NaN₃ (pH 7.5)) for 10 min on ice, blocked in 10% PBS-PBS for 15 min on ice, and then stained. For the phosphorylated Stat5 (phospho-Stat5) intracellular staining, Alexa 488-conjugated anti-phospho-Stat5 (Y694) mAb (BD Biosciences) was used. For the Gata3 intracellular staining, anti-Gata3 mouse mAb (clone HG3-31; Santa Cruz Biotechnology) and PE-conjugated goat F(ab′)₂ anti-mouse IgG (H+L) were used. Cells were cross-linked with 2% paraformaldehyde in 0.5% BSA-HBSS at 37°C for 30 min at 37°C, and then stained with the Ab. Flow cytometry was performed on FACSCalibur device (BD Biosciences) and results were analyzed by CellQuest Pro software (BD Biosciences).

In vitro CD4⁺ T cell differentiation

Splenid CD4⁺ T cells were isolated with CD4⁺ T cells isolation kit (Miltenyi Biotec) using MACS in yielding purity of >90%. In some experiments, CD4⁺ T cells were sorted using FACS Diva (BD Biosciences) in yielding purity of >99%. In effect, CD4⁺ T cells were cultured in the presence of recombinant human IL-2 (100 U/ml) and either recombinant mouse TSLP (10–30 ng/ml; R&D Systems) or recombinant mouse IL-4 (10 ng/ml; BD Biosciences), and the cells were stimulated with plate-bound anti-CD3ε mAb (clone 2C11; 10 µg/ml) and cultured with an anti-IFN-γ mAb culture supernatant (clone R4-6A2) or purified anti-IL-4 mAb (clone 11B11; 10 µg/ml; eBioscience and National Institutes of Health) for the first 2 days. The cells were maintained with rIL-2 and rIL-4 or rTSLP until day 5. On day 5, cells were harvested and resuspended on the fresh plate coated with anti-CD3ε mAb after being washed 3 times. After a 5-h incubation in the presence of monensin, cells were stained with Abs for the cell surface, cross-linked with 4% paraformaldehyde in PBS, and intracellular staining was performed with anti-IFN-γ and anti-IL-4. As indicated in Fig. 2, CD4⁺ T cells were labeled with 5 µM CFSE (Molecular Probes) for 8 min at 37°C before cultivation. Then, cells were cultured with rTSLP (10 ng/ml) or rIL-4 (10 ng/ml) for 3 days in the presence of plate-bound anti-CD3ε mAb (10 µg/ml). On day 3, the cells were re-stimulated with anti-CD3ε mAb in the presence of monensin for 5 h followed by IL-4 intra-cellular staining.

For the ELISA, naive CD4⁺ T cells or day 5 cultured cells were seeded at 0.2 million cells/200 µl of complete RPMI 1640 medium per well of 96-well flat plate in the presence or absence of plate-bound 10 µg/ml anti-CD3ε mAb. After 24 or 48 h, supernatants were harvested and ELISA was performed for IL-4, IL-5, IL-13, and IFN-γ. The concentrations were measured with clones 11B11 and BV6D-24G2 (eBioscience) for IL-4, clones TRFK5 and TRFK4 (eBioscience) for IL-5, the Ready-SET-Go! IL-13 ELISA kit (eBioscience) for IL-13, and clones XM1G1.2 and R4-6A2 (eBioscience) for IFN-γ, respectively.

For the phosphorylated Stat5 intracellular staining on day 2, CD4⁺ T cells were cultured without any cytokine or with either rTSLP (30 ng/ml) or rIL-4 (10 ng/ml) in the presence of immobilized anti-CD3ε mAb (10 µg/ml). On day 2, cells were washed twice with complete RPMI 1640 medium, resuspended in a fresh culture plate, incubated for 8 h, and then incubated with the same cytokines for 10 min at 37°C. For the Gata3 intracellular staining at early time points, CD4⁺ T cells were cultured without any cytokine or with either rTSLP (10 ng/ml) or rIL-4 (10 ng/ml) in the presence of immobilized anti-CD3ε mAb (10 µg/ml) for 2, 5, 10, 20, or 30 h. As indicated in Fig. 6C, neutralizing anti-IL-2 mAb (clone JES6-1A12 at 10 µg/ml; eBioscience) was added to the culture.

Quantitative RT-PCR

Total RNA was isolated with TRZol (Invitrogen Life Technologies) and reverse transcription was done with SuperScript II (Invitrogen) according to the manufacturer’s protocol. PCR was performed with Platinum SYBR Green qPCR SuperMix uracil-DNA glycosylase (UDG) with 6-carboxyfluorescein (Invitrogen Life Technologies) and amplified with 7900HT (Applied Biosystems). Ten nanograms of cDNA was used for a PCR. Cycling conditions were 50°C for 2 min, 95°C for 2 min, and 50 repeats of 95°C for 15 s, 57°C for 1 min, and 68°C for 1 min. All data were analyzed with an average of triplicate PCR samples by SDS2.2.1 software (Applied Biosystems). All data were normalized by cyclophilin B gene expression of each sample. The primers used are as follows: cyclophilin B gene forward, 5′-GCTACAGGAGAGAAGATTGCCC-3′; cyclophilin B gene reverse, 5′-CGGCTGTCGTCGTCGTCGTC-3′; Gata3 forward, 5′-GAAGGCTCCACGCGCAACG-3′; Gata3 reverse, 5′-ACCCTGCCGGGTCCAC-3′; Il-4 forward, 5′-GGTATCGGGAATGCGG-3′; Il-4 reverse, 5′-GATCGCACTTGAAGCCGGCTCA-3′; and Il-4ra forward, 5′-AGAGCGGTCCTGGCTAGCTCCAG-3′.

Western blot analysis

Nuclear extracts were prepared using the NE-PER nuclear and cytoplasmic extraction reagent (Pierce). Immunoblotting was performed with anti-Gata3 mouse mAb (clone HG3-31; Santa Cruz Biotechnology), anti-c-Maf rabbit polyclonal Ab (M-153; Santa Cruz Biotechnology), anti-Stat6 rabbit polyclonal Ab (Cell Signaling Technology), anti-phospho-Stat6 (Tyro-441) rabbit polyclonal Ab (Cell Signaling Technology), and rabbit anti-transcription factor IIB (TFIIB) polyclonal Ab (clone C-18; Santa Cruz Biotechnology). The levels of protein were visualized with SuperSignal West Femto maximum sensitivity substrate (Pierce) or the mixture of p-coumaric acid, luminol, and hydrogen peroxide.

Results

TSLP can directly drive Th2 development

TSLP has been associated with Th2-type inflammation in both mice and humans (16, 17, 20–22), with TSLP responsiveness being essential for the development of Ag-induced airway inflammation in mice (20, 23). However, the precise roles played by TSLP in these diseases remain to be elucidated. Recent studies have shown that in the mouse CD4⁺ T cells are capable of proliferating in response to TSLP exposure under the anti-CD3ε mAb stimulation in vitro and that TSLP can affect CD4⁺ T cell development and proliferation in vivo (24). Consistent with the in vitro data indicating that TSLP has a direct effect on CD4⁺ T cells, we found that naive CD4⁺ T cells are also capable of responding directly to TSLP to differentiate into Th2 cytokine-producing cells. To investigate mechanisms of the direct effect of TSLP on Th2 development, we used an in vitro differentiation culture system. In this system, sorted CD62Lhigh naive CD4⁺ splenic T cells (>99% pure) were cultured with exogenous IL-2, immobilized anti-CD3ε mAb, and antagonistic anti-IFN-γ mAb in the presence or absence of exogenous TSLP for 2 days. At that time, cells were transferred to a fresh well in the absence of anti-CD3ε mAb and anti-IFN-γ mAb and cultured in the same cytokine conditions for an additional 3 days. After 5 days, the cells were washed and then re-stimulated with anti-CD3ε mAb in the presence of monensin for 5 h and analyzed for cytokine production by intracellular staining and flow cytometry. The BALB/c cultures containing TSLP showed a 4- to 5-fold increase in the number of IL-4-producing CD4⁺ T cells as compared with cultures without TSLP, while there was no increase in IFN-γ-producing cells. However, this increase was not observed in Tslpr−/− mice (Fig. 1A). Similarly, TSLP treatment of naive CD4⁺ T cells induced the production of other Th2 cytokines, IL-5 and IL-13, for 24 h on days 5 and 6 upon anti-CD3ε reactivation (Fig. 1B). IL-4, IL-5, and IL-13 were not detected in the absence of anti-CD3ε reactivation (data not shown). Treatment of naive CD4⁺ T cells with increasing doses of TSLP showed that IL-4 production reached a plateau with 10 ng/ml exogenous TSLP within 48 h (data not shown), the concentration that was used for most of the following experiments. All of above data indicate that TSLP can directly drive the differentiation of CD4⁺ T cells into Th2 cytokine-producing cells.
TSLP-mediated IL-4 production is independent of cell proliferation

It has been shown that at least three to four cell divisions, following stimulation, are required for the generation of IL-4-producing Th2 cells (25, 26). Because TSLP has been shown to costimulate CD4+ T cells and enhance proliferation (24), a possible explanation for the TSLP-mediated increase in IL-4-producing cells, as shown in Fig. 1, is an enhanced cellular proliferation in the TSLP-containing cultures. To address this possibility, we tested the relationship between TCR-mediated expansion of CD4+ T cells and IL-4-production in TSLP-treated CD4+ T cells. CFSE-labeled CD4+ T cells were stimulated with plate-bound anti-CD3e mAb in the absence or presence of either exogenous TSLP or IL-4 and, after 3 days, intracellular IL-4 production in the restimulated cells was determined. Among these cultures, no cytokine- and TSLP-treated cells displayed similar kinetics of cell division, whereas IL-4-treated cells expanded more rapidly (Fig. 2). However, within each division the number of IL-4-producing cells was higher in the TSLP-containing cultures than in the cultures lacking TSLP, while the IL-4 cultures had the most IL-4-producing cells. These results show that increased levels of IL-4 production in CD4+ T cells cultured with TSLP is not due to the enhanced cell proliferation during differentiation.

TSLP-mediated IL-4 production is Stat6 and IL-4 dependent

To investigate further the mechanism by which TSLP treatment leads to IL-4 production, we examined the role of IL-4 in this process. Naïve CD4+ T cells were isolated and stimulated with anti-CD3e mAb and TSLP in the presence or absence of neutralizing anti-IL-4 mAb. The addition of the anti-IL-4 mAb completely abrogated IL-4 production in TSLP-treated CD4+ T cells, suggesting that the generation and/or maintenance of IL-4-producing CD4+ T cells depend on IL-4R signaling (Fig. 3A). To test this possibility, we examined IL-4 production in TSLP-treated CD4+ T cells from Stat6−/− mice. As seen in the IL-4-treated cultures, no IL-4-producing cells were detected when Stat6−/− CD4+ T cells were stimulated with exogenous TSLP (Fig. 3B). These data suggest that the ability of TSLP to drive the differentiation and to maintain the phenotype of IL-4-expressing CD4+ T cells is Stat6 dependent.

To determine the effect of TSLP on the generation of IL-4-producing cells, we examined whether TSLP treatment induced IL-4 transcription in the presence of TCR stimulation. CD4+ T cells were stimulated with anti-CD3e mAb in the presence of either exogenous IL-4 or TSLP, and IL-4 mRNA levels were examined at specific times following stimulation. IL-4-treated CD4+ T cells showed a marked induction of IL-4 transcripts, reaching a peak at 2.5–5 h posttreatment (Fig. 3C). Similarly, TSLP-treated cells also showed an increase in IL-4 mRNA levels, with a peak at
2.5–5 h posttreatment. In addition, IL-4 production from TSLP plus anti-CD3e mAb-treated CD4⁺ T cells was detectable by 24 h (Fig. 6B). These data suggest that TSLP treatment of CD4⁺ T cells can induce IL-4 gene transcription and IL-4 production.

Both a blockade of IL-4 and a loss of Stat6 resulted in an inability of TSLP treatment to induce IL-4-producing CD4⁺ T cells (Fig. 3, A and B). To examine the role of IL-4 signaling in TSLP-mediated IL-4 production in more detail, IL-4 mRNA levels were determined in CD4⁺ T cells from Stat6⁻/⁻/⁻ mice treated with either TSLP or IL-4. Consistent with the previous data (27), IL-4 treatment of Stat6⁻/⁻/⁻ CD4⁺ T cells resulted in the induction of IL-4 transcription. TSLP-treated Stat6⁻/⁻/⁻ CD4⁺ T cells also displayed IL-4 transcription, albeit at reduced levels as compared with CD4⁺ T cells from wild-type mice (Fig. 3D). These data suggest that induction of IL-4 transcription in CD4⁺ T cells by TSLP is partially Stat6 dependent.

Previous studies of TSLP signal transduction have shown that the engagement of the TSLP receptor leads to the activation of Stat5 (12, 28) and there is a report that the induction of IL-4 transcription in Th2-conditioned cells is Stat6 independent (27). However, our data, as shown above, demonstrated a Stat6-dependent TSLP pathway leading to transcription of the IL-4 gene. To ascertain whether TSLP is capable of directly activating Stat6 or enhancing the effect of IL-4 to induce the activation of Stat6, TSLP-stimulated CD4⁺ T cells were examined for the presence of

**FIGURE 3.** TSLP-mediated IL-4 transcript is partially Stat6 dependent. A, Splenic CD62LhighCD4⁺ T cells from BALB/c mice were cultured with exogenous IL-2 (100 U/ml) and either TSLP (10 ng/ml) or IL-4 (10 ng/ml). Cells were cultured in the presence of plate-bound anti-CD3e mAb stimulation and either anti-IFN-γ mAb or anti-IL-4 mAb for the first 2 days. On day 5, cells were restimulated with plate-bound anti-CD3e mAb for 5 h in the presence of monensin, and IL-4 and IFN-γ expressions were measured by intracellular staining. Numbers in quadrants indicate the percentage of cells in the designated gate. Three independent experiments with identical results were performed. B, Splenic CD62Lhigh CD4⁺ T cells from Stat6⁻/⁻ mice were cultured as described in A for 5 days. After restimulation with plate-bound anti-CD3e mAb and monensin, cytokine production was measured by intracellular staining. Numbers in quadrants indicate the percentage of cells in the designated gate. Three independent experiments with identical results were performed. C, D, and F, Splenic total CD4⁺ T cells from BALB/c or Stat6⁻/⁻ mice were stimulated with either exogenous TSLP (10 ng/ml) or IL-4 (10 ng/ml) in the presence of plate-bound anti-CD3e mAb stimulation. The cells were harvested at each time point (0, 2.5, 5, 10, 20, and 30 h (C); 0, 2.5, 5, and 10 h (D and F)), and IL-4 or IL-4ra (IL-4r α-chain) mRNA levels were measured by quantitative RT-PCR. All data were normalized to the cyclophilin B mRNA levels. Changes in mRNA expression are depicted as “relative change” to mRNA expression at time point 0. Three independent experiments with identical results for C and D and two independent experiments with identical results for F were performed. E, Splenic CD62LhighCD4⁺ T cells from BALB/c mice were cultured without any cytokines or with either or both TSLP (30 ng/ml) and IL-4 (1 ng/ml) for 2 days in the presence of plate-bound anti-CD3e mAb stimulation. The cells were rested in culture medium without any cytokines for 8 h, cultured with the same cytokine for 10 min, and then lysed in radioimmunoprecipitation assay buffer for immunoblotting. Blots were probed with Abs specific for phospho-Stat6 (Y641) or total Stat6, as indicated. Two independent experiments with identical results were performed.

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activated Stat6. Naive CD4⁴ T cells were isolated and stimulated with anti-CD3e mAb in the presence or absence of IL-4 or TSLP or the presence of both for 2 days, washed extensively, rested for 8 h in the absence of stimulation, and then recultured with the same cytokine alone for 10 min. At those times lysates were prepared and the presence of activated Stat6 was determined by a Western blot with an Ab specific to the phosphorylated tyrosine residue 641 of Stat6. CD4⁴ T cells initially cultured in the absence of cytokine showed the induction of phosphorylated Stat6 (Fig. 3F). Furthermore, CD4⁴ T cells treated with both IL-4 and TSLP induced higher phosphorylation of Stat6 than with IL-4 alone. The affect of TSLP treatment on Stat6 activation was not due to alterations in IL-4 receptor expression, because the expression of Il-4ra was equivalent in TSLP- and IL-4-treated CD4⁴ T cells (Fig. 3F). Taken together, these data suggest that TSLP by itself does not activate Stat6 directly but enhances the activation of Stat6 induced by IL-4, possibly through enhanced IL-4 production.

**TSLP induces Gata3 transcription in a Stat6-dependent manner**

Several transcription factors have been implicated in the regulation of IL-4 gene transcription (29, 30). Among them, the zinc finger protein Gata3 has been shown to be required for both Th2 cell development and maintenance, both at the level of individual gene transcription as well as the more global chromatin remodeling at the Th2 cytokine gene locus (31–34). Gata3 is specifically induced in Th2 cells, which is sufficient for Th2 cytokine gene expression in IL-4-producing cells (31, 33).

In addition to Gata3, c-Maf has also been shown to be important for the transcriptional regulation of the IL-4 gene (35). The expression of these Th2-specific transcription factors was assessed in CD4⁴ T cells cultured on day 5 with anti-CD3e mAb and IL-2 in the presence or absence of IL-4, TSLP, neutralizing anti-IFN-γ mAb, and anti-IL-4 mAb. Gata3 expression was increased in both TSLP- and IL-4-treated cultures, while c-Maf levels remained the same. The increased level of Gata3 in TSLP-treated cells was diminished in the presence of a neutralizing anti-IL-4 mAb (Fig. 4A).

Gata3 is expressed at a basal level in naive CD4⁴ T cells, and the up-regulation of Gata3 in primary T cells is dependent on TCR signals (27, 36). TSLP is capable of inducing IL-4 expression (Fig. 1), and the addition of neutralizing anti-IL-4 mAb to the cultures in the first 48 h abolished TSLP-mediated IL-4 production (Fig. 3A). Although Gata3 is critical for development and maintenance of the Th2 phenotype (32, 33) and for the production of initial IL-4 in TCR-stimulated naive CD4⁴ T cells (37), it was not clear from these experiments whether Gata3 in the TSLP-treated cells was induced directly by TSLP or secondarily by IL-4 in the presence of TCR stimulation. To assess whether TSLP was capable of directly inducing Gata3 expression, CD4⁴ T cells were cultured in the presence of TSLP or IL-4 upon anti-CD3e mAb cross-linking.

**FIGURE 4.** Gata3 is induced in TSLP-treated CD4⁴ T cells. A, Splenic CD62L<sup>high</sup> CD4⁴ T cells from BALB/c mice were cultured with either exogenous IL-2 (100 U/ml), IL-2 plus TSLP (10 ng/ml), or IL-2 plus IL-4 (10 ng/ml). Cells were cultured in the presence of plate-bound anti-CD3e mAb stimulation and either anti-IFN-γ mAb or anti-IL-4 mAb for the first 2 days. On day 5, the cells were harvested and levels of Gata3, c-Maf, and transcription factor IIB (TFIIB; a loading control) were detected by immunoblotting. Three independent experiments with identical results were performed. B, Splenic total CD4⁴ T cells from BALB/c mice were stimulated with either TSLP (10 ng/ml) or IL-4 (10 ng/ml) in the presence of plate-bound anti-CD3e mAb stimulation. The cells were harvested at each time point (0, 2.5, 5, 10, 20, and 30 h), and Gata3 mRNA levels were measured by quantitative RT-PCR as described in Fig. 3C. All data were normalized to cyclophilin B mRNA levels. Changes in mRNA expression are depicted as “relative change” to mRNA expression at time point 0. The data represent one of three independent experiments. C, Total splenic CD4⁴ T cells from BALB/c mice were stimulated with either TSLP (10 ng/ml) or IL-4 (10 ng/ml) in the presence of plate-bound anti-CD3e mAb stimulation and analyzed at each time point (0, 4, 24, and 48 h) for Gata3 expression by intracellular staining. Numbers indicate mean fluorescence intensity of Gata3 staining. Filled histogram, Gata3; open histogram, isotype control. Three independent experiments with identical results were performed.
cells were harvested at early time points after the cytokine stimulation together with TCR signals to assess the Gata3 transcript by quantitative RT-PCR and protein expression by flow cytometry. Gata3 mRNA was rapidly induced in both TSLP- and IL-4-treated CD4+ T cells in the presence of anti-CD3/anti-CD25 mAb stimulation, peaking within 2.5 h of treatment (Fig. 4B). Similarly, Gata3 protein was induced within 24 h of TSLP or IL-4, with the mean fluorescence intensity lower in TSLP-treated cells than in IL-4-treated cells (Fig. 4C). The reduced level of Gata3 in TSLP-treated cells, as compared with IL-4-treated cells, could be explained by the inability of TSLP to directly activate Stat6, which is critical for Gata3 transcription (Fig. 3E). To determine whether the TSLP effect on Gata3 transcription was direct or indirect, we examined CD4+ T cells from mice deficient in Stat6 or Il-4 (Fig. 5). Neither IL-4 nor TSLP were capable of inducing Gata3 transcription in CD4+ T cells from Stat6−/−/− mice (Fig. 5A), consistent with a

**FIGURE 5.** TSLP-mediated induction of Gata3 is both Stat6 and IL-4 dependent. Splenic total CD4+ T cells from BALB/c, Stat6−/− (A), or Il-4−/− (B) mice were stimulated with either TSLP (10 ng/ml) or IL-4 (10 ng/ml) in the presence of plate-bound anti-CD3e mAb stimulation. The cells were harvested at each time point (0, 2.5, 5, and 10 h), and Gata3 mRNA levels were measured by quantitative RT-PCR as described in Fig. 3C. All data were normalized to the cyclophilin B mRNA levels. Changes in mRNA expression are depicted as “relative change” to mRNA expression at time point 0. The data represent one of two independent experiments.

**FIGURE 6.** TSLP-mediated induction of IL-4 in CD4+ T cells is IL-2 independent. A, Splenic CD62LhighCD4+ T cells from BALB/c mice were cultured without any cytokines or with either exogenous TSLP (30 ng/ml) or IL-2 (100 U/ml) for 2 days in the presence of plate-bound anti-CD3e mAb stimulation. The cells were rested in culture medium without any cytokines for 8 h and restimulated with the same cytokine for 10 min, and the levels of phospho-Stat5 were measured by intracellular staining. Numbers indicate the mean fluorescence intensity of phospho-Stat5. Closed histogram, phospho-Stat5; open histogram, isotype control. Three independent experiments with identical results were performed. B, Splenic CD62LhighCD4+ T cells from BALB/c mice were cultured with different combinations of exogenous TSLP (10 ng/ml) and plate-bound anti-CD3e mAb. The culture supernatant was harvested at 24 and 48 h, and the production of IFN-γ, IL-2, and IL-4 in the supernatant was measured using ELISA. The data show concentrations of cytokines secreted by 0.2×10^5 cells per 200 μl. Three independent experiments with identical results were performed. C, Splenic total CD4+ T cells from BALB/c mice were stimulated with either exogenous TSLP (10 ng/ml) alone or TSLP plus neutralizing anti-IL-2 mAb (10 μg/ml) in the presence of plate-bound anti-CD3e mAb stimulation. The cells were harvested at each time point (0, 2.5, 5, and 10 h) and IL-4 mRNA levels were measured by quantitative RT-PCR as described in Fig. 3. All data were normalized to the cyclophilin B mRNA levels. Changes in mRNA expression are depicted as “relative change” to mRNA expression at time point 0. The data represent one of two independent experiments.
critical role of the Stat6 signaling pathway in Gata3 expression (27, 38). However, while IL-4 treatment resulted in Gata3 transcription in IL-4−/− CD4+ T cells, TSLP treatment did not (Fig. 5B). Similar results were obtained when the neutralizing anti-IL-4 mAb was added to cultures containing BALB/c CD4+ T cells and TSLP (data not shown). Taken together, these data suggest that the TSLP-mediated induction of Gata3 transcription is both Stat6 and IL-4 dependent.

Activation of Stat5 by TSLP could direct the initial IL-4 production in CD4+ T cells independently of IL-2

Stat5 has been shown to play a critical role in the induction of IL-4 production in Th2-conditioned CD4+ T cells (39). Although TSLP has been shown to be capable of inducing the phosphorylation of Stat5 in B cells (12, 28), no data were available on the effect of TSLP on Stat5 activation in CD4+ T cells. To address this issue, we examined the Stat5 phosphorylation in TSLP-treated CD4+ T cells. As shown in Fig. 6A, phosphorylated Stat5 was detected in both TSLP- and IL-2-treated CD4+ T cells, with IL-2-treated cells showing higher mean fluorescence intensity. This suggests that TSLP induces Stat5 activation in CD4+ T cells and that this activation may play an important role in initial IL-4 production. Previous work has shown that IL-4 transcription in CD4+ T cells at 24 h following stimulation with APCs and low peptide concentration is IL-2 dependent (37). Because IL-2 is present in the TSLP-treated cultures within 24 h upon TCR-engagement (Fig. 6B), we tested whether IL-2 was involved in TSLP-mediated IL-4 transcription. As shown in Fig. 6C, IL-2 blockade had no inhibitory effect on TSLP-mediated IL-4 transcription. In addition to the production of IL-2 and IL-4, TSLP-treated cells produced a small amount of IFN-γ in the 48-h cultures. However, after 5 days of culture the cells produce only IL-4, not IFN-γ (Fig. 1). In the 48-h cultures neither IL-5 nor IL-13 were detected (data not shown). In conclusion, TSLP is capable of activating Stat5 in CD4+ T cells and of producing both IL-4 and IL-2 within 48 h, but the TSLP-mediated initial IL-4 is independent of IL-2.

Discussion

Previous work has shown that TSLP treated-human peripheral CD11c+ dendritic cells exhibit activated phenotypes and are capable of priming naive CD4+ T cells to produce Th2 cytokines upon restimulation (16, 40). In mice, both bone marrow-derived dendritic cells and splenic dendritic cells can be activated by TSLP (20, 23), although the ability of these dendritic cells to prime naive CD4+ T cells has not been examined. TSLP has also been shown to have direct effects on CD4+ T cells upon TCR engagement (24). We have now shown that TSLP can drive Th2 differentiation of naive CD4+ T cells through IL-4 gene transcription. In the in vitro culture system described herein, we show that stimulation of CD62Lhigh naive CD4+ T cells with TSLP, in conjunction with TCR engagement, leads to an increase in IL-4-producing cells that are also capable of producing IL-5 and IL-13 (Fig. 1). In addition, this finding was not limited to BALB/c mice, as TSLP treatment of C57BL/6-derived naive CD4+ T cells also resulted in an increased numbers of IL-4-producing cells (data not shown). The TSLP effect was directly on CD4+ T cells as the starting population of >99% CD62L+CD4+ cells. Thus, this result is the first evidence of a direct role of TSLP on Th2 differentiation. Our data are consistent with the report that TSLP directly enhances CD4+ T cell expansion upon TCR engagement (24), demonstrating a direct influence of TSLP on a activated CD4+ T cells.

The factors that contribute to Th2 differentiation include the cytokine IL-4 and transcription factors Stat6, Gata3, and Stat5 (8, 11). Stat6 deficiency causes the impairment of proliferation and Th2 differentiation in response to IL-4 (41). In addition, overexpression of an active form of Stat6 in developing Th1 cells results in the expressions of Th2-specific cytokines and transcription factors (38). These reports suggest that Stat6 is essential for the maintenance of IL-4-mediated Th2 differentiation. In contrast, IL-4-mediated induction of Il-4 transcription is Stat6 independent at early times, showing that Stat6 is not required for initial IL-4 production (27). In this report we have shown that the maintenance of TSLP-mediated Th2 differentiation is Stat6 dependent (Fig. 3B). However, the induction of TSLP-mediated IL-4 transcription is not completely independent of Stat6 unlike that of IL-4-mediated Il-4 (Fig. 3D), suggesting that an as yet to be identified component of the TSLP signaling pathway is involved in Stat6-independent IL-4 transcription.

Gata3 also plays an important role for the maintenance of the Th2 cytokine production and chromatin remodeling at the Th2 cytokine gene loci (31, 33). IL-4 production was greatly reduced in Th2 clone D10 by expressing anti-sense Gata3, and overexpression of Gata3 leads to increased level of IL-4 and other Th2 cytokine production (31, 34, 42). In addition, early IL-4 transcript expression is Gata3 dependent (37). Thus, Gata3 has a key role in IL-4-mediated Th2 differentiation. Gata3 protein levels were elevated in TSLP- and IL-4-treated CD4+ T cells (Fig. 4A). TSLP-mediated Gata3 transcription at early time points was both Stat6 and IL-4 dependent (Fig. 5, A and B), although TSLP-mediated Gata3 protein expression was lower than that induced by IL-4 (Fig. 4C). Interestingly, TSLP-mediated early Gata3 transcription was not induced in IL-4 deficient CD4+ T cells, suggesting that TSLP exerted its effect through the induction of IL-4 and not directly on Gata3 transcription.

Stat5 also plays a critical role during Th2 differentiation. Th2 differentiation is impaired in Stat5A-deficient mice, but reconstituting Stat5A with retroviral infection restores the differentiation. This suggests that Stat5 signaling is important for Th2 polarization (43). Naïve CD4+ T cells that ectopically express a constitutively active Stat5A mutant, Stat5A*6, induce the differentiation of IL-4-producing cells under a Th2 condition in the absence of either IL-2 or Stat6. Stat5 activation is thus sufficient for IL-4-mediated Th2 differentiation (39). TSLP can activate Stat5A and Stat5B in B cells and primary CD4+ T cells (Refs. 12, 28, and this report). Thus, it is possible that TSLP mediates its effects on IL-4 transcription through the induction of IL-2, as IL-2 was also induced by TSLP-treated CD4+ T cells within 48 h (Fig. 6B). However, two factors suggest that this is not the case. First, we did not detect IL-4 production in naïve CD4+ T cells stimulated with anti-CD3e mAb in the presence of only exogenous IL-2 (Fig. 1), although we did detect activated Stat5 (Fig. 6A). Second, TSLP-mediated early IL-4 transcription was unchanged in the presence of neutralizing anti-IL-2 mAb (Fig. 6C). Taken together, these data suggest that the induction of TSLP-mediated IL-4 transcription was not due to IL-2-mediated Stat5 activation. In support of a role for Stat5 in TSLP-mediated IL-4 production, we observed histone H3 hyperacetylation of two Stat5 binding sites located in intron 2 of Il-4 gene in TSLP-treated CD4+ T cells (data not shown). These sites, H3SII and H3SIII, have been shown to be in an open chromatin conformation and to bind Stat5 following IL-2 treatment (39, 44). This observation demonstrates that TSLP is capable of remodeling the chromatin at this site in CD4+ T cells concomitant with Th2 differentiation. Taken as a whole, all data suggest that although Stat5 is likely to be an important player in TSLP-mediated Th2 differentiation, other components of the TSLP signaling pathway are likely to be critical. The nature of these components remains to be determined.
As shown in Fig. 6B, TSLP-treated naive CD4+ T cells are potentially capable of inducing IFN-γ, along with IL-4 and IL-2, within 48 h of treatment. These data suggest that TSLP might be able to also play a role in Th1-type inflammation, such as the chronic stages of atopic diseases. However, TSLP treatment for naive CD4+ T cells under both the neutralizing condition (anti-CD3ε plus IL-2) and the moderately Th2-skewed condition (anti-CD3ε, IL-2, and anti-IFN-γ mAb) results in the differentiation of naive CD4+ T cells into IL-4-producing CD4+ T cells (data not shown and Fig. 1), indicating that prolonged exposure (5 days) to TSLP leads to Th2-type differentiation. These data also suggest that the outcome of TSLP exposure may be influenced by the nature of the inflammatory milieu or the phase of the disease, whereas in allergic inflammatory responses the acute phase is Th2 dominant and the chronic phase is Th1 dominant (45, 46).

In summary, we have shown that TSLP is capable of directly driving the Th2 differentiation of naive CD4+ T cells through the direct induction of IL-4 expression. In vivo studies have shown that elevated levels of TSLP result in increased number of CD4+ T cells (Ref. 24 and unpublished data) and in Th2-polarized phenotype (20, 21). Taken together, these data demonstrated that TSLP can drive Th2 development in two ways: either through the activation of dendritic cells or by directly acting on naive CD4+ T cells. The relative role of each pathway in the normal and pathological functions of TSLP remains to be determined.

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Disclosures

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References


