Cutting Edge: Direct Action of Thymic Stromal Lymphopoietin on Activated Human CD4+ T Cells

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Thymic stromal lymphopoietin (TSLP) is a cytokine that promotes CD4+ T cell homeostasis and contributes to allergic and inflammatory responses. TSLP can act directly on mouse CD4+ T cells, but in humans, the available data have indicated that TSLP receptors are not expressed on CD4+ T cells and that TSLP instead activates dendritic cells, which in turn promote the proliferation and differentiation of CD4+ T cells. We now unexpectedly demonstrate the presence of TSLP receptors on activated human CD4+ T cells. Strikingly, whereas freshly isolated peripheral blood human T cells show little if any response to TSLP, TCR stimulation allows a potent response to this cytokine. Moreover, TSLP increases the sensitivity of human CD4+ T cells to low doses of IL-2, augmenting responsiveness of these cells to TCR engagement. Our results establish that human CD4+ T cells are direct targets for TSLP.

**Materials and Methods**

**CD4+ T cell and DC purification and culture**

CD4+ T cells from PBMC purified by negative depletion using a kit (Miltenyi Biotec) were cultured at 2 × 10^6 cells/ml in RPMI 1640 medium containing 10% FBS, 2 mM glutamine, 100 U/ml penicillin and streptomycin. DCs were purified using the blood dendritic cell isolation kit II (Miltenyi Biotec).

**RNA isolation and real-time PCR**

RNA was extracted using TRIzol (Invitrogen Life Technologies) and RNeasy (Qiagen), reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad), and hTSLPR cDNAs identified by a fluorogenic 5′-nuclease PCR assay and an ABI Prism 7900HT sequence detection system (PerkinElmer). One microgram of RNA was used for RT-PCR, and 0.2 μg of this product was amplified using internal 5′ (TGGAGGCCGTAGGTGTCAT-3′) and 3′ (5′-TGGAGGCCGTAGGTGTCAT-3′) primers and the TaqMan FAM-TAMRA probe (5′-FAM-CATGAGGAGGACAGAGGAGGACAGGAGGAGGACAGAT-3′) (Operon Biotech). TSLPR mRNA levels were measured using a standard curve relative to 18S RNA that was detected using TaqMan FAM-MGB primers (Applied Biosystems).

### References

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3. Abbreviations used in this paper: TSLP, thymic stromal lymphopoietin; KO, knockout; DC, dendritic cell; 7-AAD, 7-aminoactinomycin D.

*Cutting Edge: Direct Action of Thymic Stromal Lymphopoietin on Activated Human CD4+ T Cells*

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**TSLPR mAbs**

BALB/c mice were immunized with a human TSLPR/IL-7Rα-transfected mouse cell line, inguinal lymph node lymphocytes were fused with myeloma cells, and hybridoma supernatants evaluated. 2D10 (mouse IgG1) mAb stains TSLPR by flow cytometry and Western blotting. It was purified using Hi-Trap Protein G HP column (Amersham Bioscience). Biotin was conjugated using a kit (Invitrogen Life Technologies).

**Immunoprecipitation and immunoblotting**

Clarified lysates were immunoprecipitated overnight at 4°C with anti-Stat5 (Santa Cruz Biotechnology) plus protein A-agarose (Upstate Biotechnology), resolved on NuPAGE 4–12% Bis-Tris gels (Invitrogen Life Technologies), transferred to polyvinylidene difluoride membranes (Invitrogen Life Technologies), and Western blotted.

**Proliferation assays**

CD4+ T cells at 2 × 10^5 cells/well were activated for 5 or 6 days with plate-bound anti-CD3 or IL-2, with or without 50 ng/ml TSLP, and pulsed with 1 μCi of [3H]thymidine for the final 16 h of culture. In some experiments, cells were pre-activated for 3 days and incubated in 96-well flat-bottom plates for 2 or 6 days in medium or with TSLP, IL-2, or IL-7, and then pulsed with 1 μCi of [3H]thymidine. Proliferation was also examined by labeling cells with 2.5 μM CFSE for 8 min at room temperature and monitoring CFSE dilution.

**Results and Discussion**

**TSLPR expression on human CD4+ T cells**

As noted above, whereas mouse CD4+ T cells respond directly to TSLP (10), human TSLP has been reported to only activate peripheral blood CD11c+ DCs but to not act directly on other DCs or T or B cells (15, 16). We analyzed TSLPR expression on resting and activated human CD4+ T cells and the responsiveness of these cells to TSLP to determine whether TSLP indeed exhibited as dramatic a species variation between humans and mice as is seen with IL-7, with mice but not humans requiring IL-7 for B cell development (6–9). In freshly isolated human PBMC, as expected, TSLPR expression was readily detected on CD11c+ DCs (Fig. 1A), but not on CD19+ B cells (Fig. 1A), CD4+ T cells (Fig. 1B), or CD8+ T cells (Fig. 1B).

As TSLP production increases with inflammation (14, 15), we investigated whether cellular activation also induced TSLPR expression on rigorously purified human CD4+ T cells, which contained <1% CD11c+ DCs, approximately 1% B220+ B cells, and no detectable CD8+ T cells (Fig. 1C). Although freshly isolated CD4+ T cells had little if any TSLPR mRNA, activation by anti-CD3 plus anti-CD28 (Fig. 1D) or PHA-L (Fig. 1E) induced TSLPR mRNA within 1 day. TSLPR mRNA was determined by real-time RT-PCR, relative to 18S rRNA (mean ± SEM, n = 5). TSLPR mRNA levels determined by real-time RT-PCR, relative to 18S rRNA (mean ± SEM, n = 5), F. TSLPR mRNA expression in purified human DCs vs freshly isolated CD4+ T cells or cells that were stimulated for 15 h with anti-CD3 plus anti-CD28. G. Purified human CD4+ T cells not activated or activated with anti-CD3 plus anti-CD28 for 1 or 3 days, or 3 days activated and then 1 day rested. Expression of human TSLPR using biotinylated 2D10 mAb (solid line) or an IgG1 control mAb (dotted line) was determined by flow cytometry. Shown is the mean fluorescence intensity. H. Freshly isolated cells or cells pre-activated for 3 days and then rested for 1 day were not stimulated or stimulated for 20 min with 50 ng/ml hTSLP, 100 U/ml IL-2, or 50 ng/ml IL-7. Cell lysates were run on gels and blotted with Abs to TSLPR, phospho-Stat5 (Cell Signaling Technology), Stat5 (BD Transduction Laboratory), or β-actin (Sigma-Aldrich). I. Lysates from control or human-TSLPR transfected Jurkat cells were immunoprecipitated with 2D10 anti-TSLPR or isotype-matched mAbG1 and Western blotted with biotinylated 2D10. A–H, Each experiment was repeated at least five times.

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**FIGURE 1.** TSLPR expression in human CD4+ T cells. A and B, PBMC were stained with mAbs to CD3 and CD11c or CD19 (A), or CD3, CD4 and CD8 (B), and either anti-TSLPR 2D10 mAb (solid line) or an isotype control mAb (dotted line). C, PBMC or purified CD4+ T cells were stained with allophycocyanin-anti-CD4 or allophycocyanin-anti-CD11c, PE-anti-CD8 and FITC-anti-B220 and analyzed by FACS. D and E, Purified CD4+ T cells were not activated or activated with 5 μg/ml plate-bound anti-CD3 plus 1 μg/ml anti-CD28 (BD Pharmingen) (D) or 2 μg/ml PHA-L (Roche) (E). TSLPR mRNA levels determined by real-time RT-PCR, relative to 18S rRNA (mean ± SEM, n = 5). F, TSLPR mRNA expression in purified human DCs vs freshly isolated CD4+ T cells or cells that were stimulated for 15 h with anti-CD3 plus anti-CD28. G, Purified human CD4+ T cells not activated or activated with anti-CD3 plus anti-CD28 for 1 or 3 days, or 3 days activated and then 1 day rested. Expression of human TSLPR using biotinylated 2D10 mAb (solid line) or an IgG1 control mAb (dotted line) was determined by flow cytometry. Shown is the mean fluorescence intensity. H, Freshly isolated cells or cells pre-activated for 3 days and then rested for 1 day were not stimulated or stimulated for 20 min with 50 ng/ml hTSLP, 100 U/ml IL-2, or 50 ng/ml IL-7. Cell lysates were run on gels and blotted with Abs to TSLPR, phospho-Stat5 (Cell Signaling Technology), Stat5 (BD Transduction Laboratory), or β-actin (Sigma-Aldrich). I. Lysates from control or human-TSLPR transfected Jurkat cells were immunoprecipitated with 2D10 anti-TSLPR or isotype-matched mAbG1 and Western blotted with biotinylated 2D10. A–H, Each experiment was repeated at least five times.
levels were lower at days 2 and 3 (Fig. 1, D and E) but persisted for at least 14 days (Fig. 1D). TSLPR mRNA levels in non-activated CD4+ T cells were, as expected, lower than in DCs (16), but increased after activation (Fig. 1F). We also analyzed TSLPR expression on activated CD4+ T cells by flow cytometry (Fig. 1G; see shift in 2nd and 3rd panels compared with the control mAb) and by Western blotting (Fig. 1H, top panels, lanes 5–8 vs 1–4) using 2D10 mAb, revealing increased expression with activation that was stable after 1 day of rest (Fig. 1G). 2D10 mAb is specific for TSLPR, as revealed by its Western blotting the proper sized band from TSLPR transfected but not from control Jurkat T cells (Fig. 1I).

**FIGURE 2.** Potent activation of Stat5 but not Stat1 or Stat3 by TSLP. A, Human CD4+ T cells activated for 3 days and then rested for 1 day were labeled with PE-anti-CD4 and allophycocyanin-anti-CD56, PE-anti-CD8 and allophycocyanin-anti-CD11c, or FITC-anti-B220 and allophycocyanin-anti-CD11c and analyzed by flow cytometry. B and C, Human CD4+ T cells were activated with anti-CD3 plus anti-CD28 (B) or PHA-L (C), rested 1 day in RPMI 1640 medium/10% FBS, then 2 h in RPMI 1640, and then incubated with medium (lane 1), 50 ng/ml hTSLP (lanes 2–4), or 100 U/ml IL-2 (for 15 min, lane 5). Cellular lysates were immunoprecipitated with anti-Stat5, and the recovered proteins resolved on 4–12% NuPAGE Bis-Tris gels, transferred to polyvinylidene difluoride membranes, and analyzed by anti-phospho-Stat5 immunoblotting (Cell Signaling Biotechnology). Membranes were stripped and re-probed with mAbs to Stat1 p91 (Zymed), Stat3 (BD Transduction Laboratory) or phospho-Stat5. Membranes were stripped and re-probed with anti-Stat5 mAb (BD Transduction). D, Staining with anti-phospho-Stat5 mAb (solid line) and isotype-matched control (gray field) for phosphorylated Stat5. E, Cytotoxic T lymphocytes (CTLs) were activated for 5 days with the indicated concentration of anti-CD3 with or without 50 ng/ml TSLP and then cultured with [3H]thymidine for an additional 16 h. Each experiment was repeated at least three times. 8

**FIGURE 3.** TSLP augments proliferation of human CD4+ T cells. A, CD4+ T cells were activated for 5 days with the indicated concentration of anti-CD3 with or without 50 ng/ml TSLP and then were pulsed with [3H]thymidine for 16 h. B, CD4+ T cells were CFSE labeled, cultured in medium ± TSLP (first column) or activated with anti-CD3 ± TSLP (second column) for 6 days. Cells also were activated for 3 days, rested 1 day, labeled with CFSE, and then cultured with or without TSLP for 6 days (third column). CFSE dilution was analyzed by FACS. C, Purified CD4+ T cells were activated with anti-CD3 or anti-CD28 plus anti-CD28 with or without 50 ng/ml TSLP for 3 days, stained with FITC-annexin V and 7-AAD, and analyzed by flow cytometry. D, CD4+ T cells were activated for 3 days with anti-CD3 plus anti-CD28 and then rested for 2 d (black columns) or 6 d (open columns) in medium alone or with 50 ng/ml TSLP, 100 U/ml IL-2, or 50 ng/ml IL-7. Cells were pulsed with [3H]thymidine for an additional 16 h. Each experiment was repeated at least three times.

TSLP can activate Stat5 (17), so we evaluated whether TSLP induced Stat5 phosphorylation in human T cells. Freshly isolated CD4+ T cells responded to IL-2 and IL-7 but not to TSLP (Fig. 1H, 2nd panel from top, lane 2–4), whereas all 3 cytokines induced Stat5 phosphorylation in pre-activated CD4+ T cells (lanes 6–8), correlating with TSLPR expression in these cells (Fig. 1H, top panel, lanes 5–8 vs 1–4). Total Stat5 and actin expression were not affected by any of the treatments (bottom panel).

We next analyzed the kinetics of Stat5 phosphorylation in CD4+ T cells pre-activated with anti-CD3 plus anti-CD28 that were approximately 98.5% pure, with low to absent CD11c+, CD8+, CD56+ and B220+ cells (Fig. 2A). TSLP rapidly induced tyrosine phosphorylation of Stat5, although it was less potent than IL-2 (Fig. 2B). Similar results were obtained with cells pre-activated with PHA-L (Fig. 2C). We confirmed TSLP-induced Stat5 activation by intracellular staining of phospho-Stat5 (Fig. 2D). Using a higher dose of TSLP did not further increase Stat5 phosphorylation (data not shown).
Based on a CFSE-labeling experiment, in the presence of CD3 that by themselves induced little if any cell division (Fig. 4A), the addition of 50 ng/ml TSLP for 5 days. Strikingly, the addition of TSLP substantially increased proliferation even at low concentrations of anti-TSLP for 5 days. TSLP augments Stat5-dependent gene induction and sensitivity to IL-2. CD4+ T cells were cultured in medium for 3 days, rested for 2 days in medium containing 10% FBS, and then not pulsed with [3H]thymidine for 16 h before harvesting and analysis. Data are from one of four independent experiments. D, TSLP increases sensitivity of cells to low concentrations of IL-2. CD4+ T cells were activated for 6 days with the indicated concentrations of IL-2 with or without 50 ng/ml TSLP and then were pulsed with [3H]thymidine for 16 h before harvesting and analysis. Data are from one of five independent experiments.

Because we simultaneously stained each sample in Fig. 2D with anti-phospho-Stat5 and anti-CD4, Stat5 activation was indeed occurring in CD4+ T cells. The short stimulation period, the TSLP effect was a direct action on these cells. Because some cytokines activate more than one STAT protein (6), we also compared the ability of IL-2, IL-7, and TSLP to induce Stat1 and Stat3. In activated CD4+ T cells, TSLP activated Stat5 but mediated little if any phosphorylation of Stat1 and Stat3 (Fig. 2E, 5th vs 1st and 3rd panels), in contrast to IL-2 and IL-7.

**FIGURE 4.** TSLP augments Stat5-dependent gene induction and sensitivity to IL-2 in purified CD4+ T cells. A and B, CD4+ T cells were pre-activated for 3 days, rested for 2 days in medium containing 10% FBS, and then not stimulated or stimulated for 4 h with 50 ng/ml TSLP, 100 U/ml IL-2, or 50 ng/ml IL-7. IL-2Rα (A) and CIS (B) mRNA levels were determined by real-time RT-PCR relative to hGAPDH mRNA using gene expression arrays ready made primers (Applied Biosystems). Mean ± SEM (n = 6). C, CD4+ T cells were cultured in medium ± TSLP for 6 days or activated with anti-CD3 ± TSLP for 3 days. CD25 expression was analyzed by flow cytometry. Data are from one of four independent experiments. D, TSLP increases sensitivity of cells to low concentrations of IL-2. CD4+ T cells were activated for 6 days with the indicated concentrations of IL-2 with or without 50 ng/ml TSLP and then were pulsed with [3H]thymidine for 16 h before harvesting and analysis. Data are from one of five independent experiments.

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**TSLP**-increased proliferation of TCR-activated CD4+ T cells

To further analyze the functionality of TSLP receptors on CD4+ T cells, cells were activated with anti-CD3 with or without TSLP for 5 days. Strikingly, the addition of TSLP substantially increased proliferation even at low concentrations of anti-CD3 that by themselves induced little if any cell division (Fig. 3A). Based on a CFSE-labeling experiment, in the presence of TSLP, there was an increase in the number of responding cells, rather than simply an increase in the proliferation of a small population of cells (Fig. 3B). We also evaluated whether TSLP affected cell viability, but found little if any effect on cells treated for 3 or 5 days with medium, anti-CD3, or anti-CD3 plus anti-CD28, as evaluated by staining with 7-AAD and annexin V (Fig. 3C and data not shown).

Given that TSLP can augment the proliferation of CD4+ T cells after activation, we asked if TSLP could maintain this effect even after washing and removal of TCR stimulation. We activated cells and cultured them for 2 or 6 days in the medium, TSLP, IL-2, or IL-7 (Fig. 3, D). In each case, the presence of cytokine augmented proliferation.

**TSLP elevates sensitivity of CD4+ T cells to low dose of IL-2**

Because TSLP induces Stat5 phosphorylation, we evaluated the induction of two genes, IL2Rα (Fig. 4A) and CIS (Fig. 4B), known to be regulated by Stat5 (18–20). TSLP increased IL-2Rα and CIS mRNA expression, albeit less potently than seen with IL-2 or IL-7 (Fig. 4, A and B), consistent with the lower Stat5 phosphorylation induced by TSLP (Fig. 1H). Flow cytometry confirmed a TSLP-induced increase in IL-2Rα expression, both on non-treated or anti-CD3 activated CD4+ T cells (Fig. 4C); the effect was greater on the latter cells. Consistent with TSLP-induced IL-2Rα expression, which increases high-affinity IL-2R expression and responsiveness of cells to low concentrations of IL-2 (21, 22), TSLP increased IL-2-induced proliferation of CD4+ T cells even at low concentrations of IL-2 (Fig. 4D). In contrast, TSLP did not augment IL-7-mediated proliferation of these cells (data not shown), indicating specificity to its effect.

TSLP has pleiotropic effects in both T and B cell biology and actions related to inflammatory/allergic/asthmatic disease. As noted above, TSLP and IL-7 both are stromal factors (1, 2) and share IL-7Rα as a receptor component (3, 4). IL-7 exhibits a marked species differences, being critical for T cell development in both humans and mice but for B cell development only in mice (7, 23). Much discussion has focused on species-specific actions of TSLP (11, 15, 24). TSLP was reported to have certain actions related to inflammatory/allergic/atopic disease. As such, elevated TSLP expression, such as allergic asthma and atopic dermatitis, as well as having possible therapeutic implications.

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**Disclosures**

The authors have no financial conflict of interest.

**References**


