TSLP UPREGULATION IN HUMAN SSc SKIN AND INDUCTION OF
OVERLAPPING PROFIBROTIC GENES AND INTRACELLULAR SIGNALING
WITH IL-13 AND TGFβ

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Running head: TSLP role in inflammation and fibrosis in SSc.
Abstract:

Objective: To explore the expression of thymic stromal lymphopoietin (TSLP) in diffuse cutaneous systemic sclerosis (dcSSc) patients and compare its effects in vivo and in vitro with IL-13 and TGFβ.

Methods: Skin biopsies (dcSSc; n=14 and healthy controls; n=13) were analyzed by immunohistochemistry and immunofluorescence for TSLP, TSLP-receptor, CD4, CD8, CD31, and CD163 markers. Wild type (WT), IL4Ra1- and TSLP-deficient mice were treated with TGFβ, IL-13, Poly(l:C), or TSLP by osmotic pump. Human fibroblasts and peripheral blood mononuclear cells (PBMCs) were stimulated with TGFβ, IL-13, Poly(l:C) or TSLP. Gene expression (microarray analysis and qPCR), protein levels of Phospho-Smad2, and macrophage marker CD163 were tested.

Results: TSLP was highly expressed in skin of dcSSc patients, more strongly in perivascular areas and in immune cells, and produced mainly by CD163+ cells. Skin of TSLP-treated mice showed upregulated clusters of gene expression that overlapped strongly with IL-13 and TGFβ-treated mice. TSLP upregulated specific genes, including CXCL9, proteasome, and interferon-regulated genes. TSLP treatment in IL4Ra1-deficient mice promoted similar cutaneous inflammation as in WT, though TSLP-induced Arg-1, CCL2, and MMP12 mRNA levels were blocked. In PBMCs, TSLP upregulated TNF-alpha, MX1, IFNγ, CXCL9, and MRC1 gene expression. TSLP-deficient mice treated with TGFβ showed less fibrosis and blocked expression of PAI-1 and SPP1. Poly(l:C)-treated mice showed high levels of cutaneous TSLP.
Conclusions: TSLP is highly expressed in skin of dcSSc patients and interacts complexly with other profibrotic cytokines: TGFβ and IL-13; strongly suggesting that it might promote SSc fibrosis directly or indirectly by synergistically stimulating profibrotic genes, or production of these cytokines.
Introduction

Recruitment of T cells polarized to preferentially produce IL-4, IL-5 and IL-13, belonging to the Th2-like T subset, has been implicated in fibrosis and remodeling in systemic sclerosis (1) (2, 3). Our recent work, analyzing peripheral blood mononuclear cells (PBMCs), identified high expression of several genes critical to IL-13 signaling by PBMCs from both limited (lcSSc) and diffuse cutaneous (dcSSc) SSc patients, especially in those affected with pulmonary arterial hypertension (SSc-PAH). Mannose Receptor-1 (MRC1), an IL-13-regulated gene that is highly upregulated on alternatively activated monocytes (M2), was highly expressed by SSc-PAH patient PBMCs and its expression correlated with the pulmonary pressure and mortality (4). In addition, IL-13 has recently been strongly implicated in SSc skin disease (5). These data suggest that IL-13 is involved in both the fibrotic pathway of SSc, but also plays a key role in the vasculopathic process, leading to PAH, a deadly complication of the disease.

Despite these observations implicating IL-13 in SSc pathogenesis, little progress has been made in identifying upstream events that ultimately lead to the Th2 profibrotic signaling in SSc. Thymic Stromal Lymphopoietin (TSLP) has been shown to play a crucial role in skewing T cells to a Th2 phenotype (6). TSLP is an IL-7 cytokine family member, highly produced by epithelial cells at barrier surfaces and, consequently, the first molecule produced after many disturbances
in homeostasis (7). It strongly regulates immune cells such as dendritic cells, T and B cells, and granulocytes (8); and transgenic TSLP expression in the skin and lungs of mice leads to a fibrotic phenotype similar to atopic dermatitis and asthma, respectively (9, 10). In addition to being tightly linked to fibrosis and possibly one of the master regulators of a Th2 phenotype, TSLP is highly induced by bacteria, viruses and, Toll-like receptors (TLR) ligands (11), and thus might have a key role in activating the immune system and in stimulating fibrosis after exposure to environmental stimuli.

We show here that TSLP is highly expressed in skin of dcSSc patients, mainly by immune cells in perivascular areas. In addition, we show that chronic subcutaneous stimulation of skin by TSLP activates smad2-phosphorylation and leads to alterations in gene expression that overlap significantly with gene expression induced by TGFβ as well as IL-13. Examining the effect of these cytokines in IL4Ra1- and TSLP-deleted mice, revealed a striking overlap in induced gene expression and a complex interaction between these cytokines. As poly(I:C) induced high levels of TSLP, these observations potential link an environmental trigger, such as a TLR ligand, to SSc disease pathogenesis.
MATERIAL AND METHODS:

Study participants. The Boston University Medical Center Institutional Review Board reviewed and approved the conduct of this study. Informed consent was obtained from all patients [dcSSc (n= 14) according to diagnostic (12) and subtype (13) criteria] and healthy subjects (n= 13). Skin biopsies were performed over the dorsal midforearm and fixed with formalin or used for explant fibroblasts cultures.

PBMC isolation. Blood was collected from healthy controls in CPT tubes (Becton Dickinson). PBMCs were plated in 24- or 48- well plates at 37°C, at a concentration of 1-2 x 10^6 cells per well, in complete media (RPMI 1640 supplemented with 10% fetal bovine serum, 20 mM L-glutamine, 100 IU/ml penicillin, and 100 g/ml streptomycin) with recombinant human-IL-13 (R&D Systems, 20 ng/ml), TSLP (R&D Systems, 10 ng/ml) or Poly(I:C) (TLR3 ligand, Invivogen 2.5 μg/ml) for one hour to up to 24 hours, and lysed in RNAzol for RNA preparation.

Fibroblast culture. Primary human dermal fibroblast explant culture from healthy control subjects was established as described previously (14). Dermal fibroblasts were cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin and utilized at passage 3-6. Fibroblasts (100% confluent) were incubated in serum-free DMEM for overnight prior to the stimulation with TGFβ (R&D System;
2.5 ng/ml, recombinant human-IL-13 (R&D Systems, 20 ng/ml), or TSLP (R&D Systems; 10 ng/ml) for one hour.

RNA isolation. Total RNA from PBMCs was transferred in 600 μl of RLT buffer (Qiagen) plus β-mercapto-ethanol. RNA was purified using RNeasy total kit protocol (Qiagen). Murine skin RNA was extracted using Trizol reagent (Invitrogen). cDNAs were synthesized and used for quantitative real-time PCR using primers. qPCR was performed using an ABI PRISM 7700 sequence detection system (Applied Byosystem) and TaqMan assay.

Western-blot. Human dermal fibroblasts and mouse skin were lysed in SDS-PAGE buffer. Equal loading of protein were fractionated on SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and immunoblotted with primary antibodies, incubated with secondary antibody, and analyzed by Luminescent Image Analyzer (LAS-4000, FugiFilm). Ponceau Red and three antibodies were used: total-Smad2/3 (Cell Signaling; molecular weights 52 and 60 kDa), phospho-Smad2 (Cell Signaling; molecular weight 60 kDa), β-actin (Sigma-Aldrich; molecular weight 42) antibodies.

Histology, immunohistochemistry and immunofluorescence. Briefly for paraffinized samples, tissue sections were rehydrated, steamed with citrate buffer (Biogenex) at 99-100°C during 20 minutes, blocked and incubated with rabbit anti-human TSLP (with reactivity with mouse; LifeSpan Biosciences),
rabbit anti-human TSLP receptor (LifeSpan Biosciences), or rabbit anti-mouse CD163 (Epitomics), incubated with the secondary antibody, and developed. TSLP peptide (Lifespan Biosciences) was used as a control. Frozen skin samples were fixed in 100% acetone; blocked and then incubated with rabbit anti-human TSLP (LifeSpan Biosciences), Alexa Fluor® 488 anti-human CD8a (BioLegend), Alexa Fluor® 647 anti-human CD4, Alexa Fluor® 647 anti-human CD31, and Alexa Fluor® 488 anti-human CD163 antibodies. The secondary antibody was Rhodamine-conjugated goat anti-rabbit (Millipore). Samples were examined using a fluorescence microscope (Olympus FluoView® FV10i Compact, Self-Contained Confocal Laser Scanning Microscope).

TSLP staining scale: zero (no TSLP staining) to 3+ (strongest staining) on five different regions of the skin (connective tissue and perivascular areas, epithelial cells layer, interstitial and, endothelial cells).

In vivo experiments. C57Bl/6, BALB/c, and BALB/c-IL4Ra1-/- mice were obtained from The Jackson Laboratory. C57Bl/6-Taconic mice were obtained from Taconic Laboratory. C57Bl/6/IFNAR-/- mice were obtained from Dr. John Sprent (15). C57Bl/6/IL13-/- mice were kindly donated by Dr. Thomas Wynn. TSLP-/- mice were developed at Boston University Transgenic Core Facility with TSLP-/- embryos obtained from KOMP Repository® and the absence of TSLP mRNA level was confirmed by qPCR in the skin of all TSLP-/- mice (data not shown). Osmotic pumps (Alzet®) designed to deliver subcutaneously PBS, Poly(I:C) (0.1
mg), TGFβ (1.25 µg), IL-13 (4 µg) or TSLP (3 µg) were implanted in 4-8 weeks old mice. After 7 or 28 days mice were sacrificed and skin (~1cm²) surrounding the pump outlet was homogenized in Trizol (Invitrogen) for preparation of RNA or fixed in formalin.

Microarray analysis. All microarray data from this study has been deposited to NCBI’s Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo; Accession Number GSE34297). Microarray data was clustered using Cluster 3.0 for Macintosh OSX, using hierarchical clustering. After selecting genes showing detectable expression in 80% of the samples, genes and the arrays were centered and normalized before clustering by gene.

Flow cytometer analysis of mice: blood and spleen. Mouse spleen was digested with 1 mg/ml Collagenase D (Roche Diagnostics, Indianapolis, IN) in RPMI medium with depletion of red blood cells using lysing buffer (Sigma-Aldrich, St. Louis, MO). Digested spleen cells were incubated with antibodies. Mouse blood was collected with 0.5M EDTA. Antibodies were added directly to the blood, followed by red blood cells lysis. Fc-receptor blocker was added prior and during staining (Innovex Biosciences, Richmond, CA). The following antibodies were used: CD11b-FITC, CD11b-PE, B220-Pacific Blue, CD3-PE (all BD Biosciences, San Jose, CA), CD115-APC (eBiosciences, San Diego, CA), and CD11c-AlexaFluor 647 (Biolegend, San Diego, CA). LSRII (BD Biosciences, San Jose, CA) and Flowjo software (Treestar, San Carlos, CA) were used for the analysis.
Statistical Analysis. Comparisons of qPCR expression were analyzed by one-way ANOVA and Tukey’s multiple comparison post-tests. Two-group comparisons were analyzed by Student’s T-test.
RESULTS:

TSLP is highly expressed in the skin of diffuse cutaneous SSc patients. TSLP is an important upstream factor in Th2 skewing therefore; we analyzed TSLP protein expression in the skin of dcSSc patients. TSLP was strongly expressed in dcSSc patients compared to controls (Figure 1A to D). TSLP expression in dcSSc was highest in perivascular areas, where we observed inflammatory cell infiltrates, and was also seen in interstitial cells (Figure 1E and 1F). Expression of TSLP in the epidermis and on endothelial cells was similar between controls and dcSSc patients (data not shown). Expression of TSLP receptor was also analyzed showing stronger expression in dSSc patients compared to healthy controls (Supplementary Figure 1).

In order to localize the source of TSLP in the skin of dSSc patients, skin sections were double stained for TSLP and several cellular markers: CD4 and CD8 for T cells; CD163 cells for macrophages; CD31 cells for endothelium (Figure 1G), and DAPI for nuclei (Figure 1J). Immunofluorescence confirmed the expression of TSLP by immune cells around blood vessels (Figure 1). TSLP co-localized most strongly with CD163+ macrophages (Figure 1K) with co-localization shown in yellow. A few CD8+ (Figure 1H) and CD4+ (Figure 1I) cells co-localized with TSLP. A blocking peptide for the anti-TSLP antibody was used as a control showing clearly the specificity of the TSLP staining (Figure 1L to 1N).
TSLP shares clusters of gene expression with IL-13 and TGFβ and induces TGFβ canonical signaling in vivo. To more completely define the downstream effects of TSLP in the skin, we investigated the spectrum of the effects of TSLP in vivo using a murine model where TSLP was continuously released in the skin through an osmotic pump for 7 days (14), and comparing this with two other profibrotic cytokines implicated in SSc pathogenesis: IL-13 and TGFβ. Supervised clustering of genes expressed in skin treated with TSLP, IL-13 or TGFβ compared to control PBS-treated skin by microarray analysis showed that TSLP upregulated many genes that were also upregulated by IL-13 and TGFβ, sharing several gene clusters (Figure 2A). In particular, some genes known to be regulated by TGFβ and upregulated in SSc patient skin, were upregulated in the skin from mice treated with IL-13, TGFβ or TSLP, such as plasminogen activator inhibitor-1 (PAI-1 known also as Serpine-1), thrombospondin-1 (THS1), bone morphogenic protein-1 (BMP1) and, osteopontin-1 (SPP1) (Figure 2). We confirmed in a larger series of cytokine-treated mice upregulation of one of these TGFβ-regulated genes, PAI-1, showing that PAI-1 is induced in TGFβ-treated skin (3.28 ± 3.25-fold change; p= 0.03), but is also induced in TSLP and IL-13-treated skin (2.04 ± 0.94; and 3.28 ± 1.82-fold change; respectively; p< 0.05 for both; Figure 2B).

We also confirmed coordinate upregulation of other genes found by microarray to be induced by all three cytokines: the chemokine CXCL5, secreted frizzled related protein-2 (SFRP2), a Wnt family inhibitor upregulated in human SSc skin...
CXCL5 was highly and SFRP moderately highly induced by all three cytokines (Figure 2C, 2D, respectively). Surprisingly, NOS2 was confirmed to be highly upregulated by all three cytokines, although modestly upregulated by TGFβ (Figure 2E).

In addition, a specific cluster of upregulated genes was observed only in mice treated with TSLP, including CXCL9, proteasome genes (PSMB10 and PSMB8), guanylate binding proteins (GBP2 and GBP6) and several interferon-regulated genes (IFI203, IIGP2, and IRF7; Figure 2F and Figure 3). Although not typically associated with interferon signatures, IFNγ has been shown to regulate PSMB10, PSMB8, GBP2 and GBP6 (17). We confirmed the selective upregulation of CXCL9 by TSLP in a larger group of mice (Figure 2F), showing $9.30 \pm 5.78$-fold change expression compared to control and the other two treatments ($p= 0.03$).

We also identified two small clusters of genes mostly upregulated in IL-13, but not TSLP or TGFβ, treated skin. These clusters included genes previously shown regulated by IL-13: Arg-1 and Chi3l3 genes (Figure 2G and Supplemental Figure 1). We confirmed the upregulation of Arg-1 ($11.07 \pm 6.10$-fold change; $p= 0.002$) and Chi3l3 ($2.91 \pm 0.99$-fold change, $p< 0.01$) by IL-13 in a larger group of mice (Figure 2G and supplementary Figure 2D; respectively).

Surprisingly, we did not observe any gene cluster that was exclusively upregulated in mice treated with TGFβ.
TSLP induces MRC1 expression in healthy human PBMCs. We observed in our in vivo model that TSLP regulates overlapping clusters of genes with both IL-13 and TGFβs. We have also previously shown that MRC1, a marker for the effect of IL-13 on alternatively monocyte/macrophage activation, is associated with mortality and pulmonary artery pressure in lcSSc patients and is induced by IL-13 in healthy human PBMCs (4). In order to further understand how TSLP might regulate Th2 and consequently M2 skewing in SSc, we stimulated PBMCs with IL-13, TSLP or both and examined MRC1 mRNA expression. We observed that TSLP highly induced MRC1 expression in PBMCs (6.49 ± 4.37-fold change) to a similar degree as after IL-13 stimulation (4.50 ± 3.12-fold change; p= 0.43). Figure 4A. No statistically different additional effect was observed when both cytokines were used together (8.30 ± 4.75-fold change; p= 0.17). In order to assess the potential effect of TSLP in the skin, we then examined dermal expression of MRC1 in dcSSc patients. We observed that MRC1 was expressed at higher levels in dSSc skin (3.20 ± 1.92-fold induction) compared to control skin (1.11 ± 0.68-fold change; p<0.001), Figure 4B.

Kinetics of TSLP induced gene expression by PBMCs. We showed that TSLP stimulates a unique gene signature in vivo with increased expression of pro-inflammatory and pro-fibrotic genes (Figure 2 and Figure 3). In order to better understand the kinetics of TSLP induced gene expression, we treated PBMCs with TSLP for 1 hour up to 24 hours and examined the expression of several
genes. We confirmed that TSLP induces pro-inflammatory genes such as TNF, MX1, and IFNg at early time points, followed by induction of MRC1 and CXCL9 after 8 hours (Figure 4C). Notably, IL-13 mRNA expression was not detected at any time point during the stimulation (Figure 4C).

**TSLP induces TGFβ canonical pathway activation in skin.** In order to extend our in vivo data showing that TSLP increases expression of several genes regulated by TGFβ, we examined smad2-phosphorylation in the skin from mice treated with TSLP. Smad2-phosphorylation was induced in protein extracted from skin of mice treated with TSLP or with TGFβ; smad2 phosphorylation was weakly induced in mice treated with IL-13, but not in mice treated with PBS (Figure 4D).

**TSLP induces smad2-phosphorylation in human fibroblasts.** We then stimulated human dermal fibroblasts in vitro with TSLP and analyzed the effect on this TGFβ canonical pathway. We consistently observed strong upregulation of Smad2-phosphorylation after 1 hour of TSLP stimulation, thus with kinetics similar to that seen with TGFβ (Figure 4E).

**Cutaneous inflammatory and profibrotic gene expression in TSLP-treated mice is independent of IL-13.** Our in vivo microarray data showed that TSLP is probably involved in the remodeling process, upregulating several TGFβ and IL-13-regulated genes. Therefore, we investigated if TSLP effects in vivo are dependent on IL-13 signaling using mice deficient of IL-4Ra1 (IL4Ra1/-), a
shared co-receptor for IL-4 and IL-13. H&E analysis showed a similar inflammatory reaction in the skin in TSLP-treated mice in the absence of IL4Ra1 compared to the TSLP-treated WT mice, with a large infiltration of immune cells mainly in the subcutaneous tissue in both groups compared to PBS. In addition, TSLP treated IL4Ra1-deficient mice and WT mice showed a similar increase in CD163+ infiltrating macrophages (Figure 5A-5F).

IL4Ra1 deletion blocked expression of a subset of TSLP-induced genes. TSLP upregulated IL-13, IL1-beta and NOS2 expression in the skin of IL4Ra1-deleted and WT mice to a similar degree (IL-13–WT: 8.20 vs. IL4Ra1/-/: 11.94 fold-change, Figure 5G; IL1-beta–WT: 2.85 vs. IL4Ra1/-/: 1.72 fold-change; Figure 5K; NOS2–WT: 17.49 vs. IL4Ra1/-/: 11.58 fold-change Figure 5). Deletion of IL4Ra1 also had no significant effect on TSLP-induced expression of profibrotic genes, such as PAI-1 (WT: 4.49 ± 1.41 vs. IL4Ra1/-/: 2.66 ± 1.22 fold-change; p= 0.16; Figure 5M) and SPP1 (WT: 2.08 ± 0.64 vs. IL4Ra1/-/: 1.80 ± 0.16 fold-change; p= 0.54; Figure 5N). On the other hand, expression of several genes was blocked or partially blocked in TSLP-treated IL4Ra1-deficient mice compared to WT mice: CCL2 (WT: 26.16 ± 3.66 vs. IL4Ra1/-/: 14.36 ± 6.99 fold-change; p= 0.04), Arg-1 (WT: 6.17 ± 1.09 vs. IL4Ra1/-/: 0.71 ± 0.14 fold-change; p= 0.001), and MMP12 (WT: 158.00 ± 8.10 vs. 0.88 ± 0.72 fold-change; p< 0.01) (Figures 5H to 5J; respectively).
As IL-13 has also been reported promoting fibrosis through the IL13Ra2, we also tested the effect of TSLP-treatment in IL-13 deficient mice. TSLP stimulated expression of PAI-1, NOS2 and Arg-1 in IL-13 deficient mice to a similar level as WT mice (Supplementary Figure 3B), and that inhibition of Arg-1 seen in IL4Ra1 mice was mediated by IL-4 rather than IL-13. In contrast, CCL2 mRNA expression induced by TSLP in WT mice was not induced in IL13/-/- mice. (Supplementary Figure 3B).

We also investigated whether TSLP profibrotic effects were dependent on either type I or type II IFN by treating mice deficient of the type-I IFN receptor or IFN-gamma (IFNAR-/- and IFNg-/-) with TSLP. TSLP upregulation of PAI-1 and CXCL5 were not affected by the absence of either of these receptors (Supplementary Figure 4).

**TGFß effects in vivo is partially dependent on TSLP.** We show above that TSLP *in vivo* upregulates several TGFß and IL-13-regulated genes, and *in vitro* activates the canonical TGFß pathway. Therefore, we investigated if TGFß profibrotic effects were dependent on TSLP signaling using TSLP-deficient mice (see methods). TSLP-deficient mice had no observable phenotype and no apparent immune developmental deficiency confirmed by flow-cytometer of B, T cells and macrophages markers in the blood and spleen (Supplementary Figure 5).
Surprisingly, the profibrotic effect of TGFβ manifest by the presence of a small nodule at the outlet of the pump observed in all WT TGFβ-treated mice, was not seen in TSLP-/- mice. This macroscopic observation was confirmed by H&E analysis (Figures 6A-6C), showing less thickness of the dermis in the TSLP-/- mice treated with TGFβ (396.66 ± 134.87 µm) compared to the WT mice (504.54 ± 78.91 µm; p= 0.03; Figure 6D). Several profibrotic genes, such as PAI-1 (WT: 10.08 ± 4.76 vs. TSLP-/-: 0.62 ± 0.25 fold-change; p= 0.01; Figure 6E), osteopontin-SPP1 (WT: 20.30 ± 8.06 vs TSLP-/-: 0.78 ± 0.29 fold-change; p= 0.04; Figure 6F), and WISP1 (data not shown) were totally blocked in TGFβ-treated TSLP-deficient mice. Of note, WT mice treated with TGFβ showed an induction of TSLP skin mRNA expression compared to PBS (data not shown).

Skin of innate immune SSc murine model expresses high levels of TSLP. Consistent with the notion that SSc is triggered by an environmental stimulus, possibly infectious, we have shown previously that chronic subcutaneous Poly(I:C) causes skin fibrosis with features similar to SSc (14). Therefore, we analyzed skin TSLP expression in this model by immunohistochemistry. TSLP was highly expressed in infiltrating immune cells in the skin of poly(I:C)-stimulated skin (Figure 6H) compared to PBS-treated mice (Figure 6G), thus in similar pattern to that observed in the skin of dcSSc patients. A blocking peptide for the anti-TSLP antibody was used as a control (Figure 6I).
A complex interaction exists among IL-13, TGFβ, and TSLP. Based on our findings and on the data from the literature, PolyI:C, a TLR3 ligand, strongly induces TSLP (10). TSLP then induces inflammation independently of IL-13, induces IL-4/IL-13-regulated genes through IL-13 signaling, and ultimately lead to up-regulation of several pro-fibrotic genes similar to TGFβ. More importantly, TGFβ profibrotic effects are partially dependent on TSLP signaling and induce a TSLP positive feedback. Figure 6J.
DISCUSSION:

This study supports TSLP as a key factor for the Th2 proinflammatory and profibrotic profiles in SSc as it was highly expressed by perivascular inflammatory cells in SSc skin. In addition, our studies show that in vivo TSLP upregulates profibrotic gene expression similarly to TGFβ and activates canonical TGFβ signaling, and plays a key role in vivo in TGFβ signaling, suggesting that TSLP regulates TGFβ or that the TSLP receptor is able to cross-activate smad2 phosphorylation. Our studies show in vivo and in vitro that TSLP has a dual effect, stimulating both proinflammatory and profibrotic processes.

Epithelial cells, which highly express TSLP in allergic Th2-based disorders such as atopic dermatitis and asthma (18), were originally identified as the principal source of TSLP. More recently, several studies have shown that TSLP can be expressed by other cell types including fibroblasts, mast cells, CD68+ macrophages (19, 20), and dendritic cells (21). In our study, we observed strong expression of TSLP in the skin of dcSSc patients mainly in immune cells, co-localizing with CD163+ cells, a marker for resident activated macrophages (22) and also co-localized with a few CD4+ and CD8+ T cells. Mononuclear cell infiltration has been shown to be key in several fibrotic diseases including SSc (4, 23). Therefore, high cutaneous expression of TSLP by immune cells in SSc patients might be pivotal in perpetuating the immune system activation through TSLP-activated dendritic cells (DCs) (8), further attracting mononuclear cells, and
inducing T cell production of Th2-derived interleukins and chemokines, creating a profibrotic Th2 environment (20). Thus immune cell expression of TSLP may be more important than epithelial cell expression in fibrotic diseases such as SSc.

TSLP expression by immune cells might be triggered by an environmental stimulus, possibly infectious, and TSLP expression can be highly induced by TLR ligands, mainly Poly(I:C), a TLR3 ligand (11). Notably, we found that TSLP was primarily induced in infiltrating inflammatory cells in the skin of our murine SSc-like Poly(I:C) model, associated with skin inflammation and fibrosis. Together, these observations suggest TSLP could be induced by innate immune stimuli and subsequently, activate both inflammatory and profibrotic process involved in SSc pathogeneses.

In addition, our results in vivo and in vitro suggest that TSLP stimulates similar gene expression and intracellular pathways as TGFβ. In particular we found several clusters of genes that were upregulated by both of these cytokines in vivo. Furthermore we found that TSLP strongly upregulates smad2-phosphorylation, both in vivo and in vitro. As smad2-phosphorylation is directly downstream from the type I TGFβ receptor and is part of the canonical signaling pathway, these results strongly suggest that TSLP upregulates TGF-β activity. Strikingly, TGFβ profibrotic gene expression in vivo was strongly blocked in the absence of the TSLP signaling confirming a complex interaction between TSLP and TGFβ. Thus, a detailed understanding of the interaction between key cytokines: IL-13, TGFβ,
and TSLP, is urgent and essential for targeting these cytokines for the treatment of fibrotic diseases such as SSc.

The potential importance of TSLP in fibrotic skin disease was further reinforced by other observations. TSLP stimulated PBMCs to express MRC1, an important monocyte/macrophage alternatively activation marker, as effectively as IL-13. Supporting our results, we also observed increased mRNA levels of MRC1, suggesting that TSLP might be stimulating MRC1 expression in SSc skin. However, we cannot exclude the possibility that MRC1 expression in SSc skin is being regulated by another mediator such as IL-13, which we have shown is increased in the circulation of SSc patients and also stimulates MRC1. In addition, in mice deficient of IL-13/IL-4 signaling, TSLP failed to induce some Th2-regulated genes, showing that some effects of TSLP are mediated by IL4/IL13. However, many genes induced by TSLP, including profibrotic and proinflammatory genes were unaltered in both IL4Ra1 and IL13-deficient mice, indicating that these cytokines are not required for many TSLP effects.

A recent study in an IL-13 transgenic model of atopic dermatitis also relates directly to our findings. Increased inflammatory cells and skin remodeling with collagen deposition characterize this model. It was shown that TSLP, but no IL-13, was relevant for the induction of fibrosis (24). In IL4Ra1-deficient mice the profibrotic cutaneous effects of TSLP were not dependent on IL-13 signaling. In addition, in the absence of IL-13 signaling, TSLP still induced as strong
cutaneous inflammation and skin damage as observed in WT TSLP-treated mice. Arginase-1, an alternatively activated macrophage marker, was induced in the skin of BALB/c mice treated with TSLP, although not in C57BI/6 mice, likely a strain effect since BALB/c mice are more prone to Th2/M2 skewing compared to C57BI/6 mice (25). In accordance with the literature, Arg1 expression was dependent on the presence of the IL4Ra1 (26). Thus TSLP might be key in inducing profibrotic mediators, in amplifying IL-13 signaling, and may also be critical to inflammatory pathways that do not depend on IL-13.

In addition to its overlapping effects with IL-13 and TGFβ on gene expression, TSLP induced a distinct set of genes in murine skin, including CXCL9; a gene we had previously shown is increased in SSc skin (14). We showed that TSLP also induces CXCL9 by PBMCs in vitro, in accordance with a previous study where high levels of proteasomes, chemokines, including CXCL9, and IL-13-regulated genes were observed in PBMCs stimulated with TSLP (27). We show that TSLP rapidly stimulates pro-inflammatory gene expression by PBMCs followed by an induction of a more profibrotic marker, MRC1.

In conclusion, TSLP might represent one of the first responses to innate immune activation in dermal fibrosis, and might play a key role in SSc after exposure to an unknown trigger. TSLP might contribute to activating the immune system toward both proinflammatory and profibrotic process as observed in SSc patients, strongly interacting with IL-13 and TGFβ signaling, perpetuating the response. Of
particular note, this study highlights the importance of TSLP in TGFβ mediated fibrosis.
ONLINE SUPPLEMENTAL MATERIAL:

Immunohistochemistry of TSLP receptor in the skin of two healthy controls (HC) and two dcSSc patients are shown on Supplementary Figure 1 with a stronger TSLP receptor expression in the epidermal layer, interstitial cells, and in the perivascular areas of dcSSc patients.

Mice skin gene expression after treatment with IL-13, TSLP, and TGFβ through a 7 days subcutaneous pump are shown on Supplementary Figure 2 showing two IL-13 microarray clusters with Arg-1 gene and Chi3l3 upregulation, both confirmed by qPCR.

Mice skin gene expression after treatment with TSLP using a 7-day subcutaneous pump in mice deficient of IL13 is shown on Supplementary Figure 3. The expression of several genes was not affected the mice studied.

Mice skin gene expression after treatment with TSLP through a 7 days subcutaneous pump in mice deficient of IFNAR and IFN-gamma receptors are shown on Supplementary Figure 4. PAI-1 and CXCL5 mRNA gene expression were not affected in the absence of either of the receptors.
Flow cytometer analysis of the blood and spleen of WT and TSLP-/- mice showing a similar distribution of macrophages and both T and B cells, showed on Supplementary Figure 5.
Figure 1: Representative images of TSLP expression in dcSSc skin: A: Healthy control (HC) skin with TSLP staining in brown with a vessel on the bottom right (10x magnification). B: 20x magnification of the vessel from the HC skin on Figure 1A stained positive for TSLP on endothelial cells. C: dcSSc skin stained for TSLP in brown with positive staining on interstitial cells and on the vessels on the bottom left and upper right (10x magnification). D: 20x magnification of the left vessel from Figure 1C with positive staining on endothelial cells and in the perivascular area. E: Score of TSLP positive interstitial cells staining in HC (n=10) and SSc (n=11) skins. F: Score of TSLP positive perivascular staining in the same HC and SSc skins. Each bar represents the mean ± standard error of the mean. Representative images of immunofluorescent analysis of dcSSc skin with 60x magnification from Figure 1G to Figure 1K. G: dcSSc skin stained for DAPI in blue and CD31 in cyan. H: Similar area from Figure 1G with CD8+ positive cells stained in green, TSLP in red, and a few cells co-localizing with TSLP in yellow (white arrows). I: The same area from the dcSSc skin on Figure 1G with CD8+ positive cells stained in green, TSLP in red, and a few cells co-localizing with TSLP in yellow (white arrows). J: dcSSc skin stained for DAPI in blue, TSLP in red, and a co-localization showed in purple. K: Similar area from Figure 1J with CD163+ cells stained in green, TSLP in red, and a co-localization showed in yellow (white arrows). Specificity of anti-TSLP staining: L: TSLP positive staining in brown in two perivascular areas and in the epithelial cells layer (10x magnification). M: Perivascular area marked in the rectangle on Figure 1L with a 20x magnification showing TSLP positive staining in brown. N: Same area shown in Figure 1H stained with peptide blocked anti-TSLP control (negative staining). Rabbit anti-human Polyclonal Antibody, and TSLP Synthetic Peptide; LifeSpan Biosciences®.
Figure 2: Mouse skin gene expression after treatment with IL-13, TSLP, and TGFβ by 7 day subcutaneous pump. A: Two main clusters showing several genes highly expressed in all 3 treatment groups (IL-13, TSLP, and TGFβ) compared to control (PBS local skin, PBS distal skin). Several genes were analyzed by qPCR to confirm its high expression in the skin of mice treated with PBS, IL-13, TSLP or TGFβ (n=03 in PBS, IL-13, and TGFβ groups; n=06 in TSLP group; 2 independent experiments). B: PAI1 mRNA expression; p=0.03. C: CXCL5 mRNA expression; p<0.01. D: SFRP2 mRNA expression; p=0.03. E: NOS2 mRNA expression; p=0.03. F: CXCL9 mRNA expression TSLP vs. PBS; p= 0.03, and G: Arg1 mRNA expression IL-13 vs. PBS; p= 0.002. Data are expressed as the fold-change normalized to mRNA expression in one PBS sample. Each bar represents the mean ± standard error of the mean. Green color represents low and red color high gene expression.
Figure 3: Mouse skin gene expression after treatment with IL-13, TSLP, and TGFβ through a 7 days subcutaneous pump showing a unique TSLP cluster with several IFN-regulated genes (*) highly expressed exclusively in TSLP skin. Green color represents low and red color high gene expression.
Figure 4: A: MRC1 expression in PBMCs from 5 healthy controls stimulated for 18 hours with IL-13 (20 ng/mL), TSLP (10 ng/mL) or both using media alone as a control (* p<0.001). B: MRC1 expression in skin of 14 dcSSc patients and 13 healthy controls (HC). C: Gene expression in PBMCs from 3 healthy controls stimulated for 1 up to 24 hours with TSLP (10 ng/mL) using media alone as a control. D: Higher expression of pSmad2 in skin of mice submitted to TGFβ, IL-13, and TSLP pump compared to PBS pump. E: Increased expression of pSmad2 in healthy human dermal fibroblasts (5 independent experiments) stimulated for 1 hour with TGFβ (2.5 ng/mL) or TSLP (10 ng/mL) after overnight starvation at 100% of confluence and compared to media alone (Ct). A-C: Data are expressed as the fold-change normalized to mRNA expression in the control. Each bar represents the mean ± standard error of the mean. Each data point represents a single subject; horizontal lines show the mean. D and E: Blotted proteins were probed with monoclonal rabbit anti-pSmad2 antibody, total Smad2/3 antibody, secondary antibody, and visualized using enhanced chemiluminescence. As control for equal protein loading, the membrane was stripped and reprobed for β-actin using a monoclonal antibody for β-actin. F: Expression of pSmad2 quantified by scanning densitometry and corrected for levels of β-actin in the same samples of human dermal fibroblasts. * p<0.05 as compared with the values in samples from fibroblasts treated with media.
Figure 5: Skin involvement after 7 days of TSLP treatment in WT and IL4Ra1-deficient mice. A-C: Representative images at 10x magnification of hematoxylin and eosin (H&E) stained skin sections. A: WT PBS-treated skin showing preservation of all skin layers; B: WT TSLP-treated skin with cell infiltration; and C: IL4Ra1-/- TSLP-treated skin also showing intense cell infiltration. D-F: Representative images at 10x magnification of CD163 staining. D: WT PBS-treated skin. E: WT TSLP-treated skin with strong CD163 staining in brown on cells in the subcutaneous layer. F: IL4Ra1-/- TSLP-treated mice with similar CD163 positive staining as Figure 5E. G: IL13 mRNA expression. H: CCL2 mRNA expression. I: Arg1 mRNA expression. J: MMP12 mRNA expression. K: IL1 beta mRNA expression. L: NOS2 mRNA expression. M: PAI-1 mRNA expression. N: SPP1 (osteopontin) mRNA expression. Data are expressed as the fold-change normalized to mRNA expression in one WT PBS-treated mouse sample; n=3 in each group. Each bar represents the mean ± standard error of the mean.
Figure 6: TGFβ profibrotic in vivo effects is partially dependent on TSLP. A-C: Representative images on 10x magnification of H&E staining. A: WT PBS-treated skin with preservation of the structures. B: WT TGFβ-treated skin with thickness of the dermis represented by the arrow, which measures the distance between the muscle and the epidermal layers. C: TSLP-/- TGFβ-treated skin showing a reduction of the dermal thickness, again represented by the arrow. D: thickness of the dermis measured by the distance between the muscle and the epidermal layers (arrows). E: PAI-1 mRNA expression. F: SPP1 (osteopontin) mRNA expression. G: Skin of 28 days Poly(I:C) SSc immune model shows higher expression of TSLP mainly in immune cells. G-I: Representative images on 10x magnification of TSLP staining. G: Low TSLP expression in PBS control mouse with a preserved presence of the annexes. H: Skin of Poly(I:C)-treated skin showing strong expression of TSLP staining in brown, thickening of the dermis with loss of the annexes. I: the same area showed on Figure 6H with peptide blocked anti-TSLP control negative staining. J: Schematic representation of the complex interaction of TSLP with IL-13 and TGFβ.


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Disclosures:
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