Evidence for a Functional Thymic Stromal Lymphopoietin Signaling Axis in Fibrotic Lung Disease

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**Evidence for a Functional Thymic Stromal Lymphopoietin Signaling Axis in Fibrotic Lung Disease**

Arnab Datta,* Robert Alexander,* Michal G. Sulikowski,* Andrew G. Nicholson, † Toby M. Maher, ‡ Chris J. Scotton,*+1 and Rachel C. Chambers*+1

Thymic stromal lymphopoietin (TSLP) recently has emerged as a key cytokine in the development of type 2 immune responses. Although traditionally associated with allergic inflammation, type 2 responses are also recognized to contribute to the pathogenesis of tissue fibrosis. However, the role of TSLP in the development of non–allergen-driven diseases, characterized by profibrotic type 2 immune phenotypes and excessive fibroblast activation, remains underexplored. Fibroblasts represent the key effector cells responsible for extracellular matrix production but additionally play important immunoregulatory roles, including choreographing immune cell recruitment through chemokine regulation. The aim of this study was to examine whether TSLP may be involved in the pathogenesis of a proto-typical fibrotic disease, idiopathic pulmonary fibrosis (IPF). We combined the immunohistochemical analysis of human IPF biopsy material with signaling studies by using cultured primary human lung fibroblasts and report for the first time, to our knowledge, that TSLP and its receptor (TSLPR) are highly upregulated in IPF. We further show that lung fibroblasts represent both a novel cellular source and target of TSLP and that TSLP induces fibroblast CCL2 release (via STAT3) and subsequent monocyte chemotaxis. These studies extend our understanding of TSLP as a master regulator of type 2 immune responses beyond that of allergic inflammatory conditions and suggest a novel role for TSLP in the context of chronic fibrotic lung disease.  

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TSLP was originally identified as a growth factor capable of supporting the long-term growth of pre-B cells in vitro (8, 9). TSLP was subsequently found to be highly expressed by lung epithelial cells and epidermal keratinocytes (10, 11) and is now recognized to be a critical mediator involved in linking responses at the interface between mucosal barriers and the environment to type 2 immune responses. TSLP has been implicated in driving type 2 responses in the airways (6), skin (7), and gut (12) and mediates its effects by driving the activation of immature dendritic cells (DCs) into a type 2 polarizing phenotype (10, 13, 14), as well as via direct effects on naive and differentiated T cells (15, 16).

The biological effects of TSLP are mediated by binding to a functional heterodimeric receptor complex composed of the TSLP receptor (TSLPR) and the IL-7Ra-chain (17, 18), signaling through STAT3 (19) and STAT5 pathways (8). Recent evidence suggests that, in addition to promoting type 2 cytokine responses, TSLP plays broader homeostatic roles, including controlling regulatory T cell differentiation in the thymus (20) and contributing to intestinal homeostasis (12). The key stimuli involved in regulating TSLP expression are also beginning to be identified, with current evidence suggesting a major role for environmental stimuli (including allergen exposure (21), viral and bacterial infections (22, 23), helminth infections (24), diesel exhaust (25), and cigarette smoke (26)), proinflammatory cytokines such as TNF-α (27) and IL-1β (28), type 2 cytokines (22), and IgE (29). TSLP is now also known to be expressed by nonepithelial cell types, including mesenchymal cells (30), and recently has been implicated in promoting tumor cell growth in breast (31) and pancreatic cancer (32), joint destruction in arthritis (33, 34), and fibrocyte function in atopic dermatitis (35). Current evidence suggests that the TSLPR complex also is more broadly expressed, suggesting that the TSLPR complex also is more broadly expressed, suggesting that TSLP has a broader role in promoting disease progression.

The aim of this study was to begin to evaluate the potential importance of TSLP in nonatopic pathobiology characterized by...
type 2 cytokine responses, focussing on the prototypical fibriotic lung disease IPF. IPF is the most fatal of all fibriotic lung conditions and is characterized by a progressive decline in lung function leading to premature death as a result of respiratory failure. The pathomechanisms involved remain poorly understood, but current hypotheses propose that this condition arises as a result of chronic or repetitive lung injury (of unknown origin), followed by a highly aberrant wound healing response (reviewed in Ref. 37). The classical histopathological pattern of IPF is characterized by evidence of patchy epithelial damage, type 2 pneumocyte hyperplasia, together with abnormal proliferation of mesenchymal cells, varying degrees of fibrosis and overproduction, and disorganized deposition of collagen and extracellular matrix. Fibrotic foci are commonly observed underlying injured and reparative epithelium—these fibrotic foci comprise accumulations of fibroblasts and myofibroblasts within extensive extracellular matrix and are felt to represent the leading edge of the fibrotic lesion. Although the contribution of inflammation to disease initiation and progression in IPF remains unclear (38), current evidence supports the notion that type 2 cytokines, in particular IL-13, may contribute to fibrotic responses by amplifying the dysregulated epithelial–mesenchymal cross-talk, which is central to this condition (39, 40). The infiltration of activated DCs in the IPF lung provides further strong support for the notion that a maladaptive immune response may be responsible for driving fibrosis in this condition (41). The nature of the mediators responsible for DC migration and activation in this context remain poorly defined. However, DC trafficking to remodeling lung recently has been shown to be dependent on fibroblast-derived CCL2 (42), a chemokine implicated in facilitating type 2 immune responses (43–45), and which also has been implicated in the pathogenesis of several chronic diseases associated with tissue remodeling, including IPF. Furthermore, it is now increasingly recognized that DCs may be activated by nonantigenic endogenous danger signals, reflecting cellular injury or stress (46, 47)—supporting the notion that epithelial injury, sterile or otherwise, can initiate aberrant immune responses.

In this study, we combined the immunohistochemical analysis of human IPF biopsy material with signaling studies by using cultured primary human cells and report for the first time, to our knowledge, that TSLP and its receptor are highly upregulated in IPF. We further show that lung fibroblasts represent both a novel cellular source and target of TSLP, and that TSLP induces fibroblast CCL2 release and subsequent monocyte chemotaxis. These studies extend our understanding of TSLP as a master regulator of type 2 immune responses beyond that of allergic inflammatory conditions and suggest a novel role for TSLP in the context of chronic fibriotic lung disease.

Materials and Methods

Reagents

Recombinant human (rh)TSLP and CCL2 were purchased from R&D Systems; rhTNF-α was from PeproTech. For Western blotting, rabbit anti-human phospho-heat shock protein 27 (HSP27) (S78), rabbit anti-human phospho-p42/44 (T202/Y204), rabbit anti-human IkBα, rabbit anti-human phospho–c-Jun (S73), rabbit anti-human phospho-STAT3 (Y705), rabbit anti-human phospho-STAT5 (Y694), murine anti-human HSP27 mAb, rabbit anti-human STAT3, and rabbit anti-human STAT5 were purchased from Cell Signaling Technology. Goat anti-human ERK2 was acquired from Santa Cruz Biotechnology. For immunocytofluorescence, rabbit anti-human TSLPR and goat anti-human IL-7Rα Abs were purchased from Pro-Sci and Santa Cruz Biotechnology, respectively. Donkey anti-rabbit AF488 and donkey anti-goat AF555 were from Invitrogen. Polyclonal rabbit IgG and polyclonal goat IgG isotype controls were purchased from DakoCytomation. Sheep anti-human TSLP was purchased from R&D Systems (catalog number AF1398), and rabbit anti-human TSLPR was purchased from Pro-Sci (catalog number 4207). For inhibition studies, polyclonal anti-human CCL2 Abs were purchased from R&D Systems. The NF-κB inhibitor, SC-514; p38 inhibitor, SB203580; MEK 1/2 inhibitor, U0126; JNK inhibitors, SP600125 and TI-JIP; and STAT3 inhibitor, S3I-201, were all obtained from Calbiochem. Plasmids used for transfection were acquired from Addgene; pFUGW containing GFP only (Addgene plasmid 14883) was a gift from D. Baltimore (California Institute of Technology, Pasadena, CA) (48), whereas pcTAT3 containing a constitutively active mutant of STAT3 (Addgene plasmid 24993) was a gift from L. Cheng (The Johns Hopkins University School of Medicine, Baltimore, MD) (49). JetPRIME transfection reagent was purchased from Polyplus-Transfection. All cell culture medium (DMEM), FBS, and antibiotics (penicillin/streptomycin) were purchased from Invitrogen, aside from RPMI 1640 medium (PA). Sterile tissue culture equipment was purchased from Nunc. All other chemical reagents were from Sigma-Aldrich.

Cell culture

Primary human lung fibroblasts (pHLFs) were isolated and maintained as previously described (50, 51) and were used at no more than passage 7. Type 2 alveolar epithelial cells (AECs) were isolated from lung tissue obtained at lung transplantation using the technique modified from Thorley et al. (52). Primary cells were obtained from macroscopically healthy segments of lung from patients undergoing lung cancer resection. Approval for the use of these cells has been obtained from the Royal Brompton, Harefield, National Heart and Lung Institute, and the University College London/University College London Hospital ethics committee, and informed consent was obtained from patients. Human THP-1 monocytes were purchased from the American Type Culture Collection and grown in RPMI 1640 medium at 37°C (5% CO2), supplemented with penicillin (200 U/ml), streptomycin (200 U/ml), glutamine (4 mM), and 10% FBS (v/v), unless otherwise stated. Cells were routinely tested and found negative for mycoplasma infection.

ELISA analysis of cytokine/chemokine release by pHLFs in conditioned medium

pHLFs were grown to 80% confluence and serum-starved for 24 h prior to use in experiments. Conditioned media (CM) were collected at designated time points after exposure to varying concentrations of TNF-α, TSLP, or media alone. Samples were centrifuged at 300 × g for 5 min at 4°C to remove cell debris and stored at −80°C until analysis by ELISA. For inhibitor studies, cells were incubated with designated concentrations of inhibitors for 30 min at 37°C prior to exposure to TNF-α or TSLP. Cell viability in all inhibitor studies was >95% as assessed by trypan blue exclusion. TSLP in CM was quantified using ELISA with matched Abs, according to the manufacturer’s instructions (R&D Systems). The sensitivity limit of the TSLP ELISA is 7.8 pg/ml. Each data point represents the mean ± SEM from readings performed in triplicate from three independent assays. CCL2 in CM was quantified by ELISA as described previously (53). Paired Abs MAB679 and BAF279 for the human CCL2 ELISA were obtained from R&D Systems, and rhCCL2 protein standard was from PeproTech.

RNA isolation/RT-PCR analysis/quantitative RT-PCR analysis

Total RNA from cell cultures was isolated with TRIzol reagent as per the manufacturer’s protocol. RNA was DNase-treated using a DNasefree kit (Ambion). Reverse transcription was performed using 1 μg total RNA in a first-strand cDNA synthesis with qScript cDNA SuperMix kit (Quanta Biosciences) in a reaction volume of 20 μl as per the manufacturer’s protocol. TSLPR and IL-7Rα mRNA expression in pHLFs was analyzed by quantitative RT-PCR. Cycling conditions were as follows: activation step of 10 min; and 40 cycles of 95°C (10 s) and 62°C (45 s). The specificity of the PCR products was confirmed by melting curve analysis. Fold change in expression was calculated using the 2−ΔΔCt formula, as described previously (51). Primers are shown in Table 1.

Western blot analysis of HSP27, p42/44, IκBα, c-Jun, STAT3, STAT5, and ERK2

(Phospho-HSP27, phospho-p42/44, IκBα, (phospho-)c-Jun, (phospho-)STAT3, (phospho-)STAT5, and ERK2 expression were analyzed by
Western blotting, pHLFs were grown to 80% confluence before being quiesced for 24 h in serum-free medium. Cells were then stimulated in fresh serum-free medium with TNF-α (10 ng/ml) or TSLP (1 ng/ml) for designated times. Inhibitor and Ab studies were performed as described above. After incubation, cells were washed with ice-cold PBS and then lysed on ice in 100 μl Phosphosafe buffer (VWR) supplemented with Complete Mini protease inhibitor mixture (Roche). Equal amounts of protein were loaded onto 8–16% LongLife precast gels (Nusep), electrophoresed, transferred to nitrocellulose membranes (Hybond-ECL), and incubated with blocking buffer containing 5% nonfat dry milk in TBST for 1 h. Blots were then incubated with Abs specific for phosphorylated HSP27, p244/241, c-Jun, STAT3, and STAT5, and ERK2 (as loading controls), the blots were stripped before immunoblotting with Abs described above, using the same protocol. Blots were scanned on an Epson Perfection 4870 photo scanner, and densitometric analysis was performed using ImageJ software (National Institutes of Health) calibrated against Kodak photographic Step Tablet number 3.

### Transfection of pHLFs with small interfering RNA

pHLFs were grown to 80% confluence in antibiotic-free medium to maximize transfection efficiency. After being serum-starved for 24 h, cells were transfected with small interfering RNA (siRNA) directed against c-Jun, STAT3, or control-scrambled siRNA at final concentrations of 100 nM. Further control cells were mock-transfected at the same time. siRNA (Dharmacon) was transfected into cells using a Gemini transfection reagent (a gift from GSK) in Optimem medium. Cells were then allowed to quiesce for 24 h before subsequent stimulation. After each specific treatment, cell extracts were prepared as described above, and Western blotting was used to evaluate expression of proteins of interest. In addition, time-point matched CM were collected and analyzed for TSLP and CCL2 protein release by ELISA, as described above. siRNA sequences for c-Jun and STAT3 are listed below in Table II. siRNA sequences for c-Jun and STAT3 are listed below in Table II.

### Plasmid transfection of pHLFs

pHLFs were grown to 60% confluence (1.25 × 10^5 cells/well in 48-well plates) in DMEM/10% FBS. Cells were then transfected with 0.25 μg plasmid DNA (pFUGW as control or pSTAT3C) per well, using JetPRIME (1:2 ratio of DNA to JetPRIME, w/v), according to the manufacturer’s instructions. Four hours after addition of transfection complexes, plasmid DNA (pFUGW as control or pSTAT3C) per well, using JetPRIME reagent (1:2 ratio of DNA to JetPRIME, w/v), according to the manufacturer’s instructions. Four hours after addition of transfection complexes, pHLFs were incubated in fresh DMEM/10% FBS overnight. Cells were subsequently incubated in serum-free DMEM for 18 h to assess the effect of constitutive STAT3C activity on CCL2 production (assessed by ELISA, subsequently incubated in serum-free DMEM for 18 h to assess the effect of constitutive STAT3C activity on CCL2 production (assessed by ELISA).

### Monocyte chemotaxis

THP-1 human monocyte chemotaxis was assayed in a 48-well Boyden chamber using a 5-μm polyvinylpyrrolidone-free polycarbonate filter as described previously (54). Graded concentrations of rhCCL2 (0–100 ng/ml) were prepared in DMEM/1% BSA, and the optimum (CCL2) for inducing monocyte chemotaxis in these studies was determined to be 3 ng/ml. To determine the 50% neutralizing dose of the anti-CCL2 Ab used for inhibition studies, rhCCL2 (3 ng/ml) was incubated with graded concentrations of Ab (0–30 μg/ml) for 1 h at room temperature before being added to wells. The 50% neutralizing dose for this Ab in inhibiting CCL2 (3 ng/ml)-induced chemotaxis was determined to be 5.06 μg/ml, with complete abrogation of chemotaxis observed at 30 μg/ml. For experiments, CM from pHLFs treated with graded concentrations of TSLP as designated were added to the lower wells. For inhibition studies, CM was incubated with anti-CCL2 Ab as described above (30 μg/ml), before being added to wells. Cells were suspended in DMEM/1% BSA (1 × 10^5 cells/ml) and added to the upper wells of the chamber and allowed to migrate for 2 h at 37°C in a 5% CO2 atmosphere. After this, the filter was removed from the chamber, fixed in methanol, and stained with a Diff-Quick stain kit. Cells that migrated through the membrane were counted under light microscopy (×100 objective) on five random high-power fields (HPFs). The results are expressed as mean number of cells per five HPFs from experiments performed in triplicate on three independent occasions. Monocyte migration toward rhCCL2 (3 ng/ml) or DMEM/1% BSA was used as positive and negative controls, respectively.

### Immunocytofluorescence

Serum-fed pHLFs were grown on 8-well chamber slides (Millipore) and grown to 80% confluence. Slides were washed in PBS and fixed for 10 min with 4% paraformaldehyde in PBS. Cells were blocked with 10% PBS (v/v) 0.2% fish skin gelatin (v/v) in PBS for 1 h at room temperature. After washing twice in PBS, slides were incubated with primary rabbit anti-TSLPR and goat anti-IL-7Rα Abs or isotype control Abs for 2 h at room temperature. Slides were washed twice in PBS and incubated for 1 h at room temperature with donkey anti-rabbit IgG AF488 and donkey anti-goat IgG AF555. The cell layer was then washed twice in PBS before being mounted with Prolong Gold anti-fade reagent with DAPI (Invitrogen). Sections were visualized using a Zeiss Axioskop 2 microscope (Carl Zeiss), and images were captured using a Qicam 12-bit color fast camera using Q capture software, version 2.81 (both from QImaging).

### Human subject details

Lung biopsy specimens were obtained from 12 patients with IPF (obtained at diagnostic surgical lung biopsy) and 3 control patients (obtained from uninvolved tissue during cancer resection surgery). All biopsies used in this study were classified using the diagnostic criteria of the American Thoracic Society/European Respiratory Society Consensus (American Thoracic Society/European Respiratory Society International multidisciplinary consensus classification of the idiopathic interstitial pneumonias, 2002) demonstrating a pattern of usual interstitial pneumonia. Approval for the use of the material was obtained from the Royal Brompton, Harefield, National Heart and Lung Institute, and the University College London/University College London London Hospital ethic committee. Informed consent was obtained from each patient.

### Histologic analysis

Fresh human lung biopsy material was processed for immunohistochemical analysis as described previously (53). Briefly, after fixation in 4% formaldehyde, specimens were placed in processing cassettes, dehydrated through a serial alcohol gradient to xylene using an automated Leica Tissue Processor, and embedded in paraffin wax blocks. Before immunostaining, 3-μm-thick lung tissue sections were dewaxed in xylene and rehydrated through decreasing concentrations of ethanol to deionized water.

### Immunohistochemistry

To examine the immunolocalisation of TSLP and TSLPR in human lung tissue, Ags were unmasked by microwaving sections in 10 mM citrate buffer

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>Human Tslp</td>
<td>5′-TATGGATGGGCGAACAAAGATACC-3′</td>
<td>5′-GGATTGGAAGTTCCTGTCGG-3′</td>
</tr>
<tr>
<td>Human Tslp (long splice)</td>
<td>5′-GATTACATATATGAGTGCGAC-3′</td>
<td>5′-TTTATCGTGCGGATAGCATATTT-3′</td>
</tr>
<tr>
<td>Human Tslp (short splice)</td>
<td>5′-GGTAATCTGCCTGATCTGTA-3′</td>
<td>5′-GTCAGAACGCTACCCATTCA-3′</td>
</tr>
<tr>
<td>Human H2RA</td>
<td>5′-GAGCGCAATGACTTCTTCTGG-3′</td>
<td>5′-AGTCAGAACGCTACCCATTCA-3′</td>
</tr>
<tr>
<td>Human CΔ12</td>
<td>5′-AGCAAGGGTCCCCAAAAGAAC-3′</td>
<td>5′-CATGGAATCCTGAACCCTCA-3′</td>
</tr>
<tr>
<td>Human Hprt</td>
<td>5′-TCATTATGCCAGGATTGG-3′</td>
<td>5′-ACAGAGGGCCACAAATGTGATGTTG-3′</td>
</tr>
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</table>

Table I. Primer sequences used for RT-PCR and qualitative PCR

<table>
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<tr>
<th>Target</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>c-Jun</td>
<td>5′-GAGCCGACCUAUUGGCUAC-3′</td>
</tr>
<tr>
<td>STAT3</td>
<td>5′-CAACAGAUUGCGUCCGUAUG-3′</td>
</tr>
<tr>
<td>Scrambled controls</td>
<td>Sequences not provided by Dharmacon</td>
</tr>
</tbody>
</table>
(pH 6) (twice for 5 min, followed by a 15-min cooling step). Immuno-localization of anti-SMA to allow identification of lung (myo)fibroblasts was performed as described previously (51).

Two 30-min blocking steps with 3% H2O2 in deionized water and 3% sera corresponding to secondary Abs species made in 1% BSA/PBS were performed before incubation with primary Abs.

Immunostaining was undertaken by the avidin–biotinylated HRP enzyme complex method (Vector Laboratories) with Abs against human TSLP (0.5 μg/ml), human TSLPR (1 μg/ml), or equivalent concentrations of polyclonal nonimmune IgG controls incubated for 16 h at 4˚C. After incubation with an appropriate biotin-conjugated secondary Ab for 30 min at room temperature, and subsequently with Vector ABC complex PK-6100 (as per the manufacturer’s protocol for 30 min), 5-min color development was performed with 3,3’-diaminobenzidine (BioGenex) as a chromogen. Sections were counterstained with Gill-2 hematoxylin (Thermo Shandon), dehydrated, and coverslipped permanently. Comparative immunohistochemical analysis for TSLP, TSLPR, and anti-SMA was performed on serial sections. Sections were digitally scanned with a Hamamatsu NanoZoomer (×40 objective), and representative images are presented.

Statistical analysis

Statistical analysis was performed with GraphPad Prism software (GraphPad Software). All data are presented as mean ± SEM, unless otherwise indicated, from experiments performed in triplicate on three independent occasions. All differences in mRNA levels compared differences in ΔCp values. All Western blot data are representative of three independent experiments performed, unless otherwise specified. Statistical comparison was performed between two treatment groups by Student t test and between multiple treatment groups by ANOVA (one-way or two-way, as appropriate) with Tukey post hoc testing. A p value < 0.05 was considered significant.

FIGURE 1. TSLP, TSLPR, and anti-SMA immunostaining in IPF identifies epithelial cells and fibroblasts as major cell types displaying strong immunoreactivity. Shown is immunostaining for TSLP (A), TSLPR (B), and anti-SMA (C) in serial sections of IPF lung. (A) In IPF lung, strong immunoreactivity for TSLP is observed in AECs (arrowhead) and spindle-shaped fibroblasts (arrows) located within fibrotic foci (FF). Immunoreactivity is also associated with macrophages and airway smooth muscle cells. (B) Strong TSLPR staining is also observed in IPF lung, again localizing to AECs, fibroblasts, ASMCs, and macrophages. Spindle-shaped cells within fibrotic foci that stained positive for TSLP and TSLPR were also strongly immunoreactive for anti-SMA (C). (D and E) IFP serial tissue sections stained with isotype-specific nonimmune primary Abs as controls for TSLP and TSLPR show no discernible staining. (F and G) Immunostaining for TSLP and TSLPR, respectively, in control lung tissue demonstrated staining associated predominantly with bronchial epithelium and macrophages. Inset, Negative immunostaining with isotype control Abs on serial sections. Scale bar, 200 μm.

Results

TSLP and TSLPR expression in IPF

We first examined whether TSLP is expressed in IPF lung tissue by assessing TSLP immunoreactivity in IPF (n = 12) and control lung biopsy (n = 3) material. We found that tissue sections from IPF lung demonstrated strong TSLP immunoreactivity, which was predominantly associated with AECs and fibroblasts within fibrotic foci—the histological hallmark of IPF (Fig. 1A). Positive immunoreactivity was also detectable on airway smooth muscle cells (ASMCs) and (alveolar) macrophages. In contrast, TSLP immunoreactivity in control lung was weak and limited to occasional macrophages and bronchial epithelium (Fig. 1F). We next sought to identify potential cellular targets of TSLP in IPF. Serial sections of IPF lung demonstrated intense TSLPR immunostaining in AECs, ASMCs, and surprisingly also for fibroblasts within fibrotic foci (Fig. 1B). Activated fibroblasts within fibrotic foci were identified by their characteristic spindle-shaped morphology, demonstrating strong anti-SMA immunoreactivity on serial sections (Fig. 1C). As with TSLP, immunostaining for TSLPR in control lung was limited to macrophages and bronchial epithelium (Fig. 1G). No immunoreactivity was observed on serial sections stained with isotype control Abs for TSLP (Fig. 1D), TSLPR (Fig. 1E), or anti-SMA (data not shown). To confirm these results, additional immunohistochemical studies were undertaken using a different panel of Abs, with highly concordant results (Supplemental Figs. 1, 2). Taken together, these data raise the intriguing
possibility that lung fibroblasts may represent a novel cellular source and target for TSLP in IPF.

We next considered the potential mechanisms responsible for TSLP upregulation in IPF and focused our attention on the master cytokine, TNF-α, which has been strongly implicated in the pathogenesis of lung fibrosis (55, 56) and is a known inducer of TSLP expression (27, 28).

**TNF-α upregulates TSLP expression in pHLFs in a JNK/c-Jun dependent manner**

IPF immunohistochemistry data suggested that the hyperplastic alveolar epithelium and fibroblasts might represent potential cellular sources of TSLP in the fibrotic lung. To address this possibility, we examined whether primary human AECs (pAECs) and pHLFs express TSLP in vitro. Although the alveolar epithelium displayed strong TSLP immunoreactivity in IPF lung, we were unable to demonstrate baseline or inducible TSLP protein release by pAECs following exposure to TNF-α (data not shown). In contrast, pHLFs were found to constitutively express TSLP mRNA at baseline and this was significantly increased in response to TNF-α over time (Fig. 2A).

**FIGURE 2.** TNF-α stimulates pHLF TSLP gene expression and protein production. Serum-starved pHLFs were exposed to control medium (DMEM) or graded concentrations of TNF-α for varying durations as designated. (A) Time-course data for the effect of TNF-α (10 ng/ml) on pHLF TSLP mRNA levels. TSLP mRNA levels, at each designated time point, were assessed by qualitative PCR. Data are expressed as fold change for each time point relative to time zero, normalized to HPRT mRNA levels (mean ± SEM of triplicates from three independent experiments). (B and C) TSLP mRNA levels of the long (B) and short (C) splice variant following exposure to TNF-α (10 ng/ml) or control medium for 4 h were assessed as in (A) and data were expressed as in (A). **p < 0.01, ***p < 0.001, comparison with time-matched media controls. (D and E) Concentration-response and time-course data for the effect of TNF-α on pHLF TSLP protein levels. pHLFs were exposed to graded concentrations of TNF-α or control medium for 6 h (D) or TNF-α (10 ng/ml) or control medium for varying durations (E). TSLP protein release into CM was measured by ELISA, and the amount of secreted TSLP is expressed as picograms per milliliter (mean ± SEM of triplicates from three independent experiments). *p < 0.05, ****p < 0.001 compared with unstimulated control.

We next examined which of the two known TSLP splice-variants (57) are expressed by pHLFs. We found that pHLFs express both transcript variants constitutively but that TNF-α only increases the expression of the long splice-variant (Fig. 2B, 2C). Confirmation of TSLP induction at the protein level (ELISA) revealed that TNF-α induces TSLP protein release in both a concentration- and time-dependent manner (Fig. 2D, 2E).

To delineate the signaling pathways responsible for mediating TNF-α–induced TSLP expression by pHLFs, we used a combined pharmacological and genetic approach. Preincubation of pHLFs with SC-514 (IKK-2 inhibitor), U0126 (MEK 1/2 inhibitor), and SB203580 (p38 inhibitor) had no effect on TNF-α–induced TSLP protein release into CM despite engagement of their respective targets, as assessed by Western blot analysis of cell lysates (data not shown). Taken together, these data ruled out a significant role for NF-κB, ERK1/2, and p38. In contrast, pharmacological inhibition of JNK activity with SP600125, significantly inhibited TNF-α–induced c-Jun phosphorylation and TSLP protein release in a concentration-dependent manner from 10⁻² M onward (Fig. 3A–C). These findings were corroborated using a second structurally unrelated inhibitor of JNK activity, TI-JIP (Fig. 3D), which
FIGURE 3. TNF-α–induced TSLP expression in pHLFs requires c-Jun phosphorylation. (A–D) Serum-starved pHLFs were preincubated with graded or designated concentrations of SP600125 or TI-JIP for 30 min prior to exposure to TNF-α (10 ng/ml) or control medium (DMEM) for 30 min (A, B) or 6 h (C, D). Final concentrations of DMSO were kept constant for all experimental conditions (0.1% in DMEM). (A and B) The effect of SP600125 on TNF-α–induced c-Jun phosphorylation. Phosphorylated c-Jun was assessed by Western blot analysis of total cell lysates (A) using an anti–phospho c-Jun Ab (upper panel). The same blots were stripped, reprobed with an anti-total c-Jun Ab (lower panel), and used to verify protein loading. (Figure legend continues)
pHLFs express a functional TSLPR complex

To determine whether lung fibroblasts express a functional TSLPR complex, we first examined baseline expression of the Tslpr and IL7ra genes in pHLFs by RT-PCR. pHLFs were found to express mRNA transcripts for both constituent chains of the TSLPR complex (Fig. 4A). Examination of the expression of these chains by dual immunocytofluorescence (Fig. 4B) revealed colocalization of TSLPR and IL-7Rα in pHLFs.

Recent evidence suggests that human mesenchymal cells, such as airway smooth muscle cells, are capable of releasing chemokines, including CCL2, in response to stimulation by TSLP in vitro (59). Moreover, there is strong evidence that this chemokine, in particular, plays a pathogenic role in diseases characterized by tissue remodeling and a T-2 immune phenotype, including IPF (53). Having demonstrated increased immunoreactivity for TSLP in IPF lung, we contemplated the presence of a biologically relevant TSLP–CCL2 axis in this disease. We therefore examined whether lung fibroblasts express a functional TSLPR and were capable of upregulating expression of CCL2 following exposure to this type 2 polarizing cytokine (Fig. 5). These studies revealed that TSLP increased CCL2 mRNA levels within 4 h poststimulation; this response was transient, with levels returning back to baseline levels by 8 h (Fig. 5A). This upregulation was accompanied by the concentration- and time-dependent release of CCL2 protein into CM (Fig. 5B, 5C, respectively). Taken together, these data demonstrate that pHLFs express a functional TSLPR complex and upregulate CCL2 expression and release in response to TSLP stimulation.

TSLP-induced upregulation of CCL2 expression by pHLFs is STAT3 dependent

Signal transduction downstream of the heterodimeric TSLPR comprises functional activation of STAT3 and STAT5 (36). To investigate the potential involvement of STAT3 and STAT5 in TSLP-induced CCL2 expression in pHLFs, we performed Western blot analysis on cell lysates prepared from pHLFs exposed to TSLP using specific Abs directed against phosphorylated regulatory sites on these transcription factors. No STAT5 phosphorylation was observed in pHLFs exposed to TSLP over a period of 1 h (Supplemental Fig. 3). In contrast, TSLP induced STAT3 phosphorylation in a time-dependent manner from 15 min onward, which was maintained for 1 h (Fig. 6A).

We next determined whether STAT3 was required for TSLP-induced CCL2 protein release. Preincubation of pHLFs with the STAT3 inhibitor S3I-201 resulted in a significant concentration-
 dependent inhibition of both TSLP-induced STAT3 phosphorylation and CCL2 protein release from 10^-5 M onward (Fig. 6B, 6C, respectively). The importance of STAT3 in this response was further confirmed by siRNA knockdown of STAT3 expression, which also significantly attenuated TSLP-induced CCL2 production (Fig. 6D–F), whereas overexpression of constitutively active STAT3 enhanced baseline expression of CCL2 in pHLFs (144 ± 10% versus control vector; p < 0.05).

**TSLP induces chemotaxis of human monocytes in a CCL2-dependent manner**

It is well established that fibroblasts represent an important cellular source of chemokines, including CCL2 (60), and the generation of a stromally derived chemokine gradient is increasingly recognized as crucial to the accumulation, differentiation, and survival of immune cells in nonlymphoid target tissue (61). We therefore examined whether TSLP was indirectly capable of generating a functional fibroblast-derived chemokine gradient in vitro. Chemotaxis assays were performed in a Boyden chamber using the human monocyte cell line THP-1. First, we confirmed the chemotactic property of rhCCL2 for THP-1 cells (Supplemental Fig. 4). In subsequent studies, CM from fibroblasts exposed to TSLP (CM-TSLP) was found to promote a significant increase in monocyte migration compared with CM from unstimulated fibroblasts (Fig. 7A). To determine whether this chemotactic response was mediated by CCL2, CM-TSLP was preincubated with a neutralizing anti-rhCCL2 Ab prior to exposure to monocytes. Monocyte chemotaxis in response to CM-TSLP was significantly attenuated (∼40%) in the presence of an anti-CCL2 Ab, suggesting that the chemotactic effect of CM-TSLP was, at least in part, mediated by CCL2 (Fig. 7B). Finally, rhTSLP (1 ng/ml) exerted no chemotactic effect on THP-1 cells demonstrating that the observed chemotactic response was not due to direct stimulation by TSLP.

**Discussion**

The data presented in this article examined the potential role and regulation of TSLP in the context of fibrotic lung disease. To our knowledge, we report for the first time that TSLP and TSLPR are highly upregulated in IPF and that fibroblasts represent both a novel cellular source and target of TSLP. We further show that TNF-α is a potent inducer of TSLP expression by human lung fibroblasts and that TSLP, signaling via STAT3, induces the release of biologically relevant concentrations of the potent monocyte chemoattractant CCL2.

**Fibroblasts express and release TSLP**

Recent work has highlighted the importance of TSLP as a critical regulator of type 2–dominated inflammatory responses in the lung (6, 62). Although TSLP has been strongly implicated in the pathogenesis of atopic asthma (11) and the associated bronchial subepithelial fibrosis (6), the role of TSLP in nonatopic lung diseases associated with increased expression of type 2 cytokines, such as IPF (63), is unknown. Our report of consistently strong TSLP and TSLPR immunoreactivity within active fibrotic lesions in IPF lung supports the notion that TSLP may play a role in promoting type 2 immune responses in this condition. It is also in keeping with recent articles published during the course of our studies in the context of skin fibrosis associated with the autoimmune disorder SSc (64, 65).

Lung fibroblasts occupy a unique sentinel position in the interstitium, which enables them to relay signals of epithelial injury and modulate the immune response accordingly, for instance, by participating in chemokine regulation and immune cell trafficking. We hypothesize that following epithelial injury, increased expression of the early wave alarm-type cytokine, TNF-α, induces fibroblast TSLP expression, a notion supported by our in vitro findings demonstrating that TNF-α is a potent inducer of TSLP expression and release by primary human lung fibroblasts via activation of JNK/c-Jun. Fibroblast-derived TSLP may then in
turn promote a profibrotic type 2 cytokine microenvironment via its well-documented actions on immature DCs.

A number of recent studies have highlighted the potential importance of DCs in the pathogenesis of lung fibrosis (41, 66). In terms of their origin, it has been postulated that activated DCs in IPF originate from a pool of recruited cells which mature locally (41, 67). This is consistent with the evidence that trafficking of DCs to lymph nodes is not a prerequisite for functional T cell interactions (68), which can occur in situ (69). Although the mediators involved in local DC activation in lung fibrosis have not

FIGURE 6.  TSLP-induced upregulation of CCL2 expression by pHLFs is STAT3 dependent. TSLP induces STAT3 phosphorylation in pHLFs (A). Serum-starved pHLFs were exposed to TSLP (1 ng/ml) or control medium for the designated times, and phosphorylation of STAT3 (p-STAT3) was assessed by Western blot analysis of total cell lysates using an anti–p-STAT3 Ab [(A), upper panel]. The same blot was stripped, reprobed with anti-total STAT3 Ab [(A), lower panel], to verify protein loading. (B and C) The effect of S3I-201 on TSLP-induced STAT3 phosphorylation and CCL2 protein release. pHLFs were preincubated with graded concentrations of S3I-201 for 30 min prior to exposure to TSLP (1 ng/ml) or control medium for the designated times. Final concentrations of DMSO were kept constant for all experimental conditions (0.1% in DMEM). (B) p- and t-STAT3 following exposure to TSLP for 30 min was assessed as above. (C) CCL2 protein release into CM at 6 h was measured by ELISA. Data are presented as a percentage of the maximal response obtained with TSLP and drug vehicle alone. The first bar represents the CCL2 response to TSLP and drug vehicle alone. The second bar represents the response to control medium alone. The third bar represents highest concentration of S3I-201 examined and shows that this compound has no effect on basal CCL2 release. Negative log of the concentrations of S3I-201 is presented. Data represent the mean ± SEM of triplicates from three independent experiments. 111 p, 0.001, comparison with untreated cells. * p, 0.05, *** p, 0.001, comparison with TSLP alone. (D–F) The effect of siRNA knockdown of STAT3 expression on TSLP-induced CCL2 protein release by pHLFs. pHLFs were transfected with siRNA targeting STAT3, scrambled siRNA (final siRNA concentration, 100 nM), or mock transfected. Cells were then exposed to TSLP (1 ng/ml) or control medium for 6 h before total cell lysates were prepared for Western blot analysis (D, E); matched CM was also removed for CCL2 protein measurements by ELISA (F). Knockdown of STAT3 expression was assessed by Western blot analysis of total cell lysates from cells exposed to TSLP (1 ng/ml) (D) using an anti–t-STAT3 Ab (upper panel). The same blots were stripped, reprobed with an anti-total ERK1/2 Ab (lower panel), and used to verify protein loading. Densitometric analysis was performed on total STAT3 expression (E), and data are presented as the mean ratio of total STAT3 over total ERK2, normalized to mock-transfected control. Data represent the mean ± SEM of triplicates from three independent experiments. (F) CCL2 protein release into CM was measured by ELISA, and data are presented as picograms per milliliter (mean ± SEM of triplicates from three independent experiments). *** p < 0.001, comparison with mock-transfected cells.
yet identified, it is interesting that nonantigenic danger signals for DCs, such as uric acid and ATP (47, 70), have recently been reported to play key roles in the fibroproliferative response to tissue injury (71, 72). In light of these observations and our findings, it is now tempting to speculate that TSLP may promote the activation of DCs recruited to sites of injury. These TSLP-activated DCs (TSLP-DCs) would then be in a position to induce recruitment of effector immune cells to sites of injury (80). The subsequent functional interaction between recruited immune cells, in the presence of fibroblast-derived TSLP, may serve to promote a local type 2–dominated profibrotic cytokine milieu. During the course of this study, fibrocytes were also reported to express a functional TSLPR complex and respond to TSLP by promoting collagen deposition in a model of atop dermatitis (35). There is some evidence that (myo)fibroblasts, the key effector cells in fibrosis, may be derived from circulating collagen I⁺/CD34⁺/CD45RO⁺ fibrocytes of hematopoietic lineage (81), although whether such cells represent an important source of pathogenic fibroblasts in IPF remains a matter of debate. Interestingly, the intradermal administration of TSLP in mice has been reported to lead to the development of subcuticular fibrosis associated with a significant inflammatory cell infiltrate. Taken together, these findings in the skin and our observations in the lung support the notion that TSLP may contribute to the development of organ fibrosis in a type 2 cytokine–dominated milieu.

Chemokines are best known for their pivotal role in influencing chemotactic responses in a variety of cell types. However, they are also increasingly recognized to play additional important immunomodulatory roles, including regulating T cell differentiation. Exposure of T cells to CCL2 in vitro promotes type 2 cytokine expression (43), an effect that is enhanced in recently activated or memory T cells. This finding is consistent with the observation that the major CCL2 receptor, CCR2, is not expressed by naive T cells, but rather by recently activated CD4⁺ cells (82, 83). Although the pathogenic involvement of T cells in IPF remains an unresolved issue, the presence of T cells in fibrotic lung tissue is a consistent observation (84). Moreover, the majority of T cells organized within lymphoid aggregates in IPF display an activated

FIGURE 7. CM from TSLP-treated pHLFs induces chemotaxis of THP-1 monocytes via CCL2. CM from pHLFs exposed to TSLP induces chemotaxis of human THP-1 monocytes (A). Human THP-1 monocytes were exposed to CM collected from pHLFs treated with graded concentrations of TSLP (0–1 ng/ml) for 6 h. Chemotaxis was assessed using a Boyden chamber as described in Materials and Methods. The first bar represents the chemotactic response of THP-1 cells to DMEM/BSA (1%, w/v) only. The second bar represents the chemotactic response to rhCCL2. Data show the mean number of cells per 5 HPF ± SEM of triplicates from three independent experiments. *p < 0.05, comparison with CM from unstimulated cells. TSLP-induced CCL2 mediates monocyte chemotaxis (B). THP-1 cells were exposed to CM from pHLFs (treated with 1 ng/ml TSLP), which had been preincubated with an anti–CCL2-neutralizing Ab or isotype control (IC) Ab (both at 30 μg/ml) and chemotaxis assessed as above. The first bar represents the chemotactic response of cells to rhCCL2 (1 ng/ml). Data are presented as above. *p < 0.05, comparison with CM from pHLFs exposed to TSLP only.

Locally, thus further promoting the development of a profibrotic type 2 phenotype. Moreover, our observation that pHLFs are capable of upregulating TSLP release in response to a proinflammatory stimulus lends further support to the growing evidence that fibroblasts represent an important immunomodulatory cell type in several disease contexts. Indeed, global gene expression studies suggest that the transcriptional profile of fibroblasts may be modified toward a more immunocentric phenotype following exposure to TNF-α (75). Our data therefore lend further support to the view that fibroblasts be regarded as important sentinel cells, receptive to local tissue injury and capable of choreographing immune cell behavior (76).

Lung fibroblasts express a functional TSLP receptor complex and release CCL2

A second major novel finding reported in this article is the observation that fibroblasts constitutively express a functional TSLP receptor complex and release CCL2 downstream of TSLP signaling via STAT3. Recent studies have highlighted the importance of STAT3 (19) and STAT5 (8) in mediating functional downstream effects following TSLPR activation. We were unable to demonstrate TSLP-induced STAT5 phosphorylation in pHLFs but found instead that exogenous TSLP promoted STAT3 phosphorylation within 15 min. This pattern of STAT activation is also observed in human airway smooth muscle cells (59) and may reflect mesenchymal cell–specific signaling events downstream of TSLPR ligation. Our finding that STAT3 mediates CCL2 expression is further consistent with previous observations that STAT3 plays an important role in mediating CCL2 expression in a wide variety of cell types (77–79). Taken together, these observations identify TSLP as a novel mediator of stromal-derived chemokine expression, and are consistent with the recent notion that fibroblasts are capable of generating a “stromal address code” regulating the recruitment of effector immune cells to sites of injury (80). The subsequent functional interaction between recruited immune cells, in the presence of fibroblast-derived TSLP, may serve to promote a local type 2–dominated profibrotic cytokine milieu. During the course of this study, fibrocytes were also reported to express a functional TSLPR complex and respond to TSLP by promoting collagen deposition in a model of atop dermatitis (35). There is some evidence that (myo)fibroblasts, the key effector cells in fibrosis, may be derived from circulating collagen I⁺/CD34⁺/CD45RO⁺ fibrocytes of hematopoietic lineage (81), although whether such cells represent an important source of pathogenic fibroblasts in IPF remains a matter of debate. Interestingly, the intradermal administration of TSLP in mice has been reported to lead to the development of subcuticular fibrosis associated with a significant inflammatory cell infiltrate. Taken together, these findings in the skin and our observations in the lung support the notion that TSLP may contribute to the development of organ fibrosis in a type 2 cytokine–dominated milieu.
CCL2 is capable of modulating CD4+ T cell behavior directly. The other fibrotic conditions including SSc therefore ultimately reach beyond allergic inflammatory conditions. We propose that the therapeutic potential of anti-TSLP strategies may through the generation of immunomodulatory chemokines. We envisage several potential mechanisms by which failure of such treatment in modifying the natural history of IPF is driven by an aberrant wound healing program following initiation by the effects of TSLP itself on the trafficking of DCs and T cells. In our monocyte chemotaxis assays, neutralization of CCL2 did not completely block the chemotactic potential of CM from fibroblasts exposed to TSLP. These data suggest that TSLP may induce the release of alternative chemotactic mediators in addition to CCL2. Studies aimed at identifying these mediators are ongoing but are beyond the scope of the current work.

During the course of our studies, a profibrotic role for TSLP was also suggested in the context of the autoimmune disease, SSc (85). Although both SSc and IPF are characterized by progressive fibrosis, the pathomechanisms underlying these conditions are distinct. Whereas SSc is primarily felt to be an immune-driven disease, current evidence suggests that the fibrotic response in IPF is driven by an aberrant wound healing program following repetitive epithelial injury. Indeed, recent reports highlighting success in targeting SSc-associated lung pathology with anti-inflammatory strategies (86) are in stark contrast to the dismal failure of such treatment in modifying the natural history of IPF. Studies aimed at identifying these mediators are ongoing but are beyond the scope of the current work.

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