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Thymic Stromal Lymphopoietin Receptor-Mediated IL-6 and CC/CXC Chemokines Expression in Human Airway Smooth Muscle Cells: Role of MAPKs (ERK1/2, p38, and JNK) and STAT3 Pathways

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Thymic stromal lymphopoietin (TSLP) plays a pivotal role in allergic diseases such as asthma, chronic obstructive pulmonary disease, and atopic dermatitis. Enhanced TSLP expression has been detected in asthmatic airways that correlated with both the expression of Th2-attracting chemokines and with disease severity. Although cumulative evidence suggests that human airway smooth muscle (HASM) cells can initiate or perpetuate the airway inflammation by secreting a variety of inflammatory cell products such as cytokines and chemokines, the role of TSLP in this pathway is not known. In the current study, we sought to investigate whether HASM cells express the TSLP receptor (TSLPR) and whether it is functional. We first demonstrated that primary HASM cells express the transcript and protein of both TSLPR subunits (TSLPR and IL-7Rα). Functionally, TSLPR-mediated HASM activation induced a significant increase in CXC (IL-8/CXCL8), CC (eotaxin-1/CCL11) chemokines, and proinflammatory cytokine IL-6 expression. Furthermore, using biochemical and genetic approaches, we found that TSLP-induced proinflammatory gene expression in HASM involved the transcriptional mechanisms, MAPKs (ERK1/2, p38, and JNK), and STAT3 activation. Finally, TSLPR immunoreactivity in bronchial sections from mild allergic asthmatics suggested the potential in vivo TSLP targeting of HASM. Altogether, our data suggest that the TSLPR-mediated HASM activation induces proinflammatory cytokine and chemokines release that may facilitate inflammatory immune cells recruitment in airways. In addition, it may be inferred that TSLP is involved in the pathogenesis of allergic asthma through the activation of HASM cells by TSLP. The Journal of Immunology, 2010, 184: 7134–7143.

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Abbreviations used in this paper: AD, atopic dermatitis; ASM, airway smooth muscle; CC, C-C chemokine; COPD, chronic obstructive pulmonary disease; HASM, human airway smooth muscle; IC, isotype control; IC50, half-maximum inhibitory concentration; iNOS, inducible nitric oxide synthase; IC-mAb, isotype control mAb; p-STAT3, phosphorylated STAT3; p-ERK1/2, phosphorylated ERK1/2; p-p38, phosphorylated p38; p-JNK, phosphorylated JNK; p-IL-6Ra, phosphorylated IL-6 receptor; p-STAT1, phosphorylated STAT1; p-STAT3, phosphorylated STAT3; p-STAT5, phosphorylated STAT5; p-STAT6, phosphorylated STAT6; p-ERK1, phosphorylated ERK1; p-p38, phosphorylated p38; p-JNK, phosphorylated JNK; p-p38, phosphorylated p38; p-JNK, phosphorylated JNK; p-IL-6Ra, phosphorylated IL-6 receptor; shRNA, short hairpin RNA; SP-600125, anthra(1,9-cd)pyrazol-6(2H)-one; T, total; tGFP, turbo GFP; TSLP, thymic stromal lymphopoietin; TSLPR, thymic stromal lymphopoietin receptor; U-0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio) butadiene.

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in a latent form in the cytoplasm and gets phosphorylated at tyrosine residues upon receptor activation by cytokines such as IL-6 (19, 20). Once phosphorylated, STAT3 undergoes conformational change, dimerizes, and translocates to the nucleus where it binds the specific DNA motifs and activates the transcription of distinct groups of genes (19).

STAT3 has recently been implicated to play a critical role in promoting allergic inflammation, notably in studies where epithelial STAT3 disruption reduced the airway eosinophilia (21), and IL-17A–induced STAT3 activation mediated the eotaxin-1/CCL11 induction in HASM (22). However, despite the increased interest in pathophysiology of TSLP in inflammatory diseases such as asthma (6), cellular targets and TSLPR–associated cell signaling mechanisms and functions remain poorly understood. In this study, we first show that HASM cells express TSLPR in vitro and in vivo; TSLP stimulation induces proinflammatory cytokines and CC/CCX chemokine release; and STAT3 and MAPKs (ERK1/2, p38, and JNK) signaling is involved in TSLPR-mediated HASM activation.

Materials and Methods

Reagents

Recombinant human TSLP and TNF-α were purchased from PeproTech (Rochester Hill, NJ). Rabbit anti-human p38 MAPK mAb, rabbit anti-human ERK1/2 mAb, affinity-purified rabbit anti-phospho-p38 MAPK (T180/Y182), affinity-purified mouse anti–phospho–ERK1/2 (T202/Y204), rabbit anti-total and phospho-specific SAPK/JNK (T183/Y185) Abs, rabbit polyclonal anti-STAT5, and anti-phospho–tyrosine–specific STAT5 (Y694) mAbs were purchased from Cell Signaling Technology (Danvers, MA). PE-conjugated mouse anti-CD127R (IL-7Rα), PE-mIgG1 (MOPC21), and mouse mAb anti–phospho–tyrosine STAT5 (Y705) were purchased from BD Biosciences (Mississauga, Ontario, Canada). Affinity-purified rabbit anti-total STAT3 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse IgG1 isotype control (clone MOPC21) was purchased from Sigma-Aldrich Canada (Oakville, Ontario, Canada). All cell culture media (DMEM and F-12), antibiotics (penicillin and streptomycin), and cell culture reagents were obtained from Invitrogen Canada (Burlington, Ontario, Canada); the PBS was obtained from HyClone Laboratories (Logan, UT). Alkaline phosphatase-conjugated streptavidin was purchased from Jackson Immunoresearch Laboratories (West Grove, PA). The p38 MAPK inhibitor 4-((2-aminophenylthio)butadiene (U-0126), and the JNK inhibitor anthracene-9-carboxamide (SP-600125) were purchased from PeproTech (Rocky Hill, NJ).

Materials and Methods

Primers for housekeeping gene GAPDH and standard controls were developed in our laboratory. The forward and reverse-specific primer sequences were used, and the size of the amplified fragment and the annealing temperature for TSLPR were 5′-GAGTTGGCGAGTTAGGCACAA-3′ and 5′-ACCATCTCCATAGCTCCACC-3′, 103 bp, 62°C; for IL-7Rα were 5′-TGGAGGACTGTAATTACATC-3′ and 5′-GATGC- CCTCCGAGGCTTCTTGC-3′, 130 bp, 57°C; and for GAPDH were 5′-AACATTGCTCTCAGCAACCAAC-3′ and 5′-CCTCGAAGGGGCTACCC-3′, 137 bp, 60°C. PCR products were run on 1% w/v agarose gel electrophoresis and visualized by etidium bromide staining.

Flow cytometry analysis

A single-cell suspension of primary cultured HASM cells (10⁶ cells in 100 µl PBS/5% FCS) was prepared from serum-fed confluent cultures and then incubated with gentle agitation for 1 h on ice with mouse anti-human TSLPR mAb (R&D Systems, Minneapolis, MN) or mouse IgG1 (MOPC21) as control at final concentration of 10 µg/ml. The cells were washed twice with PBS/2% FBS and incubated in the dark for 30 min on ice with donkey FITC-conjugated anti-mouse IgG Ab (1/200) (Molecular Probes, Invitrogen, Eugene, OR). The cells were washed three times with PBS/2% FBS, resuspended in 200 µl PBS, and analyzed on FACScan (BD Biosciences, San Jose, CA). FACS analysis was performed with CellQuest software (BD Biosciences, San Jose, CA).

Immunofluorescence and confocal laser scanning microscopy

Serum-fed HASM cells grown on 24-well glass slides (Nalge Nunc International, Naperville, IL) were cultured up to semiconfluence. Slides were fixed with 4% paraformaldehyde, air-dried, and stored at −20°C until use. Briefly, after treatment with universal blocking solution for 30 min (Dako, Carpinteria, CA), slides were incubated with mouse anti-human TSLPR mAb or matched control mouse IgG1 at a final dilution of 10 µg/ml overnight at 4°C. Slides were then washed twice with PBS, followed by incubation for 1 h at room temperature with donkey anti-mouse IgG (1/200) Alexa Fluor 488 (1/200 dilution) (Molecular Probes, Invitrogen Canada). Slides were washed extensively with PBS and counterstained with nuclear stain DAPI for 10 min (Sigma-Aldrich Canada). After washing with PBS, slides were stained with PE-conjugated CD127R (IL-7Rα) Ab or isotype control PE-MOPC21 (5 µg/ml) for 2 h at room temperature. The slides were then washed twice with PBS and mounted with ProLong antifade (Molecular Probes, Invitrogen Canada). Samples were photographed on Olympus AX-70 microscope with a Photometrics PXL cooled charge-coupled device camera and Image-Pro Plus software (Carsen Group, Markham, Ontario, Canada).

Real-time RT-PCR analysis

Confluent HASM cells (passages 2–5) were grown arrested for 48 h in serum-free medium and then stimulated in fresh PBS-free medium containing recombinant human TSLP (0.1 and 1.0 ng/ml), TNF-α (10 ng/ml), or vehicle (medium alone) for 2, 6, and 24 h. Cells were harvested, total cellular RNA were extracted, and reverse transcription was performed as described above. Relative levels of IL-8/CXCL8, eosinophil-1/CCL11, and IL-6 mRNA were measured by quantitative real-time RT-PCR analysis as described previously (24, 25).

ELISA analysis of cytokine/chemokine release in cell supernatants

Semiconfluent primary HASM cells (75%, passages 2–5) were grown arrested for 48 h in PBS-free Ham’s F-12 containing antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). Cells were then cultured in fresh, serum-free Ham’s F-12 with recombinant human TSLP (0.1 ng/ml), TNF-α (10 ng/ml), or medium alone. In some experiments, cells were pretreated for 1 h with U-0126 (10 µM), SB-203580 (10 µM), or SP-600125 (50 nM) before stimulation with TSLP (0.1 ng/ml). Supernatants were then collected at 24 h, centrifuged at 1200 rpm for 7 min at 4°C to remove cellular debris, and stored at −80°C until analysis was carried out by ELISA. Immuneinactive IL-6 was quantified by ELISA using matched Abs from BioLegend (San Diego, CA), according to basic laboratory protocols. Immuneinactive IL-8/CXCL8 and eosinophil-1/CCL11 were measured by ELISA as described previously (25, 27). The sensitivity limit for both IL-8/CXCL8 and IL-6 was 10 and 7.8 pg/ml for eosinophil-1/CCL11. Each data point represents readings from a minimum of four independent assays performed in triplicate.

Luciferase reporter constructs and transient transfection

IL-8/CXCL8, eosinophil-1/CCL11, and IL-6 promoter luciferase constructs used in this study were gifted to us (pUHC13-3-IL-8-pr-wild type by Dr. M. Kracht [Medical School Hannover, Hannover, Germany]; pGL3-E02 cotxin-1/CCL11 promoter by Dr. I. Horjes-Hocek [Institute for Chemistry and Biochemistry, Salzburg, Austria]; and pGL6-luc 651 by Dr. O. Eickelberg [University of Giessen Lung Center, Department of Medicine II, Giessen, Germany]). HASM cells (4 × 10⁴) were seeded into 24-well culture
plates in fresh complete DMEM. After 24 h at 70% confluency, transient transfection of HASM cells was performed using ExGen 500 in vitro transfection reagent (MBI Fermentas, Burlington, Ontario, Canada), according to the manufacturer’s instructions. In each well, 0.8 μg promoter-luciferase DNA and 0.2 μg Renilla luciferase reporter vector-pRL-TK (Promega, Madison, WI) were cotransfected for 24 h. Then, cells were washed and stimulated with TSLP (0.1 and 1.0 ng/ml). TNF-α (10 ng/ml) was used as a positive control. After 12 h of cytokine stimulation, cells were washed twice with PBS, and cell lysates were collected with 100 μl reporter lysis buffer. The luciferase activity was measured by the Dual-Luciferase Assay System kit (Promega, Madison, WI) using a luminometer (model LB9501; Berthold Bad Wildbad, Germany), as mentioned earlier (22). Briefly, 20 μl cell lysate was mixed with 100 μl Luciferase Assay Reagent II, and firefly luciferase activity was first recorded. Then, 100 μl Stop-and-Glo Reagent was added, and Renilla luciferase activity was measured. All values are normalized to Renilla luciferase activity and expressed relative to the control transfected nonstimulated cells.

Assessment of STAT3, STAT5, and ERK1/2, p38, JNK MAPK phosphorylation

Nearly confluent HASM cells were growth arrested by FBS deprivation for 48 h as described above. Cells were then stimulated in fresh FBS-free medium with TSLP (10 ng/ml) or medium alone. In some experiments, HASM cells were also stimulated with 10 ng/ml platelet-derived growth factor-BB (R&D Systems, Minneapolis, MN), as the latter is known to be a strong inducer of STAT5 phosphorylation (28). At selected time points, the cells were washed once with cold PBS, and total proteins were extracted with lysis buffer. Harvested lysates were centrifuged for 10 min at 4°C to pellet cellular debris. The supernatants were removed and stored at −70°C. Protein lysate (10 μg) was loaded on 10% SDS-PAGE, followed by transfer to polyvinylidene difluoride membranes (Amersham Biosciences, Baie D’Urfé, Quebec, Canada). The blots were then blocked with 5% nonfat dry milk in TBS/0.1% Tween 20 (TBST) for 1 h at room temperature and then incubated overnight at 4°C with Abs specific for phosphorylated STAT3 (Y705), STAT5 (Y694), ERK1/2 (T202/Y204), p38 (T180/Y182), and JNK (T183/Y185). After washing with TBST, the blots were incubated with goat anti-mouse or goat anti-rabbit HRP-conjugated secondary antibodies and then incubated with goat anti-mouse or goat anti-rabbit HRP-conjugated secondary Abs, and bands were revealed with ECL reagents (Amersham Biosciences). After stripping, total anti-STAT3, -STAT5, -ERK, -p38, and -JNK were used as loading control. Densitometric analysis was performed on at least three gels, and integrated density value was presented as the fold increase in phosphorylated over total compared with time zero.

Lentiviral-transduced STAT3-short hairpin RNA transduction in HASM cells

Lentiviral transduction of STAT3-short hairpin RNA (shRNA) in HASM cells was performed as described earlier (22). Briefly, pseudotyped lentiviral vectors expressing specific STAT3 shRNA were obtained from Open Biosystems (Huntsville, AL). Lentiviruses were generated using 293T cell lines, and viral titer was determined by counting the puromycin-resistant colonies. A control shRNA unrelated to STAT3 sequence was used as a negative control. For shutting down STAT3 protein expression, HASM cells were transduced at a multiplicity of infection of 10 in the presence of polybrene (8 μg/ml). In brief, cells were exposed to recombinant lentivirus for 2 h at 37°C, and medium was replaced and cultured for an additional 72 h. Transduced cells were selected with puromycin. The average transduction efficiency was >98% as determined by FACS using the turbo GFP (iGFP) as the marker for cell sorting. STAT3 expression in lentivirus-transduced cells was analyzed by Western blotting using total STAT3-specific Abs. Mock and lentiviral-STAT3-shRNA-transduced HASM cells were then cultured in presence of TSLP (0.1 and 1.0 ng/ml), TNF-α (10 ng/ml), or medium alone for 2 h; IL-8/CXCL8, eotaxin-1/CCL11, or IL-6 mRNA expression was analyzed by quantitative real-time RT-PCR as described above.

Immunohistochemistry

To determine whether HASM express TSLPR in vivo, immunohistochemistry was performed using tissue sections prepared from bronchial biopsies of mild allergic asthmatic subjects with procedures approved by the Human Research Ethics Board of the Laval University (Quebec City, Quebec, Canada). Tissue sections from three mild allergic asthmatic subjects, as defined by the American Thoracic Society, were used (29). All asthma samples (mean age, 22.6 ± 7.4 years; mean FEV1, 90.33 ± 5.73%; mean PC20, 2.87 ± 2.9 mg/ml) were atopic nonsmokers and had not received inhaled or systemic corticosteroids in the last 3 mo and were not receiving medications other than inhaled β-agonists, if any. Briefly, formalin-fixed tissues were paraffin embedded, and 5-μm-thick sections were prepared, deparaffinized in xylene, and rehydrated through graded concentrations of alcohol to water and then boiled with microwave for 10 min in sodium citrate buffer (pH 6.0). Sections were washed and then incubated with blocking solution (1% BSA and 0.1% cold fish skin gelatin in TBS) for 60 min at room temperature. Mouse anti-human TSLPR mAb or control mouse IgG1 (both at 10 μg/ml) were added, and sections were incubated overnight at 4°C. Slides were then washed twice with PBS, followed by incubation for 1 h at room temperature with biotin-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Slides were then washed extensively with PBS and incubated with streptavidin-alkaline phosphatase for 30 min at room temperature. After washing with PBS, the slides were developed using Fast Red and counterstained with Mayer’s hematoxylin. Positive cells were stained red after development with Fast Red (Sigma-Aldrich Canada). Isotype-matched control mAb was used for negative control.

Statistical analysis

Results are expressed as means ± SD of three or more independent experiments. Differences between pairs were assessed by Mann-Whitney U test. Values of p < 0.05 were considered statistically significant.

Results

HASM cells express heterodimeric TSLPR

The TSLPR is heterodimeric, consisting of the IL-7Rα-chain and a common γ receptor-like chain (TSLPR) (3, 4). We first used RT-PCR to determine whether HASM express TSLPR in vivo, and TSLPR expression was analyzed by means of RT-PCR (A), FACS (B), and double immunofluorescence (C) as described in Materials and Methods. Briefly, HASM cells were stained with mouse anti-human TSLPR (Ci) and with mouse anti-human IL-7Rα mAb (Ciii). Substitution of the first Ab with isotype control mAb-MOPC21 or PE-MOPC21 eliminated the immunostaining of the positive cells (Cv, vi, respectively). Cells were also stained with DAPI for nuclear localization (Ciii, vii) staining overlap (Civ, viii). PBMC cDNA served as positive control and GAPDH as internal control for RT-PCR. IC-mAb, isotype control mAb (B, Cv, mlgG1-MOPC21; Cvii, PE-mlgG1-MOPC21). Original magnifications ×100 (C-viii).

FIGURE 1. HASM cells express TSLPR. HASM cells were cultured, and TSLPR and IL-7Rα expression was analyzed by means of RT-PCR (A), FACS (B), and double immunofluorescence (C) as described in Materials and Methods. Briefly, HASM cells were stained with mouse anti-human TSLPR (Ci) and with mouse anti-human IL-7Rα mAb (Ciii). Substitution of the first Ab with isotype control mAb-MOPC21 or PE-MOPC21 eliminated the immunostaining of the positive cells (Cv, vi, respectively). Cells were also stained with DAPI for nuclear localization (Ciii, vii) staining overlap (Civ, viii). PBMC cDNA served as positive control and GAPDH as internal control for RT-PCR. IC-mAb, isotype control mAb (B, Cv, mlgG1-MOPC21; Cvii, PE-mlgG1-MOPC21). Original magnifications ×100 (C-viii).

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PCR to search for expression of TSLPR in primary HASM cells. As shown in Fig. 1A, mRNA of TSLPR subunits was detected in all RNA preparations from three different confluent serum-fed HASM cells using specific primers for the TSLPR chain (Fig. 1A, middle panel) and IL-7Ra (Fig. 1A, upper panel). Similarly, a specific signal was also detected in human PBMCs, used as positive control (Fig. 1A). We then performed flow cytometry on single-cell suspensions to investigate whether the TSLPR is expressed on the cell surface of HASM cells. FACS analysis of HASM cells from five different donors using a mouse anti-human TSLPR mAb revealed substantial specific fluorescence on the surface of subpopulations of HASM cells (percentage of positive cells, 26 ± 3.5; n = 5) (Fig. 1B). To further investigate the protein expression of TSLPR by HASM cells, double immunofluorescence was performed with mouse anti-human-TSLPR chain mAb, followed by anti-mouse IgG F(ab')2 Alexa Fluor 488 and PE-conjugated mouse anti-CD127R (IL-7Ra). A specific immunofluorescent signal was observed in confluent serum-fed primary HASM cells stained with mouse anti-human TSLPR (Fig. 1Ci) and with mouse anti-human IL-7Ra mAb (Fig. 1Cii).

Substitution of the first Ab with isotype control mAb-MOPC21 or PE-MOPC21 eliminated the immunostaining of the positive cells (Fig. 1Cv, vi). HASM cells were also stained with DAPI for nuclear localization (Fig. 1Ci and vii). Taken together, these results demonstrate that HASM cells express both mRNA and protein coding for TSLPR.

Recombinant TSLP induces IL-6 and CC/CXC chemokines release from HASM cells

To investigate whether TSLP can influence HASM cell synthetic function, CC and CXC chemokines expression was evaluated. Serum-deprived HASM cells were stimulated over time points 2, 6, and 24 h with TSLP (0.1 and 1.0 ng/ml) and TNF-α (10 ng/ml), and mRNA expression for proinflammatory cytokine IL-6 and IL-8/CXCL8 and eotaxin-1/CCL11 (respective chemotactants for eosinophils and neutrophils) was analyzed by quantitative real-time RT-PCR. As shown in Fig. 2A–C, TSLP stimulation induced the expression of IL-8/CXCL8, eotaxin-1/CCL11, and IL-6 transcript compared with unstimulated control (p < 0.05; n = 3) at 24 h. Although IL-6 mRNA was significantly upregulated (p < 0.05) at

![FIGURE 2. TSLP induces proinflammatory cytokine and chemokine expression in HASM cells. Forty-eight-hour serum-starved HASM cells were cultured in the presence or the absence of TSLP (0.1 and 1.0 ng/ml) and TNF-α (10 ng/ml). IL-8/CXCL8, eotaxin-1/CCL11, and IL-6 expression was analyzed by real-time RT-PCR in a time-dependent manner (A–C) and ELISA after 24 h of stimulation (D–F), as described in Materials and Methods. GAPDH was used as an internal control to normalize the real-time RT-PCR data (A–C). Data represent mean ± SD of three independent experiments performed under similar conditions. *p < 0.05; **p < 0.01 compared with unstimulated control (n = 3).](https://www.jimmunol.org/content/101/12/7137/F2.large.jpg)
2 h, IL-8/CXCL8, eotaxin-1/CCL11, and IL-6 exhibited maximum transcript expression at 24 h, with almost basal level expression at 6 h. Similar results were observed with TSLP dose of 1.0 ng/ml. TNF-α served as a positive control and induced strong cytokine/chemokine expression at 2, 6, and 24 h, notably with a time dependency for eotaxin-1/CCL11 mRNA expression (p < 0.01; n = 3) (Fig. 2A–C).

Thereafter, we investigated the protein release for IL-8/CXCL8, eotaxin-1/CCL11 chemokines, and IL-6 from TSLP-stimulated HASM cells. Stimulation with 0.1 ng/ml TSLP induced a significant release of IL-8/CXCL8 (p < 0.01; n = 3), eotaxin-1/CCL11 (p < 0.05), and IL-6 (p < 0.05) at 24 h (Fig. 2D–F). Furthermore, analysis of the same supernatants revealed no increased level of TARC/CCL17 or IP-10/CXCL10 in TSLP-stimulated HASM cells compared with medium (data not shown). Taken together, these results suggest that TSLPR-mediated activation leads to selective CC and CXC chemokines and cytokine release in primary HASM cells.

FIGURE 3. TSLP induces proinflammatory gene transcription in HASM at promoter level. HASM cells were transiently transfected with IL-8/CXCL8, eotaxin-1/CCL11, and IL-6 wild-type promoter luciferase reporter constructs and stimulated with TSLP (0.1 and 1.0 ng/ml) or TNF-α (10 ng/ml); luciferase reporter activity was measured as mentioned in Materials and Methods and presented as fold increase over unstimulated control. *p < 0.05; **p < 0.01 (n = 3) compared with wild-type luciferase activity in unstimulated cells.

FIGURE 4. TSLP causes rapid STAT3 and MAPKs phosphorylation in HASM cells. Forty-eight-hour serum-deprived subconfluent HASM cells were stimulated with TSLP (10 ng/ml) for designated times; STAT3 (A, B), MAPKs (ERK1/2, p38, and JNK) (A, C), and STAT5 (D) were assessed by immunoblotting from total cell lysates. The same blots were stripped and reprobed with anti-total (T)-STAT3, anti–T-ERK1/2, anti–T-p38, anti–T-JNK, or anti–T-STAT5 Abs and used for loading control. The results represent one of similar results from three independent experiments. Densitometric analysis was performed on STAT3 (B) and MAPK (C) phosphorylation in three blots and presented as the ratio of phospho over total compared with time zero. *p < 0.05; **p < 0.01 (n = 3). p-STAT3, phosphorylated STAT3.
TSLP induces the promoter activation of IL-8/CXCL8, eotaxin-1/CCL11, and IL-6 genes

To further investigate the mechanisms by which TSLP mediates cytokine/chemokine expression, HASM cells were transiently transfected with wild-type luciferase reporter constructs for IL-8/CXCL8 (25), eotaxin-1/CCL11 (27), and IL-6 (30) carrying proximal promoter regions of respective cytokine/chemokine genes. Primary HASM cells transfected with IL-8/CXCL8, eotaxin-1/CCL11, or IL-6 promoter construct showed a significant increase in luciferase activity (p < 0.05; n = 3) in response to TSLP (0.1 ng/ml) stimulation (mean value of fold increase compared with baseline: 1.24 ± 0.19, 1.18 ± 0.13, and 1.22 ± 0.06, respectively) (Fig. 3). Surprisingly, a higher dose of 1.0 ng/ml (Fig. 3) or 10 ng/ml (data not shown) did not induce a significant increase in the promoter activity compared with a dose of 0.1 ng/ml TSLP. As expected from previous studies (31–33), TNF-α increase in the promoter activity compared with a dose of 0.1 ng/ml (Fig. 3) or 10 ng/ml (data not shown) did not induce a significant increase in the promoter activity compared with a dose of 0.1 ng/ml TSLP. As expected from previous studies (31–33), TNF-α proved to be a positive control for activation of the IL-8/CXCL8, eotaxin-1/CCL11, and IL-6 promoters and enhanced the luciferase activity significantly (1.96 ± 0.33, 1.80 ± 0.05, and 4.25 ± 0.35, respectively; p < 0.01; n = 3) (Fig. 3). Collectively, these data suggest that TSLP induces chemokines and proinflammatory cytokines expression via at least transcriptional mechanisms in HASM cells.

TSLP mediates rapid phosphorylation of STAT3 and ERK1/2, p38, and JNK MAPKs in HASM cells

A previous study in a pro-B cell line Ba/F3 showed that TSLPR signaling can activate STAT3 and STAT5 (34). To investigate the involvement of STAT3, STAT5, and/or MAPKs in TSLPR-mediated IL-6 and CC/CXC chemokine expression in HASM cells, we performed Western blot analysis using specific Abs for the phosphorylated regulatory sites on STAT3, STAT5, and MAPKs. TSLP induced a significant and reproducible increase in STAT3 tyrosine phosphorylation in HASM cells at 10 min, declining thereafter and reaching baseline levels at 120 min (Fig. 4A, 4B). However, TSLP did not induce noticeable STAT5 tyrosine phosphorylation in HASM cells (Fig. 4D). In our study, TSLP also induced a rapid and marked phosphorylation of ERK1/2, p38, and JNK MAPKs. MAPKs phosphorylation reached a maximum at 10–30 min after TSLP exposure and then gradually declined to near baseline levels over the subsequent 120 min (Fig. 4A, 4C). To investigate whether MAPKs affect the TSLP-induced cytokine/chemokine expression, we performed experiments using SB-203580, U-0126, or SP-600125, specific and potent inhibitors of p38, p42/p44 ERK, and JNK MAPKs, respectively. Treatment of HASM cells with SB-203580, U-0126, or SP-600125 before stimulation with TSLP led to a significant inhibition of IL-8/CXCL8 (p < 0.01 for SB-203580 and U0126; p < 0.05 for SP-600125), eotaxin/CCL11 (p < 0.05 for SB-203580 and SP-600125; p < 0.01 for U0126), and IL-6 (p < 0.05 for SB-203580 and U0126; p < 0.01 for SP-600125) at 24 h (Fig. 5). Taken together, our data demonstrate that the MAPK (ERK1/2, p38, and JNK) pathways activation is essential for TSLPR-mediated chemokines/chemokine expression in HASM cells.

Lentiviral-shRNA-mediated STAT3-silencing abrogates functional activation of TSLPR

To further establish the role of STAT3 activation, we used lentiviral-mediated STAT3–loss-of-function approach (22) and assessed the proinflammatory cytokine and chemokine gene expression in HASM cells. As shown in Fig. 6A, >95% of the lentiviral-transduced cells were GFP positive by FACS analysis. Lentiviral transduction of STAT3-shRNA resulted in a highly significant and reproducible decrease in STAT3 protein expression, as shown by Western blotting (Fig. 6B). However, transduction of a non-specific scramble-shRNA sequence did not affect STAT3 expression (Fig. 6B). To determine whether STAT3 inhibition functionally affect TSLPR activation, stably STAT3-silenced HASM cells were stimulated with TSLP (0.1 and 1.0 ng/ml) and TNF-α (10 ng/ml) for 2 h, and cytokine/chemokine mRNA expression was analyzed by quantitative real-time RT-PCR. Interestingly, TSLP stimulation of STAT3-shRNA–transduced HASM cells completely abrogated the IL-8/CXCL8, eotaxin-1/CCL11, and IL-6 mRNA expression (p < 0.05; n = 3) compared with the scramble-shRNA–transduced HASM cells (Fig. 6C–E). This effect was remarkable through TSLP stimulation dose of 0.1–1.0 ng/ml, depicting the importance of STAT3

FIGURE 5. MAPK inhibitors abrogate the TSLP-induced chemokine/ cytokine release. Serum-starved HASM cells were stimulated with DMSO (vehicle control) or with 0.1 ng/ml TSLP for 24 h with or without pretreatment for 1 h with inhibitors of p38 MAPK (10 μM SB-203580), p42/p44 ERK (10 μM U-0126), or JNK (50 nM SP-600125). IL-8/CXCL8 (A), eotaxin-1/CCL11 (B), and IL-6 (C) ELISA was performed as described in Materials and Methods. Error bars represent means ± SD of triplicate values from three experiments. *p < 0.01; **p < 0.05 compared with TSLP-stimulated cells, in the absence of the inhibitors.
activation in TSLP signaling in HASM cells. Notably, TNF-α stimulation of STAT3-shRNA–transduced HASM also reduced the IL-8/CXCL8 (p < 0.05) and IL-6 (p < 0.01) but not eotaxin-1/CCL11 transcript expression compared with scramble-shRNA–transduced HASM cells (n = 3) (Fig. 6B–E). Taken together, these data complement the TSLP-induced STAT3 phosphorylation (Fig. 4A) and suggests that TSLP induces proinflammatory cytokine (IL-6) and chemokines (IL-8/CXCL8 and eotaxin-1/CCL11) expression at least via involving STAT3 (Fig. 6) and MAPKs (Fig. 5) activation in HASM cells.

**TSLPR is expressed in ASM cells in vivo in allergic asthma**

Finally, we investigated whether ASM expresses the TSLPR in vivo. Bronchial sections from three mild allergic asthmatic subjects were stained with mouse anti-human TSLPR mAb, followed by biotin-conjugated goat anti-mouse IgG. Fig. 7A demonstrates clear positive red staining for TSLPR in smooth muscle tissue, as compared with control sections labeled with isotype control mAb (n = 3) (Fig. 7B). Collectively, these and the previous data demonstrate that HASM cells are a potential target of TSLP in vivo in allergic asthma.

**Discussion**

In recent years, the field of TSLP and its role in immune response regulation has gained momentum. We demonstrate in this paper that human airway structural cells express a heterodimeric TSLPR; TSLP significantly induced IL-8/CXCL8, eotaxin-1/CCL11, and IL-6 expression in HASM, involving at least transcriptional mechanisms. By using genetic and biochemical approaches, we also showed that TSLP-dependent proinflammatory gene expression in HASM requires STAT3 and MAPK (ERK1/2, p38, and JNK) activation. Moreover, ASM cells bundle within bronchial biopsies of mild allergic asthmatics demonstrated positive TSLPR immunoreactivity, suggesting that HASM is a potential target of TSLP in vivo.

TSLP is a newly discovered proallergic cytokine with pleiotropic biological functions. Earlier studies demonstrated that human monocytes and CD11c+ dendritic cells were sensitive to TSLP treatment in vitro and released TARC/CCL17, a known ligand for CCR4 expressed on polarized Th2 cells (34, 35). TSLP strongly activates and instructs the myeloid dendritic cells to induce proallergic CD4+ and CD8+ T cell responses (35, 36). In addition, TSLP can directly activate the human and mouse CD4+ and CD8+ T cells (37, 38). In a mouse model of allergen-induced asthma, TSLP activated NKT cells to enhance airway hyperresponsiveness by upregulating their IL-13 production, without affecting eosinophilia and IgE production (10). Compelling evidence from animal models suggest that TSLP may initiate asthma (9) or atopic dermatitis (AD) (39) through a dendritic cell-mediated Th2 immune response. Undoubtedly, the clinical relevance of these observations is dictated in humans by high levels of TSLP both in skin biopsies from lesional AD patients (35) and in asthmatic airways that correlated with reduced lung function (12). TSLP also enhanced the effector stages of allergic response, notably by amplifying mast cell synthetic function. In the presence of IL-1 and TNFα, TSLP activated mast cells to produce significant levels of proinflammatory cytokines (IL-5, IL-6, IL-13, and GM-CSF) and chemokines (CCL1 and CXCL8), without inducing mast cell degranulation (40). In a recent study, TSLP also induced the macrophage chemotactic protein (MCP)-1 production in intervertebral disc cells, facilitating macrophage recruitment that may be involved in the resorption of herniated disc tissue (41). The current study, besides establishing another cellular target of TSLP, provides evidence for TSLP-mediated proinflammatory cytokine and chemokines release via STAT3 activation and MAPK phosphorylation in HASM cells.

Neutrophil influx within the airways is a hallmark feature of COPD and asthma (14). Although HASM cells contribute to the perpetuation of airway wall inflammation, the exact and detailed mechanisms remain unknown. IL-8/CXCL8, a CXC family chemokine, is involved in neutrophil recruitment and activation. Increased IL-8/CXCL8 expression has been implicated in angiogenesis and metastasis of lung cancer (42) and is known to modulate HASM cell contraction in cystic fibrosis, contraction,
in HASM, unlike mast cells where it needs synergy with IL-1
and migration (43). Interestingly, we found that TSLP induces
significant level of IL-8/CXCL8 release in HASM cells in ac-
cordance with previous report in mast cells (40). In view of these
and another observation that mast cells are located within the
ASM bundle in asthma (44), our data may suggest a concerted
action of both ASM and mast cells in neutrophilic airway influx
via TSLPR-mediated IL-8/CXCL8 release.

Eotaxin-1/CCL11 is a potent chemoattractant for eosinophils
both in vitro and in vivo. Enhanced eotaxin-1/CCL11 production
has been associated with allergic diseases such as asthma, allergic
rhinitis, and AD (45). Moreover, HASM cells are known to express
eotaxin-1/CCL11 in a constitutive and TNF-α, IL-1β, IL-17A
(22, 24), and IL-9-inducible (27) manner. This study unravels a novel pathway of TSLP-induced eotaxin-1/CCL11 release from HASM that may eventually augment the recruitment of eosinophils, basophils, and Th2 lymphocytes to the airways (46).

In contrast, HASM cells are also known to produce and release
IL-6, a pleiotropic cytokine known to have proinflammatory actions in asthma (47). IL-6 is found in increased amounts in induced
sputum of asthmatic patients after mast cell activation (48) and in
the sputum, exhaled breath, and plasma of patients with COPD
(49). Our data suggest that TSLP by itself can induce IL-6 release
in HASM, unlike mast cells where it needs synergy with IL-1β or
TNF-α (40), suggesting the strength of TSLPR-mediated HASM
activation. Interestingly, TSLP induced a modest level of promoter
activity of IL-8/CXCL8, eotaxin-1/CCL11, and IL-6, suggesting
the involvement of TSLPR-mediated transcriptional mechanisms.
Taken together, our data uncover a novel role of TSLP in airway
structural cells that may eventually contribute to airway in-
flammation. However, detailed studies are required to delineate
other functions of TSLP on HASM such as effect on smooth
muscle hyperreactivity, cell contraction, or survival and/or pro-
liferation as later has been observed in a pro-B cell line Ba/F3 (34).

STAT3 was earlier defined to be a critical factor for regulating
anti-inflammatory immune responses in skin and liver pathological
models (50, 51). However, Simeone-Penney et al. (21), through
airway epithelial STAT3 disruption, reported a significantly re-
duced airway eosinophilia, suggesting a critical role for STAT3 in
allergic inflammation. Recently, STAT3 was also found to be in-
volved in IL-17A–induced eotaxin-1/CCL11 release in HASM
(22), supporting its role in promoting eosinophilic inflammation.
Our current data, in agreement with these observations, depicts the
critical involvement of STAT3 in TSLP-induced eotaxin-1/CCL11
(as well as IL-8/CXCL8 and IL-6) expression in HASM.
The involvement of STAT3 was indeed confirmed by lentivirus-
shRNA-mediated STAT3 inhibition that completely abrogated the
TSLP-induced proinflammatory cytokine/chemokine release in HASM. Notably, TSLPR ligation has previously been re-
ported to phosphorylate and activate the STAT5 in mice and hu-
mans and STAT3 in humans (52, 53). Mechanistic studies in a
pro-B cell line Ba/F3 also provide evidence of TSLP-mediated cell
proliferation, with induced phosphorylation of STAT3 and STAT5 (34). In our study, however, TSLP stimulation of HASM
induced conspicuous STAT3 phosphorylation but not STAT5.
Lack of STAT5 phosphorylation in our study may be attributed to
cell-specific signaling events induced by TSLPR activation in
HASM. Furthermore, the specific involvement of kinases, tran-
scription factors, their association with TSLPR, and the sub-
sequent gene targets remain to be settled.

MAPK family is pivotal in mediating multiple cell functions
such as cytokine expression, proliferation, and apoptosis (54). It is
well known that MAPK (at least ERK1/2) can modulate the
STAT3 activation in HASM (22, 55) and HEK293 cell line (56).
In particular, TSLP-induced IL-8/CXCL8 expression in our study may
be explained by a study in dermal fibroblasts where thrombin
activated ERK1/2, p38MAPK, and STAT3 to induce IL-8/CXCL8
release (57). A new report by Wong et al. (58) demonstrated the
TSLP-induced MAPK (ERK1/2 and p38) activation that is in-
volved in proinflammatory IL-6, CXCL8/IL-8, CXCL1, and
CCL2 release in human eosinophils. Altogether, TSLP-induced
phosphorylation of (ERK1/2, p38, and JNK) MAPK and STAT3 in
our study may suggest a complex network of regulatory cross-
talks of ERK1/2, p38, JNK MAPK, and STAT3 pathways in
mediating TSLP–TSLPR signaling in HASM cells. Further studies
are underway to delineate the cross-regulatory actions of STAT3
and MAPK in HASM synthetic response.

Finally, positive staining for TSLPR in ASM bundles from atopic
asthmatics suggest that TSLPR activation of HASM in vivo may
influence the inflammatory process via recruitment of effector cells
linked to the pathogenesis of airway inflammatory diseases such as
asthma. Enhanced TSLP expression observed in both asthmatic
(12, 13) and COPD (26) airways could possibly support such a
possibility. In conclusion, this study is the first demonstration of
the expression of a functional TSLPR in airway smooth cells that
may render the establishment of the inflammatory milieu in air-
ways. Our study also provides a better understanding of TSLP–
TSLPR complex interactions and its likely role in airway in-
flammatory response.

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**FIGURE 7.** HASM cells express TSLPR in vivo in allergic asthma. A
representative slide (n = 3) showing TSLPR immunoreactivity in bronchial
sections of mild allergic asthmatic human airways by using anti–TSLPR-
chain (A) or isotype mIgG1-MOPC21 (B), followed by biotin-conjugated
goat anti-mIgG, a tertiary layer of streptavidin-alkaline-phosphatase, and
developed with Fast Red substrate. Positive cells stained red, positive
TSLPR-immunoreactivity in muscle bundle area (A). As control, no
signal was seen with an isotype-matched control mAb on HASM cells (B).
Similar results were obtained in three other experiments. IC-mAb, isotype
control MOPC21. (original magnifications ×100.)
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Disclosures
The authors have no financial conflicts of interest.

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