Essential role of NF-κB and AP-1 transcription factors in TNF-α-induced TSLP expression in human airway smooth muscle cells

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Redhu NS, Saleh A, Halayko AJ, Ali AS, Gounni AS. Essential role of NF-κB and AP-1 transcription factors in TNF-α-induced TSLP expression in human airway smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 300: L479–L485, 2011. First published December 10, 2010; doi:10.1152/ajplung.00301.2009.—Human airway smooth muscle (HASM) cells are a rich source of inflammatory mediators that may propagate the airway inflammatory responses. Recent studies from our laboratory and others demonstrate that HASM cells express the proallergic cytokine thymic stromal lymphopoietin (TSLP) in vitro and in vivo. Compelling evidence from in vitro studies and animal models suggest that the TSLP is a critical factor sufficient and necessary to induce or maintain the allergic airway inflammation. Despite of an immense interest in pathophysiology of TSLP in allergic inflammation, the triggers and mechanisms of TSLP expression remain inadequately understood. In this study, we found that TNF-α upregulates the TSLP mRNA and induces high levels of TSLP protein release in primary human ASM cells. Interestingly, TNF-α induced the TSLP promoter activity (P < 0.05; n = 4) in HASM that was mediated by upstream NF-κB and activator protein-1 (AP-1) binding sites. Mutation in NF-κB and AP-1 binding sites completely abrogated the effect of TNF-α-mediated TSLP promoter activity and so did the expression of a dominant-negative mutant construct of IκB kinase. Furthermore, the peptide inhibitors of IκB kinase or NF-κB inhibited the TNF-α-induced TSLP protein release (P < 0.05; n = 3) in HASM. Collectively, our data suggest a novel important biological role for NF-κB pathway in TNF-α-induced TSLP expression in HASM and recommend this as a prime target for anti-inflammatory drugs.

Asthma is a complex chronic disease of the lungs, clinically characterized by reversible airflow obstruction, enhanced bronchial responsiveness, and airway inflammation (18). Airway inflammation is a critical multicellular process involving mainly eosinophils, neutrophils, CD4+ T lymphocytes, and mast cells (21). However, airway smooth muscle (ASM) cells have also emerged recently as key “inflammatory cells” with significant contribution to initiating or perpetuating the airway wall inflammation by virtue of secreting and responding to multiple inflammatory cell products (32). Compelling evidence highlights the potential of ASM to contribute towards airway inflammation, airway wall remodeling, and airflow obstruction in this multifaceted disease syndrome (17).

TNF-α, a proinflammatory cytokine, has been demonstrated to play a central role in asthma pathogenesis through direct immunomodulatory actions on ASM (16). TNF-α by itself or in combination with other cytokines, such as IL-1β or IL-13, augments the expression of various “pro-asthmatic” mediators, such as cytokines (IL-6 and GM-CSF), chemokines (eotaxin-1, eotaxin-3, RANTES, TARC, and IL-8/CXCL8), adhesion molecules (VCAM-1 and ICAM-1), and other molecules (cyclooxygenase-2/COX-2: generates proinflammatory prostaglandins; Refs. 16, 17). Notably, TNF-α is one of the most potent cytokine activators of NF-κB, an ubiquitously expressed transcription factor that modulates the expression of a number of cellular genes involved in immune inflammatory processes (24). In a recent mechanistic study (22), TNF-α was shown to induce the expression of multiple NF-κB-sensitive genes, including IL-6, IL-8/CXCL8, and eotaxin-1 via NF-κB acetylation, transactivation, and histone acetyl transferase function in human ASM that could potently be inhibited by IFN-γ treatment. Taken together, TNF-α-mediated ASM activation has emerged as a detrimental element in asthma pathogenesis.

Thymic stromal lymphopoietin (TSLP), a novel proallergic, IL-7-like cytokine, is known to activate myeloid dendritic cells and induces proallergic CD4+ and CD8+ T-cell responses in vitro (15, 35) and in vivo (1, 43), respectively. TSLP expression in airways of asthma patients correlates with the disease severity (41) and has also been detected in chronic obstructive pulmonary disease airways in vitro and in vivo (40, 42), suggesting that it is involved in the development of airway inflammation. Mice expressing TSLP in the lungs develop a spontaneous airway inflammation with features similar to human asthma (43). On the contrary, mice deficient in the TSLP receptor exhibit suppressed airway inflammation upon challenge with allergens (1). Collectively, TSLP has gained momentum over past few years as a critical factor sufficient and necessary to induce or maintain the allergic inflammation (26, 33). However, little is known about the triggers and mechanisms of TSLP expression.

We and others (2, 34, 42) have recently demonstrated that human ASM (HASM) cells are capable of expressing constitutive TSLP in vitro and in vivo. We reported that TNF-α and IL-1β, alone or in combination, considerably upregulated the HASM TSLP expression. Due to increasing interest in the role of TSLP in pathophysiology and the hunt for novel therapeutics of allergic inflammatory disorders such as atopic dermatitis, asthma, and chronic obstructive pulmonary disease, it is of utmost importance to decipher the triggers and associated mechanisms of TSLP expression. In this study, we explore the mechanisms of NF-κB and activator protein-1 (AP-1) activation in TNF-α-induced TSLP expression in HASM.

MATERIALS AND METHODS

Reagents. Recombinant human TNF-α, sheep polyclonal anti-human TSLP antibody and recombinant human TSLP were purchased from R&D Systems (Minneapolis, MN). FBS was from HyClone Laboratories (Logan, UT), DMEM, Ham’s F-12, trypsin-EDTA, and...
antibiotics (penicillin and streptomycin) were from Invitrogen Life Technologies (Grand Island, NY). All other reagents were procured from Sigma-Aldrich Canada (Oakville, ON), unless specified.

Inhibitors of NF-κB activation pathway. The following cell-permeable peptide inhibitors and control peptides were purchased from CalBiochem (EMD; San Diego, CA): I) P1- IκB kinase (IKK) inhibitor peptide (Ac-Ala-Ala-Val-Alu-Leu-Pro-Ala-Leu-Leu-Leu-Ala-Leu-Ala-Leu-Pro-Asp-Arg-His-Asp-Ser-Gly-Leu-Asp-Ser-Met-Lys-Asp-Glu-NH2; cat. no. 401477) and P2-IKK inactive control peptide (Ac-Ala-Ala-Val-Ala-Leu-Pro-Ala-Leu-Leu-Leu-Ala-Leu-Leu-Pro-Asp-Asp-Arg-His-Asp-Gly-Leu-Asp-Ala-Leu-Met-Lys-Asp-Glu-NH2; cat. no. 401478). P1 contains the IKK recognition sequence for IKKs and a cell-permeable peptide sequence, derived from the hydrophobic region of the signal peptide fibroblast growth factor. P2 is a control peptide containing a mutated IKK recognition sequence for IKKs and a cell-permeable peptide sequence, derived from the hydrophobic region (h-region) of the signal peptide of Kaposi fibroblasts. P1- IKK inhibitor peptide (H-Ala-Ala-Val-Ala-Leu-Leu-Pro-Ala-Ala-Ala-Pro-Val-Gln-Arg-Lys-Arg-Gln-Lys-Ala-Pro-Val-Ala-Pro-Ala-Glu-Val-Ala-Leu-Pro-Val-Met-Pro-OH; cat. no. 481486). P3 contains the nuclear localization sequence for NF-κB (K-RG) and prevents NF-κB activation.

Preparation, culture, and stimulation of HB/TSM cells. Primary human bronchial smooth muscle (HBSM) cells were prepared as we described previously (30). Primary human tracheal smooth muscle (HTSM) cells were obtained from macroscopically healthy segments of the trachea during postmortem in Respiratory Hospital at Health Sciences Centre (Winnipeg, MB, Canada) and were isolated and cultured, as were the primary HBSM cells (28, 30). Serum-fed subconfluent HASM cells (used collectively for HBSM/HTSM cells (passages 2–5) were synchronized for 48 h in FBS-deficient Ham’s F-12 medium containing 5 μg/ml human recombinant insulin, 5 μg/ml human transferrin, 5 ng/ml selenium, and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Cells were then stimulated in fresh FBS-free F-12 medium containing recombinant human TNF-α (10 ng/ml) or medium alone.

RT-PCR analysis. HASM cells were harvested by TRIZol method (Invitrogen Canada, Burlington, ON, Canada) for total cellular RNA extraction, and TSLP mRNA expression was analyzed by RT-PCR and ethidium bromide staining, as we described previously (42).

TSLP release ELISA. Supernatants were collected from HASM cells 24, 48, and 72 h after stimulation with TNF-α or medium alone, followed by centrifugation at 1,200 rpm for 7 min at 4°C to remove cellular debris, and they were stored at −80°C until analysis by ELISA. In some experiments, cell-permeable peptide inhibitors for IKK (P1), NF-κB (P3), and related control peptides (P2, P4) were used at 10 μg/ml concentration for 1 h at 37°C before stimulation with TNF-α (10 ng/ml). Immunoreactive TSLP within the supernatants was quantified using ELISA with matched antibodies according to basic laboratory protocol provided by the manufacturer (R&D Systems) and described earlier (42). TSLP protein was quantified in reference to serial dilutions of recombinant standards falling within the linear part of the standard curve for each specific TSLP point measured. The sensitivity limit of TSLP assay was 3.5 pg/ml. Each data point represents readings from a minimum of three independent assays performed in duplicate.

 Luciferase reporter constructs and cell transfection. The plasmid pGL3-basic vector (Promega, Madison, WI) encoding wild-type human TSLP promoter and all other constructs containing mutations in the human TSLP promoter were kindly provided by Dr. Steven F. Ziegler (23). The mutations in the binding sites for human NF-κB (GggGAACTCCA changed to GttcAACTCCA) and human AP-1 (TGtCTTTG changed to TGtGTGTG) were verified by DNA sequencing analysis (23). HASM cells (4 × 10^4) were plated into 12-well culture plates in fresh complete DMEM. At 50–70% confluency, cells were transfected with wild-type human TSLP promoter construct or different mutations of TSLP promoter. Transient transfection of HASM cells was performed using ExGen 500 in vitro transfection reagent (MBI Fermentas, Ontario, Canada) according to the manufacturer’s instructions. In some experiments, HASM cells were transfected with only the dominant-negative mutant of IκB (referred to as dnIKKβ) construct using ExGen 500; the cells were washed with serum-free Ham’s F-12 after 24 h, replenished with fresh F-12 media, and stimulated with TNF-α. After 24 h, the supernatants were collected and subjected to TSLP ELISA as mentioned above. To assess the TSLP promoter luciferase activity, 1.6 μg of wild-type TSLP promoter DNA and 0.4 μg of Renilla luciferase reporter vector pRL-TK (Promega) were cotransfected in each well for 24 h. The medium was changed, and the cells were washed and stimulated with recombinant TNF-α (10 ng/ml) or medium alone. After 12 h of cytokine stimulation, cells were washed twice with PBS and cell lysates were collected with 100 μl of reporter lysis buffer (Promega). The luciferase activity was measured using the dual-luciferase assay system kit (Promega, Madison) using a luminometer (model LB9501; Berthold, Bad Wildbad, Germany), as we described earlier (30). Briefly, 20 μl of cell lysate were mixed with 100 μl of luciferase assay reagent II and firefly luciferase activity was first recorded. Then, 100 μl of Stop-and-Glo reagent were added, and Renilla luciferase activity was measured.

RESULTS

TNF-α upregulates the TSLP expression at mRNA and protein level. We (42) earlier reported that TNF-α upregulates the TSLP expression in HASM cells. In this study, we first ascertained that HASM cells express TSLP constitutively and TNF-α enhances the TSLP expression at mRNA level (Fig. 1A). TSLP measurement by ELISA confirmed that TNF-α stimulation enhanced the TSLP protein release compared with the control in a time-dependent manner (Fig. 1B). Collectively, our previous and current data establish the fact that TNF-α upregulates the TSLP expression in HASM cells.

TNF-α-induced TSLP transcriptional activation involves NF-κB and AP-1. To investigate the underlying mechanisms in TNF-α-induced TSLP expression, we employed the transient transfection of subconfluent HASM cells with the proximal TSLP promoter fused to a luciferase reporter gene, and the subsequent effect of TNF-α stimulation was assessed on TSLP promoter activation. In contrast to the control, TNF-α stimulation induced a 3.702 ± 0.031-fold increase in luciferase reporter activity compared with control (P < 0.05; n = 3; Fig. 2). This suggests that the TNF-α induces TSLP gene expression in HASM cells, at least via modulating the TSLP promoter activity.

To further characterize the promoter region(s) involved in TSLP transcriptional activation by TNF-α exposure, we examined the TSLP promoter region for binding sites for transcription factors known to be involved in the TNF-α-induced TSLP expression. No significant changes were noted in the proximal promoter region. We then employed a transfection assay using an artificial TSLP promoter containing specific mutations in the NF-κB and AP-1 binding sites and compared the luciferase activity with that of the wild-type TSLP promoter. The proximal TSLP promoter fused to a luciferase reporter gene, and the subsequent effect of TNF-α stimulation was assessed on TSLP promoter activation. In contrast to the control, TNF-α stimulation induced a 3.702 ± 0.031-fold increase in luciferase reporter activity compared with control (P < 0.05; n = 3; Fig. 2). This suggests that the TNF-α induces TSLP gene expression in HASM cells, at least via modulating the TSLP promoter activity.

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TNF-α-INdUcED TSLP EXPRESSION VIA NF-κB IN HUman ASM

Fig. 1. TNF-α upregulates thymic stromal lymphopoietin (TSLP) mRNA and protein expression in human airway smooth muscle (HASM) cells. A: serum-deprived HASM cells from 3 donors were cultured in presence of TNF-α (10 ng/ml) for 2 h and lysed in TRIzol, total mRNA was extracted, and reverse transcription-PCR was performed using specific TSLP primers. B: TSLP protein release from TNF-α-stimulated HASM cells at 24, 48, and 72 h was measured by ELISA. Data represent at least 3 independent experiments performed under similar conditions. *P < 0.05, **P < 0.01, compared with the respective unstimulated control.

Fig. 2. TNF-α-induced TSLP promoter activity is mediated by NF-κB and AP-1 sites. HASM cells were transfected with wild-type or mutant TSLP promoter constructs, followed by incubation with TNF-α inhibitor peptides and further stimulation with TNF-α. As shown in Fig. 2A and B, TNF-α-induced TSLP promoter activity in HASM cells was significantly reduced (P < 0.05; n = 3) by both NF-κB (SN50) and IKK inhibitory peptides. However, both inhibitory pep-
Tides did not change the NF-κB/H9260B-mutated TSLP promoter activity in HASM cells, suggesting the critical requirement of intact NF-κB binding site in TNF-α-induced TSLP expression.

**DISCUSSION**

TSLP has recently emerged as a critical proallergic cytokine that is necessary and sufficient to initiate or perpetuate the Th-2 type allergic inflammation. We show here that the proinflammatory cytokine TNF-α induces TSLP expression in HASM via a transcriptional mechanism regulated by NF-κB and AP-1. NF-κB is a ubiquitously expressed transcription factor known to mediate the expression of many inflammatory mediators including cytokines, adhesion molecules, chemokines, and growth factors. An essential step in the “canonical pathway” of NF-κB activation is the phosphorylation of IκB proteins by IKKs, followed by the migration of NF-κB dimers from cytoplasm to the nucleus where they mediate the gene transcription (24, 39). NF-κB-dependent proinflammatory genes are implicated to play a critical role in airway inflammatory diseases such as asthma. Increased NF-κB activation and the resultant inflammatory milieu have been demonstrated in the airways of asthmatic subjects as well as in rodent models of asthma (9). This has kept NF-κB on the list of attractive targets for asthma and inflammatory airway disease therapy. Indeed, the strategies targeting NF-κB pathway have shown promising results in inhibiting multiple aspects of the allergic inflammation in animal models of asthma (9).
With respect to ASM, various studies have demonstrated the NF-κB-mediated inflammatory gene expression such as IL-17-induced IL-8/CXCL8 release (12), neutrophil elastase-induced IKKβ expression, and one regulatory subunit IKK-α including two catalytic subunits IKK-α and -β (12). IKK complex is one of the most critical among these and has been targeted to develop therapeutic strategies (9). IKK activation has been subjected to the development of pharmacological and genetic inhibitors for therapeutic intervention (13). Interestingly, we also found that the expression of a dnIKKβ mutant inhibits the TNF-α-induced TSLP expression in HASM cells. Moreover, the increase in NF-κB/p65 overexpression-mediated TSLP transcription in our study confirmed the significance of this critical pathway in NF-κB activation, as also demonstrated in studies where TNF-α was shown to phosphorylate both NF-κB/p65 (10) and IKKβ (22) in HASM. Various small molecule inhibitors, such as TP53-1, PS-1145, and ML120B, or molecular interventions using genetic IKKβ-knockdown demonstrate a role for this kinase in the expression of multiple inflammatory gene expression in ASM (7, 8), reviewed in Ref. 9. Likewise, IKK (36)- or NF-κB peptide inhibitor (SN50) (25)-mediated and dnIKKβ expression-directed reduction in TNF-α-induced TSLP expression in our study insists on considering NF-κB as a potential target for therapeutic interventions to suppress the allergic inflammation in airway diseases such as asthma.

TNF-α exerts its pleiotropic actions by binding to two receptors designated as p55 (TNFR1) and p75 (TNFR2), both shown to be coexpressed on most cell types including HASM (6). Although ASM express both of these receptors, TNF-α exerts its effects on ASM primarily via activation of TNFR1, including synthetic functions (IL-6 and RANTES; Ref. 4), augmentation of agonist-induced calcium signals, cell proliferation, and expression of adhesion molecule ICAM-1 (5). Of note, TNF-α has been well documented as critical factor in modulating mitogen-activated protein kinases (MAPKs) activation in ASM (4). The MAPKs play an essential role in modulating contractile, proliferative, and synthetic responses in HASM (14).

MAPK-mediated transcriptional regulation has also been shown to involve transcription factors such as NF-κB and AP-1. Specifically, TNF-α-induced MAPK activation can mediate the nuclear translocation and enhancement of the transcriptional activity of NF-κB and c-Jun (38), the latter being known as AP-1 following heterodimerization with c-fos. In cardiac myocytes, p38 MAPK regulates the NF-κB activation to mediate TNF-α-induced IL-6 expression (11). A recent study (37) in HASM demonstrates the regulation of CD38 gene expression through p38 and JNK MAPKs involving NF-κB and AP-1 activation. Similarly, IL-8/CXCL8 expression in HBECs involved p38 and JNK MAPKs, besides NF-κB and AP-1-like transcription factor activation (31). Earlier, we (12) also demonstrated that IL-17 enhances IL-1β-mediated IL-8/ CXCL8 expression that is regulated by p38, ERK1/2 MAPKs, and phosphatidylinositol 3-kinase pathways and is dependent on the cooperation of the AP-1 and NF-κB transcription factors upstream of the IL-8/CXCL8 gene. These studies provide the potential evidence for cross talk between the MAPK signaling pathways and transcription factors (e.g., NF-κB and AP-1) associated with the regulation of gene expression. Indeed, in one of our previous studies (42), pharmacologic inhibitors of MAPK (p38 and p42/p44 ERK) but not phosphatidylinositol 3-kinase abolished the TNF-α-induced TSLP expression in HASM. Ablation of TNF-α-induced TSLP promoter activity and TSLP release following pharmacologic inhibition of IKK or NF-κB (SN50 peptide) in our current study may further demonstrate a role for this kinase in the expression of multiple transcription factors such as asthma.

TSLP is known to be produced by epithelial cells in the lungs, gut, skin, fibroblasts, airway smooth muscle, and mast cells (2, 3, 23, 40, 42). Besides activating myeloid dendritic cells and inducing proallergic CD4+ or CD8+ responses, TSLP can also synergize with IL-1β and TNF-α to induce Th-2 cytokine and chemokine (IL-5, IL-13, IL-6, GM-CSF, CXCL8, and CCL1) expression in mast cells (3). TSLP expression is under the control of differential regulatory mechanisms under different stimulations such as p38 and Jun kinase (JNK) in response to respiratory syncytial virus (RSV) (33); cooperation between NF-κB and JAK/STAT following combination of TNF-α and Th-2 cytokines (20); IL-1β- and TNF-α-induced NF-κB activation in HBECs (23); and IL-1β- and TNF-α-induced MAPK (p38 and p42/p44 ERK) activation in HASM (42). Notably, the NF-κB activation has been reported to be involved in TSLP expression in airway epithelial cells (23).
synovial fibroblasts (33), intervertebral disc cells (29), and human corneal epithelial cells (27). Our data are in agreement with these findings and suggest a critical role of NF-κB in TNF-α-induced TSLP expression in human ASM cells. In one of our experimental strategies, we also performed EMSA to demonstrate the NF-κB and AP-1 binding to their respective sites on TSLP promoter. Our data revealed a weak binding signal (data not shown), suggesting the involvement of other transcription factors in regulating NF-κB and AP-1 binding to TSLP promoter. Notably, the human TSLP promoter has other binding sites such as IRF-1 and Opaque 2 (23) that may have a negative regulatory effect on NF-κB and AP-1 binding, which we are investigating in more detail.

Collectively, we provide multiple lines of evidence to establish the involvement of NF-κB activation in TNF-α-induced TSLP expression in HASM: 1) mutation in NF-κB binding site completely abrogated the TNF-α-induced TSLP promoter activity (Fig. 2), 2) overexpression of NF-κB/p65 subunit enhanced the TSLP promoter activity (Fig. 3A), 3) expression of dnIKKδ abrogates the TSLP promoter activation and protein release (Figs. 3B and 4C), 4) peptide inhibitors of IKK or NF-κB inhibit the TNF-α-induced TSLP protein release (Fig. 4), and 5) NF-κB or IKK inhibitory peptides abrogate the TSLP promoter activity when NF-κB binding site is intact (Fig. 5). Altogether, NF-κB appears to be a critical factor in HASM TSLP expression and warrants potential therapeutic interventions in allergic inflammatory diseases such as asthma.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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