

Inducible expression of the proallergic cytokine thymic stromal lymphopoietin in airway epithelial cells is controlled by NF κ B

Hai-Chon Lee, and Steven F. Ziegler

PNAS published online Jan 9, 2007;
doi:10.1073/pnas.0607305104

This information is current as of January 2007.

E-mail Alerts

This article has been cited by other articles:
www.pnas.org#otherarticles

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#).

Rights & Permissions

To reproduce this article in part (figures, tables) or in entirety, see:
www.pnas.org/misc/rightperm.shtml

Reprints

To order reprints, see:
www.pnas.org/misc/reprints.shtml

Notes:

Inducible expression of the proallergic cytokine thymic stromal lymphopoietin in airway epithelial cells is controlled by NF κ B

Hai-Chon Lee[†] and Steven F. Ziegler^{†‡§}

[†]Immunology Program, Benaroya Research Institute, 1201 Ninth Avenue, Seattle, WA 98101; and [‡]Department of Immunology, University of Washington School of Medicine, 1959 Northeast Pacific Street, Seattle, WA 98195

Edited by Tak Wah Mak, University of Toronto, Toronto, ON, Canada, and approved November 21, 2006 (received for review August 22, 2006)

The epithelial-derived cytokine thymic stromal lymphopoietin (TSLP) is important for the initiation of allergic airway inflammation through a dendritic cell-mediated T helper 2 response. To identify the factors that control TSLP expression, we examined the ability of inflammatory mediators to regulate TSLP production in human airway epithelial cells. We found that both IL-1 β and TNF- α were capable of inducing rapid TSLP production in primary human bronchial airway epithelial cells. We further characterized the human TSLP gene promoter, using two human epithelial cell lines, 16HBE⁺ and A549, and showed that IL-1 β - and TNF- α -mediated human TSLP promoter activation in these cells was mediated by an upstream NF κ B site. Mutation of this NF κ B site abolished activation, as did overexpression of a dominant-negative version of I κ B kinase (IKK) β (a kinase acting on I κ B, the inhibitor of NF κ B). Interestingly, human TSLP mRNA levels were also increased after exposure to Toll-like receptor (TLR) 2, TLR8, and TLR9 ligands, further supporting an important role for NF κ B in TSLP gene regulation. Similarly, analysis of the mouse TSLP gene promoter revealed the presence of a similarly situated NF κ B site that was also critical for IL-1 β -inducible expression of mouse TSLP. Taken together, these results demonstrate that the inflammatory mediators IL-1 β and TNF- α regulate human TSLP gene expression in an NF κ B-dependent manner.

proinflammatory cytokine | T helper 2 inflammation | transcriptional regulation

Thymic stromal lymphopoietin (TSLP) is a cytokine that was originally identified in culture supernatants of a mouse thymic stromal cell line and has been shown to support the development of pre-B cells to B220⁺IgM⁺ immature B cells (1–4). The TSLP receptor complex is a heterodimer that consists of a low-affinity ligand binding chain, the TSLP receptor (TSLPR), and the IL-7 receptor α chain (IL-7R α) (5, 6). TSLP and TSLPR have also been identified and characterized in humans, and the human TSLP receptor complex has been shown to also be a heterodimer that is composed of the TSLPR and IL-7R α chains (7, 8). Although the degree of sequence homology between human and mouse TSLP and between human and mouse TSLPR is quite low (43% and 39%, respectively), the expression profiles are similar in the two species, with B cells and CD11c⁺ dendritic cells being the primary cell populations expressing the TSLPR (ref. 9 and S.F.Z. and D. R. J. Rawlings, unpublished results). TSLP is produced primarily by epithelial cells in the lungs, gut, and skin (8). Recent work (e.g., refs. 10 and 11) has shown that TSLP levels are increased at sites of inflammation. For example, lesional skin from patients with atopic dermatitis displayed markedly elevated TSLP expression, whereas uninvolved skin did not (10). Similarly, airway epithelium from asthmatics showed increased TSLP mRNA expression (11) and supported a role for TSLP in promoting T helper 2-type allergic inflammation. CD4⁺ T cells, primed by TSLP-treated dendritic cells, produce the proallergic cytokines IL-4, IL-13, IL-5, and TNF- α , but not IL-10 and IFN γ , upon restimulation (10).

TSLP has also been shown to be critically important for airway inflammation in mice. Mice expressing a lung-specific TSLP transgene developed a spontaneous airway inflammatory disease with all of the characteristics of human asthma (12). In addition, TSLP levels were elevated in the lungs of mice with antigen-driven airway inflammatory disease (12). On the other hand, TSLPR-deficient mice developed a greatly attenuated airway disease in the same antigen-driven model, consistent with the finding that TSLP responses are both necessary and sufficient for the development of airway inflammation in mice (12, 13). Finally, mice containing an inducible skin-specific TSLP transgene developed an atopic dermatitis-like inflammatory disease after induction of TSLP expression (14). Taken as a whole, these data are consistent with the hypothesis that TSLP plays an important role in the development of allergic inflammatory disorders. Although TSLP plays an important role in the development of allergic inflammation, little is currently known about the factors that control its expression.

To begin an analysis of the factors that control TSLP gene expression, we have dissected the human TSLP promoter and characterized the cis-regulatory factors that control transcription of the human TSLP gene. We demonstrate that TSLP expression is induced in airway epithelial cells (AECs) exposed to proinflammatory mediators, including IL-1 β , TNF- α , and selected TLR agonists. Analysis of the human TSLP gene promoter identified an NF κ B site 3.7 kb upstream of the start of transcription that was critical for IL-1 β - and TNF- α -induced TSLP transcription. An orthologous NF κ B binding site was found in the promoter of the mouse TSLP gene and was shown to be essential for inducible expression. Thus, the regulation of TSLP gene transcription is similar in humans and mice, and activation of NF κ B is critical for inflammation-induced expression of TSLP.

Results

IL-1 β and TNF- α Induce Human TSLP Expression from Normal Human Bronchial Epithelial (NHBE) Cells. Although TSLP has been associated with inflammatory responses, the factors that regulate its expression remain unclear. Studies showed that epithelial cells, primarily in the lung, skin, and gut, are the primary sources of TSLP in humans (7). Because AECs in human asthmatics were found to have increased TSLP mRNA (11), and TSLP levels in the lungs of mice with antigen-induced airway inflammation

Author contributions: H.-C.L. and S.F.Z. designed research; H.-C.L. performed research; H.-C.L. and S.F.Z. analyzed data; and H.-C.L. and S.F.Z. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS direct submission.

Abbreviations: TSLP, thymic stromal lymphopoietin; TSLPR, TSLP receptor; TLR, Toll-like receptor; AEC, airway epithelial cell; NHBE, normal human bronchial epithelial; RXR, retinoic acid X receptor; I κ B, inhibitor of NF κ B; MLE12, mouse type II airway epithelial cell line; IKK, I κ B kinase.

[§]To whom correspondence should be addressed. E-mail: sziegler@benaroyaresearch.org.

© 2007 by The National Academy of Sciences of the USA

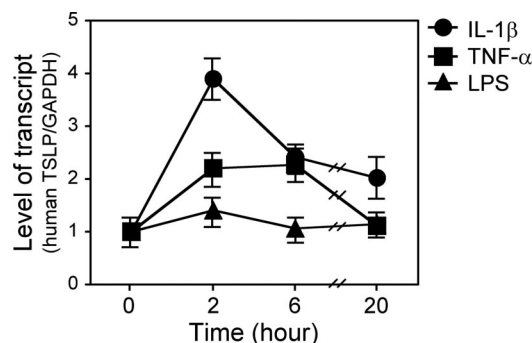


Fig. 1. IL-1 β and TNF- α induce human TSLP expression on NHBE cells. NHBE cells were cultured with IL-1 β (1 ng/ml), TNF- α (10 ng/ml), or LPS (10 μ g/ml) for the indicated time course. Total RNA was extracted, and human TSLP mRNA was measured by real-time quantitative PCR. TSLP mRNA level was normalized with GAPDH mRNA. Data are the mean $\pm$ SE of triplicate data points from a representative experiment. Results are representative of at least three independent experiments.

displayed elevated TSLP levels (12), we chose to study the regulation of TSLP gene expression in AECs. Initially, NHBE cells were treated with cytokines associated with pulmonary inflammation, including IL-1 β , TNF- α , IL-4, and IL-13, along with other known inflammatory agents (e.g., LPS), and TSLP expression was examined by using real-time quantitative PCR. IL-4, IL-13, and LPS did not affect TSLP gene expression (Fig. 1 and data not shown). However, NHBE cells cultured with IL-1 β or TNF- α showed increased TSLP transcription (Fig. 1). Compared with unstimulated cells, IL-1 β induced an almost 4-fold increase of human TSLP mRNA by 2 h after stimulation, whereas a similar increase was seen at 6 h of stimulation with TNF- α . TSLP mRNA levels gradually decreased afterward in both cultures. In contrast, TSLP mRNA levels were unaffected in NHBE cells treated with LPS. Consistent with these results, TSLP levels were increased in the supernatant of NHBE cells treated with IL-1 β or TNF- α but not in supernatants from LPS-treated cells, as measured by the ELISA method (data not shown). TSLP mRNA and protein levels were also found to be increased in two human airway epithelial cell lines, 16HBEo⁻ and A549 (data not shown). These results demonstrate that human TSLP is induced in AECs by proinflammatory mediators.

Transcriptional Activation of the Human TSLP Promoter by IL-1 β and TNF- α Is Mediated by NF κ B. To define the regions of the human TSLP promoter involved in IL-1 β and TNF- α -mediated transcription, a nested series of deletion constructs of the human TSLP gene promoter were generated. All constructs included the transcriptional start codon. These constructs were transiently transfected into 16HBE⁺ and A549 cells, and the response to IL-1 β and TNF- α was determined by measuring luciferase activity. Very little activity was seen in any of the constructs in the absence of IL-1 β and TNF- α . Stimulation of epithelial cell lines transfected with the -4.5- and -4.0-kb constructs showed induction of luciferase activity, and the response to IL-1 β was more robust. In contrast, further deletion constructs did not respond to IL-1 β or TNF- α (Fig. 2). These results suggest that the region between -4.0 and -3.0 kb of the human TSLP gene promoter contains cis-regulatory elements involved in IL-1 β and TNF- α -induced expression of TSLP.

To identify the elements involved in the activation of human TSLP transcription by IL-1 β , we made additional 5'-deletion constructs between -4.0 and -3.3 kb. With these constructs, 16HBEo⁻ were transfected, and luciferase activity was measured after IL-1 β stimulation. As shown in Fig. 3a, constructs containing sequences between -4.0 and -3.8 kb were highly

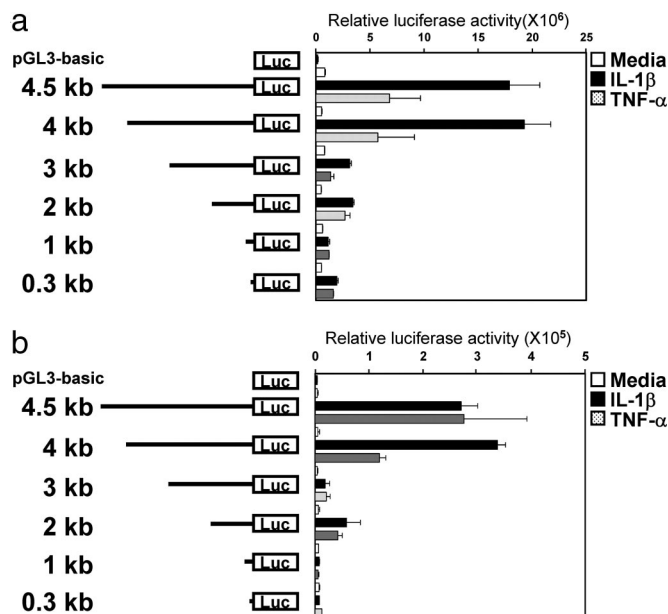


Fig. 2. Transcriptional activation on 5' deletion of the human promoter by IL-1 β and TNF- α . Transfection of 16HBEo⁻ (a) and A549 (b) cells was performed with the mixture of the luciferase constructs containing various 5' deletions of the human TSLP promoter and pRSV- β -Gal luciferase control vector. Schematic representations of promoter elements used are shown on the left. Nineteen hours after transfection, cells were incubated for 5 h in IL-1 β (1 ng/ml) or TNF- α (10 ng/ml). At that time, cells were harvested, and lysates were prepared for determination of luciferase activity. Luciferase activity in the whole-cell lysate was normalized with β -galactosidase activity. Data are the mean $\pm$ SE of triplicate data points from a representative experiment of five independent experiments performed.

inducible by IL-1 β , whereas all deletions from -3.7 to -3.3 kb were at background levels. This indicated that ≈ 120 bp of human TSLP gene promoter from -3.86 to -3.74 kb contained a cis element that was required for IL-1 β induction. Similar results were seen with a second human airway epithelial cell line, A549 (data not shown). Inspection of this ≈ 120 -bp sequence revealed the presence of the NF κ B, IRF-1, Opaque-2 consensus binding sites, and a putative AP-1 binding site (Fig. 3*b*). Next, we tested whether these motifs were important for IL-1 β activation by generating a series of site-directed mutations in each binding site. As shown in Fig. 3*c*, mutations in either the IRF-1 or the Opaque-2 motif had no effect on the activation of the human TSLP promoter by IL-1 β , whereas mutation in the putative AP-1 motif resulted in slightly lowered activity. In contrast, promoter activity was significantly decreased in 16HBE $^{-}$ cells transfected with the construct containing a mutation of the NF κ B motif. These results indicated that the induction of TSLP gene expression seen in cells stimulated with IL-1 β was likely mediated through NF κ B.

NFκB p65/p50 Binds to the NFκB Motif in the Human TSLP Promoter. NFκB is composed of either homo- or heterodimeric subunits of the NFκB/Rel family members, and different combinations of NFκB subunits contribute to the cell type- and stimulant-specific transcriptional activation (15, 16). To determine the nature of the NFκB complex involved in TSLP transcription, we generated nuclear extracts from IL-1β-treated A549 cells and tested them, using an oligonucleotide containing the NFκB binding motif from the human TSLP gene promoter. EMSA, using extracts from IL-1β stimulated cells but not unstimulated cells, induced binding activity to the hTSLP/NFκB probe corresponding to the NFκB motif. Further, cocubination of nuclear extracts from

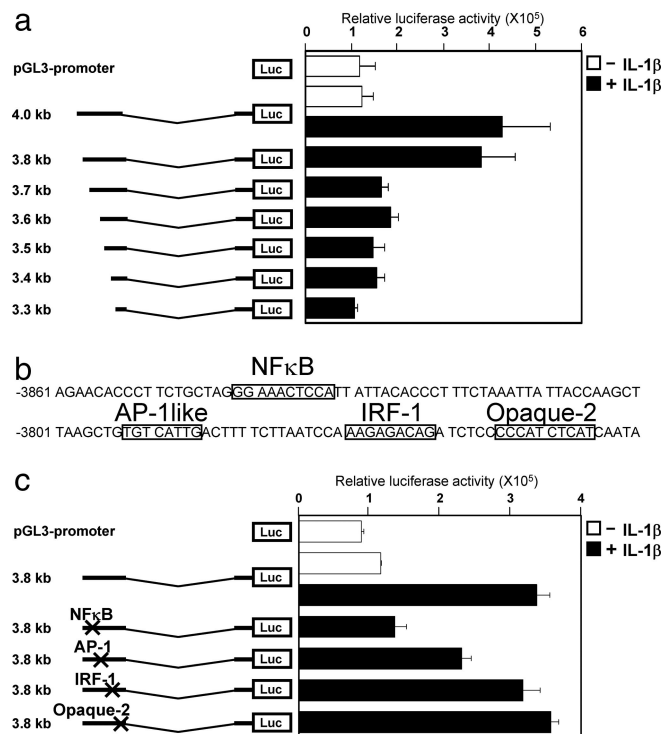


Fig. 3. Identification of an upstream cis-positive element involved in the activation of human TSLP transcription by IL-1 β stimulation. The 16HBE $^{-}$ cells were transfected with the mixture of the luciferase constructs containing additional 5' deletion constructs (a) in -4.0 to -3.3 kb of the human TSLP promoter or various mutation constructs (c) and pRSV- β -Gal luciferase control vector. The experiments were carried out as described in the legend to Fig. 2. Schematic representations of human TSLP promoter reporter constructs are shown on the left. (b) Nucleotide sequence from -3.86 to -3.74 kb relative to the start site of human TSLP. The position of the NF κ B, IRF-1, Opaque-2, and AP-1 binding site are indicated. All results are representative of three independent experiments.

IL-1 β -treated cells with Abs against p65 and p50 each induced supershift of the DNA binding complex (Fig. 4a). The specificity of NF κ B binding to this motif was confirmed two ways. First, EMSAs performed by using extracts from IL-1 β -treated cells

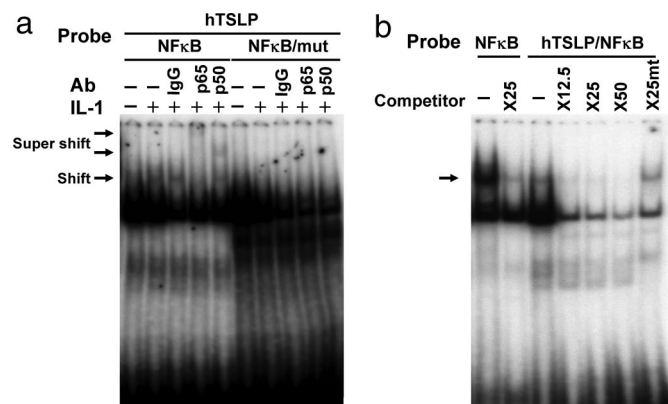


Fig. 4. NF κ B binds to the NF κ B motif of the human TSLP promoter. Nuclear extracts prepared from A549 cells stimulated with IL-1 β were incubated with labeled oligonucleotide probes and subjected to EMSA. Arrows indicate specific binding activity. For supershift assay (a), nuclear extract was preincubated with 1 μ g of normal rabbit IgG, rabbit anti-NF κ B p65, or rabbit anti-NF κ B p50 for 1 h before the addition of labeled oligonucleotide probe. For competition assay (b), the reaction was preincubated with 12.5 - to 50 -fold molar excess of unlabeled oligonucleotide for 5 min before the addition of labeled oligonucleotide probe.

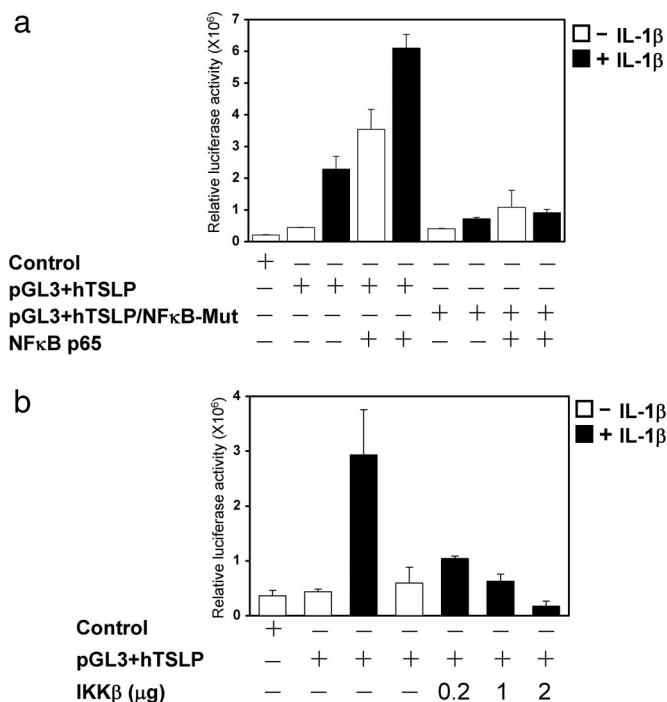


Fig. 5. IL-1 β regulates NF κ B activation and transcription from the human TSLP promoter via the IKK pathway. The A549 cells were transfected with the mixture of the luciferase constructs containing human TSLP promoter, NF κ B expression plasmid (a) or dominant-negative mutant of IKK β (b) and pRSV- β -Gal luciferase control vector. The experiments carried out as described in the legend to Fig. 2. The A549 cells were stimulated with IL-1 β (1 ng/ml) for 5 h. All results are representative of three independent experiments.

and a probe containing a mutated version of the human TSLP gene promoter NF κ B site failed to generate the shifted complex (Fig. 4a). Second, formation of the NF κ B complex could be blocked by competition with unlabeled probe of the same sequence but not with an unlabeled probe containing the mutant NF κ B site (Fig. 4b). These data show that IL-1 β stimulation results in the activation of NF κ B, and that a p65/p50 heterodimer of NF κ B binds to the human TSLP gene promoter.

One of the major pathways for NF κ B activation involves the phosphorylation of the inhibitor of NF κ B (I κ B) α , which is followed by I κ B α degradation and the subsequent migration of NF κ B dimers from cytoplasm to the nucleus (17, 18). To determine whether this pathway is involved in the transcriptional activation of human TSLP, we investigated the effect of overexpressing wild-type NF κ B or a dominant-negative mutant of I κ B kinase (IKK) β . The reporter constructs of the human TSLP gene promoter, containing either a wild-type or mutated NF κ B site, were transfected with an expression vector for the p65 subunit of NF κ B in A549 cells. After 19 h, cells were stimulated with IL-1 β for 5 h. As shown in Fig. 5a, overexpression of the p65 subunit resulted in increased expression of the reporter containing the wild-type NF κ B motif. Furthermore, treatment of the cells with IL-1 β further enhanced expression. This induction was diminished in the construct with mutation in NF κ B motif in human TSLP promoter (Fig. 5a). Conversely, cotransfection of the wild-type reporter construct with a cDNA encoding a dominant-negative mutant of IKK β inhibited IL-1 β -mediated transcription in a dose-dependent fashion (Fig. 5b).

Engagement of TLRs on NHBE Cells Induces Human TSLP Expression. Similar to receptors for IL-1 β and TNF- α , engagement of TLRs also activates NF κ B (19, 20). Therefore, we ascertained whether treatment of NHBE cells with TLR ligands would activate TSLP

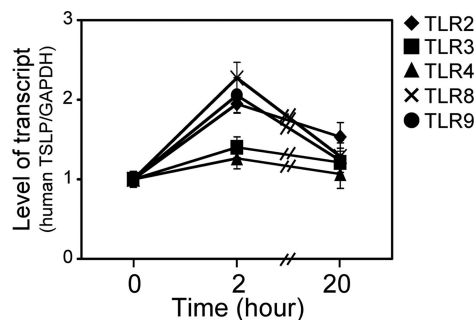


Fig. 6. TLR2, TLR8, and TLR9 induce human TSLP expression on NHBE cells. NHBE cells were incubated with the TLR2 agonist lipoteichoic acid (10 μ g/ml), the TLR3 agonist poly(I-C) (5 μ g/ml), the TLR4 agonist LPS (10 μ g/ml), the TLR8 agonist poly(CpG) (5 μ g/ml), and the TLR9 agonist CpG-B (5 μ g/ml) for 2 or 20 h. Total RNA was extracted, and human TSLP mRNA was measured by real-time quantitative PCR. TSLP mRNA level was normalized with GAPDH. Data are the mean $\pm$ SE of triplicate data points from a representative experiment and represent cells treated with agonist for indicated TLR. Results are representative of three independent experiments.

gene expression. Using RT-PCR analysis, we have determined that NHBE cells express TLR2, TLR3, TLR4, TLR8, and TLR9 (data not shown). To examine TSLP expression, NHBE cells were stimulated with agonists for TLR2, TLR3, TLR4, TLR8, and TLR9, and TSLP gene expression was assessed by real-time quantitative PCR. Agonists for TLR2, TLR8, and TLR9 led to an increase in TSLP gene expression at both 2 and 20 h of stimulation (Fig. 6 and data not shown). In contrast, TSLP mRNA levels were only marginally increased or were unaffected in cells treated with TLR3 and TLR4 ligands. Taken together, these data indicated that TLR2, TLR8, and TLR9 can induce human TSLP expression in AECs.

Mouse TSLP Gene Is also Regulated by NF κ B. To examine the regulation of the mouse TSLP gene, the mouse lung epithelial cell line (MLE12) was used (21). To determine the region of the TSLP promoter responsible for inducible expression, the -5.0 -, -4.0 - and -3.0 -kb regions from the start of translation were amplified by PCR and subcloned into pGL-2 basic vector. This construct was transfected into MLE12 cells, and luciferase activity was measured after treatment with IL-1 β . As shown in Fig. 7a, the -5.0 - and -4.0 -kb constructs had activity in the MLE12 cells without treatment. However, after treatment with IL-1 β cytokine, the level of activity increased ≈ 2 -fold. Thus, both the human and mouse TSLP genes show inducible expression in AECs after exposure to the inflammatory cytokine IL-1 β . To further characterize the sequences responsible for this inducible expression, a series of 5' deletions were generated from the -4.0 - to -3.0 -kb promoter fragment and tested in MLE12 cells for inducible expression after cytokine treatment. Constructs that contain sequence 5' of -3.6 kb showed inducible expression, whereas those lacking this region did not (Fig. 7b). Therefore, a region between -3.6 - and -3.8 -kb 5' of the start of transcription of the mouse TSLP gene controls cytokine-mediated transcription. An inspection of the sequence showed the presence of two putative NF κ B binding sites. The sites were mutated individually or together to determine their potential role in cytokine-mediated TSLP transcription. Mutation of the 3' site, but not the 5' site, eliminated IL-1 β -mediated induction of the promoter construct (Fig. 7c). From these data we can conclude that the inducible expression of the human and mouse TSLP genes requires activation of NF κ B and subsequent binding to a site around -3.6 kb upstream of the start of transcription.

Previous work has shown that epidermal ablation of the nuclear receptors Retinoid X receptor (RXR) α and RXR β

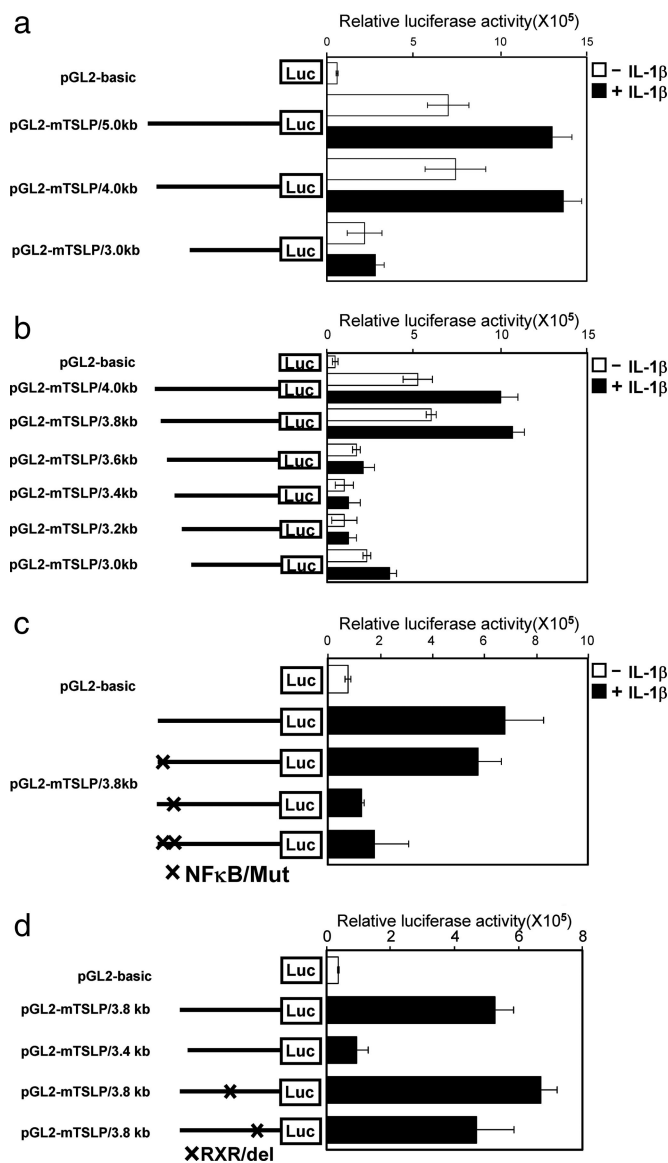


Fig. 7. Identification of an upstream cis-positive element involved in the activation of mouse TSLP transcription by IL-1 β stimulation. The MLE12 cells were transfected with the mixture of the luciferase constructs containing -5.0 -, -4.0 -, or -3.0 -kb of the mouse TSLP promoter (a), 5' deletion constructs in -4.0 to -3.0 kb of the mouse TSLP promoter (b), NF κ B mutation constructs (c), or retinoid X receptor (RXR) deletion constructs (d); in every case, pRSV- β -Gal luciferase control vector was added. The experiments were carried out as described in Fig. 2 legend. Schematic representations of mouse TSLP promoter reporter constructs are shown on the left. All results are representative of three independent experiments.

resulted in development of an atopic dermatitis-like disease that included elevated expression of TSLP (22). These authors also identified two possible RXR binding sites upstream of the TSLP gene (base pairs -1049 to -1062 and base pairs -2164 to -2175). To determine whether these sites were involved in regulating TSLP gene expression, promoter constructs were generated containing deletions of each site, using the mTSLP/3.8-kb promoter construct as template. These constructs were transfected into MLE12 cells, and luciferase activity was measured. As shown in Fig. 7d, deletion constructs have no effect in transcriptional activity of mouse TSLP gene, whereas a construct lacking the NF κ B site showed a marked decrease in activity. This result suggested that these sites are not required for the regulation of mouse TSLP gene expression.

Discussion

Many studies have shown an important role for AECs in the progression and attenuation of inflammatory diseases (23–25). AECs are intimately associated with both dendritic cells and macrophages, and interactions between AECs and these cell populations shape the responses in airways to infection and allergen challenge (26). The cytokine TSLP, which is expressed in the lung primarily by AECs, has been shown to be necessary and sufficient for the development of airway inflammation in mice (12). In addition, elevated levels of TSLP mRNA were found in the airway epithelium from asthmatics (11). Despite its importance in allergic inflammatory responses, the factors that control TSLP gene expression are poorly understood.

In this study, we demonstrate that the inflammatory mediators IL-1 β and TNF- α can induce TSLP expression in human AECs via an NF κ B-mediated pathway. NHBE cells have a low basal expression of TSLP, which can be elevated after exposure to IL-1 β or TNF- α . This increase in expression was seen at both the mRNA and protein levels, which suggests that a transcriptional mechanism was responsible. This was confirmed through analyses mapping the sequences in the TSLP promoter involved in IL-1 β and TNF- α -mediated induction, using the human lung epithelial cell lines A549 and 16HBE $^{-}$. Although we cannot rule out the presence of additional regulatory elements further upstream, these studies revealed a single NF κ B site 3.7 kb upstream of the start of transcription that was required for inducible expression of the reporter. Several lines of evidence support the importance of this site, and of NF κ B, in TSLP gene regulation. First, mutation of this site in the TSLP promoter-reporter construct eliminated IL-1 β -mediated induction. Second, EMSA experiments showed that this site was capable of binding NF κ B in nuclear extracts from IL-1 β -treated cells, and that the complex consisted of p50/p65 NF κ B. Third, overexpression of p65 in A549 cells resulted in increases in basal TSLP expression and IL- β -induced expression. Finally, expression of a dominant-negative version of IKK β resulted in an inhibition of IL-1 β -mediated induction of TSLP gene expression. These data demonstrate that activation of NF κ B and subsequent binding to the -3.7-kb element is critical for induction of TSLP gene expression by IL-1 β and TNF- α .

We have found that TSLP expression is induced by inflammatory stimuli, including the cytokines IL-1 β and TNF- α and ligands for TLR2, TLR8, and TLR9 (27). A common element in the signaling pathways from each of these stimuli is the activation of NF κ B. However, given the broad nature of NF κ B activation, it seems likely that other factors are involved in regulating TSLP expression. In fact, Li *et al.* (22) have shown that mice with targeted deletion of RXR α and RXR β in the epidermis develop a skin inflammatory disease similar to atopic dermatitis. This disease development is accompanied by increased TSLP expression in the epidermis, suggesting that RXRs are involved in repressing transcription of the TSLP gene. Consistent with this, they found two nuclear receptor binding sites in the mouse TSLP gene promoter (22). RXRs function as binding partners for a wide variety of nuclear receptors, including members of the retinoic acid receptor family (28, 29) and the vitamin D receptor (30) and can act as either transcriptional activators or repressors, depending on the binding partner and the presence or absence of ligands (31–33). Recently, this same group showed that topical administration of 1 α ,25-(OH) $_2$ D $_3$, the active form of vitamin D $_3$, led to induction of TSLP expression (34). Li *et al.* (34) interpreted this finding to suggest that an RXR/vitamin D receptor heterodimer binds to the TSLP promoter, and that in the absence of ligand, it represses transcription; however, in the presence of ligand, it activates expression. However, our data argue against this interpretation. As shown in Fig. 7*d*, deletion of the putative RXR binding sites in the mouse TSLP promoter

did not affect activity of a reporter containing the mouse TSLP gene promoter. In addition, we have found that treatment of 16HBE $^{-}$ cells with IL-1 β and 9-*cis*-retinoic acid (a ligand for RXRs) inhibited both IL-1 β -induced and basal expression of TSLP (data not shown), suggesting that RXRs, when bound to ligand, may play a role in repressing TSLP gene transcription, possibly through direct effects on NF κ B activation.

In summary, we have shown that TSLP gene expression is induced in both human and mouse AECs after exposure to proinflammatory cytokines IL-1 β and TNF- α and TLR2, TLR8, and TLR9 ligands. The effect of these cytokines and inflammatory stimuli in both species is mediated by NF κ B. Thus, TSLP gene regulation is similar in human and mouse, which suggests a common function for TSLP in the initiation and/or maintenance of T helper 2-type inflammatory responses.

Materials and Methods

Reagents. Antibodies to normal rabbit IgG, NF κ B p65 (sc-109X) and NF κ B p50 (sc-114X) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant human IL-1 β and TNF- α were purchased from R&D Systems (Minneapolis, MN). LPS from *Salmonella enterica* Re595 and lipoteichoic acid from *Staphylococcus aureus* were purchased from Sigma-Aldrich (St. Louis, MO). Poly(I-C) was purchased from Invitrogen (Carlsbad, CA). Poly(G10) (G*GGGG*G*G*G*G*G) and CpG-B (T*C*G*T*C*G*T*T*T*G*T*C*G*T*T*T*G*T*C*G*T*T*T) were synthesized by Operon Biotechnologies (Huntsville, AL). Asterisks denote phosphorothioate linkage.

Cell Lines. NHBE were purchased from Clonetics (Walkersville, MD). The human bronchial epithelial cell line 16HBE $^{-}$ was kindly provided by Dieter C. Gruenert (California Pacific Medical Research Institute, San Francisco, CA). These cells were grown in bronchial/tracheal epithelial-cell basal medium (Cambrex, East Rutherford, NJ), to which bovine pituitary extract (13 μ g/ml), hydrocortisone (0.5 μ g/ml), human recombinant epidermal growth factor (0.5 μ g/ml), epinephrine (0.5 μ g/ml), transferrin (10 μ g/ml), insulin (5 μ g/ml), retinoic acid (0.1 μ g/ml), triiodothyronine (6.5 μ g/ml), gentamicin (50 μ g/ml), and amphotericin B (50 μ g/ml) were added. The human lung epithelial cell line A549 was purchased from ATCC (Manassas, VA) and maintained in MEM supplemented with 10% FBS, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. MLE12 cells were grown in Hite's medium supplemented with 2% FBS, 100 units/ml penicillin, and 0.1 mg/ml streptomycin.

Plasmid Construction. Original mouse and human promoter fragments were isolated by PCR, using primers generated from genomic sequences of the respective genes. Promoter length was determined from the translational start site (data not shown). All constructs that contain deletions of the human TSLP promoter were subsequently amplified from this cloned genomic DNA, using Pfx DNA polymerase (Invitrogen), MluI-ended primers, and NheI-ended antisense primers. Amplified fragments were then cloned into the pGL2 or pGL3-basic vector (Promega, Madison, WI). A series of internal deletion fragments were generated by digestion with EcoRI (base pairs -2998 and -136) and then cloned into pGL3-promoter vector. Mutant constructs were performed by using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The luciferase reporter construct that contained the human or mouse TSLP promoter was used as the DNA template. The mutations in the binding sites for human NF κ B (GGGAACTCCA changed to Gt-tCAACTCCA), human AP-1 (TGTCATTG changed to TGTt-gTTG), human IRF-1 (AAAGAGACAG changed to ggctAGA-CAG), human Opaque-2 (CCATCTCATC changed to CCaTCTtGTC), mouse NF κ B 1 (GAAAATTACC changed to GAAAATTgaa), and mouse NF κ B 2 (AGGAATTTCC

changed to AttcATTTC) were verified by sequence analysis. Two putative sequences that resembled RXR binding sites (GGGTCAGGGGACA and AGCTCAACAGGTCA) were deleted in the mouse TSLP promoter to generate RXR-del1 and RXR-del2, respectively. To generate the human NF κ B expression vector, cDNA encoding NF κ B was amplified by PCR and cloned into pcDNA3.1 expression vector (Invitrogen). Dominant-negative I κ B was kindly provided by Christopher B. Wilson (University of Washington School of Medicine).

Transfection and Luciferase Assay. Cells (3×10^5) were plated in 35-mm or 60-mm dishes and transfected with 1 μ g of luciferase reporter plasmid driven by wild-type or mutated TSLP promoter and 1 μ g of a β -galactosidase plasmid driven by the Rous sarcoma virus long-terminal repeat promoter (pRSV- β -Gal) using Mirus transfection reagent (Mirus, Madison, WI) according to the manufacturer's protocol. After transfection, cells were cultured for 19 h, then stimulated for 5 h with cytokines IL-1 β or TNF- α . In the experiment using cDNA expression plasmids, a mixture containing 1 μ g of luciferase plasmid, 0.2 μ g of cDNA expression plasmid, and 1 μ g of pRSV- β -Gal plasmid was used to transfect the cells, which were then cultured for 24 h. The total amount of DNA was kept constant with control plasmid. Cells were harvested and lysed in 100 μ l of lysis buffer (Promega). Luciferase activity was measured by using a Lumat LB9507 luminometer (Berthold, Bad Wildbad, Germany). Relative luciferase activity was given as the ratio of relative light units to relative β -galactosidase units. In each experiment, samples were analyzed in triplicate, and each experiment was repeated at least twice.

Real-Time Quantitative PCR. Total RNA was prepared with an RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. PCR amplification was performed on ABI 7700 Sequence Detector (Applied Biosystems, Foster City, CA). For

each sample, the PCR was performed in duplicate with the Platinum SYBR Green qPCR Super Mix-UDG with ROX (Invitrogen). Melting curves of the amplified products were determined to identify the amplicon. Threshold cycle numbers were analyzed by using Sequence Detection System, version 2.2 (Applied Biosystems) and transformed by using the Δ Ct or $\Delta\Delta$ Ct method as described by the manufacturer. The level of human TSLP mRNA was normalized with that of GAPDH (data not shown).

EMSA. Nuclear extract was prepared as described in ref. 35. Nuclear extract (5 μ g) was incubated for 20 min at room temperature with 2 ng of labeled oligonucleotide probe in 10 μ l of binding buffer [20 mM Tris-HCl (pH 7.5)/0.1 mM EDTA/4 mM DTT/40 mM NaCl/0.05% BSA/10% glycerol/0.125% Nonidet P-40] containing 1 μ g of poly(dI-dC). The reaction mixtures were applied to 4.5% gel (19:1 acrylamide/bisacrylamide) in $0.5\times$ TBE buffer at 4°C. The gels were dried and exposed to Kodak BioMax MS film (Kodak, Tokyo, Japan). For competition assay, the reaction was preincubated with 12.5- to 50-fold molar excess of unlabeled oligonucleotide for 5 min before the addition of labeled oligonucleotide probe. For supershift experiments, the nuclear extract was preincubated with 1 μ g of normal rabbit IgG antibody (Santa Cruz Biotechnology) or NF κ B p65 (sc-109X) or NF κ B p50 (sc-114X) for 1 h on ice. The sequences of double-stranded oligonucleotides used as probes were as follows: NF κ B consensus motif, 5'-AGAGATCTGTACAGGATGT-TCTAGAT-3'; hTSLP NF κ B motif, 5'-CTGCTAGGGA-AACTCCATTATTAC-3'; hTSLP NF κ B mutated motif, 5'-CTGCTAAATAAACTCCATTATTAC-3'. Consensus nucleotides are underlined. Mutated nucleotides are in boldface.

We thank Theingi Aye for technical assistance and Drs. Daniel Campbell, Baohua Zhou, and Jane H. Buckner for critical discussion of manuscript. This work was partially supported by National Institutes of Health Grants AI44259 and AI068731 (to S.F.Z.).

- Friend SL, Hosier S, Nelson A, Foxworth D, Williams DE, Farr A (1994) *Exp Hematol* 22:321–328.
- Ray RJ, Furlonger C, Williams DE, Paige CJ (1996) *Eur J Immunol* 26:10–16.
- Levin SD, Koelling RM, Friend SL, Isaksen DE, Ziegler SF, Perlmutter RM, Farr AG (1999) *J Immunol* 162:677–683.
- Sims JE, Williams DE, Morrissey PJ, Garka K, Foxworth D, Price V, Friend SL, Farr A, Bedell MA, Jenkins NA, et al. (2000) *J Exp Med* 192:671–680.
- Pandey A, Ozaki K, Baumann H, Levin SD, Puel A, Farr AG, Ziegler SF, Leonard WJ, Lodish HF (2000) *Nat Immunol* 1:59–64.
- Park LS, Martin U, Garka K, Gliniak B, Di Santo JP, Muller W, Largaespada DA, Copeland NG, Jenkins NA, Farr AG, et al. (2000) *J Exp Med* 192:659–670.
- Quentmeier H, Drexler HG, Fleckenstein D, Zaborski M, Armstrong A, Sims JE, Lyman SD (2001) *Leukemia* 15:1286–1292.
- Reche PA, Soumelis V, Gorman DM, Clifford T, Liu M, Travis M, Zurawski SM, Johnston J, Liu YJ, Spits H, et al. (2001) *J Immunol* 167:336–343.
- Ziegler SF, Liu YJ (2006) *Nat Immunol* 7:709–714.
- Soumelis V, Reche PA, Kanzler H, Yuan W, Edward G, Homey B, Gilliet M, Ho S, Antonenko S, Lauerma A, Smith K, et al. (2002) *Nat Immunol* 3:673–680.
- Ying S, O'Connor B, Ratoff J, Meng Q, Mallett K, Cousins D, Robinson D, Zhang G, Zhao J, Lee TH, Corrigan C (2005) *J Immunol* 174:8183–8190.
- Zhou B, Comeau MR, De Smedt T, Liggitt HD, Dahl ME, Lewis DB, Gyarmati D, Aye T, Campbell DJ, Ziegler SF (2005) *Nat Immunol* 6:1047–1053.
- Al-Shami A, Spolski R, Kelly J, Keane-Myers A, Leonard WJ (2005) *J Exp Med* 202:829–839.
- Yoo J, Omori M, Gyarmati D, Zhou B, Aye T, Brewer A, Comeau MR, Campbell DJ, Ziegler SF (2005) *J Exp Med* 202:541–549.
- Karin M, Ben-Neriah Y (2000) *Annu Rev Immunol* 18:621–663.
- Li Q, Verma IM (2002) *Nat Rev Immunol* 2:725–734.
- Baeuerle PA, Baltimore D (1988) *Cell* 53:211–217.
- Baeuerle PA, Baltimore D (1988) *Science* 242:540–546.
- Bonizzi G, Karin M (2004) *Trends Immunol* 25:280–288.
- Akira S, Takeda K (2004) *Nat Rev Immunol* 4:499–511.
- Wikenheiser KA, Vorbroke DK, Rice WR, Clark JC, Bachurski CJ, Oie HK, Whitsett JA (1993) *Proc Natl Acad Sci USA* 90:11029–11033.
- Li M, Messaddeq N, Teletin M, Pasquali JL, Metzger D, Chambon P (2005) *Proc Natl Acad Sci USA* 102:14795–14800.
- Kuperman DA, Huang X, Koth LL, Chang GH, Dolganov GM, Zhu Z, Elias JA, Sheppard D, Erle DJ (2002) *Nat Med* 8:885–889.
- Grunig G, Warnock M, Wakil AE, Venkayya R, Brombacher F, Rennick DM, Sheppard D, Mohrs M, Donaldson DD, Locksley RM, Corry DB (1998) *Science* 282:2261–2263.
- Brusselle G, Kips J, Joos G, Bluethmann H, Pauwels R (1995) *Am J Respir Cell Mol Biol* 12:254–259.
- Lambrecht BN (2001) *Clin Exp Allergy* 31:206–218.
- Medzhitov R (2001) *Nat Rev Immunol* 1:135–145.
- Egea PF, Rochel N, Birck C, Vachette P, Timmins PA, Moras D (2001) *J Mol Biol* 307:557–576.
- Gampe RT, Jr, Montana VG, Lambert MH, Wisely GB, Milburn MV, Xu HE (2000) *Genes Dev* 14:2229–2241.
- Kliwer SA, Umesono K, Mangelsdorf DJ, Evans RM (1992) *Nature* 355:446–449.
- Zelcer N, Tontonoz P (2006) *J Clin Invest* 116:607–614.
- Szanto A, Narkar V, Shen Q, Uray IP, Davies PJ, Nagy L (2004) *Cell Death Differ* 11(Suppl 2):S126–S143.
- Glass CK, Ogawa S (2006) *Nat Rev Immunol* 6:44–55.
- Li M, Hener P, Zhang Z, Kato S, Metzger D, Chambon P (2006) *Proc Natl Acad Sci USA* 103:11736–11741.
- Lee HC, Shibata H, Ogawa S, Maki K, Ikuta K (2005) *J Immunol* 174:7800–7806.