Letter to the Editor

IgE induces transcriptional regulation of thymic stromal lymphopoietin in human airway smooth muscle cells

To the Editor:

Human airway smooth muscle cells (ASMCs) are considered "inflammatory-like cells" by virtue of expressing cell adhesion, costimulatory molecules, and secretion of cytokines and chemokines that may initiate or perpetuate the airway inflammation and the development of airway remodeling.[1] Recent data suggest that ASMCs are capable of producing and responding to the proallergic thymic stromal lymphopoietin (TSLP) in asthma and chronic obstructive pulmonary disease.[2,3] In this report, we demonstrate that IgE induces TSLP in ASMCs via a transcriptional mechanism. These effects are dependent on spleen tyrosine kinase (Syk), nuclear factor-κB (NF-κB), and activator protein 1 (AP-1) transcription factors.

TSLP has emerged as a key player in the pathogenesis of allergic diseases such as asthma, atopic dermatitis, and allergic rhinitis (see reference E9 in this article's Online Repository at www.jacionline.org). It activates the myeloid dendritic cells to induce proallergic $CD4^+$ and $CD8^+$ T-cell responses and activates the mast cells to facilitate their cross talk with ASMCs.[4] In human ASMCs, IL-1β, TNF-α, and cigarette smoke extract can induce elevated levels of TSLP.[2-4] Interestingly, β_2 agonists enhanced the cytokine-induced TSLP in ASMCs, an effect that was abolished by the concurrent use of glucocorticoids, suggesting a novel mechanism of adverse effects associated with the prolonged use of β_2 agonists in the absence of glucocorticoids in asthma (see reference E10 in this article's Online Repository at www.jacionline.org). Traditionally, bronchial hyperresponsiveness has been shown to be associated with serum IgE levels and to be transferable from asthmatic to nonasthmatic subjects by IgE-rich serum. Indeed, IgE-rich serum alters the contractile and synthetic function of ASM. We and others have demonstrated the expression of the high-affinity IgE receptor (FcεRI) in human ASMCs *in vitro* and *in vivo*.[5,6] FcεRI activation on ASM induced rapid and transient intracellular Ca^{2+} mobilization, T_H2 (IL-4, IL-5, and IL-13) cytokines, and chemokine (eotaxin-1/CCL11) release.[5]

In the current report, we found that the IgE stimulation (10 μg/mL) of cultured primary human ASMCs induced a significant increase in TSLP transcript at 12 hours ($P = .017$, n = 4; Fig 1, *A*) and also enhanced the TSLP protein release at 24 hours ($P = .014$; Fig 1, *B*) (see the Methods section in this article's Online Repository at www.jacionline.org). In corroboration with earlier reports,[3,4] TNF-α induced a stronger and sustained TSLP expression in ASMCs. Interestingly, IgE activated the human TSLP gene promoter and induced a 2.07 ± 0.02-fold increase in luciferase reporter activity ($P < .001$, n = 4; Fig 1, *C*). In inflammatory cells, Syk activation is an indispensable upstream signaling component known to facilitate the FcεRI activation–induced signaling events and transcription factors' activation. To test this possibility in ASM FcεRI activation, we employed a novel pharmacological Syk inhibitor R112, which has been shown to completely abrogate the mast cell degranulation and mediator release following FcεRI cross-linking (see references E5 and E6 in this article's Online Repository at www.jacionline.org). Incubation of ASM cells

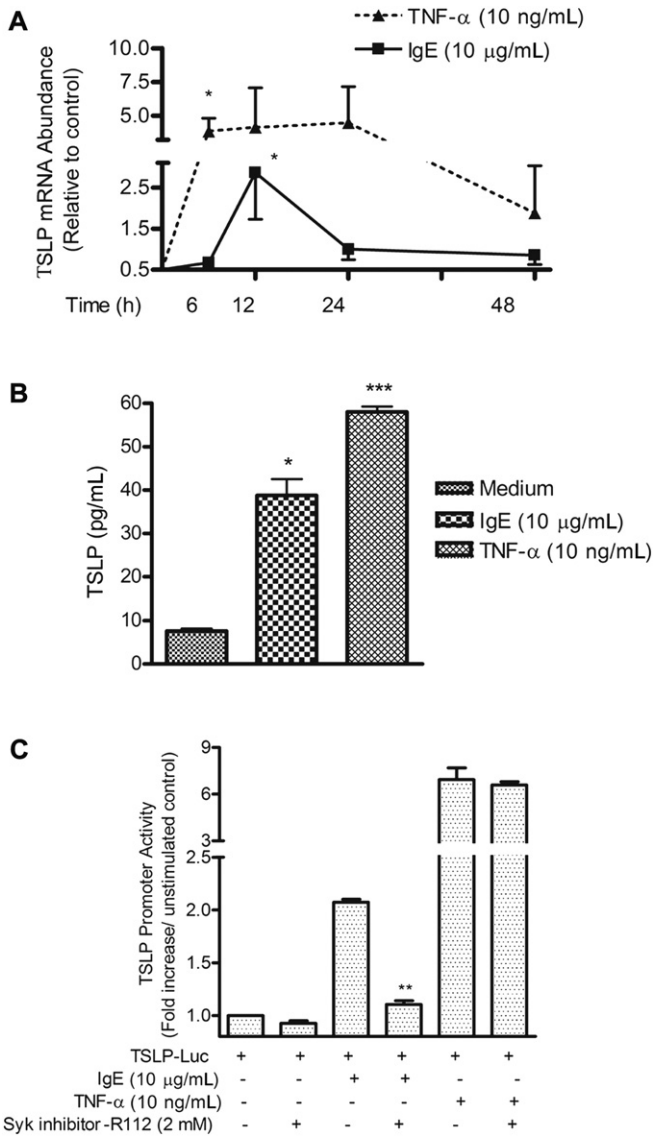

FIG 1. TSLP mRNA **(A)** and protein expression **(B)** were measured from ASM cells stimulated with IgE or TNF-α by using real-time RT-PCR and ELISA, respectively. **C,** ASM cells were transiently transfected with human TSLP promoter luciferase construct and stimulated with IgE or TNF-α in the presence of a Syk inhibitor, R112, or vehicle (dimethyl sulfoxide). TSLP promoter activity was measured. n = 4, $*P < .05$, $**P < .01$, $***P < .001$ compared with the unstimulated (Fig 1, *A,* Mann-Whitney test; Fig 1, *B,* unpaired *t* test) or stimulated (Fig 1, *C,* unpaired *t* test) control.

with R112 prior to IgE stimulation abrogated the IgE induction of TSLP promoter activity (n = 3, $P = .002$, Fig 1, *C*). However, R112 did not affect the constitutive or TNF-α–induced TSLP promoter activity in ASMCs. We then knocked down the Syk expression in ASMCs by using lentiviral short hairpin RNA (shRNA) strategy (Fig 2). As revealed by fluorescence-activated cell sorting analysis, more than 95% of the lentivirus-transduced cells were turbo-green fluorescent protein positive (Fig 2, *A*). Transduction of cells with Syk-shRNA clones resulted in a significant decrease in Syk expression (Fig 2, *B*). We then tested the effect

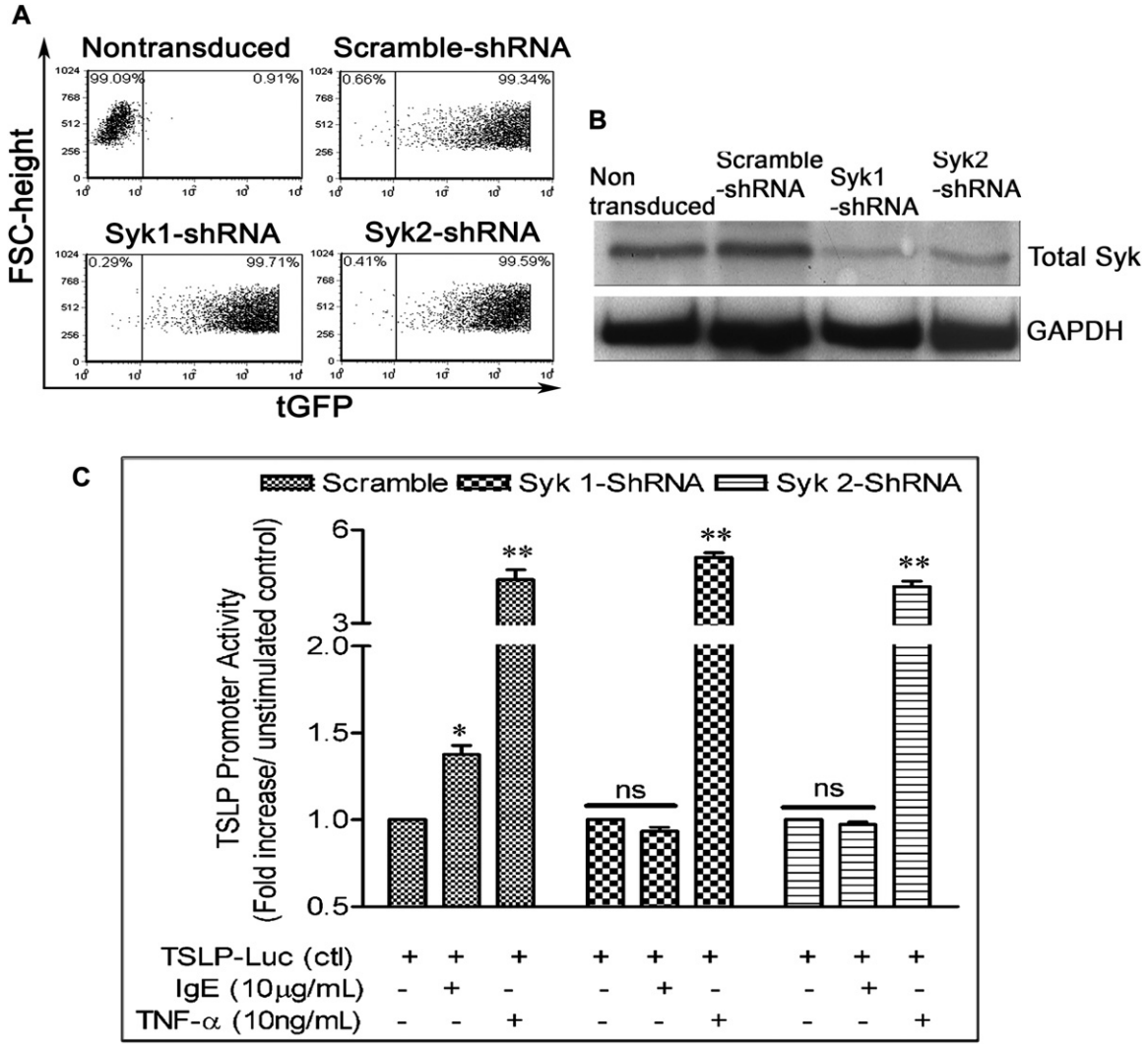

FIG 2. **A,** Lentiviral transduction efficiency in ASM for the Syk-shRNAs as determined by FACS by using turbo-green fluorescent protein as a marker. **B,** Lentiviral Syk-shRNA transduction silenced the ASM Syk expression efficiently as observed by Western blotting. **C,** Syk-silenced and scramble ASM cells were transfected with a TSLP promoter construct, stimulated with IgE or TNF-α for 12 hours, and TSLP promoter activity was measured. n = 3, $*P < .05$, $**P < .01$ (Fig 2, *C*, unpaired *t* test) compared with the unstimulated control.

of IgE or TNF-α on ASMCs transiently transfected with luciferase-encoding TSLP promoter constructs. As shown in Fig 2, *C*, IgE-induced TSLP promoter activity was abrogated in Syk-shRNA–transduced cells but was unaffected in scramble-shRNA–transduced cells ($P = .019$). In contrast, the TNF-α induction of TSLP promoter activity was unaffected in lentivirus-mediated Syk-silenced ASMCs (Fig 2, *C*). Collectively, consistent with our pharmacological approach, these results reveal an essential role for Syk in IgE-induced TSLP gene promoter activation in ASMCs (Fig 1, *B*, and Fig 2, *C*).

To identify the promoter region(s) involved in transcriptional activation by IgE, we employed reporter plasmids containing TSLP promoter with mutations in NF-κB or AP-1–like transcription factors. Transient transfection of these constructs revealed a baseline reporter activity, confirming the constitutive expression of TSLP in ASMCs. Compared with wild-type (WT) promoter constructs, IgE or TNF-α exposure resulted in a markedly lower activation of NF-κB (M1) or AP-1 (M2) mutant luciferase reporter constructs (n = 3, $P = .014$), suggesting an important role for these transcription factors (Fig 3, *A*). An essential step in the canonical pathway of NF-κB activation is the phosphorylation of the inhibitor of NF-κB (IκB) proteins by IκB kinases (IKKs), followed by the migration of NF-κB dimers from cytoplasm to nucleus. To determine the role of this pathway in the current study, we investigated the effect of overexpressing WT NF-κB/p65 or dominant-negative mutant of IKKβ (referred to as dnIKKβ) in ASM cells (see references E3 and E4 and the Methods section in this article's Online Repository at www.jacionline.org). Interestingly, overexpression of the proactive NF-κB/p65 subunit enhanced the basal transcriptional activity of the TSLP promoter (Fig 3, *B*). Moreover, both IgE ($P = .045$) and TNF-α ($P = .021$) stimulation further augmented the TSLP promoter activity that was slightly improved upon by WT NF-κB/p65 overexpression (Fig. 3, *B*). Importantly, Fig 3, *C*, demonstrates a dose-dependent inhibition of the IgE– or TNF-α–mediated TSLP promoter activity

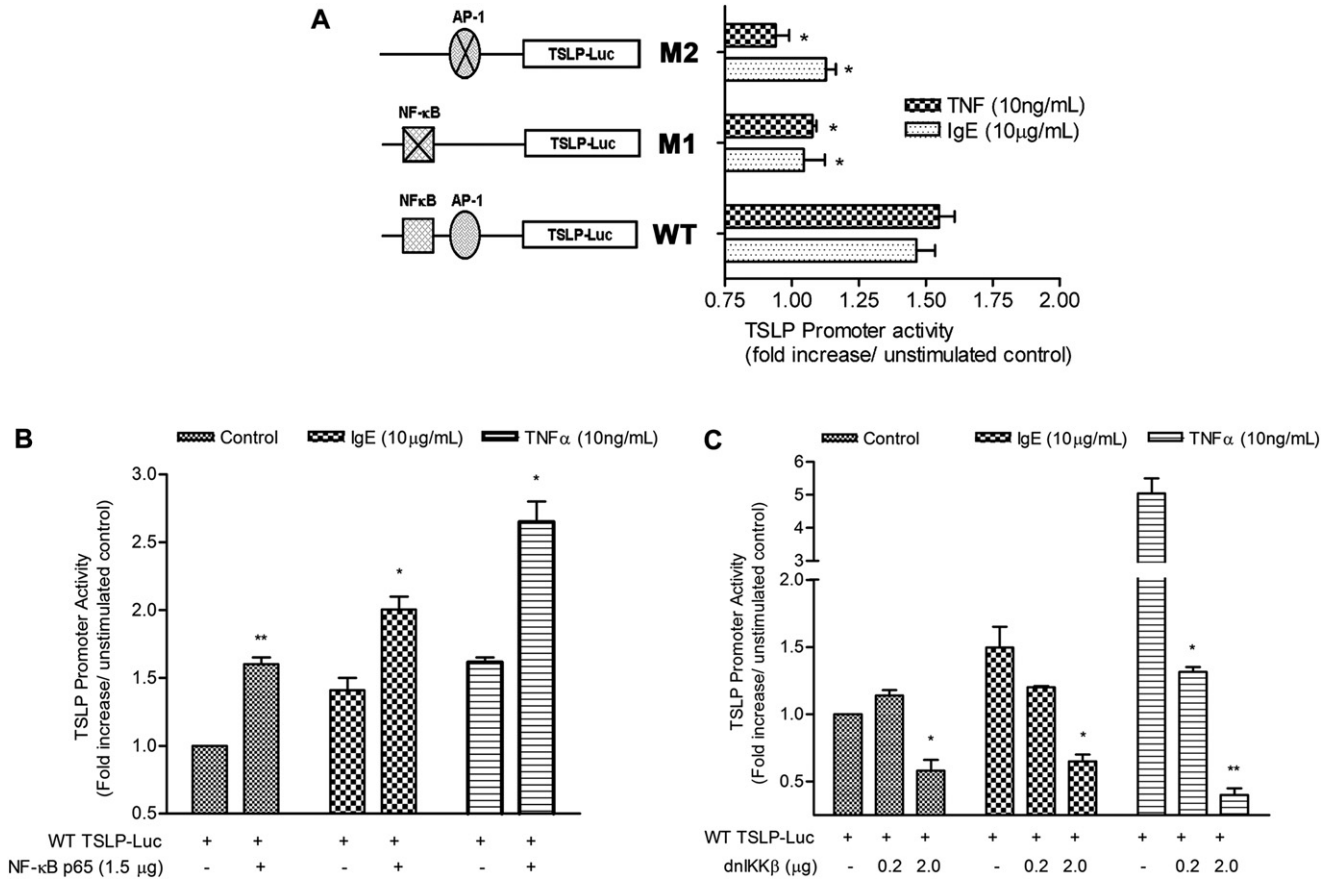

FIG 3. Primary ASM cells were transfected with **(A)** mutant constructs of NF-κB (M1), AP-1 (M2), or WT TSLP promoter (WT); **(B)** WT TSLP promoter construct and NF-κB/p65 subunit overexpressing plasmid; and **(C)** WT TSLP promoter and dnIKKβ. Cells were stimulated with IgE or TNF-α for 12 hours, and luciferase reporter activity was measured. n = 3, $*P < .05$, $**P < .01$ (Fig 3, *A*, Mann-Whitney test; Fig 3, *B* and *C*, unpaired *t* test) compared with the stimulated WT control.

following expression of the dnIKKβ subunit. Collectively, these results suggest that the IgE– or TNF-α–induced TSLP expression in ASM is mediated by NF-κB and AP-1 transcription factors' activation (Fig 3).

Classically, IgE binding to FcεRI in inflammatory cells has been considered a "passive sensitization" step and requires multivalent antigens to promote cross-linking of FcεRI-bound IgE. However, in the last decade, IgE sensitization alone has been shown to confer synthetic and prosurvival effects on mast cells, monocytes, and neutrophils from asthmatic subjects (see references E11-13 in this article's Online Repository at www.jacionline.org). Along the same line, we have recently reported that FcεRI expression in ASM is regulated by proinflammatory and T_H-2 cytokines and IgE sensitization induces the expression of CC (CCL11, CCL5) and CXC (CXCL8, CXCL10) chemokines.[7] In agreement with our data, IgE-induced *de novo* synthesis of IL-4, IL-6, TNF-α, and IL-8/CXCL8 in ASM was shown to be abolished by the therapeutic anti-IgE mAb Omalizumab/Xolair without affecting FcεRI expression.[6]

In vivo, the local IgE expression has been demonstrated in the airways of atopic subjects,[8] which could modulate the ASM synthetic function leading to TSLP expression without allergen/antigen cross-linking. This ongoing TSLP expression via the IgE/FcεRI pathway may in fact explain the "atopic" or "proallergic" state of ASM in allergic asthma that has been linked to the development of airway hyperresponsiveness in large epidemiological studies, particularly the Tucson Children's Respiratory Study.[9] In fact, early antigen sensitization of ASM without subsequent airway challenge or resensitization may confer a distinctive and persistent hypercontractile phenotype that can arise neonatally and persist into adult life.

We thank Dr Aaron J. Marshall for critical review of the manuscript and Dr Sam Kung Laboratory members for assistance with the lentivirus experiments. We gratefully acknowledge the Department of Chemistry of Rigel Pharmaceuticals, Inc, for kindly providing the Syk inhibitor R112. The help of Dr Rasheda Rabbani, Biostatistician at the Manitoba Institute of Child Health, is acknowledged.

Naresh Singh Redhu, MSc[a]*
Ali Saleh, PhD[a]*‡
Hai-Chon Lee, PhD[c]‡
Andrew J. Halayko, PhD[b]
Steven F. Ziegler, PhD[c]
Abdelilah S. Gounni, PhD[a]

From [a]the Departments of Immunology and [b]Physiology, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada, and [c]the Immunology Program, Benaroya Research Institute, Seattle, Wash. E-mail: gounni@cc.umanitoba.ca.

*These authors contributed equally to this work.

‡Dr Saleh is now with St. Boniface General Hospital Research Centre, Winnipeg, Manitoba, Canada, and Dr Lee is now with Carter Immunology Center, University of Virginia, Charlottesville, Va.

A.S.G. is supported by funds from the Canadian Institutes of Health Research and the Manitoba Research Chairs program. N.S.R. is supported by studentships from the Canadian Thoracic Society–Canada Lung Association. A.J.H. is supported by funds from the Canada Research Chairs Program.

Disclosure of potential conflict of interest: A.S. Gounni receives research support from the Canadian Institutes of Health Research and the Manitoba Health Research Council. A.J. Halayko receives research support from the Canadian Institutes of Health Research and the Canada Research Chairs Program. S. Ziegler is a stockholder in Amgen Corporation. The rest of the authors have declared that they have no conflict of interest.

REFERENCES

1. Tliba O, Panettieri RA Jr. Noncontractile functions of airway smooth muscle cells in asthma. Annu Rev Physiol 2009;71:509-35.
2. Shan L, Redhu NS, Saleh A, Halayko AJ, Chakir J, Gounni AS. 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. J Immunol 2010;184:7134-43.
3. Zhang K, Shan L, Rahman MS, Unruh H, Halayko AJ, Gounni AS. Constitutive and inducible thymic stromal lymphopoietin expression in human airway smooth muscle cells: role in chronic obstructive pulmonary disease. Am J Physiol Lung Cell Mol Physiol 2007;293:L375-82.
4. Allakhverdi Z, Comeau MR, Jessup HK, Delespesse G. Thymic stromal lymphopoietin as a mediator of crosstalk between bronchial smooth muscles and mast cells. J Allergy Clin Immunol 2009;123:958-960.e2.
5. Gounni AS, Wellemans V, Yang J, Bellesort F, Kassiri K, Gangloff S, et al. Human airway smooth muscle cells express the high affinity receptor for IgE (Fc epsilon RI): a critical role of Fc epsilon RI in human airway smooth muscle cell function. J Immunol 2005;175:2613-21.
6. Roth M, Tamm M. The effects of omalizumab on IgE-induced cytokine synthesis by asthmatic airway smooth muscle cells. Ann Allergy Asthma Immunol 2010;104:152-60.
7. Redhu NS, Saleh A, Shan L, Gerthoffer WT, Kung SK, Halayko AJ, et al. Proinflammatory and Th2 cytokines regulate the high affinity IgE receptor (FcepsilonRI) and IgE-dependant activation of human airway smooth muscle cells. PLoS One 2009;4:e6153.
8. Gould HJ, Sutton BJ, Beavil AJ, Beavil RL, McCloskey N, Coker HA, et al. The biology of IGE and the basis of allergic disease. Annu Rev Immunol 2003;21:579-628.
9. Taussig LM, Wright AL, Holberg CJ, Halonen M, Morgan WJ, Martinez FD. Tucson Children's Respiratory Study: 1980 to present. J Allergy Clin Immunol 2003; 111:661-75; quiz 76.

doi:10.1016/j.jaci.2011.06.045

METHODS

Reagents

Recombinant human TNF-α was purchased from R&D Systems (Minneapolis, Minn). Human IgE was purchased from Diatec (Norway, Sweden). FBS was purchased from HyClone Laboratories (Logan, Utah). Dulbecco modified Eagle medium (DMEM), Ham's F12, trypsin-EDTA, and antibiotics (penicillin, streptomycin) were purchased from Invitrogen Life Technologies (Grand Island, NY). All other reagents were procured from Sigma-Aldrich Canada Ltd (Oakville, Ontario) unless specified otherwise.

Preparation of human airway smooth muscle cells

Bronchial human ASM cells were obtained from macroscopically healthy segments of 2nd- to 4th-generation lobar or main bronchus of patients undergoing surgery for lung carcinoma in accordance with the procedures approved by the Ethics Committee of the University of Manitoba, Winnipeg, Manitoba, Canada. Primary human ASMCs were isolated from explants as described previously.[E1,E2] Cells were cultured in DMEM supplemented with 10% FBS, L-glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37°C with 5% CO_2.

Cell stimulation

ASMCs (passage 2-5) were grown for 48 hours 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 human IgE (10 μg/mL), TNF-α (10 ng/mL), or medium alone.

Real-time RT-PCR analysis

ASMCs were harvested by using the TRIzol method (Invitrogen Canada, Inc, Burlington, Ontario, Canada) for total cellular RNA extraction and TSLP mRNA expression analysis by real-time RT-PCR, as described previously.[E2] Briefly, oligonucleotide primers were synthesized on the basis of the entire coding region of the human TSLP gene (GenBank accession no. NM_033035): forward primer, 5′-TATGAGTGGGACCAAAAGTACCG-3′; and reverse primer, 5′-GGGATTGAAGGTTAGGCTCTGG-3′. Primers for the housekeeping gene glyceraldhyde-3-phosphate dehydrogenase (GAPDH) are forward primer 5′-AGCAATGCCTCCTGCACCACCAAC-3′ and reverse primer 5′-CCGGA GGGGCCATCCACAGTCT-3′. Real-time relative quantitative PCR for TSLP and *GAPDH* was performed by using ABI 7500 Real-Time PCR System and analyzed by using 7500 System SDS software version 1.3.1 (Applied Biosystems, Foster City, Calif) according to the manufacturer's instructions. Product specificity was determined by melting curve analysis and by visualization of PCR products on agarose gels. The amplification of the *TSLP* gene in stimulated cells was calculated first as the ratio of *TSLP* per copy of *GAPDH* and then expressed as normalized values of fold increase over the value obtained with unstimulated control cells.

TSLP ELISA

Supernatants were collected from human ASM cells 24 and 48 hours after stimulation with IgE, TNF-α, or medium alone, followed by centrifugation at 1200 rpm for 7 minutes at 4°C to remove cellular debris, and stored at –80°C until analysis by using ELISA. Immunoreactive TSLP within the supernatants was quantified by using ELISA as described previously.[E2] 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.

Luciferase reporter constructs and cell transfection

The plasmid encoding WT human TSLP promoter and all other constructs containing mutations in the human TSLP promoter were used as described earlier.[E3,E4] The mutations in the binding sites for human NF-κB (GUGGAUAACTCCA changed to GUTTCUAACTCCA) and human AP-1 (TGTUCAUTTG changed to TGTUTGUTTG) were verified by sequence analysis (data not shown). ASMCs (4×10^4) were plated into 12-well culture plates in fresh complete DMEM. At 50% to 70% confluency, cells were transfected with WT human TSLP promoter construct or different mutations of the TSLP promoter. Transient transfection of ASM cells was performed by using ExGen 500 *in vitro* transfection reagent (MBI Fermentas, Burlington, Ontario, Canada) according to the manufacturer's instructions. In each well, 1.6 μg of WT TSLP promoter DNA and 0.4 μg of *Renilla* luciferase reporter vector-pRL-TK (Promega) were cotransfected for 24 hours. The medium was changed, and cells were washed and stimulated with human IgE (10 μg/mL) or TNF-α (10 ng/mL). Since TNF-α (10 ng/mL) has been shown to induce TSLP expression in ASM cells,[E2,E4] it was used as a positive control throughout our experiments. After 12 hours of cytokine stimulation, cells were washed twice with PBS and cell lysates were collected with 100 μL of reporter lysis buffer (Promega, Madison, Wis). In some experiments, cells were pretreated for 40 minutes with R112 (2 μmol/L), an ATP-competitive Syk inhibitor (inhibitory constant [K(i)] = 96 nmol/L) (provided by the Department of Chemistry of Rigel Pharmaceuticals, Inc),[E5] or with dimethyl sulfoxide vehicle before stimulation with IgE (10 μg/mL) for 12 hours. We also used another related Syk inhibitor R406, which is more potent than R112 and has shown efficacy in animal models of allergic asthma[E6] (data not shown). The luciferase activity was measured by using the Dual-Luciferase Assay System kit (Promega, Madison, Wis) with a luminometer (model LB9501, Berthold, Bad Wildbad, Germany). Briefly, 20 μL of cell lysate was mixed with 100 μL of Luciferase Assay Reagent II and firefly luciferase activity was first recorded. Then, 100 μL of Stop-and-Glo reagent was added, and *Renilla* luciferase activity was measured. All values were normalized to *Renilla* luciferase activity and expressed relative to the transfected nonstimulated cells.

Lentiviral vector transduction in ASM cells

For shRNA studies, 2 pseudotyped lentiviral vectors (clone IDs: V2LHS_2250 and V2LHS_153702) expressing specific Syk shRNA were obtained from Open-Biosystems (Huntsville, Ala). In these vectors, microRNA-adapted shRNA (shRNAmir or shRNA) are cloned into pGIPZ lentiviral vector to create a powerful RNAi trigger capable of producing RNAi in most cell types including primary and nondividing cells. Lentiviruses were generated by using 293T cell lines, and viral titer was determined by counting the puromycin- resistant colonies, as described elsewhere.[E7,E8] A control shRNA unrelated to Syk sequences (scramble-shRNA) was used as a negative control. For silencing Syk protein expression, ASMCs 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 hours at 37°C and the medium was replaced and cultured for an additional 72 hours. Transduced cells were selected with puromycin. The average transduction efficiency was more than 95% as determined by FACS using the turbo-green fluorescent protein as the marker for cell sorting. Syk-shRNA–transduced and scrambled-shRNA–transduced ASMCs were then used for TSLP promoter activity measurement, as mentioned above.

Statistical analysis

Data were obtained from experiments performed in duplicate and repeated at least 3 times, and results are expressed as mean ± SEM. Statistical analysis was performed by using the professional software GraphPad Prism 4.00. Statistical significance was determined by using Mann-Whitney *U* test and unpaired *t* test, and *P* values of <.05 were considered statistically significant.

REFERENCES

E1. Hamann KJ, Vieira JE, Halayko AJ, Dorscheid D, White SR, Forsythe SM, et al. Fas cross-linking induces apoptosis in human airway smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 2000;278:L618-24.

E2. Zhang K, Shan L, Rahman MS, Unruh H, Halayko AJ, Gounni AS. Constitutive and inducible thymic stromal lymphopoietin expression in human airway smooth

muscle cells: role in chronic obstructive pulmonary disease. Am J Physiol Lung Cell Mol Physiol 2007;293:L375-82.

E3. Lee HC, Ziegler SF. Inducible expression of the proallergic cytokine thymic stromal lymphopoietin in airway epithelial cells is controlled by NFkappaB. Proc Natl Acad Sci U S A 2007;104:914-9.

E4. Redhu NS, Saleh A, Halayko AJ, Ali AS, Gounni AS. Essential role of NF-kappaB and AP-1 transcription factors in TNF-alpha-induced TSLP expression in human airway smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 2010;300:L479-85.

E5. Rossi AB, Herlaar E, Braselmann S, Huynh S, Taylor V, Frances R, et al. Identification of the Syk kinase inhibitor R112 by a human mast cell screen. J Allergy Clin Immunol 2006;118:749-55.

E6. Matsubara S, Li G, Takeda K, Loader JE, Pine P, Masuda ES, et al. Inhibition of spleen tyrosine kinase prevents mast cell activation and airway hyperresponsiveness. Am J Respir Crit Care Med 2006;173:56-63.

E7. Kung SK, An DS, Chen IS. A murine leukemia virus (MuLV) long terminal repeat derived from rhesus macaques in the context of a lentivirus vector and MuLV gag sequence results in high-level gene expression in human T lymphocytes. J Virol 2000;74:3668-81.

E8. Redhu NS, Saleh A, Shan L, Gerthoffer WT, Kung SK, Halayko AJ, et al. Proinflammatory and Th2 cytokines regulate the high affinity IgE receptor (FcepsilonRI) and IgE-dependant activation of human airway smooth muscle cells. PLoS One 2009;4:e6153.

E9. Ziegler SF. The role of thymic stromal lymphopoietin (TSLP) in allergic disorders. Curr Opin Immunol 2010;22:795-9.

E10. Smelter DF, Sathish V, Thompson MA, Pabelick CM, Vassallo R, Prakash YS. Thymic stromal lymphopoietin in cigarette smoke-exposed human airway smooth muscle. J Immunol 2010;185:3035-40.

E11. Kalesnikoff J, Huber M, Lam V, Damen JE, Zhang J, Siraganian RP, et al. Monomeric IgE stimulates signaling pathways in mast cells that lead to cytokine production and cell survival. Immunity 2001;14:801-11.

E12. Katoh N, Kraft S, Wessendorf JH, Bieber T. The high-affinity IgE receptor (FcepsilonRI) blocks apoptosis in normal human monocytes. J Clin Invest 2000;105:183-90.

E13. Saffar AS, Alphonse MP, Shan L, Hayglass KT, Simons FE, Gounni AS. IgE modulates neutrophil survival in asthma: role of mitochondrial pathway. J Immunol 2007;178:2535-41.