Published in final edited form as:

J Allergy Clin Immunol. 2012 November ; 130(5): 1187–1196.e5. doi:10.1016/j.jaci.2012.07.031.

Thymic stromal lymphopoietin is induced by respiratory syncytial virus–infected airway epithelial cells and promotes a type 2 response to infection

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Abstract

Background—Respiratory viral infection, including respiratory syncytial virus (RSV) and rhinovirus, has been linked to respiratory disease in pediatric patients, including severe acute bronchiolitis and asthma exacerbation.

Objective—The study examined the role of the epithelial-derived cytokine thymic stromal lymphopoietin (TSLP) in the response to RSV infection.

Methods—Infection of human airway epithelial cells was used to examine TSLP induction after RSV infection. Air–liquid interface cultures from healthy children and children with asthma were also tested for TSLP production after infection. Finally, a mouse model was used to directly test the role of TSLP signaling in the response to RSV infection.

Results—Infection of airway epithelial cells with RSV led to the production of TSLP via activation of an innate signaling pathway that involved retinoic acid induced gene I, interferon promoter-stimulating factor 1, and nuclear factor-κB. Consistent with this observation, airway epithelial cells from asthmatic children produced significantly greater levels of TSLP after RSV infection than cells from healthy children. In mouse models, RSV-induced TSLP expression was found to be critical for the development of immunopathology.

Conclusion—These findings suggest that RSV can use an innate antiviral signaling pathway to drive a potentially nonproductive immune response and has important implications for the role of TSLP in viral immune responses in general.

Keywords

TSLP; RSV; asthma; epithelium; T_H2

Asthma is a chronic airway inflammatory disease characterized by T_H2-type lung immune infiltrate, airway hyperresponsiveness (AHR), goblet cell metaplasia, and mucus hypersecretion. Importantly, early childhood infection with respiratory viruses such as respiratory syncytial virus (RSV) and rhinovirus (RV) strongly correlates with subsequent asthma development. Furthermore, respiratory viral infection is a leading cause of exacerbated disease in preexisting asthmatic patients.^{1–3} Murine studies have found that production of the T_H2-associated cytokine, IL-13, in response RSV can serve as both an exacerbating and predisposing factor during subsequent allergen exposure.⁴ It remains unclear as to the mechanism by which this uncharacteristic T_H2-like response is stimulated.

Thymic stromal lymphopoietin (TSLP) is a key factor in the development of allergic asthma. It is expressed at elevated levels in the lungs of humans with asthma and mice with antigen-induced airway inflammation.⁵ Mice lacking TSLP responses fail to develop antigen-driven airway disease. In contrast, elevating TSLP levels in the lungs of mice drive the development of disease akin to human asthma.^{6,7} Importantly, both *in vitro* and *in vivo* studies have shown a strong link between TSLP expression and the production of T_H2-associated effector cytokines IL-4, IL-5, IL-13, and TNF α .^{5,7–12}

The strong association of both TSLP and respiratory viruses with asthma development suggests that respiratory viral infection may stimulate pathways that lead to TSLP expression. Here, we report that viral activation of retinoic acid induced gene I (RIG-I) induced rapid TSLP production in primary human bronchial airway epithelial cells (AECs). In addition, we show that this is through activation of the nuclear factor- κ B (NF- κ B) arm of the RIG-I signaling pathway. Interestingly, bronchial epithelial cells (BECs) from asthmatic children produced significantly more TSLP than BECs from healthy children. Furthermore, TSLP promotes T_H2 skewing during RSV infections and participates in the development of immunopathology by promoting by mucus secretion and AHR. This study found that activation of the RIG-I innate signaling pathway serves to induce the T_H2-promoting cytokine TSLP and may provide a link between respiratory viral infection and asthma.

METHODS

Cells and virus

Normal human BECs (NHBEs) were maintained in appropriate medium according to the manufacturer's instruction (Lonza, Walkersville, Md). The human lung epithelial cell line A549 was maintained in Dulbecco modified Eagle medium (DMEM) with 10% FBS and antibiotics. Murine air-liquid interface (ALI) cultures were grown from mouse tracheal epithelial cells and differentiated as described.¹³ Generation of RIG-I knockout (KO) and

IFN- α receptor KO mouse embryonic fibroblasts (MEFs) was previously described.^{14,15} Sendai virus (SeV) was obtained from Charles River Laboratories (Wilmington, Mass). Viral infections were done with SeV at 50 hemagglutinating unit (HAU)/mL for A549 and NHBECs and at 100 HAU/mL for MEFs. RSV virus (Line 19, A strain) was originally isolated from an infected patient at the University of Michigan. Virus was propagated as previously published.¹⁶ In brief, Hep2 cells were infected with RSV for approximately 2 days until syncytia were visible. Cells were then frozen at 80°C overnight to lyse, and the supernatant fluid was harvested, clarified, and divided into aliquots. To determine viral titers in culture supernatant fluids, an immunoplaque assay was performed as previously described.¹⁷ Infections were performed at a multiplicity of infection (MOI) of 1 unless otherwise indicated. IL-1 receptor antagonist (anakinra) was kindly provided by Dr Srinath Sanda (Benaroya Research Institute, Seattle, Wash), and anti-human TNF α antibody was purchased from BioLegend (San Diego, Calif).

ALI cultures

Primary BECs were obtained by unsheathed bronchoscope cytologic brushings via an endotracheal tube during elective surgical procedures as described by Lane et al.¹⁸ Written consent was obtained from parents of all subjects. The protocol was approved by the Seattle Children's Hospital Institutional Review Board. Primary cultures were established by seeding freshly brushed BECs into hormonally supplemented bronchial epithelial growth medium (BEGM; Clonetics; Lonza) that contained gentamicin and amphotericin B and further supplemented with penicillin/streptomycin (100 μ g/mL). When confluent, the cells were passaged (P0) with trypsin and were allowed to further split into 3 new passage 1 (P1) T25 flasks. At passage 2, cells were seeded onto transwells and screened negative for evidence of mycoplasma infection. Cells were grown submerged in BEGM until 100% confluent, at which time apical medium was removed, and basolateral medium was replaced with ALI medium. ALI medium consisted of a 1:1 mixture of BEGM and DMEM supplemented with *all-trans* retinoic acid (30 ng/mL), human recombinant epidermal growth factor (0.5 ng/mL), MgCl₂ (0.6 mmol/L), CaCl₂ (1 mmol/LM), and penicillin/streptomycin (100 μ g/mL). Differentiated BECs were exposed at the apical surface of transwells with RSV strains A2 and Line 19 at a MOI of 0.5 or an equivalent volume of control Vero cell supernatant fluid for 2 hours. Sampling of basolateral supernatant fluid was performed 96 hours after RSV or control exposure. Each experimental condition per cell line consisted of triplicate transwells. More detailed analysis of subjects and cultures are provided (see this article's Methods section in the Online Repository at www.jacionline.org).

Real-time quantitative PCR and ELISA

Total RNA and cDNA preparation, and PCR conditions and TSLP primers, were as previously described.¹⁹ TSLP ELISAs were performed with either human or murine TSLP ELISA kits (R&D Systems, Minneapolis, Minn). Human primers used included RIG-I (NM_014314.3) purchased from InvivoGen (San Diego, Calif), Toll-like receptor 3 (TLR3; NM_003265.2; forward, 5'-CTCCATCTCATGTCCAACCTC-3'; reverse, 5'-TCCAGCTGAACCTGAGTTCC-3'), and GAPDH (NM_002046.3; forward, 5'-CATCCCTGCCTCTACTGGCG-3'; reverse, 5'-TAGACGGCAGGTCAGGTC CAC-3')

manufactured by Invitrogen (Carlsbad, Calif). RSV infection of human epithelial cultures was confirmed by examining RSV-F expression by real-time PCR as previously described.²⁰

Transfection and luciferase assay

A549 cells (2×10^5) were seeded into 6-well plates and transfected with Mirus transfection reagent (Mirus Bio Corp, Madison, Wis) with various plasmids (see figure legends for details) and pRSV- β -Gal as normalization control. After 19 hours, cells were infected with SeVor RSV for 6 hours. The lysates from these cells were assayed for luciferase activity as described.¹⁹ Small interfering RNA (siRNA) against human RIG-I and control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif). NHBEs were transfected at 100 nmol/L siRNA with the use of Lipofectamine 2000 (Invitrogen). After transfection for 30 hours, cells were infected with SeVor RSV. The efficiency of siRNA silencing was evaluated with real-time PCR.

In vivo RSV infection

Wild-type (WT) Balb/c or TSLPRKO²¹ mice (backcrossed to Balb/c for 12 generations) were infected with 4×10^4 plaque-forming unit (pfu) RSV (Line 19²²) via intratracheal injection in 40 μ L of total volume or appropriate mock control on day 1. Mice were analyzed 8 to 10 days later for cytokine production, for AHR, and histologically for mucus production. Airway hyperreactivity was assessed as previously described with the use of the direct ventilation method and airway resistance measurements.¹⁷ Briefly, mice were anesthetized, intubated via cannulation of the trachea, and ventilated (0.3 mL of tidal volume, 120 breaths/minute). AHR was measured with mouse plethysmography (Buxco, Wilmington, NC) and software for calculation of the measurements. After baseline measurements, mice were injected intravenously with 7.5 mg of methacholine (Sigma-Aldrich, St Louis, Mo), and the peak airway resistance was recorded as a measure of AHR. For histologic examination of mucus production, sections from formalin-fixed and paraffin-embedded lungs were stained with periodic acid–Schiff (PAS). For RSV-specific CD4 T-cell responses, single cell suspensions from mediastinal lymph nodes of infected TSLPR^{-/-} and control mice were plated at 10^6 cells/well and infected with 4×10^4 pfu of RSV. Twenty-four hours later supernatant fluids were collected for analysis on the Bio-Rad Bioplex 200 system (Bio-Rad, Hercules, Calif), according to the manufacturer's protocol. Kits (Bio-Rad) containing antibody beads to T_H cytokines (IL-17, IFN- γ , IL-4, IL-5, and IL-13) were used to assay for cytokine production in each of the samples.

Statistical analysis

Experimental results were analyzed for statistical significance (Student *t* test or ANOVA with Tukey posttest when appropriate). Statistical significance is indicated in the figures with **P* < .05 and NS = not statistically different, unless noted otherwise.

RESULTS

Induction of human TSLP expression by RSV infection of primary AECs

Severe infection with the family *Paramyxoviridae* virus during childhood was found to strongly correlate with subsequent asthma development, and infection in adults with asthma

commonly led to significant bouts of exacerbated disease. Further, a significant body of work has now reported the critical nature of TSLP expression by AECs in promoting the asthma phenotype.^{5,7} In addition, several studies have now reported that infection of AECs with a variety of viruses or treatment with the viral mimetic poly(I:C) can induce TSLP secretion by these cells.^{10,23} Given these data, we first determined whether RSV infection induced the expression of human TSLP in primary NHBECs and the optimal viral titer for this effect. As shown in Fig 1, A, induction of TSLP mRNA level depended on the amount of virus and reached maximal response in 4×10^6 pfu/mL RSV. Importantly, TSLP expression required productive infection because there was no significant induction of TSLP mRNA levels in infection of UV-irradiated RSV. We used this dose (4×10^6 pfu/mL) of RSV for subsequent experiments. RSV (Fig 1, B and C) infection of NHBECs markedly increased TSLP mRNA transcription from about 2 hours and reached maximal levels at 12 hours after infection (Fig 1, B). Next, infection with RSV resulted in high production of TSLP at 24 hours after infection (Fig 1, C). Similar results were obtained with the human AEC line, A549 (data not shown). These results are in agreement with prior work that found TSLP induction by RSV in rat AECs²⁴ but extended this by highlighting TSLP induction by RSV in primary human AECs for the first time.

Previous studies from our laboratory have found that the inflammatory cytokines, TNF α and IL-1 β , are strong inducers for TSLP production.²⁵ We next addressed the possibility that RSV-induced TSLP expression was a downstream effect of viral induction of these factors, rather than a direct effect of the virus itself. NHBECs were infected with RSV alone, or together with anti- TNF α (Fig 1, D) or anti-IL-1 β antibodies (Fig 1, E). After 24 hours, the supernatant fluids were collected for ELISA. The level of TSLP was not decreased when either anti-TNF α or anti-IL-1 β antibodies were added. These results argue that TSLP production is a direct result of viral infection and not secondarily induced downstream of TNF α .

RIG-I viral recognition pathway mediates human TSLP gene expression during SeV infection

The innate immune system initially recognized RNA viruses through activation of TLRs and cytoplasmic RNA helicases (RIG-I and melanoma differentiation-associated gene 5), leading to the production of type I interferon and inflammatory cytokines.^{15,26,27} Importantly, the response to both RSV and SeV infection was shown to be primarily mediated by the RIG-I pathway.¹⁵ To determine whether *Paramyxoviridae* activation of the RIG-I signaling pathway controlled TSLP expression, A549 cells were cotransfected with a human TSLP promoter reporter²⁵ and a dominant-negative form of RIG-I (DN-RIG-I).²⁸ Expression of DN-RIG-I inhibited RSV-mediated TSLP promoter activation (Fig 2, A). We next tested interferon promoter-stimulating factor 1 (IPS-1), an essential factor in the RIG-I pathway.²⁹ IPS-1 overexpression led to enhanced TSLP promoter activity after RSV infection (Fig 2, B). Further, IPS-1 overexpression alone, which has been shown to activate downstream elements of the RIG-I pathway,²⁹ was sufficient to induce activation of the TSLP promoter in the absence of RSV infection. To further evaluate the requirement for RIG-I and IPS-1 signaling in *Paramyxovirus*-induced TSLP expression, MEFs from RIG-I KO mice were infected with RSV. Although MEFs from WT mice expressed TSLP after

RSV infection, MEFs from RIG-I KO mice expressed dramatically reduced levels of TSLP (Fig 2, C). Similar results were seen for SeV in all of these settings (see Fig E1, A–D, in this article's Online Repository at www.jacionline.org). In addition, no difference was found in the ability of IFN- α receptor-deficient (lacking responsiveness to type I interferon) and WT MEFs to express TSLP in response to SeV (see Fig E1, F), suggesting that virus-induced TSLP is not a secondary effect because of type I interferon signaling.

To confirm the role of RIG-I in inducing TSLP in primary cells, we used siRNA to knockdown endogenous RIG-I in NHBEs before infection with RSV. Endogenous TSLP mRNA and protein expression in response to RSV infection were markedly reduced by RIG-I siRNA (Fig 2, D, and data not shown). Similar results were seen with SeV infection (see Fig E1, E). Taken as a whole, these data indicated that activation of the RIG-I pathway is both necessary and sufficient for robust *Paramyxoviridae*-induced TSLP expression.

RIG-I–induced activation of NF- κ B mediates SeV-induced expression of TSLP

The transcriptional effect of RIG-I pathway activation was mediated by the transcription factors interferon regulatory factor 3 (IRF-3), IRF-7, and NF- κ B.^{30,31} We have previously shown that the human TSLP promoter has putative IRF and NF- κ B binding sites, and we and others have also shown that NF- κ B is critical for TSLP gene induction by inflammatory cytokines.^{25,32} We next assessed which pathway mediated RIG-I–induced TSLP expression. Overexpression of NF- κ B alone resulted in activation of TSLP promoter activity, which was further increased after SeV infection (Fig 3, A). Consistent with these data, expression of a DN form of I- κ B kinase subunit β (IKK β), which blocks NF- κ B activation, strongly inhibited TSLP-promoter activity in response to SeV (Fig 3, B). The human TSLP gene promoter has 3 potential NF- κ B binding sites 3.8 kb, 1.3 kb, and 0.2 kb upstream of the start of transcription.²⁵ Mutational analysis showed that the sites at –3.8 and –0.2, but not the –1.3 site, were critical for TSLP promoter activation (Fig 3, C). In addition, chromatin immunoprecipitation (ChIP) analysis of the endogenous promoter showed NF- κ B binding at the –3.8 and –0.2 sites (see Fig E2 in this article's Online Repository at www.jacionline.org). In contrast, overexpression of constitutive active IRF-3 or IRF-7 failed to activate the TSLP promoter, although both strongly activated the IFN- β promoter (Fig 3, D and E). As a whole, these results indicated that NF- κ B acts as the crucial transcription factor down-stream of RIG-I in mediating TSLP expression.

Response of asthmatic and nonasthmatic BECs to RSV infection

The above-mentioned data indicated that RSV infection of normal BECs, grown in submerged cultures, can induce the production of TSLP. To extend these studies we compared the response of BECs isolated from healthy and asthmatic children to RSV infection. These studies used ALI cultures generated from cells isolated from bronchial brushings. Once established the cultures were infected on the apical surface with RSV (both the Line 19 and A2 strains), and 4 days later the level of TSLP in the basolateral supernatant fluid was determined. As shown in Fig 4, cultures generated from cells isolated from asthmatic subjects showed significantly higher levels of TSLP production than those from healthy subjects in the absence of infection, consistent with previous reports of increased TSLP levels in the lungs of patients with asthma.^{5,33,34} As a whole, cultures from the

asthmatic subjects displayed a marked increase in TSLP after RSV infection, whereas cultures from the healthy subjects did not (Fig 4). In addition, when analyzed individually, cultures from the asthmatic subjects also displayed a significant within-subject increase in TSLP after RSV infection compared with uninfected cultures (RSV A2: 392 vs 33 pg/mL, $P = .007$; RSV Line 19: 137 vs 33 pg/mL, $P = .01$; see Fig E3 in this article's Online Repository at www.jacionline.org). We observed smaller increases in TSLP production by most epithelial cell lines from healthy children in response to RSV infection, which reached statistical significance for RSVA2 (RSVA2: 27 vs 13 pg/mL, $P = .02$; RSV Line 19: 26 vs 13 pg/mL, $P = .1$; Fig E3). There was not a significant difference in RSV-F expression between asthmatic and healthy epithelial cultures, indicating that the RSV infection status was not different between cells from the 2 groups (data not shown). These data were consistent with a role for TSLP in both the development of asthma as well as in RSV-induced exacerbations.

As described earlier, the response of AECs to virus infection involves 2 recognition pathways, TLR3 and RIG-I. As shown in Fig 2, we have found that RIG-I signaling was critical for RSV-induced TSLP gene expression. To determine whether these RNA sensors could account for the differences seen in the response of ALI cultures from healthy or asthmatic children to RSV infection we examined RIG-I and TLR3 expression in control and virus-infected cultures. We found that, although both sets of cultures showed a significant increase in TLR3 expression after infection, only the asthmatic cultures showed a significant increase in RIG-I expression (Fig 5). These results potentially implicated RIG-I in the differential responses seen for polarized AECs from asthmatic and healthy children to RSV infection.

TSLP contributes to RSV-induced T_H2 inflammation *in vivo*

Infection of Balb/c mice with RSV leads to immunopathology that includes AHR and increased mucus production due to elevated levels of IL-13.^{4,35} To evaluate the contribution of TSLP to the immune response to RSV we first examined whether RSV infection *in vivo* led to elevated TSLP expression. At late times during infections (days 8–10; the peak of immunopathology) RSV infected mice showed elevated TSLP protein levels in lung tissue homogenate relative to uninfected controls (Fig 6A). To determine whether TSLP responses were involved in RSV-mediated immunopathology, TSLPR-deficient mice were infected with RSV. Although WT mice displayed significant increases in lung resistance, indicative of the development of AHR, and displayed enhanced mucus overproduction due to elevated IL-13 levels, RSV-infected TSLPR^{-/-} mice had reduced AHR and significantly reduced mucus overproduction (Fig 6, B–D). The latter was likely because of a marked diminution in the induction of IL-13 expression in the lungs of RSV-infected TSLPR^{-/-} mice, leading to a decrease in Gob-5 expression and in overall mucus production, as shown by PAS staining (Fig 6, B and D). Consistent with these data, when CD4 T cells from the mediastinal lymph nodes of infected mice were restimulated with RSV, T cells from TSLPR^{-/-} mice showed a significant decrease in IL-13 production (as well as IL-5), whereas T_H1 and T_H17 responses (measured by IFN γ and IL-17A production, respectively) were unchanged (Fig 6, E). Taken together, these data indicated that TSLP is involved in promoting the T_H2 response to RSV infection and in promoting associated immunopathology.

DISCUSSION

The important role of respiratory virus infection on the onset of asthma, as well as the exacerbation of disease in patients with established asthma, has become clear.^{36–38} However, the mechanism responsible for these effects has remained unclear. In this study, we report that infection of airway epithelium with *Paramyxoviridae* family viruses induces robust expression of TSLP, a T_H2-promoting cytokine. This finding is particularly interesting given that RSV infection in young children has been shown to generate a mixed T_H1/T_H2 response.^{39,40} Our data suggest that it is the ability of RSV to induce TSLP expression by AECs that is responsible for the subsequent T_H2 aspects of the response.

We report RIG-I as a novel pathway that leads to TSLP expression after respiratory virus infection of AECs. This finding is consistent with several recent studies, including treatment of NHBEs with poly(I:C) or with RV,¹⁰ and HIV and simian immunodeficiency virus infection,⁴¹ showing potent TSLP induction. Our finding that RSV and SeV are capable of inducing TSLP suggests that this may be a common feature of the host immune response to viruses. TSLP has been shown to both promote T_H2^{6,7,9} and inhibit T_H1 responses.^{42,43} Thus, virus-induced TSLP production may be either a mechanism for increasing viral clearance (eg, through increased mucus production) or a viral evasion mechanism to dampen the T_H1 response to infection. Data in support of the latter hypothesis come from studies that suggest the T_H2 response to respiratory virus infection blunts memory development.^{3,44} We have found that TSLPR-deficient mice infected with RSV have significantly reduced immunopathology, supporting the notion that RSV-induced TSLP is involved in promoting the T_H2 aspect of the response.

Respiratory viruses such as RSV and RV have been found to significantly affect both the development and exacerbation of allergic airway diseases.^{1,2,4} Although several factors, including IL-4, IL-13, CCR4, and CCL17,^{4,45} have been implicated in this phenomenon, the driver of this seemingly paradoxical T_H2 response to infection remains unclear. Our findings suggest that TSLP provides the mechanistic link between respiratory viral infections and the development and exacerbation of allergic airway disease.

Numerous studies have now implicated various TLR pathways in the pathogenesis of asthma.^{10,46–48} However, a link between RIG-I and asthma has not been established. Our finding that the innate immune pathway downstream of RIG-I induces TSLP expression suggests a possible role for RIG-I in asthma development. Indeed, the finding that only the ALI cultures from asthmatic children displayed increased RIG-I expression after RSV infection is consistent with a possible role. Further, because TSLP plays important roles in bridging the innate and adaptive immune response,^{6,7,9,49} RIG-I-induced TSLP expression may be an aspect of the adaptive response to virus infection.

The final important aspect of this study is the differential response of AECs from healthy and asthmatic children to RSV infection. It is becoming clear that the airway epithelium is more than a physical barrier; it is an important player in the response to barrier insult.^{50,51} It is also apparent that this population of cells plays an important role in the development and progression of asthma.^{52,53} Airway epithelium defects in patients with asthma leads to

decreased barrier function, which serves to enhance the access of environmental allergens and viruses.^{54,55} In addition, patients with asthma displayed impaired airway repair, leading to the remodeling of the airways seen in patients with chronic asthma.^{56,57} Consistent with these observations, we have found that ALI cultures established from bronchial brushings from asthmatic children produce significantly more TSLP after infection with RSV than cultures that used cells from healthy children. This finding provides a possible mechanistic explanation for the ability of RSV to drive disease exacerbations in persons with established asthma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported by the National Institutes of Health (grants AI068731, HL098067, AR055695, and AR056113 to S.F.Z.; grant HL102708 to S.F.Z. and J.S.D.; grants AI060389 and AI083019 to M.G. Jr; and grant HL059178 to N.W.L.).

M. Headley, M. Gale, J. S. Debley, and N.W. Lukacs have received research support from the National Institutes of Health (NIH). S. F. Ziegler has received research support from the NIH, has received lecture fees from the American Academy of Allergy, Asthma & Immunology, and has received travel expenses from the Federation of Clinical Immunology Services.

We thank John Mcguire and William Parks (University of Washington) for murine ALI cultures, Theingi Aye and Whitney Xu for excellent technical assistance, and Drs Campbell and Hamerman for critical discussion of the manuscript prior to submission. We thank Matt Warren for his administrative support.

Abbreviations used

AEC	Airway epithelial cell
AHR	Airway hyper responsiveness
ALI	Air-liquid interface
BEC	Bronchial epithelial cell
BEGM	Bronchial epithelial growth medium
ChIP	Chromatin immunoprecipitation
DMEM	Dulbecco modified Eagle medium
DN	Dominant-negative
HAU	Hemagglutinating unit
IKKβ	I- κ kinase subunit β
IPS-1	Interferon promoter-stimulating factor 1
IRF-3	Interferon regulatory factor 3
KO	Knockout
MEF	Mouse embryonic fibroblast
MOI	Multiplicity of infection

NF-κB	Nuclear factor-κB
PAS	Periodic acid–Schiff
Pfu	Plaque-forming unit
RIG-I	Retinoic acid induced gene I
RSV	Respiratory syncytial virus
RV	Rhinovirus
SeV	Sendai virus
siRNA	Small interfering RNA
TLR	Toll-like receptor
TSLP	Thymic stromal lymphopoietin
WT	Wild-type

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Key messages

- TSLP is induced by RSV infection of AECs through the antiviral RIG-I pathway.
- TSLP contributes to the type-2 response to RSV infection, and loss of TSLP signaling during infection decreases immunopathology.
- BECs from asthmatic children produce significantly more TSLP after RSV infection than the same cells from healthy children, showing a potential role for TSLP in viral exacerbations and providing a model for therapeutic intervention.

FIG 1.

RSV induces TSLP expression in primary AECs. **A**, NHBEs were exposed to UV-irradiated RSV or RSV with indicated virus titers for 12 hours, and TSLP mRNA was measured by real-time quantitative PCR for virus titer. **B** and **C**, NHBEs were exposed to UV-irradiated RSV or RSV (MOI, 1) for indicated time course. Cell or culture supernatant fluids were harvested, and TSLP mRNA was measured by real-time quantitative PCR (Fig 1, **B**). TSLP protein levels were measured by ELISA (Fig 1, **C**). **D** and **E**, NHBEs were exposed to RSV (MOI, 1) without or with anti-TNF α antibodies (10, 50, or 100 ng/mL) (Fig 1, **E**), anti-IL-1R (1, 10, or 50 μ g/mL) (Fig 1, **E**) for 24 hours, and culture supernatant fluids were harvested. TSLP protein levels were analyzed by ELISA. Data represent means $\pm$ SDs of 3 independent measurements. Similar results were obtained for 5 independent experiments (Fig 1, **A–E**). * $P < .05$.

FIG 2.

RIG-I and IPS-1 activation mediates *Paramyxovirus*-induced TSLP expression. **A** and **B**, A549 cells were transfected with control plasmid or luciferase constructs containing the human TSLP promoter alone or in combination with expression vectors for DN-RIG-I (Fig 2, A) or IPS-1 (Fig 2, B) followed by infection with RSV (MOI, 1). **C**, Normal littermate control (NLC) or RIG-I KO MEFs were infected with or without RSV, and TSLP protein expression was measured by ELISA. *n.d.* indicates not detected. **D**, NHBEs were transfected with control or RIG-I siRNA, followed by RSV infection, and TSLP mRNA was assessed at 8 hours after infection. Data represent means $\pm$ SDs of 3 independent measurements. Similar results were obtained for 5 independent experiments (Fig 2, A–D).

**P* .05.

FIG 3.

RIG-I-mediated NF-κB activation controls SeV-induced TSLP expression. **A** and **B**, A549 cells were transfected with NF-κB p65 (Fig 3, A) and DN-IKKβ expression vectors with a human TSLP promoter construct (Fig 3, B). Cells were subsequently infected with SeV as above and analyzed for luciferase activity. Data are the means ± SDs with 3 for each group. **C**, A549 cells were transfected with control or constructs containing the WT hTSLP promoter or promoter NF-κB site mutants. WT, Nonmutated human TSLP promoter; 1, deletion of -3.2-kb site, 2, deletion of -1.3-kb site, 3, deletion of -0.2-kb site. Cells were

subsequently infected with SeV as above and analyzed for luciferase activity. **D** and **E**, Human TSLP or IFN β promoter reporters were cotransfected with expression vectors encoding IRF-3 (Fig 3, *D*) or IRF-7 (Fig 3, *E*) and infected with SeV. After 6 hours cell lysates were analyzed for luciferase activity. Data are the means $\pm$ SDs with 3 for each group. **P* < .05.

FIG 4.

Human asthmatic epithelium produces greater levels of TSLP in response to RSV infection. ALI cultures were generated from primary BECs isolated from healthy or asthmatic children via bronchial brushing. Data show TSLP protein levels, measured by ELISA, in basolateral culture supernatant fluids after infection with either RSV Line 19 or RSV A2 or control at an MOI of 0.5 ($n = 12$ patients for all groups). P values were calculated with an ANOVA with Tukey posttest.

FIG 5.

RSV-mediated expression of RIG-I and TLR3 in ALI cultures from healthy and asthmatic children. RIG-I and TLR3 expression (log₂ scale) by BECs from healthy (*open plots*; n = 9) and asthmatic children (*solid plots*; n = 12) in response to RSV infection by A2 and Line 19 strains or exposure to control vero cell supernatant fluid (VC). RIG-I expression: ANOVA $P < .001$ between groups; asthma RSV A2 group 3-fold greater RIG-I expression than asthma VC group ($*P < .001$); asthma RSV 19 group 4-fold greater RIG-I expression than asthma VC group ($*P < .001$); no significant change in RIG-I expression by healthy cells with RSV A2 or RSV 19; no significant difference in RIG-I expression between asthmatic and healthy VC groups. TLR3 expression: ANOVA $P = .003$ between groups; healthy RSV A2 group 1.4-fold greater TLR3 expression than by healthy VC group ($**P = .05$); healthy RSV 19 group 1.8-fold greater TLR3 expression than by healthy VC group ($***P = .03$); asthma RSV A2 group 1.5-fold greater TLR3 expression than by asthma VC group ($†P = .002$); asthma RSV 19 group 1.8-fold greater TLR3 expression than by asthma VC group ($††P = .002$); no significant difference in TLR3 expression between asthmatic and healthy VC groups. RIG-I and TLR3 expression normalized to GAPDH. Bottom and top of box plots represent 25th and 75th percentiles, respectively; *dotted band* represents the median and *whiskers* represent minimums and maximums.

FIG 6.

TSLP promotes RSV-mediated immunopathology *in vivo*. **A**, WT Balb/c mice were infected intratracheally with RSV and evaluated for TSLP expression in lung homogenate by ELISA at 6, 8, and 10 days after infection. **B–D**, WT or TSLPRKO mice were infected with RSV intratracheally and analyzed for immunopathology at day 6 after infection. **B**, IL-13 and mucin (Muc5AC, Gob5) mRNA expression between WT (filled bars) and TSLPRKO (open bars) mice. **C**, Airway resistance after methacholine challenge, measured by plethysmography, in RSV infected WT (filled bars) and TSLPRKO (open bars) mice

infected with RSV for 8 days. **D**, PAS stain of lung section from WT or TSLPRKO mice after RSV infection. **E**, Cytokine production in supernatant fluids from restimulated mediastinal lymph node cultures from RSV-infected WT (*filled bars*) and TSLPRKO (*open bars*) mice. Data are representative of 3 experiments with 5 mice per group per experiment. * $P < .05$.