

ORIGINAL ARTICLE

TSLP from RSV-stimulated rat airway epithelial cells activates myeloid dendritic cells

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The respiratory syncytial virus (RSV) is a primary cause of lower respiratory tract infections in children, the elderly and in people who are immune suppressed, and is also the cause for the development of asthma primarily in infants. However, the immunological mechanisms by which RSV enhances allergic sensitization and asthma remain unclear. The aim of this study was to examine the influence of RSV-infected airway epithelial cells on the activation and functions of rat myeloid dendritic cells (mDCs). We found that the exposure of primary rat airway epithelial cells (PRAECs) to RSV induced a rapid (6 h), high (12 h) and persistent (18 h) increase in thymic stromal lymphopoietin (TSLP) mRNA compared with untreated PRAECs. TSLP protein expression was also enhanced by RSV infection. Functional maturation of mDCs was induced by RSV-treated PRAECs, as shown by their enhanced levels of OX40L and thymus- and activation-regulated chemokine (TARC) mRNAs, which increased the expressions of major histocompatibility complex II (MHCII) and CD86 costimulatory molecules and promoted enhanced T-cell proliferation in mixed lymphocyte reactions. These activities were inhibited in cocultures with RSV-infected RTECs (rat tracheal epithelial cells, an immortalized cell strain) that had been pretreated with TSLP-targeted small interfering RNA. These results suggest that RSV can induce epithelial cells to produce TSLP, which in turn promotes the maturation of mDCs that might support Th2 cell polarization.

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The respiratory syncytial virus (RSV), a member of the pneumovirus genus within the family Paramyxoviridae, primarily infects infants and young children, although RSV infections are also significant health problems among the elderly and people who are immune suppressed.^{1,2} Exposure to RSV, particularly in infants, has been proposed as a mechanism for the development of asthma. Although clinical evidence has shown that RSV can worsen asthma status, its possible role in asthma development remains controversial. RSV exposure may directly result in asthma, or RSV may trigger changes in pulmonary pathophysiology in people who are predisposed to asthma.³ The susceptibility to asthma could be increased by RSV infection by its activities during immune responses.^{4,5}

The primary target of RSV infection is the airway epithelium. The airway epithelial cell surface expresses numerous Toll-like Receptors (TLRs) that can recognize RSV. RSV recognition by cell surface TLRs promotes the secretion of inflammatory cytokines. TLR4 is the main extracellular receptor that can recognize RSV by means of binding to the fusion protein present on the viral envelope.⁶ In addition, TLR3 in respiratory epithelial cells can also recognize RSV.⁷ As an intracellular receptor, TLR3 can recognize viral replication intermediates, such as dsRNA. As with TLR4, increased TLR3 expression is also observed on RSV-infected epithelial cells. This suggests an

increased sensitivity and secretion of inflammatory cytokines on contact with RSV.⁷

When exposed to RSV, respiratory epithelial cells can express many potent immunomodulatory and inflammatory mediators.^{8,9} Chemokines produced by RSV-infected airway epithelium promote the recruitment of neutrophils, monocytes, memory T cells and eosinophils from the peripheral blood or infected tissues. These recruited immune cells secrete proinflammatory cytokines, and increased secretion of these cytokines leads to airway damage.^{10,11}

Yet, these activities are not definitive for the development of asthma. Asthma is a disease characterized by chronic airway inflammation that is mediated by Th2 cells. First, however, DCs are essential for the priming of naive T cells and their differentiation into Th2 cells. DCs are considered to be the most powerful antigen-presenting cells and have a central role in the initiation of primary immune responses and in the enhancement of secondary immune responses. RSV-stimulated myeloid DCs (mDCs) may have immune stimulatory effects on both Th1 and Th2 responses, whereas RSV-stimulated plasmacytoid DCs have direct antiviral activity through the release of interferon- α .¹² There are some DCs in close proximity to bronchial epithelial cells, and these epithelial cells can regulate the recruitment, maturation and even the activation of these DCs. Although DC maturation and

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polarization have been well studied during responses to pathogens, cell–cell interactions (epithelium and DC) may also instruct DC responses.

Thymic stromal lymphopoietin (TSLP), a member of the interleukin (IL)-7 family of cytokines,¹³ was originally identified as a growth factor secreted by Z210R.1 thymic stromal cells that supported the proliferation and survival of the NAG8/7 pre-B cell line.¹⁴ TSLP is produced by epithelial cells, skin keratinocytes, stromal cells, smooth muscle cells, lung fibroblasts and mast cells.¹⁵ In humans, DCs rapidly respond to TSLP by TSLP receptors.¹⁶ After TSLP stimulation, matured mDCs induce Th2 cell polarization and sustain the Th2 memory response.^{13,17}

The expression of TSLP by airway epithelial cells is increased in asthmatic airways.¹⁸ However, whether RSV can induce airway epithelial cells to produce TSLP is controversial. Kato *et al.* showed that TSLP could be induced by dsRNA for normal human bronchial epithelial (NHBE) cells.¹⁹ However, dsRNA is an intermediate product of viral genome duplication and not the virus itself, and NHBEs are not primary airway epithelial cells. In addition, these authors did not consider the possible involvement of DCs. Again, RSV-stimulated DCs may promote both Th1 and Th2 responses.¹²

In this study, we first analyzed whether TSLPs could be induced in primary rat airway epithelial cells (PRAECs) that were stimulated by RSV. We subsequently examined whether this epithelial cell-derived TSLP could promote DC maturation and their polarization to promote Th2-type immune responses.

RESULTS

RSV-induced TSLP expression by PRAECs

There is increasing information regarding the stimuli necessary for and the regulation of TSLP production, although whether RSV can induce the expression of TSLP by airway epithelial cells is not well known. PRAECs were treated with RSV (MOI=10) for varying times (6–24 h) and RNA was isolated. As shown in Figure 1a, PRAEC exposure to RSV induced a rapid (6 h), high (12 h) and persistent (18 h) increase in TSLP mRNA compared with untreated PRAEC (about 3.5-, 8.4- and 6.0-fold induction at 6, 12 and 18 h, respectively, $n=3$; $P<0.05$). Later, PRAECs were infected with different doses of RSV (MOI=2.5, 5 and 10) for 12 h. We established the optimal amount of virus at an MOI=10, as RSV stimulation for 12 h at an MOI=10 induced an obvious upregulation of TSLP mRNA (about 5.4-fold, $n=3$, $P<0.05$, Figure 1b). RSV-induced TSLP protein secretion in the supernatant of PRAEC culture was further confirmed by enzyme-linked immunosorbent assay detection (Figure 1c, 35.1 ± 58 vs 12.9 ± 2.2 pg/ml, $P<0.05$); TLR-3 and -4 are required for RSV infection and amplification in airway epithelial cells. Before RSV exposure, rat tracheal epithelial cells (RTECs) were treated with siRNA duplex pools for TLR-3 and -4. As shown in Figures 1d and e, knockdown of TLR3, but not of TLR4, by siRNA could significantly inhibit the RSV-induced production of TSLP mRNA and protein by RTECs. The efficiency and specificity of small interfering RNA-induced TSLP, TLR3 and TLR4 gene knockdown in RTECs were confirmed; these results are shown in the supplementary figures (Supplementary Figure S1–S2). Taken together, these results indicate that RSV can directly stimulate TSLP expression by PRAECs through TLR-3.

Effect of TSLP from RSV-infected PRAEC on maturation of mDCs

On day 8 of culture, mDCs were cocultured with RSV-infected or medium-exposed PRAECs (2 or 6 h, MOI=10) using a transwell culture system. After 48 h of stimulation, RSV-infected PRAECs

enhanced the expressions of major histocompatibility complex II (MHCII) and CD86 on mDCs compared with medium-exposed PRAECs, but CD80 expression did not change (Figures 2a and b). When TSLP expression was knocked down in RTECs by siRNA silencing, the expressions of MHCII and CD86 on mDCs were reduced, whereas CD80 expression was not altered (Figure 2c). This suggested that the production of TSLP by RSV-infected PRAECs could stimulate the maturation of mDCs, which are adjacent to PRAECs.

TSLP from RSV-infected PRAECs induces Th2, but not Th1, markers in mDCs

We next examined whether TSLP from RSV-infected PRAEC could activate mDCs to produce thymus- and activation-regulated chemokine (TARC) and OX40L, which are important Th2-polarizing molecules of mDCs, and TNF- α , which is a Th1-polarizing molecule of mDCs, in coculture. The mRNA expressions for OX40L (Figure 3a) and TARC (Figure 3b) were upregulated, whereas TNF- α mRNA expression was downregulated (Figure 3c). Gene silencing for RSV-infected TRECs and coculture with mDCs showed that both siTSLP and siTLR-3 resulted in downregulated mRNA expressions for OX40L (Figure 3d) and TARC (Figure 3e), whereas TNF- α mRNA was upregulated (Figure 3f). Silencing with siTLR-4 had no effect on the expressions of mRNAs of these markers. This indicated that TSLP released from PRAECs could induce mDCs to express molecules that induce Th2 polarization.

Influence of RSV-infected PRAECs on mDC function

Finally, to confirm functional mDC maturation, allogeneic mixed leukocyte reactions were performed. mDCs isolated after exposure to RSV-treated PRAECs supported increased T-cell proliferation compared with mDCs exposed to untreated PRAECs ($OD=0.477 \pm 0.015$ vs 0.376 ± 0.011 , $n=4$, $P<0.01$; Figure 4a). To confirm that these results were due to TSLP released from epithelial cells, after exposure to siTSLP and RSV-treated RTECs, mDCs exhibited decreased T-cell proliferation compared with mDCs exposed to RTECs without siRNA treatment (Figure 4b).

DISCUSSION

Associations between RSV infection and asthma have been recognized for many years.²⁰ RSV infection can upregulate the expressions of several asthma-related cytokines and chemokines of cultured airway epithelial cells,²¹ including RANTES, iNOS, MUC-1, endothelin-1, 5-lipoxygenase, IL-10 and IL-13. These mediators have direct effects on cells during inflammatory responses. By comparison, TSLP, a cytokine mainly produced by the epithelium, has an important role in DC-mediated activation of Th2 inflammatory responses. However, whether RSV can stimulate TSLP expression by human bronchial epithelial cells is controversial, and no previous study has tested RSV effects on PRAECs.

Kato *et al.* showed that TSLP could be induced by dsRNA for NHBE cells.¹⁹ However, dsRNA is an intermediate product of viral genome duplication and not of the virus itself. In addition, these authors did not consider the possible involvement of DCs. Lee *et al.* considered that an inflammatory mediator (IL-1 β or TNF- α) was required for TSLP production by NHBE cells.²² Yet, NHBEs are not primary airway epithelial cells. Wang *et al.* found that healthy, but not RSV-infected lung epithelial cells had T-cell inhibitory activities, although they used ELISAs for inflammatory molecules in supernatants only at 24 h after stimulation.²³ Using reverse transcriptase (RT)-PCR analyses at multiple time points (6–24 h), we found that TSLP mRNA expression could be upregulated in RSV-infected PRAECs at earlier time points after

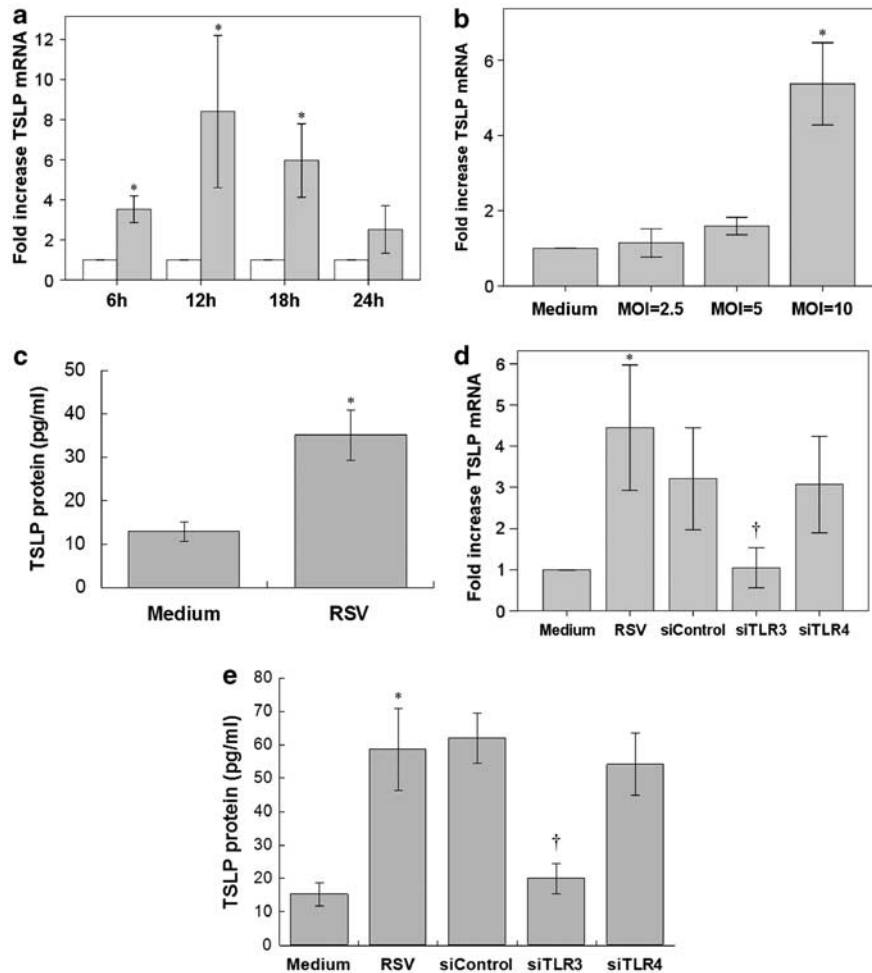


Figure 1 RSV-induced upregulation of TSLP in rat airway epithelial cells by TLR3. (a) TSLP mRNA (6–24 h) was analyzed in response to RSV (MOI=10) using quantitative RT-PCR. Results are given as fold changes relative to medium; gray bars indicate RSV-treated cells and white bars indicate cells in medium (mean $\pm$ s.d., $n=3$, $*P<0.05$ compared with non-treated PRAEC). (b) TSLP mRNA at 12 h after stimulation (MOI=10, 5 or 2.5, respectively) was measured as above. Results are representative of three independent experiments. (mean $\pm$ s.d., $n=3$, $*P<0.05$). (c) TSLP protein secreted into the supernatant of PRAECs at 24 h after RSV stimulation (MOI=10) was measured by ELISA (mean $\pm$ s.d., $n=4$, $*P<0.05$). (d) RTECs were transfected with siTLR3, siTLR4 or siCONTROL duplex pools for 24 h, and subsequently treated with RSV (6 h, MOI=10) or medium. TSLP mRNA was analyzed as above. (e) TSLP protein secreted into culture supernatants by RSV-infected RTEC was assayed by ELISA (mean $\pm$ s.d., $n=4$, $*P<0.05$, TSLP derived from RSV-treated RTEC vs medium-treated RTECs, and $^{\dagger}P<0.05$, TSLP derived from siControl-treated RTECs compared with siTLR3-treated RTECs).

stimulation (6–18 h). Further, PRAECs-derived TSLP promoted the functional maturation and Th2 polarization capability of mDCs.

We also showed that, as expected, stimulation of TSLP expression in rat airway epithelial cells by RSV was mainly induced by TLR3, but not TLR4. Both TLR-3 and TLR-4 can recognize RSV or viral dsRNA.²⁴ TLR-4 on cell surfaces recognizes the fusion protein present on the viral envelope, whereas TLR-3 is an intracellular receptor that can recognize viral genome products. Our results suggest that TSLP is activated only when RSV replication occurs within airway epithelial cells. This proposal was confirmed by our RSV-infected PRAECs and by mDC coculture experiments in which PRAECs were first treated with RSV (MOI=10) for 2 or 6 h, washed 3–4 times with phosphate-buffered saline, and then could still influence mDCs by TSLP. Although it has not been proven that RSV can induce asthma directly, it can induce airway hyperresponsiveness and amplify the responses to allergen exposure.²⁵

An allergic reaction is induced by an overactive Th2 immune response. The strength of this response depends on the inflammatory conditions at the sites of induction and the interactions between

epithelial cells and dendritic cells. Epithelial cells enhance the maturation and antigen-presenting capabilities of DCs by secreting relevant cytokines and then indirectly affect the subsequent immune response. TSLP has a role to link epithelial cells, DCs and effector lymphocytes. Thus, TSLP induction may partially explain these observations.

The role of lung DCs in virus-induced pulmonary inflammation is less clear. Lambrecht and Hammond found that bone marrow-derived DCs could sensitize mice to allergen, leading to Th2 responses, and that mDCs in the lung were required for allergic sensitization by the airways.²⁶ On allergen challenge, mDCs are essential for the induction of allergic airway inflammation, as well as for its maintenance. We studied the influence of RSV-infected airway epithelium on the maturation and Th2-polarization capacity of mDCs using cell–cell coculture with a transwell system.

RSV-infected airway epithelium induced the upregulation of maturation markers MHCII and CD86 on rat mDCs, although CD80 was not altered and mDC OX40L mRNA was upregulated. OX40L can skew an immune response toward a Th2 type. Increased expressions of OX40L can enhance the Th2-polarizing function of

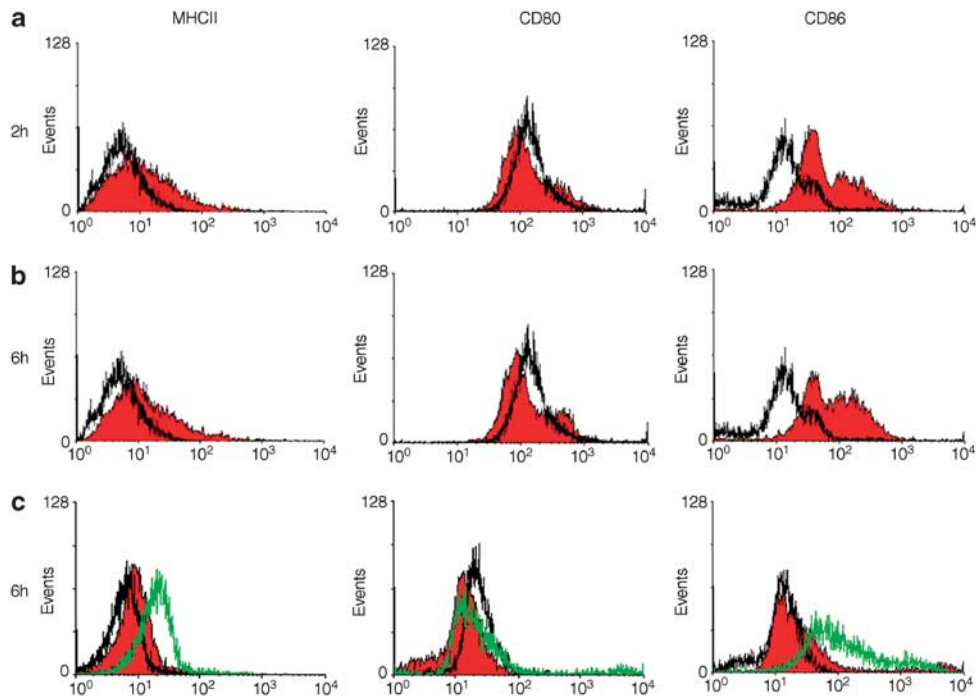


Figure 2 Maturation of mDCs by RSV-infected rat airway epithelial cells. (a–c) Expressions of MHCII, CD80 and CD86 on rat mDCs were determined after coculture with RSV-infected (2 or 6 h, MOI=10) PRAECs (red line) or medium-exposed PRAECs (black line) by transwell for 48 h. (c) Expressions of MHCII, CD80 and CD86 on rat mDCs were determined after coculture with RSV-infected (6 h, MOI=10) RTECs (green line), siTSLP-transfected and RSV-infected RTECs (red line) or medium-exposed RTECs (black line) by transwell for 48 h. Experiments for (a–c) were carried out three times; results of one representative experiment are shown.

OX40L/OX40 on CD4⁺ T cells and cause an imbalance of Th1/Th2 cells to the Th2 subtype. The imbalance of Th1/Th2 cells is an abnormal immune response during the onset and development of asthma, which includes an abnormally high number of hyperactive Th2 cells. Jember *et al.* reported that blockade of CD28/B7 interactions only partially inhibited asthma and that OX40/OX40L interaction also had an important function.²⁷ Salek-Ardakani *et al.* found that memory CD4⁺ T cells expressed OX40.²⁸ Sensitized animals that inhaled a nebulized antigen that blocked OX40/OX40L signaling showed diminished draining lymph nodes in the lung and an accumulation and long-term survival of memory T cells in lung tissue. This also prevented the production of eosinophils, mucus secretion and airway hyperresponsiveness, and inhibited Th2 cytokine production. This suggested that OX40/OX40L signal transduction had an important role in atopic asthma regulated by memory Th2 cells. In our study, RSV-infected PRAECs could enhance OX40L expression on the surface of rat mDCs. We speculate that this might be an important mechanism for RSV to induce or exacerbate asthma.

CD80 and CD86 seem to support T-cell activation equivalently.²⁹ CD86 is expressed constitutively at low levels and is rapidly upregulated on mDCs, whereas CD80 induction occurs later after activation. This may explain why CD80 expression did not seem to be altered on rat mDCs in our experiments. By comparison, the earlier expression of CD86 implies that CD86 is an important costimulator for initiating T-cell responses. Blocking CD86 by administering an mAb can inhibit allergic airway manifestations in mice in different experimental contexts.^{30,31} In humans, CD86 on peripheral blood mononuclear cells seems to be the principal costimulatory ligand for allergen-induced Th2 cytokine production.^{32,33} However, the contribution of CD80 to the initiation and development of allergic airway response is more controversial.³¹ In fact, we found that when RSV was added to PRAEC

and mDC coculture systems directly (cell-to-cell mixing or by transwell), expressions of CD80, CD86 and MHCII were increased simultaneously, although only very slightly (data not shown).

TSLP can promote a rapid response by human DCs. In our study, TARC expression was induced, whereas TNF- α was inhibited. TARC is a chemokine associated with Th2 recruitment, whereas TNF- α is a Th1 cytokine. TARC is also designated as CCL17. Binding of TARC to its receptor CCR4 can activate downstream phospholipase C, which causes high concentrations of intracellular Ca²⁺ and the activation of protein kinase C. It has also been found that TARC was related to the accumulation of eosinophils. TARC can also selectively induce cell migration, particularly Th2 cells.³⁴ Serum TARC levels are increased in children with asthma and are related to IgE levels.³⁵ We found that RSV-infected PRAECs could increase TARC expressions by rat mDCs. This indicated that the ability of rat mDCs to induce accumulations of eosinophils and Th2 cells increased after interactions with RSV-infected PRAECs. TNF- α is a typical Th1 cytokine. The fact that the expressions of TNF- α by mDCs were decreased in coculture was not surprising.

The results of TSLP siRNA gene silencing tests confirmed the role of TSLP derived from RSV-infected PRAEC on mDCs. Our study shows that the exposure of myeloid DCs to RSV-infected PRAECs, but not RSV itself, can induce a Th2-polarization capacity by mDCs. However, when RSV was added directly to PRAEC and mDC coculture systems, TARC, IL-12 and TNF- α were upregulated simultaneously (data not shown). We speculate that RSV has a direct effect on mDCs and that this contribution partly interacts with (reinforces or weakens) the effects that RSV-infected PRAECs have on mDCs.

To further investigate DC maturation, we showed that mDCs induced by RSV-treated epithelial cells could support T-cell proliferation, as determined by a mixed leukocyte reaction. In the coculture

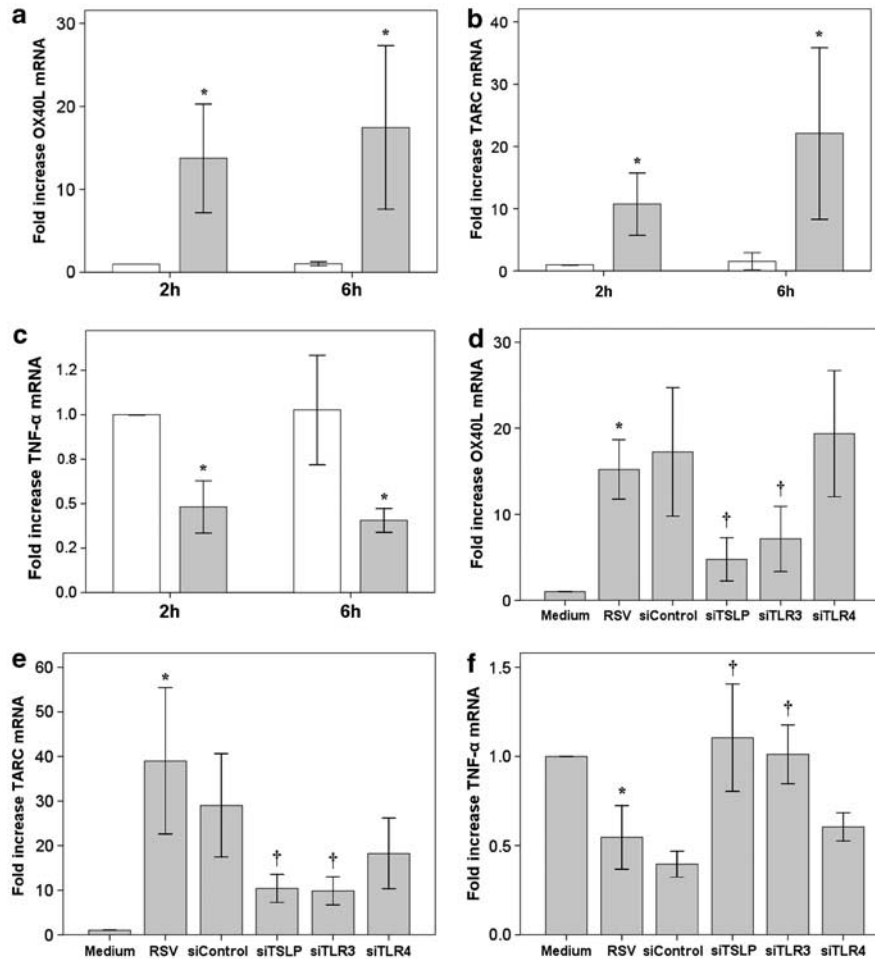


Figure 3 RSV-treated rat airway epithelial cells support mDC expressions of Th2, but not Th1, priming cytokines, and this effect is inhibited when epithelial cells are pretreated with siTSLP or siTLR3, but not siTLR4. (a–c) Immature myeloid DCs were exposed to RSV-treated PRAECs (2 or 6 h, MOI=10) or medium-exposed PRAEC by transwell for 48 h. mDCs were sorted by fluorescence-activated cell sorting, and RNA was isolated from mDCs and quantified for OX40L (a), TARC (b) and TNF- α (c) by RT-PCR (gray bars are RSV-treated cells and white bars are for cells in medium; mean $\pm$ s.d., $n=4$, * $P<0.05$ compared with nontreated PRAECs). (d–f) RTECs were transfected with siControl, siTSLP, siTLR3 or siTLR4 duplex pools for 24 h, and subsequently treated with RSV (6 h, MOI=10). Immature myeloid DCs were exposed to RSV-treated RTEC by transwell for 48 h. mDCs were sorted by fluorescence-activated cell sorting, and RNA was isolated and quantified for OX40L (d), TARC (e) and TNF- α (f) by RT-PCR (mean $\pm$ s.d., $n=4$, * $P<0.05$ compared with medium, † $P<0.05$ compared with siControl).

system, rat mDCs were activated and became functionally mature. In the subsequent mixed leukocyte reaction assay, infected PRAECs could increase the capabilities of mDCs to enhance T-cell proliferation. This effect could be inhibited by TSLP knockdown. This suggested that PRAEC-released TSLP had a critical role in activating mDCs. The high expression of TSLP by RSV-infected PRAECs was mediated by TLR3, but not by TLR4.

This is an interesting finding, as RSV-infected mDCs inhibit T-cell proliferation by producing suppressing soluble factors such as interferon- α .^{36,37} Similarly, we found that when RSV was added to PRAEC–mDC coculture systems, T-cell proliferation was impaired compared with similar cultures using uninfected mDCs (data not shown).

Clinical data suggest low-level interferon- γ -associated Th-1 responses and strong production of Th-2 cytokines in RSV bronchiolitis.^{38,39} It is known that in severe bronchiolitis caused by RSV, numerous airway epithelial cells are infected by virus and these cells influence DCs, including uninfected mDCs. Accordingly, there is a possibility that many more mDCs are directly exposed to RSV

epithelium without being infected by RSV. Our study shows that mDCs cocultured with RSV-treated airway epithelial cells, but not RSV itself, can induce a Th2-like pattern of DC activation. Our study partly explains those Th2-biased immune responses found in bronchiolitis in infants, which are quite different from responses to other respiratory viruses, such as influenza virus and adenovirus that are characterized by strong interferon- γ production. This may be a pathway or a connection between RSV bronchiolitis and asthma.

In summary, our study indicates that RSV can induce airway epithelial cells to express TSLP by TLR3 and that TSLP subsequently activates mDCs and enhances their maturation, which supports Th2 differentiation and their local polarization and accumulation. This suggests that RSV may provoke asthma by inducing epithelial cells to produce TSLP, which in turn promotes the maturation and migration of DCs. TSLP could also skew an immune response to a Th2 type. Th2-associated cytokines would exacerbate asthma. Thus, it may be possible to ameliorate asthma induced by RSV by decreasing TSLP secreted by respiratory epithelial cells.

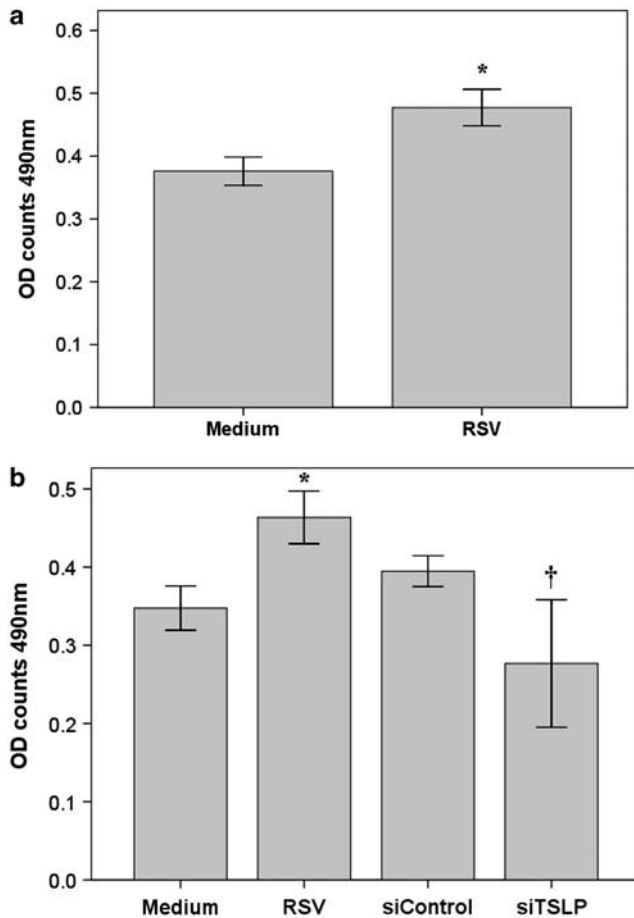


Figure 4 T-cell proliferation in response to mDCs derived from RSV-treated rat airway epithelial cells. T cells prepared from mesenteric lymph nodes of Wistar rats were stimulated with mitomycin C-treated mDCs (DC to T cell ratio, 1:50; 3 days), which had been pretreated for 48 h with RSV-treated PRAECs (6 h, MOI=10) (a) or with RSV-infected (6 h, MOI=10) RTECs, with siControl-transfected and RSV-infected RTECs, or with siTSLP-transfected and RSV-infected RTECs (b). After 72 h, cells were incubated with 20 μ l per well MTT (5 g l^{-1} , 4 h), centrifuged at 1000 r.p.m. for 5 min and supernatants were removed. Dimethyl sulfoxide (150 μ l per well) was added and dimethyl sulfoxide incorporation by proliferating responder cells was measured using a dimethyl sulfoxide ELISA according to the manufacturer's instructions. Results are mean $\pm$ s.d., $n=4$, * $P<0.05$ compared with medium, † $P<0.05$ compared with siControl.

METHODS

RSV culture and titration

RSV Strain Long, an A subtype RSV, was obtained from GuangZhou Biotest Bioengineering, Guangzhou, China. The virus was cultured on monolayers of Hep-2 cells in RPMI-1640 medium containing 2 mM glutamine, 100 IU/ml penicillin, 100 U/ml streptomycin and 2% fetal calf serum. When the infected monolayers exhibited approximately 80% syncytial formation, cells and medium from the monolayers were harvested and centrifuged for 10 min at 1000 g at 4 °C. The supernatant was passed through a 0.45 μ m filter and stored at -80 °C. Virus titers were determined by plaque assay using Hep-2 cells as previously described.⁴⁰ The virus titer consisted of 1.5×10^7 plaque-forming units (pfu) ml^{-1} .

Animals

We used 3- to 4-week-old Wistar rats. All animals were purchased from the Experimental Animal Center of Shanghai Institute, Shanghai, China, for Biological Sciences and maintained under SPF conditions. Experiments using animals were approved by the Institutional Ethics Committee.

In vitro generation of rat mDCs

Rat mDCs were generated as described previously.¹² Bone marrow cells from the femurs and tibiae of Wistar rats were grown at a starting concentration of 1×10^6 cells ml^{-1} in RPMI 1640 supplemented with 10% fetal bovine serum, L-glutamine (2 mmol l^{-1}), penicillin/streptomycin (50 units ml^{-1}) and 50 ng ml^{-1} of recombinant granulocyte/monocyte colony-stimulating factor. Fresh medium supplemented with granulocyte/monocyte colony-stimulating factor was added on days 2 and 4, and all of the loosely adherent cells were transferred to Petri dishes on day 6. The following day, nonadherent cells were harvested. DC purity and maturation were analyzed by flow cytometry using markers for CD80, CD86 and MHCII.

Isolation and in vitro culture of PRAECs

PRAEC harvesting from 3- to 4-week-old Wistar rats used methods slightly modified from those previously described.⁴¹ The dissociated cells from two tracheae (1.2×10^6 cells) were seeded onto one tissue culture insert semipermeable support membrane (Costar Transwell clear (Costar Transwell, New York, NY, USA), tissue culture-treated polyester membrane 12-well plate inserts, 0.4 μ m pore) in 200 μ l of culture medium, with 600 μ l outside the insert. To precoat these inserts, 200 μ l of a collagen solution (filter-sterilized 50 $\mu\text{g ml}^{-1}$ type I rat-tail collagen in 0.02 N acetic acid) was added to the semipermeable membrane, air-dried overnight and later washed twice with phosphate-buffered saline before use. The cells were incubated at 37 °C with 5% CO_2 for 5 days. On day 6, the medium on the apical surface of the cultured cells was removed along with any nonadherent cells and debris. Primary cultures grown at the air-liquid interface remained viable for up to 30 days.

PRAEC monolayers were infected with RSV (MOI=10, 5 or 2.5) for 6, 12, 18 and 24 h.

RSV-infected PRAECs and mDC coculture

PRAECs on the upper layer of an insert of a transwell culture were treated with RSV (MOI=10) for 2 or 6 h, after which the entire supernatant (upper layer) and culture medium (lower layer outside the insert) were removed, and then washed with phosphate-buffered saline 3–4 times. mDCs (nonadherent cells on day 6) were added to the lower layer of a transwell (filled with 600 ml airway epithelial cell medium) at a cell:cell ratio of 1:1 for 48 h. The culture medium was added between the upper layer and the lower layer, which helped in establishing a contact between cells in the upper and lower layers. No medium was added in the upper layer so that the rat airway epithelial cells could contact the air and be polarized. The airway epithelial cells in the upper layer were washed and rinsed with fresh medium every 6 h to get rid of possible virus released by rat airway epithelial cells.

Quantitative RT-PCR

RNA was isolated from airway epithelial cells and mDCs using the Micro-to-Midi total RNA purification System (Invitrogen, Foster City, CA, USA). Quantification by RT-PCR used a One-Step Quantitect SyberGreen Real Time PCR kit (Toyobo, Shanghai, China) following the manufacturer's instructions. *In vitro* transcription used T7/SP6 RNA polymerase (Fermentus, Shenzhen, China), followed by complementary DNA digestion and purification. Levels of transcripts for OX40L, TARC and TNF- α were normalized to the β -actin transcript level as an internal control ($\Delta\text{Ct}(\text{target}) = \text{Ct}(\text{target}) - \text{Ct}(\beta\text{-actin})$). Changes in respective transcript levels after treatment were assessed by $2^{-\Delta\Delta\text{Ct}}$ ($\Delta\Delta\text{Ct}(\text{target}) = \Delta\text{Ct}(\text{target, stimulated}) - \Delta\text{Ct}(\text{target, resting})$).

Mixed leukocyte reaction

T cells were prepared from spleens of Wistar rats by passing spleen cell suspensions through nylon wool without further purification for T-cell subsets. Mitomycin C-treated mDCs (1.5×10^5) and T cells (3×10^5) were cocultured in triplicate in a round-bottomed 96-well plate in a total volume of 200 μ l per well at mDC/T-cell ratios of 1:50. After 72 h, cells were incubated with 20 μ l per well of MTT (5 g l^{-1} , 4 h), centrifuged at 1000 r.p.m. for 5 min and supernatants were removed. Dimethyl sulfoxide (150 μ l per well) was added and MTT incorporation by proliferating responder cells was measured using an ELISA reader according to the manufacturer's instructions.

siRNA gene silencing

For small interfering RNA studies, RTECs, an immortalized cell strain, were obtained from the Institute of Basic Medical Sciences of the Chinese Academy of Medical Sciences, Beijing, China. These were cultured in MEM-EBSS with 10% fetal calf serum on the upper layer of a transwell insert. After 48 h, cells were transfected with a siRNA duplex pool targeted against rat TLR3 (5'-GGUGUCUCCACAAACCAATT-3', 5'-UUGGUUUGUGGAACACCTT-3'), TLR4 (5'-GCAUAGAGGUACUCCUAATT-3', 5'-UUAGGAAGUACCUCUAUGCTT-3') or TSLP (5'-CCUAAAAGACCACGUGUUTT-3', 5'-AAACACGUGGUCUUUAGGTT-3') (Invitrogen) using lipofectamine (Invitrogen) according to the manufacturer's instructions. After 24 h, transfected RTECs were exposed to RSV and a portion of them were cocultured with DCs, as described previously.

TSLP ELISA assay

Supernatants from RTEC cultures were assayed for TSLP protein concentrations using an ELISA kit. The monoclonal antibody for rat TSLP for ELISA was purchased from R&D Systems (Minneapolis, MN, USA).

Flow cytometry

The expressions of costimulatory molecules on DCs after RSV stimulation were determined using fluorescence-activated cell sorting analysis. Cells were stained in phosphate-buffered saline containing 5% fetal calf serum and 5 mM sodium azide (fluorescence-activated cell sorting wash) using monoclonal antibodies directed against the following antigens: CD80 labeled with Biotin anti-rat CD80 and streptavidin-fluorescein isothiocyanate, as well as CD86 and MHCII labeled with fluorescein isothiocyanate. To reduce nonspecific antibody binding, anti-FcγRII antibody was included in all cell surface staining processes. All antibodies were purchased from eBioscience.

Statistical analysis

All experiments were performed at least in triplicate. Values for all measurements are expressed as mean ± s.d. unless stated otherwise. Statistical analysis was performed using the *t*-test or one-way ANOVA with Bonferroni's adjustment. Data were analyzed using SAS 9.0 (SAS Institute Cary, NC, USA.). All *P*-values were two-sided and were considered significant if *P* was less than 0.05.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Immunology and Cell Biology website (<http://www.nature.com/icb>)