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*Exp Biol Med* (Maywood) published online 24 April 2014

DOI: 10.1177/1535370214523889

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# Original Research

## Azithromycin inhibits double-stranded RNA-induced thymic stromal lymphopoietin release from human airway epithelial cells

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### Abstract

Thymic stromal lymphopoietin (TSLP) is elevated in asthma and triggers dendritic cell-mediated activation of TH2 inflammatory responses. Viral stimuli, a major cause of asthma exacerbations, have been shown to induce over-expression of TSLP in asthmatic epithelium. Azithromycin has various effects such as antimicrobial and anti-inflammatory effects. However, the effect of azithromycin on the production of TSLP has not been studied. Here we have explored effects of azithromycin on dsRNA-induced TSLP in normal human bronchial epithelial (NHBE) cells. NHBE were stimulated with poly (I:C) in the presence of azithromycin. The effects of azithromycin on dsRNA-induced inflammatory responses in NHBE cells were analyzed. We demonstrated that azithromycin inhibited the production and mRNA expression of TSLP in NHBE cells. Azithromycin also inhibited the nuclear factor- $\kappa$ B luciferase activity induced by poly (I:C), and it prevented dsRNA-induced loss of the NF- $\kappa$ B repressor protein I $\kappa$ B $\alpha$ . These results demonstrate the azithromycin has anti-TSLP activity in normal human bronchial epithelial cells.

**Keywords:** Azithromycin, thymic stromal lymphopoietin, bronchial epithelial cells, double-stranded RNA

*Experimental Biology and Medicine* 2014; 0: 1–7. DOI: 10.1177/1535370214523889

### Introduction

Exacerbations of asthma are the main cause of asthma morbidity and mortality and account for significant health-care costs.<sup>1</sup> The deterioration and exacerbations in children and adults are frequently associated with viral infection, and the bronchial epithelium is the major target of the infection.<sup>2,3</sup> This infection could induce the production of proinflammatory cytokines and antiviral INF- $\beta$ .<sup>4</sup>

Thymic stromal lymphopoietin (TSLP) is an interleukin 7 (IL-7)-like cytokine with its ability to regulate numerous physiological processes. These include conditioning of dendritic cells (DCs) to drive TH2 skewing of T cells, enhanced class switching in B cells, and induction of Foxp3<sup>+</sup> Treg cells in humans.<sup>5–7</sup> Recent findings indicate that TSLP plays an important role in allergic inflammation.<sup>8</sup> It is highly expressed by airway epithelial cells of patients with asthma.<sup>9–11</sup> TSLP-activated DCs initiate adaptive allergic immune responses by triggering differentiation of naive T cells into inflammatory TH2 cells. Thus, TSLP represents a critical factor linking responses at interfaces between the body and environment to allergic TH2 immune reactions.<sup>12,13</sup>

Double-stranded RNA (dsRNA) is produced during viral replication and induces innate immune responses including epithelial generation of interferons and inflammatory cytokines.<sup>14</sup> Recently, it was demonstrated that polyinosinic-polycytidylic acid (poly I:C), a synthetic dsRNA recognized by dsRNA sensors including Toll-like receptor 3 (TLR3), was a strong trigger for TSLP production in human bronchial epithelial cells, and the TSLP release can be synergistically enhanced with an atopic cytokine milieu.<sup>13</sup> These findings imply that respiratory viral infection, through epithelial TLR3 stimulation, may amplify TH2 inflammation via the induction of TSLP in the asthmatic airway.

The use of macrolides has been proposed both in chronic and acute asthmatic inflammation.<sup>15,16</sup> Besides direct antimicrobial activity against gram-positive cocci and atypical pathogens, C14 macrolides such as azithromycin also have immune modifying effects including inhibition of IL-5 production in TH2 cells, suppression of proinflammatory cytokines, and modification of DC maturation.<sup>17–19</sup> However, their direct action on TSLP production in human bronchial epithelial cells is not well understood. It is reported that azithromycin has less cytotoxicity than roxithromycin,

erythromycin estolate, and other macrolides in cell protein concentration.<sup>20</sup> Therefore, the objective of this study was to evaluate the effect of azithromycin on the dsRNA-induced TSLP expression in human bronchial epithelial cells.

## Materials and methods

### Reagents and cell culture

Poly (I:C) was purchased from InvivoGen (San Diego, CA, USA); 100 ng/mL IL-4 and 100 ng/mL IL-13 were purchased from R&D Systems (Minneapolis, MN, USA). Azithromycin was obtained from Sigma (St Louis, MO, USA). Antibodies against IκBα and β-actin were purchased from Cell Signaling Technology (Danvers, MA, USA). Normal human bronchial epithelial (NHBE) cells (Lonza, Cleveland, USA) were maintained in serum-free bronchial epithelial cell growth medium (Cambrex, East Rutherford, NJ, USA). Cells were seeded in flat-bottomed 96-well micro-culture plates and cultured until they reached 100% confluence, and then the medium was changed to fresh medium. After further cultivation for 24 h, cells were stimulated with poly (I:C) and T<sub>H</sub>2 cytokines (100 ng/mL IL-4 and 100 ng/mL IL-13) in the absence or presence of azithromycin.

### ELISA

Concentration of TSLP, IL-8, and IFN-β were quantified by ELISA using commercially available paired antibodies and standards, following the manufacturer's instructions (R&D Systems).

### RNA isolation and real-time RT-PCR

RNA was isolated and purified using the RNeasy Mini kit (Qiagen, Hilden, Germany). For real-time RT-PCR analyses, 1 μg of DNase-treated total RNA was reverse transcribed. The amplification of the cDNA was accomplished using the ABI Prism 7900HT sequence detection system in the presence of the commercially available SYBR Green PCR Master Mix (Takara, Dalian, China) in a 40-cycle PCR. The primer sequences were as follows: GAPDH (Forward, 5'-GGT CGG AGT CAA CGG ATT TG-3'; Reverse, 5'-ATG AGC CCC AGC CTT CTC CAT-3'); TSLP (Forward, 5'-TAT GAG TGG GAC CAA AAG TAC CG-3'; Reverse, 5'-GGG ATT GAA GGT TAG GCT CTG G-3'); IL-8 (Forward, 5'-AAG CTG GCC GTG GCT CTC TTG-3'; Reverse, 5'-AGC CCT CTT CAA AAA CTT CTC-3'); IFN-β (Forward, 5'-CCA ACA AGT GTC TCC TCC AAA T-3'; Reverse, 5'-AAT CTC CTC AGG GAT GTC AAA G-3'); TLR3 (Forward, 5'-AAA TTG GGC AAG AAC TCA CAG G-3'; Reverse, 5'-GTG TTT CCA GAG CCG TGC TAA-3'); PKR (Forward, 5'-AAA GCG AAC AAG GAG TAA G-3'; Reverse, 5'-TTC AGA AGG GCT CTA ACA-3'); RIG-I (Forward, 5'-TAT TCT GAT TGC CAC CTC-3'; Reverse, 5'-TAA ATA CTG CTT CGT CCC-3'); MDA5 (Forward, 5'-GTT ATC CGT TAT GGT CTC G-3'; Reverse, 5'-GGA AAG TTA TTA GTG ATG GGT T-3'). The denaturing, annealing, and extension conditions of each PCR cycle were 95°C for 5 s, 60°C for 20 s, and 72°C for 34 s, respectively. The relative expression was calculated using the 2<sup>-ΔΔCT</sup> method. The mRNA levels of

each target gene were normalized to the levels of GAPDH and were represented as fold induction.

### Transient transfection and luciferase assay

For the transfection, we seeded NBHE cells (1 × 10<sup>7</sup>) in a 100-mm culture dish. We then used Lipofectamine<sup>TM</sup>2000 purchased from Invitrogen (Carlsbad, CA, USA) to transiently transfect pGL4.32 reporter gene constructs into NBHE cells. To measure the luciferase activity, we used a luminometer Gene-Light 55 (Microtech Nichion, Chiba, Japan) in accordance with the manufacturer's protocol. All the transfection experiments were performed in at least three different experiments, with similar results. The relative luciferase activity was defined as the ratio of firefly luciferase activity to renilla luciferase activity.

### Western blotting

NHBE cell lysates were separated by SDS-PAGE (10% acrylamide gels) and transferred to polyvinylidene difluoride (PVDF) membranes, following detailed protocols described previously.<sup>21</sup> The blots were probed with IκBα, and β-actin antibodies and then proteins were visualized by the ECL Western Blotting System (Pierce, Rockford, IL, USA). To quantify the expression, the intensities of the bands were measured using Image-Pro Plus version 4.5 and are expressed as intensities relative to actin.

### Statistical analysis

Results are expressed as the mean ± SD. A Student's t-test was used to determine significance among the groups. A value of *P* < 0.05 was considered significant. Analyses and graphical representation were performed using Graph-Pad Prism 5.01 software (Graphpad).

## Results

### Effect of azithromycin on dsRNA-induced expression of TSLP in NHBE cells

To assess the inhibitory effect of azithromycin on the production of TSLP, we pretreated normal human bronchial epithelial (NHBE) cells with increasing concentrations of azithromycin for 4 h and subsequently stimulated the cells with poly (I:C) plus T<sub>H</sub>2 cytokine milieu (IL-4 and IL-13) for 48 h in the presence of azithromycin. As shown in Figure 1(a), the stimulation with poly (I:C) increased TSLP production from NHBE cells. The levels of TSLP that increased due to poly (I:C) were significantly decreased by azithromycin treatment in a dose-dependent way.

To determine whether azithromycin can modulate poly (I:C)-induced mRNA expression of TSLP, we pretreated the cells with azithromycin for 4 h before the poly (I:C) stimulation. We then stimulated the cells with poly (I:C) for 2, 8, 16, and 24 h in the absence of IL-4 and IL-13. The dsRNA-induced gene expression of TSLP peaked at 8 h, and azithromycin showed an inhibitory effect at 8, 16, and 24 h (Figure 1b).

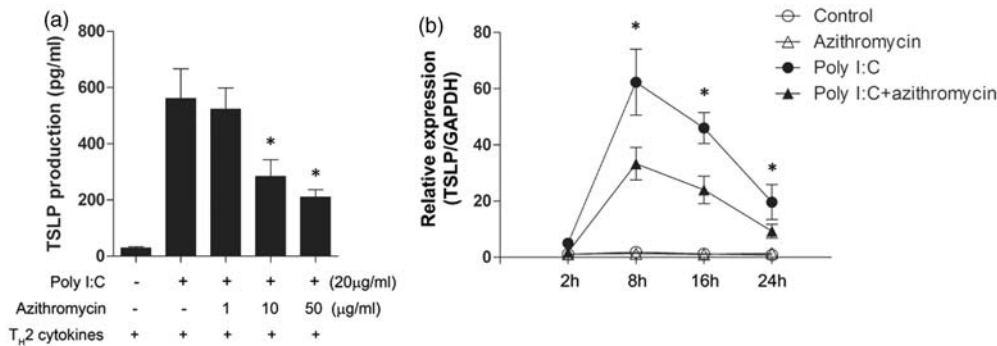


**Figure 1** Azithromycin decreases dsRNA-induced expression of TSLP in NHBE cells. (a) NHBE cells were pretreated with increasing concentrations of azithromycin (0, 1, 10, and 50 µg/mL) for 4 h and subsequently were stimulated with poly (I:C) (20 µg/mL) plus T<sub>H</sub>2 cytokine milieu (IL-4 and IL-13) in the presence of azithromycin. Concentrations of TSLP in the culture supernatant collected 48 h after stimulation were measured. Data shown are the means for two wells and are representative of three independent experiments (\**P* < 0.05). (b) NHBE cells were pretreated with azithromycin (50 µg/mL) for 4 h, and then the cell were stimulated with poly (I:C) (20 µg/mL) for 2, 8, 16, and 24 h in the absence of 100 ng/mL IL-4 and 100 ng/mL IL-13. TSLP gene expression was analyzed by real-time RT-PCR and is presented as the fold change relative to the expression in the negative control at 2 h. The data are presented as the mean ± SD of a total of two independent experiments (\**P* < 0.5)

### Effect of azithromycin on dsRNA-induced expression of IL-8 and IFN-β in NHBE cells

The activation of TSLP receptor could enhance release of IL-8,<sup>22</sup> a proinflammatory mediator that is closely related with asthma. Thus, if azithromycin could down-regulate the expression of IL-8, this will give a clue on the mechanism of how azithromycin could relieve asthma. Asthma deterioration and exacerbations in children and adults are frequently associated with viral infection. Thus, it is important to assay the IFN-β expression level after azithromycin treatment. To investigate whether azithromycin suppresses the dsRNA-induced innate inflammatory response, NHBE cells were pretreated with azithromycin in concentrations ranging from 1 to 50 µg/mL for 4 h before they were challenged by poly (I:C) 20 µg/mL for 12 h in the absence of T<sub>H</sub>2 cytokines. Azithromycin displayed a concentration-dependent inhibitory effect on dsRNA-stimulated mRNA expression of IL-8, whereas the expression of IFN-β was not significantly modified (Figure 2a and b). Furthermore, the effects of azithromycin treatment on poly (I:C)-induced cytokine production were confirmed by ELISA. It was demonstrated that co-incubation with azithromycin for 48 h significantly inhibited secretion of IL-8, while the IFN-β production was not influenced (Figure 2c and d).

### Effect of azithromycin on the expression of RNA-recognizing receptors in NHBE cells

Several intracellular receptors have been described to bind to dsRNA, such as Toll-like receptor 3 (TLR3), PKR, RIG-I, and MDA5.<sup>23</sup> To reveal the inhibitory mechanisms of azithromycin, we examined the mRNA expression of these RNA-recognizing receptors. As shown in Figure 3, stimulation of NHBE cells with poly (I:C) leads to increases in the different receptors at a variable degree, whereas azithromycin treatment did not affect the mRNA level of these genes.

### Suppressive effects of azithromycin on dsRNA-stimulated TSLP production via NF-κB signaling pathway

NF-κB is one of the key transcription factors mediating TSLP release from NHBE cells.<sup>24</sup> Next, we examined whether azithromycin could regulate the luciferase expression specifically via NF-κB activation. As shown in Figure 3, the poly (I:C) stimulation increased the reporter gene activity. However, this increased NF-κB luciferase activity was significantly decreased by azithromycin treatment (Figure 4a).

The activity of NF-κB is controlled by IκB, we examined whether azithromycin influenced the degradation of IκBα by means of western blot analysis. In this study, treatment of NHBE cells with poly (I:C) showed increased degradation of IκBα in the cytoplasm extracts measured by immunoblotting. Azithromycin treatment (50 µg/mL) partially restored the level of IκBα in dsRNA-stimulated NHBE cells (Figure 4b).

### Discussion

The current treatment of asthma consists of corticosteroids and/or β<sub>2</sub>-agonists and only partially prevents asthma exacerbations.<sup>25</sup> A vital need exists for new treatment regimes for this form of asthma. For more than 20 years macrolide antibiotics have been used to treat chronic inflammatory airway diseases based on their immunomodulatory activity.<sup>26</sup> Clinical studies have shown that macrolides have beneficial effects in chronic inflammatory lung diseases including diffuse panbronchiolitis, bronchiectasis, bronchiolitis obliterans syndrome, and cystic fibrosis.<sup>16,27,28</sup> Macrolides exert their anti-inflammatory actions through multiple mechanisms. Macrolide antibiotics down-regulate damaging prolonged inflammation as well as increase mucus clearance. Macrolide therapy appears to suppress the secretion of proinflammatory cytokines and chemokines such as IL-1β, IL-6, IL-8, and TNF-α from bronchial epithelial cells, lung fibroblasts, macrophages, and leukocytes.<sup>29,30</sup> In the present study, we showed that azithromycin

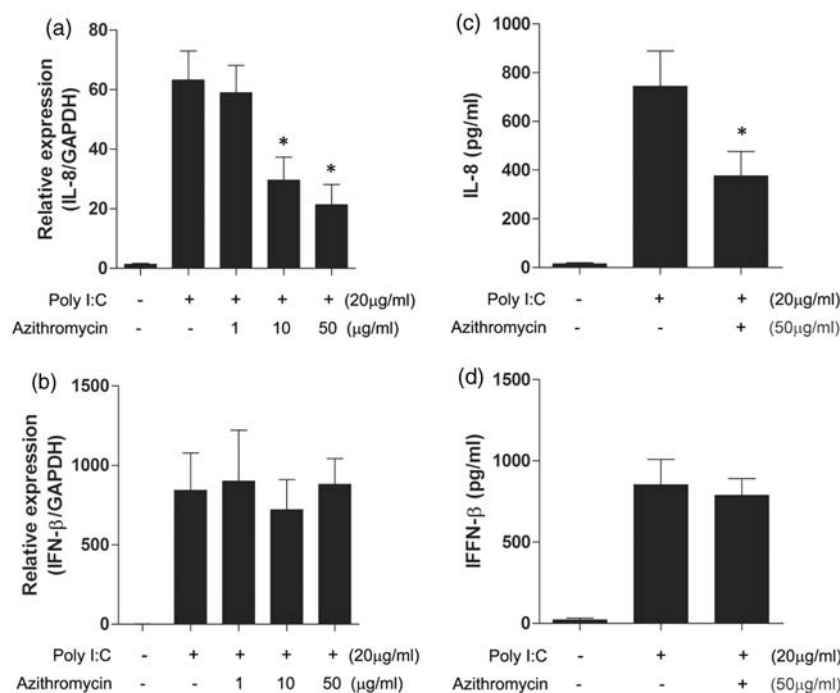


**Figure 2** Azithromycin inhibits dsRNA-induced upregulation of IL-8 but not IFN- $\beta$  expression. NHBE cells were pretreated with increasing concentrations of azithromycin (0, 1, 10, and 50 µg/mL), and then the cells were stimulated with poly (I:C) (20 µg/mL) for 8 h. IL-8 (a) and IFN- $\beta$  (b) gene expression was analyzed by real-time RT-PCR and is presented as the fold change relative to the expression in the negative control. The data are presented as the mean  $\pm$  SD of a total of two independent experiments (\* $P$  < 0.05). (c, d) NHBE cells were stimulated with poly (I:C) (20 µg/mL) in the presence or absence of azithromycin (50 µg/mL). Concentrations of IL-8 and IFN- $\beta$  in the culture supernatant collected 48 h after stimulation were measured. Data shown are the means for two wells and are representative of three independent experiments (\* $P$  < 0.05).

inhibited dsRNA-induced TSLP from normal human bronchial epithelial cells by inhibiting NF- $\kappa$ B signaling, while expression of antiviral cytokine IFN- $\beta$  was not significantly influenced.

The observation that viral products induce TSLP production by bronchial epithelium may be related to the well-documented aggravating role of infection in allergy as well as asthma.<sup>31</sup> A subset of TLRs are localized to the endosomal compartment, where they recognize nucleic acids from viruses that have entered the cell by the endocytic pathway. Besides of TLR3, these nucleotide-sensing TLRs include TLR7, and TLR8, which recognize single-stranded RNA, and TLR9, which recognizes DNA. Rhinovirus infection has been identified as a main trigger associated with exacerbations of both asthma and COPD. dsRNA is produced during rhinovirus replication and causes innate immune responses, while non-replicative rhinovirus and ssRNA are poor stimuli for epithelial interferon and cytokine generation. So it will be interesting to investigate the expression of ssRNA-recognizing TLR7 not only in immune cells but also in bronchial epithelial cells. Therefore, in the previous reports and present study, dsRNA was adopted as a mimic of danger responses of viral RNA, it will be important to extend these studies towards virus infection models and the consequence of epithelial infection on DC activation and T cell polarisation.

Previous studies have shown that exposure to rhinovirus infection or dsRNA *in vitro* induces TSLP production in bronchial epithelial cells,<sup>32</sup> suggesting that this TSLP may

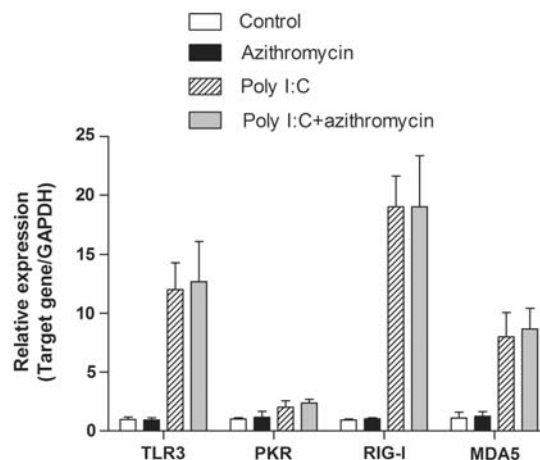


**Figure 3** Azithromycin does not change the expression levels of TLR3, PKR, RIG-I, and MDA-5 mRNA in NHBE cells. NHBE cells were treated or not with azithromycin (50 µg/mL) in the presence or absence of poly (I:C) stimulation (20 µg/mL) for 8 h, and then TLR3, PKR, RIG-I, and MDA5 mRNA expression were analyzed by real-time RT-PCR and is presented as the fold change relative to the expression in the negative control. The data are presented as the mean  $\pm$  SD of a total of two independent experiments.

link the innate antiviral response and the T<sub>H</sub>2 adaptive immune response. TSLP was originally reported to exert its T<sub>H</sub>2-promoting properties through a DC-mediated pathway in human beings that involved induction of the OX40 ligand on DCs.<sup>33</sup> There has therefore been great interest in targeting the pathways downstream of TSLP such as

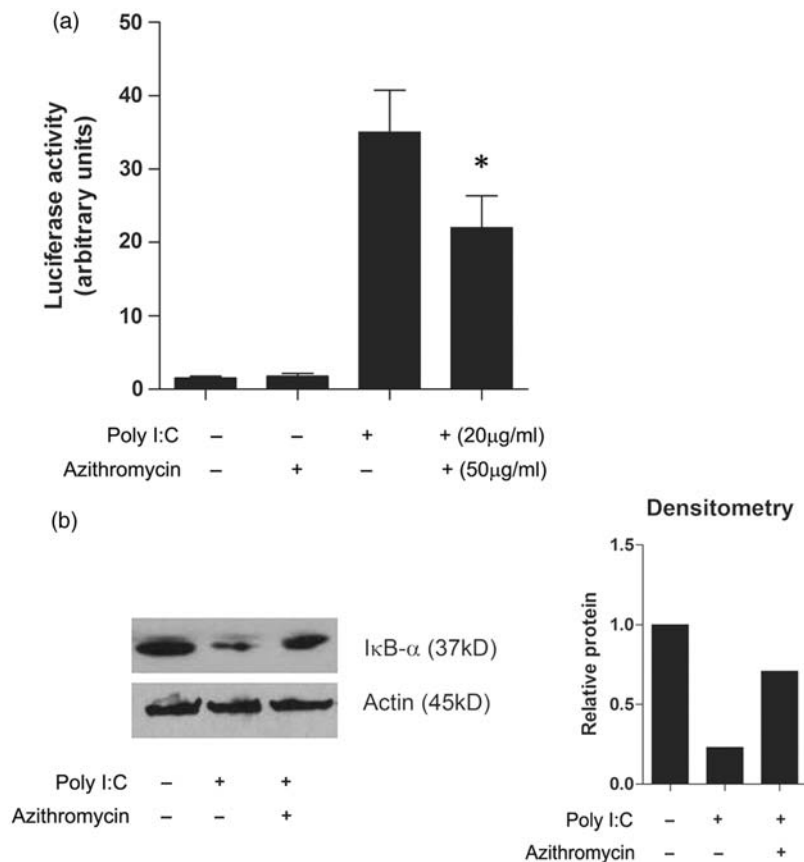


**Figure 4** Azithromycin inhibits dsRNA-induced NF- $\kappa$ B activation in NHBE cells. (a) NHBE cells ( $1 \times 10^7$ ) were transiently transfected pGL4.32 and treated with or without azithromycin (50  $\mu$ g/mL) for 2 h and then stimulated with or without poly (I:C) (20  $\mu$ g/mL) for 48 h. The NF- $\kappa$ B activity was assessed with a luciferase assay. The data are presented as the mean  $\pm$  SD of a total of two independent experiments (\* $P < 0.5$ ). (b) NHBE cells were incubated with or without poly (I:C) (20  $\mu$ g/mL) in the presence or absence of azithromycin (50  $\mu$ g/mL) for 18 h, and degradation of I $\kappa$ B $\alpha$  in cytoplasmic extract from NHBE cells was assessed by western blot and densitometric analysis. Data shown are representative of two independent experiments

TSLP-TSLP receptor interaction and OX40L-OX40 interaction. In the present study, azithromycin inhibited the production and mRNA expression of TSLP. To our knowledge, this is the first study showing an inhibition of TSLP by azithromycin in bronchial epithelial cells.

Induction of immune responses by dsRNA and RNA viruses involves endosomal receptor TLR3 or present in the cytoplasm including the RNA helicases RIG-I and MDA5 and the serine-threonine kinase PKR.<sup>23</sup> Our data demonstrated that none of these cytoplasmic dsRNA-recognition receptors were modulated by azithromycin treatment, however the roles of MDA5, RIG-I, or PKR could not be completely excluded. Activation of TLR3 by dsRNA RNA viruses transduces its signals to NF- $\kappa$ B and IRF-3 and induces proinflammatory genes and type I IFNs including IFN- $\beta$ .<sup>34</sup> However, the effects of macrolide antibiotics on TLR3/IRF3 signaling and IFN- $\beta$  production have shown contradictory results. Previous data showed that erythromycin treatment down-regulated TLR3/IRF3 signaling and IFN- $\beta$  production, while in another report, azithromycin significantly increased rhinovirus-induced interferons.<sup>35,36</sup> Here, we showed that secretion of IFN- $\beta$ , which may have protective role in asthma exacerbations,

was not compromised by azithromycin treatment, suggesting a positive role of macrolides in the antiviral ability, which is independent of TSLP production.

The transcription factor NF- $\kappa$ B mediates cytokine gene activation downstream of TLR3 signaling. Drugs that inhibit NF- $\kappa$ B by stabilizing its binding to I $\kappa$ B have also been shown to be effective inhibitors of TSLP generation in dsRNA-stimulated bronchial epithelial cells.<sup>37</sup> Additionally, such drugs have produced a less desirable inhibition of the antiviral IFN- $\beta$ .<sup>38</sup> The present findings suggest possibilities of separating anti-TSLP and anti-IFN- $\beta$  effects since azithromycin reduced expression of TSLP without inhibiting the IFN- $\beta$  production.

In conclusion, we demonstrated that azithromycin suppress the dsRNA-induced release of TSLP from normal human bronchial epithelial cells. TSLP is considered the master switch for T<sub>H</sub>2 responses. Therefore, it is a promising strategy to shut down the release of TSLP in the bronchial epithelial cells of asthma patients. Azithromycin could therefore be effective in the treatment of asthma exacerbation through the suppression of TSLP release from bronchial epithelial cells, when exogenous dsRNA is involved in the pathogenesis or exacerbation.

**Authors' contribution:** Conceived and designed the experiments: Jun-bo Xia and Jing Zhu. Performed the experiments: Jun-bo Xia, Jing Zhu, and Yan Wang. Analyzed the data: Jing Zhu and Yan Wang. Contributed reagents/materials/analysis tools: Jun-bo Xia, Jing Zhu and Yan Wang. Wrote the paper: Jun-bo Xia and Jing Zhu. Approve the paper: Jun-bo Xia

## ACKNOWLEDGEMENTS

This study was funded by Hangzhou Science and Technology Development Planning Project (Grant No. 20100633B07), and Hangzhou Medical Science and Technology Development Planning Project (Grant No. 2012A001).

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(Received October 29, 2013, Accepted December 31, 2013)