

Short Thymic Stromal Lymphopoietin Attenuates Toluene Diisocyanate-induced Airway Inflammation and Inhibits High Mobility Group Box 1-Receptor for Advanced Glycation End Products and Long Thymic Stromal Lymphopoietin Expression

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ABSTRACT

Short thymic stromal lymphopoietin (short TSLP), one of TSLP variants, exerts anti-inflammatory activities in endotoxin shock and colitis mouse models. Our latest work reported that short TSLP prevented house dust mite-induced epithelial barrier disruption. Yet the role of short TSLP in toluene diisocyanate (TDI)-induced asthma is unknown. Male BALB/c mice were sensitized and challenged with TDI to generate a chemical-induced asthma model. Synthetic short TSLP peptides were given intranasally or intraperitoneally before each challenge. TDI significantly increased inflammation and hyperresponsiveness of airway, which were suppressed by short TSLP treatment. Levels of mouse TSLP, high mobility group box 1 (HMGB1), and receptor for advanced glycation end products (RAGE) in airway epithelium and whole lung tissues were markedly increased in TDI group compared with control mice, which were decreased after administration of short TSLP. Meanwhile, short TSLP also inhibited STAT5(Y694) phosphorylation, which was highly expressed in airways of TDI-exposure mice. *In vitro*, both TDI-human serum albumin (HSA) and recombinant human (rh) HMGB1 promoted long TSLP but not short TSLP gene production in human bronchial epithelial cells (16HBE). Cells pre-treated with short TSLP exhibited less expression of RAGE and long TSLP and lower phosphorylation of Akt(S473), p38 MAPK(T180/Y182), and STAT5(Y694) than stimulated with TDI-HSA or rhHMGB1 alone. Results suggest that short TSLP prevents airway inflammation in a chemical-induced asthma model, which might be associated with the inhibitions of HMGB1-RAGE and long TSLP expression and STAT5(Y694) phosphorylation.

Key words: toluene diisocyanate; asthma; short TSLP; HMGB1-RAGE; long TSLP; STAT5.

Thymic stromal lymphopoietin (TSLP) is a likely IL-7 cytokine that highly expressed in barrier epithelial cells such as lung (Shikotra *et al.*, 2012), skin (Leyva-Castillo *et al.*, 2013), and gut

(Biancheri *et al.*, 2015). It can drive the development of strong allergic Th2 responses with release of IL-4, IL-5, IL-13, and TNF- α (Soumelis *et al.*, 2002) through upregulation of OX40 ligand

expression on TSLP-treated DCs (Ito et al., 2005). TSLP is overexpressed in the airways of asthmatic patients and is associated with Th2 inflammation (Ying et al., 2005). Human TSLP has two variants: long TSLP (lTSLP) and short TSLP (shTSLP). Long TSLP encodes a 159 amino acid (aa) protein, whereas the short TSLP encodes a sequence that is identical in the C-terminal region of long TSLP and consists of 63 aa and/or a 60 aa (Bjerkan et al., 2015). In most of previous studies, they did not distinguish between these two TSLP variants. On one hand, in human beings, long TSLP, that is full-length TSLP, was always named "TSLP". On the other hand, although mouse TSLP is the ortholog of human long TSLP (Fornasa et al., 2015), TSLP variants in mice are currently uncertain as a murine short TSLP variant has not been described or annotated in Reference Sequence so far (Bjerkan et al., 2016). Harada et al. (2009) first demonstrated the functional analysis of TSLP variants in human bronchial epithelial cells, indicating the long TSLP variant highly expression after poly(I:C)/dsRNA stimulation, contributed to Th2-polarized immunity. In contrast to long TSLP, short TSLP has been rarely described at the protein level or functionally studied, but the latest two reports showed short TSLP was constitutively expressed in healthy skin, gut, oral mucosa, and primary oral keratinocytes (Bjerkan et al., 2015; Fornasa et al., 2015), which were in parallel with earlier observations in keratinocytes (Xie et al., 2012). Besides, short TSLP exerted an antimicrobial activity (Bjerkan et al., 2015) and strongly impaired the capacity of both peripheral blood mononuclear cells and monocyte-derived dendritic cells to produce inflammatory cytokines such as IFN- γ (Fornasa et al., 2015). Moreover, short TSLP prevented LPS-induced endotoxin shock and ameliorated experimental colitis (Fornasa et al., 2015). Most importantly, our latest study showed short TSLP prevented house dust mite (HDM)-induced airway epithelial barrier disruption (Dong et al., 2016), suggesting a protective role of short TSLP in HDM-induced asthma model. However, the role of short TSLP in prevention and treatment of toluene diisocyanate (TDI)-induced airway inflammation is currently not evaluated.

Diisocyanates, such as TDI, are important sensitizers that are used in the production of rigid or flexible polyurethane foam. They have been the most common cause of occupational asthma in many industrialized areas (Tarlo and Lemiere, 2014). TDI is a hapten and requires conjugation to autologous or heterologous proteins to produce a functional allergen. Although considerable controversy remains regarding its pathogenesis, TDI-induced asthma is characterized by Th2-dominated airway inflammation along with neutrophil and eosinophil infiltrating into the airways (De Vooght et al., 2013; Folmsbee et al., 2015; Liang et al., 2015a; Long et al., 2016; Yao et al., 2016b). It is distinct from the complete antigens such as HDM-induced atopic asthma, which are characterized by eosinophilic airway inflammation (Verheijden et al., 2015; Vroman et al., 2016). The other major difference between 2 asthma models is that TDI, being a low-molecular-weight sensitizer, could result in an irritant-induced occupational asthma in the absence of sensitization. Injury to the airway epithelium is likely to play a central role in the pathogenesis of irritant-induced asthma (Tarlo and Lemiere, 2014).

Many studies have identified the upregulation of high mobility group box 1 (HMGB1) in the lungs of asthma models (Lee et al., 2013; Liang et al., 2015a; Tang et al., 2014; Yao et al., 2015a) and in the sputum of asthmatic patients (Cuppari et al., 2015; Hou et al., 2011; Zhou et al., 2012). Receptor for advanced glycation end products (RAGE) is a member of the immunoglobulin superfamily of cell surface receptors and HMGB1 is one of the

its ligands (Liang et al., 2015b; Sukkar et al., 2012). Studies suggested that absence of RAGE or inhibition of HMGB1 significantly attenuated ovalbumin (OVA) and HDM induced airway inflammation (Lee et al., 2013; Shim et al., 2012; Ullah et al., 2014). Recently, we have also reported that HMGB1-RAGE axis played a critical role in TDI-induced airway inflammation by using specific RAGE or HMGB1 antagonists (Tang et al., 2014; Yao et al., 2015a, 2016a). These findings prompted us to investigate whether short TSLP could interfere with HMGB1-RAGE axis in TDI-induced murine asthma model.

MATERIALS AND METHODS

Special reagents. TDI (toluene-2, 4-diisocyanate, $\geq 98.0\%$), methacholine and acetone were obtained from Sigma-Aldrich (Shanghai, China). The vehicle (AOO) used to dissolve TDI consists of a mixture of 2 volumes of acetone and 3 volumes of olive oil for dermal sensitization, and 1 volume of acetone and 4 volumes of olive oil for airway challenge. Synthetic short TSLP peptides (63aa: MFAMK TKAAL AIWCP GYSET QINAT QAMKK RRRK VTTNK CLEQV SQLQG LWRRF NRPLL KQQ, molecular weight 7.43 kDa) (Bjerkan et al., 2015) were custom-prepared by ChinaPeptides company (Shanghai, China) and resuspended in sterile water. The purity of the peptides ($>95\%$) was confirmed by high performance liquid chromatography (HPLC) analysis.

Animals and protocols. 6–8-Week-old specific-pathogen-free (SPF) male BALB/c mice (± 20 g) were purchased from Southern Medical University. The mice were housed in a SPF house with 12 h dark/light cycles and fed with sterile water and irradiated food *ad libitum*. All studies were conducted in accordance with the committee of Southern Medical University on the use and care of animals. The protocols were approved by the Animal Subjects Committee of Nanfang Hospital (NFYY-2016-19). Animal models were generated as described previously (Liang et al., 2015a; Yao et al., 2015a,b; Yao et al., 2016a,b). Briefly, male BALB/c mice were dermally sensitized with 0.3% TDI on the dorsum of both ears ($20 \mu\text{l}$ per ear) on days 1 and 8. On days 15, 18, and 21, mice were individually placed in a horizontal cylindrical chamber and challenged through the airways with 3% TDI dissolved in acetone/olive oil (1:4) by means of compressed air nebulization (NE-C28; Omron, Tokyo, Japan) for 3 h at each time. As a control, mice were sensitized and challenged by the same procedures with the same amount of AOO.

Synthetic short TSLP peptides treatment. To assess the role of short TSLP on airway inflammation and hyperresponsiveness, and to verify the effects of two different administration routes with short TSLP peptides in asthma model, synthetic short TSLP peptides were given by either i.n. or i.p. administration based in part on dosing regimens reported by Fornasa et al. (2015). A total of 40 mice were used ($n = 10$ for each group). For i.n. treatment, mice received $50 \mu\text{g}$ of short TSLP i.n. on days 15, 18, and 21. For i.p. treatment, mice received $200 \mu\text{g}$ of short TSLP i.p. on days 15, 18, and 21. The i.n. and i.p. administrations of short TSLP peptides were given 30 min before each challenge. The equal volume sterile water was given by i.n. administration in AOO and TDI group. Briefly, mice were randomized to the following four groups: (1) AOO-sensitized, AOO-challenged, and i.n. sterile water-treated (AOO group); (2) TDI-sensitized, TDI-challenged, and i.n. sterile water-treated (TDI group); (3) TDI-sensitized, TDI-challenged, and i.n. short TSLP-treated (TDI + i.n. short TSLP group); (4) TDI-sensitized, TDI-challenged, and i.p. short TSLP-treated (TDI + i.p. short TSLP group).

Assessment of airway hyperresponsiveness. Airway reactivity to methacholine was assessed 24 h after the third challenge

according to the previously described method (Liang *et al.*, 2015a). Briefly, mice were placed in a barometric plethysmographic chamber (Buxco Electronics, Troy, NY) and challenged with vehicle (sterile saline) followed by increasing concentrations of methacholine (3.125, 6.25, 12.5 mg/ml) by means of ultrasonic nebulization. Aerosols were generated with an matching ultrasonic nebulizer (Aeroneb Laboratory Nebulizer; Buxco Electronics, Inc, Troy, NY), and then nebulized into the main chamber through its inlet for 3 min. Pressure fluctuations caused by breathing of the mice were continuously monitored for 3 min after each nebulization, and subsequently these pressure fluctuations were quantified using the algorithm for enhanced pause (Penh, dimensionless parameter), which represented an accurate index of airway resistance (Lundblad *et al.*, 2002). Penh is a dimensionless value that represents a function of the ratio of peak expiratory flow (PEF) to peak inspiratory flow (PIF) and a function of the timing of expiration (Pause) ($\text{Penh} = \text{PEF}/\text{PIF} \times \text{Pause}$) (Ye *et al.*, 2012).

Quantification of total serum IgE. The mice were sacrificed as described previously (Liang *et al.*, 2015a). Blood samples collected were rested for 1 h at room temperature, then centrifuged ($3000 \times g$, 20 min) and supernatants were harvested and stored at -80°C . Total serum IgE was measured by ELISA (eBioscience, San Diego, CA) according to the manufacturer's instructions.

Analysis of bronchoalveolar lavage fluids. The recovered bronchoalveolar lavage (BAL) fluids were pooled. Total cells in the BAL fluids were counted, and the BAL fluids were centrifuged ($1000 \times g$, 10 min). The supernatant was frozen (-80°C) for further analysis of IFN- γ , IL-4, IL-5, IL-13, and TSLP by multiplex immunoassays (eBioscience) according to the manufacturer's instructions. The upper and lower limit of quantification of IFN- γ , IL-4, IL-5, IL-13 and TSLP are 4250/1.04, 4450/1.09, 9800/2.39, 9500/2.32 and 35700/8.72 pg/ml, respectively. For differential cell counts, a cytospin sample (Shandon Scientific, Runcorn, UK) was prepared and stained using hematoxylin and eosin (H&E) (Solarbio, Beijing, China). For each sample, a total of 200 cells were counted for the numbers of macrophages, eosinophils, neutrophils, and lymphocytes.

Measurement of IFN- γ , IL-4, IL-5, IL-13 in supernatants of cultured lymphocytes. Cervical lymph nodes were dissected from the mice and processed for each mouse separately, then kept on ice in RPMI-1640 medium. Cell suspensions were obtained by pressing the lymph nodes through a cell strainer ($40 \mu\text{m}$) (BD Bioscience, Erembodegem, Belgium) and rinsing with 10 ml of tissue culture medium (RPMI-1640). Cells were counted using a Bürker hemocytometer. Lymphocytes were then washed three times and suspended (10^7 cells/ml) in complete tissue culture medium (RPMI-1640 supplemented with 10% heat-inactivated FBS). Cells were seeded into 48-well culture plates at a density of 10^6 cells/ml and incubated in complete RPMI-1640 medium for 43 h with $5 \mu\text{g}/\text{ml}$ concanavaline A (Sigma-Aldrich), then centrifuged ($1000 \times g$, 4°C , 10 min). Supernatants were harvested for quantitative assessment of IFN- γ , IL-4, IL-5 and IL-13 by multiplex immunoassays (eBioscience) according to manufacturer's recommendations. The upper and lower limit of quantification of IFN- γ , IL-4, IL-5, and IL-13 were described above.

Pulmonary histopathological examination. Left lungs were dissected from mice, then fixed in 4% neutral formalin, dehydrated, and paraffin-embedded. Sections ($4 \mu\text{m}$) were cut with Leica microtome 2030 (Leica Microsystems Nussloch GmbH, Nussloch, Germany). Lung tissues were stained with H&E and Masson's trichrome (Solarbio) for blinded histopathologic assessment. As previously described (Yao *et al.*, 2016a), peribronchial and perivascular inflammation in H&E stained slides was

assessed using a scoring standard: 0 = normal; 1 = infrequent inflammatory cells; 2 = a ring of inflammatory cells 1 cell layer deep; 3 = a ring of inflammatory cells 2–4 cells deep; 4 = a ring of inflammatory cells >4 cells deep. Scoring was performed at a magnification of $200\times$ by examining at least 40 image fields of 20 slices from 6 mice per group. We measured the amount of collagen as previously described (Mizuguchi *et al.*, 2004). Briefly, the presence of collagen fibril deposition was estimated at a magnification of $200\times$ by scanning 10 no-repeated fields in each specimen with Masson staining from 6 mice per group. Blue-stained fibrotic areas were quantified by the Image-Pro plus 6.0 software. The results were expressed by the proportion of the relative volume of the scanned blue area (normalized to AOO group). Sections were assigned a random code to blind the examiner to the identity of each specimen.

Immunohistochemistry and Western blot. Lung sections were deparaffinized, then submerged in citrate buffer (pH 6.0) for antigen retrieval. Samples were treated with H_2O_2 for 10 min to block endogenous peroxidase, blocked with 5% BSA for 20 min at room temperature and then stained with monoclonal rabbit anti-HMGB1 antibody (ab79823, 1:600, Abcam, Cambridge, MA), polyclonal rabbit anti-RAGE antibody (ab37647, 1:100, Abcam), rabbit monoclonal to p-STAT3(Y705) (9145P, 1:50, CST, Danvers, MA), rabbit monoclonal to p-STAT5 (Y694) (ab32364, 1:50, Abcam) and polyclonal rabbit anti-TSLP antibody (ab188766, 1:100, Abcam) respectively and incubated overnight at 4°C . After washing with PBS, slices were incubated with anti-rabbit IgG (Boster, Wuhan, China) for 20 min and then stained with SABC (Boster) for 20 min at room temperature. Signals were visualized with a DAB peroxidase substrate kit (Boster). Proteins localization in the lung was assessed under light microscopy.

To evaluate the protein expression of HMGB1, RAGE, STAT3, STAT5, and TSLP, whole lung tissues were collected from the experimental mice, and lysed in buffer containing protease inhibitor, calcineurin inhibitors and PMSF. Protein extracts were subjected to 10% or 12% SDS-PAGE, transferred to PVDF membrane (Millipore, Bedford, MA), and then probed with the following antibodies: HMGB1, RAGE, STAT3, p-STAT3(Y705), STAT5, p-STAT5(Y694), and TSLP, then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (1:4000, CST) as the secondary antibody for 1 h at room temperature. The membranes were visualized by enhanced chemiluminescence (Millipore). Quantitative image analysis was performed with an Image J software.

Preparation of TDI-HSA conjugates. TDI-human serum albumin (HSA) (both TDI and HSA are from Sigma-Aldrich) conjugates were prepared by a modification of Son's method (Son *et al.*, 1998) previously described (Zhao *et al.*, 2009). The molar ratio of TDI to HSA was 5.32. Each concentration of TDI-HSA we used had no detectable effect on cell viability (data not shown).

Cell culture and treatment. Human bronchial epithelial cells line 16HBE14o-(16HBE) cells (Bio-Rad Laboratories (Shanghai) Co, Ltd, ATCC, Portland, Oregon) were cultured in RPMI-1640 medium (Hyclone) containing 10% fetal calf serum (Hyclone) at 37°C and 5% CO_2 . Medium was changed every other day, and cells passaged to five to eight generations were used for the following experiments. When reaching 90% confluence, the cells were passaged and seeded to proper culture plates at a density of 10^4 – 10^5 cells/ cm^2 . After 24 h incubation, the culture medium was changed to serum free RPMI-1640. Different concentrations of TDI-HSA conjugates (0–100 $\mu\text{g}/\text{ml}$) or rhHMGB1 (0–1000 ng/ml, Sigma-Aldrich) were then added to the culture medium for indicated times with or without the pre-treatment of short TSLP peptides (50 ng/ml, 12 h). The concentrations of TDI-HSA (Song

et al., 2013) and rhHMGB1 (Liang et al., 2015b; Ojo et al., 2015) used in this experiment were partly based on previous studies. We compared the effects of different concentrations of HSA control reagent (0–100 µg/ml) on TSLP variants mRNA and the long TSLP protein expression to the blank control. There were no statistical differences (data not show). Hence we used the blank control as the control group for the following experiments.

Western blot. To evaluate the protein expression of HMGB1, RAGE, long TSLP and the activation of signaling pathways in vitro, cells protein extracts were subjected to 10% or 12% SDS-PAGE, transferred to PVDF membrane (Millipore), and then probed with antibodies as follows: anti-HMGB1 (ab190377, 1:2000, Abcam), anti-RAGE (MAB5328, 1:500, Millipore), anti-Akt, anti-p-Akt(S473), anti-p38 MAPK, anti-p-p38 MAPK(T180/Y182), anti-JNK, anti-p-JNK, anti-STAT3, anti-p-STAT3(Y705), anti-STAT5 (1:1000, all from CST) and anti-p-STAT5 (Y694) (ab32364, 1:1000, Abcam) and anti-TSLP antibody (ab47943, 1:1000, Abcam), then with HRP-conjugated donkey anti-rabbit or anti-mouse IgG antibody (1:4000, CST) as the secondary antibody for 1 h at room temperature. Because the short TSLP encodes a sequence that is identical in the C-terminal region of long TSLP as described in the introduction, the anti-TSLP antibody could recognize both short TSLP and long TSLP. Based on their different molecular weight (short TSLP < 10 kDa, long TSLP is ~25 kDa), we cut away the lower molecular weight field from the SDS-PAGE (<10 kDa), finally there was only one obvious band in the field of 15–55 kDa just as the instruction of ab47943 indicated, which was the long TSLP (25 kDa). The membranes were visualized by enhanced chemiluminescence. Quantitative image analysis was performed with an Image J software.

Measurement of human TSLP variants mRNA expression by real-time quantitative PCR. Real-time quantitative PCR (qPCR) method was used for the determination of the differential gene expression of long TSLP and short TSLP in 16HBE cells. Total cellular RNA was isolated from 16HBE cells using TRIzol reagent (Takara, Otsu, Japan), and cDNA was synthesized using the PrimeScript RT Reagent Kit (Takara). The qPCR reactions were performed on the Mx3005P (USA) using SYBRPremix Ex Taq II (Takara). The primer sequences were as follows: long TSLP: 5'-CAC CGT CTC TTG TAG CAA TCG-3' and 5'-TAG CCT GGG CAC CAG ATA GC-3'. Short TSLP: 5'-CGT AAA CTT TGC CGC CTA TGA-3' and 5'-TTC TTC ATT GCC TGA TTT AT-3'. β -Actin: 5'-TCA CCC ACA CTG TGC CCA TCT ACG A-3' and 5'-CAG CGG AAC CGC TCA TTG CCA ATG G-3'. The cycling conditions used were 95 °C for 15 s, 60 °C for 1 min, for 40 cycles, followed by a melting point determination or dissociation curves. Relative gene expression to control was calculated using the $2^{-\Delta\Delta CT}$ method.

Immunofluorescence microscopy. To examine the localization of HMGB1 and RAGE after TDI-HSA stimulation, we performed double immunofluorescent techniques. 16HBE cells (2×10^5 cells/well) were cultured in RPMI-1640 containing 10% (v/v) FBS on Glass Bottom Cell Culture Dish (NEST, China) and were treated with TDI-HSA (80 µg/ml) for 8 h at 37 °C with or without the short TSLP pre-treatment (50 ng/ml, 12 h). Then 16 HBE cells were fixed with 4% (v/v) formaldehyde in PBS for 10 min and treated with 0.5% (v/v) Triton X100 for 5 min, and blocked with 5% (w/v) BSA for 2 h at room temperature, then incubated with mouse monoclonal anti-HMGB1 antibody (ab190377, 1:500, Abcam) and rabbit polyclonal anti-RAGE antibody (ab37647, 1:500, Abcam) overnight at 4 °C. The cells were incubated with Alexa 488-labeled goat anti-rabbit IgG antibody and Alexa 594-labeled goat anti-mouse IgG antibody (Life Technologies, Grand Island, NY) for 1 h in the dark at room temperature. The cells nuclei were

stained with 4', 6-diamidino-2-phenylindole (DAPI) dihydrochloride (Beyotime Biotechnology, China) for 10 min. A laser-scanning confocal microscope (Olympus, Tokyo, Japan) was used to examine the distribution of RAGE and HMGB1.

Statistical analysis. SPSS 20.0 software was used for the statistical analyses. Statistical analysis was performed by one-way analysis of variance (ANOVA) and post hoc tests were analyzed by Bonferonni (equal variances assumed) or Dunnett's T3 (equal variances not assumed) post hoc tests for multiple comparisons. Results were presented as means $\pm$ SD. $P < 0.05$ was considered statistically significant.

RESULTS

Short TSLP Decreased AHR and Total Serum IgE in TDI Mice

Airway reactivity was assessed 24 h after the third challenge. Results showed that TDI-induced asthmatic mice exhibited a significant increase in airway hyperresponsiveness (AHR) compared with AOO group when provoked by methacholine (3.125, 6.25, and 12.5 mg/ml; Figure 1A, $P < 0.05$), which could be partly recovered by i.n. administration with short TSLP (Figure 1A, $P < 0.05$). And the i.p. administration of short TSLP also ameliorated AHR when provoked by methacholine at the concentration of 12.5 mg/ml (Figure 1A, $P < 0.05$), indicating an antagonistic role of short TSLP for TDI-induced AHR. Similarly, the level of total serum IgE increased by TDI was markedly inhibited by i.n. but not by i.p. administration of short TSLP (Figure 1B, $P < 0.05$).

Short TSLP Inhibited the Levels of Th1/2 Cytokines in Supernatants of Cultured Lymphocytes

TDI-induced occupational asthma is characterized by a mixture of Th1 (IFN- γ) and Th2 (IL-4, IL-5, IL-13) airway inflammation along with neutrophil and eosinophil infiltrating into the airways (Song et al., 2013; Yao et al., 2015b). To evaluate the effect of short TSLP on TDI-induced allergic airway inflammation, we detected the levels of Th1/2 cytokines in BAL fluids and in supernatants of cultured lymphocytes. Results showed that both i.p. and i.n. administration of short TSLP peptides decreased the secretion of IFN- γ , IL-4, IL-5, and IL-13 in supernatants of cultured lymphocytes in TDI-induced asthmatic mice (Figure 1C, $P < 0.05$). Disappointedly, we failed to detect the cytokines (IFN- γ , IL-4, IL-5, IL-13, and TSLP) in BAL fluids, which is possibly related to the low concentrations of these cytokines in BAL fluids. Consistently, study showed chemical-induced asthma is characterized by airway hyperreactivity and little airway inflammation (Devos et al., 2016).

Short TSLP Ameliorated TDI-Induced Airway Inflammation

Total cell counts, as well as percentages of different inflammatory cells were assessed in BAL fluids (Figure 1D–F). In line with total cell counts (Figure 1E), higher amounts of neutrophils and eosinophils were found after sensitization and challenge with TDI ($P < 0.05$), which were significantly decreased by two form administrations with short TSLP peptides ($P < 0.05$). Similarly, histologic analysis revealed typical pathologic features of TDI-induced airway disease. Numerous inflammatory cells infiltrated around the bronchus, and the lining airway epithelium was thickened and proliferated in TDI group. Mice treated with short TSLP showed marked reductions in extravasation of inflammatory cells in peribronchial and perivascular regions and the proliferation of bronchial epithelial cells but had no

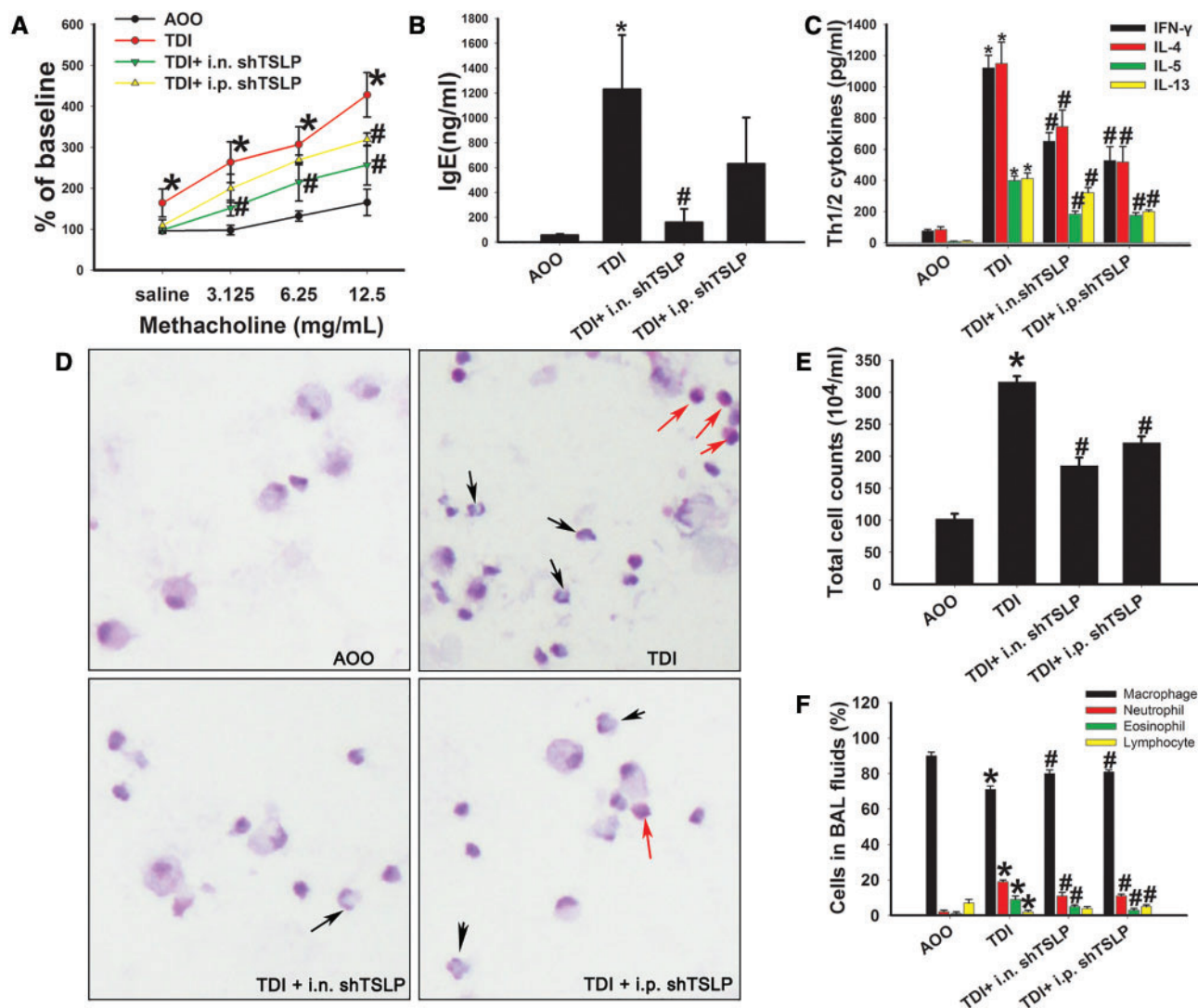


FIG. 1. TDI-induced airway hyperresponsiveness, total serum IgE, Th1/2 cytokines, and inflammatory cells accumulation in BAL fluids. A, Penh was used to assess airway reactivity to methacholine. Results are expressed as the percentage of baseline Penh value for each concentration of methacholine. B, Total IgE in the sera. C, Levels of Th1-related IFN- γ and Th2-related IL-4, IL-5, IL-13 in lymphocyte culture supernatants. D, Representative cell smears from BAL fluids stained with H&E. Black arrows indicate neutrophils, and red arrows indicate eosinophils. Original magnification was $\times 200$. E and F, Total and differential inflammatory cell counts in BAL fluids. $n = 6$ per group. * $P < 0.05$ vs AOO group; # $P < 0.05$ vs TDI group. Abbreviation: H&E, hematoxylin and eosin.

effect on the thickening of the epithelial layer and subepithelial collagen fibrils deposition (Figure 2A–C).

Short TSLP Suppressed RAGE, HMGB1 and Mouse TSLP Expression in Lungs of TDI-Treated Mice

To evaluate the effect of short TSLP on HMGB1-RAGE and mouse TSLP expression in this model, Western blot and Immunohistochemistry methods were used. As expected, HMGB1, RAGE and mouse TSLP expression were increased in lungs of TDI-treated mice and decreased after short TSLP administration (Figure 3A–E, $P < 0.05$). Immunohistochemistry showed that expression of HMGB1 was faint and mainly located in the nucleus of all cell types in AOO group. In consistent with our previous study (Liang et al., 2015a), TDI exposure leads to nucleocytoplasmic translocation of HMGB1 in airway epithelia and infiltrating inflammatory cells (Figure 3B). In TDI-exposed mice, RAGE was also abundantly expressed in the bronchial epithelial and much less in the infiltrating inflammatory cells (Figure 3A). The short TSLP treatment obviously ameliorated

these changes. Results showed that mouse TSLP was also most abundantly expressed in bronchial epithelia and infiltrating inflammatory cells in the lungs of TDI-exposed mice (Figure 3C). As expected, the increased mouse TSLP induced by TDI was dramatically inhibited by i.n. or i.p. administration of short TSLP peptides (Figure 3C).

Short TSLP Inhibited STAT5(Y694) but Not STAT3(Y705)

Phosphorylation in TDI-Asthmatic Mice

STAT3 and STAT5 are the critical downstream molecules of TSLP-TSLP receptor signaling (Wohlmann et al., 2010). Inhibitions of STAT3 (Gavino et al., 2016) and STAT5 (Hausding et al., 2011) could prevent lung inflammation and remodeling. In this model, we also evaluated the phosphorylation of STAT3(Y705) and STAT5(Y694) in lungs exposed to TDI. It was the p-STAT5 (Y694) that significantly increased in TDI-treated mice and decreased after short TSLP treatment (Figure 4B, C and E, $P < 0.05$). As shown in immunohistochemistry, p-STAT5 (Y694) was obviously increased in airway epithelia and

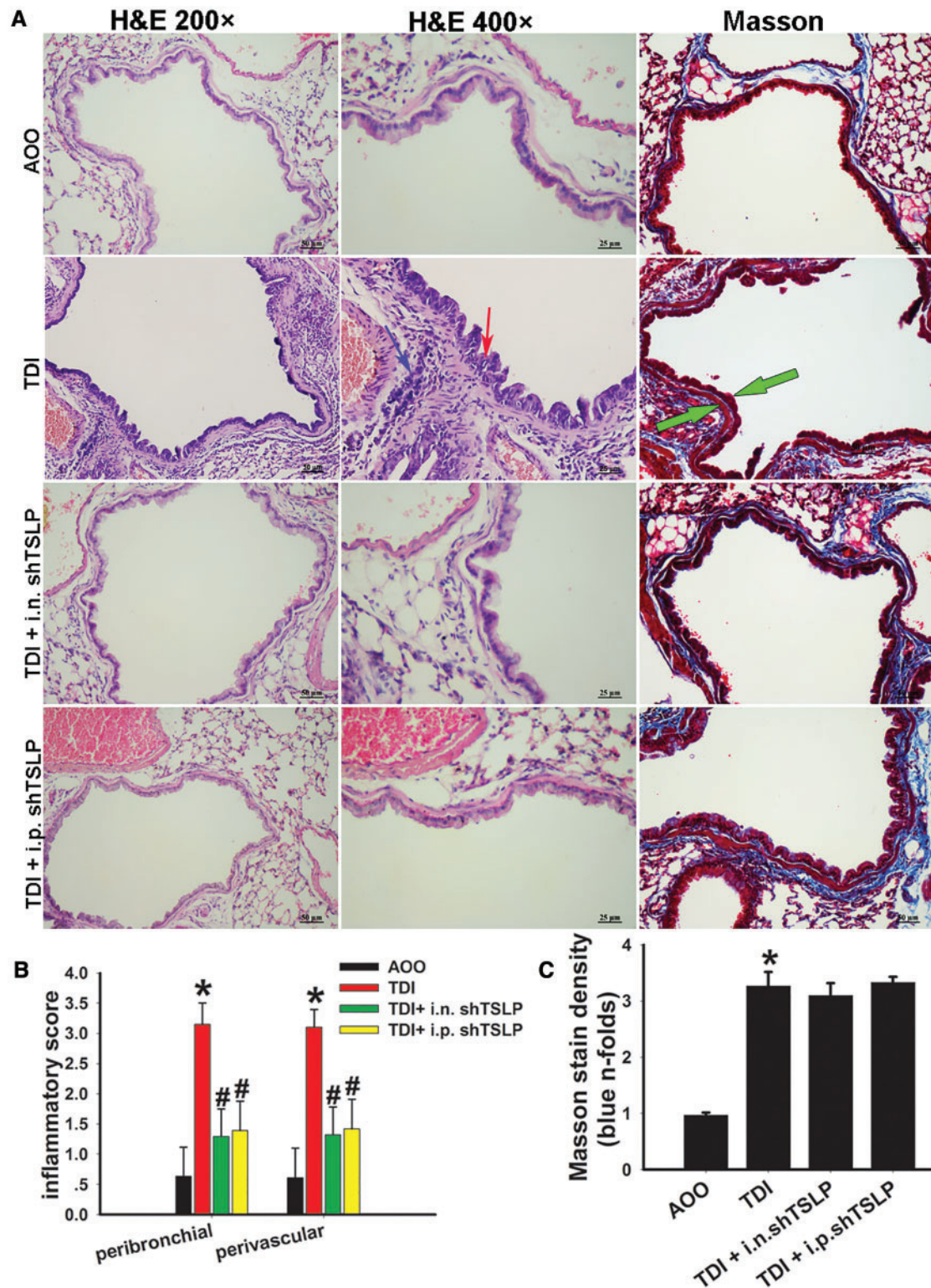


FIG. 2. TDI-induced pulmonary pathological changes. A, Representative H&E-stained lung sections of different treatment groups at $\times 200$ (left panel) and $\times 400$ (middle panel) original magnification and Masson trichrome-stained sections at $\times 200$ magnification (right panel). Blue arrow indicates inflammatory infiltration in peribronchial regions; red arrow indicates the proliferation of bronchial epithelial cells; green arrows indicate the thickening of the epithelial layer. B and C, Semi-quantification of peribronchial and perivascular inflammation as well as Masson's trichrome staining. $n = 6$ per group. $^*P < 0.05$ vs AOO group; $^{\#}P < 0.05$ vs TDI group. Abbreviation: TDI, toluene diisocyanate.

infiltrating inflammatory cells, showing cytoplasm localization in TDI mice. The phosphorylation of STAT5(Y694) was largely decreased after two form administrations with short TSLP

peptides (Figure 4B, C and E, $P < 0.05$). However, the level of p-STAT3(Y705) was not changed in each group (Figure 4A, C and D).

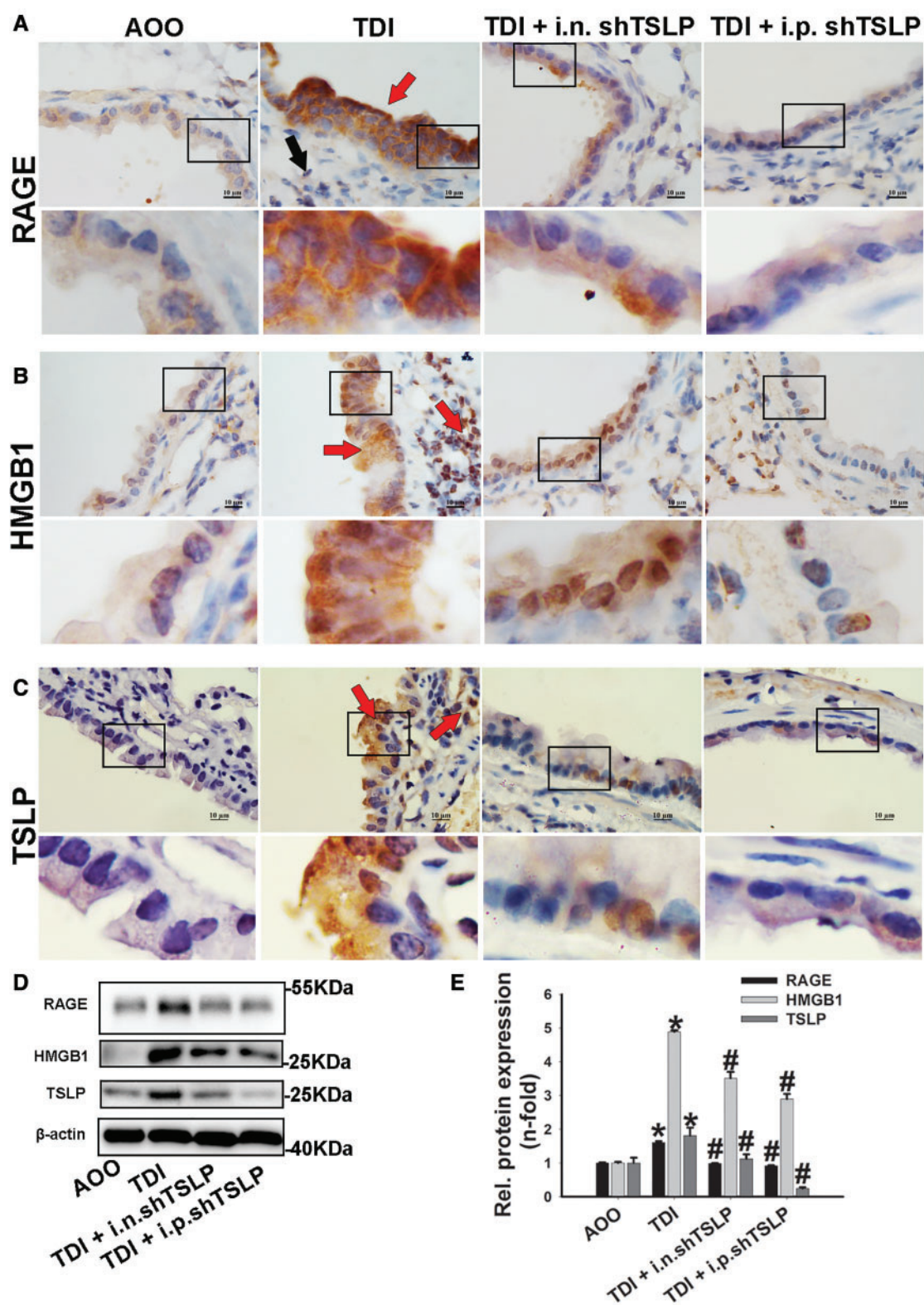


FIG. 3. Pulmonary expressions of RAGE, HMGB1 and mouse TSLP. A–C, Representative immunohistochemistry of RAGE, HMGB1 and mouse TSLP in lung (upper panel) and the amplified view of them in lung (lower panel). Red arrows indicate most abundant expression of proteins. Black arrows indicate less expression of proteins. Original magnification was $\times 1000$. D, Western blot analysis of RAGE, HMGB1 and mouse TSLP in whole lung and E, desitometric analysis of the blots. $n = 6$. * $P < 0.05$ compared with AOO group; # $P < 0.05$ compared with TDI group. Abbreviations: HMGB1, high mobility group box 1; RAGE, receptor for advanced glycation end products; TSLP, thymic stromal lymphopoietin.

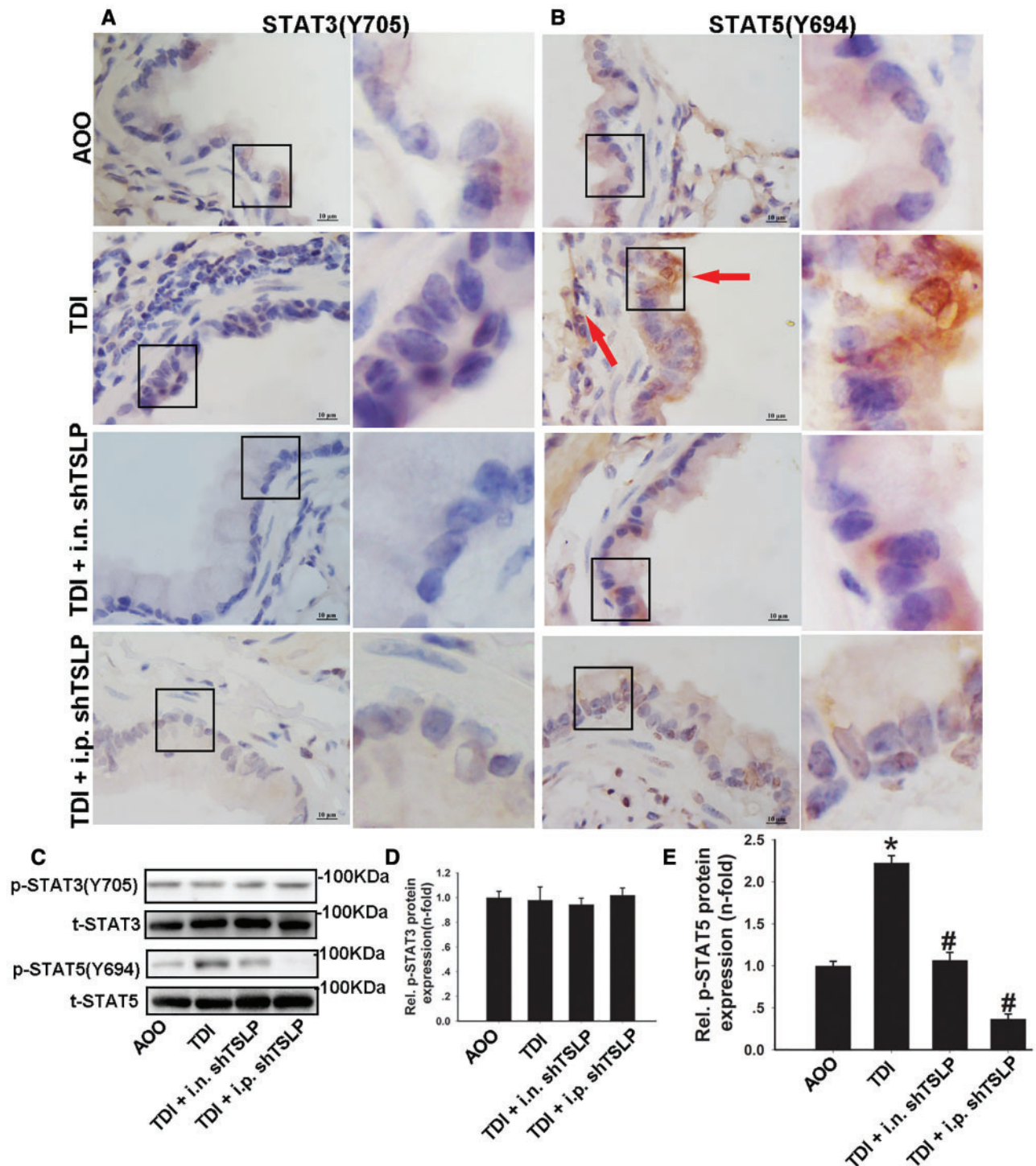


FIG. 4. Phosphorylation of STAT3(Y705) and STAT5(Y694) in lung tissues. A and B, Representative immunohistochemistry of p-STAT3(Y705) (A) and p-STAT5(Y694) (B) in lung. Original magnification was $\times 1000$ (left panel). Amplified view in the bronchial epithelia (right panel). Red arrows indicate most abundant expression of p-STAT5 in airway epithelia and infiltrating inflammatory cells. C, Western blot analysis of STAT3(Y705) and STAT5(Y694) in whole lung and D and E, densitometric analysis of the blots. $n = 6$. * $P < 0.05$ compared with AOO group; # $P < 0.05$ compared with TDI group.

Effect of Short TSLP on the Pulmonary Histopathology Changes in AOO Mice

To address the toxicity effect of short TSLP on normal BALB/c mice, we detected the pulmonary histopathology changes in AOO mice treated by short TSLP. H&E-stained analysis revealed that little inflammatory cells infiltration and the lining airway epithelium was not thickened and proliferated in each group

(Figure 5). This indicated short TSLP had no significant toxicity effect on the pulmonary histopathology changes in AOO mice.

Both TDI-HSA and rhHMGB1 Promoted RAGE and Long TSLP Expression in 16HBE Cells

To further clarify the effect of TDI-HSA and rhHMGB1 on human TSLP variants, we detected long TSLP and short TSLP gene

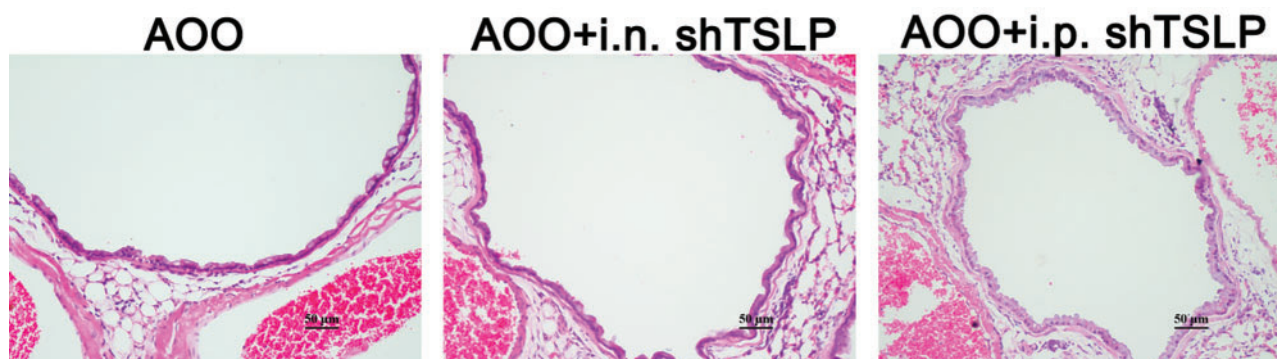


FIG. 5. Effect of short TSLP on the pulmonary histopathology changes in AOO mice. Representative H&E-stained lung sections of different treatment groups at $\times 200$ original magnification. AOO mice received sterile water i.n. (AOO group) or 50 μg of short TSLP i.n. (AOO + i.n. shTSLP) or 200 μg of short TSLP i.p. (AOO + i.p. shTSLP) on days 15, 18, and 21. The administrations of short TSLP peptides were given 30 min before each challenge.

expression in 16HBE cells by real time qPCR after stimulation with TDI-HSA and rhHMGB1, respectively. Results showed that both TDI-HSA and rhHMGB1 significantly upregulated long TSLP mRNA expression (6.1 times and 15.9 times, respectively) but not short TSLP (0.9 times and 1.04 times, respectively) compared with control group (Figure 6A and B). Similarly, Western blot also showed that both TDI-HSA (Figure 6C–F, $P < 0.05$) and rhHMGB1 (Figure 6G–J, $P < 0.05$) increased RAGE and long TSLP protein expression. In addition, TDI-HSA also stimulated HMGB1 production in 16HBE cells (Figure 6C–F, $P < 0.05$).

Both TDI-HSA and rhHMGB1 Activated STAT5(Y694), Akt(S473) and p38 MAPK(T180/Y182) Signals in 16HBE Cells

Except for the STAT3(Y705) and STAT5(Y694) signals described above, sensitized mice exposed to inhaled allergens show increased activation of p38 MAPK (Liang et al., 2013), JNK (Nath et al., 2005), PI3K/Akt (Liang et al., 2015a) in airway epithelial cells and lung. Here we further detected the phosphorylation of STAT3(Y705), STAT5(Y694), Akt(S473), p38 MAPK(T180/Y182) and JNK in 16HBE cells. Western blot showed that STAT5(Y694), Akt(S473) and p38 MAPK(T180/Y182) phosphorylation were increased (Figure 7A–D, $P < 0.05$), but STAT3(Y705) and JNK were not changed after TDI-HSA or rhHMGB1 stimulation (Figure 7A–D).

Short TSLP Inhibited Long TSLP and RAGE Expression and STAT5(Y694), Akt(S473) and p38 MAPK(T180/Y182) Activation in 16HBE Cells

To evaluate the effect of short TSLP on RAGE and long TSLP expression induced by both TDI-HSA and rhHMGB1, 16HBE cells were pre-treated by short TSLP peptides (50 ng/ml) for 12 h. Western blot indicated that short TSLP decreased RAGE and long TSLP protein expression induced by both TDI-HSA and rhHMGB1 (Figure 8A and B, $P < 0.05$). Furthermore, double immunofluorescent (Figure 8C) indicated that in control 16HBE cells, HMGB1 was only expressed in the nucleus. In TDI-HSA-treated cells, HMGB1 staining was observed not only in the nucleus but also in the cytosol. Interestingly, after short TSLP pre-treatment, the localization of HMGB1 in the cytosol in TDI-HSA-treated cells disappeared. In addition, results showed the short TSLP pre-treatment can also inhibit the levels of STAT5(Y694), Akt(S473) and p38 MAPK(T180/Y182) phosphorylation induced by TDI-HSA or rhHMGB1 *in vitro* (Figure 8D and E, $P < 0.05$).

DISCUSSION

To the best of our knowledge, it is the first time to imply a critical role for synthetic short TSLP variant in attenuating TDI-induced airway inflammation and inhibiting HMGB1-RAGE and long TSLP expression and STAT5(Y694), Akt(S473) and p38 MAPK(T180/Y182) phosphorylation.

Whether mouse TSLP exhibits two variants have not yet been investigated (Bjerkkan et al., 2016). However, Fornasa et al. (2015) proved that human short TSLP had anti-inflammatory properties in the mouse models of endotoxin shock and experimental colitis. It could be attributed to the fact that the C-terminal region of mouse TSLP shares 40% identity with human short TSLP. Here we further confirmed that human short TSLP prevents airway inflammation in a chemical-induced asthma model. We found that two form administrations of short TSLP (i.n. and i.p.) significantly reduced inflammatory cells infiltration in peribronchial and perivascular regions and the proliferation of bronchial epithelial cells, resulted in a significant decrease in BAL fluid leukocytes numbers, and inhibited the levels of Th1-related IFN- γ and Th2-related IL-4, IL-5, IL-13 in supernatants of cultured lymphocytes. However, in this model, neither the thickening of the epithelial layer nor subepithelial collagen fibrils deposition was rescued. A possible explanation is that the repair of airway remodeling needs longer treatment period.

Although the sequence of short TSLP is identical to the C-terminal portion of long TSLP, they seemed to exert exactly opposite functions (Fornasa et al., 2015). The long TSLP is well known as a proinflammatory cytokine (Cheng et al., 2013; Leyva-Castillo et al., 2013) and the short TSLP could have anti-inflammatory properties (Fornasa et al., 2015). For the moment, the biological character of human short TSLP is still largely unknown. It is more like an antimicrobial peptide (AMP) from its special structures characterized as α -helical regions, structural features shared with many classical AMPs (Sonesson et al., 2011). As an AMP, short TSLP may also have toxic effects, especially systemic administration with a larger concentration. On the other hand, lung as a therapeutic target of asthma, local intrapulmonary administration of short TSLP could predict a better anti-inflammatory effect than systemic intraperitoneal administration. These may at some degree explain some of our findings that mice received 200 μg of short TSLP by i.p. administration exhibited less effective on AHR and total serum IgE than mice received 50 μg of short TSLP by i.n. administration. However, two different administration routes with two different dosages limited the direct compare of their effects, and using

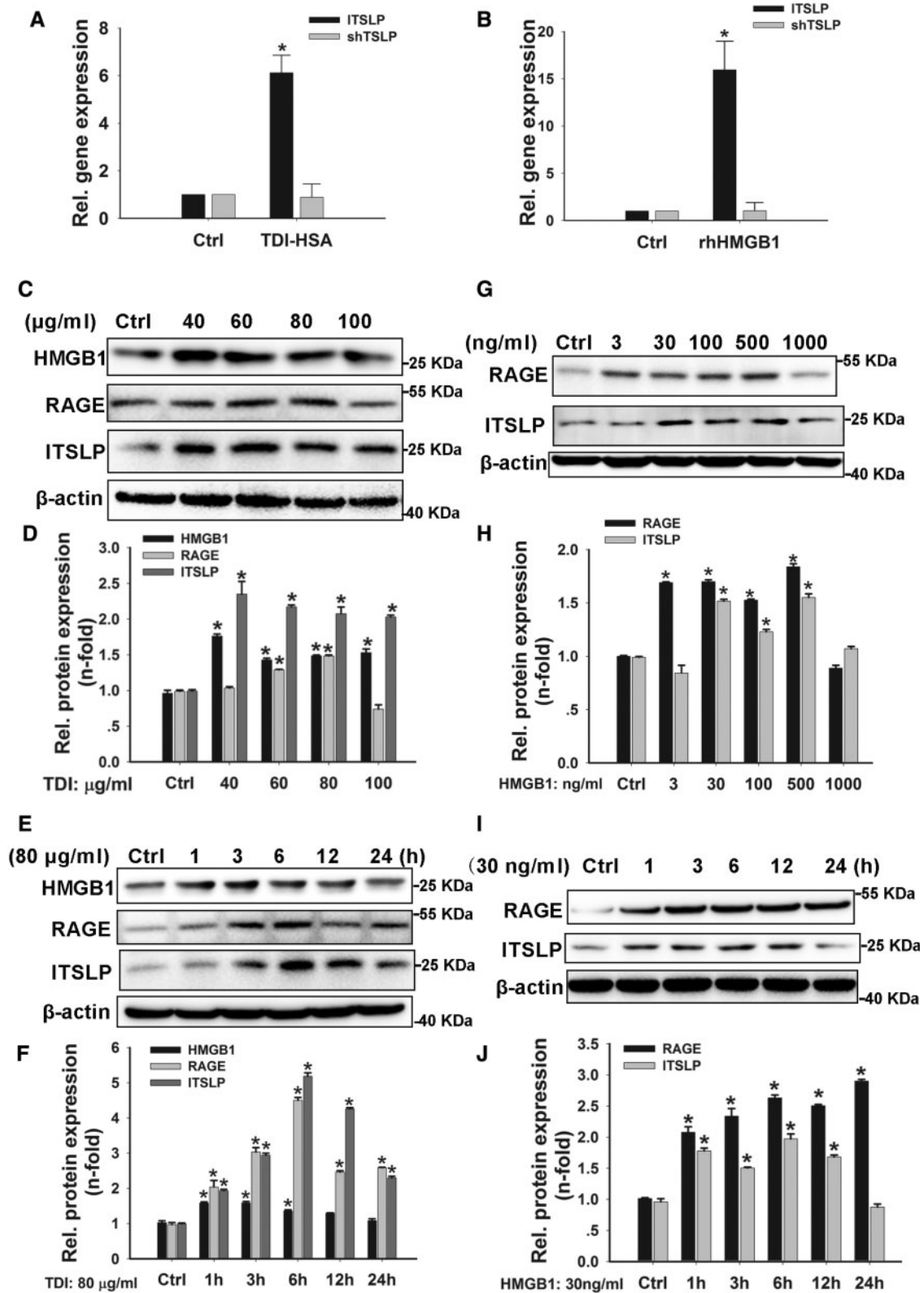


FIG. 6. Expressions of human TSLP variants mRNA and HMGB1, RAGE and long TSLP proteins in 16HBE cells. A and B, 16HBE cells were stimulated with TDI-HSA (80 µg/ml) or rhHMGB1 (30 ng/ml) for 4 h. Both TDI-HSA(A) and rhHMGB1(B) upregulated the long TSLP but not short TSLP mRNA production. C, E, G and I, Western blot analysis of HMGB1, RAGE and long TSLP expression in 16HBE cells and D, F, H and J, densitometric analysis of the blots. C, D and G, H, Different concentrations of TDI-HSA (C and D) or rhHMGB1 (G and H) stimulated cells for 6 h, and E, F and I, J, cells were treated by TDI-HSA (80 µg/ml) (E-F) or rhHMGB1 (30 ng/ml) (I-J) for different times, and the control cells were treated by PBS for 24 h. Data are mean $\pm$ SD of three independent experiments. * P < 0.05 compared with control group. Abbreviation: HSA, human serum albumin.

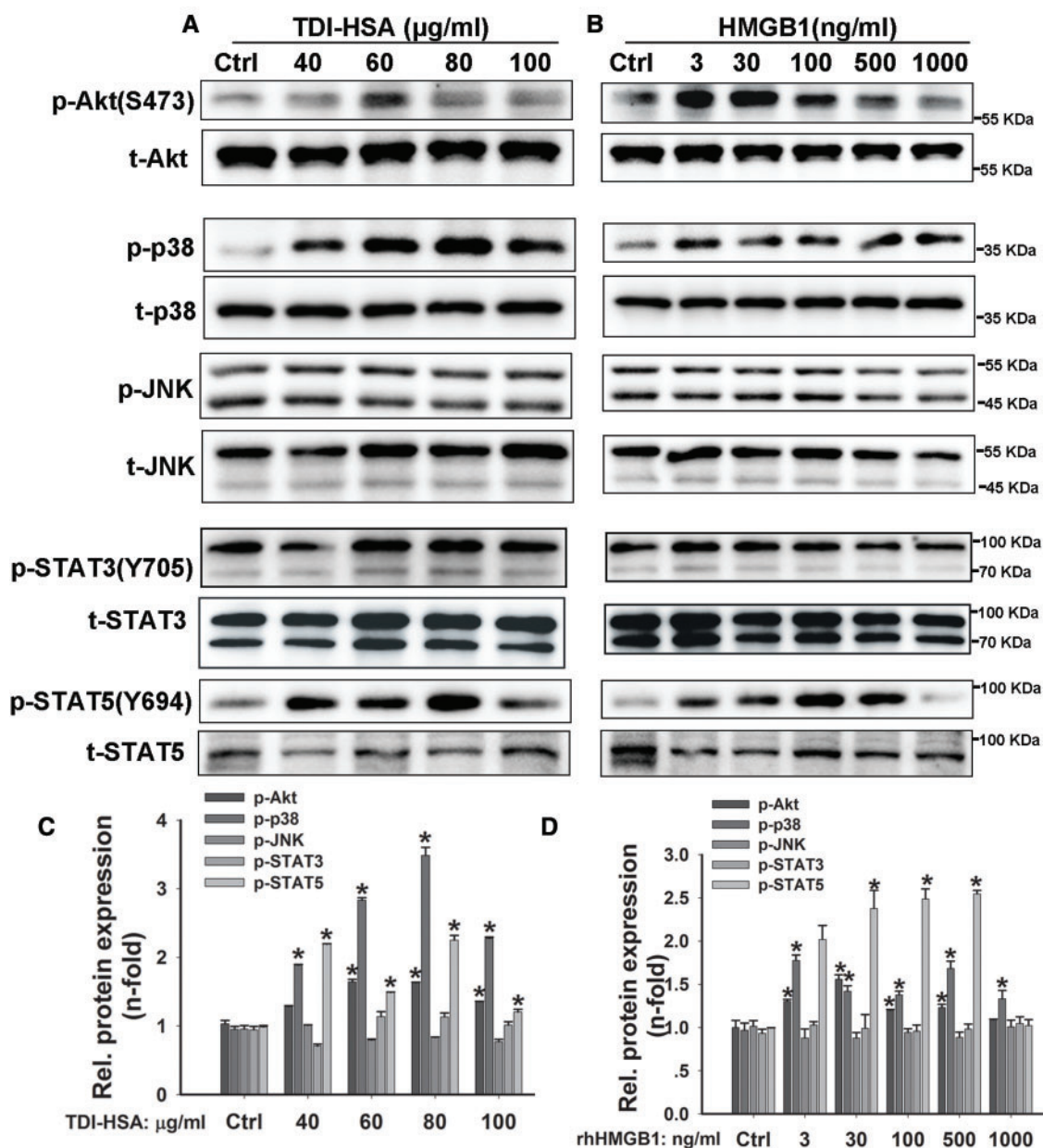


FIG. 7. Effects of TDI-HSA or rhHMGB1 on signaling systems activation in 16HBE cells. A and B, Western blot analysis of STAT3(Y705), STAT5(Y694), Akt(S473), p38 MAPK(T180/Y182) and JNK expression in 16HBE cells after treatment by different concentrations of TDI-HSA(A) or rhHMGB1(B) for 20 min and C and D, densitometric analysis of the blots. Data are mean $\pm$ SD of three independent experiments. * $P < 0.05$ compared with control group.

more concentrations of short TSLP would be helpful to draw a conclusion between the two different treatments.

Short TSLP and long TSLP are not alternatively spliced isoforms but derive from the activity of 2 putative promoter regions (Fornasa *et al.*, 2015). Studies indicated that TLR ligands such as poly I:C (Harada *et al.*, 2009; Xie *et al.*, 2012) or cytokines (TNF- α + IL-4 + IL-13) pre-dominantly upregulated the long TSLP expression. In contrast, vitamin D receptor agonists promoted the short TSLP expression (Fornasa *et al.*, 2015; Xie *et al.*, 2012). Here we found that mouse TSLP expression was significantly elevated in bronchial epithelia and infiltrating inflammatory cells of the lungs exposed to TDI, although we failed to detect the release of mouse TSLP in BAL fluids. And we further observed that both TDI-HSA and rhHMGB1 increased long TSLP but not short TSLP production in human bronchial epithelial cells. Mouse TSLP as a key initiator

of allergic airway inflammation in mice, lung-specific expression of a *tslp* transgene induced airway inflammation and hyperreactivity characterized by Th2 cytokines and increased IgE (Zhou *et al.*, 2005). Inhibitions of mouse TSLP and long TSLP expression by short TSLP peptides may be responsible for its anti-inflammatory actions in this asthma model. Although no study defined the exact role of mouse TSLP or long TSLP in TDI-induced murine asthma model so far. Recent investigations (Bjerkkan *et al.*, 2015; Fornasa *et al.*, 2015) showed that in healthy conditions the only isoform expressed was the short TSLP, whereas under inflammation, the long TSLP appeared. However, whether the ratio of short TSLP and long TSLP expression is aberrant in patients with occupational or atopic asthma still remains unknown.

Evidence suggests that HMGB1 acts as a key molecule of innate immunity that is passively released upon cell necrosis/

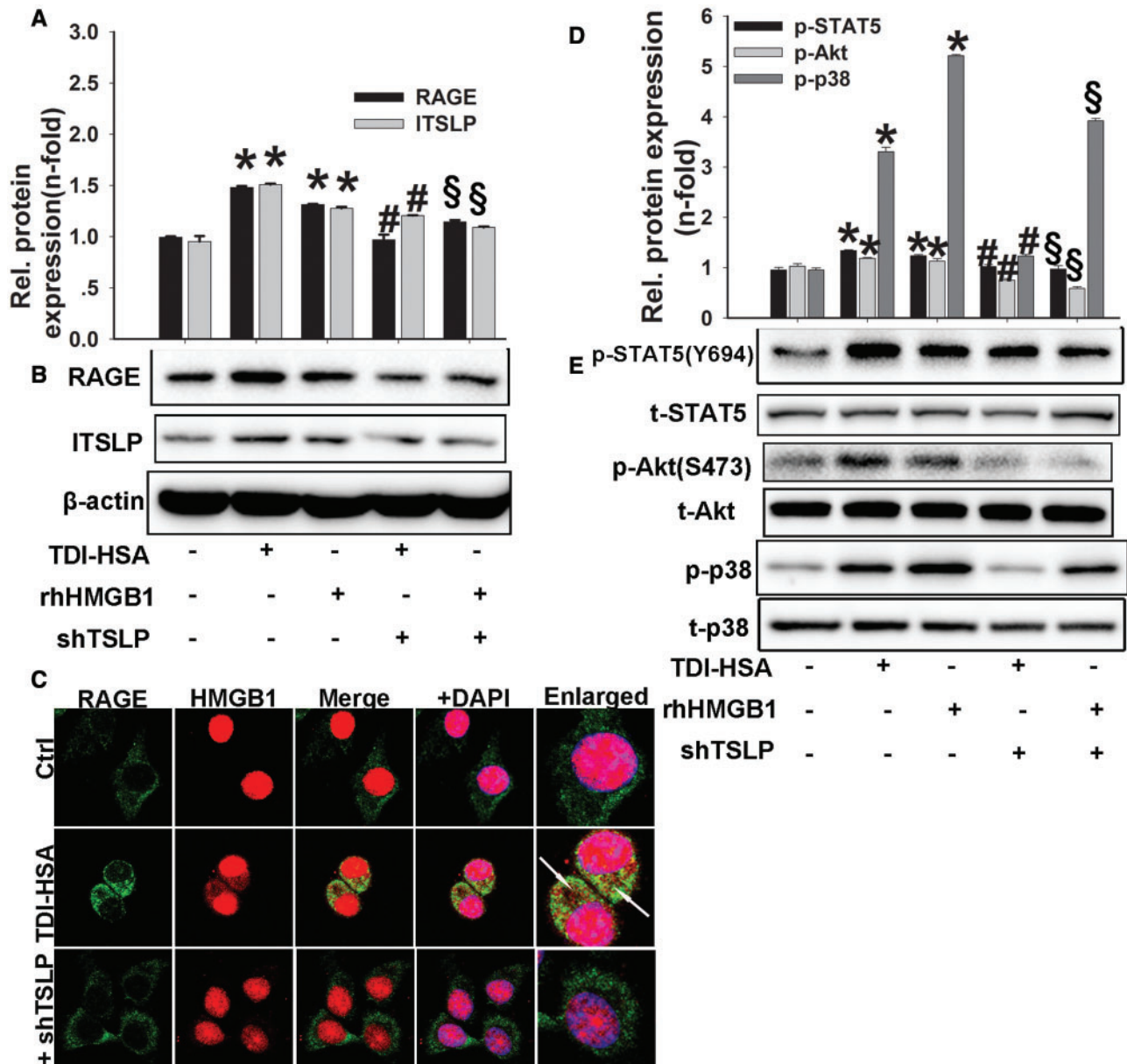


FIG. 8. Effects of short TSLP on RAGE and long TSLP proteins expression, HMGB1 and RAGE localization and signals activation in 16HBE cells. Western blot and densitometric analysis of RAGE and long TSLP proteins expression (A and B) and STAT5(Y694), Akt(S473) and p38 MAPK(T180/Y182) phosphorylation (D and E) and double immunofluorescent analysis of the localization of HMGB1 and RAGE (C). 16HBE cells were stimulated with TDI-HSA (80 μ g/ml) or rhHMGB1 (30 ng/ml) with or without the pre-treatment of short TSLP peptides (50 ng/ml, 12 h) as indicated for 6 h (A and B) or 8 h (C) or 20 min (D and E). C, RAGE was stained with the rabbit polyclonal antibody and Alexa 488-labeled goat anti-rabbit IgG antibody. HMGB1 was stained with the mouse monoclonal antibody and Alexa 594-labeled goat anti-mouse IgG antibody. The cells nuclei were stained with DAPI. Original magnification was $\times 100$. Arrows indicate the localization of RAGE and HMGB1 in the cytosol. Data are mean $\pm$ SD of three independent experiments. * $P < 0.05$ compared with control group. # $P < 0.05$ compared with TDI-HSA group. \$ $P < 0.05$ compared with rhHMGB1 group.

pyroptosis and is actively produced by immune cells (Castiglioni et al., 2011) as well as airway epithelium cells (Ullah et al., 2014). In this experiment, we observed HMGB1 was increased at the cytoplasm of airway epithelium, paralleled by a significant increase of RAGE protein expression in lungs of TDI-treated mice (Figure 3). The similar results can also be found in vitro, coupled with the colocalization of HMGB1 with RAGE in the cytosol (Figure 7C). These are consistent with our previous study (Liang et al., 2015a), which indicated PI3K pathway mediated HMGB1 production in the cytoplasm of airway epithelium in TDI-treated mice. Interestingly, all of these were rescued by treatment with short TSLP. Our results also showed the

inhibition of short TSLP on the phosphorylation of Akt (S473), the direct downstream substrate of PI3K. This may provide a mechanism that PI3K/Akt pathway could mediate the cytoplasmic expression of HMGB1. Because nucleocytoplasmic translocation is an important process for HMGB1 secretion and exerting alarmin activities (Gauley and Pisetsky, 2009), the increased cytoplasmic expression of HMGB1 not only enables release but also can actively promote its own secretion, which could mediate chronic inflammation (Bertheloot and Latz, 2016).

Meanwhile, we evaluated different signals activation in this study including STAT3(Y705), STAT5(Y694), Akt(S473), p38

MAPK(T180/Y182) and JNK. Increased expression of p-STAT5(Y694) was observed in lungs of TDI-exposed mice and in 16HBE cells stimulated by TDI-HSA or rhHMGB1, which could be inhibited by short TSLP. In addition, Akt(S473) and p38 MAPK(T180/Y182) were activated by both TDI-HSA and rhHMGB1 *in vitro*, which is concordance with our previous studies that PI3K/Akt was phosphorylated and definitely related to the airway inflammation in TDI-induced murine asthma model (Liang *et al.*, 2015a; Yao *et al.*, 2015b). While the remarkably decreased levels of the p-Akt(S473) and p-p38 MAPK(T180/Y182) were detected after short TSLP pre-treatment. These were also partly coincident with the work of Dong *et al.* (2016), which also identified that short TSLP attenuated the phosphorylation of p38 MAPK induced by HDM. Taken together, these findings suggest that short TSLP might attenuate airway inflammation through modulation of these signals. However, more evidences are needed to clarify the functions of Akt(S473), p38 MAPK(T180/Y182) and STAT5(Y694) by using specific inhibitors in this model.

Two limitations of this study should be noted. One is the use of monolayer cultures rather than air-liquid interface differentiated human airway epithelial cells. Secondly, although previous studies didn't used a control peptide group (Dong *et al.*, 2016; Fornasa *et al.*, 2015; Liu *et al.*, 2017; Sonesson *et al.*, 2011), an appropriate control peptide such as a same aa composition control peptide with a scrambled sequence would clearly demonstrate the effect was specifically due to synthetic short TSLP peptide but not the off-target effect of peptide.

In conclusion, our results support that synthetic short TSLP exerts a significant anti-inflammatory property in TDI-induced asthma model, which may be associated with the inhibitions of HMGB1-RAGE and long TSLP expression and STAT5(Y694), Akt(S473) and p38 MAPK(T180/Y182) phosphorylation.

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AUTHORS' CONTRIBUTIONS

H.Z. and S.C. conceived the design of this study and supervised the scientific work. Y.W. and Y.L. performed the experiments and wrote the first drafts of the manuscript. W.Z., Y.L., Y.W., C.Y., J.X., H.D. and F.Z. contributed to the research plan and design. All of the authors contributed to and approved the full manuscript.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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