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Original Article

Prevention of IL-6 signaling ameliorates toluene diisocyanate-induced steroid-resistant asthma

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ABSTRACT

Background: Accumulating evidence indicated the crucial role for interleukin 6 (IL-6) signaling in the development of allergic asthma. Yet, the role of IL-6 signaling in toluene diisocyanate (TDI)-induced mixed granulocytic airway inflammation still remains unclear. Thus, the aims of this study were to dissect the role of IL-6 signaling and to evaluate the effect of tocilizumab on TDI-induced steroid-resistant asthma.

Methods: TDI-induced asthma model was prepared and asthmatic mice were respectively given IL-6 monoclonal antibody, IL-6R monoclonal antibody (tocilizumab, 5 mg/kg, *i.p.* after each challenge) for therapeutic purposes or isotype IgG as control.

Results: TDI exposure just elevated IL-6R expression in the infiltrated inflammatory cells around the airway, but increased glycoprotein 130 expression in the whole lung, especially in bronchial epithelium. Moreover, TDI inhalation increased airway hyperresponsiveness (AHR) to methacholine, coupled with mixed granulocytic inflammation, exaggerated epithelial denudation, airway smooth muscle thickening, goblet cell metaplasia, extensive submucosal collagen deposition, dysregulated Th2/Th17 responses, as well as innate immune responses and raised serum IgE. And almost all these responses except for raised serum IgE were markedly ameliorated by the administration of IL-6 neutralizing antibody or tocilizumab, but exhibited poor response to systemic steroid treatment. Also, TDI challenge induced nucleocytoplasm translocation of HMGB1 and promoted its release in the BALF, as well as elevated lung level of STAT3 phosphorylation, which were inhibited by anti-IL-6 and anti-IL-6R treatment.

Conclusions: Our data suggested that IL-6 monoclonal antibody and tocilizumab might effectively abrogate TDI-induced airway inflammation and remodeling, which could be used as a clinical potential therapy for patients with severe asthma.

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Introduction

Asthma constitutes a global healthy problem, currently affecting approximate 347 million people of all ages around the world.¹

Although corticosteroids act as the cornerstone of asthma therapy, still 5%–20% of asthmatic patients that respond poorly to high-doses of steroids were diagnosed severe asthma, which lead to heavier healthcare costs, rendering severe asthma a substantial

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socioeconomic burden.^{1,2} Work-relatedness accounts for 15%–33% of adult-onset asthma, but delays in diagnosis remain common and lead to declined lung function and worse prognosis.³ As one of the most commonly reported inducers of asthma, toluene diisocyanate (TDI) has a capacity to induce airway inflammation characterized by copious amounts of neutrophils and a handful of eosinophils recruitment around the airway lumen.⁴ Evidence from clinical studies and animal models revealed that TDI-induced asthma responds poorly to corticosteroid and even removal from diisocyanate exposure couldn't completely prevent the progression of asthma.^{5–7} Thus, in-depth understanding of the pathogenesis underlying TDI-induced neutrophil-dominated airway inflammation is of great importance to the management of severe asthma.

Interleukin 6 (IL-6) is a pleiotropic cytokine produced by many different cells from innate immune system (macrophages, neutrophils, mast cells and dendritic cells), adaptive immune system (B cells and T cells), and structural cells such as epithelial cells, fibroblasts, and endothelial cells.⁸ The specificity in IL-6 signaling is determined by the ligation of IL-6 to membrane IL-6 receptor (mIL-6R) or a soluble form of the IL-6R (sIL-6R), and the subsequent association of this complex with glycoprotein 130 (gp130), thereby mediating a vast array of inflammatory responses.⁹ A growing body of evidences have demonstrated that IL-6 signaling is critically involved in the development of asthma. Elevated IL-6 levels have been detected in serum,¹⁰ induced sputum¹¹ and bronchoalveolar lavage fluid (BALF)^{12,13} of asthmatic patients, and correlated to impaired lung function and asthma severity.^{11,14} Initial studies demonstrated IL-6^{-/-} mice displayed exaggerated ovalbumin (OVA)-induced airway hyper-responsiveness (AHR), eosinophilia and Th2 responses, which were inhibited by endogenous and exogenous IL-6.^{15,16} However, other researchers reported that inhibition of IL-6 signaling using knockout mice or neutralizing antibodies markedly attenuated house dust mite (HDM)- or OVA-induced AHR and inflammation.^{17,18} Even in a same study, IL-6R blocking antibody relieved cockroach-induced mixed granulocytic airway inflammation, but had no effect on HDM-induced airway inflammatory responses and even caused a trend for increased eosinophil infiltration.¹⁹ These conflicted results emphasized the controversial but vital role of IL-6 signaling in the pathogenesis of asthma. Previously, others and we have also reported increased levels of IL-6 were detected in the BALF or lung of TDI-induced asthma models,^{6,20,21} yet how IL-6 signaling contributes to the pathology of TDI-elicited asthma was still unclear.

Significant advances have been made in the generation of monoclonal antibodies that target IL-6 signaling directly or indirectly.⁹ Without neglecting the importance of other neutralizing antibodies for IL-6 signaling, tocilizumab, a humanized monoclonal antibody that inhibits IL-6 signaling by completely binding to both mIL-6R and sIL-6R, is approved in more than 100 countries worldwide for the treatment of Rheumatoid Arthritis (RA).⁹ The effectiveness of tocilizumab in RA has sparked a series of clinical trials to examine whether this approach is effective in other refractory diseases.^{22,23} But the therapeutic potential of inhibiting IL-6 and its receptors in severe asthma is still in its infancy. In allergic asthmatics, DCs differentiated from monocytes treated with tocilizumab had poor capacity for eliciting Th2 polarization as compared to DCs without treatment.¹⁷ In 2019, Esty *et al.* reported that 2 refractory asthmatic patients demonstrated clinical and immunological responses to tocilizumab monotherapy, with restricted Th2/Th17 responses.²⁴ However, the effect of tocilizumab on *in vivo* TDI-induced asthmatic inflammation has not been assessed. Thus, the aims of this study were: (1) to explore the role of IL-6 signaling and (2) to further evaluate the effect of tocilizumab on TDI-induced steroid-resistant asthma.

Methods

Animals

6–8 week old male BALB/c mice (20–22g) were purchased from Guangdong Medical Laboratory Animal Center. All experimental mice were housed in a specific pathogen-free facility with 12 h dark/light cycles [temperature $23 \pm 2^\circ\text{C}$, humidity range 40–70%, 12 h light/dark cycle (lighting: 7:00–19:00)] and with sterile water and irradiated food available *ad libitum*. All animal care and experimental procedures studies complied with the guidelines of the Committee of Jinan University on the use and care of animals and were approved by the Animal Subjects Committee of Shenzhen People's Hospital.

Reagents

Toluene diisocyanate (toluene-2, 4-diisocyanate, $\geq 98.0\%$), methacholine, prednisone and acetone were obtained from Sigma–Aldrich (Shanghai, China). Humanized IL-6R monoclonal antibody (tocilizumab, #A2012) was brought from Selleck Chemicals (Shanghai, China). Rat anti-mouse IL-6 monoclonal antibody (anti-IL-6 mAb, #BE0046), rat IgG1 isotype control (#BE0088) and human IgG1 isotype control (#BE0297) were from Bio X Cell (Lebanon, NH, USA). Multiplex immunoassays for IL-4, IL-5, IL-13, IL-17A, IL-17F, IL-6, IL-18, CCL11, CXCL1, CSF-3 and ELISA kit for total IgE were bought from eBioscience (San Diego, CA, USA). ELISA detection kits for mouse TSLP, IL-25, IL-33 and IFN- γ were brought from eBioscience (San Diego, CA, USA). ELISA detection kit for mouse high mobility group box 1 (HMGB1) was brought from Chondrex (Redmond, WA, USA). Primary antibody against HMGB1 was from Abcam (Shanghai, China). Rabbit anti-STAT3 and anti-p-STAT3 were obtained from Cell Signaling Technology (Danvers, MA, USA). Rabbit anti-gp130 antibody was bought from ABclonal (Wuhan, China). Rabbit anti-IL6R antibody was obtained from Proteintech (Rosemont, IL, USA).

TDI-induced asthma model

TDI-induced experimental asthma model was generated based on our previous work. All mice were randomized to the following groups: (1) vehicle-sensitized, vehicle-challenged, PBS-treated mice (control group); (2) TDI-sensitized, TDI-challenged, PBS-treated mice (TDI group); (3) TDI-sensitized, TDI-challenged, 5 mg/kg Prednisone-treated mice (TDI + Pred group); (4) TDI-sensitized, TDI-challenged, 5 mg/kg human or rat isotype IgG1-treated mice (TDI + isotype IgG group, the effect of human IgG1 isotype control on TDI-exposed mice was similar to that of rat IgG1 isotype control, as shown in [Supplementary Fig. 1](#)); (5) TDI-sensitized, TDI-challenged, 5 mg/kg IL-6 monoclonal antibody-treated mice (TDI + anti-IL-6 group); (6) TDI-sensitized, TDI-challenged, 5 mg/kg tocilizumab-treated mice (TDI + Tocilizumab group). Briefly, on days 1 and 8, the mice were dermally treated with 0.3% TDI on the dorsum of both ears (20 μL per ear). On days 15, 18 and 21, the mice were placed in a horizontal rectangle chamber and challenged for 3 h each time via the airways with 3% TDI by means of compressed air nebulization (NE-C28; Omron, Tokyo, Japan). Control mice were treated by the same procedures with the same amount of vehicle. The vehicle used to dissolve TDI consisted of a mixture of two volumes of acetone and three volumes of olive oil for sensitization, and one volume of acetone and four volumes of olive oil for inhalation. Anti-IL-6 mAb, rat IgG1 isotype control, tocilizumab (anti-IL-6R mAb) or human IgG1 isotype control were diluted in 250 μL PBS. IL-6 mAb (5 mg/kg), tocilizumab (5 mg/kg), rat IgG1 isotype control (5 mg/kg) or human

IgG1 isotype control (5 mg/kg) were administered²⁵ separately via intraperitoneal injection route once immediately after each inhalation. Prednisone (5 mg/kg) was administered via intraperitoneal injection route for continuous 7 days after first inhalation.

Assessment of airway hyperresponsiveness

Airway reactivity to methacholine was assessed 24 h after the final challenge with the FinePointe Resistance and Compliance system (DSI, Buxco, USA). Measurements of lung resistance (R_L) were performed in response to increasing doses of aerosol methacholine (6.25, 12.5, 25 and 50 mg/mL) every 5 min following each nebulization step until a plateau phase was reached. Results were expressed as R_L value for each concentration of methacholine.

Measurement of serum IgE

Mice were sacrificed with overdose of pentobarbital (100 mg/kg, i.p.) one day after the last airway challenge. Blood samples were taken from the retroorbital plexus/sinus, allowed to rest at room temperature for 1 h, then centrifuged (3000 g, 20 min), and supernatants were collected for detection of total IgE with ELISA kit according to the manufacturer's instructions.

Bronchoalveolar lavage fluid and detection of cytokines

After blood was taken, the lungs were lavaged *in situ*, twice with 0.8 mL sterile saline (0.9% NaCl, prewarmed), and the recovered fluid was pooled. Total cells in BALF were counted, and a cytospin sample was prepared and stained with haematoxylin and eosin (H&E) for blinded assessment of differential cell percentages in BALF. Then the remaining fluids were centrifuged (1000 g, 10 min), and supernatants were stored for further analysis of IL-4, IL-5, IL-13, IL-17A, IL-17F, IL-6, IL-18, CCL11, CXCL1, CSF-3 and HMGB1.

Pulmonary histopathological examination

Left lungs were dissected from mice, and then fixed in 4% neutral buffered formalin for 24 h, dehydrated, and embedded in paraffin. Lung sections (4 μ m) were prepared with a Leica microtome 2030 (Leica Microsystems Nussloch GmbH, Nussloch, Germany). Lung tissues were stained with H&E, periodic acid-Schiff (PAS), and Masson's trichrome for blinded histopathologic assessment. Peribronchial and perivascular inflammation in H&E-stained sections were assessed using a scoring standard as previously described.²⁶ Airway epithelial denudation was measured by assessing the percentage of the denuded area in the entire circumference of the bronchus. Thickness of airway smooth muscle (ASM) was measured by a modification of Cho's method.⁶ Briefly, the thickness of the peribronchial smooth muscle layer (the transverse diameter) in large airways was measured from the innermost aspect to the outermost aspect of the circumferential smooth muscle layer. The presence of collagen fibril deposition with Masson staining was analyzed by ImageJ software.²⁶ Scoring was performed at a magnification of 200 $\times$ by examining at least 8–10 image fields of 12 slices from 6 mice per group.

Western blot and immunohistochemistry

Pulmonary expressions of IL-6R, gp130, p-STAT3 and HMGB1 were detected by western blot. Whole lung extracts mixed with 5 $\times$ SDS loading buffer were separated by 10% SDS-polyacrylamide gel and transferred onto PVDF membranes. Membranes were then probed with anti-mIL-6R, anti-sIL-6R, anti-gp130, anti-pSTAT3, and anti-HMGB1 antibodies with indicated

dilutions. After incubation with an IRDye-680WC-conjugated secondary antibody, immunoreactive bands were exposed to Odyssey CLx Imager (LI-COR Biosciences, Lincoln, NE) for image capture. For immunohistochemistry of IL-6R, gp130 and HMGB1, deparaffinized lung sections subject to antigen retrieval, and then were treated with H₂O₂ for 15 min to block endogenous peroxidase, and incubated overnight at 4 °C in recommended dilutions of anti-IL-6R, anti-gp130 and anti-HMGB1 antibodies. After washing with PBS, slices were incubated with a secondary antibody for 20 min at room temperature. Signals were visualized with DAB.

Statistical analysis

Prism (version 8, GraphPad) and SPSS version 22.0 (SPSS Inc., Chicago, Illinois, US) were used for statistical analysis and graphing. Data were expressed as mean $\pm$ SD, and comparisons among groups were analyzed via one-way analysis of variance (ANOVA) accompanied by LSD-*t* for multiple comparisons. *P* < 0.05 was regarded as statistically significant.

Results

Pulmonary expression of IL-6 signaling in TDI-induced asthma model

Pulmonary expression of IL-6 signaling in TDI-exposed mice were analyzed. Immunohistochemistry of IL-6R showed that there's relatively weak staining in the lungs of control mice, and it seems that TDI sensitization and challenge did not alter IL-6R expression in the airway epithelia, but increased the positive staining in the infiltrating inflammatory cells and alveolar regions (Fig. 1A). Fit with this, western blot revealed TDI exposure did not raise mIL-6R and sIL-6R expressions in the whole lung (Fig. 1C), and treatment with IL-6 blocking antibody or IL-6R neutralizing antibody showed no effects on IL-6R expression. As for gp130, a key downstream factor of sIL-6R, there is relatively faint staining in bronchial and alveolar regions of the lung in control mice. TDI exposure led to increased gp130 expression pattern in the airway epithelia but not alveolar regions, and there seems that anti-IL-6 mAb or tocilizumab at the doses of 5 mg/kg had no effect on TDI-induced increased gp130 expression (Fig. 1B). Similar results were indicated by western blot (Fig. 1D). In addition, higher IL-6 levels were detected in BALF of TDI-elicited mice, which were remarkably repressed by administration of anti-IL-6 mAb or tocilizumab (Fig. 1E), to some extent suggesting that IL-6 signaling was effectively inhibited by IL-6 neutralizing antibody and IL-6R blocking antibody. The effects of anti-IL-6 mAb and anti-IL-6R blocking antibody on naive mice were shown in Supplementary Figure 2.

Inhibition of IL-6 signaling decreased airway hyperreactivity and inflammation in TDI asthma

Mixed granulocytic airway inflammation was induced by exposing male BALB/c mice to TDI. As expected, airway exposure to TDI induced increased airway hyperresponsiveness to methacholine, heavier epithelium injury and ASM thickening, evident peribronchial inflammation with copious amounts of neutrophils and a handful of eosinophils infiltration in the airway lumen, and raised serum IgE, which were poor response to Prednisone (See Supplementary Fig. 3, 4). However, almost all these responses were blunted by treatment with anti-IL-6 mAb or IL-6R blocking antibody, except for the increased serum IgE, indicating a crucial role for IL-6 signaling in the initiation of TDI-elicited steroid-insensitive airway inflammation (Fig. 2).

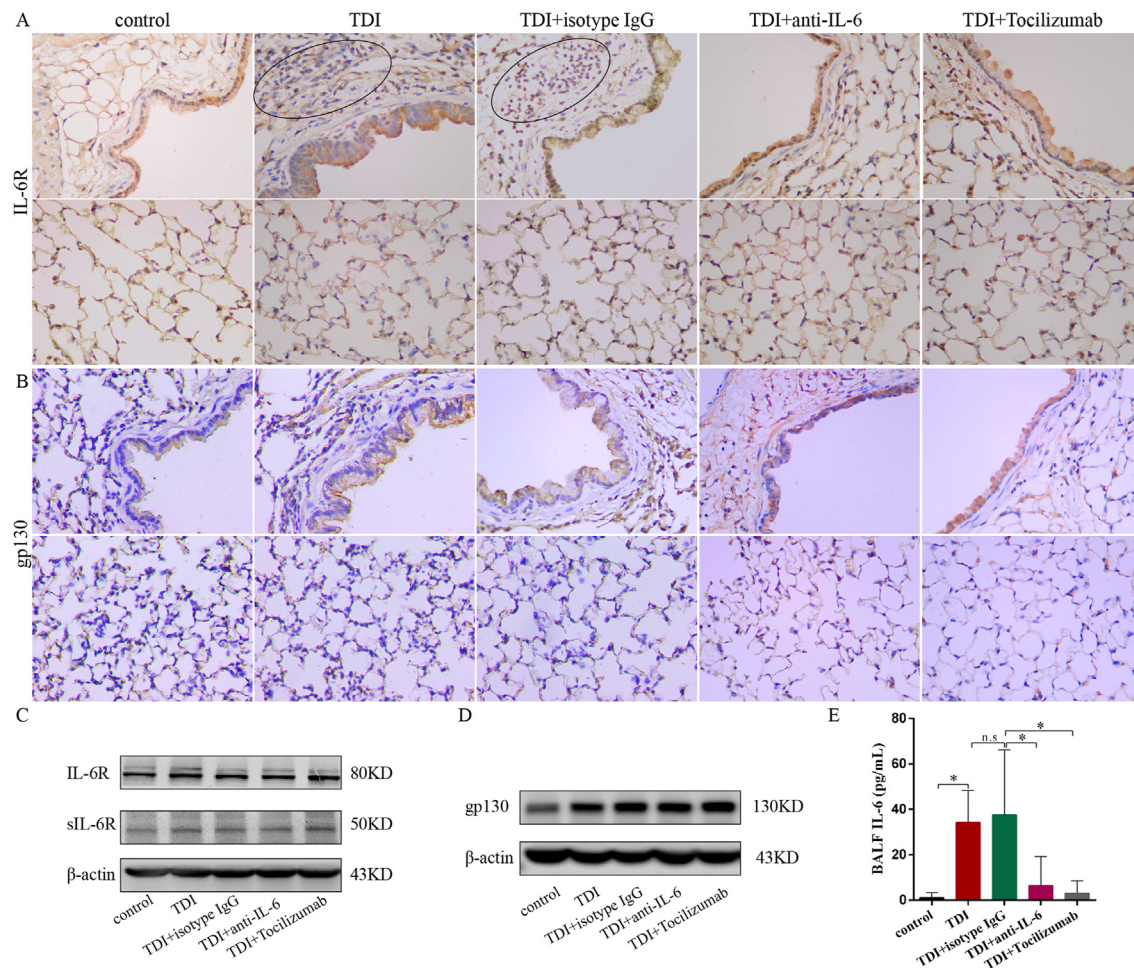


Fig. 1. Pulmonary expression of IL-6 signaling in TDI-induced asthma. **A**, Immunohistochemical staining of IL-6R in the bronchial (upper panel) and alveolar regions (lower panel) of lungs in different treatment groups. TDI exposure increased the expression of mIL-6R in the infiltrated inflammatory cells around the airway (the circles marked) but not the airway epithelium, which was inhibited by IL-6 blocking antibody or tocilizumab. Original magnification was 400 $\times$. **B**, Immunohistochemical staining of gp130 in the bronchial (upper panel) and alveolar regions (lower panel) of lungs in different treatment groups. Original magnification was 400 $\times$. **C-D**, Protein expressions of mIL-6, sIL-6 and gp130 in lung homogenates were detected by Western blotting. n = 4-6. **E**, Levels of IL-6 in BALF was detected. n = 7-10. *P < 0.05; **P < 0.01; ***P < 0.001. n.s. represents none significant differences.

Blocking IL-6 signaling restrained Th2/Th17 dysfunction and innate immune responses in TDI-exposed mice

Dysregulation of Th2 and Th17 responses were observed in TDI-exposed mice, with higher levels of IL-4, IL-5, IL-13, IL-17A, IL-17F, IL-18, eosinophil attractant CCL11, neutrophil-active chemokine CXCL1 and CSF-3 in the BALF. And treatment with IL-6 blocking antibody or tocilizumab decreased the hypersecretion of IL-4, IL-5, IL-13, IL-17A, CXCL1, and CSF-3, but did not affect the release of IL-18, IL-17F and CCL11 in TDI-induced asthma (Fig. 3). These results suggest that IL-6 signaling is a critical regulator of Th2 and Th17 responses in TDI-induced steroid-insensitive asthma.

Moreover, to explore the role of IL-6 signaling in innate immune responses, we detected BALF levels of TSLP, IL-33, and IL-25. TDI exposure increased the production of TSLP and IL-25 but not IL-33 in the BALF, of which TDI-induced IL-25 secretion was inhibited by blocking IL-6 signaling treatment, indicating that IL-6 signaling occupied an important role in the regulation of innate immune responses in TDI-induced asthma (Fig. 4). Yet, we failed to detect the levels of IFN- γ in BALF.

Ablation of IL-6 signaling attenuated TDI-induced asthmatic airway remodeling

Airway remodeling is another essential feature in the pathophysiology of TDI-induced asthma. To further explore the role of IL-6 signaling in the development of TDI-induced airway remodeling, we evaluated the extent of collagen deposition and goblet cell metaplasia by Masson's trichrome staining and PAS staining, respectively. As indicated by histopathology and morphometry, TDI sensitization and challenge led to extensive submucosal collagen deposition and pronounced goblet cell metaplasia in the airway epithelium compared with vehicle control, of which TDI-induced collagen deposition were reduced by IL-6 blocking antibody and tocilizumab. As for TDI-induced goblet cell metaplasia, was significantly attenuated by tocilizumab, but not anti-IL-6 mAb (Fig. 5).

IL-6 signaling promoted HMGB1 nucleocytoplasmic translocation in TDI-exposed mice with pSTAT3 upregulation

HMGB1 has been proven to play a crucial role in the pathogenesis of TDI-induced asthma. Yet, the effect of IL-6 signaling on

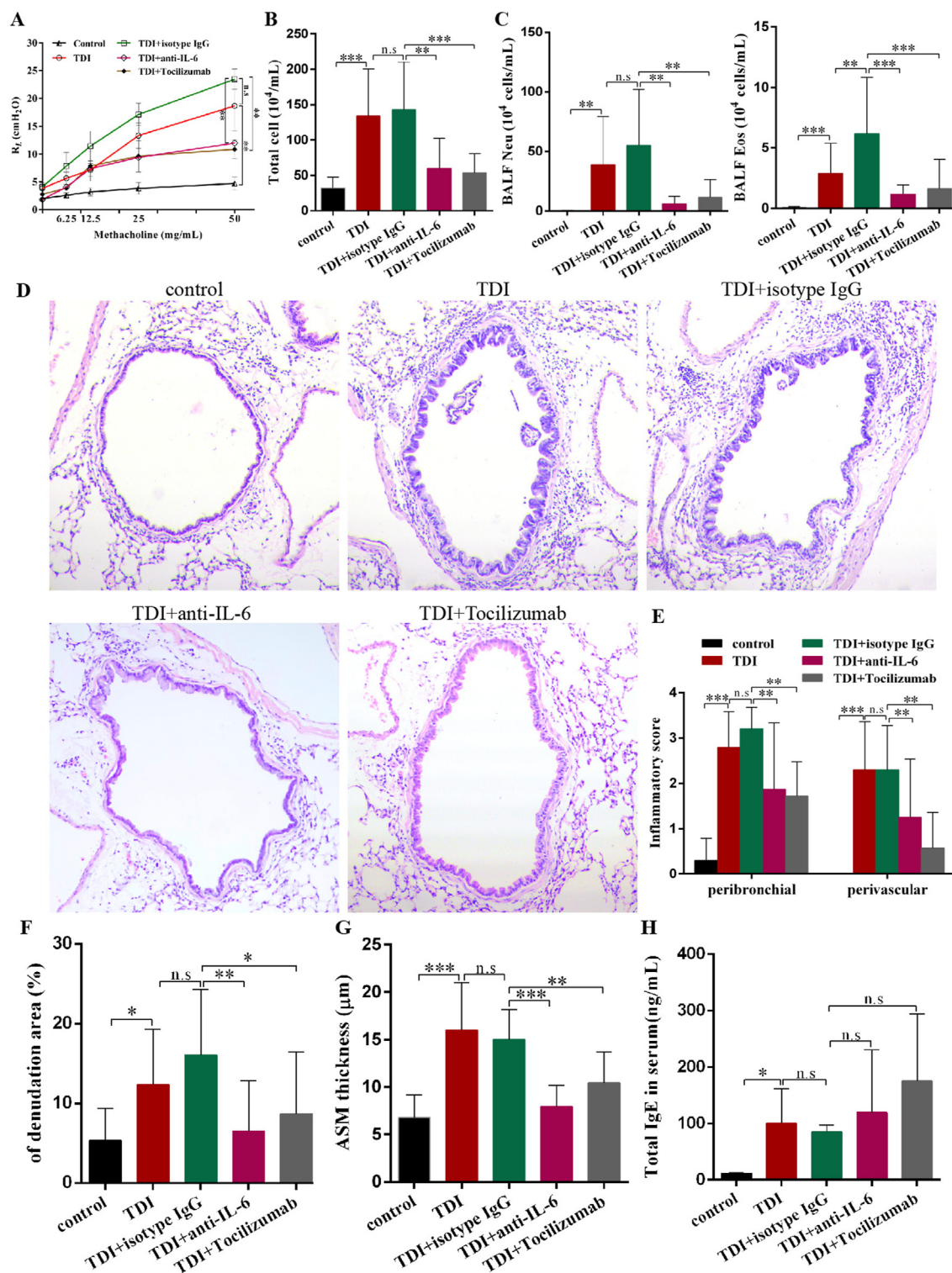


Fig. 2. Prevention of IL-6 signaling attenuated airway hyperresponsiveness (AHR) and inflammation in TDI-induced asthma model. **A**, AHR was measured by lung resistance (R_L). $n = 4$. **B-C**, Numbers of total inflammatory cells, neutrophils and eosinophils in BALF. $n = 10-12$. **D-E**, Representative H&E-stained lung sections of different groups. And semiquantification of airway inflammation was performed. Original magnification was $200 \times$. $n = 9-12$. **F**, Semiquantification of epithelial denudation was performed. $n = 10-12$. **G**, Analysis of ASM thickness was performed. $n = 10-12$. **H**, levels of total IgE in serum. $n = 5-6$. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. n.s represents none significant differences.

TDI-induced HMGB1 production was still unclear. Thus, we assessed the expression of HMGB1 by immunohistochemistry, western blot and ELISA. Immunohistochemistry indicated HMGB1 was detected almost only in the nuclei of airway epithelium and alveolar regions. However, we found that TDI sensitization and

challenge resulted in HMGB1 translocation from the nuclei to the cytoplasm, especially in bronchial epithelium, and scattered in alveolar epithelium and infiltrating inflammatory cells around the airway (Fig. 6A). Similarly, TDI inhalation increased the protein expression of HMGB1 in the whole lung (Fig. 6B). Furthermore,

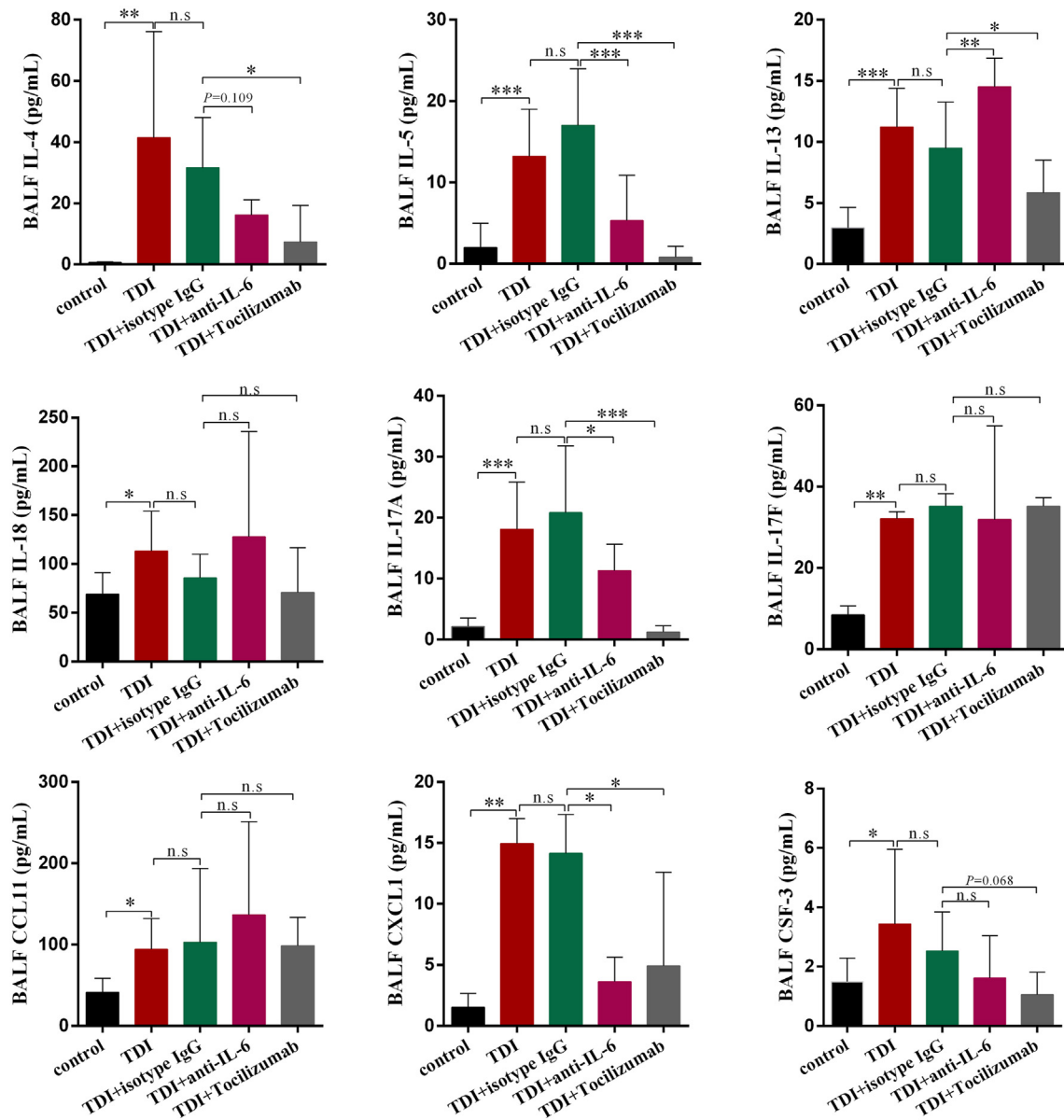


Fig. 3. Blocking IL-6 signaling restricted TDI-induced Th2/Th17 responses. BALF levels of IL-4, IL-5, IL-13, IL-17A, IL-17F, IL-18, CCL11, CXCL1 and CSF-3. $n = 7-10$. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. n.s. represents none significant differences.

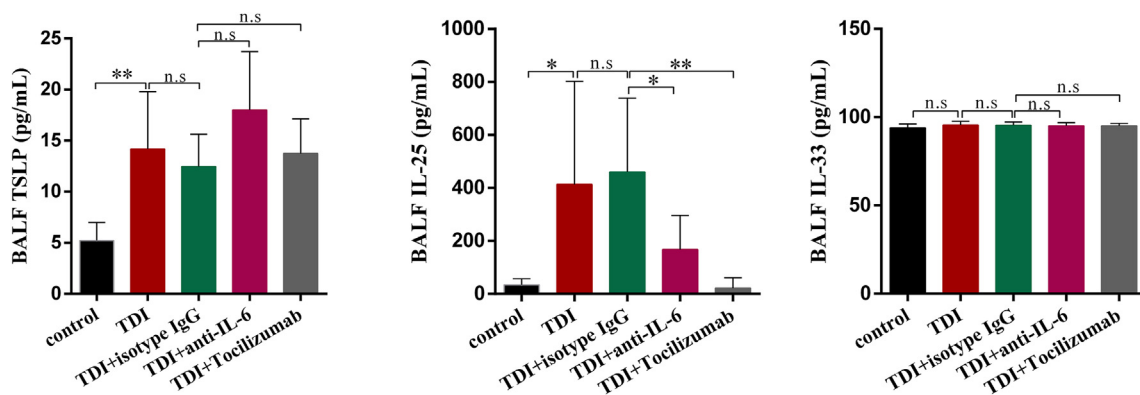


Fig. 4. Ablation of IL-6 signaling restricted TDI-induced innate immune responses. BALF levels of TSLP, IL-33 and IL-25. $n = 7-10$. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. n.s. represents none significant differences.

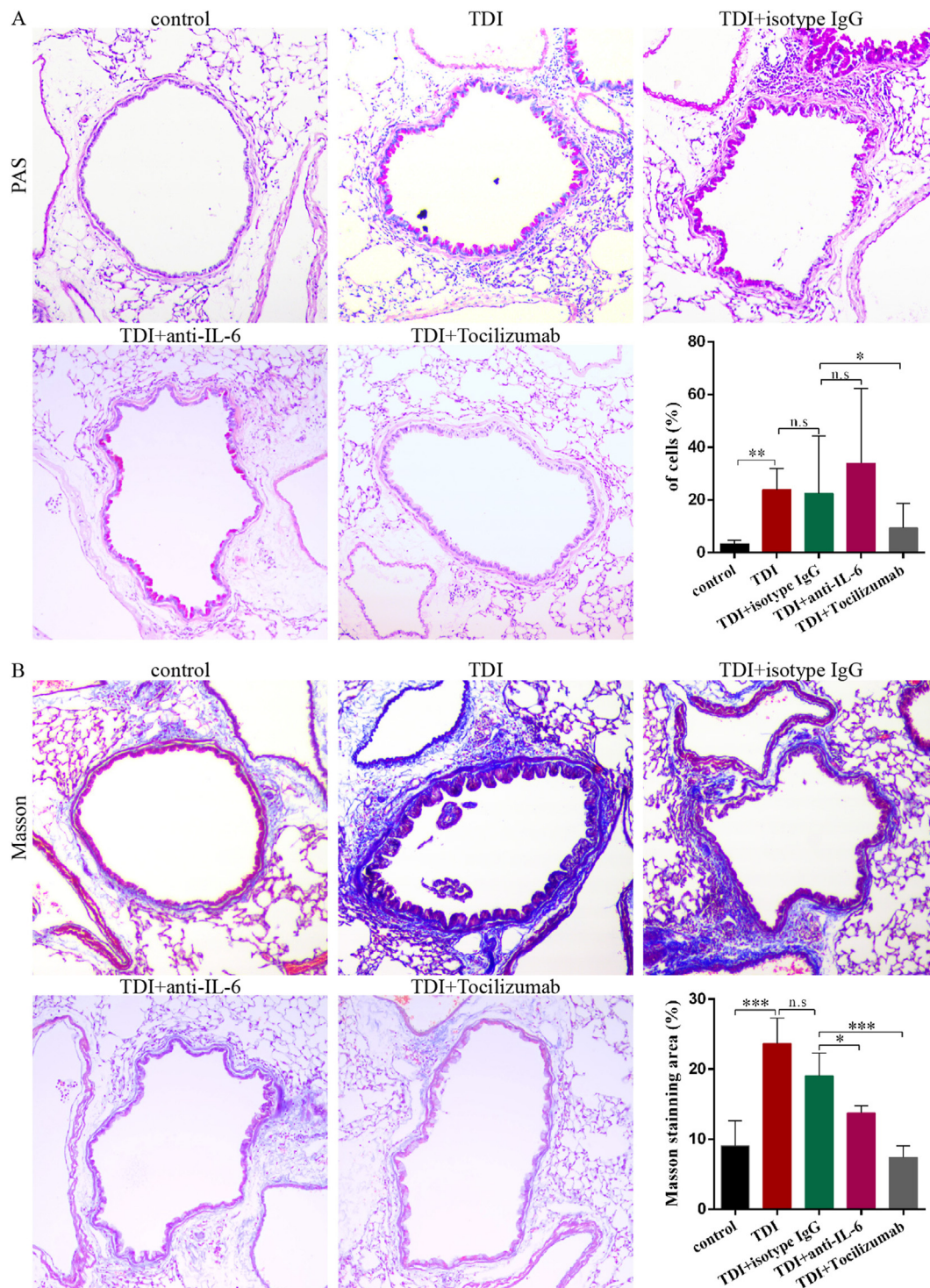


Fig. 5. Inhibition of IL-6 signaling alleviated airway remodeling induced by TDI. **A**, Representative PAS-stained lung sections showing goblet cells metaplasia of different groups. And tocilizumab markedly ameliorated TDI-induced goblet cells metaplasia. Original magnification was 200 $\times$. Semiquantification of PAS staining was performed. The number of PAS-positive epithelial cells to be counted to obtain a percentage to quantify the PAS-positive staining. $n = 4-6$. **B**, Representative Masson-stained lung sections showing submucosal collagen deposition of different groups. And IL-6 mAb or tocilizumab ameliorated TDI-induced collagen deposition. Original magnification was 200 $\times$. Semiquantification of Masson staining was performed by ImageJ software. $n = 4-6$. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. n.s. represents none significant differences.

elevated levels of HMGB1 were detected in the BALF of TDI-treated mice (Fig. 6C). Markedly, all these increased protein expression, BALF levels and nucleocytoplasmic translocation of HMGB1 were largely alleviated by blocking IL-6 signaling using IL-6 mAb or tocilizumab (Fig. 6A-C), which indicated IL-6 signaling occupied a

pivotal role in the TDI-induced HMGB1 translocation and release. At the same time, we also found that TDI exposure upregulated pulmonary expression of a classic downstream mediator of IL-6 signaling, p-STAT3, which was inhibited by IL-6 blocking antibody and anti-IL-6R treatment (Fig. 6D). Taken together, these findings

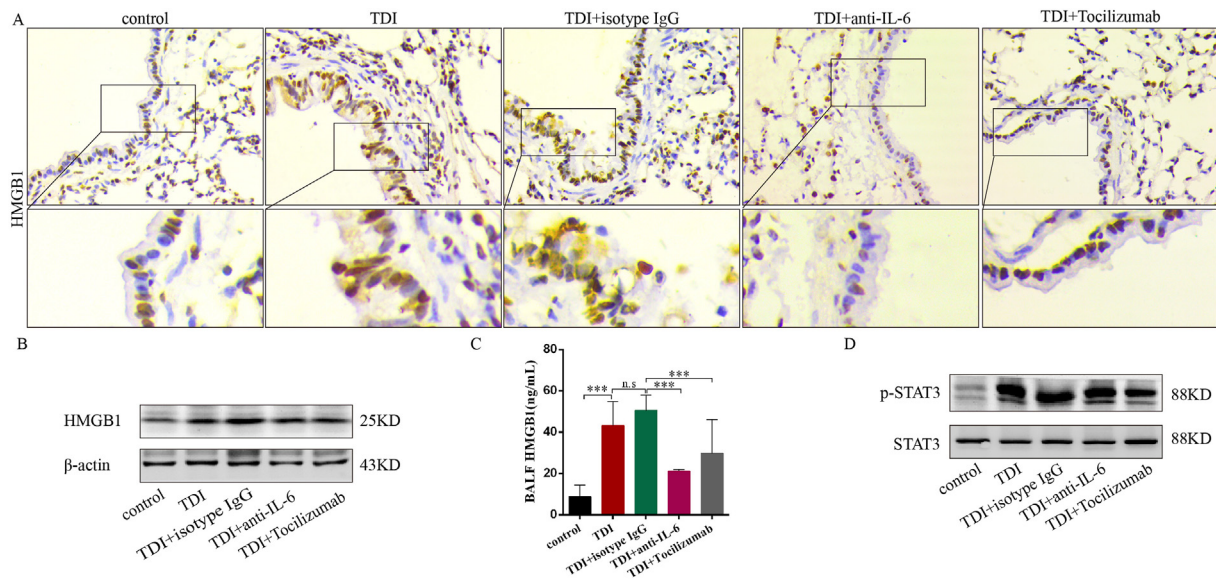


Fig. 6. Blockade of IL-6 signaling repressed the production of HMGB1 and STAT3 phosphorylation in TDI-exposed mice. **A**, Immunohistochemical staining of HMGB1 around the bronchial regions in different groups. Original magnifications were 400× (upper panel) and 1000× (lower panel). **B**, Protein expression of HMGB1 in lung homogenates was detected by Western blotting. n = 4–6. **C**, Levels of HMGB1 in BALF was detected by ELISA. n = 7–10. **D**, Protein expression of p-STAT3 in lung homogenates was detected by Western blotting. n = 4–6. *P < 0.05; **P < 0.01; ***P < 0.001. n.s. represents none significant differences.

implied that IL-6 signaling orchestrated airway inflammation and remodeling partly via inhibiting HMGB1 release and STAT3 phosphorylation in TDI-induced steroid-resistant asthma.

Discussion

Granulocyte chemotaxis is an important aspect of the pathogenesis of the complex inflammatory responses in the airway.¹⁵ Although eosinophils occupied a crucial role in the pathogenesis of allergic asthma, increasing evidences indicated neutrophil-predominated mixed airway inflammation is also recognized as a hallmark feature of refractory asthma. As one of the most commonly reported occupational asthmogens, TDI exhibits an ability to induce mixed granulocytic airway inflammation, with neutrophils dominated and a slight infiltration of eosinophils around the airway lumen, which has been proved to be steroid-insensitive.⁶ Researchers demonstrated that depletion of neutrophils by blocking antibodies can prevent TDI-induced airway hyperresponsiveness (AHR) and epithelial injury, and reduce airway inflammation,²⁷ suggesting that neutrophil might be a potential therapeutic target in TDI-induced asthma.

Aberrant activation of IL-6 pathway has been associated with neutrophilic and mixed granulocytic airway inflammation in severe asthmatics, and resulted in poor lung function.^{28,29} In the meanwhile, neutrophils also have been proposed as an important source of IL-6 and sIL-6R generated at sites of inflammation in the lungs of asthmatics.^{30,31} These supported that IL-6 signaling is critically involved in the pathology of neutrophilic asthma. In the present study, we prepared a steroid-insensitive murine asthma model as previously described,⁶ providing us with a unique opportunity to explore the role of IL-6 signaling in the development of mixed granulocytic asthma in response to TDI. As expected, we demonstrated that IL-6 signaling is activated, together with higher BALF IL-6 levels and IL-6R expression in the infiltrated inflammatory cells, in a TDI-induced asthma model. Though several researchers have reported that tocilizumab failed to inhibit IL-6 signaling in mice through IL-6R,^{32,33} in recent years other studies found that tocilizumab alleviated inflammatory responses in mice by

suppressing IL-6 secretion and/or STAT3 phosphorylation.^{34–36} Indeed, in line with these, immunohistochemistry or western blot in our study also indicated that both tocilizumab and IL-6 mAb didn't alter the patterns of mIL-6R, sIL-6R and gp130 in the lung of TDI-exposed mice (Fig. 1), but markedly repressed the BALF IL-6 level and p-STAT3 in the whole lung, to some extent implying that IL-6 signaling was inhibited by these two neutralizing antibodies through their anti-inflammatory effects. And to the best of our knowledge, for the first time we uncovered that ablation of IL-6 signaling with anti-IL-6 mAb or tocilizumab significantly relieved TDI-induced AHR, airway inflammation, and dysregulation of Th2/Th17 responses as well as innate immune response. This is in agreement to what was found in OVA- or HDM-induced model by using blocking antibodies or gene-knockout mice,^{17,37} but in disagreement with the data published by other researches reporting that IL-6 deficiency resulted in exaggerated OVA-induced AHR, airway eosinophilia and Th2 responses, while endogenous and exogenous IL-6 restricted OVA-induced inflammatory response and Th2-cell differentiation.^{15,16} Actually, IL-6 exerts distinct effects on Th2 inflammation and eosinophils influx into the airway via different underlying mechanisms, including inducing apoptosis of lung T cells, inhibiting Th1 responses and impairing function of macrophages.^{8,38} In addition to the dual effects on the eosinophilia, IL-6 is a potent trigger of IL-17A, which attracts granulocytes into mucosal surface and promotes neutrophils aggregation.³⁹ In the present study, anti-IL-6 mAb or tocilizumab markedly suppressed TDI-induced release of Th17-related cytokines IL-17A, IL-6, and neutrophil chemokines CXCL1, CSF-3 in BALF, which might contribute to the alleviation of airway neutrophilia. Indeed, our TDI-induced asthma model is characterized by neutrophil-dominant mixed airway inflammation, with dysfunctional Th2/Th17 responses, similar to the HDM-induced model in the work of Lin *et al.*,¹⁷ but differed from OVA-induced allergic asthma in other studies mentioned above.^{15,16} Also, ligation of IL-6 to receptors by IL-6R blocking antibodies or antagonists would only interfere with the downstream of IL-6 signaling, which might differ from inhibition of IL-6 signaling by using transgenic mice.⁴⁰ Thus, this might be attributed to the different intervention routes and inflammatory

phenotypes among different asthma models, but the exact mechanism is still open to further investigation.

Apart from airway inflammation, another characteristic feature of TDI-induced steroid-insensitive asthma is airway remodeling, featuring epithelial denudation, ASM thickening, goblet cell metaplasia and extensive collagen deposition. Yet currently, there are limited evidences suggesting a direct link between IL-6 signaling and airway remodeling. In 2000, Kuhn *et al.* found for the first time that transgenic mice overexpressing IL-6 displayed increased airway wall remodeling when compared with littermate control mice.⁴¹ Subsequently, other researchers reported IL-6 trans-signaling in ASM has relevance to the development of airway remodeling and tumor necrosis factor (TNF- α)-induced ASM cell proliferation depends on IL-6.^{42,43} Their contributions shed light on a probably underestimate function of IL-6 signaling in airway remodeling. In this study, IL-6R monoclonal antibody not only exhibited a capacity to blunt TDI-induced airway inflammation, but also gave rise to a reduction of epithelial denudation and ASM thickening, as well as an alleviation of goblet cell metaplasia and collagen deposition, with lower levels of BALF IL-4, IL-5 and IL-13, indicating that activation of IL-6 signaling is critically engaged in TDI-induced airway remodeling, though anti-IL-6 mAb exerted no effects on TDI-induced goblet cell metaplasia. Likewise, Schmit *et al.* uncovered that IL-6 deficiency resulted in reduced goblet cell hyperplasia, but with exaggerated airway inflammation and submucosal collagen deposition in an *Aspergillus fumigatus*-induced allergic asthma model.⁴⁴ Their finding seems opposite to ours in spite of the crucial role of IL-6 signaling in airway remodeling. Actually, consistent with our results, IL-6 is also proposed to contribute to mucus metaplasia in the airways likely through its effect on IL-13 production.⁸ Besides, of note, as mentioned above, blockade of IL-6 to receptors by neutralizing antibodies might obtained results different from what was found by using gene-knockout mice.⁸ Thus, it is easy to understand our results seems different from those in the work of Schmit *et al.*

Although IL-6 signaling is proposed to participate in TDI-induced airway inflammation and remodeling, the mechanisms underlying were still obscure. High mobility group box 1 (HMGB1) is an identified proinflammatory molecule and when localized in the extracellular space, acts as an alarmin through diverse receptors to orchestrate airway inflammation, attract cells recruitment and eventually lead to tissue remodeling. Previously we detected an increased pulmonary expression of HMGB1 in TDI-exposed mice, contributing to neutrophil infiltration around the airway.²⁶ Several studies demonstrated that inhibition of HMGB1 can reduce the secretion of IL-6 in the inflammatory process,^{45,46} suggesting a link between HMGB1 and IL-6, but the effect of IL-6 on HMGB1 have been rarely reported. In 2015, several Japanese researchers reported that anti-IL-6R mAb markedly prevented HMGB1-mediated inflammatory responses *in vivo*.⁴⁷ In the current study, we found that TDI induced nucleocytoplasmic translocation of HMGB1 in the lung and promoted its release in the BALF, which were recovered by anti-IL-6 mAb and anti-IL-6R treatment. These findings suggested that IL-6 signaling may be responsible for TDI-induced HMGB1 release in the lung, which needs to be further confirmed in our future studies. Besides, downstream mediators of IL-6 pathway (e.g. STAT3, PI3K) were strongly correlated with the pathobiology of asthma.^{8,48} We found that increased level of STAT3 phosphorylation (p-STAT3) was inhibited by tocilizumab in TDI-inhaled mice, suggesting tocilizumab is a potent inhibitor of p-STAT3. Taken together, it is possible that the effect of tocilizumab on TDI-elicited asthma *in vivo* is partly mediated by blockade of STAT3 signaling or inhibition of HMGB1.

In conclusion, our results revealed that inhibition of IL-6 signaling by IL-6 monoclonal antibody or tocilizumab might

effectively prevent the development of TDI-induced airway inflammation and remodeling, which could be used as a clinical potential therapy for patients with severe asthma in the future.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.alit.2021.07.004>.

Conflict of interest

The authors have no conflict of interest to declare.

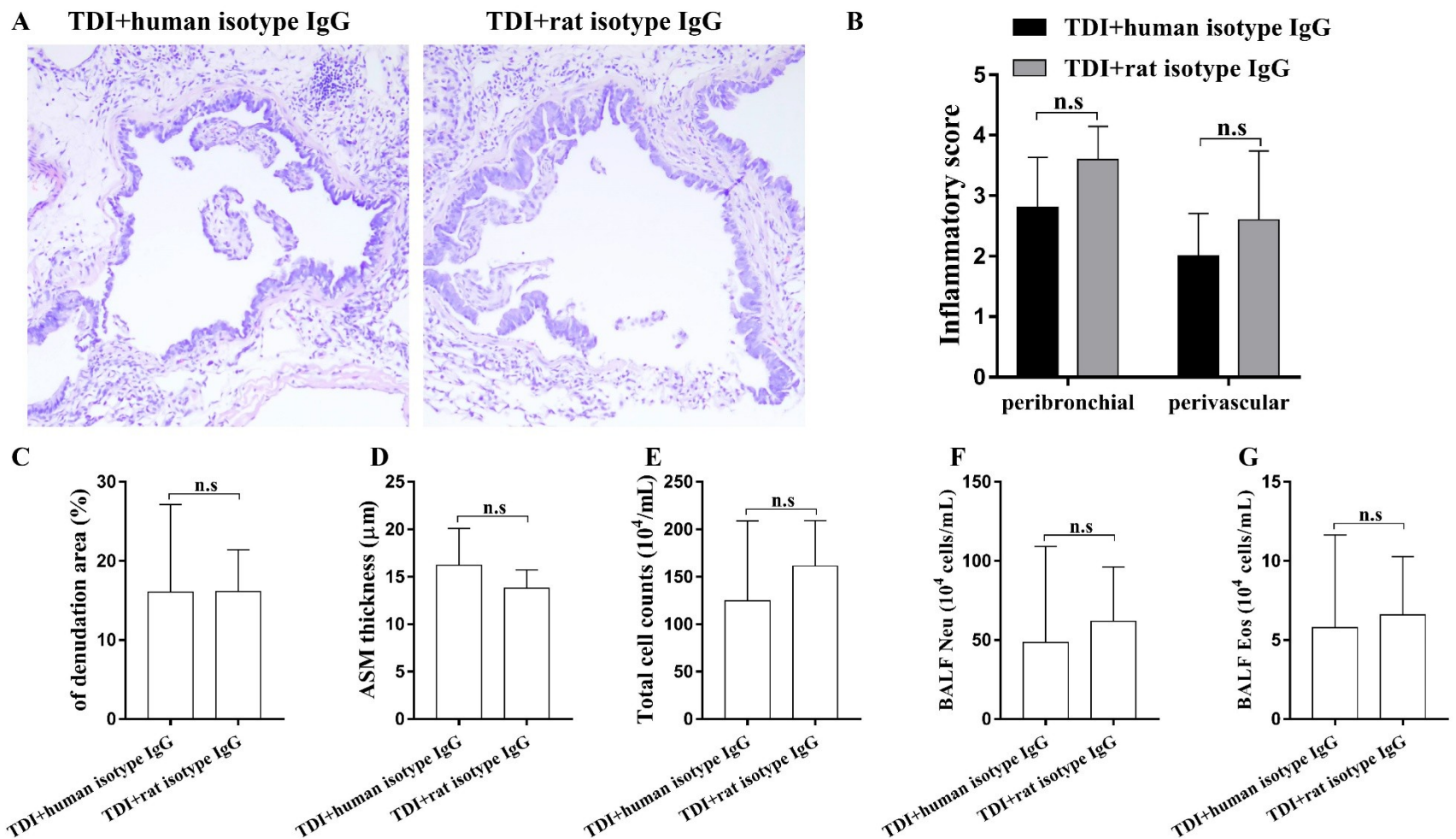
Authors' contributions

SC, RC, and SL designed the experiment, analyzed data and wrote the manuscript. SC, ZC, and YD performed the experiments. ZL, SZ, LY, DL, and KY helped with the experiments. RC and SL helped with data analysis and revised the manuscript. All the authors agreed that the final approval of the version to be published and ensured questions relating to the accuracy or integrity of any part of the work were appropriately investigated and resolved.

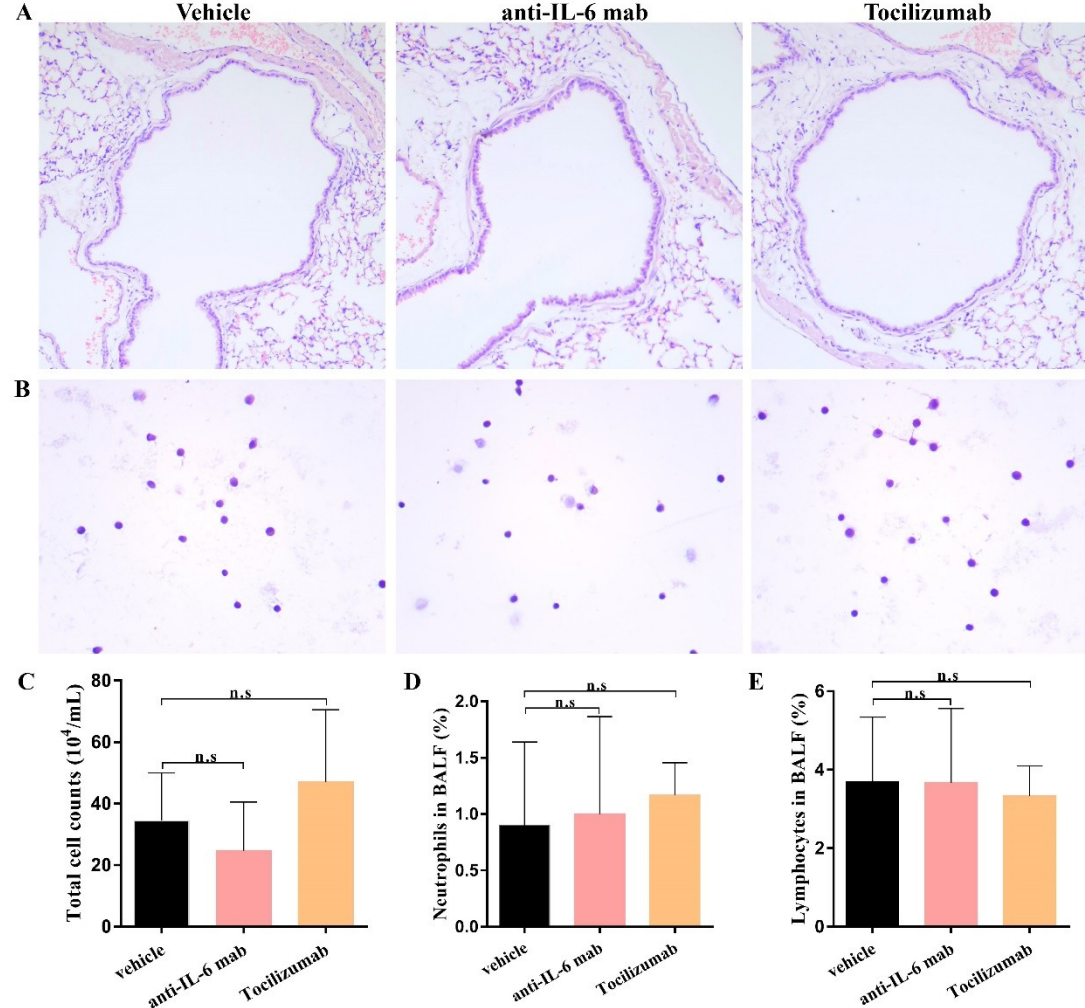
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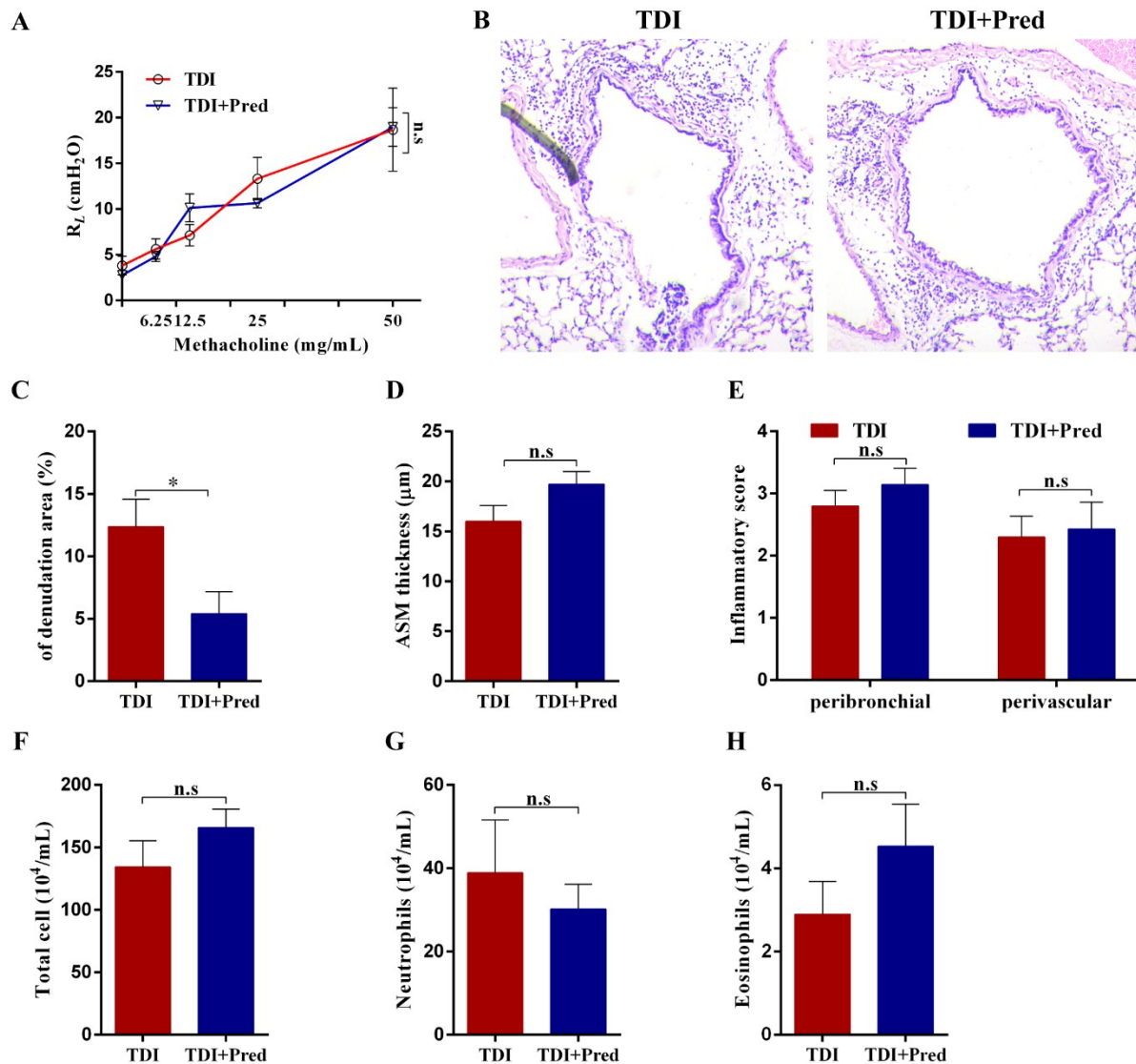
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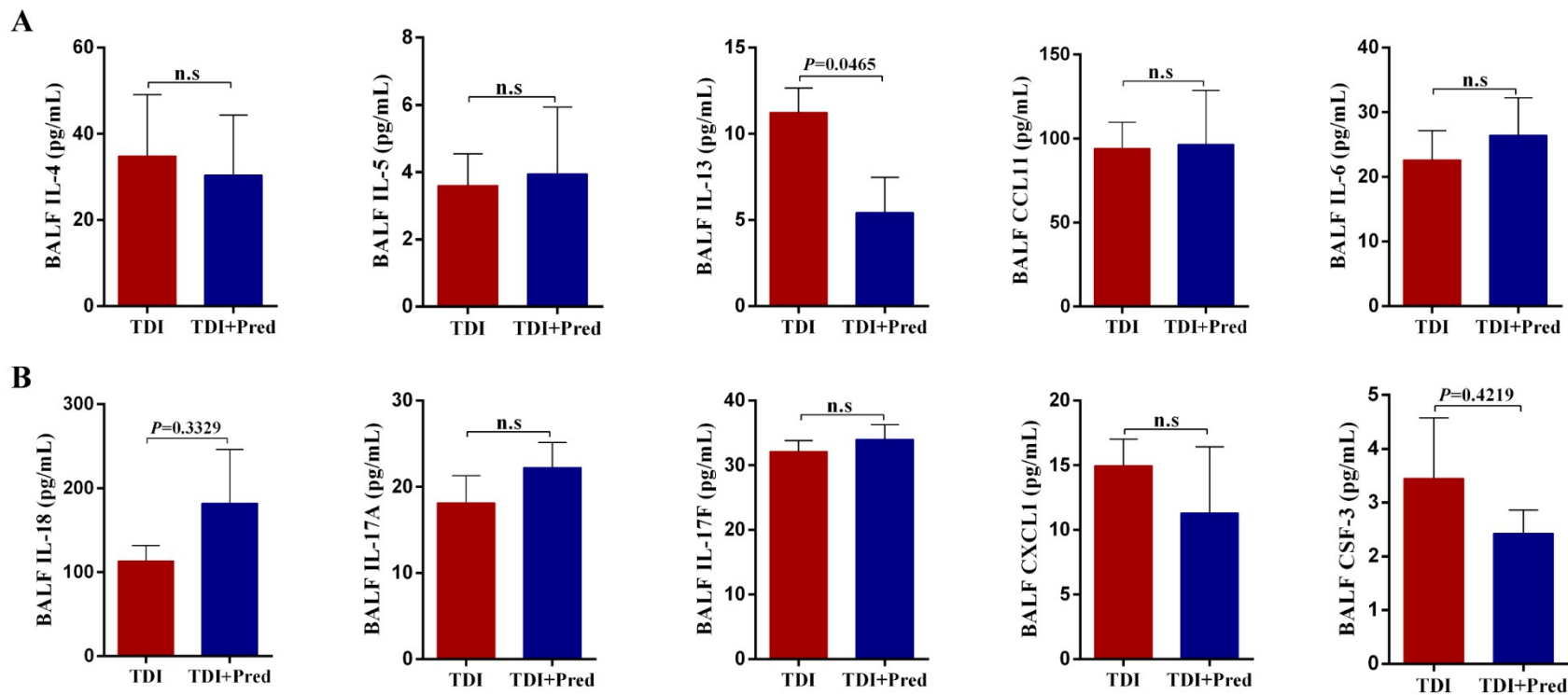
Supplementary Fig. 1. The effect of human isotype IgG on TDI-induced airway inflammation was similar to that of rat isotype IgG control. TDI-exposed mice were treated with human isotype IgG1 (5mg/kg, i.p.) or rat isotype IgG1 (5mg/kg, i.p.) after every challenge for a total of three times. The vehicle used to dissolve the isotype IgG controls was PBS. Analysis was performed one day after the last treatment. **A**, Representative H&E stained lung sections of two groups. Original magnification was 200×; **B-D**, Semiquantification of peribronchial, perivascular inflammation, as well as epithelial denudation and airway smooth muscle (ASM) thickness; n=5. **E-G**, Total and differential inflammatory cell counts in BALF. n=5. There were no significant differences between two groups. **n.s** indicated not significant.



Supplementary Fig. 2. None of the neutralizing antibodies affected airway inflammation in naive mice. BABL/C mice were treated with anti-IL-6 mAb (5mg/kg, i.p.), tocilizumab (5mg/kg, i.p.), or vehicle control once daily every other two days for a total of three times. The vehicle used to dissolve the blocking antibodies was PBS. Analysis was performed one day after the last treatment. **A**, Representative H&E stained lung sections of different treatment groups. No inflammation was seen in the airways of all enrolled mice and the epithelia were intact. Original magnification was 200 $\times$; **B**, Representative H&E stained cytospin samples from BALF of different treatment groups. Original magnification was 400 $\times$. **C-E**, Total and differential inflammatory cell counts in BALF. $n=6\sim8$. There were no significant differences among all groups. **n.s** indicated not significant.



Supplementary Fig. 3. Systemic prednisone (Pred) treatment had no effects on TDI-induced airway hyperreactivity and inflammation. **A**, Airway hyperresponsiveness was measured by lung resistance (R_L). $n=4\sim5$. **B**, Representative H&E-stained lung sections of mice treated with TDI alone or TDI+Pred. Original magnification was 200 $\times$. **C-E**, Semiquantification of epithelial denudation, airway smooth muscle (ASM) thickness as well as peribronchial and perivascular inflammation, $n=7\sim8$. **F-H**, Numbers of total inflammatory cells and neutrophils, eosinophils in BALF. $n=8\sim10$. **n.s** indicated not significant.



Supplementary Fig. 4. BALF levels of IL-4, IL-5, IL-6, IL-18, IL-17A, IL-17F, CCL11, CXCL1 and CSF-3 were not significantly different between TDI-exposed mice treated with and without Pred. $n=7\sim8$. **n.s** indicated not significant.