

Blockade of the NLRP3/Caspase-1 Axis Ameliorates Airway Neutrophilic Inflammation in a Toluene Diisocyanate-Induced Murine Asthma Model

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ABSTRACT

Multiple studies have addressed the vital role of Nod-like receptor protein 3 (NLRP3)/caspase-1/IL-1 β signaling in asthma. Yet, the role of NLRP3/caspase-1 in toluene diisocyanate (TDI)-induced asthma is still obscure. The aim of this study is to investigate the role of the NLRP3/caspase-1 axis in TDI-induced asthma. Using an established murine model of TDI-induced asthma as described previously, we gave the asthmatic mice a highly selective NLRP3 inhibitor, MCC950, as well as the specific caspase-1 inhibitors VX-765 and Ac-YVAD-CHO for therapeutic purposes. Airway resistance was measured and bronchoalveolar lavage fluid was analyzed. Lungs were examined by histology, immunohistochemistry, Western blotting, and flow cytometry. TDI exposure elevated the expression of NLRP3 and caspase-1 that was coupled with increased airway hyperresponsiveness (AHR), neutrophil-dominated cell infiltration, pronounced goblet cell metaplasia, extensive collagen deposition, and increased T_H2/T_H17 responses. Both VX-765 and Ac-YVAD-CHO effectively inhibited the activation of caspase-1 in TDI-asthmatic mice that was accompanied by dramatic attenuation of AHR, airway inflammation, and airway remodeling, in addition to a decreased T_H2 response and lower levels of IL-18 and IL-1 β . MCC950 blocked the activation of NLRP3 and downregulated protein expression of caspase-1, IL-1 β , and IL-18 in TDI-exposed mice. Furthermore, MCC950 remarkably alleviated AHR, airway inflammation, airway remodeling, and significantly suppressed T_H2/T_H17 responses. These findings suggested that blockade of the NLRP3/caspase-1 axis effectively prevents the progression of TDI-induced asthma and could be used as therapeutic targets for asthmatics.

Key words: asthma; toluene diisocyanate; neutrophilic inflammation; caspase-1, NLRP3.

Nearly 10% of adult-onset asthma can be attributed to occupational asthma, with exposure to diisocyanate being the most prevalent cause due to its worldwide use in industrial manufacturing (Gautrin et al., 2003). Toluene diisocyanate (TDI)-induced asthma is often characterized by cell infiltration dominated by neutrophils and a small number of eosinophil aggregating in the airway (De Vooght et al., 2013; Tang et al., 2014), which responds poorly to corticosteroids and often persists even after removal from diisocyanate exposure (Lemiere et al., 1996; Piirila et al., 2000). Poor understanding of the intrinsic mechanisms underlining the development of TDI-induced asthma is hindering the development of therapeutic strategies (Hansbro et al., 2013; Wang et al., 2010). It is generally known that a series of pathogenic mechanisms, including immunologic, inflammatory, and neurogenic pathways, as well as genetic factors, have been proved to contribute to TDI-induced asthmatic inflammation (Wisniewski and Redlich, 2001). Accumulating evidence has demonstrated that increased neutrophil infiltration was associated with steroid insensitivity and impaired lung function (Moore et al., 2014; Uddin et al., 2010). Moreover, depletion of neutrophils significantly blunted TDI-induced airway hyperresponsiveness (AHR) and airway inflammation (De Vooght et al., 2013). Thus, there is an urgent need to find more specific therapeutic targets for TDI-induced neutrophilic asthma.

In recent years, studies regarding the roles of inflammasomes in airway disorders have escalated. The Nod-like receptor protein 3 (NLRP3) inflammasome, which consists of an NLRP3 scaffold, apoptosis-associated speck-like protein containing a CARD (ASC) adaptor and caspase-1, has emerged as a pivotal mediator of chronic inflammatory diseases (Schroder and Tschopp, 2010). The NLRP3 inflammasome is often activated by damage-associated molecular patterns (DAMPs), pathogen-associated molecular patterns (PAMPs), or environmental irritants resulting in the auto-activation of caspase-1, followed by the cleavage and activation of proIL-1 β and proIL-18 to their mature forms and promotion of their release (Schroder and Tschopp, 2010). A multitude of evidence has suggested that NLRP3, the most widely characterized inflammasome, is involved in the pathogenesis of asthmatic inflammation and airway remodeling. Elevated gene expression of NLRP3, caspase-1, and IL-1 β in sputum but not in biopsy specimens was found in patients with neutrophilic asthma (Rossios et al., 2018; Simpson et al., 2014) and was correlated with disease severity and steroid resistance (Kim et al., 2017). Blockade of NLRP3 with the specific inhibitor MCC950 or gene knockout mice demonstrated dramatically alleviated AHR and inflammation in mouse models of house dust mite (HDM)-induced, ovalbumin (OVA)-induced, or infection-induced severe asthma (Besnard et al., 2011; Kim et al., 2017; Rossios et al., 2018). Using genetic knockout mice, Kim et al. demonstrated that NLRP3 facilitated airway hyper-reactivity in obesity-associated asthma (Kim et al., 2014). In addition, caspase-1 knockout mice exhibited markedly reduced eosinophilia despite increasing neutrophilia (Menzel et al., 2017). However, others discovered that caspase-1-deficient and NLRP3-deficient mice exhibited prominent HDM-induced allergic inflammation with pronounced eosinophil recruitment (Madouri et al., 2015). These opposite results pointed out the controversial but critical role of the NLRP3/caspase-1 axis in the pathogenesis of asthma. Increased expression of caspase-1 and IL-1 β in TDI-induced asthma has been already reported

(Johnson et al., 2005; Liang et al., 2015). Inhibition of IL-1 signaling through deletion of the IL-1 receptor or neutralizing antibodies abrogated the development of TDI-induced asthma (Johnson et al., 2005). Yet, the role of the well-established upstream NLRP3/caspase-1 axis in TDI-induced asthma is still elusive. Thus, the aim of this study is to investigate the role of the NLRP3/caspase-1 axis in a TDI-induced asthma model.

MATERIALS AND METHODS

Reagents. TDI (toluene-2,4-diisocyanate, $\geq 98.0\%$), methacholine, and acetone were obtained from Sigma-Aldrich (Shanghai, China). The vehicle (acetone and olive oil, AOO) used to dissolve TDI consisted of a mixture of 2 volumes of acetone and 3 volumes of olive oil for sensitization and 1 volume of acetone and 4 volumes of olive oil for the challenge. Multiplex immunoassays for IL-4, IL-5, IL-13, IL-17A, IL-17F, IL-6, IL-18, tumor necrosis factor (TNF)- α , CCL11, CXCL1, and CSF3 and ELISA kits for total IgE and IL-1 β were purchased from eBioscience (San Diego, California). The NLRP3 inhibitor MCC950 (no. s7809) and caspase-1 inhibitor VX-765 (no. s2228) were purchased from Selleck Chemicals (Houston, Texas). Another caspase-1 inhibitor, Ac-YVAD-CHO (no. A8954), was purchased from ApexBio Technology (Houston, Texas). Antibodies used are listed as follows: primary antibodies against NLRP3 (no. ab214185) and caspase-1 (no. ab1872) were purchased from Abcam (Shanghai, China), rabbit anti-IL-18 antibody (no. A16737) was purchased from ABClonal Biotech (Shanghai, China), and mouse anti-IL-1 β antibody (no. 12242) from Cell Signaling Technology (Danvers, Massachusetts).

Animals. Six-to-eight-week-old specific-pathogen-free male C57BL/6 mice (20–22 g) were purchased from Southern Medical University. The mice were housed in a specific pathogen-free facility with 12 h dark/light cycles (temperature $23^{\circ}\text{C} \pm 2^{\circ}\text{C}$, humidity range 40%–70%, 12 h light/dark cycle [lighting: 7: 00–19: 00]). Sterile water and irradiated food were available ad libitum. All animal care and experimental procedures studies complied with the guidelines of the Committee of Guangzhou Medical University on the use and care of animals and were approved by the Animal Subjects Committee of Guangzhou Medical University.

TDI-induced asthma model. TDI-induced asthmatic mice were produced as described elsewhere (Yao et al., 2016). In the first protocol, all mice were randomized to 4 groups: (1) vehicle-sensitized, vehicle-challenged, and DMSO-treated mice (control group), (2) TDI-sensitized, TDI-challenged, and DMSO-treated mice (TDI group), (3) TDI-sensitized, TDI-challenged, and 100 mg/kg VX-765-treated mice (TDI+VX-765 group), and (4) TDI-sensitized, TDI-challenged, and 1 mg/kg Ac-YVAD-CHO-treated mice (TDI+Ac-YVAD-CHO group). In the second protocol, mice were randomly allocated to 3 groups: (1) vehicle-sensitized, vehicle-challenged, and PBS-treated mice (control group), (2) TDI-sensitized, TDI-challenged, and PBS-treated mice (TDI group), and (3) TDI-sensitized, TDI-challenged, and 200 mg/kg MCC950-treated mice (TDI+MCC950 group). Briefly, on days 1 and 8, mice were dermally sensitized 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 with 3% TDI through 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 2 volumes of acetone and 3 volumes of olive oil for sensitization and 1 volume of acetone and 4 volumes of olive oil for inhalation. The selective caspase-1 blockers, VX-765 (100 mg/kg, i.p.) (Maroso et al., 2011; Zhao et al., 2016), and Ac-YVAD-CHO (1 mg/kg i.n.) (Kim et al., 2017), were dissolved in DMSO and diluted with PBS (pH = 7.4). Either blocker was given to the mice once daily for 7 consecutive days, beginning immediately after the first challenge and continuing until the last day of challenge. Sham mice received the same volume of solvent for comparison. The specific selective NLRP3 inhibitor, MCC950 (200 mg/kg, i.p.) (Rossios et al., 2018) dissolved in PBS (pH = 7.4), was given to the mice immediately after each inhalation. Sham mice received the same volume of solvent for comparison.

Assessment of airway hyperresponsiveness. Twenty-four hours after the last challenge, measurements of lung resistance (R_L) were performed on anesthetized and mechanically ventilated (DSI, Buxco FinePointe) mice in response to increasing doses of methacholine by means of ultrasonic nebulization (6.25, 12.5, 25, and 50 mg/ml). Measurements of R_L were performed every 5 min following each nebulization step until a plateau phase was reached. Results were expressed as percentage of baseline R_L value for each concentration of methacholine.

Cell isolation and flow cytometry analysis. About 80 mg of right lung tissue was diced and incubated in RPMI 1640 medium with collagenase (200 U/ml, Sigma) at 37°C for 40 min. About 100-mm nylon filters were used to obtain single-cell suspensions from lung tissue, and ACK lysis buffer was used to remove the red blood cells. Isolated cells from lung and bronchoalveolar lavage fluid (BALF) were stimulated with phorbol 12-myristate 13-acetate (5 ng/ml) and ionomycin (1 mg/ml) in the presence of brefeldin A (10 mg/ml) for 5 h. BALF and lung single-cell suspensions were fixed, permeabilized (Cytofix/Cytoperm buffer, BD Biosciences, San Diego, California), and stained for 30 min with antibodies for CD4, IFN- γ , IL-4, and IL-17A (BD Biosciences, San Diego, California). Data were collected on a BD Biosciences Fortessa flow cytometer and analyzed using FlowJo software. Cell counts were performed before staining for flow cytometry analysis.

Measurement of serum total IgE and quantitation of cytokines in BALF and lung. Mice were euthanized with overdoses of pentobarbital (100 mg/kg, i.p.) 24 h after the last airway challenge. Blood samples were taken from the retroorbital plexus/sinus, allowed to rest at room temperature for 1 h, centrifuged (3000 g, 20 min) and supernatants harvested for detection of total IgE with an ELISA kit according to the manufacturer's instructions.

After blood was taken, the lungs were lavaged twice in situ with 0.8 ml prewarmed sterile saline (0.9% NaCl) and the recovered fluid was pooled. Total cells in BALF were counted, and a cytospin sample was prepared and stained with hematoxylin and eosin (H&E) for blinded assessment of differential cell percentages in BALF. The remaining fluids were centrifuged (1000 g, 10 min) and supernatants were stored at -80°C for further analysis. The levels of cytokines and chemokines in BALF and lung homogenates, including IL-4, IL-5, IL-13, IL-17A, IL-17F, IL-1 β , IL-6, IL-18, as well as eosinophil chemokine CCL11 and neutrophil chemokine CXCL1 and CSF3, were measured using multiplex immunoassay or ELISA kits (eBioscience, San Diego, California) according to the manufacturer's specifications.

Lung histopathological examination. The left lungs were infused with 4% neutral buffered formalin to fully inflate all lobes (judged visually), then fixed and embedded in paraffin. Lung sections (4 μ m) were cut with a Leica microtome 2030 (Leica Microsystems Nussloch GmbH, Nussloch, Germany). Lung tissues were stained with H&E and periodic acid-Schiff (PAS) for blinded histopathologic assessment. Lung inflammation and airway goblet cell metaplasia were semi-quantified as described previously (Yao et al., 2016). A value of 0 was scored when no inflammation was detectable, a value of 1 for infrequent cuffing with inflammatory cells, a value of 2 for most bronchi or vessels infiltrated by 1 layer (1–10 cells) of inflammatory cells, a value of 3 for most bronchi or vessels were surrounded by 2 layers (10–20 cells) of inflammatory cells, and a value of 4 for most bronchi or vessels were surrounded by more than 2 layers (more than 20 cells) of inflammatory cells. PAS-stained lung sections were examined under light microscopy for PAS-positive staining in the upper airway epithelium in fields at 200 $\times$ magnification. PAS-positive epithelial cells of the total epithelial cells were counted to obtain a percentage and compare groups. About 16–20 image fields of 8 sections from 8 to approximately 10 mice per group were analyzed. Sections were assigned a random code to blind the examiner to the identity of each specimen.

Western blotting and immunohistochemistry. Pulmonary expression of NLRP3, caspase-1, IL-1 β , and IL-18 was detected by Western blotting. The right lung extracts were mixed with 5 $\times$ SDS loading buffer and separated by 10% or 12% SDS-polyacrylamide gel and transferred onto PVDF membranes. Membranes were then probed with anti-NLRP3, anti-caspase-1, anti-IL-1 β , and anti-IL-18 antibodies at the indicated dilutions according to the manufacturer's recommendation. After incubation with an IRDye680WC-conjugated secondary antibody (LI-COR Biosciences, Lincoln, Nebraska), immunoreactive bands were exposed to Odyssey CLx Imager for image capture. Data analysis was performed with ImageJ software. For immunohistochemistry of NLRP3, caspase-1, IL-1 β , and IL-18, lung sections were deparaffinized then subjected to antigen retrieval. Samples were treated with H₂O₂ for 15 min to block endogenous peroxidase and then incubated overnight at 4°C in recommended dilutions of anti-NLRP3, anti-caspase-1, anti-IL-1 β , and anti-IL-18 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 6, GraphPad) and SPSS (version 20.0, SPSS Inc., Chicago, Illinois) were used for statistical analysis and graphing. Data were expressed as mean $\pm$ SEM, and comparisons among groups were analyzed via 1-way analysis of variance (ANOVA) accompanied by Bonferroni post hoc test (equal variances assumed) or Dunnett's T3 (equal variances not assumed) post hoc tests for multiple comparisons. $p < .05$ was regarded as statistically significant. At least 3 independent experiments were repeated.

RESULTS

Blocking Caspase-1 Ameliorated Airway Hyperresponsiveness, Airway Remodeling, and Inflammation Induced by TDI

TDI-exposed mice exhibited increased airway reactivity to methacholine when compared with the control, together with a denser accumulation of inflammatory cells around the airway and pronounced goblet cell metaplasia as shown by BALF

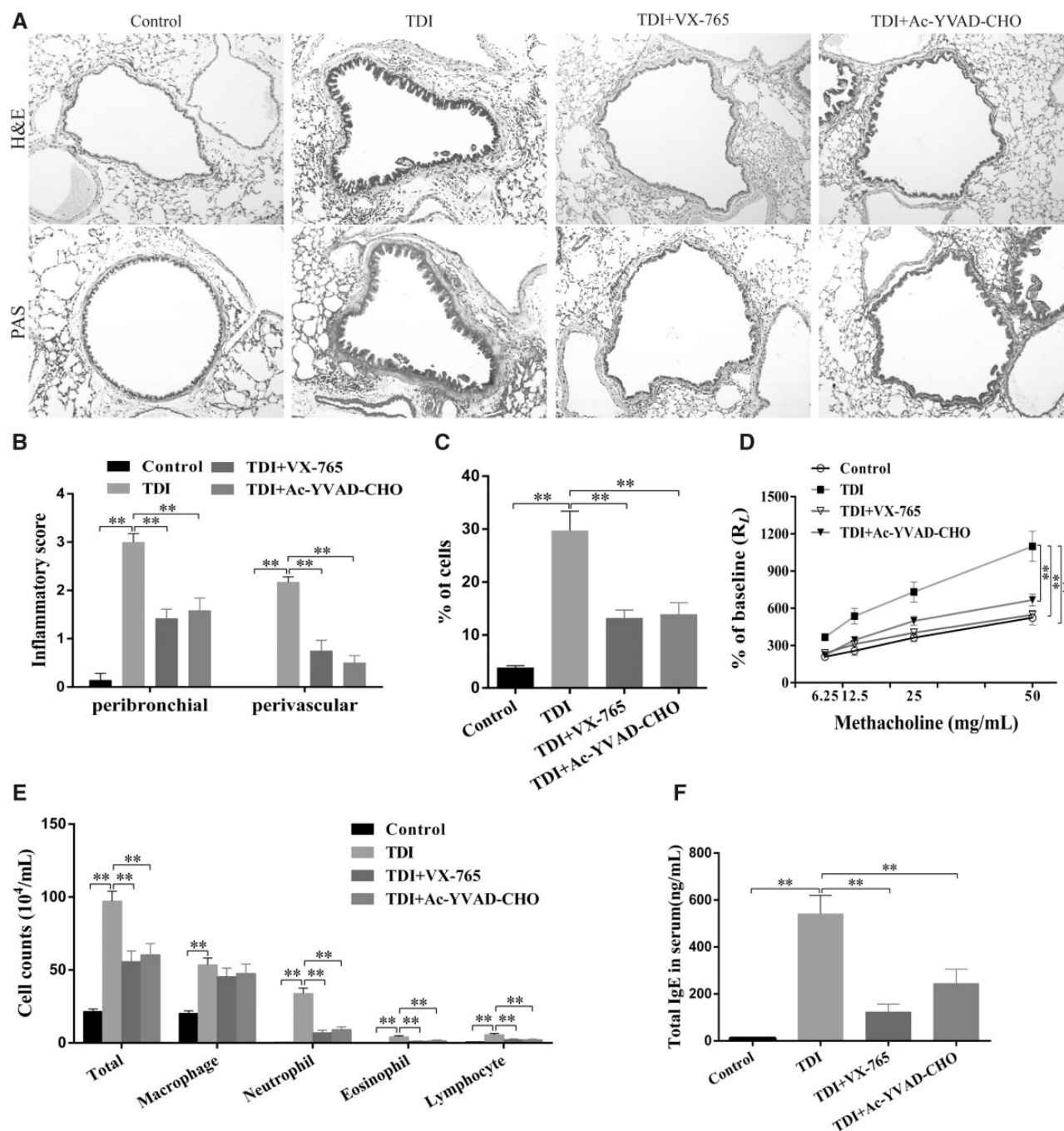


Figure 1. Caspase-1 inhibition led to decreased airway hyperresponsiveness (AHR), airway inflammation, and airway remodeling in TDI-induced asthma. **A**, Representative H&E-stained and periodic acid-Schiff (PAS)-stained lung sections of different groups. Original magnification was 200 $\times$. **B–C**, Semi-quantification of airway inflammation and PAS staining was performed. The number of PAS-positive epithelial cells to counted to obtain a percentage to quantify the PAS-positive staining. $n = 8\sim 12$. **D**, AHR was measured by lung resistance (R_L). Results were shown as percentage of baseline. $n = 5$. **E**, Number of total inflammatory cell, macrophage, neutrophil, eosinophil, and lymphocyte in BALF. $n = 8\sim 12$. **F**, Concentrations of total IgE in serum were determined. $n = 6\sim 8$. * $p < .05$; ** $p < .01$.

analysis, pathology, and morphology, respectively (Figs. 1A–E). Treatment with VX-765 (100 mg/kg) or Ac-YVAD-CHO (1 mg/kg), beginning from the first challenge of TDI and continuing daily for 7 consecutive days, significantly ameliorated AHR, airway inflammation around the airways, and epithelial goblet cell hyperplasia as assessed by H&E and PAS staining (Figs. 1A–E). In line with this, there was a marked increase in total IgE in the serum after TDI inhalation that was significantly reduced by both antagonists (Figure 1F).

Caspase-1 Depletion Diminished TDI-Induced T_H1/T_H2 Responses With a Lesser Effect on T_H17 Cells

Clinical and experimental evidence indicates that $T_H1/T_H2/T_H17$ dysfunction was involved in TDI-induced asthma (Johnson et al., 2005; Matheson, 2005; Pollaris et al., 2016; Yao et al., 2015). Using flow cytometry, we explored the percentage of $T_H1/T_H2/T_H17$ cells in the BALF and lung tissue, and found that T_H17 response is dominant in TDI-induced asthma model. We used CD4 to mark all the T cells roughly, and then adopted different

markers (IFN- γ , IL-4, and IL-17A) to mark $T_H1/T_H2/T_H17$ cells in CD4-positive cells (Choy et al., 2015; Gurung et al., 2015). Dysregulation of T_H1 , T_H2 , and T_H17 responses were observed in TDI-sensitized and challenged mice, with significantly increased percentages of CD4 IFN- γ^+ (T_H1), CD4 IL-4 $^+$ (T_H2), and CD4 IL-17A $^+$ (T_H17) cells in both BALF and lung. Treatment with VX-765 or Ac-YVAD-CHO robustly decreased the number of T_H1/T_H2 cells, although they had no significant effect on T_H17 cells (Figs. 2A and 2B). In addition, $T_H1/T_H2/T_H17$ -associated cytokines were analyzed. TDI exposure notably upregulated the levels of T_H2 -related IL-4, IL-5, and IL-13 as well as T_H17 -related IL-17A and IL-17F in both BALF and lung homogenates when compared with control (Figs. 2C and 2D). Other cytokines including IL-6, IL-18, and TNF- α , as well as the eosinophil chemoattractant CCL11 and neutrophil chemoattractants CXCL1 and CSF-3, were also notably raised in TDI-induced asthma. VX-765 or Ac-YVAD-CHO blunted the release of all these except IL-13, IL-17A, and IL-17F in BALF and lung homogenates (Figs. 2C and 2D). However, we failed to detect the T_H1 -related cytokine IFN- γ , T_H2 -related cytokine IL-25, T_H17 -related cytokines IL-22, and IL-23 in both BALF and lung homogenates.

Caspase-1 Inhibition Dramatically Inhibited TDI-Upregulated Expression of IL-1 β and IL-18

To confirm the effects of caspase-1 inhibitors, we measured caspase-1 by immunohistochemistry and Western blotting. We discovered that after TDI inhalation, caspase-1 was markedly increased with positive staining mainly distributed in the cytoplasm of bronchial and alveolar epithelial cells, alveolar macrophages, and infiltrating inflammatory cells (Figure 3A). Moreover, pulmonary expression of procaspase-1 (45KD) and cleaved caspase-1 (20KD) were significantly increased (Figs. 3C and 3D). Both VX-765 and Ac-YVAD-CHO diminished protein expression of caspase-1 and procaspase-1, indicating that TDI-induced caspase-1 activation was effectively inhibited. Consistent with this, the increased pulmonary activity of caspase-1 induced by TDI sensitization and inhalation was also suppressed by VX-765 or Ac-YVAD-CHO (though not significant) (Figure 3E). Furthermore, we also detected NLRP3, the upstream of caspase-1. TDI exposure markedly raised the protein expression of NLRP3 that was observed mainly in bronchial epithelium and was weakened by treatment with VX-765 or Ac-YVAD-CHO (Figs. 3B–D). These data suggested that exposure to TDI triggered the activation of caspase-1 (Figs. 3B–D).

To reconfirm the role of caspase-1 and its downstream mechanism involved in TDI-induced asthma, we examined the production of IL-18 and IL-1 β , two classic downstream cytokines. After TDI challenge, IL-18 and IL-1 β were remarkably increased as shown by dense staining throughout the cytoplasm of bronchial and alveolar epithelial cells as well as in infiltrating inflammatory cells. Treatment with either inhibitors almost completely reversed the TDI-induced expression of IL-1 β although IL-18 was only slightly reduced (Figs. 4A and 4B). By Western blotting analysis, the protein level of cleaved IL-1 β (17KD) was significantly suppressed by blockade of caspase-1, with unchanged levels of proIL-1 β and proIL-18 (Figs. 4C and 4D). Moreover, TDI-induced elevation of IL-18 in BALF (Figure 2C) and increased IL-1 β in lung homogenates were alleviated by caspase-1 inhibition (Figure 4E). However, IL-1 β was not detected in BALF. These data suggested that proIL-18 and proIL-1 β were cleaved in a caspase-1-dependent manner (Schroder and Tschopp, 2010).

MCC950 Abrogated TDI-Induced Airway Hyperresponsiveness, Airway Inflammation, and Remodeling

Subsequently, we tested whether blocking NLRP3 inflammasome (upstream of caspase-1) with MCC950 (200mg/kg) could attenuate TDI-induced inflammation. Similarly, TDI-induced asthmatic mice demonstrated increased airway reactivity, as well as significantly increased neutrophils with lesser numbers of eosinophils infiltrating into the airway and pronounced bronchial epithelial hyperplasia (Figs. 5A–F). Treatment with MCC950 nearly abolished each of these responses, implying a crucial role for NLRP3 inflammasome activation in the pathogenesis of TDI-induced asthma.

Inhibition of NLRP3 Prominently Restricted $T_H1/T_H2/T_H17$ Responses

Treatment with MCC950 dramatically suppressed the elevated cell percentages of CD4 IFN- γ^+ , CD4 IL-4 $^+$, and CD4 IL-17A $^+$ cells in both BALF and lung of TDI-exposed mice (Figs. 6A and 6B). In addition, MCC950 largely inhibited the release of all aforementioned cytokines except CCL11 in BALF (Figs. 6C and 6D).

MCC950 Inhibited the Activation of NLRP3 and Downregulated the Expression of Caspase-1, IL-1 β , and IL-18 in TDI-Induced Asthma

To confirm the effect of MCC950, we measured protein levels of NLRP3 and its most important effector, caspase-1. Treatment with MCC950 nearly eliminated the protein expression of caspase-1, although it upregulated procaspase-1 (Figs. 7C and 7D). Similar results were obtained by immunohistochemistry (Figs. 7A and 7B). Consistent with this, there was a robust increase of caspase-1 activity in lung homogenates after TDI inhalation, which was reduced by MCC950 (Figure 7E).

Finally, we measured the typical downstream proteins of the NLRP3/caspase-1 axis, IL-18, and IL-1 β , of which IL-1 β played a critical role in the differentiation and maturation of T_H17 cells (Chung et al., 2009). TDI challenge following dermal sensitization remarkably increased the pulmonary expression of proIL-18, proIL-1 β , and IL-1 β , yet only IL-1 β was conspicuously reduced by MCC950 (Figs. 8C and 8D). Immunohistochemistry showed similar results (Figs. 8A and 8B). In accordance with this, IL-18 in BALF (Figure 8C) and IL-18 and IL-1 β in lung homogenates were elevated after TDI exposure (Figure 8E), all of which were decreased by MCC950.

DISCUSSION

The large number of neutrophils infiltrating into the airways is a central feature of TDI-induced asthma (De Vooght et al., 2009) and is the major cause for steroid resistance and decreased lung function (Moore et al., 2014; Uddin et al., 2010). Blocking neutrophils with Ly6G-specific antibody abrogated AHR, bronchial epithelial damage, and airway inflammation in TDI-exposed mice (De Vooght et al., 2013). Here, we established a TDI-induced asthma model as described previously, with typical asthmatic features including AHR and neutrophil-predominant airway inflammation, along with a small number of eosinophils. It is well-documented that neutrophil infiltration involves a vast array of chemoattractants and cytokines. Without diminishing the importance of others, IL-1 β and IL-18 are crucially important mediators for neutrophil recruitment (de Oliveira et al., 2016), both of which belong to the IL-1 family and share some structural similarities (Xu et al., 2017). Emerging evidence has suggested that IL-1 β and IL-18 play preeminent roles in driving the pathogenesis of neutrophilic asthma (Kim et al., 2015). Prevention of IL-1 β activity through deletion of the IL-1 receptor type I or administration of neutralizing antibody abrogated the

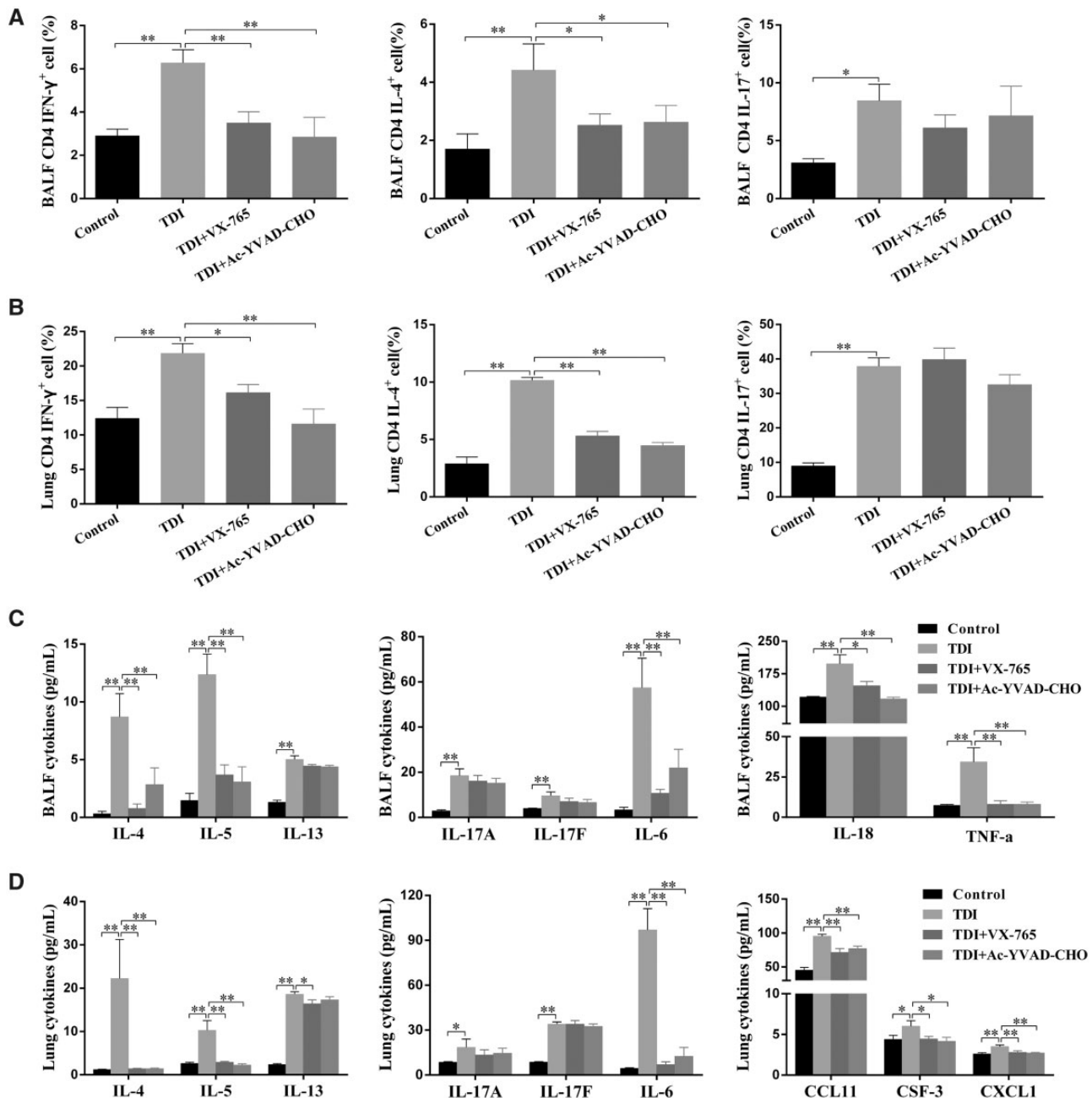


Figure 2. Blocking caspase-1 robustly attenuated TDI-induced T_H2 responses with a lesser effect on T_H17 cells. A-B, Number of CD4 IFN- γ ⁺, CD4 IL-4⁺, CD4 IL-17A⁺ cells in BALF cells (A) and lung single cell suspensions (B). Data were presented as percentages of CD4⁺ cells. $n = 4$. C, Levels of IL-4, IL-5, IL-13, IL-17A, IL-17F, IL-6, IL-18, and TNF- α in BALF were quantified by multiplex immunoassays. $n = 6-8$. D, Levels of IL-4, IL-5, IL-13, IL-17A, IL-17F, IL-6, IL-18, IL-1 β , CCL11, CXCL1, and CSF-3 in lung homogenates were quantified by multiplex immunoassays or ELISA. $n = 6-8$. * $p < .05$; ** $p < .01$.

progression of TDI-induced asthma (Johnson et al., 2005), although administration of recombinant IL-1 β replicated the hallmarks of steroid-resistant neutrophilic asthma (Kim et al., 2017). Similarly, IL-18-deficient mice exhibited diminished neutrophilic airway inflammation and remodeling in OVA-induced asthma (Yamagata et al., 2008). In the present study, along with the increased airway neutrophils, we detected upregulated pulmonary expression of IL-1 β and IL-18 after TDI challenge following dermal sensitization, with denser and aberrant distribution in the cytoplasm and nucleus of infiltrating inflammatory cells, as well as in bronchial and alveolar epithelia, which agrees with the findings of other studies (Liang et al., 2015; Yao et al., 2017,

2018), implying that IL-1 β and IL-18 are involved in TDI-induced neutrophilic inflammation. Yet, the upstream mechanisms are still obscure.

Caspase-1 is an endogenous cysteine protease, which is required for the maturation and release of IL-1 β and IL-18, and plays a crucial role in inflammation (Schroder and Tschopp, 2010). Recent studies have highlighted the central role for caspase-1 in the pathogenesis of asthma (Kim et al., 2017; Madouri et al., 2015; Menzel et al., 2017). In 2015, Madouri et al. reported enhanced airway eosinophilic inflammation in caspase-1-deficient mice exposed to HDM (Madouri et al., 2015). Conversely, Menzel et al. discovered that lack of caspase-1

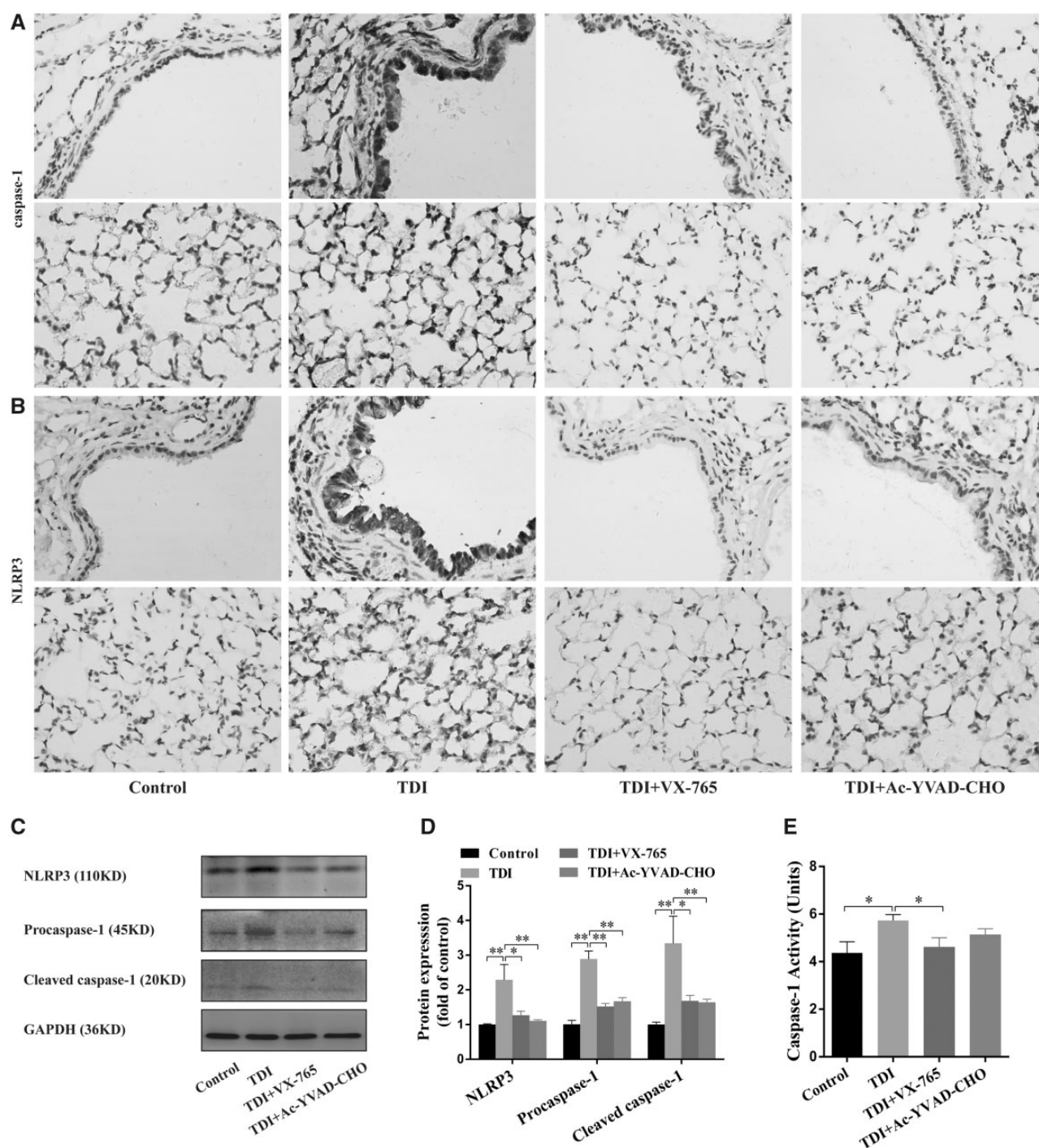


Figure 3. Treatment with either VX-765 or Ac-YVAD-CHO inhibited activation of caspase-1 and weakened pulmonary protein expression of NLRP3. **A**, Representative immunohistochemistry of caspase-1 in the bronchial regions (upper panel) and alveolar regions (lower panel). Treatment with VX-765 or Ac-YVAD-CHO significantly inhibited caspase-1 expression. Original magnification was 400 $\times$. **B**, Representative immunohistochemistry of NLRP3 in the bronchial regions (upper panel) and alveolar regions (lower panel) revealed much more staining after TDI exposure when compared with the control. Original magnification was 400 $\times$. **C-D**, Protein expression of NLRP3, procaspase-1 (45KD), and cleaved caspase-1(20KD) in lung homogenates was detected by Western blotting and densitometric analysis was performed. $n = 6$. **E**, Levels of caspase-1 activity in lung homogenates. $n = 6 \sim 8$. * $p < .05$; ** $p < .01$.

reduces eosinophilia and T_H2 cytokine production in a HDM-induced and rhinovirus-boosted asthma exacerbation model (Menzel et al., 2017). Such contradictory findings led us to seek the role of caspase-1 in our TDI-induced asthma model, which is neutrophil rather than eosinophil predominant. As reported previously (Liang et al., 2015), a higher level and an increased protease activity of caspase-1 were detected in lungs of TDI-

exposed mice, indicating that caspase-1 activation might mediate TDI-asthmatic inflammation. So we set off to investigate the effects of blocking caspase-1 in this model. VX-765 is the prodrug of a potent and selective competitive inhibitor of the caspase-1 subfamily, although Ac-YVAD-CHO is a peptidomimetic small molecule with an aspartic acid that shows high affinity for caspase-1. Both of these can act as a decoy, binding

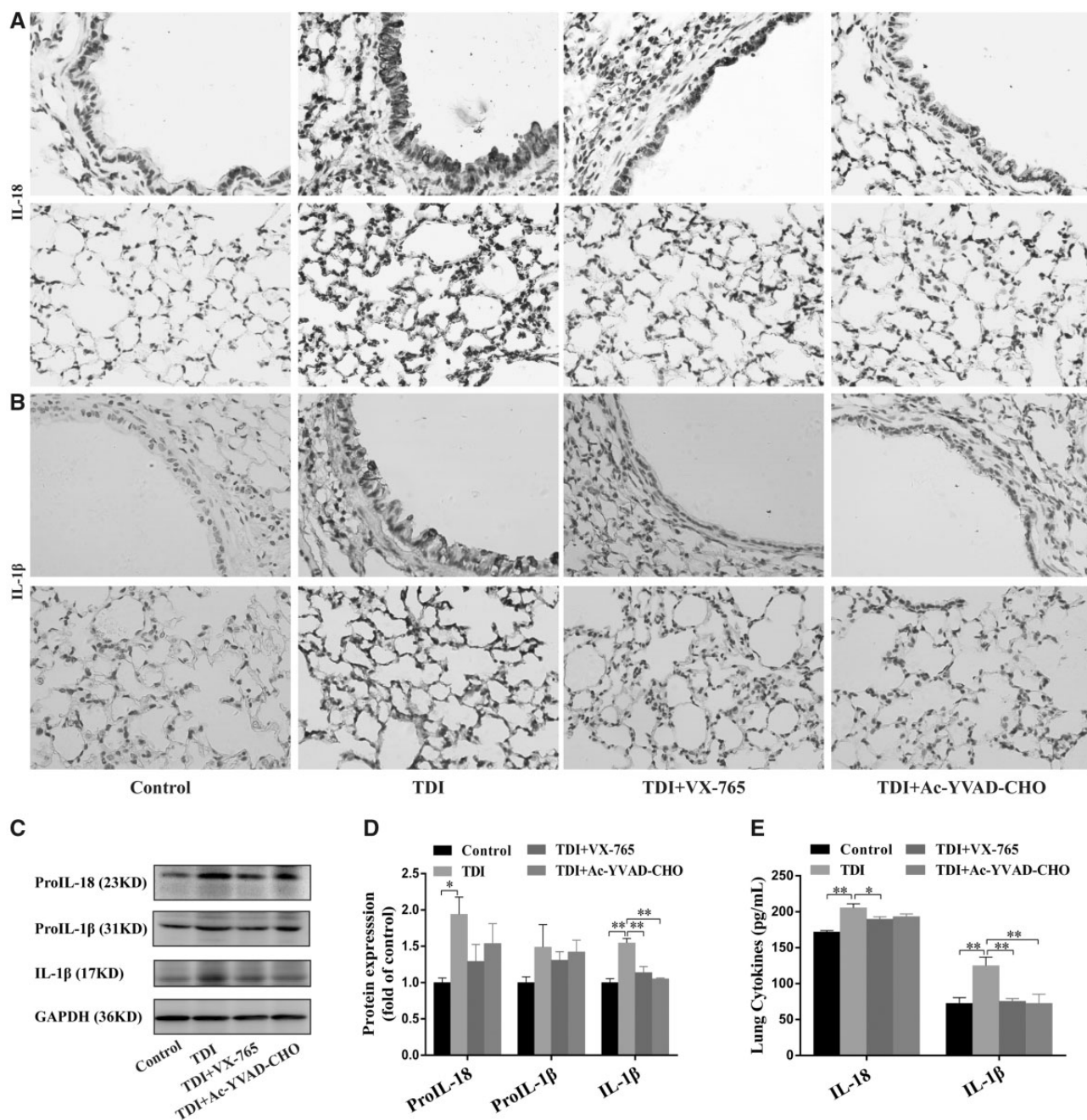


Figure 4. Blockade of caspase-1 suppressed expression of IL-1 β and IL-18 in TDI-exposed asthma. A-B, Representative immunohistochemistry of IL-18 (A) and IL-1 β (B) in the bronchial regions (upper panel) and alveolar regions (lower panel). Original magnification was 400 $\times$. C-D, Protein expression of proIL-18 (23KD), pro IL-1 β (31KD), and IL-1 β (17KD) in lung homogenates was detected by Western blotting and densitometric analysis was performed. $n = 6$. E, Levels of IL-18 and IL-1 β in lung homogenates. $n = 6 \sim 8$. * $p < .05$; ** $p < .01$.

specifically to caspase-1, and thus inhibit subsequent signaling pathways. *In vivo* and *in vitro* studies have already verified the safety and effectiveness of these 2 inhibitors in other disease models (Kim et al., 2017; Rossios et al., 2018; Zhao et al., 2016). As expected, treatment with either VX-765 or Ac-YVAD-CHO markedly abolished TDI-induced AHR and remodeling, decreased airway neutrophilia and eosinophilia, and suppressed the T_H2 response. At the same time, we also observed that the TDI-induced increases of IL-18 and IL-1 β were markedly inhibited after administration of VX-765 or Ac-YVAD-CHO, in spite of the unchanged protein levels of proIL-18 and proIL-1 β . These observations suggested that the TDI-induced increase of IL-18 and IL-

1 β was caspase-1 dependent (Schroder and Tschopp, 2010), which is responsible for airway neutrophil as well as eosinophil infiltrates (Gurung et al., 2015; Jorgensen et al., 2016; Yamagata et al., 2008).

Caspase-1 is the mature form of pro-caspase-1 and the effector of a protein complex named inflammasome (Schroder and Tschopp, 2010). As caspase-1 activating platforms, inflammasomes were involved in a variety of inflammatory diseases, including COPD, neutrophilic asthma, respiratory infections, and cystic fibrosis (Kim et al., 2015). Among them, the NLRP3 inflammasome, which controls the major auto-activation of caspase-1, is the most fully characterized. Blocking NLRP3 in HDM- and

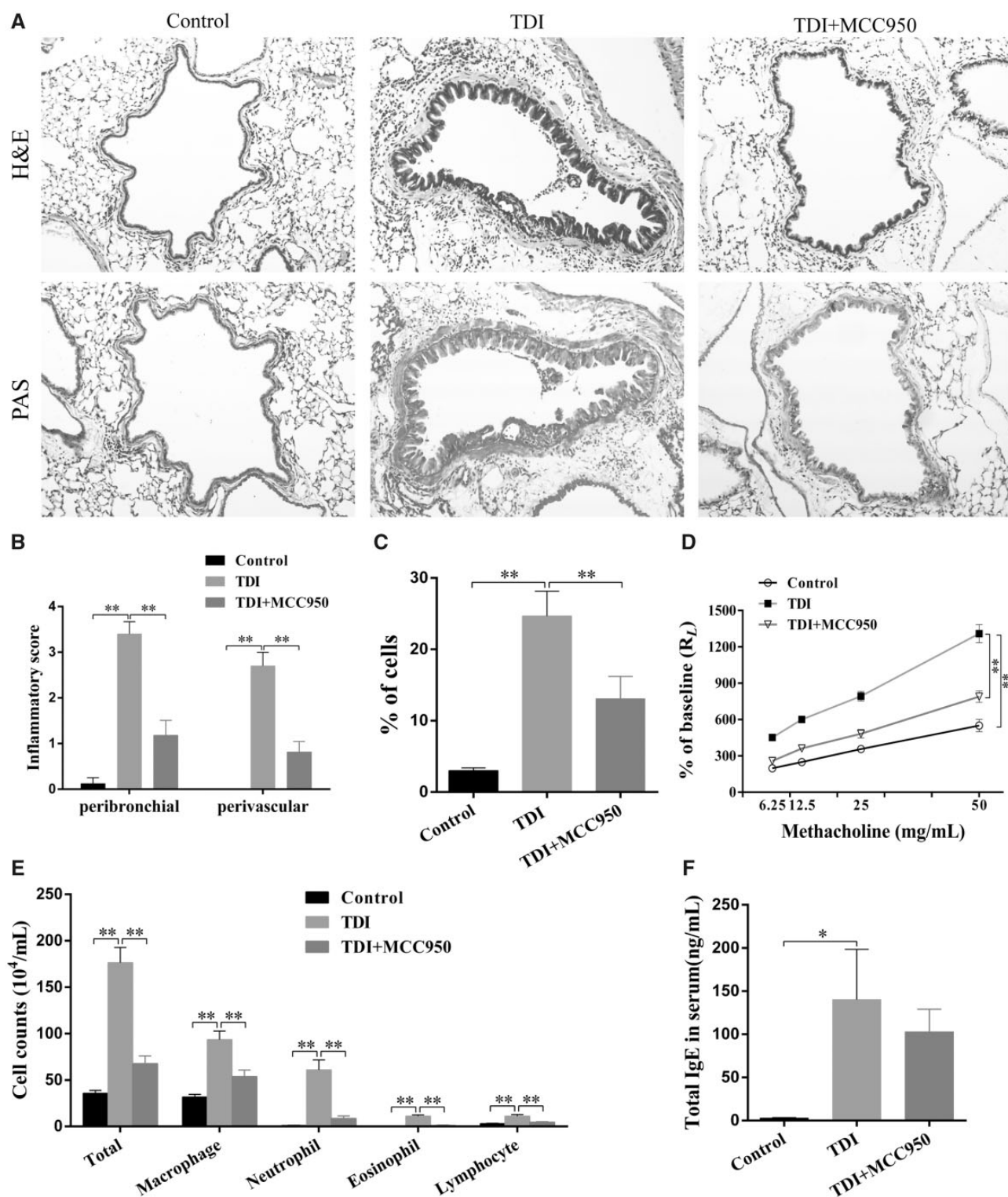


Figure 5. MCC950 ameliorated TDI-asthmatic airway inflammation, airway remodeling, and airway hyperreactivity. **A**, Representative H&E- and PAS-stained lung sections of different groups. Original magnification was 200 $\times$. **B-C**, Semi-quantification of airway inflammation and PAS staining was performed. Count the number of PAS-positive epithelial cells to quantify the PAS-positive staining. $n = 8\sim 11$. **D**, Airway hyperresponsiveness was measured by lung resistance (R_L). Results were shown as percentage of baseline. $n = 5$. **E**, Number of total inflammatory cell, macrophage, neutrophil, eosinophil, and lymphocyte in BALF. $n = 8\sim 11$. **F**, Concentrations of total IgE in serum were determined. $n = 5\sim 7$. * $p < .05$; ** $p < .01$.

infection-induced severe, steroid-resistant murine asthma models potentially suppressed neutrophilic inflammation and AHR, indicating pivotal roles for NLRP3/caspase-1 in the pathogenesis of refractory asthma (Kim et al., 2017; Rossios et al.,

2018). In the current study, we investigated the protein expression and distribution of NLRP3 in lungs of TDI-sensitized and challenged mice. As described previously (Liang et al., 2015), immunohistochemistry revealed that NLRP3 was extensively

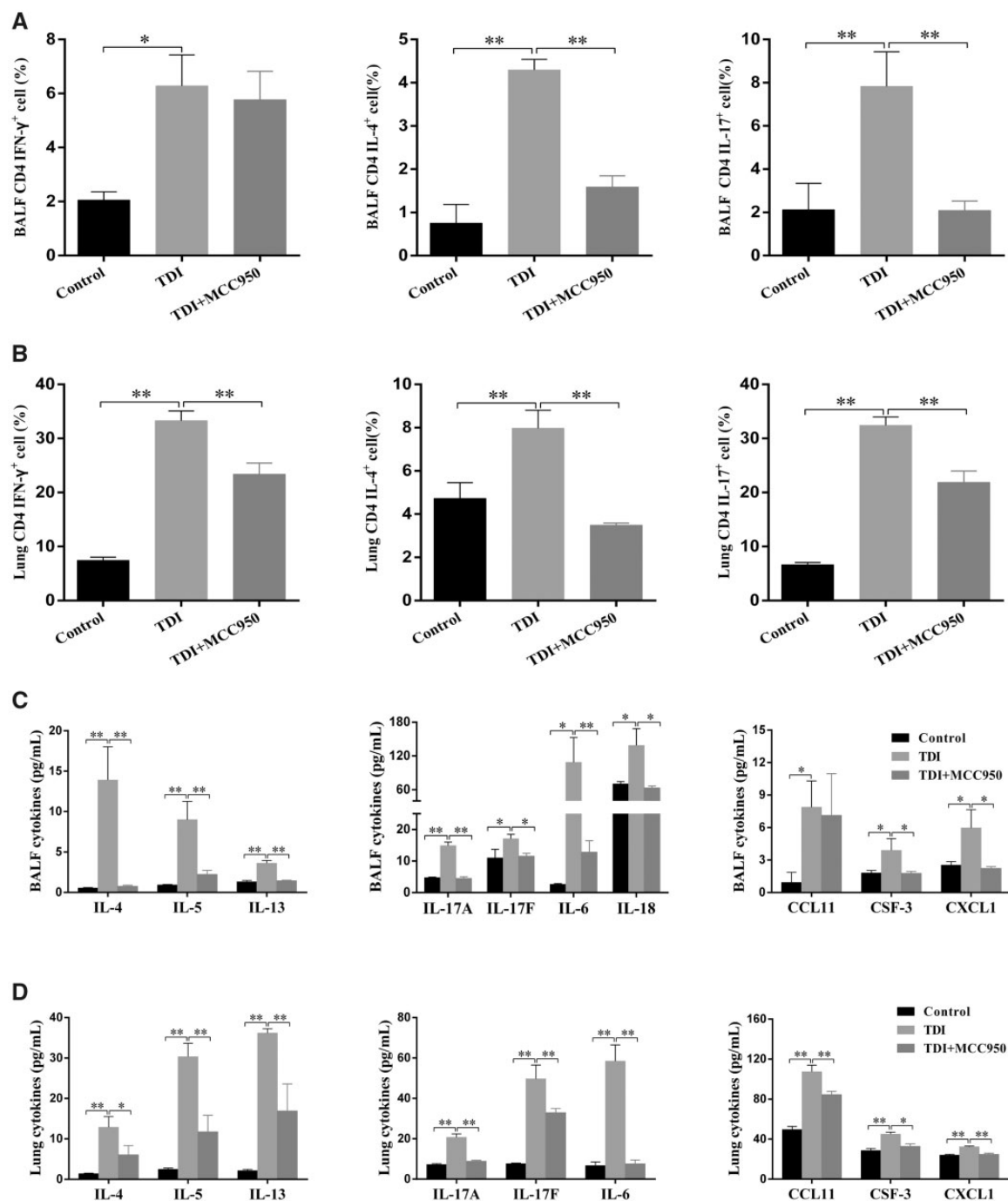


Figure 6. MCC950 prominently suppressed T_H17/T_H2 responses with minor T_H1 responses in TDI-exposed mice. A, Number of CD4 IFN- γ^+ , CD4 IL-4 $^+$, and CD4 IL-17A $^+$ cells in BALF cells and lung single cell suspensions. Data were presented as percentages in CD4 $^+$ cells. $n=4$. B, Number of CD4 IFN- γ^+ , CD4 IL-4 $^+$, and CD4 IL-17A $^+$ cells in lung single cell suspensions. $n=4$. C, Levels of IL-4, IL-5, IL-13, IL-17A, IL-17F, IL-6, IL-18, CCL11, CXCL1, and CSF-3 in BALF were quantified by multiplex immunoassays. $n=7-9$. D, Levels of IL-4, IL-5, IL-13, IL-17A, IL-17F, IL-6, IL-18, IL-1 β , CCL11, CXCL1, and CSF-3 in lung homogenates were quantified by multiplex immunoassays or ELISA. $n=7-9$. * $p < .05$; ** $p < .01$.

distributed in the bronchial and alveolar epithelium, with less expression in the surrounding infiltrated inflammatory cells, which coincides with the results seen from clinical patients with neutrophilic asthma (Rossios et al., 2018; Simpson et al., 2014). Here, we treated the TDI-exposed mice with a specific antagonist of NLRP3. Though the mechanism is elusive, MCC950, as a potent highly specific small-molecule inhibitor, binds to NLRP3 and affects a key step in its activation that might

interfere with post-translational modifications (Coll et al., 2015; Perera et al., 2018). Similar to caspase-1 inhibition, treatment with MCC950 significantly inhibited NLRP3 expression and caspase-1 activity, reduced IL-18 and IL-1 β production, and finally, resulted in the alleviation of hyperresponsiveness, inflammation, and remodeling of the airways in TDI-exposed mice, indicating that TDI induces caspase-1 activation in a NLRP3 inflammasome-dependent manner (Yazdi et al., 2010),

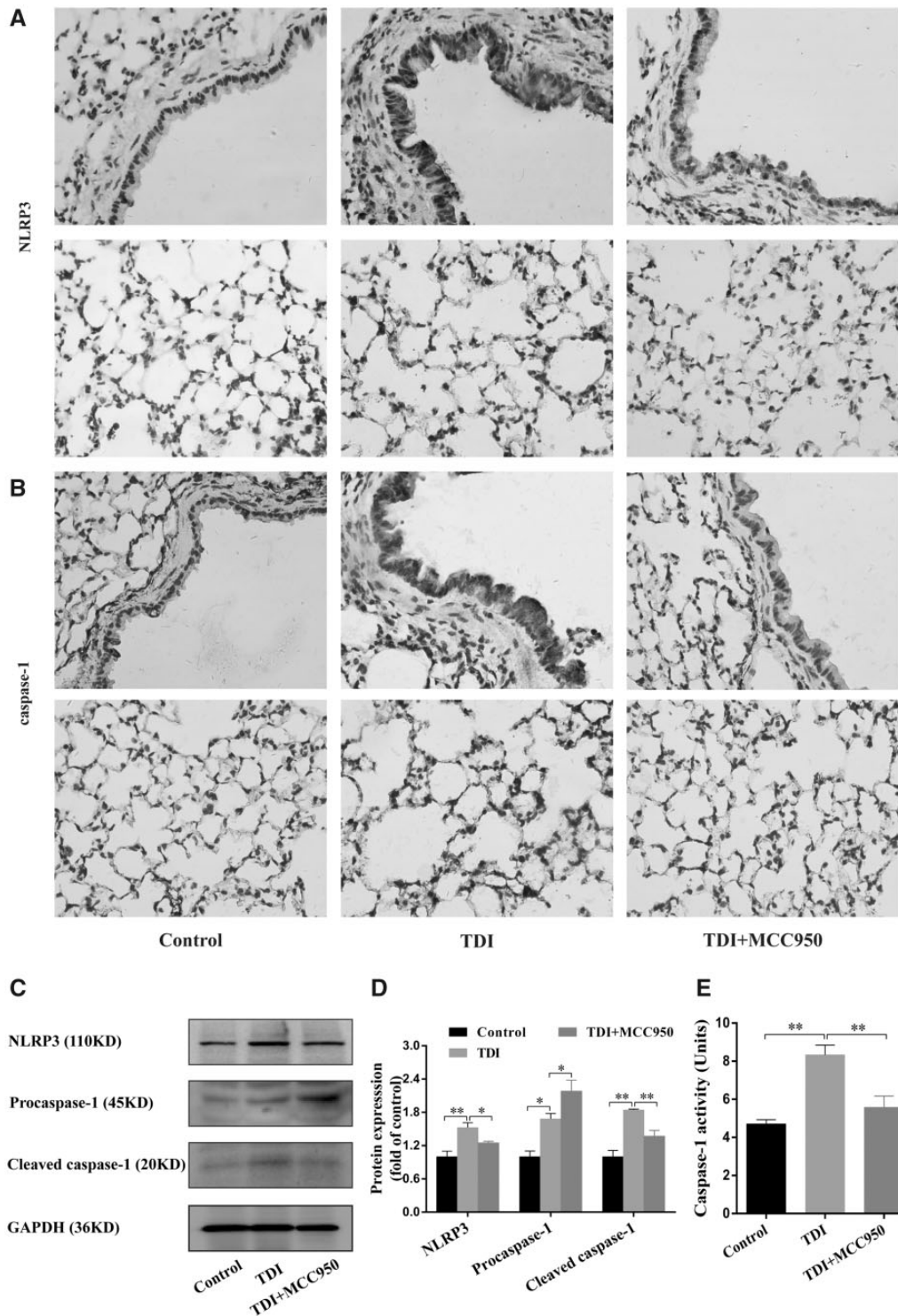


Figure 7. MCC950 inhibited activation of NLRP3 and downregulated pulmonary expression of caspase-1. A, Representative immunohistochemistry of NLRP3 in the bronchial regions (upper panel) and alveolar regions (lower panel) revealed much more staining after TDI exposure when compared with control. Treatment with MCC950 significantly inhibited NLRP3 expression. B, Representative immunohistochemistry of caspase-1 in the bronchial regions (upper panel) and alveolar regions (lower panel). Original magnification was 400 $\times$. C-D, Protein expression of NLRP3, procaspase-1, and cleaved caspase-1 in lung homogenates was detected by Western blotting and densitometric analysis was performed. $n = 6$. E, Levels of caspase-1 activity in lung homogenates. $n = 7-11$. * $p < .05$; ** $p < .01$.

therefore contributing to the production of IL-18 and IL-1 β and ultimately to airway neutrophilia as well as eosinophilia (Gurung et al., 2015; Jorgensen et al., 2016; Yamagata et al., 2008).

Researchers have linked NLRP3-dependent, IL-1 β -mediated IL-17 response with neutrophilic airway inflammation and AHR

(Kim et al., 2014; Martin et al., 2013). Furthermore, the large numbers of neutrophils in the airway represent a T_H17 dominated response with a weaker T_H2 response in TDI-induced asthma. In this study, we found that blocking the NLRP3 inflammasome with MCC950 significantly suppressed both T_H2 and T_H17

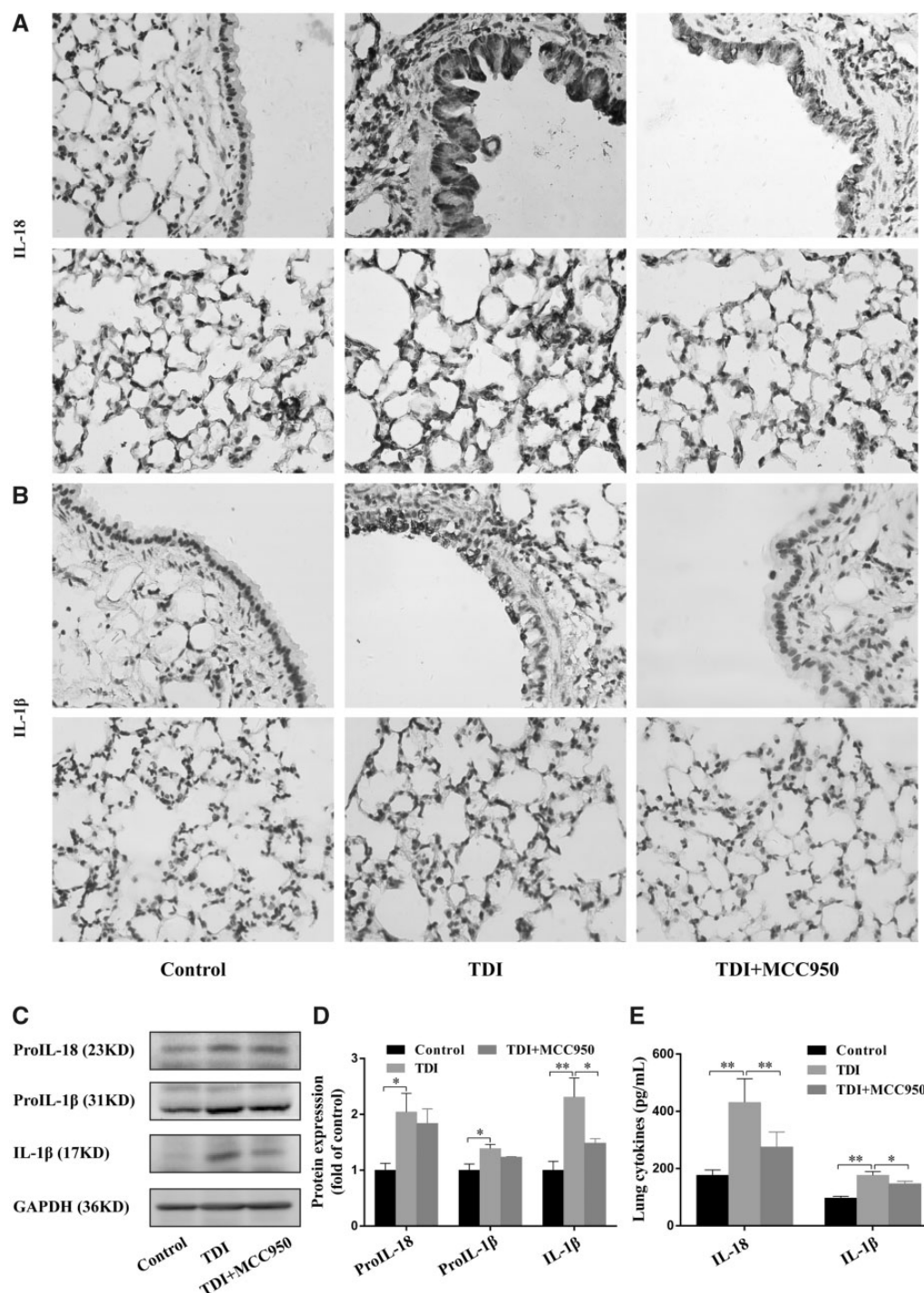


Figure 8. Blocking NLRP3 remarkably reduced expression of IL-18 and IL-1β in TDI-induced asthma. A-B, Representative immunohistochemistry of IL-18 (A) and IL-1β (B) in the bronchial regions (upper panel) and alveolar regions (lower panel). Original magnification was 400×. C-D, Protein expression of proIL-18, proIL-1β, and IL-1β in lung homogenates was detected by Western blotting and densitometric analysis was performed. $n = 6$. E, Levels of IL-18 and IL-1β in lung homogenates. $n = 7 \sim 9$. * $p < .05$; ** $p < .01$.

responses in a TDI-induced asthma model, paralleled with reduced infiltration of eosinophils and neutrophils into the airway. Though MCC950 had slightly different effects with caspase-1 inhibition on T_H2/T_H17 responses, they both decreased TDI-induced AHR, which agrees with the finding that targeting T_H2 alone or combined T_H17 -abrogated AHR in asthma (Choy et al., 2015). Anyway, MCC950 treatment displayed similar effects to caspase-1 inhibition (Kim et al., 2017) and IL-1

prevention (Johnson et al., 2005), suggesting that the NLRP3/caspase-1 axis is engaged in both T_H2 and T_H17 responses induced by TDI. This agrees with several published studies demonstrating that inhibition of NLRP3 suppressed both neutrophilic and eosinophilic inflammation in HDM- and infection-induced severe asthma (Kim et al., 2017; Rossios et al., 2018). In addition, absence of NLRP3 has also been demonstrated to repress the T_H2 -dominant allergic airway

inflammation induced by OVA (Besnard *et al.*, 2011; Bruchard *et al.*, 2015) though it failed to control eosinophil recruitment and T_H2 response induced by HDM (Madouri *et al.*, 2015). Taken together, our data demonstrated that blocking the NLRP3 axis abrogated TDI-induced airway inflammation by restricting T_H2 / T_H17 responses.

In conclusion, our data demonstrated a direct contribution of the NLRP3/caspase-1 axis in driving TDI-induced neutrophilic and eosinophilic airway inflammation. Importantly, these data suggested that blockade of the NLRP3/caspase-1 axis may effectively prevent the progression of TDI-induced asthma and could be used as potential therapeutic targets for asthmatics.

DECLARATION OF CONFLICTING INTERESTS

The author/authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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AUTHORS CONTRIBUTIONS

T. A., C. S., and Y. L. designed the experiment and wrote the manuscript. C. S., Y. L., H. Q., and H. P. performed the experiments. L. Y., Z. Z., and H. G. helped with the experiments. T. A., Y. L., Y. J. and Z. Q. 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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