



Bronchial epithelial pyroptosis promotes airway inflammation in a murine model of toluene diisocyanate-induced asthma



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ABSTRACT

Airway epithelial injury in response to allergens such as toluene diisocyanate (TDI) leads to persistent airway inflammation. Pyroptosis is recognized as a strong proinflammatory cell death process. However, the role of pyroptosis in bronchial epithelial injury and airway inflammation in TDI-induced asthma remains unknown.

In this study, cytotoxic effect of TDI on 16HBE cells (a human bronchial epithelial cell line) was detected. Then a TDI-induced experimental asthma mouse model was established for *in vivo* study.

Here we found that TDI induced pyroptosis in 16HBE cells, as evidenced by enhanced expressions of caspase-1 and elevated levels of LDH, IL-1 β and HMGB1. As expected, TDI-induced inflammatory cell death was significantly blocked by a specific NLRP3 inflammasome inhibitor. Intriguingly, in asthmatic mice, the increased cleavages of caspase-1 and pyroptotic executioner gasdermin D (GSDMD) in bronchial epithelial cells were decreased by NLRP3 inflammasome inhibitor. Furthermore, inhibition of NLRP3 inflammasome attenuated airway hyper-responsiveness and airway inflammation, accompanied by lower levels of IL-1 β , IgE and Th2-related cytokines.

Our data suggest that bronchial epithelial pyroptosis exacerbates airway inflammation and hyper-responsiveness in TDI-induced asthma via NLRP3 inflammasome activation and GSDMD cleavage. Therefore, NLRP3 inflammasome-mediated pyroptosis may be a potential treatment target for TDI-induced asthma.

1. Introduction

Asthma is a chronic inflammatory airway disease mainly due to occupational or environmental exposure to industrial products, microorganisms and other allergens. Airway epithelial cells act as the first line of immune defense against pathogen-associated and damage-associated molecular patterns, as well as environmental allergens, such as silica, smoke and asbestos. Importantly, airway epithelial cells also participate in regulating inflammatory and immune responses to irritants [1]. Evidences in bronchial biopsy samples from patients with

asthma revealed that impaired airway epithelium was a hallmark of asthma pathogenesis [2–4]. Moreover, damaged airway epithelium attracts inflammatory cells by releasing cytokines and chemokines [5] and the recruited cells contribute to airway hyperresponsiveness (AHR) [6]. Persistent airway epithelial injury maintains the airway in hyper-inflammatory conditions and irreversibly remodels airway tract [7]. Hence, it is essential to understand mechanisms of epithelial damage in response to various allergens.

Pyroptosis, a special process of cell death, is characterized by cell lysis and proinflammatory cytokine release. Recent studies have

Abbreviations: TDI, toluene diisocyanate; NLRP3, NOD-like receptor protein 3; PAMPs, pathogen-associated molecular patterns; DAMPs, damage-associated molecular patterns; ASC, apoptosis-associated speck-like protein; GSDMD-N, N-terminal fragment of gasdermin D; LDH, lactate dehydrogenase; IL-1 β , interleukin 1 β ; AHR, airway hyper-responsiveness; BALF, bronchoalveolar lavage fluid; TLR, toll-like receptor

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identified the molecular basis of pyroptotic process [8,9]. Specifically, most PAMPs or DAMPs are recognized via pattern recognition receptors that recruit apoptosis-associated speck-like protein (ASC) and pro-caspase-1 to assemble complexes termed inflammasomes [10]. Upon inflammasome activation, activated caspase-1 cleaves pro-IL-1 β into bioactive IL-1 β that triggers inflammatory processes in local tissues. In addition, activated caspase-1 also leads to gasdermin D (GSDMD) cleavage to generate an active N-terminal fragment (GSDMD-N). As the pyroptotic executioner, GSDMD-N oligomerizes on the cell membrane to form pores [8,9], causing rapid cell rupture and release of IL-1 β and HMGB1 [7,11]. Moreover, intracellular materials discharged from pyroptotic cells are regarded as potential source of antigens to induce immune responses. Both inflammatory mediators and pyroptosis-associated immune responses can drive tissue damage and structural changes [12].

Toluene diisocyanate (TDI), a kind of material for many kinds of industrial and consumer products, has been recognized as the most common cause of occupational asthma [13]. Upon a long-term exposure to TDI, the incidence of asthma increases to nearly 15 % [14]. Previously, loss of airway epithelium integrity was found in patients with asthma and in a murine model of TDI-induced asthma [15]. Importantly, TDI-induced asthma shows prominent airway inflammatory injury. It has been reported that expression of caspase-1 is enhanced in TDI-induced asthma mice [16]. Bronchial biopsies from airway mucosa of patients with TDI-induced asthma showed a higher level of IL-1 β [17]. These suggest that pyroptosis, a kind of proinflammatory cell death, may be a cause of airway epithelial injury in response to TDI. As we have described in the "cell death immune recognition model" [18], pyroptosis drives inflammatory and immune process [19]. Therefore, the aim of this study is to investigate whether TDI induces bronchial epithelial pyroptosis, and to determine its role in the airway inflammation in asthmatic mice [20].

2. Material and methods

2.1. Materials

TDI was purchased from Sigma-Aldrich (Shanghai, China). TDI is a hapten and requires conjugation to human serum albumin (HSA) for *in vitro* study. TDI was dissolved in the vehicle which was comprised of acetone and human serum albumin to produce a functional stimuli as described previously [21]. The HSA concentration (50–200 μ g/ml) of the conjugates was considered as the concentration of TDI-HSA. For asthma model, TDI was dissolved in different vehicles for sensitization and challenge, respectively. For dermal sensitization, 0.3 % TDI means the TDI concentration in the vehicle comprised of 2 volumes of acetone and 3 volumes of olive oil. For airway challenge, 3 % TDI means TDI concentration in the vehicle consisted of 1 vol of acetone and 4 volumes of olive oil [22]. LPS and ATP were purchased from Sigma-Aldrich (St. Louis, MO, USA), as classical pyroptosis inducers. Nigericin was obtained from InvivoGen (San Diego, USA). MCC950, the specific inhibitor of NLRP3 inflammasome, was purchased from Selleck Chemicals (#s7809, Houston, TX, USA). MCC950, LPS, ATP and Nigericin were dissolved in the ultrapure water for *in vitro* study. Cell culture reagents were obtained from Gibco (Paisley, UK).

2.2. Cell culture

Human bronchial epithelial cell line 16HBE14o-(16HBE) cells were obtained from Bio-Rad Laboratories (Shanghai, China). 16HBE cells were cultured in RPMI-1640 media containing FBS and penicillin-streptomycin. 16HBEs were treated with different concentrations of TDI (0, 50, 100, 150 and 200 μ g/ml) for 3 h, 6 h or 12 h, with or without pretreatment with MCC950 (10 μ M) for 1 h. As positive controls, cells were pretreated with LPS (1 μ g/ml) for 4 h, and treated with ATP (5 mM) or Nigericin (10 μ M) for another 1 h. The cells and supernatants

were harvested for the following experiments.

2.3. Cell viability assay

16HBE cells were harvested after stimulation with different concentrations of TDI and then cell viability tested by Cell Counting Kit-8 (CCK-8) (Dojindo, Beijing, China), following the manufacturer's instructions.

2.4. LDH release assay, IL-1 β and HMGB1 detection

After stimulation with TDI for 3 h and 6 h, the lactate dehydrogenase (LDH) levels in the culture supernatants were detected using a LDH assay kit (Promega, Madison, Wisconsin, USA). In addition, cells were pretreated with MCC950 (10 μ M), and treated with TDI (150 μ g/ml), LPS + ATP or LPS + Nigericin. Then the concentration of IL-1 β in the supernatants were measured by ELISA kit (Raybiotech, San Diego, USA). Similarly, the levels of HMGB1 in the culture supernatants were measure by ELISA kit (Cusabio, Wuhan, China).

2.5. Flow cytometric analysis of 16HBE cells

Cells were primed with TDI (150 μ g/ml) for 6 h or LPS (1 μ g/ml) + ATP (5 mM) for 5 h, with or without pretreatment with MCC950 (10 μ M) for 1 h. Then cells were stained with FAM-YVAD-FLICA caspase-1 Assays (ImmunoChemistry Technologies, Bloomington, USA) and PI (BD, Franklin Lakes, New Jersey, USA), and the ratios of caspase-1 positive and PI positive cells were analyzed by FACSVerse™, respectively.

2.6. IL-1 β gene expression analysis

RT-qPCR analysis was performed to confirm the expression of candidate genes in 16HBEs after TDI treatment. Cells were primed with TDI (150 μ g/ml) for 6 h, with or without pretreatment with MCC950 (10 μ M) for 1 h. Total cell RNA was extracted using TRIzol reagent (Vazyme, Nanjing, China). The complementary DNAs (cDNAs) were synthesized using the PrimeScript RT reagents Kit (Perfect Real Time, TaKaRa) according to the manufacturer's instructions. And then, RT-qPCR analysis was performed in triplicate in an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control for quantification of target gene expression. The following PCR primers were used: IL-1 β , forward, 5'-CTGTCCTCGGTGTTGAAAGA-3', and reverse, 5'-TTGGGTAATTTTGGGATCTACA-3'; GAPDH, forward, 5'-TGGAAGTCCAGACGCTACTCAG-3', and reverse, 5'-CGGGAAGCTTGTCAATCAATGGAA-3'. To quantify gene expression, 2-step qRT-PCR was performed using hot start Taq at 95 °C (15 s), with annealing and extension at 60 °C (60 s) for 40 cycles, followed by a melting curve analysis. All qRT-PCR data were analyzed using the $2^{-\Delta\Delta Ct}$ method.

2.7. Immunofluorescence analysis of HMGB1

After treatment with TDI, the distribution of HMGB1 was detected using immunofluorescence. For staining preparation, cells were fixed with 4 % paraformaldehyde, permeabilized with 0.2 % Triton X-100, and blocked with 5 % fetal bovine serum (FBS). Then, cells were stained with rabbit anti-HMGB1 antibody (Abcam, catalog ab18256, Cambridge, UK), followed by incubation with a Cy3-conjugated goat secondary antibody against rabbit IgG (Servicebio, catalog GB21303, China). For deoxyribonucleic acid (DNA) visualization, the cells was stained with 4', 6'-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific, USA). Images were collected with a fluorescence microscope at original $\times 400$ magnification. And the fluorescence values were analyzed by Image J Software.

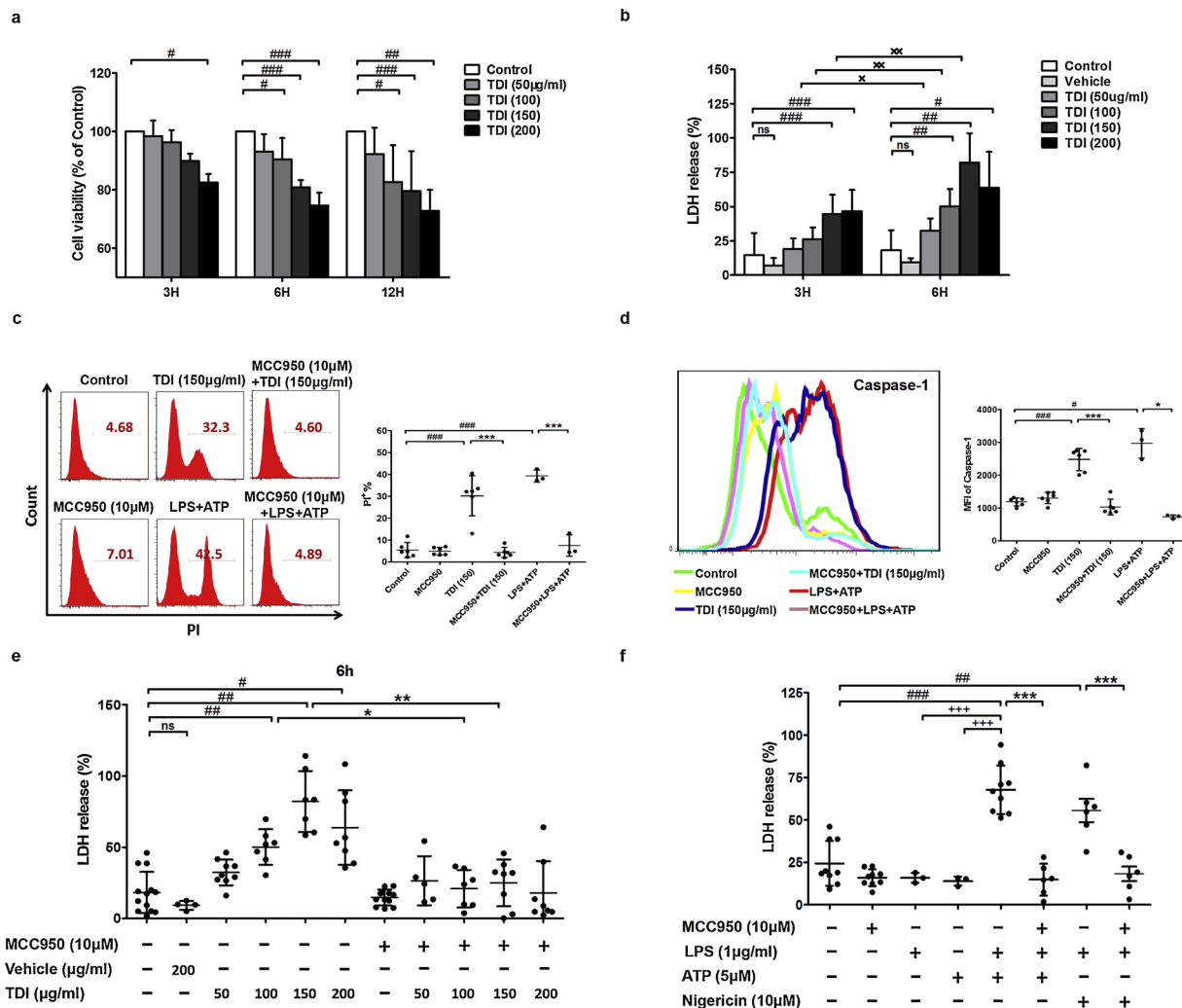


Fig. 1. TDI induced cell death in 16HBE cells.

(a, b) Effects of TDI on the cell viability and LDH release in 16HBE cells. Cells were treated with TDI at different concentrations (0, 50, 100, 150 and 200 µg/ml) for 3 h, 6 h or 12 h. Then the cell viability was detected using CCK-8 or LDH release. (c) Cell death was analyzed by flow cytometry after PI staining. The ratio of PI positive cells was detected after TDI exposure for 6 h or LPS + ATP treatment. (d) Cells were analyzed by FAM-FLICA caspase-1 staining using flow cytometry. (e, f) Cells were pretreated with MCC950 and then exposed to TDI for 6 h or LPS + ATP or LPS + Nigericin for 5 h. Levels of LDH were detected. For all experiments, data were shown as mean $\pm$ SD. # p < 0.05, ## p < 0.01, ### p < 0.001 compared with Control; * p < 0.05, ** p < 0.01, *** p < 0.001 compared with LPS + ATP, LPS + Nigericin or TDI-treated groups; $\times p$ < 0.05, $\times \times p$ < 0.01 compared with the same concentration of TDI for 3 h; +++ p < 0.001 compared with LPS or ATP alone group; ns, no significant difference compared with Control. 16HBE cells, a human bronchial epithelial cell line; TDI, toluene diisocyanate.

2.8. Mice

Male BALB/c mice (6–8 weeks old) were obtained from Southern Medical University (Guangzhou, China) and housed in the Laboratory Animal Center of Southern Medical University under SPF condition. This study was approved by the Ethics Committee of the Southern Medical University and under an experimental license approved by Animal Subjects Committee of Southern Medical University (L2017177).

2.9. Asthmatic model and MCC950 administration

Mice were randomized to three groups as follows: Vehicle-sensitized, vehicle-challenged and saline-treated mice as Control + Saline group (n = 12); TDI-sensitized, TDI-challenged and saline-treated mice as TDI + Saline group (n = 12); TDI-sensitized, TDI-challenged and MCC950-treated mice as TDI + MCC950 group (n = 12). As described previously [16], mice were sensitized with 0.3 % TDI on the back of each ear on day 1 and day 8. Then TDI-sensitized mice were challenged

on day 15, day 18 and day 21 respectively with 3 % TDI nebulization for 3 h. Control + Saline group was sensitized and challenged with vehicle. In the TDI + MCC950-treated group, MCC950 were dissolved in saline and injected intraperitoneally at a dose of 10 mg/kg as described previously [23], on every other day from the day before the first TDI challenge. Similarly, the TDI + Saline group and Control + Saline group were administrated with an equal volume of saline.

2.10. Airway hyper-responsiveness assessment

Airway reactivity in response to different concentrations of methacholine was measured in the Guangzhou Institute of Respiratory Disease on the day after the last challenge with TDI. 6 mice per group were randomly challenged with increasing concentration of methacholine (3.125, 6.25, 12.5, 25 and 50 mg/ml) via ultrasonic nebulization in the barometric plethysmographic chamber. Pressure fluctuations were quantified by algorithm for enhanced pause called Penh [22].

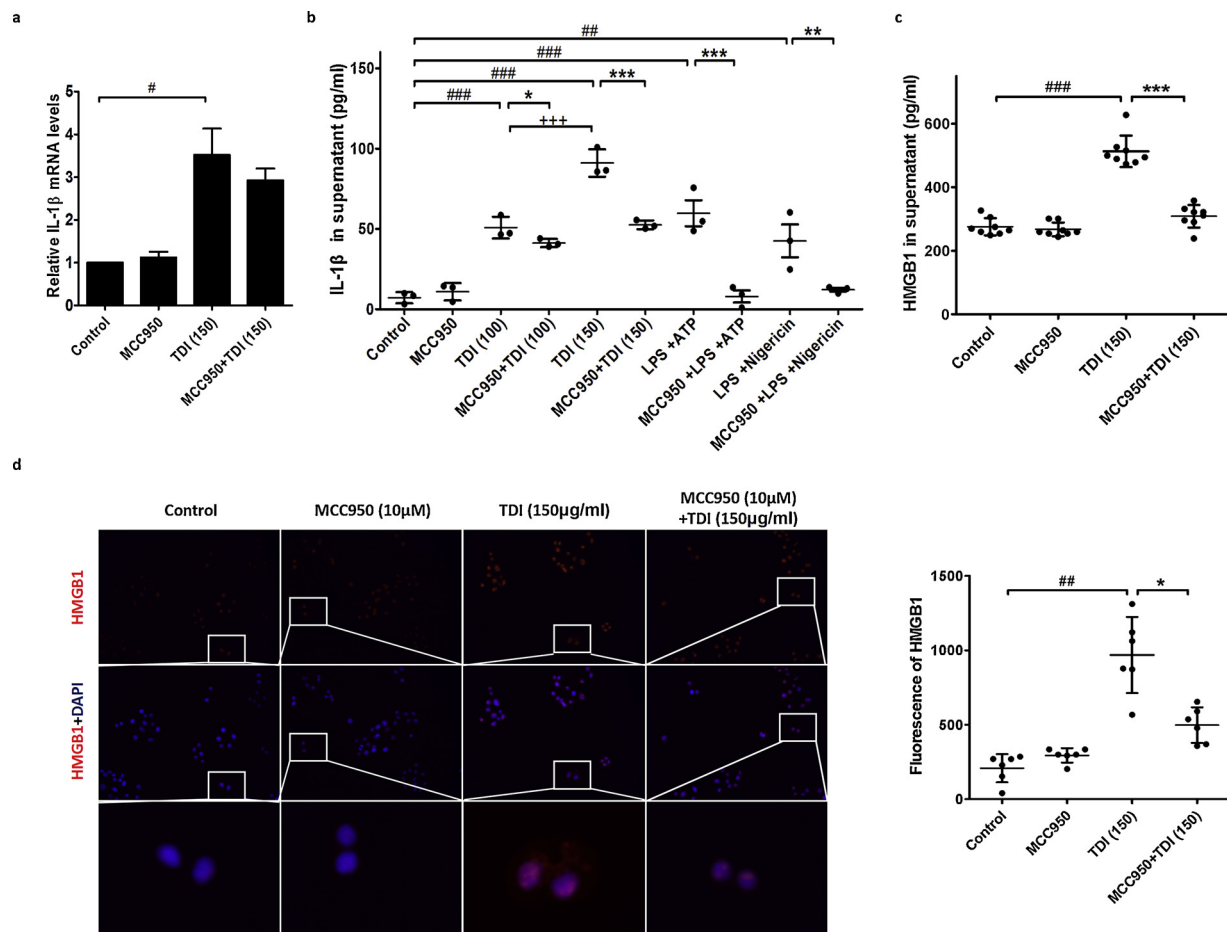


Fig. 2. TDI triggered bronchial epithelial pyroptosis in a NLRP3 inflammasome-dependent manner.

TDI induced pyroptosis in 16HBE cells, as determined by caspase-1 activation and IL-1 β release. LPS + ATP was used as positive control for NLRP3 inflammasome-dependent pyroptosis. Specific inhibitor for NLRP3 inflammasome (MCC950, 10 μ M) was used. Cells were pretreated with MCC950 before the treatment with TDI or LPS + ATP. Then (a) the mRNA expression of IL-1 β were detected using RT-qPCR. (b, c) The concentrations of IL-1 β and HMGB1 were tested using ELISA. (d) The distribution of HMGB1 (red) was analyzed at x400 magnification using immunofluorescence. DAPI, DNA-binding dye (blue). The fluorescence values of HMGB1 were analyzed by Image J Software. For experiments, data were shown as mean $\pm$ SD. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared with Control; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with TDI, LPS + ATP-treated groups; + + + $p < 0.001$ compared with TDI (100 μ g/ml).

2.11. Plasma IgE and IL-1 β detection

After AHR assessment, mice were euthanized to obtain blood samples. The plasma was harvested and stored at -80°C . The plasma from other mice without assessing AHR was collected on the day after the last challenge of TDI. The plasma levels of IgE in all mice and IL-1 β in mice without AHR assessment were detected by ELISA kits (BOSTER, Wuhan, China and Raybiotech, San Diego, USA), respectively.

2.12. Analysis of bronchoalveolar lavage fluid

On the day after the last TDI challenge, the BALF of mice ($n = 6$ per group) without AHR assessment were collected and total cells in the BALF counted. The supernatants of BALF were collected and the concentrations of IL-1 β , IL-4, IL-5, IL-6 and IL-13 detected using ELISA kits (Raybiotech, San Diego, USA and BOSTER, Wuhan, China) respectively. Then the cells of different categories were counted via smearing and H&E staining. In order to verify the inflammatory infiltration, cells in the BALF were stained with APC-anti-mouse Ly-6G (labeling neutrophils) and PerCP-CY5.5-anti-mouse Siglec-F (labeling eosinophils). Percentage of Ly-6G $^{+}$ neutrophils or Siglec-F $^{+}$ eosinophils detected using flow cytometric analysis.

2.13. Pulmonary histological examination

On day 23, mice were sacrificed and lungs removed for further analysis. For histological analysis, lungs were fixed with 10 % formalin and decalcified with 5 % formic acid. Then the samples were embedded in paraffin and cut into 4 μ m sections. For assessment of pulmonary histological changes, sections were stained with hematoxylin and eosin (H&E). Peribronchial and perivascular inflammation were graded as previously described [24] and scored at $\times 200$ magnification.

2.14. Immunofluorescence analysis of caspase-1 p20 and GSDMD

The lung sections were stained with mouse monoclonal anti-caspase-1 p20 antibody (Santa Cruz Biotechnology, USA) and checked under fluorescence microscope for the expression of caspase-1 p20. Similarly, the expression of GSDMD in the bronchial epithelial cells was assayed using anti-GSDMD antibody (Abcam, catalog ab219800, Cambridge, UK).

2.15. Western blot of GSDMD in the lung tissues

Expressions of full length-GSDMD and cleaved GSDMD in the lungs from three groups were detected using anti-GSDMD (Abcam, catalog ab209845, Cambridge, UK). Data were analyzed by Image J Software.

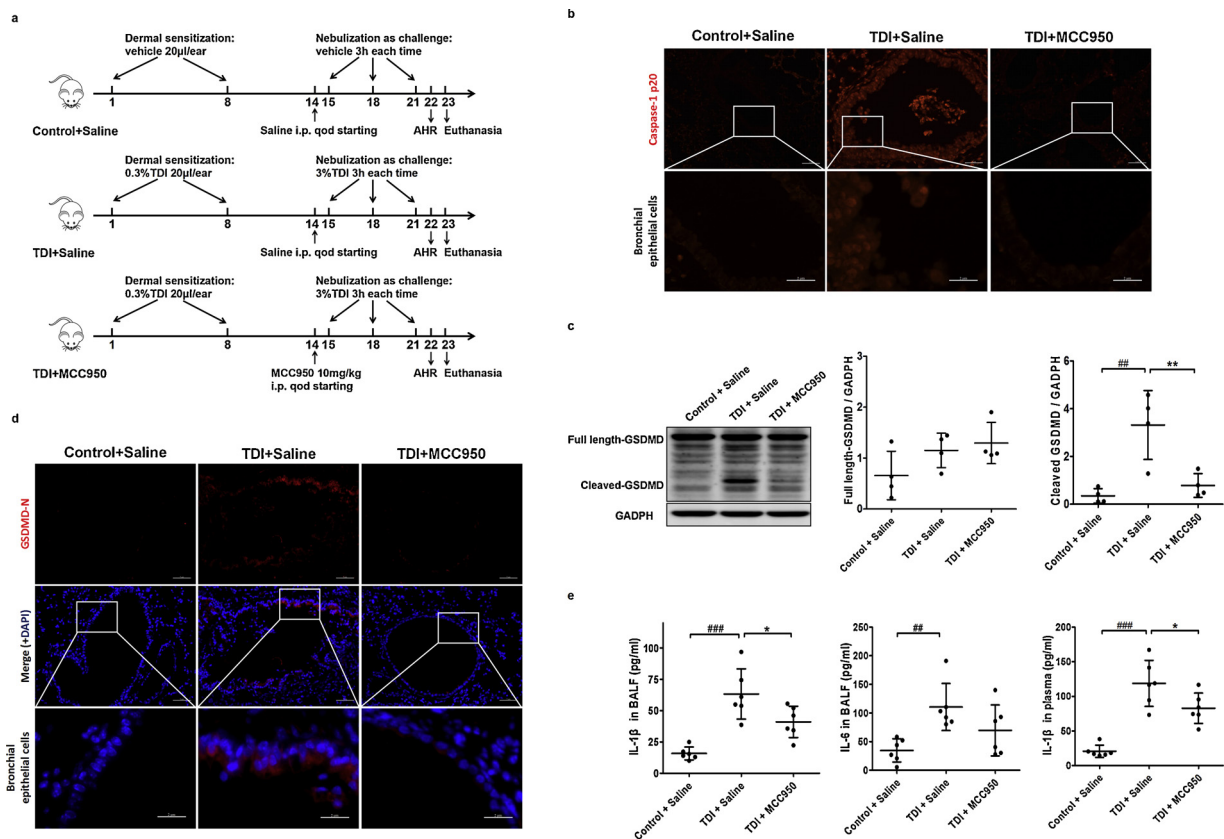


Fig. 3. Increased bronchial epithelial pyroptosis in TDI-induced asthma mice was mediated by NLRP3 inflammasome. (a) Schematic diagram of the protocol for in vivo study. Experimental groups were Control + Saline, TDI + Saline and TDI + MCC950, $n = 12$ per group. (b, d) Immunofluorescence staining for cleaved caspase-1 (caspase-1 p20) and cleaved GSDMD (red, upper panel) and the amplified view (low panel) in airway epithelial cells were assessed. (c) Expression of full length-GSDMD and cleaved GSDMD in the lungs from three groups were detected by western blot. (e) Levels of IL-1 β and IL-6 in the BALF, and IL-1 β in the plasma were tested using ELISA. Data were mean $\pm$ SD ($n = 6$ per group). ## $p < 0.01$, ### $p < 0.001$ compared with Control + Saline group; * $p < 0.05$, ** $p < 0.01$ compared with TDI + Saline group.

2.16. Statistical analysis

Data were analyzed by SPSS software (version 16.0) and presented as the means $\pm$ SD. Intergroup comparisons were evaluated using one-way ANOVA, which is followed by Least Significant Difference test (equal variances assumed) or Dunnett-t test (equal variances not assumed). The value of $P < 0.05$ was considered significant.

3. Results

3.1. TDI induced pyroptosis in 16HBE cells

The inflammatory injury of bronchial epithelium is a characteristic feature in allergic diseases [25]. Therefore we firstly assessed the effect of TDI on the cell viability of the human bronchial epithelial cell line. 16HBE cells were exposed to TDI at different concentrations (0, 50, 100, 150 and 200 $\mu\text{g/ml}$) for 3 h, 6 h and 12 h. As shown in Fig. 1a, a significant decrease in viability was found in cells treated with TDI at 200 $\mu\text{g/ml}$ over 3 h or at 100 $\mu\text{g/ml}$ over 6 h.

TDI-induced cell death in 16HBEs was also verified by other methods. As shown in Fig. 1b and c, TDI-treated cells released LDH or showed PI staining in a dose and time-dependent manner, indicating that TDI induced bronchial epithelial cell death by disrupting cell membrane integrity.

As pyroptosis is a kind of programmed necrotic cell death by caspase-1 activation [26], we next determined whether TDI-induced cell death in 16HBE cells belonged to pyroptosis. As shown in Fig. 1d, higher expression of caspase-1 was also found in TDI-treated cells,

indicating that TDI triggered pyroptosis in bronchial epithelial cells.

3.2. TDI-induced 16HBE pyroptosis is NLRP3 inflammasome-dependent

In order to understand the mechanisms of TDI in inducing bronchial epithelial pyroptosis, we explored the role of NLRP3 inflammasome, a key molecule in pyroptosis induction through triggering caspase-1 expression and downstream caspase-1-mediated effects. We used NLRP3 inflammasome activators LPS + ATP and LPS + Nigericin as positive controls [27]. LPS + ATP and LPS + Nigericin facilitated the release of LDH. Furthermore, LPS + ATP enhanced the ratio of PI positive cells and the expression of caspase-1 (Fig. 1).

Importantly, increased ratios of PI positive cells (Fig. 1c), enhanced expression of caspase-1 (Fig. 1d) and elevated level of LDH (Fig. 1e and f) in TDI or LPS + ATP-treated cells were abolished by a specific NLRP3 inflammasome inhibitor MCC950. These results confirmed that TDI-induced pyroptosis in 16HBE cells was NLRP3 inflammasome-dependent.

3.3. TDI induced hyper-inflammatory state in 16HBE cells

As inflammasome-mediated pyroptotic process drove inflammatory response by releasing IL-1 β and other cellular ingredients [18], we next confirmed IL-1 β expression and release by TDI treatment. 16HBE cells were exposed to TDI at 150 $\mu\text{g/ml}$ for 6 h, then the mRNA expressions of IL-1 β in 16HBEs and the levels of IL-1 β in the supernatants detected. As shown in Fig. 2a, TDI up-regulated mRNA expression of IL-1 β . Furthermore, TDI dose-dependently increased the release of IL-1 β that

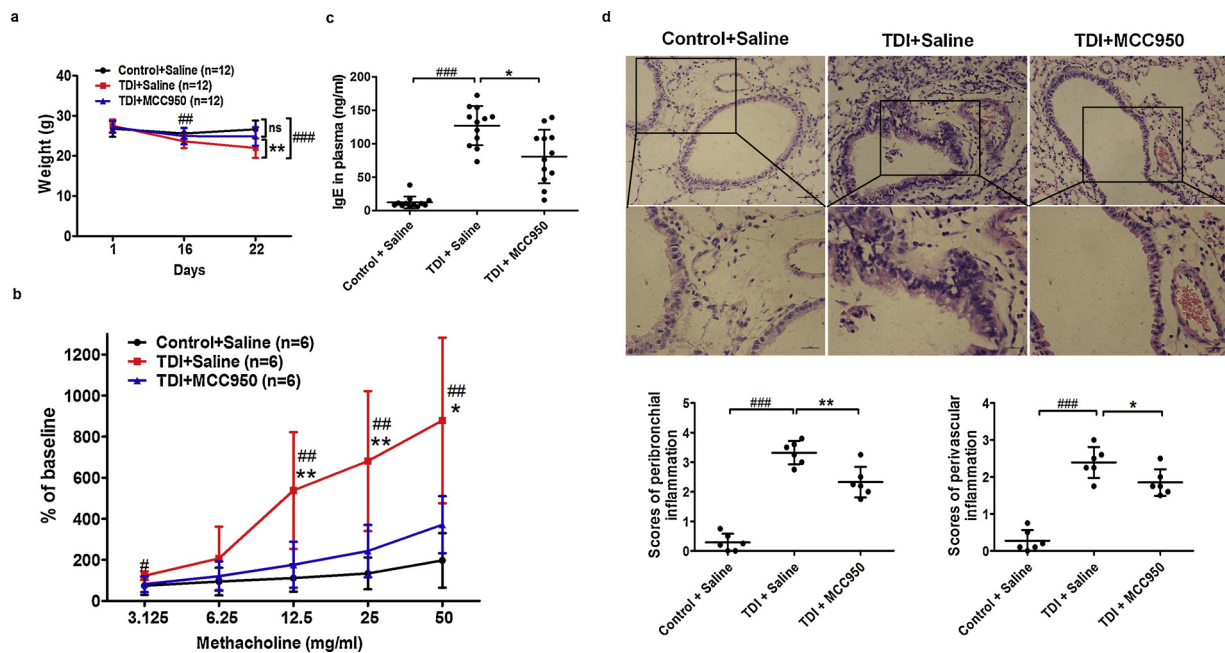


Fig. 4. Inhibition of NLRP3 inflammasome decreased AHR, IgE level and pulmonary pathological changes in TDI-induced asthma.

(a) Mouse weights of three groups were measured on day 1 (dermal sensitization), day 16 (the day after first challenge) and day 22 (the day after last challenge). (b) Penh value was determined in response to different concentrations of methacholine. (c) Plasma levels of IgE were tested using ELISA. (d) H&E staining of lung sections showed at x200 magnification (upper panel) and the amplified view (low panel). And histological changes were graded respectively. Data were mean $\pm$ SD (n = 6–12 per group). [#]p < 0.05, ^{##}p < 0.01, ^{###}p < 0.001 compared with Control + Saline group; ^{*}p < 0.05, ^{**}p < 0.01 compared with TDI + Saline group; ns, no significant difference compared with Control + Saline group.

was blocked by inhibition of NLRP3 inflammasome (Fig. 2b).

In addition, HMGB1 is also released from necrotic cells [28], and serves as a key DAMP in chronic airway inflammation [29]. Then we investigated whether the release of HMGB1 was also enhanced in TDI-induced pyroptosis. As shown in Fig. 2c, TDI increased the concentrations of HMGB1 in the culture supernatants, which were decreased by NLRP3 inflammasome inhibitor. Correspondingly, immunofluorescence analysis showed that more HMGB1 translocated into the cytosol after TDI treatment. Increased fluorescence intensity of HMGB1 in TDI-treated cells was again reduced by MCC950 treatment (Fig. 2d).

Taken together, these results confirmed that TDI induced inflammatory epithelial injury through induction of pyroptosis with activation of NLRP3 inflammasome.

3.4. Enhanced bronchial epithelial pyroptosis in TDI-induced asthma was mediated by NLRP3 inflammasome activation

To confirm the involvement of bronchial epithelial pyroptosis in TDI-induced asthma, we established the TDI-induced experimental asthma mice and administrated as schematically shown in Fig. 3a. Firstly, we detected the expression and activation of pyroptosis-related molecules in the airway. In TDI + Saline-treated mice, expression of activated caspase-1 (caspase-1 p20) was enhanced in bronchial epithelial cells (Fig. 3b). Increased expression of cleaved GSDMD was also found in the lungs of asthmatic mice (Fig. 3c and supplementary figure). These findings were further confirmed by immunofluorescence analysis for cleaved GSDMD in bronchial epithelial cells (Fig. 3d). Moreover, TDI + Saline-treated mice showed higher concentrations of IL-1 β in the BALF and plasma (Fig. 3e).

As expected, inhibition of NLRP3 inflammasome suppressed the expression of caspase-1 p20 and cleaved GSDMD in airway epithelium (Fig. 3b and d), decreased the cleavage of GSDMD in the lungs (Fig. 3c and supplementary figure), and reduced the concentrations of IL-1 β in the BALF and plasma (Fig. 3e). These results confirmed that NLRP3 inflammasome mediated bronchial epithelial pyroptosis in TDI-induced

asthma mice.

3.5. Inhibition of bronchial epithelial pyroptosis attenuated airway hyper-responsiveness and airway inflammation in TDI-induced asthma

We next determined the pathogenic effect of NLRP3 inflammasome-mediated pyroptosis in asthmatic mice. In TDI-treated mice, MCC950 alleviated the loss of weight (Fig. 4a) and attenuated AHR (Fig. 4b), suggesting a protective role of inhibition of NLRP3 inflammasome in TDI-induced asthma. In addition, increased plasma level of IgE in TDI-treated mice was also decreased by MCC950 (Fig. 4c).

Moreover, we investigated the effect of NLRP3 inflammasome inhibitor on airway inflammatory response in asthmatic mice. Histological analysis showed that bronchial epithelial hyperplasia and massive inflammatory cell infiltration around the bronchi in TDI-treated mice were effectively decreased by MCC950, as evaluated by scores of peribronchial and perivascular inflammation (Fig. 4d).

Then cells in BALF were collected and differentially counted. In TDI-treated mice, increased number of total cells (Fig. 5a), and percentages of neutrophil and eosinophil (Fig. 5b) were markedly decreased by MCC950 treatment. These findings were also confirmed by flow cytometry of Ly-6G⁺ neutrophils and Siglec-F⁺ eosinophils (Fig. 5c). Interestingly, a significant reduction in the percentage of macrophage was also found in BALF from TDI-treated mice, while it was not influenced by the treatment of MCC950 (Fig. 5b).

Since T cell response in TDI-induced asthma is Th2 dominant [30], we also analyzed Th2 cytokines in the BALF. As shown in Fig. 5d, enhanced releases of IL-4, IL-5 and IL-13 in the BALF from asthmatic mice were eliminated by inhibition of NLRP3 inflammasome (Fig. 5d).

4. Discussion

Allergen-induced airway inflammation is a hallmark of asthma pathogenesis [31]. Environmental allergens are well-established triggers for airway inflammatory epithelial injury [32]. Studies in human and

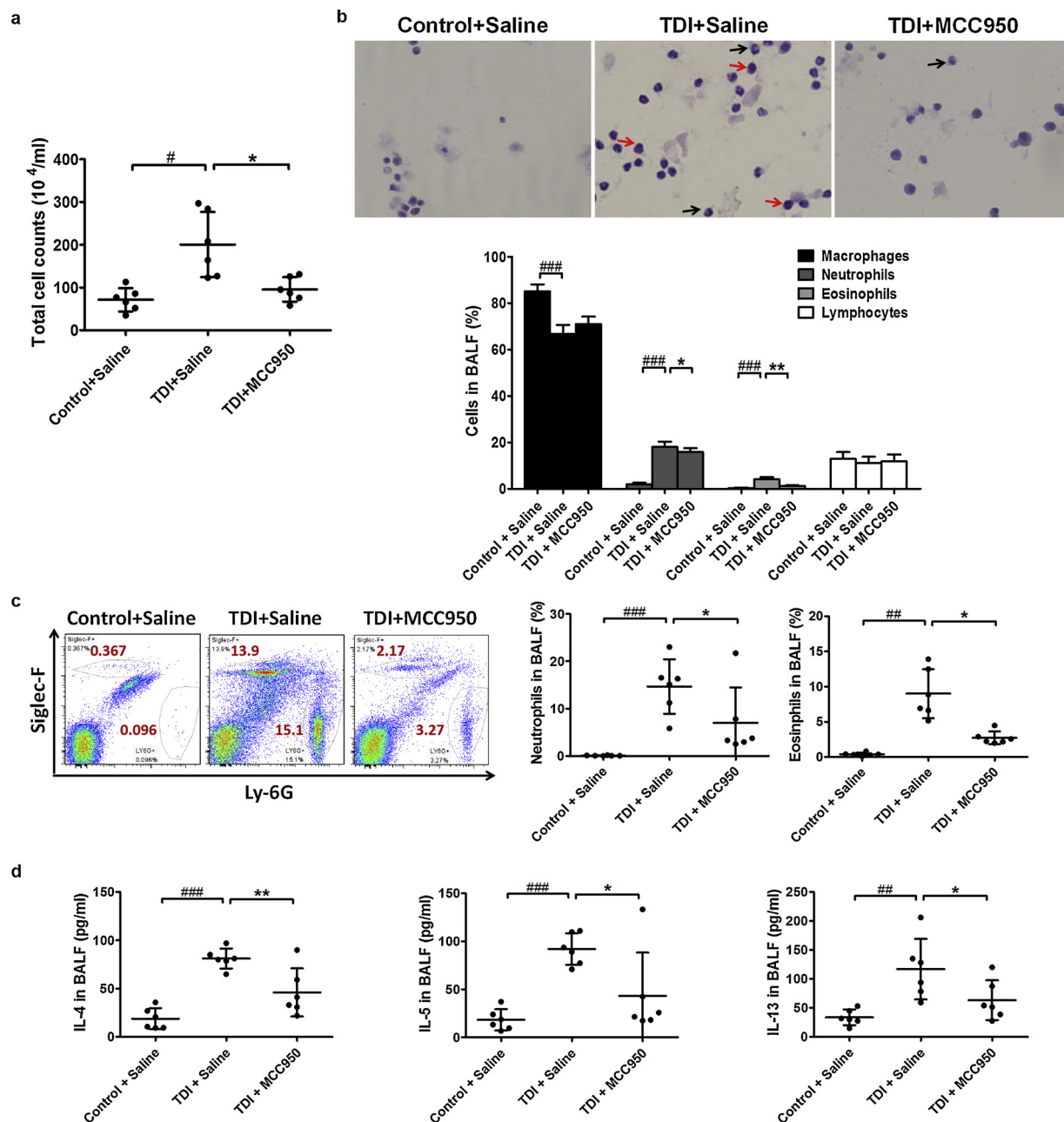


Fig. 5. Inhibition of NLRP3 inflammasome alleviated airway inflammatory response in TDI-induced asthma.

(a–c) Cellular profiles of BALF were analyzed in the three groups. (a) Total cell count was performed. (b) H&E staining of cell smears from BALF showed at x400 magnification, and different inflammatory cells were counted. Red arrows indicate infiltrating eosinophils; black arrows indicate infiltrating neutrophils. (c) Infiltrated cells in the BALF were verified by labeling with Ly-6G and Siglec-F cells using flow cytometry. Ly-6G⁺ Siglec-F⁺ cells indicate neutrophils; Ly-6G⁺ Siglec-F⁺ cells indicate eosinophils. (d) Levels of IL-4, IL-5 and IL-13 in the BALF were tested using ELISA. Data were presented as mean $\pm$ SD (n = 6 per group). #p < 0.05, ##p < 0.01, ###p < 0.001 compared with Control + Saline group; *p < 0.05, **p < 0.01 compared with TDI + Saline group.

murine asthmatic model have revealed airway epithelial barrier disruption, and evident sloughing of airway epithelial cells in the bronchoalveolar lavage [33]. In this study, we found that TDI induced pyroptosis in a human bronchial epithelial cell line (Figs. 1 and 2) and in bronchial epithelial cells of TDI-induced asthma mice (Figs. 3–5). To the best of our knowledge, this is the first study demonstrating that TDI induces pyroptosis in 16HBE cells and asthmatic mice. And these findings may explain mechanisms underlying the loss of airway epithelial integrity in TDI-induced asthma. Airway epithelial injury contributes to the enhanced sensitivity and responsiveness of the airway tract, causing persistent airway inflammation [34]. In this study, TDI-induced asthmatic model mice showed enhanced AHR and airway

inflammation, which was inhibited by the blockade of pyroptosis (Fig. 3–5). These findings suggest that bronchial epithelial pyroptosis plays a vital role in the pathogenesis of TDI-mediated asthma. An overview of these findings is presented in Fig. 6.

Pyroptosis is mediated by activated inflammasome caused by infectious or non-infectious stimuli. Importantly, we found that TDI-induced bronchial epithelial pyroptosis was NLRP3 inflammasome-dependent. NLRP3 inflammasome inhibitor abolished bronchial epithelial pyroptosis by suppressing the expression of caspase-1, and downstream cleavage of GSDMD (Fig. 3). In addition, we showed that NLRP3 inflammasome activation contributed to the airway inflammation in TDI-treated mice (Figs. 4 and 5), which were in line with observations in the

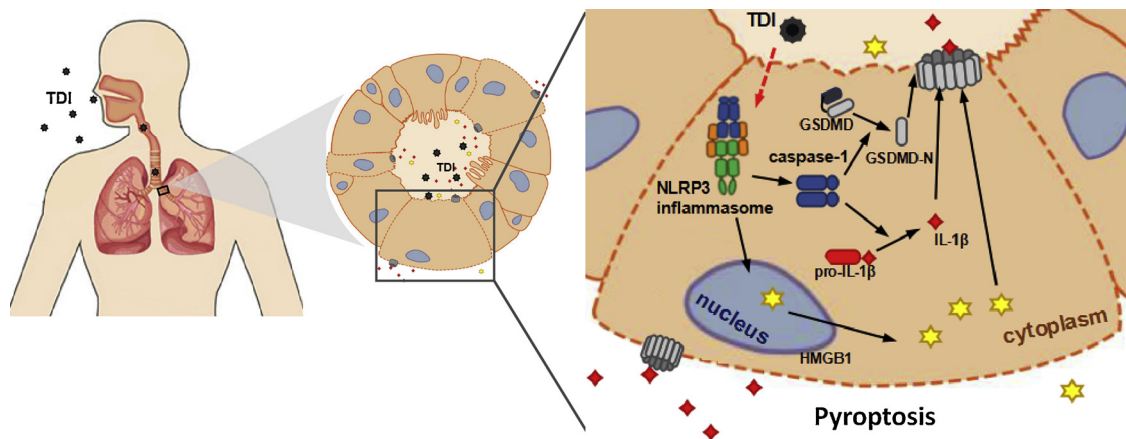


Fig. 6. Schematic diagram of TDI induced NLRP3 inflammasome-mediated pyroptosis in bronchial epithelial cells.

Inhaled TDI triggers NLRP3 inflammasome-mediated caspase-1 activation in bronchial epithelial cells. Activated caspase-1 leads to IL-1 β maturation and GSDMD cleavage. Then cleaved GSDMD executes lytic cell death through forming pores on the cell membrane. Following pyroptosis, increased IL-1 β releases into the airway and peribronchovascular interstitium. In addition, HMGB1 is produced in the nucleus, translocates into the cytoplasm and then releases into the airway. Furthermore, these mediators lead to persistent inflammatory injury.

recent literature [35].

Pyroptosis was first observed in macrophages triggered by microbial infection [8]. However, we found that in TDI-treated mice, the decreased ratio of macrophage in the BALF could not be abolished by specific NLRP3 inflammasome inhibitor MCC950, suggesting that the reduction of the ratio of macrophage was probably not relevant to NLRP3 inflammasome-mediated pyroptosis. Furthermore, TDI-induced asthma is characterized by eosinophil and neutrophil infiltration in the airway [24]. Here we found a similar result in the BALF from asthmatic mice (Fig. 5). In this respect, our findings support the viewpoint that the decrease of macrophage proportion is due to dramatically increased extrusion of neutrophils and eosinophils in the BALF. These results suggest that TDI-induced epithelial pyroptosis deteriorates airway inflammatory epithelial injury by recruiting neutrophils and eosinophils.

IL-1 signal is implicated in recruiting inflammatory cells, especially in eosinophils and neutrophils [7,36]. More than that, IL-1 β affects airway constriction and dilation in airway smooth muscles [37]. Our current data showed that MCC950 reduced the infiltration of neutrophils and eosinophils, as well as Th2-related cytokines. This agrees with the findings that inhibition of NLRP3 repressed the inflammatory infiltration in the airway in infection-induced severe asthma [20]. In addition, IL-1 β -mediated IL-17 response was also related to neutrophilic airway inflammation. NLRP3 inflammasome activation aggravated TDI-induced airway inflammation by regulating Th2 and Th17 responses [35]. Other study also supported that MCC950 suppressed both Th2 and Th17 responses that mediated chronic inflammation in a murine model of autoimmune disease [23].

Our present study demonstrated that TDI-induced IL-1 β release from 16HBEs (Fig. 2) and bronchial epithelial cells via pyroptosis (Fig. 3). These results were in line with observations in other irritants-induced asthma [38,39]. Correspondingly, inhibition of pyroptosis reduced the level of IL-1 β in the supernatant of TDI-treated cells and in BALF of TDI-induced asthma model. Our results also showed that TDI increased mRNA expression of IL-1 β in 16HBE cells (Fig. 2a). MCC950 did not affect the increased mRNA expression of IL-1 β , indicating that MCC950 specifically targeting the maturation of IL-1 β , other than gene expression. Other *in vivo* study reported that PI3K signal pathway might also contribute to IL-1 β expression, but not pro-caspase-1 expression [16], which might partly explain why IL-1 β was not completely blocked by inhibition of NLRP3 inflammasome (Fig. 2b).

HMGB1 acts as an important molecule on the airway inflammation. In this study, we found an increased level of HMGB1 in the supernatant of TDI-treated 16HBE cells. Inhibition of pyroptosis with MCC950 could block HMGB1 release from TDI-treated cells (Fig. 2c). These findings

supported the notion that HMGB1 was also released from pyroptotic cells. Importantly, our current data showed that HMGB1 was produced in the nucleus and translocated into the cytoplasm, a key process for HMGB1 release, in TDI-treated 16HBE cells (Fig. 2d). Similar results have been described in the lungs of TDI-induced asthmatic mice [16]. In addition, we found NLRP3 inflammasome activation mediated HMGB1 production and release after TDI treatment. Other study also supported that HMGB1 secretion occurred downstream of inflammasome assembly and caspase-1 activation [40]. Therefore the NLRP3 inflammasome-mediated pyroptosis may explain why conventionally nuclear located HMGB1 was able to be rapidly released.

Evidences are accumulating that inflammasome contributes to the development of respiratory inflammatory diseases, including asthma, acute respiratory distress syndrome (ARDS) [41], and chronic obstructive pulmonary disease (COPD). Furthermore, inflammasomes were activated in response to allergens such as silica [42], asbestos and particles. Although TLR signal mediated NF- κ B activation is generally believed important to trigger the production of NLRP3 protein and pro-IL-1 β , as well as a second signal needs to assemble NLRP3 inflammasome, our results show that TLR priming is not indispensable. We show that TDI exposure is necessary and sufficient for the activation of NLRP3 inflammasome, which is in agreement with emerging evidences that silica [43] and particulate [44] induced NLRP3 inflammasome activation without TLR priming. These results suggest that inflammasome activation and successive pyroptosis induction with release of proinflammatory DAMPs may be a common and fundamental pathway of allergic diseases.

A limitation of this study should be noted. TDI is a hapten and requires conjugation to proteins in the airway to induce airway inflammation and hyper-responsiveness. Hence it is difficult to directly detect the concentration of TDI in the airway.

5. Conclusions

Our results demonstrates an important role of bronchial epithelial pyroptosis in the pathogenesis of TDI-induced asthma. Therefore, inhibition of NLRP3 inflammasome-mediated pyroptosis may be a novel therapeutic strategy in the treatment of inhaled stimuli-induced asthma.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

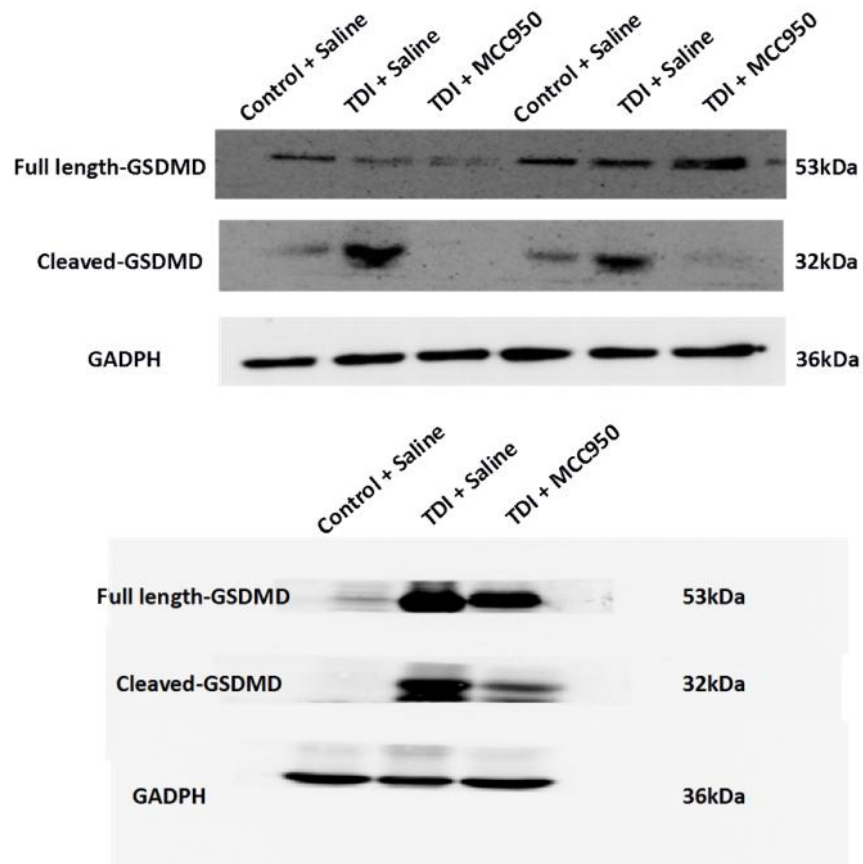
Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.biopha.2020.109925>.

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Supplementary Figure.



Enhanced expression of cleaved GSDMD in the lungs from TDI-induced asthma mice. Expression of full length GSDMD and cleaved GSDMD in the lungs from TDI-induced asthma mice were detected by western blot.