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To cite this article: Jiafu Song, Lihong Yao, Jiaxin Shi, Jiashu Li & Caiyun Xu (2020): Protective effects of N-acetylcysteine on a chemical-induced murine model of asthma, Journal of Asthma, DOI: [10.1080/02770903.2020.1781166](https://doi.org/10.1080/02770903.2020.1781166)

To link to this article: <https://doi.org/10.1080/02770903.2020.1781166>



Accepted author version posted online: 17 Jun 2020.
Published online: 26 Jun 2020.



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Protective effects of N-acetylcysteine on a chemical-induced murine model of asthma

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ABSTRACT

Introduction: Oxidative stress is involved in the pathophysiology of inflammatory airway diseases, including asthma. In this study, we elucidated the possible protective effects of the antioxidant N-acetylcysteine (NAC) on a toluene diisocyanate (TDI)-induced murine asthma model.

Methods: Male BALB/c mice were sensitized and challenged with TDI to generate a chemical-induced asthma model. NAC was given intraperitoneally to mice immediately after each TDI challenge. Airway reactivity to methacholine and bronchoalveolar lavage fluid was analyzed. Lungs were examined by histology.

Results: NAC treatment dramatically reduced the increased airway hyperresponsiveness, inflammatory infiltration, and goblet cell metaplasia in TDI-exposed mice. Numbers of total cells, neutrophils, and eosinophils in the bronchoalveolar lavage fluid of TDI-challenged mice were significantly higher than vehicle control, but the administration of NAC decreased these inflammatory cell counts. TDI exposure led to significantly increased levels of interleukin 4 (IL-4) and IL-5, which were also suppressed by NAC. In addition, diminished lung reduced oxidized glutathione ratio and superoxide dismutase activity were observed after TDI challenge, and these changes were attenuated by NAC.

Conclusion: NAC treatment has beneficial effects in TDI-induced asthma.

ARTICLE HISTORY

Received 19 February 2020
Revised 24 May 2020
Accepted 7 June 2020

KEYWORDS

Asthma; toluene diisocyanate; airway inflammation; N-acetylcysteine

Introduction

Toluene diisocyanate (TDI) is a highly reactive small molecular chemical that is widely used for polyurethane foam manufacturing, autobody painting and repair, plastics manufacturing, and home decoration. It is one of the leading causes of occupational asthma, accounting for 10–25% of all adult-onset asthma (1). It is estimated that as many as 5% of workers exposed to diisocyanates develop asthma that may continue for a lifetime even after the exposure is discontinued (2). TDI-induced asthma is characterized by hyperresponsiveness, inflammation, and remodeling of the airways, often associated with marked infiltration of neutrophils and slight infiltration of eosinophils into the airways, which are resistant to steroid treatment (3). Clinically, TDI-induced asthma shares similar manifestations with allergic asthma induced by high molecular weight allergens, illustrating common pathogenic processes.

N-acetylcysteine (NAC) was first recognized for its role in acetaminophen overdose and as a mucolytic. Over the past decade, there has been growing evidence addressing the effectiveness of NAC in treating a wide range of physical disorders such as chronic obstructive pulmonary disease (COPD), lung fibrosis, cancer, schizophrenia, and memory impairment (4–8). The therapeutic effects of NAC are largely associated with their marked capacity for maintaining intracellular concentrations of reduced glutathione (GSH) (9). As one of the major antioxidants in the lung, GSH is involved in response to TDI exposure. Studies have demonstrated that TDI is able to bind to GSH in the mucosal surfaces (10,11). A rapidly decreased level of GSH has also been observed in TDI-exposed murine lung and bronchial epithelial cells (12,13), suggesting that replenishing GSH can attenuate TDI-induced asthmatic responses. Asthma, which is commonly thought to be associated with increased oxidative

Table 1. Group assignments.

Group	Sensitization	Challenge	Treatment
AOO	AOO	AOO	PBS
TDI	TDI	TDI	PBS
TDI + NAC	TDI	TDI	NAC

Group assignments were made via random number sequence. TDI Group: the mice were dermally sensitized with 0.3% TDI on days 1 and 8 and challenged via the airways with 3% TDI on days 15, 18, and 21. The mice were treated with PBS after each TDI challenge. AOO Group: the mice were sensitized and challenged by the same procedures with the same amount of AOO and treated with PBS. TDI+NAC Group: the mice were sensitized and challenged by the same procedures with the same amount of TDI and treated with NAC (100 mg/kg; *i.p.*) dissolved in PBS.

stress, is a heterogeneous disease (14–16). In recent studies, it has been demonstrated that NAC reduces inflammation in an eosinophilic asthma model induced by ovalbumin (OVA) or German cockroach extract (GCE) (17–19). However, its effectiveness remains unknown in a neutrophil-predominant asthma model developed by TDI.

In this study, we investigated whether NAC has therapeutic effects on a TDI-induced murine asthma model.

Methods and materials

Animals and reagents

Male BALB/c mice (20–22 g) aged 6–8 weeks were purchased from Southern Medical University Experimental Animal Center (Guangzhou, China). The mice were housed in a specific pathogen-free facility with 12 h dark/light cycles and with sterile water and irradiated food available *ad libitum*. The Ethics Committee of The First People's Hospital of Lianyungang (Jiangsu, China) approved the Animal Experiments of the study (Allowance No. lw2019.10.10.001). TDI ($\geq 98.0\%$), acetone, methacholine, and NAC were purchased from Sigma-Aldrich (Shanghai, China). The vehicle (acetone: olive oil [AOO]) used to dissolve TDI consisted of a mixture of two volumes of acetone and three volumes of olive oil for dermal sensitization, and one volume of acetone and four volumes of olive oil for airway challenge.

Experimental procedures

Mice were randomized to the following three groups: (1) AOO-sensitized, AOO-challenged, and phosphate-buffered saline (PBS)-treated mice (AOO group); (2) TDI-sensitized, TDI-challenged, and PBS-treated mice (TDI group); and (3) TDI-sensitized, TDI-challenged, and NAC-treated mice (TDI + NAC group). Animal models were established as described elsewhere (20). Briefly, on days 1 and 8, the mice were dermally treated with 0.3% TDI on the dorsum of both ears

(20 μ L 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). As a control, mice were sensitized and challenged by the same procedures with the same amount of AOO. NAC (100 mg/kg; *i.p.*) dissolved in PBS was given to the mice immediately after each TDI challenge. Sham mice received the same volume of PBS for comparison (Table 1).

Measurement of airway hyperresponsiveness

Airway reactivity to methacholine was assessed the day after the third TDI challenge. Mice were anesthetized and mechanically ventilated (Buxco Electronics, Inc., Troy, NY, USA) before measurements of lung resistance (R_L) in response to increasing doses of aerosolized methacholine (3.12, 6.25, 12.5, and 25 mg/mL) were collected (21). R_L was recorded every 5 min following each nebulization step until a plateau phase was reached. Results were expressed as a percentage of baseline value (value at 0 mg/mL methacholine) for each concentration of methacholine.

Assessment of immunoglobulin E and bronchoalveolar lavage fluid

One day after the last airway challenge, mice were sacrificed with an overdose of pentobarbital. Blood samples were immediately taken from the retro-orbital plexus/sinus. After resting for 1 h at room temperature, they were centrifuged ($3000 \times g$, 20 min), and supernatants were stored for further detection of total immunoglobulin E (IgE). For bronchoalveolar lavage fluid (BALF), mice were cannulated through the trachea and lavaged twice *in situ* with pre-warmed 1 ml sterile saline, and the recovered fluid was pooled. Total cell numbers 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. Then the remaining fluids were centrifuged ($1000 \times g$, 10 min), and supernatants were stored for further detection of interleukin 4 (IL-4) and IL-5.

Pulmonary histopathological examination

After bronchoalveolar lavage, the left lungs were isolated and fixed in 4% neutral formalin, paraffin-embedded, cut in 4 μ m sections, and stained with H&E and periodic acid-Schiff (PAS) for blinded

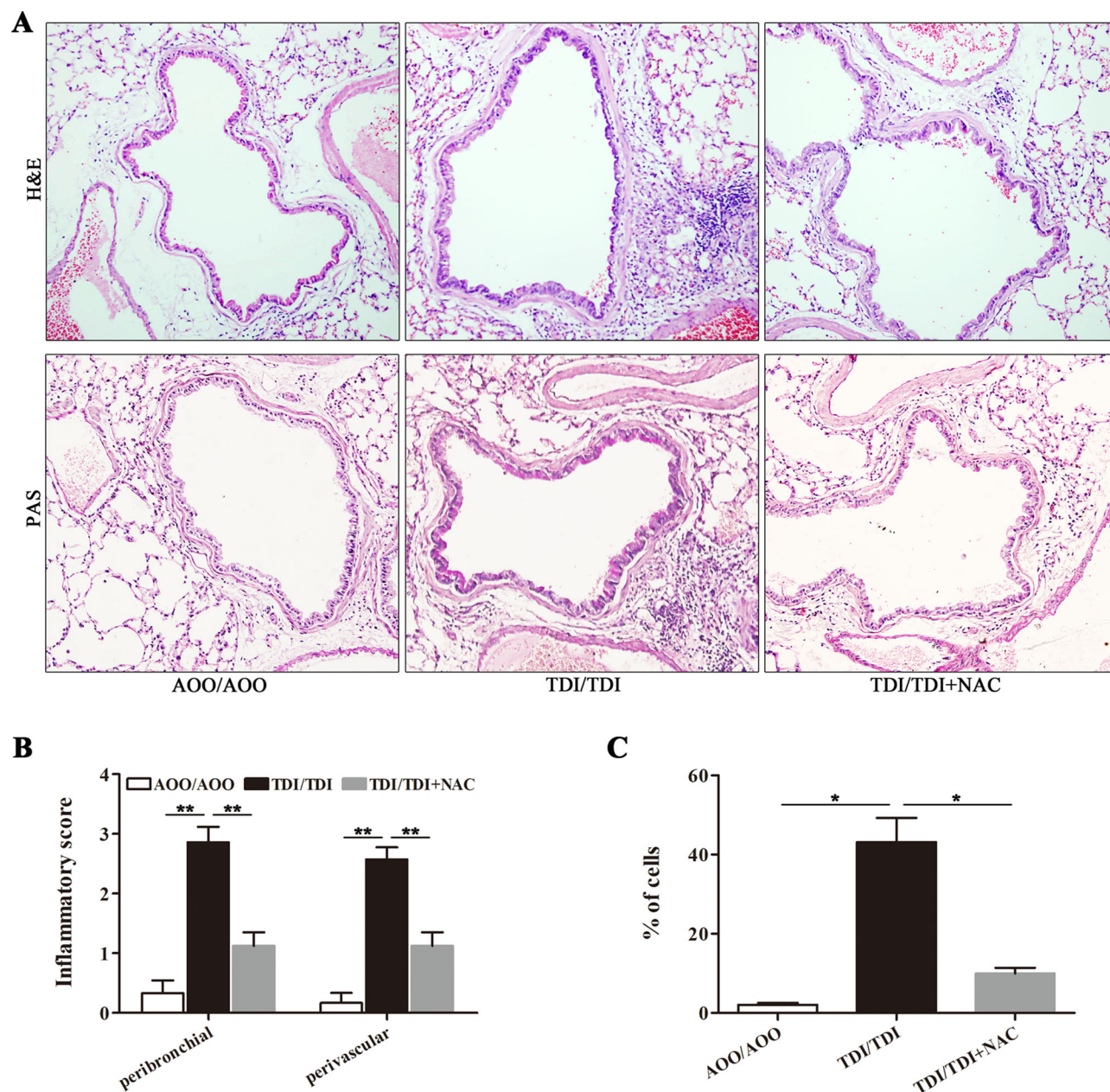


Figure 1. N-acetylcysteine (NAC) attenuates TDI-induced bronchial inflammation and epithelial goblet cell metaplasia. A-B, Representative H&E- and PAS- stained lung sections of different groups. Original magnification was 200 $\times$. Semiquantification of airway inflammation (C) as well as airway mucus production (D) was also performed. $n = 8 \sim 10$. * $p < 0.05$; ** $p < 0.01$.

histopathologic assessment. Lung inflammation and airway goblet cell metaplasia were semiquantified as previously described (20). Scoring was performed at a magnification of 200 $\times$ by examining at least 40 image fields of 20 slices from eight mice per group. Sections were assigned a random number to blind the examiner to the identity of each specimen.

Quantification of reduced glutathione and oxidized

The right lungs isolated from the experimental mice were first snap frozen in liquid nitrogen and then

stored at -80°C . Lung homogenates were obtained for the determination of reduced glutathione disulfide (GSH) and oxidized glutathione (GSSG) according to the manufacturers' instructions (Beyotime Biotechnology, Beijing, China).

Assessment of superoxide dismutase activity

Lung tissue homogenates were used to detect superoxide dismutase (SOD) activity by colorimetric methods according to the manufacturer's recommendations (Beyotime Biotechnology).

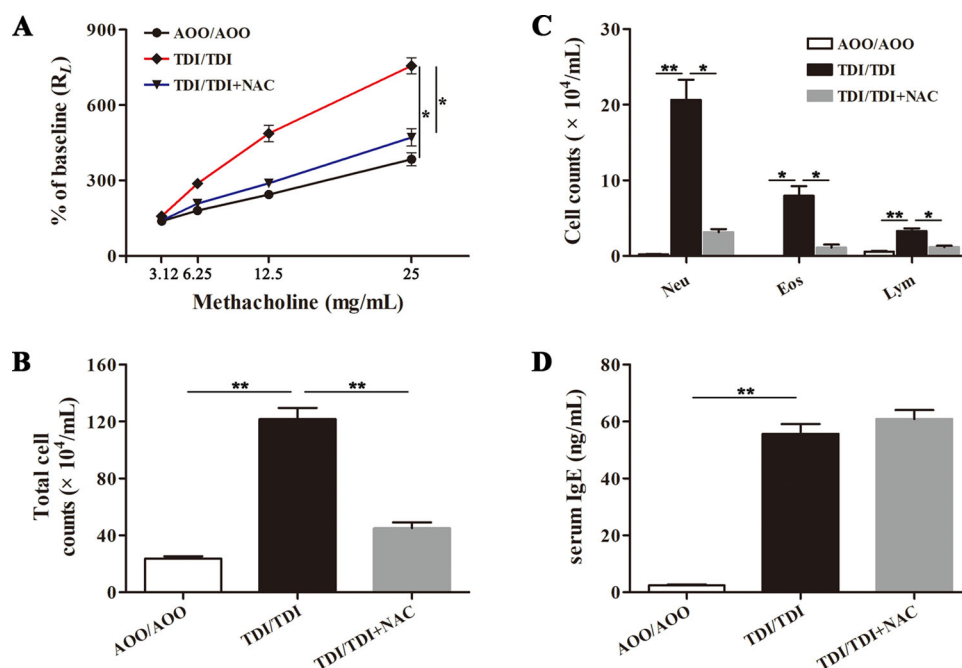


Figure 2. NAC decreases TDI-induced airway hyperresponsiveness and neutrophil and eosinophil accumulation. **A**, Airway hyperresponsiveness was measured by lung resistance (RL). Results are shown as a percentage of the baseline value. $n = 5$. **B**, **C**, Numbers of total and differential inflammatory cells in BALF were counted. $n = 8-10$. **D**, Total serum IgE was also measured. $n = 8-10$. * $p < 0.05$; ** $p < 0.01$.

Statistical analysis

Data are expressed as the mean $\pm$ standard error of the mean. Results were interpreted using one-way analysis of variance and Bonferroni's difference post-hoc test with SPSS 22.0. Differences were considered statistically significant when $p < 0.05$.

Results

NAC treatment attenuates TDI-induced bronchial inflammation and epithelial goblet cell metaplasia

Histological examination revealed significant peribronchial and perivascular inflammation and pronounced epithelial goblet cell metaplasia in the TDI group compared to the AOO group. These symptoms were alleviated in the NAC group (Figure 1).

NAC decreases TDI-induced airway hyperresponsiveness, and neutrophil and eosinophil accumulation

Higher reactivity to methacholine was observed in TDI-sensitized and aerosolized mice compared with the controls, whereas TDI-exposed NAC-treated mice had lower airway hyperresponsiveness than the TDI/TDI group (Figure 2A). BALF analysis showed that antigen-challenged mice had markedly larger amounts

of total inflammatory cell, neutrophil, eosinophil, and lymphocyte accumulation in the airway, which were markedly inhibited by NAC treatment (Figure 2B,C). The concentration of serum IgE was elevated in TDI-sensitized and challenged mice but was not affected by treatment with NAC (Figure 2D).

NAC inhibits airway T helper 2 response in TDI-induced asthma

Allergic airway inflammation is thought to be dominated by the T helper 2 (Th2) response. Therefore, we detected the levels of Th2-related IL-4 and IL-5 in the supernatants of BALF to determine if NAC affects the production of these cytokines. As expected, intraperitoneal injection of NAC at a dose of 100 mg/kg significantly inhibited TDI-triggered release of IL-4 and IL-5, suggesting that NAC is able to counteract the Th2 response (Figure 3).

NAC reverses GSH status and oxidative stress in the lungs of TDI-exposed mice

GSH and GSSG were measured in the lung tissue after the last challenge. A decrease in GSH accompanied by an increase in GSSG, which translates into a decrease of the GSH/GSSG ratio, an index of the tissue redox state, was observed in TDI-exposed mice. These

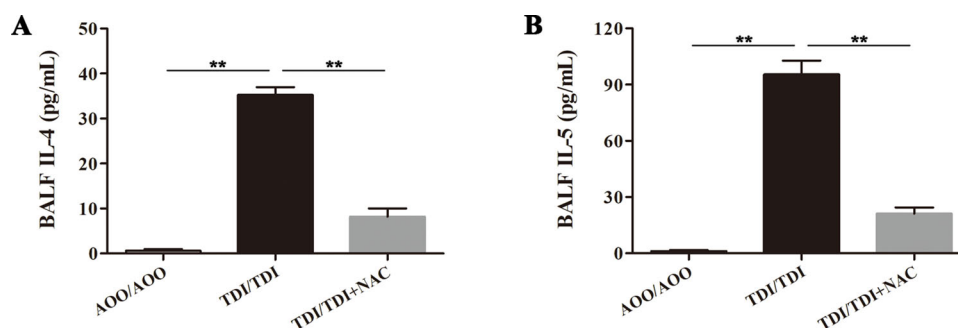


Figure 3. NAC inhibits TDI-induced Th2 response in the airway. Th2-related cytokines IL-4 and IL-5 in the supernatants of BALF were detected by ELISA. $n = 8 \sim 10$. * $p < 0.05$; ** $p < 0.01$.

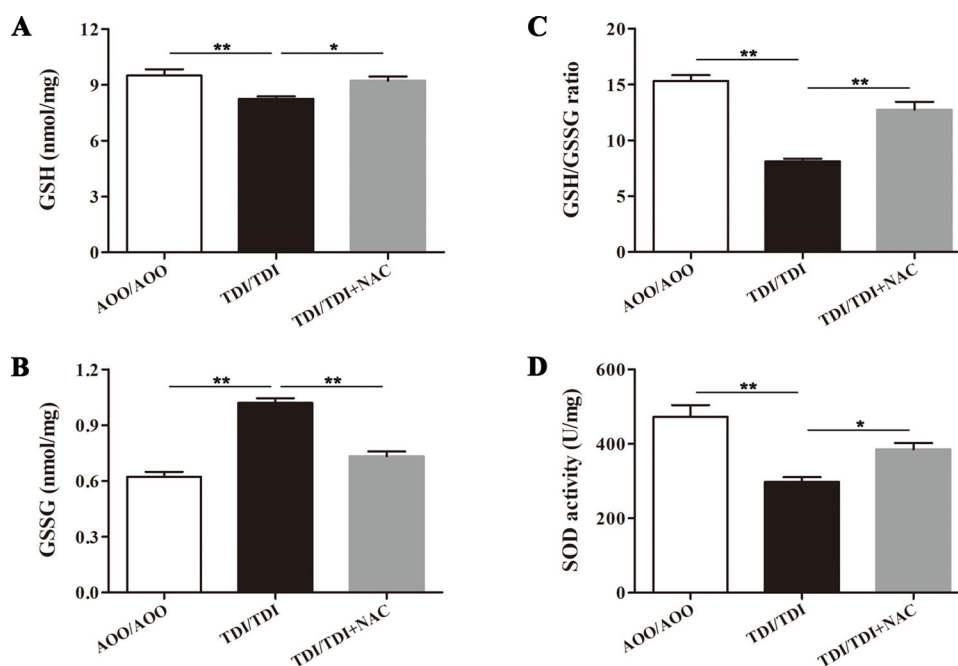


Figure 4. Effects of NAC on the glutathione content and superoxide dismutase (SOD) activity of lung tissue of TDI-exposed mice. $n = 6 \sim 8$. * $p < 0.05$; ** $p < 0.01$.

changes were partially reversed in mice treated with NAC (Figure 4A–C).

SOD is an important antioxidant enzyme that catalyzes the dismutation of the superoxide radical into either ordinary molecular oxygen or hydrogen peroxide, sequentially scavenging detrimental reactive oxygen species (ROS) (22). We detected the decreased activity of SOD in TDI-sensitized and challenged mice, which was markedly restored by NAC administration (Figure 4D). These data suggest that NAC treatment can protect TDI asthmatic mice from oxidative damage.

Discussion

In this study, we demonstrated that NAC treatment could ameliorate TDI-induced hyper-responsiveness

and inflammation of the airway, which could be attributed to its mitigation of oxidative damage.

The family of diisocyanates encompasses a large set of highly reactive chemicals that are commonly used in industrial production including TDI, hexamethylene diisocyanate, and methylene diphenyl diisocyanate. They are all well-established respiratory allergens and account for the majority of work-related asthma cases (23). As small molecular compounds, diisocyanates elicit asthmatic responses that differ from large molecular proteins (17,24). To mimic the characteristics of clinical asthma patients, we developed a TDI-induced murine asthma model that is featured by airway hyperresponsiveness, bronchial neutrophil and eosinophil infiltration, epithelial goblet cell metaplasia, and increased Th2 response and IgE production. In recent years, researchers have revealed the promising

therapeutic effects of NAC in respiratory diseases, including cystic fibrosis and COPD (25). Similarly, in this study, we found that intraperitoneal injection of NAC in TDI-sensitized and challenged mice could reverse most of the asthmatic features, which agrees with the findings in other studies showing the beneficial effects of NAC in different allergen-induced asthma models (17–19,26–28). Importantly, the model induced by TDI is not sensitive to glucocorticosteroid, as shown in our previous study and other research works (3,29); therefore, it provides a potential therapy for steroid-resistant asthma. However, we did observe that TDI-induced overproduction of Th2-related cytokines (IL-4 and IL-5) was inhibited by NAC treatment, whereas the increased production of IgE was not suppressed. Though it is well known that the Th2 cytokines IL-4 and IL-13 are mandatory for class-switch recombination of IgE, a lot of other signals are also involved in the production of IgE such as the transcription factors B-cell maturation protein 1 and X-box protein 1 (30).

Oxidative stress is thought to play a key role in the pathogenesis of TDI-induced asthma. TDI exposure induces oxidative stress, which causes inflammation of the epithelium, induces airway epithelial cells to produce cytokines, and recruits inflammatory cells, such as neutrophils, into the airways, which then increase airway inflammation and lead to more severe asthma with airway remodeling (31,32). Predominant neutrophilic airway inflammation is a hallmark feature of TDI-induced asthma, which is responsible for corticosteroid insensitivity and decreasing lung function (33,34). Interestingly, it has been reported that activated neutrophils infiltrating to the inflamed tissues can generate oxidative stress by producing excess amounts of ROS (35,36), whereas the released ROS can act as direct cell toxins and second messengers that modulate intracellular signaling and activate a cascade of inflammation and neutrophil infiltration, creating a positive feedback that facilitates neutrophilia and tissue damage (37). Thus, antioxidants can be developed as therapeutic candidates for TDI-induced asthma. NAC, a type of antioxidant, is a potent scavenger of oxygen-free radicals that can interact directly with ROS and nitrogen species (25), and has been proven effective in COPD patients and a fibrosis mouse model (6,38). In this study, we observed that the TDI-induced increased neutrophil accumulation in the airway was markedly inhibited in NAC-treated mice. This could be attributed to a restored balance between oxidants like GSSG and antioxidant SOD in TDI-exposed mice.

Aberrations in alveolar and mucosal GSH metabolism are widely recognized as a central feature of many inflammatory lung diseases such as idiopathic pulmonary fibrosis, cystic fibrosis, and asthma (39). Maintaining GSH/GSSG homeostasis is crucial for antioxidant defense in the lung. Researchers have proven that TDI can react readily with GSH, leading to depletion of the antioxidant GSH in bronchial epithelial cells (40). We also detected a decreased level of GSH in the lungs of TDI-sensitized and challenged mice, accompanied by a decreased ratio of GSH/GSSG. Therefore, GSH supplementation might be protective. Nevertheless, administration of GSH is not considered optimal considering its poor bioavailability and limited ability to cross the phospholipid bilayer of cells. NAC is the most enduring delivery mode for cysteine. In addition to being a potent ROS scavenger, it is also frequently used as a precursor to GSH, supports the synthesis of GSH, and helps to replenish GSH when endogenous stores are compromised (9). Therefore, in this study, we treated TDI-exposed mice with NAC. As expected, NAC administration significantly restored the decreased level of GSH and GSH/GSSG, which was paralleled by eliminated airway inflammation, indicating that NAC attenuates TDI-induced asthma through supplementing GSH. A recent study indicated that the inflammasome may play a vital role in the pathogenesis of TDI-induced asthma (41); moreover, ROS can mediate the NLRP3 inflammasome on bronchial epithelial cells (42). Consequently, we believe that NAC can attenuate inflammation by suppressing the ROS-NLRP3 inflammasome axis. In addition, researchers have shown that ROS is associated with eosinophil extracellular traps, as demonstrated in a mouse asthma model (17), and enhances mononuclear cell mitochondrial function and neutrophil activation in asthma patients (36,43). Therefore, we consider that the effect of NAC may be involved in various cell types and intracellular signaling pathways.

In summary, our data suggest that a high dose of NAC has protective effects by acting as an antioxidant in TDI-induced asthma. The main limitation of this study is that the underlying mechanisms were not explored. Thus, additional studies are required to further delineate the mechanisms underlying the beneficial effect of NAC on asthma, as well as to explore the potential for using NAC administration as a concomitant therapy for neutrophilic asthma patients. Additionally, the concentration of TDI is difficult to directly detect in the airway, as it is a hapten that

requires conjugation to proteins in the airway to induce inflammation.

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