

Chicken IgY facilitates allergic airway inflammation in a chemical-induced murine asthma model by potentiating IL-4 release



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HIGHLIGHTS

- Chicken IgY anti-HMGB1 antibody exacerbates TDI-induced allergic airway inflammation.
- IgY aggravates the asthmatic reaction and anti-HMGB1 counteracts the effects of IgY.
- It is IgY that facilitates TDI-induced allergic airway inflammation by promoting the release of IL-4.

ARTICLE INFO

Article history:

Received 23 May 2015

Received in revised form 25 August 2015

Accepted 29 August 2015

Available online 1 September 2015

Keywords:

Asthma

Toluene diisocyanate

Airway inflammation

IgY anti-HMGB1

ABSTRACT

High mobility group box 1 (HMGB1) is a DNA-binding protein that is abundantly expressed in most tissues. Recently, HMGB1 has gained much attention for its regulation of immunity and inflammation. Yet its role in toluene diisocyanate (TDI)-induced asthma still remains poorly characterized. In this study, mice were sensitized and challenged with TDI to establish a TDI-induced asthma model. An IgY anti-HMGB1 antibody or isotype IgY was given intraperitoneally after each challenge. Airway reactivity to methacholine, airway inflammation, bronchial epithelial hyperplasia and shedding were unexpectedly aggravated after administration of the anti-HMGB1 antibody and was accompanied by increased pulmonary expression of HMGB1, especially in those mice treated with IgY. Levels of IL-4, IL-5, IL-13 and TNF- α were also elevated with TDI-induction. Primary lymphocytes from TDI sensitized and challenged mice demonstrated increased secretion of IL-4 after IgY stimulation. To confirm the effect of IgY, a cohort of mice exposed to TDI or vehicle was injected with IgY and the same results were observed after IgY treatment as in TDI asthmatic mice. Taken together, these results show that the IgY anti-HMGB1 antibody can facilitate TDI-induced allergic airway inflammation. Specifically, IgY, rather than anti-HMGB1, plays an important role in the process of exacerbated asthma, shedding light on an underappreciated role of avian IgY.

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1. Introduction

High mobility group box 1 (HMGB1) is a highly conserved, ubiquitous DNA-binding protein present in the nucleus of almost all eukaryotic cells (Javaherian et al., 1978). In response to infection or sterile injury, it can be actively or passively released into the extracellular milieu and acts as a proinflammatory mediator (Yang

et al., 2010). The emergence of various selective antagonists targeted against HMGB1 activity has shed new light on studying the role of HMGB1 in a vast array of inflammatory disorders (Yang and Tracey, 2010). Among the antagonists in development, the IgY anti-HMGB1 antibody is the most widely used and has been proved to be safe and protective in mouse models of acute lung injury (Kim et al., 2005), pulmonary fibrosis (Hamada et al., 2008), acute pancreatitis (Sawa et al., 2006) and colitis (Maeda et al., 2007).

More recently, in both asthmatic patients and animal models, the level of HMGB1 in induced sputum as well as lung tissues was also found to be higher than control (Hou et al., 2011; Shim et al., 2012; Watanabe et al., 2011). And inhibition of

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HMGB1 significantly attenuated airway hyperresponsiveness, airway inflammation and remodeling in experimental asthma models (Lee et al., 2013; Shim et al., 2012), indicating that HMGB1 contributes to the pathogenesis of asthma. We have previously reported increased expression of HMGB1 in a toluene diisocyanate (TDI)-induced asthma model (Tang et al., 2014). We found that inhibiting HMGB1 by a non-specific antagonist, ethyl pyruvate (EP), could alleviate airway inflammation. Yet the role of HMGB1 in TDI-induced asthma still needs to be further elucidated. So our initial goal was to investigate the role of HMGB1 in TDI-induced asthma with the IgY anti-HMGB1 antibody. Unexpectedly, we found that the TDI-challenged mice developed increased airway hyperresponsiveness and inflammation after administration of the neutralizing antibody, especially for those treated with the control IgY antibody. Those surprising findings drove us to test the hypothesis that IgY, rather than anti-HMGB1, might exacerbate TDI-induced asthma.

2. Materials and methods

2.1. Reagents

Toluene-2,4-diisocyanate, methacholine, and acetone were obtained from Sigma–Aldrich (Shanghai, China). Sodium pentobarbitone was obtained from Weijia Biotechnology Company (Guangzhou, China). The vehicle (AOO) used to dissolve TDI consists 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. The ELISA kit for IgE quantification was obtained from ALPCO (Windham, NH).

2.2. Animals and treatment with anti-HMGB1 antibody

Male BALB/c mice aged 6–8 weeks were obtained from Southern Medical University. The mice were housed in filter-top cages with free access to sterile acidified water and irradiated food in a specific pathogen-free facility with 12-h dark/light cycles in Southern Medical University. All studies were conducted in accordance with the Animal Subjects Committee of Nanfang Hospital on the use and care of animals. Mice were randomly divided into the following four groups: (1) AOO group, AOO-sensitized/AOO-challenged, and PBS treated; (2) TDI group, TDI-sensitized/TDI-challenged, and PBS treated; (3) TDI + anti-HMGB1 group (“TDI + anti-H” for short in figure legends), TDI-sensitized/TDI-challenged, and chicken IgY anti-HMGB1 antibody treated; (4) TDI + IgY group, TDI-sensitized/TDI-challenged, and chicken IgY treated.

Briefly, on days 1 and 8, mice were dermally sensitized with 0.3% TDI on the dorsum of both ears (20 μ L per ear). On days 15, 18, and 21, mice were individually placed in a horizontal cylindrical chamber and challenged through the airways with 3% TDI dissolved in acetone/olive oil (1:4) by means of compressed air nebulization (NE-C28; Omron, Tokyo, Japan) for 3 h at each treatment. As a control, mice were sensitized and challenged by the same procedures with the same amount of TDI solvent. In TDI-inhaled mice, chicken IgY anti-HMGB1 antibody or control IgY (200 μ g/mouse, intraperitoneally; Shino-Test Corp.) dissolved in 200 μ L PBS was administered 1 h after each airway challenge. Sham mice were injected with a similar volume of PBS by comparison.

2.3. Determination of airway responsiveness, serum IgE and analysis of bronchoalveolar lavage fluid (BALF)

Airway reactivity to methacholine, serum IgE and BALF were assessed as previously described (Tang et al., 2014). The

supernatant of BALF was used for detecting IL-4, IL-5 and IL-13 (eBioscience, San Diego, US).

2.4. Measurement of IFN γ , IL-4, IL-5, IL-13, IL-17A and TNF- α in supernatants of cultured lymphocytes

Cervical lymph nodes were dissected from the mice and processed for each mouse separately, then kept on ice in RPMI-1640 medium. Cell suspensions were obtained by pressing the lymph nodes through a cell strainer (40 μ m) (BD Bioscience, Erembodegem, Belgium) and rinsing with 10 mL of tissue culture medium (RPMI-1640). Cells were counted using a Bürker hemocytometer. Lymphocytes were then washed three times and suspended (10^7 cells/mL) in complete tissue culture medium (RPMI-1640 supplemented with 10% heat-inactivated FBS). Cells were seeded into 48-well culture plates at a density of 10^6 cells/mL and incubated in complete RPMI-1640 medium for 43 h with 5 mg/mL concanavaline A (Sigma–Aldrich, Shanghai, China), then centrifuged (1000 $\times$ g, 4 $^{\circ}$ C, 10 min). Supernatants were harvested for quantitative assessment of IFN γ , IL-4, IL-5, IL-13, IL-17A and TNF- α by multiplex immunoassays (eBioscience) according to manufacturer's recommendations.

2.5. Pulmonary histopathological examination

Left lungs were dissected from mice, then fixed in 4% neutral formalin, dehydrated, and paraffin-embedded. Sections (4 μ m) were cut with Leica microtome 2030 (Leica Microsystems Nussloch GmbH, Nussloch, Germany). Lung tissues were stained with H&E and Masson's trichrome for blinded histopathologic assessment. Peribronchial and perivascular inflammation in H&E stained slides was assessed using an scoring standard: 0 = normal; 1 = infrequent inflammatory cells; 2 = a ring of inflammatory cells 1 cell layer deep; 3 = a ring of inflammatory cells 2–4 cells deep; 4 = a ring of inflammatory cells more than 4 cells deep. The presence of collagen fibril deposition with Masson staining was analyzed by Image-Pro Plus software. Scoring was performed at a magnification of 200 $\times$ by examining at least 40 image fields of 20 slices from 6 mice per group. Sections were assigned a random code to blind the examiner to the identity of each specimen.

2.6. Immunohistochemistry and western blot

Lung sections were prepared as previously described (Tang et al., 2014), then stained with polyclonal rabbit anti-HMGB1 antibody (ab79823, Abcam, 1:500). After washing with PBS, slices were incubated with anti-rabbit secondary antibody (Zhongshanjinqiao, Beijing, China) for 30 min at room temperature. Signals were visualized with a DAB peroxidase substrate kit (Zhongshanjinqiao). HMGB1 localization in the lung was assessed under light microscopy.

For western blot of HMGB1, lung tissues were collected from mice receiving TDI or vehicle, and lysed in buffer containing protease inhibitor, calcineurin inhibitors and PMSF. Protein extracts were subjected to 10% SDS-PAGE, transferred to PVDF membrane (Millipore, Bedford, MA), and then probed with rabbit anti-HMGB1 antibody (Abcam). After incubation with the IRDye[®] 800WC-conjugated secondary antibody, immunoreactive bands were exposed to Odyssey[®] CLx Imager for image capture. Data analysis was done with Odyssey Software.

2.7. Examination of IgY-potentiated release of TNF- α and Th2 cytokines in primary murine lymphocytes

To examine the potential proinflammatory role of IgY, we performed a second protocol. Mice were exposed to TDI or AOO

with aforementioned procedures and lymphocyte suspensions from TDI sensitized/TDI inhaled mice as well as AOO sensitized/AOO inhaled mice were prepared as above. One-milliliter portions of the cell suspension were transferred in quadruplicate to 48-well, flat-bottomed tissue culture plates at a density of 10^6 cells/mL in RPMI-1640 supplemented with 10% FBS. The inducer, chicken IgY with LPS removed, at doses of 1 μ g, 10 μ g and 50 μ g, in the volume of 50 μ L PBS, were added to the wells with cell suspensions. Wells containing PBS (50 μ L)-treated lymphocyte samples were used as negative control. The plates were incubated at 37 °C for 43 h in a 5% CO₂ atmosphere. After incubation, the cells were centrifuged at 200 $\times$ g at room temperature for 15 min. The supernatants were collected and used for cytokine determination.

TNF- α and IL-4, IL-5, IL-13 were quantified by multiplex immunoassays (eBioscience) according to procedures recommended by the manufacturer.

2.8. In vivo administration of IgY

To test the effect of IgY in control mice and to confirm its effect on TDI asthmatic mice, a third protocol was included. In this protocol, mice received one of the following four treatments with aforementioned procedures: AOO sensitized/challenged and PBS

treated, AOO sensitized/challenged and IgY treated, TDI sensitized/challenged and PBS treated, TDI sensitized/challenged and IgY treated. IgY (200 μ g per mouse dissolved in 200 μ L PBS) or PBS (200 μ L) was injected intraperitoneally 1 h after each inhalation. Airway reactivity to methacholine was measured. Cellular analysis of BAL fluid and pulmonary histological examination were performed. Lymphocyte culture supernatants from TDI sensitized/inhaled as well as AOO sensitized/inhaled mice were collected as above for measurements of TNF- α and IL-4, IL-5, IL-13 (Multiplex immunoassays; eBioscience). Lung tissues were homogenized for detecting p-ERK expression by western blot (anti-p-ERK, Cell Signaling Technology).

2.9. Statistical analysis

Statistical analysis was performed using SPSS version 16.0. Data were expressed as mean $\pm$ standard error (SE). The differences in TDI-induced asthma manifestations among groups were determined by one-way analysis of variance (ANOVA) and post hoc tests were analyzed by Bonferonni (equal variances assumed) or Dunnett's T3 (equal variances not assumed) post hoc tests for multiple comparisons. The effect of chicken IgY on the release of cytokines was assessed by two-way ANOVA accompanied by

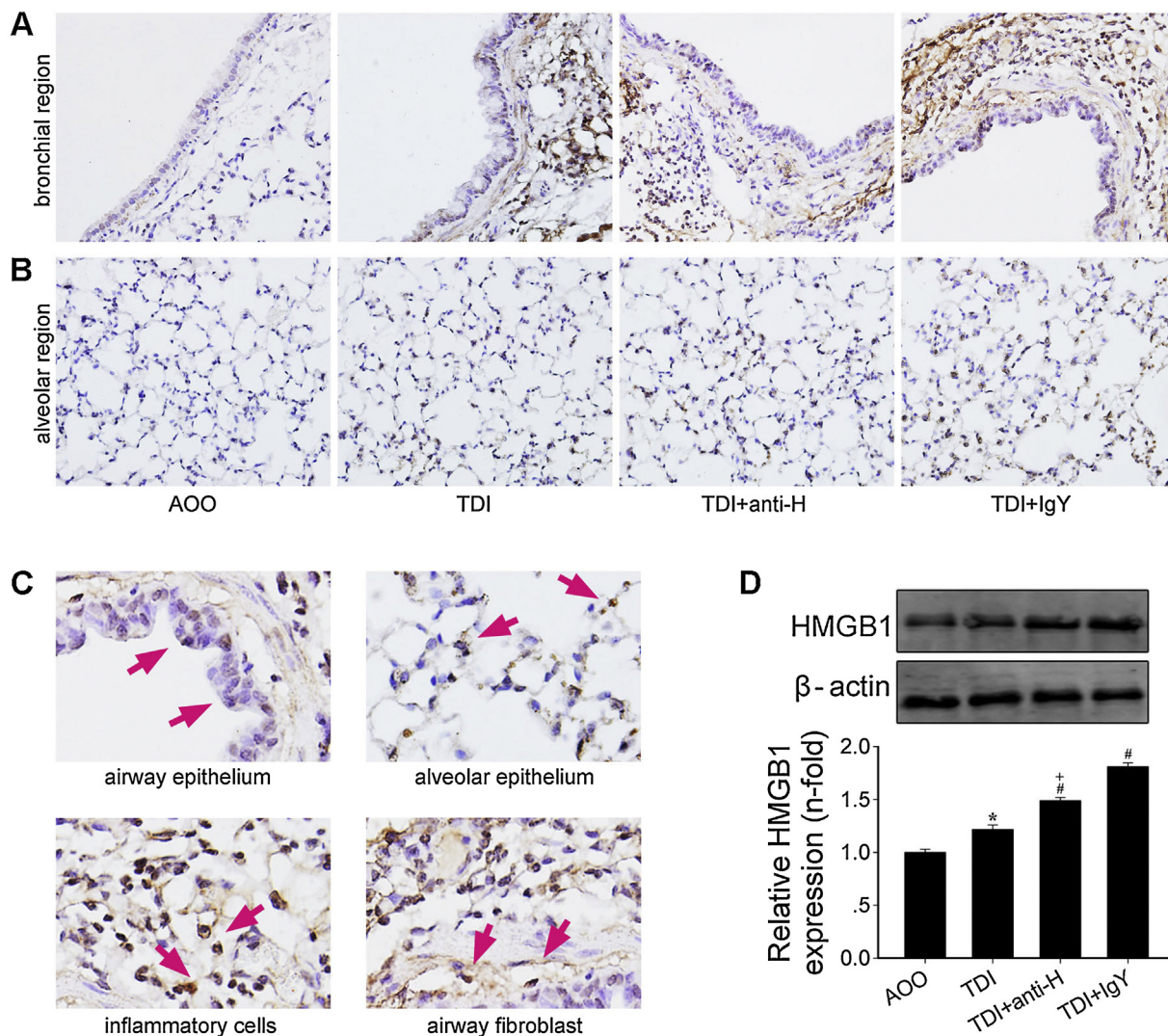


Fig. 1. Pulmonary expression of HMGB1. Representative immunohistochemical staining of HMGB1 in bronchial (A) and alveolar (B) regions. Original magnification was 400 $\times$. (C) Amplified view of cells expressing HMGB1 in the cytoplasm in the lungs of TDI-exposed mice treated with IgY. (D) Pulmonary HMGB1 expression was determined by western blot and subsequent densitometric analysis of the blots ($n=6$). * $P < 0.05$ vs AOO group; # $P < 0.05$ vs TDI group; + $P < 0.05$ vs TDI + IgY group.

Bonferonni post hoc test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Pulmonary expression of HMGB1 was suppressed by IgY anti-HMGB1 antibody

In contrast to our previous study (Song et al., 2013), we used compressed air nebulization for the airway challenge. In spite of this, pulmonary expression of HMGB1 was still upregulated in TDI-inhaled mice. As shown in Fig. 1, both the location and amount of HMGB1 were changed by TDI. In AOO group, HMGB1 was observed almost only in the nuclei. After TDI challenge, we found that bronchial epithelium, alveolar epithelium, airway fibroblasts and infiltrating inflammatory cells all expressed HMGB1 in the cytoplasm. Similar results were obtained by western blot. Surprisingly, the IgY-treated mice did not show similar expression of HMGB1 as mice in TDI group; instead, their protein levels of HMGB1 were significantly higher. Mice injected with IgY anti-HMGB1 antibody had increased expression of HMGB1 compared with TDI group, but decreased levels compared with mice treated with IgY, indicating that the neutralizing antibody did effectively inhibit HMGB1.

3.2. IgY anti-HMGB1 antibody aggravates TDI-induced airway hyperresponsiveness and has no effect on total serum IgE

Airway reactivity was determined 24 h after the last inhalation. Sensitized mice challenged with TDI for 3 h daily for a total of 3 times developed hyperreactivity of the airway to inhaled methacholine as compared to AOO-challenged group (3.12, 6.25 and 12.5 mg/mL; $p < 0.05$; Fig. 2A). Yet airway hyperresponsiveness was exacerbated ($p < 0.05$) after administration of chicken anti-HMGB1 antibody or control IgY. Compared with IgY group, anti-HMGB1 antibody treatment attenuated airway sensitivity to methacholine (6.25 and 12.5 mg/mL; $p < 0.05$). The level of total serum IgE was markedly raised by TDI, while the IgY anti-HMGB1 antibody or IgY alone had no effect on it (Fig. 2B).

3.3. IgY anti-HMGB1 antibody facilitates airway accumulation of neutrophils and eosinophils

Total cell counts, as well as percentages of different inflammatory cells were assessed in BAL fluid (Fig. 2C and D). In line with total cell counts (Fig. 2C), higher amounts of neutrophils and eosinophils were found after sensitization and challenge with TDI ($p < 0.05$), which were significantly increased with chicken anti-HMGB1 antibody and control IgY ($p < 0.05$). In comparison with the anti-HMGB1 group, mice in IgY group had even greater numbers of neutrophils and eosinophils in BAL fluid (Fig. 2D).

3.4. IgY anti-HMGB1 antibody exacerbates peribronchial inflammation, epithelial hyperplasia and shedding

Histological examination of lung sections from TDI group revealed large numbers of infiltrating inflammatory cells and increased collagen deposition around the airways as well as evident epithelial hyperplasia compared to AOO group. While treatment with either IgY anti-HMGB1 antibody or IgY resulted in substantially more inflammatory cell recruitment, heavier epithelial hyperplasia and shedding was observed in TDI-inhaled mice (Fig. 3A–C). The anti-HMGB1 antibody-treated mice displayed less neutrophil and eosinophil infiltration around the bronchus and ameliorated epithelial shedding when compared with IgY group. Collagen deposition did not differ in any of the TDI-challenged mice (Fig. 3A and D).

3.5. Dysregulation of Th1, Th2, Th17 and TNF- α is involved in the detrimental role of IgY anti-HMGB1 antibody

TDI asthma is thought to be a mixture of a Th1 and Th2 response (De Vooght et al., 2009). At the same time, Th17, TNF- α and IL-1 β have also been identified as important factors in TDI asthma pathogenesis (Johnson et al., 2005; Kim et al., 2007; Matheson et al., 2002). Therefore, we measured levels of those cytokines in supernatants of cultured lymphocytes and BAL fluid. Mice in TDI group exhibited increased release of Th2-related factors, including IL-4, IL-5 and IL-13, Th17-related IL-17A, and the proinflammatory

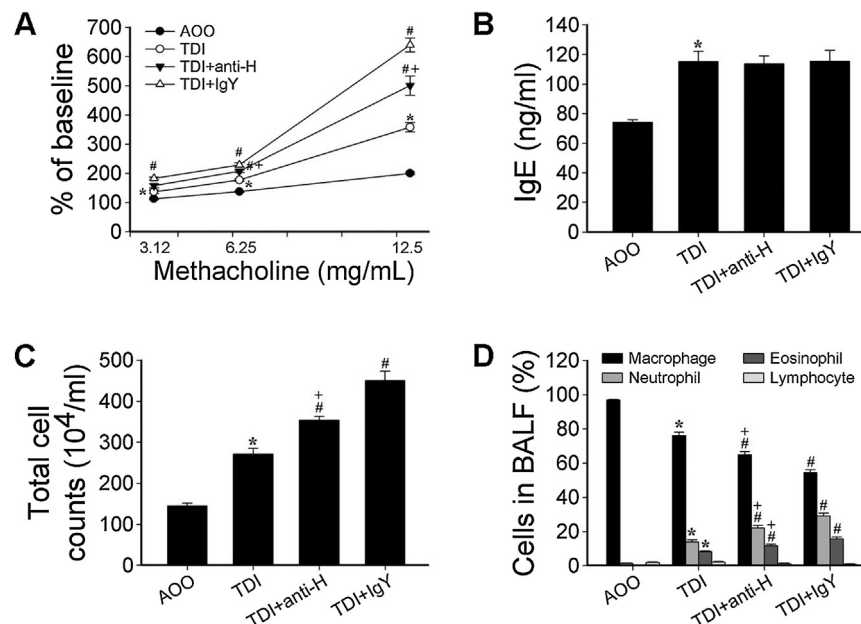


Fig. 2. TDI-induced airway hyperresponsiveness, serum IgE, and neutrophil and eosinophil accumulation in bronchoalveolar lavage fluid (BALF). (A) Penh was used to assess airway reactivity to methacholine. Results are expressed as the percentage of baseline Penh value for each concentration of methacholine. (B) Total IgE in the sera. Total (C) and differential (D) inflammatory cell counts in BALF. $n = 6-10$ per group. * $P < 0.05$ vs AOO group; # $P < 0.05$ vs TDI group; + $P < 0.05$ vs TDI + IgY group.

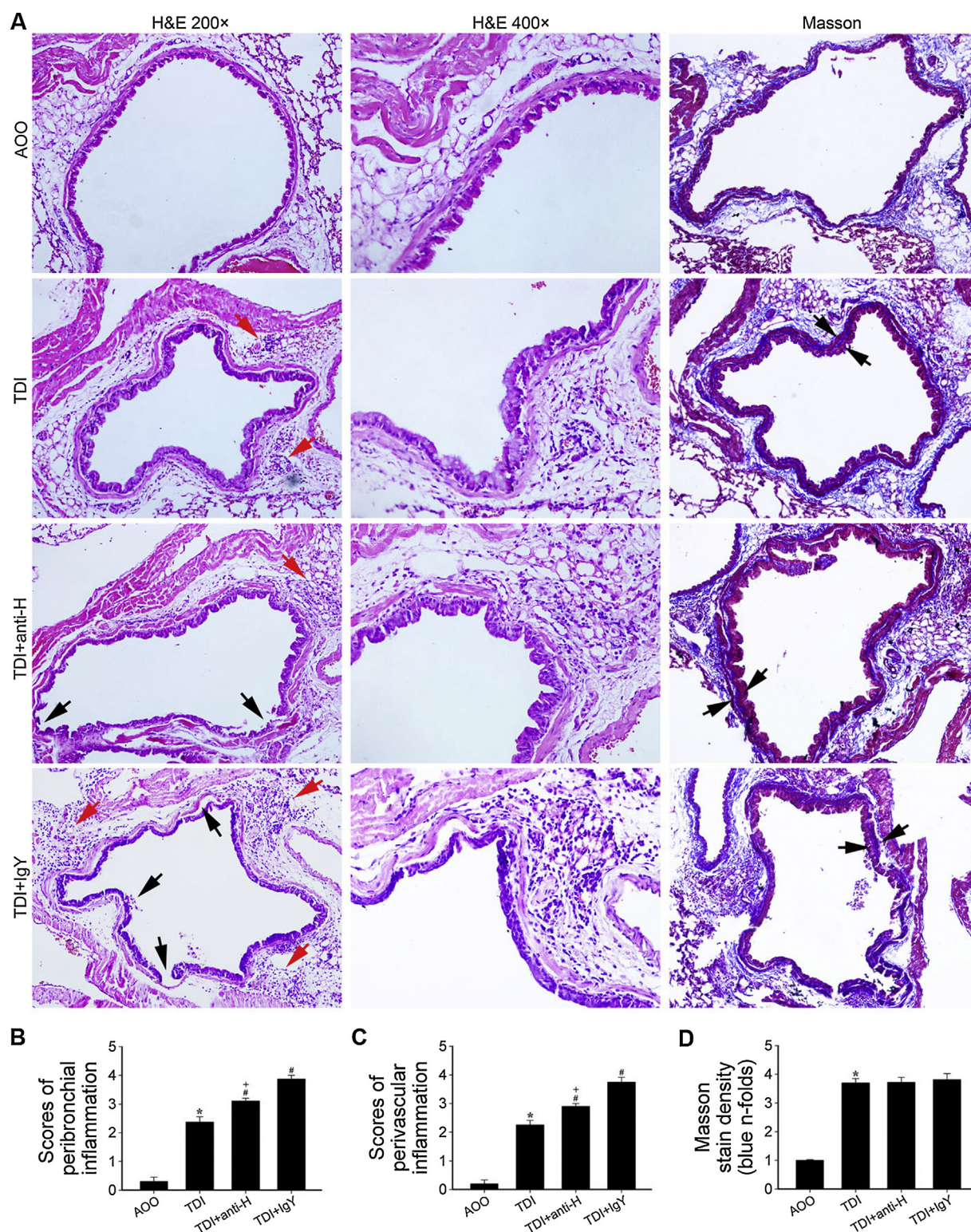


Fig. 3. TDI-induced pulmonary pathological changes. (A) Representative H&E-stained lung sections of different treatment groups at 200 $\times$ (left panel) and 400 $\times$ (middle panel) original magnification and Masson trichrome-stained sections at 200 $\times$ magnification (right panel). Red arrows indicate inflammatory infiltration in peribronchial regions; black arrows indicate epithelial shedding or collagen deposition around the airways. (B–D) Semi-quantification of peribronchial and perivascular inflammation as well as Masson's trichrome staining. $n = 6$ –10 per group. * $P < 0.05$ vs AOO group; $^{\#}P < 0.05$ vs TDI group; $^{+}P < 0.05$ vs TDI + IgY group (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

cytokine, TNF- α , in lymphocyte culture supernatants, as well as elevated levels of IL-4 and IL-5 in BAL fluid compared with AOO group (Fig. 4; $p < 0.05$). The levels of IFN- γ , IL-17A, IL-5 and TNF- α were all markedly raised following treatment with the anti-

HMGB1 antibody, especially with IgY in comparison with TDI. Yet neither IFN γ , IL-17A, TNF- α , nor IL-1 β were detected in BAL fluid. These data suggest that IgY might aggravate cytokine release in asthma.

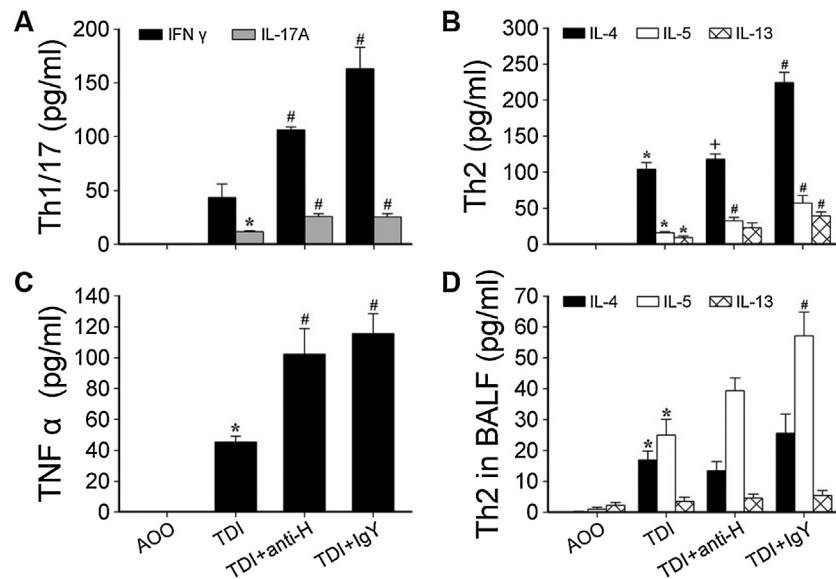


Fig. 4. Levels of Th1, Th2, and Th17-related cytokines and TNF- α . (A–C) Levels of Th1-related IFN- γ ; Th2-related IL-4, IL-5, and IL-13; Th17-related IL-17A; and TNF- α in lymphocyte culture supernatants. (D) IL-4, IL-5 and IL-13 levels in BALF. Data are expressed as the mean $\pm$ SE ($n = 6-10$). * $P < 0.05$ vs AOO group; # $P < 0.05$ vs TDI group. + $P < 0.05$ vs TDI + IgY group.

3.6. IgY promotes IL-4 release in TDI-induced asthma both in vitro and in vivo

As HMGB1 is an identified proinflammatory mediator (Yang and Tracey, 2010), we hypothesized that the exacerbated asthma manifestations after treatment with IgY anti-HMGB1 antibody might be attributed to IgY. So in a second protocol we examined whether IgY could promote the release of inflammatory cytokines in vitro. Different doses of IgY or control PBS were added to cultured murine primary lymphocytes. We observed significantly increased secretion of IL-4 when stimulated with IgY at the dose of 50 μ g only in lymphocytes from TDI asthmatic mice, while no differences of IL-5, IL-13 nor TNF- α were seen (Fig. 5). IL-4, IL-5, IL-13 and TNF- α were not detected in IgY- or PBS-treated lymphocyte supernatants of AOO sensitized/challenged mice.

To confirm the role of IgY, a third group of mice were exposed to TDI or AOO and IgY was injected intraperitoneally. In line with the above results, IgY had few effects on airway reactivity and airway inflammation in mice challenged with AOO, but exacerbated both of TDI-challenged mice and increased levels of related cytokines. Interestingly, in AOO-challenged mice, we found pronounced, though mild, elevation of IL-4 and TNF- α in supernatants of cultured lymphocytes after administration of IgY, while the other cytokines were not detected. At the same time, we found that

when compared with AOO-inhaled mice, pulmonary expression of phospho-ERK in TDI-inhaled mice was distinctly upregulated, which was further elevated after administration of IgY (Fig. 6).

The results indicate that IgY promoted the release of IL-4, therefore exacerbating TDI asthma.

4. Discussion

In the present study, we observed enhanced airway hyper-responsiveness and airway inflammation in TDI asthmatic mice after treatment with chicken IgY anti-HMGB1 antibody. These are in disagreement with the results of studies in hemorrhage shock, sepsis, and other disease syndromes (Andersson and Tracey, 2011; Yang et al., 2004, 2006), and contradictory to those obtained using OVA-immunized mice where the anti-HMGB1 antibodies used were not of the IgY isotype (Lee et al., 2013; Shim et al., 2012).

HMGB1 is a nuclear protein that has been identified as a pathogenic inflammatory mediator during infection or sterile tissue injury (Lu et al., 2014). As an important member in the family of damage-associated molecular patterns (DAMPs), it can be largely secreted into the extracellular milieu from immune cells such as monocytes or macrophages after stimulation with lipopolysaccharide (LPS) or nitric oxide (Andersson and Tracey,

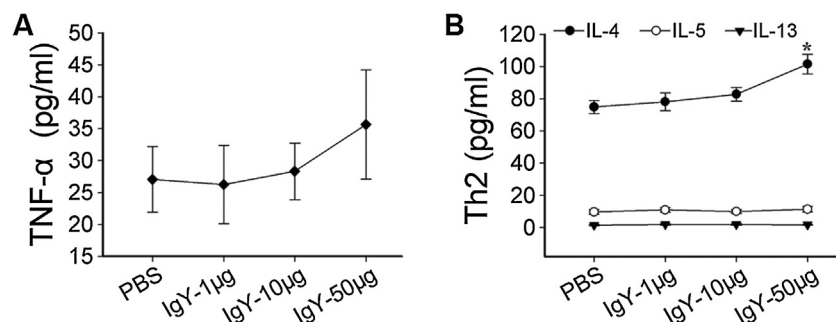


Fig. 5. Chicken IgY promotes the release of IL-4 in primary lymphocytes from TDI sensitized and challenged mice. (A) Levels of TNF- α secreted by cultured lymphocytes from TDI sensitized and challenged mice. (B) Levels of Th2 cytokines (IL-4, IL-5 and IL-13) secreted by cultured lymphocytes from TDI sensitized and challenged mice. $n = 8$ per group. Neither TNF- α , IL-4, IL-5, nor IL-13 was detected in cell supernatants of AOO sensitized/challenged mice. * $P < 0.05$ vs PBS-treated.

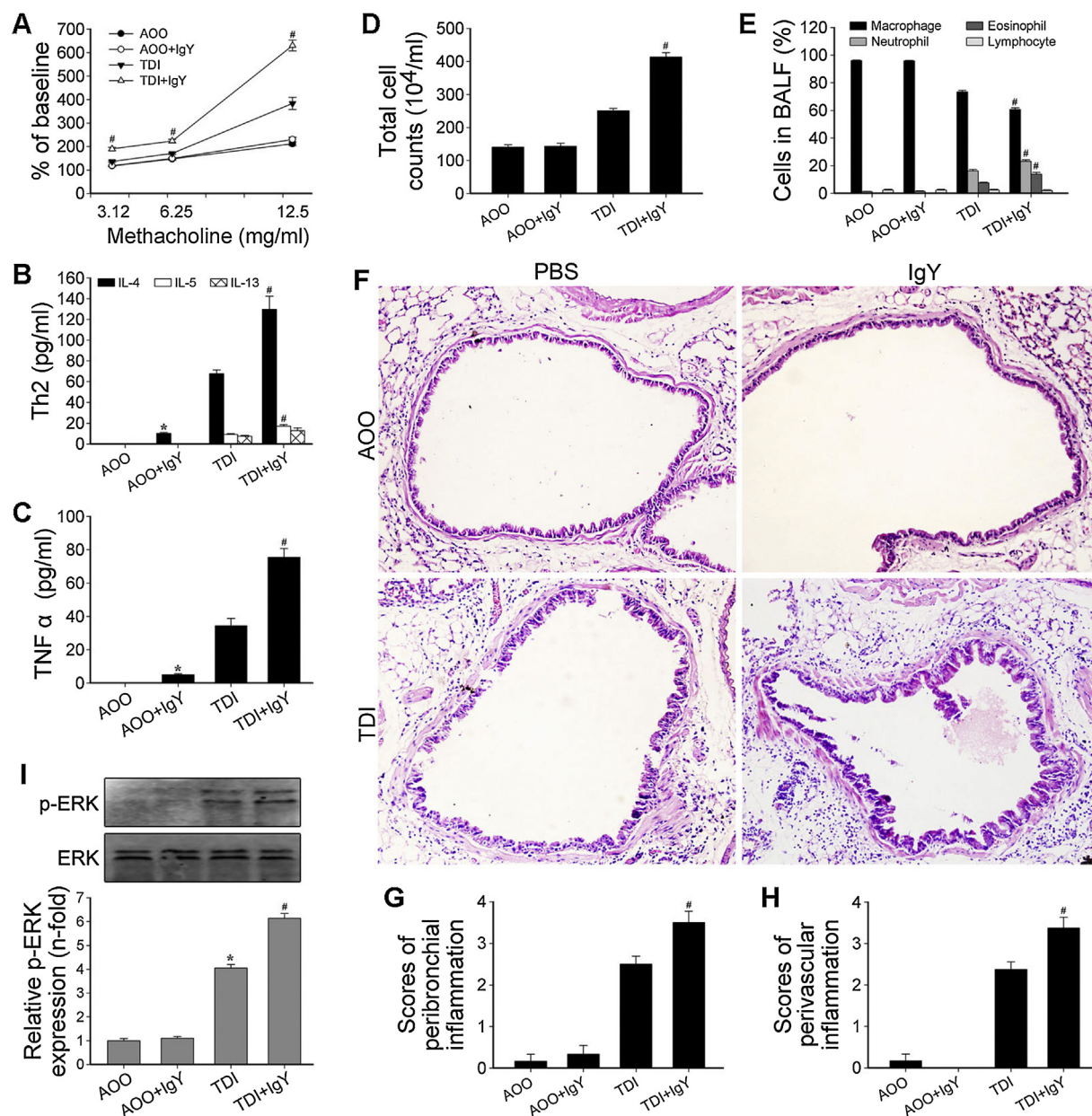


Fig. 6. Effect of IgY on airway reactivity, cytokine release and airway inflammation in TDI asthmatic or control mice. (A) Penh was utilized to determine airway reactivity. Results are expressed as the percentage of baseline Penh value for each concentration of methacholine. (B and C) IL-4, IL-5, IL-13 and TNF- α levels in supernatants of cultured primary lymphocytes. (D and E) Total cell counts and percentages of different inflammatory cells in BAL fluid. (F) Representative H&E stained lung sections. Original magnification was 200 $\times$. (G and H) Semi-quantification of peribronchial and perivascular inflammation. $n = 6$ –10 per group. (I) Pulmonary expression of p-ERK determined by western blot and subsequent densitometric analysis; $n = 6$. * $P < 0.05$ vs AOO group; # $P < 0.05$ vs TDI group.

2011; Gardella et al., 2002). Secreted or released, HMGB1 is known to participate in the activation of innate cell surface immune receptors, typically the Toll-like receptors (TLR) 2 and TLR4, and the receptor for advanced glycation end products (RAGE), thereby triggering inflammation (Andersson and Tracey, 2011; Gardella et al., 2002; Harris et al., 2012; Sims et al., 2010). HMGB1 stimulates the release of TNF- α , IL-1 β and other inflammatory cytokines from macrophages and mediates acute lung injury and lethality (Andersson et al., 2000). Blocking HMGB1 with either the nonspecific inhibitor EP or specific antagonists, such as neutralizing antibody or A box, significantly decreases the release of proinflammatory mediators such as TNF, IL-1 β , IL-6 and confers protection against sepsis as well as ischemia–reperfusion associated organ injury (Andrassy et al., 2008; Tsung et al., 2005; Ulloa et al., 2002). Our previous study also demonstrated an increased

level of HMGB1 in TDI-induced asthma model, and showed that EP can ameliorate TDI-induced airway inflammation, partly via downregulation of HMGB1 expression (Tang et al., 2014).

In order to further explore roles of HMGB1 in TDI-induced asthma, in this study we originally inhibited its activity with the IgY anti-HMGB1 neutralizing antibody. This selective antagonist has been justified to be safe and effective in other disease models (Hamada et al., 2008; Herzog et al., 2014; Takamiya et al., 2009). Despite different means of airway challenge, pulmonary expression of HMGB1 was still upregulated in TDI-inhaled mice, accompanied by similar asthma manifestations. We anticipated that the IgY anti-HMGB1-treated mice would demonstrate ameliorated asthma after inhalation of TDI. However, we observed aggravated airway hypersensitivity, worsened bronchial epithelial hyperplasia and shedding, heavier accumulation of neutrophils

and eosinophils into the airway, and higher levels of IL-4, IL-5, IL-13, IL-17A and TNF- α in IgY anti-HMGB1-treated mice, especially for those treated with IgY. These are in striking contrasts to the results of aforementioned studies. As HMGB1 is an identified proinflammatory mediator, and in this study the antibody-treated mice did exhibit suppressed expression of HMGB1, decreased airway hyperreactivity, inhibited infiltration of granulocytes and lower levels of IL-4 compared with IgY group which partly supports the pathogenic role of HMGB1 in TDI asthma, here we inferred that it is IgY, rather than anti-HMGB1, that promotes the production of proinflammatory mediators and therefore exacerbates asthma responses in this model.

IgY is the principal antibody present in the serum and egg yolk of amphibians, reptiles, and birds, and shares a common ancestor with both mammalian IgG and IgE (Flajnik, 2002; Warr et al., 1995). Similar to IgG, it is the major defence mechanism against systemic infections (Qureshi et al., 2000). In the past decades, passive immunization using chicken egg yolk immunoglobulin (IgY) to produce antibiotics, antivirals or antitoxins for the treatment and control of infectious or noninfectious disorders in both animals and humans has become an attractive alternative with considerable attention as it possesses a variety of advantages over mammalian IgG such as convenience, high yield, cost-effectiveness and low reactivity with human Fc receptors, which should result in a lesser chance of severe adverse reactions (Diraviyam et al., 2014; Larsson et al., 1993; Rahman et al., 2012). Despite all its merits, researchers have also proposed the potential harm of IgY. More than 40 years ago, studies revealed that IgY possesses a similar ability of IgE to mediate cutaneous anaphylactic reactions (Faith and Clem, 1973; Grey, 1967). Later, it was found to activate chicken mast cells to drive local anaphylaxis in the gut (Rose et al., 1980). Antibody-dependent hypersensitivity and fatal systemic anaphylactic shock have also been validated in chickens (Bellavia et al., 1992; Chand and Eyre, 1976; Faith and Clem, 1973). However, all these observations were seen only in birds; whether IgY could also bring about similar responses in mammals remains inconclusive. In 2012, Sevcik et al. demonstrated an interaction of IgY with the murine lymphocyte plasma membrane in vivo using 3-D deconvolution fluorescent microscopy. The interaction did not result in endocytosis of the labeled protein, suggesting the potential capacity of triggering adaptive immunity against IgY in mammals (Sevcik et al., 2012). At the same time, Polanowski and coworkers found that IgY isolated from hen egg yolk could induce the release of TNF- α and IL-1 β in a murine macrophage-like cell line and human whole blood cell cultures, indicating strong cytokine-inducing activity of IgY in animals (Polanowski et al., 2012). In this study, it is worth noting that TDI-inhaled mice treated with IgY displayed the highest levels of IL-4, IL-5, IL-13, and TNF- α in supernatants of cultured lymphocytes in all enrolled groups, while IL-1 β was not detected. It has been well established that TNF- α is a major initiator and propagator of airway inflammation and hyperresponsiveness in TDI-induced asthma. TNF- α deficiency also markedly abrogates TDI-induced Th2 cytokine release in airway tissues (Matheson et al., 2002), indicating its role in the development of Th2 responses. As such, we hypothesized that IgY may exacerbate TDI-induced asthma by promoting the release of TNF- α .

To further evaluate the effect of IgY on TNF- α , primary lymphocytes from TDI-inhaled mice and control mice were prepared and then stimulated with chicken IgY or control PBS. We found no change of TNF- α after treatment with IgY in both groups of cultured lymphocytes. Instead, a surprising increase of IL-4 was seen when lymphocytes from TDI asthmatic mice were stimulated by 50 μ g IgY. Yet none of these cytokines was detected in cell supernatants from control mice. Another independent group of mice were exposed to TDI or AOO and injected with IgY to

confirm the vicious effect of IgY. Though administration with IgY resulted in elevation of TNF- α and IL-4 in AOO-exposed mice, it was a mild increase and not strong enough to elicit visible hypersensitivity nor inflammation of the airway. Similarly, we also found that IgY had no effect on airway inflammation in non-sensitized mice that inhaled TDI (data not shown). For mice sensitized and challenged with TDI, we observed the same results before and after IgY treatment. Hence, we concluded that IgY can potentiate IL-4 release in TDI-induced asthma model, which could also account for the discordance between our in vivo results and other reports in nonallergic disease models (Hamada et al., 2008; Herzog et al., 2014; Takamiya et al., 2009) using the same neutralizing antibody.

The importance of IL-4 in the generation of allergic inflammation has long been ascertained. IL-4-deficient mice exposed to ovalbumin displayed ameliorated bronchial hyperresponsiveness and airway morphology (Brusselle et al., 1995). Moreover, inhalation of IL-4 stimulated airway eosinophilia, AHR, and goblet cell hyperplasia, all of which can be ablated by blocking the IL-4 receptor (Perkins et al., 2006). For these reasons, our current finding that IgY can augment the release of IL-4 presents valid evidence for the unexpected observations in IgY anti-HMGB1 antibody-treated mice, and could account for the inconsistency with the OVA-induced mouse asthma model in which cases the anti-HMGB1 antibodies used were both IgG isotypes (Lee et al., 2013; Shim et al., 2012). To further explore the mechanism involved in IgY-potentiated IL-4 release induced by TDI, we assessed pulmonary expression of phospho-ERK, which is critical in the regulation of IL-4 release in OVA-induced asthma (Duan et al., 2004). In this study, after administration of IgY in TDI-exposed mice, protein levels of phospho-ERK was increased, indicating that IgY may promote IL-4 release through the ERK pathway. In contrast, a recent study by Xu et al. using an allergic asthma model of guinea pigs demonstrated therapeutical effectiveness of anti-IL-1 β IgY (Wei-xu et al., 2014). This could be explained in part by the notion that the potential detrimental responses of IgY are dampened by the potent protection of anti-IL-1 β against allergic inflammation.

A second explanation for the IgY response is the IgE-like structure of IgY (Taylor et al., 2008). Though the role of IgE in TDI-induced asthma has not been fully elucidated, it is well documented that IgE plays a central role in induction and maintenance of allergic airway inflammation and asthma-related symptoms (Holgate et al., 2005). Some have attributed the most crucial function of IgE in allergic diseases to its ability to sensitize mast cells to release biologically active mediators in an antigen-specific manner (Galli and Tsai, 2012). Intriguingly, previous studies have shown that IgY can also activate local mast cells and drive anaphylactic responses in chicken (Rose et al., 1980). As existing evidence only suggests interactions of IgY with murine lymphocytes and erythrocytes (Sevcik et al., 2012), further studies are required to verify whether IgY could cross-react with mammalian mast cells.

One additional explanation for the IgY response could be related to its possible interaction with TDI. As a low molecular weight compound, TDI is considered a hapten and should bind with proteins to form functional antigens (Tarlo and Lemiere, 2014). The injected IgY may interact with TDI and act as an adjuvant, thereby amplifying subsequent inflammatory responses. These points need to be clarified in future studies.

5. Conclusions

In summary, we found that chicken IgY anti-HMGB1 neutralizing antibody can facilitate TDI-induced allergic

inflammation, wherein IgY aggravates the asthmatic reaction and anti-HMGB1 inhibits the effects of IgY, shedding light on an underappreciated role of avian IgY.

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This study was funded by National Natural Science Foundation of China (81270089, 81270087, 81300029, 81470228), National Program on Key Basic Research (973 Program, 2012CB518203), Special Project on the Integration of Industry, Education and Research of Guangdong Province (2012B091100153), and Science and Technology Program of Guangdong Province (2011B031800245).

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