



Maternal exposure to NO₂ enhances airway sensitivity to allergens in BALB/c mice through the JAK-STAT6 pathway

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HIGHLIGHTS

- Maternal NO₂ exposure enhances OVA-IgE and AHR in offspring to allergens.
- Maternal NO₂ exposure disrupts the balance of Th1/Th2 in offspring to allergens.
- Maternal NO₂ exposure activates the JAK-STAT6 pathway in offspring to allergens.

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ABSTRACT

Previous studies have indicated that nitrogen dioxide (NO₂) exposure could increase airway sensitivity to allergens for children. Recently, fetal stress was proposed as a crucial factor for allergic airway response occurring in offspring. Considering that there is inadequate evidence linking maternal NO₂ exposure to offspring airway sensitivity to allergens, pregnant Balb/c mice were exposed daily to 2.5 ppm NO₂ throughout the gestation period; then, the offspring were challenged to an allergen (ovalbumin, OVA) to evaluate airway sensitivity. For air + saline group and air + OVA group, offspring mice were maternally exposed to clean air followed by treatment with saline and OVA, respectively, in adulthood. For NO₂ + saline group and NO₂ + OVA group, offspring mice were maternally exposed to NO₂ followed by treatment with saline and OVA, respectively, in adulthood. The results showed that maternal NO₂ exposure increased the level of OVA-immunoglobulin (Ig) E in serum and caused airway hyper-responsiveness and pathological changes in offspring. Furthermore, maternal NO₂ exposure altered the expression of pro-inflammatory factors and impaired the T helper (Th) 1/Th2 balance. In addition, janus kinase-signal transducer and activator of transcription 6 pathway participated in OVA-induced airway sensitivity of offspring. Our study showed that the potential risk of airway sensitivity to allergens in offspring is enhanced by maternal NO₂ exposure and proposed a possible mechanism for preventing, alleviating, and evaluating the outcomes in polluted environments.

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1 Introduction¹

In China, the concentration of nitrogen dioxide (NO₂), a ubiquitous air pollutant, is considerably increasing, thus exhibiting an accelerating trend in annual growth rate (Kuenen, 2006; Lamsal

et al., 2013). Outdoors, coal burning, and exhaust emissions are the main sources of ambient NO₂. Indoors, the use of coal (or gas) in fire stoves and smoking contribute to personal NO₂ exposure. In addition, high NO₂ levels are present in certain occupational exposure sites, including mining, garages, and ferries (Ezratty et al.,

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¹ Abbreviations: NO₂: nitrogen dioxide; FOAD: fetal origins of adult disease; PM: particulate matter; POPs: persistent organic pollutant; RR: relative risk; IgE: immunoglobulin E; Th: T helper; IL: interleukin; IFN-γ: interferon-gamma; OVA: ovalbumin; RT: room temperature; AS: air + saline; AO: air + OVA; NS: NO₂ + saline; NO: NO₂ + OVA; ELISA: enzyme-linked immunosorbent assay; AHR: airway hyper responsiveness; PBS: phosphate buffer saline; SDS: sodium dodecyl sulfonate; PAGE: polyacrylamide gel; JAK: janus kinase; STAT: signal transducer and activator of transcription; GATA: t-cell-specific transcription factor; T-bet: t-box transcription factor; TNF-α: tumor necrosis factors α; PND: postnatal day; MCH: methacholine; H&E: hematoxylin and eosin; R_L: resistance of lung; R_E: resistance of expiration; C_{dyn}: dynamic compliance of respiratory; IL-4Rα: interleukin-4 receptor α.

2014). Monitoring data have shown that NO₂ concentrations along roads with heavy traffic are less than 0.2 ppm (Yin et al., 2010), but many reach 2 ppm indoors or even 4 ppm in occupational exposure sites (WHO, 2003). NO₂ enters the body primarily through inhalation and then penetrates deeply into the lungs because of its hydrophobicity, thus affecting airways, bronchioles, and alveoli. Both long-term and short-term NO₂ exposure can increase the risk for allergic reaction in the respiratory tract by enhancing immune response, thus causing a range of harmful effects, such as increased airway inflammation and reduced lung function (Chen et al., 2007; U.S. EPA, 2008). Previous reports confirmed that even low concentrations of NO₂ could lead to allergic respiratory reactions (Dell et al., 2014; Klumper et al., 2015), particularly in children (Belanger et al., 2013; Kattan et al., 2007; Liu et al., 2014). Subsequent animal studies demonstrated that acute NO₂ exposure enhanced airway inflammation, led to histopathological changes in the lung, and augmented the degree of allergic reaction (Ji et al., 2015; Martin et al., 2013).

Allergic airway disease is characterized by aberrant adaptive immune responses to allergens resulting in airway inflammation, mucus hyper-secretion, and airway hyper-responsiveness (AHR) (Rutledge et al., 2015). In most countries, it has been confirmed that the prevalence of allergic airway diseases has dramatically increased over the past decades (Andiappan et al., 2014). Recent studies have suggested that the increase in allergic airway disease morbidity and mortality may be associated with early life stress, particularly from maternal environmental pollutant exposure. Dr. David Barker was the first to popularize the concept of fetal origins of adult disease (FOAD) (Barker, 1990), which has since received considerable attention. The FOAD hypothesis proposes that events during early life have a profound impact on the risk for the development of future adult diseases. Furthermore, considering that pollutants can reach fetus through the umbilical cord, it is reasonable that respiratory diseases can occur during pregnancy following the initiation of respiratory system development (Vieira, 2015). Among common environmental pollutants, some epidemiological studies have shown that maternal exposure to PM_{2.5} (Jedrychowski et al., 2013), persistent organic pollutants (POPs) (Noakes et al., 2006), and second-hand smoke (Singh et al., 2011) increases the risk for allergic airway diseases in neonates. With regard to NO₂, a birth cohort study found an increased risk of lower respiratory tract infection (relative risk (RR) = 1.05; 95% CI: 0.98–1.12) following a 10-μg/m³ increase in maternal exposure to NO₂ during pregnancy (Aguilera et al., 2013). Allergic airway disease is a chronic immune system disorder, characterized by increases in immunoglobulin (Ig) E levels, unbalanced levels of T helper (Th)-cell cytokines, and decreased lung function (Agrawal and Shao, 2010). Previous studies indicated that in response to inhaled antigens, Th2 or Th17 cytokines (interleukin (IL)-4, IL-13, IL-17, etc.) are released, while Th1 cytokines (interferon-gamma (IFN-γ)) are suppressed (Choy et al., 2011; Unni et al., 2014). The resulting imbalance in cytokines stimulates B cells to synthesize IgE (Barnes, 2008) and, in turn, causes unbalanced Th1/Th2 or Th1/Th17 differentiation, eventually provoking the development of airway allergy (Paul and Zhu, 2010; Akdis et al., 2012). Previous studies were mainly focused on the relationship between direct NO₂ exposure and individual allergic airway diseases. However, experimental studies about the relationship between maternal NO₂ exposure and airway sensitivity to allergens in offspring are lacking, and further investigations on this topic are required. In the present study, we exposed pregnant Balb/c mice to NO₂ followed by postnatal allergic challenge to address (1) whether maternal NO₂ exposure enhances airway sensitivity to allergens in offspring and (2) if so, how maternal NO₂ exposure causes these responses following allergen challenge.

2. Materials and methods

2.1. Animal housing and NO₂ exposure of pregnant mice

Mature male and female Balb/c mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and maintained on a 12-h light/dark cycle in a temperature-controlled (22–24 °C), moderate-humidity (40% ± 5%) room with unrestricted access to ovalbumin (OVA)-free food and water. Female mice were housed overnight with male mice (male: female = 1:2) during the proper period for fertilization. The following day, plug-positive mice were considered pregnant, with the day on which a vaginal plug was observed being defined as gestation day 0 (GD0). Pregnant mice were exposed to NO₂ (2.5 ppm, 5 h/day) monitored using a gas detector (FIX550-NO2-A, WANDI Technology co., Wuhan, China) or untreated air throughout gestation (approximately 21 days). Each female mouse was fed separately in wire cages until weaning of offspring. All the animal protocols were approved by the Shanxi University's Institutional Animal Care and Use Committee (Approved Animal Use Protocol Number: HZZ20140503).

2.2. OVA challenge

OVA challenge was performed when offspring were 7 weeks old. According to a previous study's description (Penn et al., 2007) and manufacturer's instructions, mice were placed in a plastic box (Bestlab Technology co., Beijing, China) and challenged to sterilized 0.9% (wt/vol) saline aerosol or 1% OVA (Grade V > 98% pure; Sigma Chemical Co., St. Louis, USA) in saline (30 min/day, 14 days). An ultrasonic nebulizer (JSC-OK, Anshan City Electronic Medical Instruments co., Anshan, China) was used to maintain a stable flow rate. At 9 weeks of age, mice were sacrificed to determine the effect of maternal NO₂ exposure on OVA-induced airway allergy (Fig. 1). For air + saline (AS) group and air + OVA (AO) group, offspring mice were maternally exposed to clean air followed by treatment with saline and OVA, respectively, in adulthood. For NO₂ + saline (NS) group and NO₂ + OVA (NO) group, offspring mice were maternally exposed to NO₂ followed by treatment with saline and OVA, respectively, in adulthood.

2.3. Measurement of OVA-specific IgE in serum

Blood was collected 24 h after the last OVA aerosol exposure using evacuated tubes (Taizhou Tianyi Medical Devices Co., Ltd, Taizhou, China) and kept at room temperature (RT) for 30 min. The collected blood was centrifuged at 1000 g for 10 min at RT. The supernatant (serum) was stored at –80 °C until analysis. OVA-specific IgE levels in the serum (1:100 diluted) were measured using an enzyme-linked immunosorbent assay (ELISA) reagent kit (Mouse OVA-Specific IgE ELISA Kit with Pre-coated Plates, Bio-Legend Inc., San Diego, CA, USA) according to the manufacturer's instructions.

2.4. Measurement of AHR

Offspring AHR was measured using the AniRes2005 Lung Function System (Version 3.5, Bestlab Technology co., Beijing, China) according to the manufacturer's instructions. Briefly, after anesthetizing mice with 50 mg/kg pentobarbital sodium (Beijing Solarbio Technology Co., Ltd, Beijing, China), the skin of the neck was carefully incised and the trachea was intubated and quickly connected to a small computer-controlled ventilator through the tracheal cannula (You et al., 2014). The time ratio of expiration/inspiration and the respiratory rate were preset to 1.5:1 and 90/

min, respectively. Gradient (0.025, 0.05, 0.1, and 0.2 mg/kg body weight) methacholine (O-acetyl- β -methylcholine chloride, MCH, Sigma, USA) was injected into the jugular vein at 5-min intervals, and the resistance of lung (R_L), resistance of expiration (R_E), and dynamic compliance of respiration (C_{dyn}) were recorded to evaluate the lung function of mice.

2.5. Bronchoalveolar lavage fluid (BALF) collection and differential cell counts

Following narcosis by i.p. injection of pentobarbital sodium (50 mg/kg), lungs were lavaged with 1 mL 0.9% (weight/volume (wt/vol)) warm sterilized saline three times per mouse. After centrifugation (4 °C, 250 g, 10 min), the cell pellet was resuspended in sterilized saline and stained with Wright–Giemsa for differential BALF counts (You et al., 2014; Hao et al., 2015). Numbers of eosinophils (EOS), macrophages (MAC), and total cells were counted.

2.6. Histological examination

A fresh fraction of each lung tissue was quickly extracted, washed with 0.1 M phosphate buffer saline (PBS, pH 7.4), and fixed in 10% buffered formalin overnight at RT. The next day, tissues were embedded in paraffin, and then, 5–6 μ m sections were prepared for staining with hematoxylin and eosin (H&E) to estimate inflammatory cell infiltration and airway structure alteration. Ten random fields per section were observed by light microscopy (Olympus, Japan) at 200 $\times$ magnification (Ji et al., 2015). We used the Image-Pro plus 6 (<http://www.mediacy.com/imageproplus>) for measuring the total airway area (μ m²) and luminal area (μ m²) (Weibel et al., 1963; Said et al., 2007; Leite et al., 2010). Medial area (μ m²) was calculated as the difference between total and luminal areas. The ratio of medial area to total airway area was used to evaluate luminal area changes.

2.7. Measurement of cytokine expression in the lung

Thirty milligrams of each lung tissue was homogenized in 300 μ L of 0.9% sterilized saline. The homogenate was then

centrifuged (4 °C, 250 g, 10 min), and 200 μ L supernatant was used for the measurement of TNF- α , IL-1 β , IFN- γ , IL-4, IL-13, and IL-17 using the corresponding ELISA reagent kit (Westang Biotechnology Co., Shanghai, China) according to the manufacturer's instructions. Furthermore, protein (in microgram) present in each sample was measured by the Bradford assay (Bradford, 1976). Cytokine levels in each sample were normalized to the protein content, as described previously (Wen et al., 2006).

2.8. Western blotting

Fifty milligrams lung tissue of each lung was homogenized in 400 μ L ice-cold lysis buffer, and the concentrations of the protein extracts were quantified, as described previously (Sang et al., 2011). Then, the same amounts of protein obtained from each sample were electrophoresed on sodium dodecyl sulfonate (SDS)-polyacrylamide gel (PAGE), transferred onto nitrocellulose membranes, and blocked with 3% bovine serum albumin for 1.5 h. After that, the blocked membranes were incubated with primary antibodies (1:200) for janus kinase (JAK) 1, signal transducer and activator of transcription (STAT) 6, p-STAT, T-box transcription factor (T-bet), and T-cell-specific transcription factor (GATA) 3 (Bioss, Beijing Biosynthesis Biotechnology co., LTD., Beijing, China) at 4 °C overnight. Following three washes, membranes were incubated with secondary antibody (1:5000) at RT for 2 h. Finally, the membranes were scanned and detected using the LI-COR Odyssey Infrared Fluorescent System (LiCor Biosciences Inc., NE, USA).

2.9. Statistical analysis

Data are expressed as mean $\pm$ standard error. One-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test were used to determine significant differences for comparisons of all the groups to a control group. A significant difference was defined as $p < 0.05$. Data analysis and figure generation were conducted using Origin 8.0.

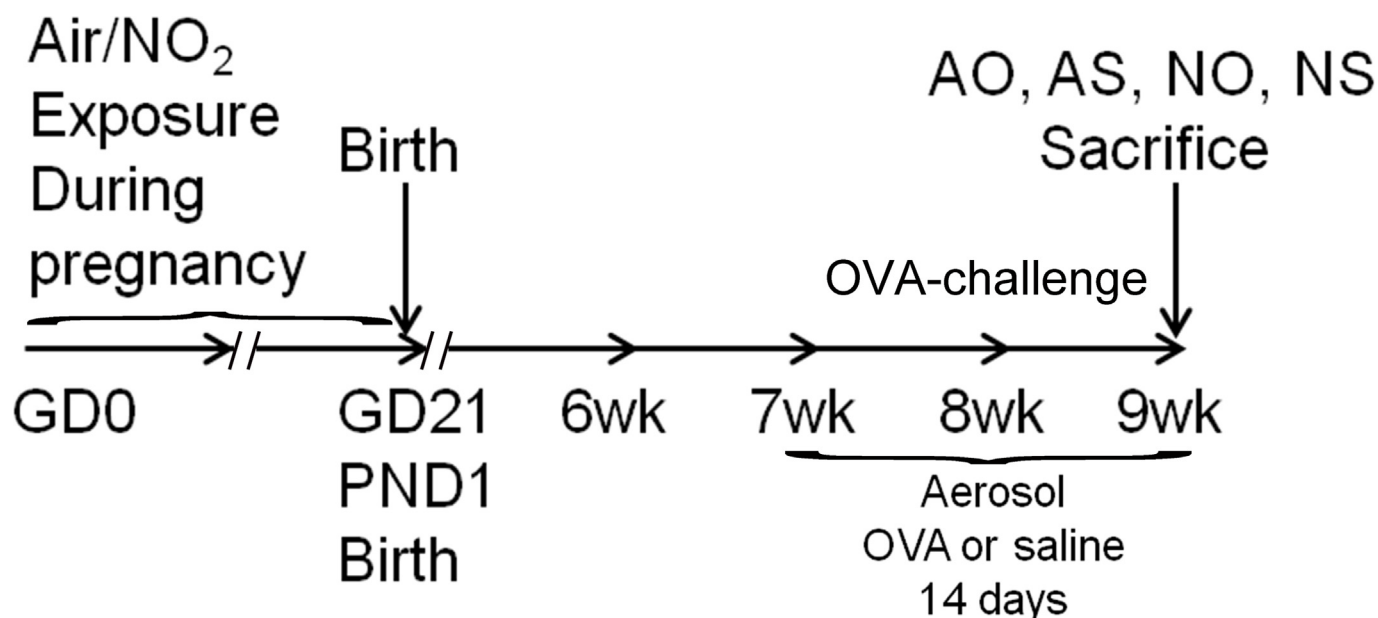


Fig. 1. Prenatal exposure and postnatal treatments and sacrificing showing time line. Gestation day: GD; postnatal day: PND; wk: week.

3. Results

In the present study, we examined the effects of maternal NO₂ exposure on offspring lungs. There were no significant differences in delivery rate or litter size between NO₂ and control air dams. There were 76 offspring from 11 litters in control groups and 71 offspring from 10 litters in NO₂ groups. None of the offspring died.

3.1. Maternal exposure to NO₂ enhances OVA-IgE level and AHR in OVA-challenged offspring

To address whether maternal exposure to NO₂ increases sensitivity to allergens, we exposed pregnant Balb/c mice to NO₂ followed by postnatal allergic challenge. Fig. 2 shows that OVA challenge significantly increased OVA-IgE level subsequent to maternal NO₂ exposure compared to control dams, with the level in the NO group being 2.58-fold ($p < 0.01$) higher than that in the AO group. These findings suggest that maternal NO₂ exposure induces a more sensitive reaction to allergens in offspring.

Furthermore, as shown in Fig. 3, offspring AHR was enhanced by OVA challenge. R_L was significantly increased with higher level of MCH treatment in OVA groups compared to that in saline groups (AO vs. AS, NO vs. NS, respectively). Similarly, R_L was also significantly increased in the NO group vs. the AO group at 0.2 mg/kg MCH ($p < 0.05$). In addition, R_e in OVA groups was higher than that

in saline groups (AO vs. AS, NO vs. NS, respectively), while R_e measurements were not significantly different between NO and AO groups. We observed that decreased Cdyn levels were significantly decreased between NS and NO groups and between NO and AO groups at higher levels of MCH treatment. As shown by histology in Fig. 4A and B, OVA challenge induced significant remodeling of the bronchial airway wall and increased secretion of mucus and inflammatory cell infiltration compared with those in saline groups (AO vs. AS, NO vs. NS, respectively). Furthermore, histopathological changes observed in the NO group were more serious than those in the AO group. Cell count result (Fig. 4C) showed that EOS, MAC, and total cell numbers in OVA groups were significantly higher than those in the saline groups (AO vs. AS, NO vs. NS, respectively), and EOS, MAC, and total cell numbers in the NO group were 2.35-, 1.29-, and 1.48-fold higher than those in the AO group (EOS, $p < 0.05$; MAC, $p < 0.05$; total cell, $p < 0.01$), respectively. These findings imply that maternal NO₂ exposure promotes OVA-induced airway remodeling and inflammation, thereby exacerbating AHR in offspring upon initial allergen exposure.

3.2. Maternal exposure to NO₂ leads to abnormal Th1/Th2 differentiation in offspring

After OVA aerosol exposure ended, we measured airway allergy-related cytokines, including pro-inflammatory cytokines TNF- α and

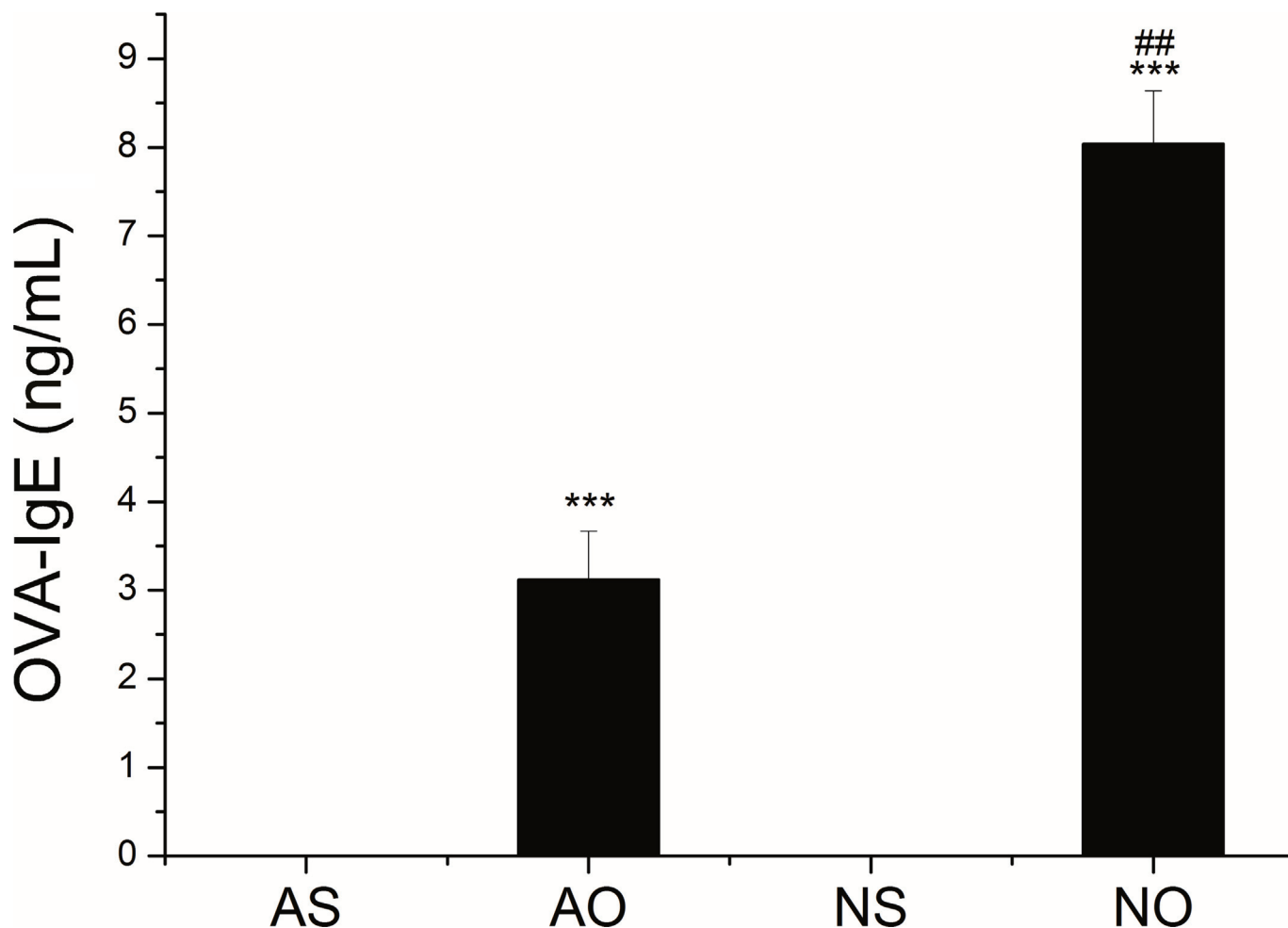


Fig. 2. Effect of maternal NO₂ exposure on OVA-special IgE in serum of offspring. The vertical axis shows the levels of OVA-special IgE concentration of mice model of allergic disease (for AO group, $n = 6$; for AS group, $n = 6$; for NO group, $n = 6$; and for NS group, $n = 7$). Values are recorded as the mean $\pm$ SE. *** $p < 0.001$, compared with saline groups, respectively; ## $p < 0.01$, compared with AO group.

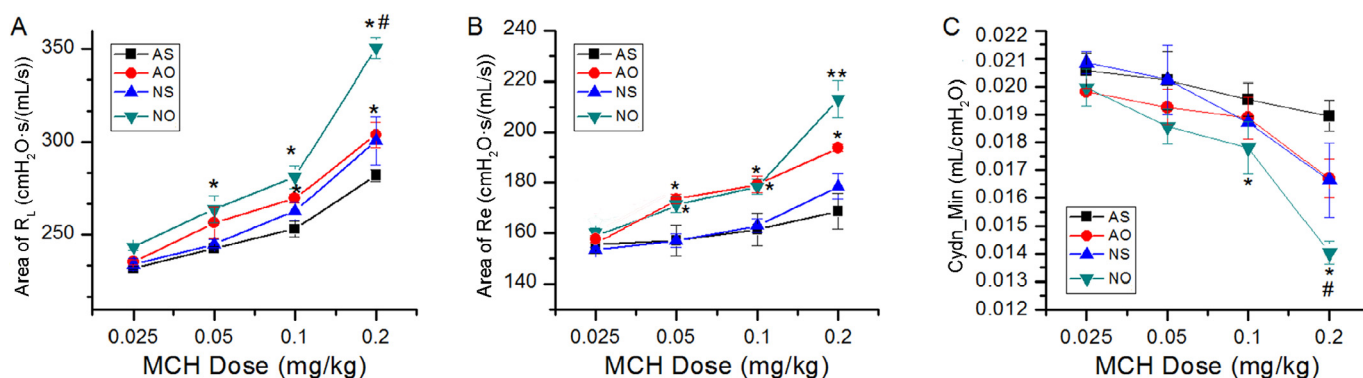


Fig. 3. Effect of maternal NO₂ exposure on AHR of offspring. The vertical axis (A: area of R_L ; B: area of R_e ; C: Cdyn_Min) shows AHR of mice model of allergic disease (for AO group, $n = 10$; for AS group, $n = 9$; for NO group, $n = 11$; and for NS group, $n = 9$). Values are recorded as the mean $\pm$ SE. * $p < 0.05$, ** $p < 0.01$, compared with saline groups, respectively; # $p < 0.05$, compared with AO group.

IL-1 β , Th1 cytokine IFN- γ , Th2 cytokines IL-4 and IL-13, and Th17 cytokine IL-17. As shown in Fig. 5A, TNF- α levels increased significantly in OVA groups compared to those in saline groups (AO vs. AS, NO vs. NS, respectively), and maternal NO₂ exposure enhanced this response (NO group vs. AO group). IL-1 β displayed a similar trend to TNF- α (Fig. 5B). With regard to Th1 cytokines (Fig. 5C), IFN- γ expression in OVA groups was suppressed compared to that in saline groups (AO vs. AS, NO vs. NS, respectively), and the IFN- γ content in the NO group was only 74.6% of that in the AO group ($p < 0.05$). As shown in Fig. 5D and E, IL-4 levels in the NO group were significantly higher than those in the NS group; however, no significant changes were observed between AO and AS groups or between NO and AO groups. OVA challenge enhanced IL-13 expression in OVA groups compared to that in saline groups (AO vs. AS, NO vs. NS, respectively), with an obvious increase observed between NO and AO groups ($p < 0.05$). IFN- γ /IL-4 ratios (Fig. 5F) in AO and NO groups were significantly higher than those in AS and NS groups, respectively, and the ratio in NO group was 1.41-fold higher than that in AO group ($p < 0.05$). Concerning Th-17 cytokines (Fig. 5G), no significant difference was found between any two groups. Thus, maternal NO₂ exposure enhanced Th2 cytokine rather than Th17 cytokine expression in offspring.

As shown in Fig. 5H, OVA challenge significantly suppressed T-bet expression in OVA groups compared to that in saline groups (AO vs. AS, NO vs. NS); T-bet level in the NO group decreased 24.9% compared to the AO group ($p < 0.05$). GATA-3 was upregulated in OVA groups compared to that in saline groups (AO vs. AS, NO vs. NS) (Fig. 5I), and GATA-3 in the NO group was 1.37-fold higher than that in the AO group ($p < 0.05$).

3.3. Maternal exposure to NO₂ activates the biomarkers for the JAK-STAT pathway

We investigated whether the JAK-STAT pathway was involved in the mechanism of abnormal Th1/Th2 differentiation in offspring to OVA allergen following maternal exposure to NO₂. To accomplish this, we further measured the expression of JAK-STAT-related proteins in the lung. As shown in Fig. 6, JAK-1 and p-STAT6 were significantly increased in OVA groups compared to those in saline groups (AO vs. AS, NO vs. NS). Furthermore, JAK-1 increased 1.28-fold in the NO group vs. the AO group ($p < 0.05$); p-STAT6 increased 1.20-fold in the NO group vs. the AO group ($p < 0.05$).

4. Discussion

Over the past decades, the prevalence of childhood airway

allergic diseases has significantly increased, the cause of which is believed to include both changes in dietary habits and residential environments (Okada et al., 2010). In addition to exposure during childhood and adulthood, epidemiological studies have shown that fetal stress during pregnancy can lead to impaired lung function and increase the risk of developing allergic airway diseases in later life (Penn et al., 2007; Prescott, 2010; Wang and Pinkerton, 2008). However, there are few equivalent experimental studies that investigate the effect of maternal NO₂ exposure on subsequent postnatal airway sensitivity to allergens in offspring.

OVA is a commonly employed allergen to establish animal models and induces robust, allergic pulmonary inflammation in laboratory rodents (Nials and Uddin, 2008). In the present study, pregnant female mice were exposed to 2.5 ppm NO₂ or control air throughout gestation, and subsequently, 7-week-old offspring were exposed to aerosol OVA or saline for 2 weeks to evaluate airway sensitivity to that allergen. Previous studies indicate that increase in IgE levels may cause various immune effector cells to release a variety of mediators promoting AHR (Fahy, 2000), which is associated with histopathologic changes of lung (Al-Muhsen et al., 2011). As expected, significant changes in airway allergic symptoms, such as increases in OVA-specific IgE, AHR, and appearance of histopathology occurred. Compared to AHR and OVA-specific IgE in AO mice, those in NO mice were significantly increased, demonstrating NO₂-dependent enhancement of AHR and OVA-specific IgE upon initial allergen exposure. However, in the absence of subsequent OVA stimulation, maternal NO₂ exposure did not alter basic lung histology or lung function as indicated by the lack of differences between NO₂ and control air mice (NS vs. AS). Together, our results suggest that maternal NO₂ exposure likely enhances airway sensitivity to allergens in offspring, similarly to the results observed after maternal exposure to environmental tobacco smoke and nicotine, which also significantly augmented AHR and allergen-IgE (Wongtrakool et al., 2012).

Cytokines are important effectors involved in the immune reaction of offspring (Nesin and Cunningham-Rundles, 2000). Similar to AHR, cytokines released from Th cells are also important biomarkers that indicate allergic airway diseases (Yang et al., 2014). In general, the mechanism of allergic airway disease includes the imbalanced differentiation of Th cells, which can secrete different cytokines that aggravate the airway allergy. The Th2 subtype is characterized by the secretion of IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 (Romagnani, 2000). A body of animal studies supports the hypothesis that Th2 cytokines such as IL-4 and IL-13 induce airway remodeling (Atamas et al., 2013; Leigh et al., 2004). Conversely, IFN- γ produced by Th1 cells plays an important role in suppressing the

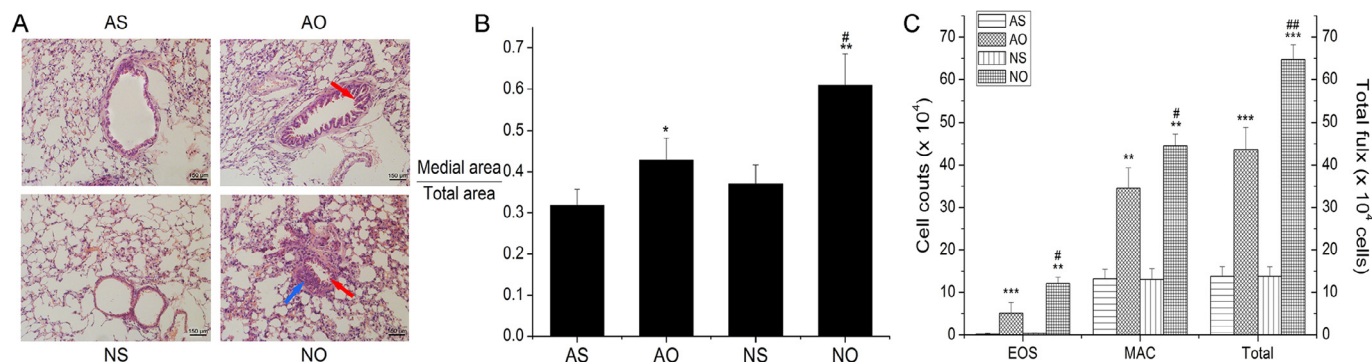


Fig. 4. Effect of maternal NO_2 exposure on histopathologic changes and differential cell counts of offspring. (A) Typical histopathologic images of different groups. Scale bar = 150 μm . Red arrows represent airway remodeling, and blue arrows represent inflammatory cell infiltration. (B) Luminal area in bronchial airways in lungs of offspring. The vertical axis shows the ratio of medial area to total airway area in lungs in offspring (for AO group, $n = 3$; for AS group, $n = 3$; for NO group, $n = 3$; and for NS group, $n = 3$). (C) Differential cell counts in lungs of different groups. Values are recorded as the mean $\pm$ SE. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with saline groups, respectively; # $p < 0.05$, ## $p < 0.01$, compared with AO group.

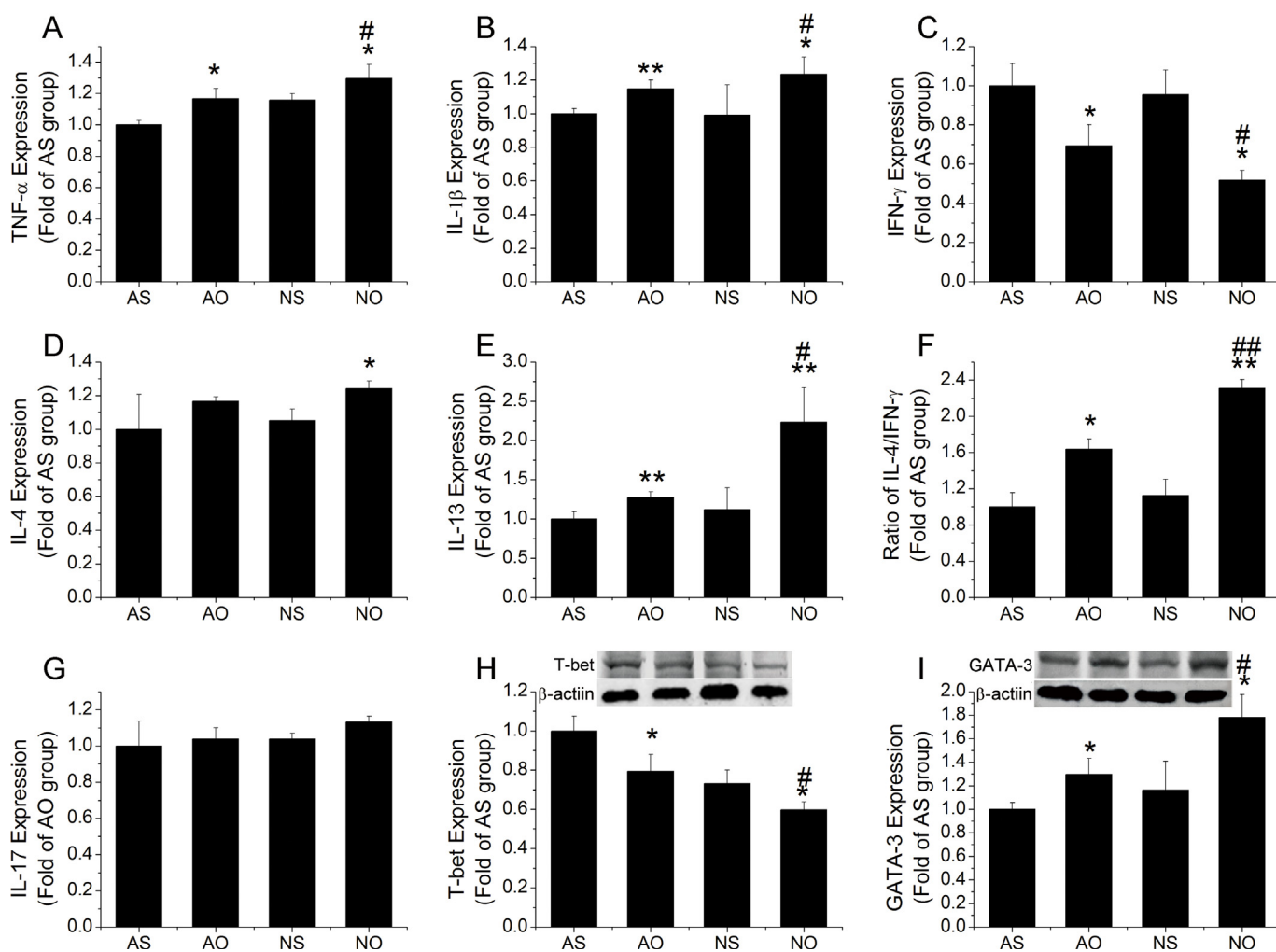


Fig. 5. Effect of maternal NO_2 exposure on cytokine level of TNF- α (A), IL-1 β (B), IFN- γ (C), IL-4 (D), IL-13 (E), IL-4/IFN- γ ratio (F), and IL-17 (G), and protein expression of T-bet (H) and GATA-3 (I) in lungs of offspring. The vertical axis shows the levels of cytokine or protein concentration in lungs (for AO group, $n = 7$; for AS group, $n = 8$; for NO group, $n = 9$; and for NS group, $n = 9$). Values are recorded as the mean $\pm$ SE. * $p < 0.05$, ** $p < 0.01$, compared with saline groups, respectively; # $p < 0.05$, ## $p < 0.01$, compared with AO group.

proliferation of Th2 cells *in vitro* and antagonizes the *in vivo* Th2-type responses, such as IL-4-dependent IgE antibody production (Fernandez-Botran et al., 1988; Gajewski and Fitch, 1988). Thus, the IFN- γ /IL-4 ratio reflects the Th1/Th2 differentiation ratio. In

addition to the IFN- γ /IL-4 ratio reflecting the imbalance of Th1/Th2 differentiation, the balance between T-bet and GATA-3 expression also indicates the balance between Th1 and Th2 cytokines (Chakir et al., 2003; Ji et al., 2015). In our study, maternal NO_2 exposure

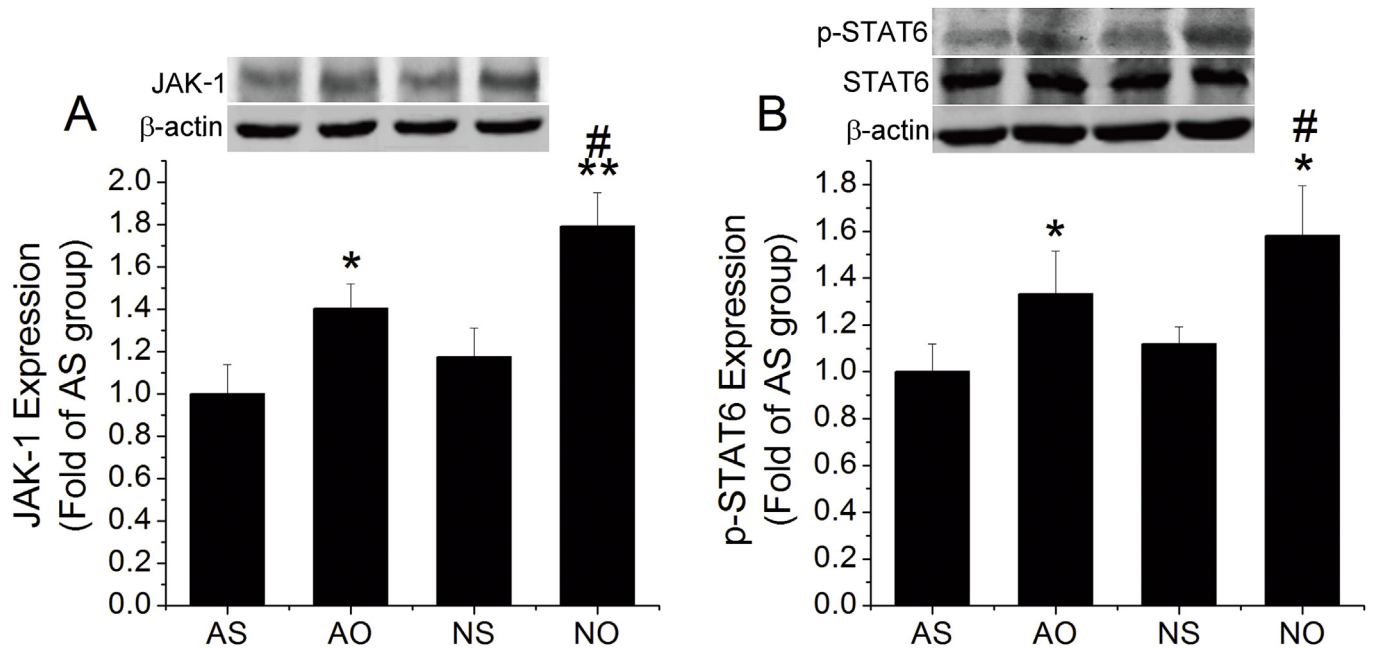


Fig. 6. Effect of maternal NO₂ exposure on JAK-STAT6 pathway. Protein expression of JAK-1 (A) and p-STAT6 (B) were measured (for AO group, $n = 7$; for AS group, $n = 8$; for NO group, $n = 9$; and for NS group, $n = 9$). Values are recorded as the mean $\pm$ SE. * $p < 0.05$, ** $p < 0.01$, compared with saline groups, respectively; # $p < 0.05$, compared with AO group.

significantly enhanced the release of IL-4, IL-13, and GATA3 and suppressed the release of IFN- γ and T-bet, thus disrupting the balance of respiratory immune responses in adult offspring after challenge with OVA. Recently, Th17 cells, a Th-cell lineage distinct from Th1 and Th2 cells, have also been found to participate in the development of airway allergic symptoms in animals. However, our study indicated that the release of IL-17 was not enhanced after maternal NO₂ exposure and OVA challenge. Additionally, we observed some pro-inflammatory factors (TNF- α and IL-1 β , for example) participating in the allergic process (Besnard et al., 2012; Nakae et al., 2007). These data strongly indicate that maternal exposure to NO₂ enhances the airway sensitivity to allergens predominantly through the induction of Th2 differentiation. Although there are no equivalent experimental airway allergy studies with which these responses can be directly compared, a similar study has indicated that maternal exposure to diesel exhaust pollutants increase serum OVA-specific IgE levels, airway inflammation, AHR, and Th2 and Th17 cytokines in offspring (Manners et al., 2014).

Several studies have focused on STAT-6, given its involvement in directing Th2 responses and IgE production (Darcan-Nicolaisen et al., 2009; Li et al., 2012). With regard to JAK, the activation of any of the four JAK family members induces the phosphorylation of one or more of seven STAT family members, thus leading to their dimerization and translocation into the nucleus, where they activate gene transcription (Morales et al., 2010). As one of the important pathways participating in allergic airway diseases, several groups have examined the role of JAK-STAT6 signaling in murine models of pulmonary inflammation and AHR (Matsunaga et al., 2011; Morales et al., 2010). Briefly, binding of IL-4 and IL-13 to their receptor interleukin-4 receptor α (IL-4R α) induces the upregulation of JAK-1/JAK-3, thereby leading to the phosphorylation of STAT6 (Morlacchi et al., 2014; Walford and Doherty, 2013). Then, p-STAT6 upregulates GATA-3 expression and activates the expression of Th2-specific genes, such as IL-4 and IL-13, and suppresses the expression of Th1-specific genes, such as IFN- γ and T-bet, culminating in imbalanced Th1/Th2 differentiation (Vale-Pereira et al., 2011). Furthermore, previous studies have suggested that

airway allergies could be induced by the disruption of the balance of Th1/Th2 differentiation and activation of the JAK-STAT pathway (Chapoval et al., 2010). Additional studies have also demonstrated that prenatal air pollution promotes Th2 polarization through the Th2-polarization pathway, GATA-3/STAT-6 (Singh et al., 2011). In our study, maternal exposure to NO₂ upregulated the expression of JAK-1 and led to the phosphorylation of STAT-6, which induced the expression of the Th2 master regulator GATA-3 and Th2 signature cytokines.

5. Conclusion

Taken together, our findings indicate that maternal NO₂ exposure promotes symptoms of allergic airway sensitivity to allergens, such as OVA-IgE, AHR, and pathological changes. In addition, these actions are likely attributed to JAK-STAT6-associated abnormal Th1/Th2 differentiation. These data imply that maternal exposure to NO₂ decreases the threshold for induction of airway allergy symptoms in response to allergens.

Conflicts of interest

We have no conflicts of interests to declare.

Acknowledgments

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