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Prenatal O_3 exposure increases the severity of OVA-induced asthma in offspring

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ABSTRACT

Background: Accumulating epidemiological studies showed that prenatal and early life exposure to ambient air pollution was important contributor to the development of childhood asthma. However, the effects and mechanisms of prenatal exposure to ozone (O_3), a type of ambient air pollution, on the progression of asthma in offspring remain unclear.

Objective: This study aimed to determine the effects and mechanism of asthma in offspring after prenatal O_3 exposure.

Methods: Pregnant BALB/c mice were exposed to O_3 or air on gestational days (GDs) 13–18. Their offspring were sensitized and challenged to ovalbumin (OVA) to establish asthma model, and the asthma features were evaluated. The splenic natural killer (NK) cells in the offspring were measured to explore the mechanism on the effects of asthma in the offspring. The responses of the pregnant mice and dams after O_3 exposure were evaluated.

Results: Airway inflammation, mucus secretion, OVA-specific immunoglobulin (Ig) E, T helper (Th) 2-skewed response, the frequency of $CD3\varepsilon^-CD49b^+$ splenic NK cells, the expression of tumor necrosis factor (TNF)-α, and IL (interleukin)-17 were significantly exacerbated in the OVA-induced asthma offspring after prenatal O_3 exposure. In addition, airway inflammation, a lower number of $CD3\varepsilon^-CD49b^+$ splenic NK cells, and systemic oxidative stress were caused at the end of pregnancy after O_3 exposure, which did not recover at the end of lactation for the first two responses.

Conclusions: Prenatal O_3 exposure increased the severity of OVA-induced asthma in the offspring, which might be directly induced by $CD3\varepsilon^-CD49b^+$ splenic NK cells in the offspring and indirectly related to the damaged maternal immune system.

1. Introduction

As the largest developing country in the world, China may have the worst air pollution (Kan et al., 2009; Song et al., 2017). With the rapid development of the economy, the number of motor vehicles in China has increased dramatically in recent decades, and air pollution has gradually changed from the conventional coal combustion type to the mixed coal combustion/motor vehicle emission type (Kan et al., 2009). Nitrogen oxides (NOX), volatile organic compounds (VOCs), and other primary air pollutants that were produced by fossil fuel combustion, which generated ozone (O_3) through atmospheric photochemistry. As a pivotal component of the photochemical ambient air pollution mixture, tropospheric O_3 is commonly considered one of the most prevalent air pollutants (Bell et al., 2004). Long-term O_3 exposure is significantly

Abbreviations: O_3, ozone; GD, gestational day; OVA, ovalbumin; NK, natural killer; IgE, immunoglobulin E; Th2, T helper cell; IFN, interferon; TNF, tumor necrosis factor; IL, interleukin; NOX, nitrogen oxides; VOCs, volatile organic compounds; HR, hazard ratio; AHR, airway hyperreactivity; PM, particulate matter; SPF, specific pathogen free; PBS, phosphate-buffered saline; PND, postnatal day; BALF, bronchoalveolar lavage fluid; WBC, white blood cells; ELISA, enzyme-linked immunosorbent assay; 8-IP, 8-isoprostanes; RBC, red blood cell; H&E, hematoxylin and eosin; PAS, periodic acid-schiff; Pbm, perimeter of the bronchial basement membrane; BSA, bovine serum albumin; DAB, 3, 3-diaminobenzidine; AOD, average optical density; NO_2, nitrogen dioxide; DCs, dendritic cells

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associated with the risk of death, especially for cardiovascular (Day et al., 2017) and respiratory diseases (Malley et al., 2017). More importantly, O_3 was the sole air pollutant considered as a risk factor in the Global Burden of Disease Study because of its independent influence on mortality due to respiratory diseases in the American Cancer Society Cohort Study (Turner et al., 2016). In 2016, the number of deaths attributable to O_3 pollution worldwide reached 1.04 million, and in China, it accounted for 28.0% of the deaths from respiratory diseases (Malley et al., 2017). A prospective cohort study in the US also showed that O_3 exposure was notably associated with the risk of death from respiratory diseases (hazard ratio (HR) = 1.04; 95%CI: 1.00–1.09), and that the risk of death from respiratory diseases attributable to O_3 exposure increased significantly in high-temperature areas (Lim et al., 2019). A large number of epidemiological studies have confirmed that O_3 exposure caused respiratory inflammation and even asthma in humans, especially in sensitive people (Niu et al., 2018) and children (McConnell et al., 2002). Subsequent animal model studies proved that O_3 exposure caused airway hyperreactivity (AHR) (Larsen et al., 2010), airway inflammation, and histopathological changes in mice sensitized with ovalbumin (OVA) (Funabashi et al., 2004).

Allergic asthma is one of the most common public health problems. More than 300 million people suffer from allergic asthma worldwide, resulting in a substantial disease burden (Masoli et al., 2004). It is characterized by AHR, bronchial inflammation, T helper (Th) 2 cytokine skewing response, immunoglobulin (Ig) E synthesis, mucus hypersecretion and even airway remodeling (Cohn et al., 2004). Additionally, the potential role of Th17 response in some asthma models is also emphasized (Brandt et al., 2013). The incidence of asthma has dramatically risen over the past few decades and currently affects one in ten children (Braman, 2006). Although genetic factors contribute to susceptibility and development of asthma, the increase in asthma prevalence is too rapid to be ascribed simply to genetic factors. Environmental factors, especially ambient air pollution, may contribute to the incidence and development of asthma. Epidemiological studies suggested that exposure to O_3 in early life was significantly and positively associated with pulmonary function development (Hwang et al., 2015) and an increased risk of asthma hospital admissions (Moore et al., 2008). However, those studies were mainly focused on the O_3 exposure to asthma in exposed individuals. Fewer studies have explored the association between prenatal O_3 exposure to the development of offspring asthma. Herein, we investigated whether prenatal O_3 exposure was associated with an increased risk of exacerbation in offspring asthma using a model of OVA allergen sensitization and challenge and explored the underlying mechanism.

2. Materials and methods

2.1. Animals housing and grouping

2.1.1. Animals housing

Mature male and female BALB/c mice were purchased (Beijing Vital River Laboratory Animal Technology Co, Ltd, Beijing, China, animal batch number:11400700366095) and raised in specific pathogen free (SPF) animal rooms at 21 ± 2°C under 12 h light/dark cycle with unrestricted access to OVA-free food and water.

2.1.2. Grouping of pregnant mice

Male and female mice were housed overnight at a 1:2 ratio for fertilization. The day of vaginal plug positive presentation was considered as the first day of gestation (designated as gestational day (GD) 1). At this time, the pregnant mice were randomized into O_3- or Air-exposed groups. Each group was randomly divided into two subgroups according to various end time, namely, an OP group (O_3 exposure, the observed end time was the end of pregnancy), AP group (Air exposure, the end of pregnancy), OL group (O_3 exposure, the observed end time was the end of lactation), and AL group (Air exposure, the ending of lactation).

2.1.3. Grouping of offspring

The offspring in the OL and AL groups were divided into two groups according to their mothers, an O_3/PBS group and O_3/OVA group for the OL group, and an Air/PBS group and Air/OVA group for the AL group. The offspring were treated with phosphate-buffered saline (PBS) (as the control) and OVA (as the model), respectively.

2.2. Exposure and the observed end time for the pregnant mice

2.2.1. Exposure treatment

The pregnant mice were exposed to O_3 (7.3 mg/m^3, 1 h/day) in the OP and OL groups or air in the AP and AL groups using a HOPE-MED 8050 dynamic poisoning experimental device (Tianjin Hope Industry & Trade Co, Ltd, Tianjin, China) from GD13 to GD18. The concentration of O_3 was monitored by Multi Component Gas Analyzer (GT-2000, Shenzhen Korno Electronic Technology Co., Ltd, Shenzhen, China). O_3 was generated by an O_3 generator (CQ-801S, Chongqing Electromechanical Trading Co., Ltd. Anhui, China).

2.2.2. The observed end time for the pregnant mice

At GD19, 24 h after the last O_3 exposure, the pregnant mice in the OP and AP groups were sacrificed and samples collected. The animals in the AL and OL groups lived freely with their offspring in cages until the dams were sacrificed and samples collected when their offspring were weaned (postnatal day (PND) 21).

2.3. OVA sensitization and challenge and the observed end time for the offspring

2.3.1. OVA sensitization and challenge

Two-week-old offspring were sensitized with three intraperitoneal injections of 0.1 ml PBS containing 50 μg OVA (Grade V; Sigma-Aldrich, St. Louis, MO, USA) and 2 mg alum adjuvant (Imject Alum; Thermo Fisher Scientific, Waltham, MA, USA) at PND9, 11, and 13. At four weeks of age, the offspring were placed in HOPE-MED 8050 dynamic poisoning experimental device and challenged by PBS (the O_3/PBS and Air/PBS groups) or 3% OVA (Grade II; Sigma-Aldrich, St. Louis, MO, USA) in PBS (the O_3/OVA and Air/OVA groups). The challenge was 30min/day and for 7 consecutive days.

2.3.2. The observed end time for the offspring

The offspring were sacrificed and samples collected 24 h after the last OVA or PBS exposure. The experimental protocol and grouping are illustrated in Fig. 1. All of the animal protocols used in this study were approved by the Ethics Committee of Preventive Medicine of Shandong University (Approved number: 20170315).

2.4. Measurement of bronchoalveolar lavage fluid (BALF) cellularity and cytokines

The lungs of the offspring, pregnant mice, and dams in each group were lavaged with 0.4 ml precooled PBS three times each. The recovered lavage fluid (80%–85%) was centrifuged (4°C, 100 g, 10 min), the supernatant was rapidly frozen (−80°C), and the cell pellet resuspended in PBS (4°C, without $Ca^{2+}Mg^{2+}$). BALF total white blood cells (WBCs) and differential cell counts were conducted using a HEMAVET 950 multi-species hematology analyzer (Drew Scientific, Dallas, TX, USA). The cytokine levels of interleukin (IL)-4, IFN (interferon)-γ, IL-17 (Novus Biologicals, Centennial, MO, USA), and tumor necrosis factor (TNF)-α (OmnimAbs, Alhambra, CA, USA) in the cell-free BALF were measured using enzyme-linked immunosorbent assay (ELISA) reagent kits according to the manufacturer's protocols.

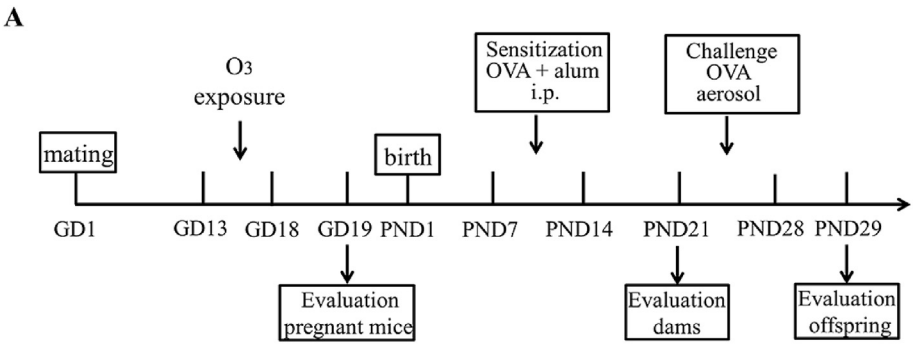


Fig. 1. The experimental protocol. (A) Pregnant BALB/c mice were exposed to either O_3 (7.3 mg/m^3 for 1 h/day) or air on GD13-18. The AP and OP groups were then evaluated 24 h after the final O_3 exposure (GD19). After weaning, the OL and AL dams were evaluated. The offspring were given intraperitoneal (i.p.) injections of PBS or a mixture of OVA and alum in PBS and then exposed to aerosols of 3% OVA or PBS (30min/day, 7 days) on postnatal day (PND) 22–28. The offspring were evaluated 24 h after the final exposure to aerosols. (B) The experimental groups were shown in Table 1.

B

Groups	Pregnant mice exposure	Offspring asthma model
Air/PBS	Air	PBS
Air/OVA	Air	OVA
O_3/PBS	O_3	PBS
O_3/OVA	O_3	OVA

2.5. Measurement of 8-isoprostanes (8-IP) and OVA-specific IgE in serum

Blood was collected from the offspring, pregnant mice, and dams at the observed end times shown in Fig. 1. After an hour of clotting, the serum were isolated (850 g for 15 min at 4°C), quick frozen in liquid nitrogen, then stored at −80°C until further analysis. The levels of 8-IP and the levels of OVA-specific IgE were measured using ELISA reagent kits (Cayman Chemical Company, Ann Arbor, MI, USA) as per the manufacturer's instructions.

2.6. Flow-cytometric analysis of splenic natural killer (NK) cells

The spleens of the offspring, pregnant mice, and dams in each group were ground in RPMI-1640 (KeyGen, Nanking, China), and the grinding solutions were centrifuged at 250 g for 5 min at 4°C. Red blood cells were removed using red blood cell (RBC) lysis buffer (BioLegend, San Diego, CA, USA) and the splenocytes were washed twice in PBS using centrifugation at 250*g* for 5 min at 4°C each time. Cells were resuspended in PBS and counted with a hemocytometer. Then, spleen cells were stained for 30 min at 4°C in the dark with antibodies, including APC-anti-mouse CD3ε and PE-anti-mouse CD49b (BioLegend, San Diego, CA, USA), according to the manufacturer's instructions. Next, cells were washed twice in PBS using centrifugation at 350*g* for 5 min at 4°C each time, and then resuspended in 400 μL PBS. CytoFLEX (Beckman Coulter, Inc., Brea, CA, USA) was used for detection and CytExpert software (Beckman Coulter, Inc., CA, USA) was used for analysis.

2.7. Pulmonary histopathology

The lung tissues of the offspring, pregnant mice, and dams in each group were excised and fixed in 4% paraformaldehyde at room temperature. After 24 h, the fixed lungs were embedded in paraffin, then, 5 μm sections were cut for staining with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS). The lung tissues were scored semi-quantitatively for peribronchial inflammation and goblet cell hyperplasia as described elsewhere (Corson et al., 2010; Rickel et al., 2008). We also used the Image-Pro Plus 6.0 software to determine the thickness of the goblet cells to assess the goblet cells area and the perimeter of the bronchial basement membrane (Pbm) of the mice, which represented the degree of epithelial cell transformation to mucus secretion phenotype (Zhang et al., 2017). Under blinded conditions, all of the scoring and measurements were performed by the same researcher.

2.8. Immunohistochemical detection of IL-4 protein expression

The lung tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5 μm sections. After dewaxing, the tissue sections were placed in a repair box filled with EDTA antigen repair buffer (pH9.0) for antigen repair. Then, the sections were incubated with 3% H_2O_2 for 25 min, and were blocked with 3% bovine serum albumin (BSA) for 30 min. The sections were incubated with IL-4 antibody (abcam, Cambridge, UK) overnight at 4°C, followed by incubation with secondary antibody for 50 min at room temperature. Chromogenic agent 3, 3-diaminobenzidine (DAB) was added to form the stain for microscopic examination of the IL-4 in the lung tissues. The sections were imaged, and the average optical density (AOD) (Wang et al., 2015) of the positive area was evaluated using Image-Pro Plus 6.0 software.

2.9. Statistical analysis

We used the SPSS 22.0 software for data entering and statistical analysis. If the data were normally distributed, measurement data were presented as mean ± standard deviation (SD) to represent centralized and discrete trends. If the variances were homogenous, one-way analysis of variance (ANOVA) was used with post hoc comparisons using Tukey test to analyse differences between multiple groups. If the variances were not homogenous, the data were analyzed via nonparametric ANOVA. Data comparing two groups were analyzed using the independent sample *t*-test if the variances were homogenous. The Fisher exact probability method was used when the variances had uneven homogeneity. Values of $P < 0.05$ were considered to be statistically significant. Figures were constructed using GraphPad Prism 5 Software. The attributable risk proportion (AR%) was to calculate the proportion attributed to O_3 exposure to indicate the extent of asthma exacerbation.

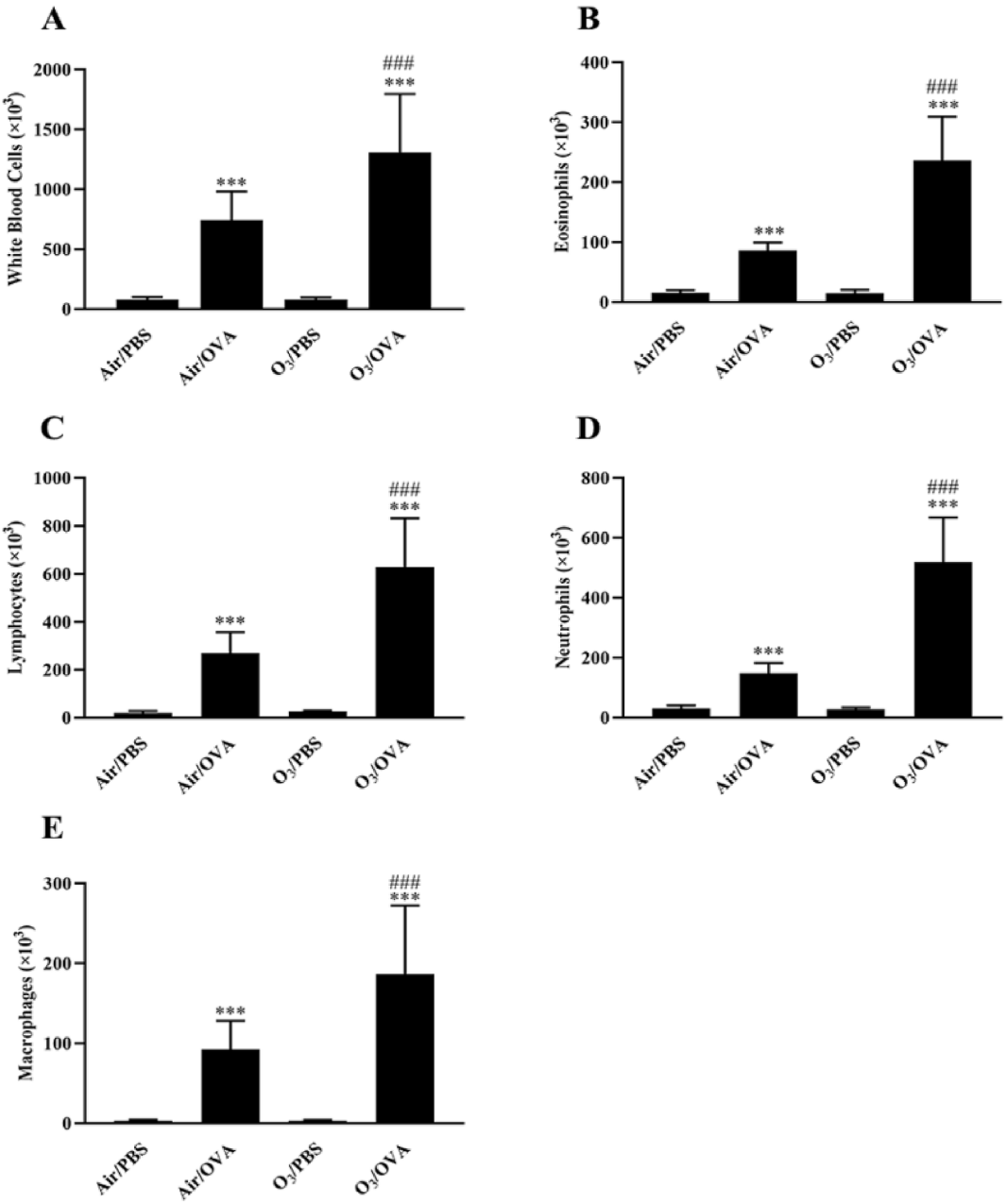


Fig. 2. The total white blood cells and differential cell counts of the offspring. The numbers of (A) total white blood cells (WBCs), (B) eosinophils, (C) lymphocytes, (D) neutrophils, and (E) macrophages were counted. The results were recorded as the mean ± SD (n = 10–16 mice per group). $^{***}p < 0.001$, compared with the PBS groups, respectively; $^{\#\#\#}p < 0.001$, compared with the Air/OVA group.

3. Results

3.1. Airway inflammation and mucus production in the offspring

The total WBC in BALF levels (Fig. 2A) were significantly increased in both the Air/OVA and O_3/OVA groups compared to each PBS control ($P_{\text{Air/OVA vs. Air/PBS}} < 0.001$; $P_{\text{O3/OVA vs. O3/PBS}} < 0.001$, respectively). In the O_3/OVA group, these levels were greater than in the Air/OVA group ($P < 0.001$), and the AR% was 43.2%. Eosinophils, lymphocytes, neutrophils, and macrophages were all significantly increased in both the Air/OVA and O_3/OVA groups compared to each PBS control (P values were all less than 0.001). In O_3/OVA group, they were 236 ± 73.3, 630 ± 202, 519 ± 149, and 187 ± 85.3 ($\times 10^3$), which were markedly increased compared to the Air/OVA group (the P values were all less than 0.001). The AR% of eosinophils, lymphocytes, neutrophils, and macrophages were 63.6%, 57.2%, 71.5%, and 50.6%, respectively (B, C, D, and E in Fig. 2).

Similarly, the score for peribronchial inflammation infiltration markedly increased in the Air/OVA and O_3/OVA groups compared to each PBS control ($P_{\text{Air/OVA vs. Air/PBS}} < 0.001$; $P_{\text{O3/OVA vs. O3/PBS}} < 0.001$, respectively), and this score markedly increased due to prenatal O_3 exposure ($P_{\text{O3/OVA vs. Air/OVA}} < 0.01$) as measured using the semi-quantitative scoring system. The AR% of the score for peribronchial inflammation infiltration was 28.8%. The results of H&E staining and the score for peribronchial inflammation infiltration in the lung tissues are shown in Fig. 3.

In addition, the phenomenon of the goblet cells hyperplasia and mucus secretion in the lung tissues measured via PAS staining qualitatively increased in both the Air/OVA and O_3/OVA groups compared to each PBS group (Fig. 3C). The score of the goblet cells hyperplasia

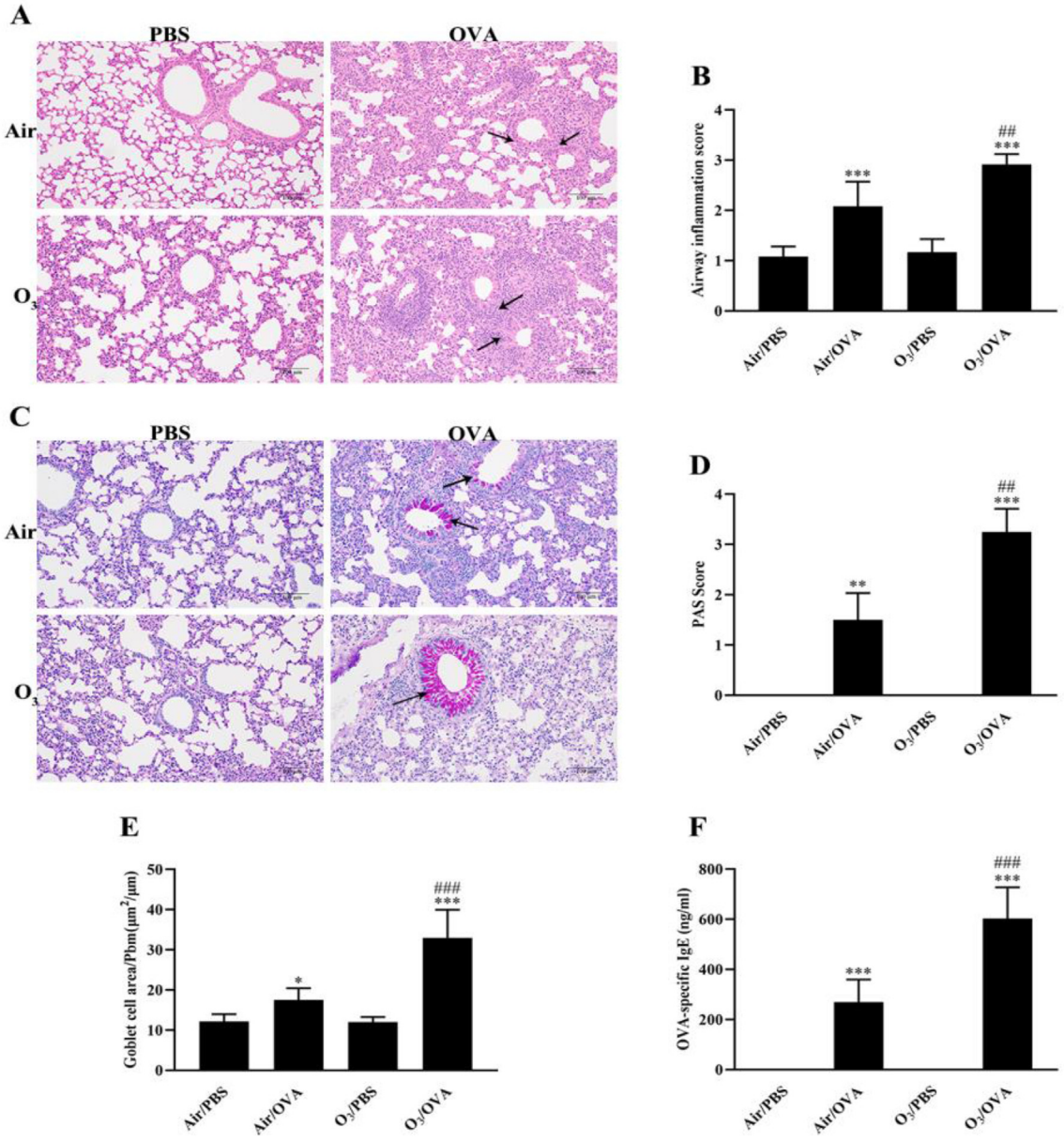


Fig. 3. Airway inflammation, mucus secretion, and the concentrations of OVA-specific IgE in offspring. (A) Peribronchial inflammation was determined via histological analysis of the H&E-stained lung segments (200 ×). (B) Scoring of H&E-stained sections. (C) Goblet cell hyperplasia and mucus secretion were evaluated via histological analysis of the PAS-stained lung segments (200 ×). (D) PAS score of goblet cells hyperplasia. (E) Score of goblet cell area/Pbm in lung sections. (F) The concentrations of OVA-specific IgE were determined by ELISA. The results were presented as the mean ± SD (n = 6–16 mice per group). Arrows point to inflammatory cells infiltration or goblet cells hyperplasia. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared with the PBS groups, respectively; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared with the Air/OVA group.

(Fig. 3D) and goblet cells area/Pbm (Fig. 3E) were all significantly increased in both the Air/OVA and O_3/OVA groups compared to each PBS control (the PAS score of goblet cells hyperplasia, $P_{Air/OVA\ vs.\ Air/PBS} < 0.01$ and $P_{O3/OVA\ vs.\ O3/PBS} < 0.001$; the score of goblet cells area/Pbm, $P_{Air/OVA\ vs.\ Air/PBS} < 0.05$ and $P_{O3/OVA\ vs.\ O3/PBS} < 0.001$, respectively). They were also significantly increased in the O_3/OVA group compared to the Air/OVA group ($P_{O3/OVA\ vs.\ Air/OVA} < 0.01$, and $P_{O3/OVA\ vs.\ O3/PBS} < 0.001$, respectively), according to semi-quantitative scoring system. The AR% of the PAS score of goblet cells hyperplasia and the score of goblet cells area/Pbm were 53.8% and 46.9%, respectively.

3.2. OVA-specific IgE production in the offspring

The concentrations of OVA-specific IgE in the serum were notably increased in both the Air/OVA and O_3/OVA groups compared to each PBS control ($P_{Air/OVA\ vs.\ Air/PBS} < 0.001$, $P_{O3/OVA\ vs.\ O3/PBS} < 0.001$, respectively). In the O_3/OVA group, it was 603 ± 125 ng/ml, which was markedly increased compared to that in Air/OVA group ($P_{O3/OVA\ vs.\ Air/OVA} < 0.001$) (Fig. 3F). The AR% of the OVA-specific IgE was 55.4%.

3.3. The cytokines of Th cell subsets and pro-inflammatory factor secretion in the BALF of the offspring

The Th1 cytokine IFN-γ levels demonstrated no significant difference in the different groups ($P_{Air/OVA\ vs.\ Air/PBS}$, $P_{O3/OVA\ vs.\ O3/PBS}$, and $P_{O3/OVA\ vs.\ Air/OVA} > 0.05$) (Fig. 4A). Regarding the Th2 cytokine, the concentrations of IL-4 were notably increased in both the Air/OVA and O_3/OVA groups compared to each PBS control ($P_{Air/OVA\ vs.\ Air/PBS} <$

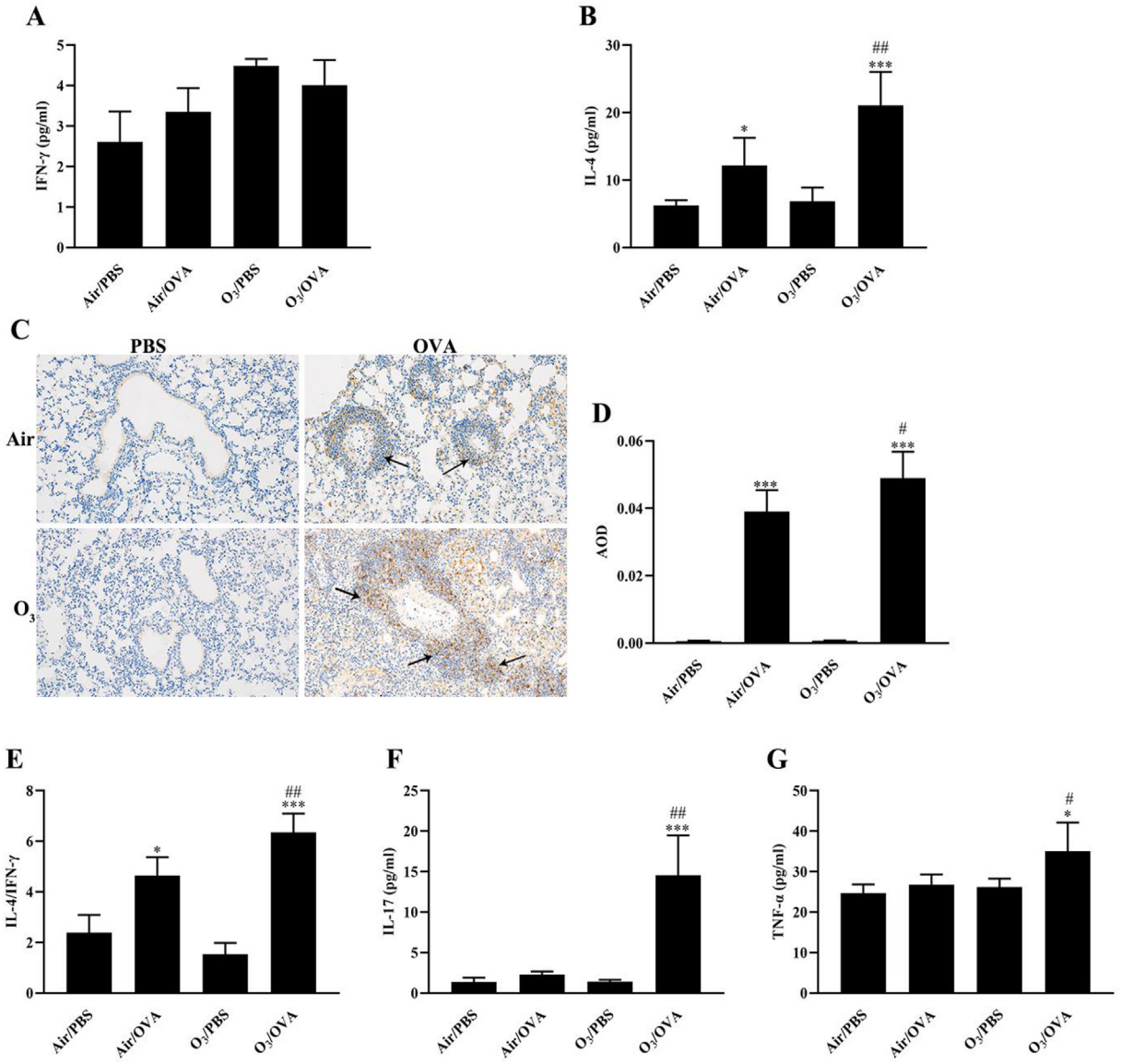


Fig. 4. The Th cytokines IL-4, IFN-γ, and IL-17 and pro-inflammatory cytokine TNF-α in the offspring. The concentration of (A) IFN-γ, (B) IL-4, (F) IL-17, and (G) TNF-α in BALF. (E) The ratio of IL-4/IFN-γ in BALF. The immunohistochemical staining of IL-4 protein expression in the lung tissues (200 ×) (C) and the average optical density (AOD) scoring of IL-4 protein expression staining in lung tissues via immunohistochemistry (D). The black arrows point to the expression of IL-4. The data were presented as the mean ± SD (n = 6–14 mice per group). $^{*}p < 0.05$, $^{***}p < 0.001$, compared with the PBS groups, respectively; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with the Air/OVA group.

0.05, $P_{O3/OVA\ vs.\ O3/PBS} < 0.001$, respectively). In O_3/OVA group, it was 21.1 ± 4.97 pg/ml, which was markedly increased compared to that in Air/OVA group ($P_{O3/OVA\ vs.\ Air/OVA} < 0.01$) (Fig. 4B), and the AR% was 42.3%. In addition, the results and AOD of the IL-4 protein expression staining in the lung tissues via immunohistochemistry are shown in Fig. 4C and D. The AOD for IL-4 protein expression markedly increased in both the Air/OVA and O_3/OVA groups compared to each PBS control ($P_{Air/OVA\ vs.\ Air/PBS} < 0.001$, $P_{O3/OVA\ vs.\ O3/PBS} < 0.001$, respectively), and the AOD markedly increased due to prenatal O_3 exposure ($P_{O3/OVA\ vs.\ Air/OVA} < 0.05$). The AR% of the AOD was 20.4%. Moreover, the L-4/IFN-γ ratio, which is an index of Th2 and Th1 balance, were significantly higher in both the Air/OVA and O_3/OVA groups compared to each PBS control ($P_{Air/OVA\ vs.\ Air/PBS} < 0.05$, $P_{O3/OVA\ vs.\ O3/PBS} < 0.001$, respectively), and this ratio markedly increased due to prenatal O_3 exposure ($P_{O3/OVA\ vs.\ Air/OVA} < 0.01$) (Fig. 4E). The AR% of this ratio was 26.8%.

The levels of IL-17 were markedly increased in the O_3/OVA group compared to the O_3/PBS group ($P_{O3/OVA\ vs.\ O3/PBS} < 0.001$). In the O_3/OVA group, it was 14.6 ± 4.92 pg/ml, which was markedly increased compared to the Air/OVA group ($P_{O3/OVA\ vs.\ Air/OVA} < 0.01$) (Fig. 4F), and the AR% was 84.3%.

The concentrations of TNF-α, a pro-inflammatory factor, were significantly increased in the O_3/OVA group compared to the O_3/PBS group ($P_{O3/OVA\ vs.\ O3/PBS} < 0.05$). In the O_3/OVA group, it was 35.1 ± 7.06 pg/ml, which was markedly increased compared to that in Air/OVA group ($P_{O3/OVA\ vs.\ Air/OVA} < 0.05$) (Fig. 4G), and the AR% was 23.6%. The levels of IL-17 and TNF-α did not reach statistical significance in the Air/OVA group compared to the Air/PBS group.

3.4. Splenic NK cells in the offspring

The frequency and the numbers of the $CD3\varepsilon^{-}CD49b^{+}$ splenic NK cells were significantly increased in both the O_3/PBS and O_3/OVA groups compared to each Air control ($P_{O3/PBS\ vs\ Air/PBS} < 0.01$, $P_{O3/OVA\ vs.\ Air/OVA} < 0.001$, respectively), as shown in Fig. 5A and B.The AR% values of the $CD3\varepsilon^{-}CD49b^{+}$ splenic NK cells in the two groups were

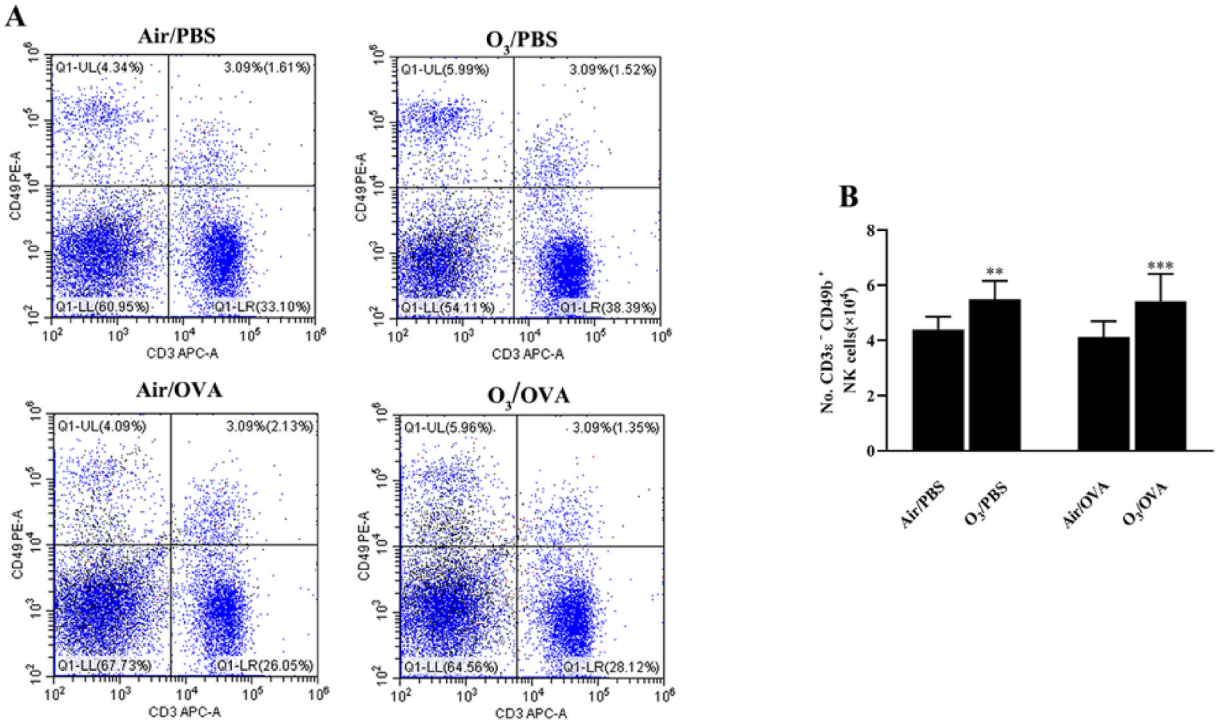


Fig. 5. The frequency and the numbers of the CD3ε$^-$CD49b$^+$ splenic NK cells in offspring. (A) The frequency of NK cells and (B) the numbers of CD3ε$^-$CD49b$^+$ splenic NK cells were determined using flow cytometry. The data were presented as the mean ± SD (n = 10–17 mice per group). $^{**}p < 0.01$, $^{***}p < 0.001$, compared with the Air groups, respectively.

20.0% and 24.1%, respectively.

3.5. The responses to the maternal exposure to O_3

There were no significant difference in AP, OP, AL, and OL groups of body weight at grouping which indicated the balance between groups. There were no significant difference between the groups for the body weight at the beginning (GD13) and the end (GD18) of O_3 exposure, and at the end of lactation (PND21) (Table 1).

Regarding the maternal airway inflammation after O_3 exposure, the numbers of total WBC in the BALF were significantly increased in the OP group compared to the AP control ($P_{OP\ vs.\ AP} < 0.05$) (Fig. 6A), which were increased by 33.4%. The numbers of eosinophils, lymphocytes, neutrophils, and macrophages in the OP group were 69.6 ± 7.92, 44.2 ± 12.3, 60.8 ± 13.1, and 5.60 ± 1.13 (× 10^3), respectively, which were markedly increased by 56.0%, 51.8%, 71.5%, and 42.9% compared to the AP group ($P_{eosinophils} < 0.001$, $P_{lymphocytes} < 0.05$, $P_{neutrophils} < 0.01$, and $P_{macrophages} < 0.05$). After the dams finished lactating, the total WBC numbers were significantly increased in the OL group compared to the AL control ($P_{OL\ vs.\ AL} < 0.01$). The levels of eosinophils, neutrophils, and macrophages in the OL group were 35.5 ± 9.02, 36.5 ± 7.34, and 5.00 ± 2.20 (× 10^3), respectively, which were markedly increased by 46.2%, 47.1%, and 58.0% compared to the AL control ($P_{eosinophils} < 0.01$, $P_{neutrophils} < 0.01$, and $P_{macrophages} < 0.05$). The lymphocytes did not reach statistical significance in the OL group compared to the AL group (Fig. 6B). Similarly, the score for peribronchial inflammation infiltration of H&E staining (Fig. 6C and D) markedly increased in both the pair (OP group vs AP group, $P < 0.01$) and other (OL group vs AL group, $P < 0.05$) using the semi-quantitative scoring system. PAS staining did not show mucus secretion and goblet cell hyperplasia in the different groups (Fig. 6E).

The frequency and the numbers of the CD3ε$^-$CD49b$^+$ splenic NK cells were significantly decreased in both the OP and OL groups compared to each Air control ($P_{OP\ vs.\ AP} < 0.05$, $P_{OL\ vs.\ AL} < 0.05$, respectively), which were markedly reduced by 22.2% and 20.1%, respectively, as shown in Fig. 6 (F and G).

Relatively, the levels of 8-IP in the serum, a sensitive index of oxidative stress, were notably increased by 32.5% in the OP group compared to the AP group (1190 ± 183 vs. 798 ± 138 pg/ml, $P_{OP\ vs.\ AP} < 0.01$). After the dams finished lactating, the levels of 8-IP difference between the OL group and the AL group (904 ± 144 vs. 946 ± 129 pg/ml) were not statistically significant (Fig. 6H).

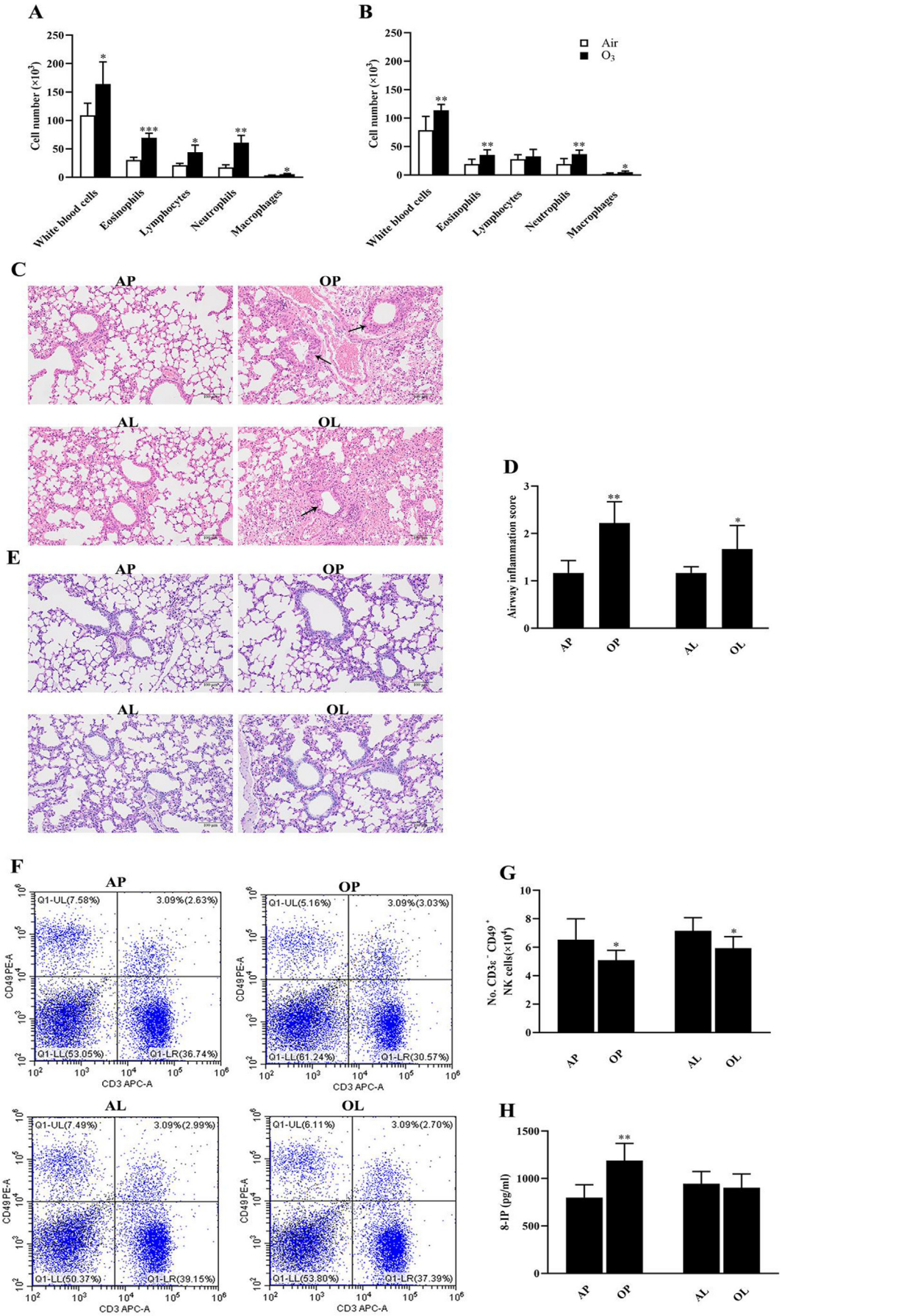
Table 1
The maternal body weight change.

Groups	n	Grouping (g)	GD13 (g)	GD18 (g)	PND21 (g)
AP	9	21.6 ± 0.64	26.26 ± 1.08	32.6 ± 1.84	–
OP	9	21.57 ± 0.76	25.43 ± 1.09	32.1 ± 0.84	–
AL	8	21.3 ± 0.97	26.01 ± 0.93	33.94 ± 1.65	28.49 ± 2.99
OL	9	21.4 ± 0.75	26.51 ± 1.06	32.64 ± 1.76	27.06 ± 1.71
F	–	0.28	1.74	2.07	2.33
P	–	0.84	0.18	0.12	0.15

Grouping (g): maternal body weight at grouping, GD13 (g): maternal body weight at the beginning of O_3 exposure, GD18 (g): maternal body weight at the end of O_3 exposure, PND21 (g): maternal body weight at the end of lactation.

4. Discussion

Previous studies have proved that prenatal exposure to diesel exhaust particles (Brandt et al., 2015) and house dust mite (Richgels et al., 2017) promoted the development of asthma in offspring, suggesting that exposure to environmental pollutants or allergens during pregnancy has significant impacts on asthma in offspring. Although O_3 exposure has adverse effects on pulmonary function and was important contributor to asthma in exposed individuals (Funabashi et al., 2004; Larsen et al., 2010),fewer studies have explored the influences of the prenatal O_3 exposure on the development or exacerbation of offspring asthma. Our data showed that prenatal O_3 exposure exacerbated airway inflammation, mucus production, the levels of OVA-specific IgE, and



(caption on next page)

Fig. 6. The effects of prenatal O_3 exposure on the pregnant mice and dams. (A), (B) The numbers of total WBC and differential inflammatory cells were counted after prenatal exposure to O_3 at GD19 and PND21, respectively. (C) Peribronchial inflammation was determined via histological analysis of the H&E-stained lung segments (200 ×). (D) Scoring of the H&E-stained sections. (E) PAS-stained of lung segments (200 ×). (F) The levels of 8-IP were measured by ELISA at GD19 and PND21. (G) The frequency of $CD3\varepsilon^-CD49b^+$ splenic NK cells and (H) the number of $CD3\varepsilon^-CD49b^+$ splenic NK cells were determined using flow cytometry at GD19 and PND21. The arrows point to inflammatory cells infiltration. The data were presented as the mean ± SD (n = 4–9 mice per group). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared with the Air groups, respectively.

the imbalance of Th2 and Th1 differentiation in offspring, all indicators demonstrating that prenatal O_3 exposure increased the severity of OVA-induced asthma in offspring. To the best of our knowledge, this is the first study to explore the effects of prenatal O_3 exposure on asthma in offspring.

Asthma has a variety of inflammatory cell-dominated airway inflammation types. Research on prenatal exposure to residual oil fly ash (Hamada et al., 2007) and combustion-derived PM (Saravia et al., 2014), reported that offspring were affected by eosinophilic inflammation. The characteristic of inflammation types induced by O_3 in our study included neutrophilic-dominated airway inflammation. The AR% of neutrophils after prenatal O_3 exposure in BALF were 71.5% accompanied by a higher rate of eosinophilic inflammation (the AR% of the neutrophils was 63.6%). This indicated that prenatal O_3 exposure was significantly increased neutrophilic and eosinophilic inflammation in the offspring. It was worth noting that the phenomenon of neutrophils substantially increased in the offspring, consistent with maternal inflammation, which also was in agreement with the findings from other studies of mice exposed to O_3 (Duan et al., 2017; Michaudel et al., 2018). Collectively, we demonstrated that the characteristics in airway inflammation of asthma caused by O_3 was mainly increased due to neutrophilic inflammation accompanied eosinophilic inflammation.

The mechanism of asthma remains unclear and controversial. Studies have shown that the imbalanced differentiation of the Th cells played pivotal role in respiratory diseases (Legg et al., 2003) and asthma (Liang et al., 2017). In other words, the pathogenesis of asthma included the numbers and functional imbalance of Th2 and Th1 cell subsets, which can excrete relevant cytokines that exacerbate the development of asthma (Mei et al., 2018). Th2 subtypes mainly secrete IL-4, IL-5, and IL-13 (Tibbitt et al., 2019). IL-4 has the greatest biological effects on the IgE regulatory cytokines and is universally recognized as the most important cytokine in the pathogenesis of asthma (Russkamp et al., 2019). Besides, IL-4 and IFN-γ were considered to be positive and negative regulators of IgE (Gajewski and Fitch, 1988; Horwitz and Busse, 1995) and were suggested as the representative cytokines to reflect the Th2 and Th1 immune response pattern (Trop and Ilan, 2002; Zhao et al., 2019). In our research, the ratio of IL-4/IFN-γ was notably higher in the O_3/OVA mice than the Air/OVA mice, which was in agreement with previous studies of mice exposed to O_3 (Duan et al., 2017) and the offspring immune reaction to prenatal exposure to nitrogen dioxide (NO_2) (Yue et al., 2018) or house dust mite (Richgels et al., 2017). Therefore, we inferred that the Th2 dominant response was the primary cause of prenatal O_3 exposure exacerbating asthma in offspring. Moreover, the relationship between the Th2 dominant response and the types of asthma-causing substances was weak in the mechanism of asthma.

Additionally, some studies found Th17 cells, a Th-cell lineage distinct from Th1 and Th2 cells, play an important role in the pathogenesis of asthma (Doe et al., 2010; Shi et al., 2011). Our data showed that there were no statistical differences in the IL-17 levels between the Air/OVA mice and the Air/PBS mice, however, prenatal O_3 exposure significantly exacerbated IL-17 secretion. The TNF-α, a pro-inflammatory factor in allergic reaction process (Nakae et al., 2007), demonstrated similar trend of IL-17 in our study. Therefore, we inferred that prenatal O_3 exposure was a necessary condition for the significantly high concentrations of IL-17 and TNF-α in the offspring.

More importantly, NK cells initially described as lymphocytes involved in innate immunity, but recent studies have shown that it also participated in the regulation of the adaptive immune responses (Lee et al., 2007). Research indicated that NK cells can regulate the outcome of Th2-mediated immune responses by stimulating dendritic cells (DCs) maturation during allergic airway disease and modulating the magnitude of antigen presentation (Mathias et al., 2014), because the DCs upregulate costimulatory molecules and induce $CD4^+$T cells priming. Mathias et al. (2014) showed that $CD4^+$T cells derived from OVA sensitized wild-type mice can fully induce eosinophilia in $RAG^{-/-}$ recipients, while $CD4^+$ T cells from NK cells deficient mice were unable to induce eosinophilia in $RAG1^{-/-}$ recipients. Besides, researchers used depleting antibodies of anti-NK1.1 mAb to deplete NK cells in vivo to produce an OVA-induced asthma model. The results indicated that the decrease in the NK cells significantly reduced airway inflammation, OVA-specific IgE, and the secretion of Th2 cytokines, suggesting that NK cells were required at initiation of asthma (Korsgren et al., 1999). In a word, NK cells were likely necessary to activate $CD4^+$T cells, differentiate native T cells into Th2 cells, and initiate adaptive type 2 immune response. In this study, prenatal O_3 exposure significantly increased the frequency and the numbers of $CD3\varepsilon^-CD49b^+$ splenic NK cells and upregulated the level of IL-4, which directly increased the severity of OVA-induced asthma in offspring.

We further explored the origins of the exacerbation of asthma in offspring exposed to prenatal O_3. In addition to causing airway inflammation, O_3 exposure significantly damaged the maternal immune system during pregnancy and lactation. Since early life (including the embryonic stage) is a period of rapid immune system development, the destruction of the immune system in offspring may be related to the maternally damaged immune system during pregnancy and lactation (Simon et al., 2015). Meanwhile, based on Barker's hypothesis (Barker and Osmond, 1986) and previous findings (Heindel and Vandenberg, 2015), we inferred that the maternally damaged immune system was probably an important cause of the development of offspring asthma by affecting the fetal immune system programming.

In the present study, the concentration of O_3 exposure was calculated according to the annual average concentration, including during the high-temperature seasons, which simulated the conditions that mimicked actual O_3 exposure in humans.

5. Conclusions

In summary, we found that prenatal O_3 exposure exacerbated the severity of OVA-induced asthma in offspring, which might be directly caused by the increased $CD3\varepsilon^-CD49b^+$ splenic NK cells in offspring. O_3 exposure damaged the maternal immune system, which may indirectly contribute to the exacerbation of asthma in offspring. These findings are important for regulating the risks of progression of asthma in offspring, which caused by prenatal exposure to O_3.

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Declaration of competing interest

We have no conflicts of interests to declare.

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Zhiping Wang, Lingling Fu, and Xiwei Yang designed this study.Lingling Fu, Xiwei Yang, Xinai Liu, and Gongchang Yu conducted the experiments. Lingling Fu and Xiwei Yang analyzed the data and wrote the manuscript. All of the authors reviewed and revised the manuscript.

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