



Antagonistic effect of vitamin E on nAl₂O₃-induced exacerbation of Th2 and Th17-mediated allergic asthma via oxidative stress[☆]

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ABSTRACT

Some basic research has shown that nanomaterials can aggravate allergic asthma. However, its potential mechanism is insufficient. Based on the research that alumina nanopowder (nAl₂O₃) has been reported to cause lung tissue damage, the purpose of this study was to explore the relationship between nAl₂O₃ and allergic asthma as well as its molecular mechanism. In this study, Balb/c mice were sensitized with ovalbumin (OVA) to construct the allergic asthma model while intratracheally administered 0.5, 5 or 50 mg kg⁻¹·day⁻¹ nAl₂O₃ for 3 weeks. It was observed that exposure to nAl₂O₃ exacerbated airway hyperresponsiveness (AHR), airway remodeling, and inflammation cell infiltration, leading to lung function damage in mice. Results revealed that nAl₂O₃ could increase ROS levels and decrease GSH levels in lung tissue, promote the increases of the T-IgE, TGF-β, IL-1β and IL-6 levels, stimulate the over-expression of transcription factors GATA-3 and RORγt, decrease the levels of IFN-γ and IL-10 and increase the levels of IL-4 and IL-17A, resulting in the imbalance of Th1/Th2 and Treg/Th17 immune responses. In addition, antioxidant Vitamin E (Vit E) could alleviate asthma-like symptoms through blocking oxidative stress. The study displayed that exposure of nAl₂O₃ deteriorated allergic asthma through promoting the imbalances of Th1/Th2 and Treg/Th17.

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1. Introduction

Nanoparticles refer to materials with at least one dimension of less than 100 nm in the three-dimensional space (National Nanotechnology Initiative (U.S.), 2017). The application of nanomaterials has become more and more extensive, due to their unique physical and chemical properties such as their particle size, distribution, chemical composition, surface area and surface charge (Oberdörster et al., 2005; Saleh, 2011; Saleh, 2015; Saleh, 2016; Saleh, 2018; Xia et al., 2006). Thanks to its high hardness, high strength, heat resistance, corrosion resistance and other excellent characteristics, aluminum oxide nanopowder (nano-alumina or nAl₂O₃) is widely utilized in aerospace, electronics, chemical

industry, medicine, catalysts and carriers, rubber, insulation materials, ceramics and other fields (Duoren, 2012; Pidgeon et al., 2004; Hood, 2004). Inevitably, people today are exposed to nAl₂O₃ at an increasing level. Therefore, it is urgent to reveal the possible health risk of nAl₂O₃.

Some studies have shown that nAl₂O₃ has certain toxic effects. For example, the exposure of nAl₂O₃ with the concentration of 8.1 mg L⁻¹ can induce neurotoxicity to affect motor behaviors in nematode *Caenorhabditis elegans* (Yinxia et al., 2012). It has also been reported 100 μg L⁻¹ nAl₂O₃ has toxic impacts on human neuroblastoma SH-SY5Y and mouse hippocampal HT22 cells *in vitro* and on ICR female mice *in vivo* (Shah et al., 2015a). Moreover, research shows that exposure to nAl₂O₃ has potential innate immune system toxicity in rat brain (Xiaobo et al., 2009). nAl₂O₃ also has a certain toxic effect on the histological structure, hematology and biochemistry of male albino rats (Morsy et al., 2016). There is also a recent research revealing that 50 and 13 nm particle size alumina resulted genotoxic effect via introducing oxidative stress

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(Qinli et al., 2017). It is worth noting that study has found exposure to $80 \mu\text{g cm}^{-3}$ nAl_2O_3 can cause intracellular accumulation of nAl_2O_3 in A549 human pneumocytes, so that inducing elevated levels in oxidative stress and partial cell death (Simondeckers et al., 2008; Lin et al., 2014).

Inhalation is the primary exposure route for nanomaterials, and most studies have shown that inhalation of toxic metals and metal oxide nanomaterials can lead to lung cancer (Borm et al., 2006; Sikkeland et al., 2016; Donaldson et al., 2004). Jixuan Ma et al. has used one-time airway instillation of nAl_2O_3 suspension, and found that exposure of nAl_2O_3 can cause damage to lung tissue structure (Jixuan et al., 2014). In addition, nAl_2O_3 can reduce the phagocytic function of neutrophils and affect the non-specific immune function in mice (Wenhui et al., 2016). More importantly, our previous research found that exposure to nAl_2O_3 induced oxidative stress and inflammation response in lung of Balb/c mice (Haiyan et al., 2019). Moreover, Related studies have shown that exposure of nanomaterials can aggravate the further deterioration of allergic asthma, resulting in impaired lung function in mice (Bing et al., 2011; Jinquan et al., 2013). However, experimental studies on the respiratory toxicity of nanomaterials are not sufficient, so further exploration and in-depth research are needed (Meldrum et al., 2017). To date, the study on the links between nAl_2O_3 and allergic asthma have not been reported in the literature.

Asthma belongs to chronic airway inflammation, with symptoms such as the excessive mucus secretion, reversible airway obstruction, airway remodeling as well as inflammatory cell infiltration (Fajt and Wenzel, 2015; Lynch et al., 2016). Airway hyper-responsiveness (AHR) is a key mark of allergic asthma, and it changes are usually used to evaluate the severity of asthma (Hargreave et al., 1980). AHR is closely related to serum total immunoglobulin E (T-IgE) levels, which can be elevated by the occurrence of airway hyperresponse (Burrows et al., 1989; Sears et al., 1991a; Smith, 1978). On the other hand, Asthma is mainly dominated by the Th2-type immune response. As a typical Th2 cytokine, IL-4 could aggravate allergic asthma with elevated levels of T-IgE, eosinophil infiltration and lung pathological changes (Ray et al., 2016). Meanwhile, studies have revealed that Th17-type immune response also plays an important role in the development of asthma, especially for severe allergic asthma. Cytokine IL-17 induced by Th17 cell can recruit a large number of neutrophils to worsen allergic asthma (Martin et al., 2014; Poynter, 2016).

Therefore, This study aimed at investigating the relationship between exposure of nAl_2O_3 and allergic asthma as well as their underlying mechanisms, especially the role of nAl_2O_3 on the imbalance of Th2-type and Th17-type immune responses. Hopefully, this study could reinforce nAl_2O_3 -induced respiratory toxicity and provide evidence for determining the relationship of nAl_2O_3 to the occurrence of allergic asthma.

2. Materials and methods

2.1. Experimental animals

Specific pathogen free (SPF) male Balb/c mice (5–6 weeks; 18 ± 2 g) were purchased from the Hubei Province Experimental Animal Center (Wuhan, China) and fed in SPF level experimental animal center of School of Life Sciences of Central China Normal University. They were maintained in 12 h photoperiod at $20\text{--}25^\circ\text{C}$ and 50–70% humidity, provided ad libitum access to a commercial diet and filtered water and adapted to the environment for a week before any administrations. All experimental procedures were approved by the Office of Scientific Research Management of Central China Normal University (Wuhan, China), with a certificate of Application for the Use of Animals (approval ID: CCNU-IACUC-

2016-003). The mice were treated humanely with regard for alleviation of suffering.

2.2. Main experimental reagent and kits

$\gamma\text{-nAl}_2\text{O}_3$ (<50 nm, particle size) was obtained from Sigma-Aldrich (Shanghai, China). The particle size and morphology of the nAl_2O_3 in saline were analyzed by scanning electron microscope (SEM, FE-SEM, JSM-6700F-Japan).

Mouse enzyme-linked immunosorbent assay (ELISA) kits for total immunoglobulin E (T-IgE) in serum, interferon- γ (IFN- γ) and interleukin-10 (IL-10) in BALF, interleukin-1 β (IL-1 β) and interleukin-6 (IL-6) in lung were obtained from eBioscience (San Diego, CA, USA). GSH test kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The protein test kits were provided by Beijing Dingguo Changsheng Biotechnology Co. LTD (Beijing, China). Vitamin E (Vit E), Ovalbumin (OVA) and 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich (St Louis, MO, USA). $\text{Al}(\text{OH})_3$ (>99%) were purchased from Strem Chemicals (Newburyport, MA, USA).

2.3. Experimental design and animal exposure

nAl_2O_3 was dispersed in distilled water. Then the suspensions were stirred in constant temperature magnetic stirrer (MI-902, Shanghai pujiang analytical instrument factory) for an hour following by a 15-min sonication in water bath (JN-3200DT, Ningbo jiangnan instrument factory). The suspensions of nAl_2O_3 were resuspended for 15 min before each exposure.

Mice for experiment were randomly divided into 9 groups of eight each: (1) saline group (control); (2) ovalbumin sensitization group (OVA); (3) OVA combined with $100 \text{ mg kg}^{-1} \cdot \text{day}^{-1}$ Vit E group (OVA + Vit E); (4) $0.5 \text{ mg kg}^{-1} \cdot \text{day}^{-1}$ nAl_2O_3 combined with OVA group (nAl_2O_3 0.5 + OVA); (5) $5 \text{ mg kg}^{-1} \cdot \text{day}^{-1}$ nAl_2O_3 combined with OVA group (nAl_2O_3 5 + OVA); (6) $50 \text{ mg kg}^{-1} \cdot \text{day}^{-1}$ nAl_2O_3 combined with OVA group (nAl_2O_3 50 + OVA); (7) $0.5 \text{ mg kg}^{-1} \cdot \text{day}^{-1}$ nAl_2O_3 combined with OVA along with $100 \text{ mg kg}^{-1} \cdot \text{day}^{-1}$ Vit E group (nAl_2O_3 0.5 + OVA + Vit E); (8) $5 \text{ mg kg}^{-1} \cdot \text{day}^{-1}$ nAl_2O_3 combined with OVA along with $100 \text{ mg kg}^{-1} \cdot \text{day}^{-1}$ Vit E group (nAl_2O_3 5 + OVA + Vit E); (9) $50 \text{ mg kg}^{-1} \cdot \text{day}^{-1}$ nAl_2O_3 combined with OVA along with $100 \text{ mg kg}^{-1} \cdot \text{day}^{-1}$ Vit E group (nAl_2O_3 50 + OVA + Vit E).

The mice were instilled intratracheally with three different nAl_2O_3 suspensions ($0.5 \text{ mg kg}^{-1} \cdot \text{day}^{-1}$, $5 \text{ mg kg}^{-1} \cdot \text{day}^{-1}$ and $50 \text{ mg kg}^{-1} \cdot \text{day}^{-1}$) once every two days, for a total of 11 instillations, and sensitized with $50 \mu\text{g}$ OVA plus 1.75 mg $\text{Al}(\text{OH})_3$ in $300 \mu\text{L}$ saline by intraperitoneal injection on day 7, 14, and 21. Then mice were followed by an aerosol challenge of 1% OVA ($30 \text{ min} \cdot \text{day}^{-1}$) from days 24–30 (7 times) using an ultrasonic nebulizer (Yuyue 402A type1, Shanghai Yu Yue Medical Equipment Co. Inc, Shanghai, China). Intragastric administration of Vit E ($100 \text{ mg kg}^{-1} \cdot \text{day}^{-1}$) was given 3 h after administration of nAl_2O_3 as an antioxidant in OVA treated and combined-OVA nAl_2O_3 exposure groups.

2.4. Preparation of serum and T-IgE measurement

Within 24 h of the last atomization, the mice were anesthetized intraperitoneally with pentobarbital sodium ($100 \text{ mg kg}^{-1} \cdot \text{day}^{-1}$). Then serum samples were collected from heart blood by centrifugation (3000 rpm at 25°C for 15 min) and stored at -80°C . The concentrations of T-IgE in serum were measured by ELISA kits, according to manufacturer's instructions. Sensitivities of ELISA kits were 4 ng/mL for T-IgE.

2.5. Collection of bronchoalveolar lavage fluid (BALF) and inflammatory cells count

After serum collection, the BALF of each mouse was collected using a syringe with saline. The total quantity of each sample was about 1.2 mL. The samples of BALF were centrifuged at 3000 rpm at 4 °C for 10 min and the supernatant fluid was frozen at –80 °C for the assessment of protein IFN- γ and IL-10 levels. The cell sediment was suspended in saline for counting inflammatory cells. Total cells, lymphocytes, neutrophils, and eosinophils in BALF were assayed by a Blood Cell Analysis system (MTN-21, Matee3nu Technology Corp, Jinan, China).

2.6. Preparation of lung tissue homogenate

The mice were sacrificed by cervical dislocation and the whole lung were taken out and rinsed in ice-cold PBS. Then lung tissues were homogenized in a glass homogenizer for 10% tissue homogenate on ice and all centrifuged at 10,000 rpm and 4 °C for 10 min. Next, all supernatant were collected and frozen at –80 °C for later assessment. Lung tissue homogenate was used for measurement of reactive oxide species (ROS), glutathione (GSH), IL-1 β and IL-6.

2.7. Measurement of ROS and GSH

Dichlorofluorescein (DCF) fluorescence method was used for the determination of ROS content. 100 μ L diluted sample and 100 μ L 0.2 μ mol/L 2', 7'-Dichloro-dihydrofluorescein diacetate (DCFH-DA) were added into 96-well plates respectively. Then the 96-well plates were incubated at 37 °C for 10 min away from light and measured using the FLx 800 fluorescence reader (BioTek Instruments, Winooski, VT, USA) under the excitation wavelength of 480 nm and emission wavelength of 520 nm. GSH content was measured using ELISA kit according to the manufacture's protocol. The protein concentration was determined using BCA protein assay kit.

2.8. Measurement of cytokines

The concentration of IFN- γ , IL-10 in BALF, IL-1 β and IL-6 in lung were measured using ELISA kits according to the manufacturer's instructions. The sensitivities of ELISA kits were 15 pg/mL for IFN- γ , 30 pg/mL for IL-10, 8 pg/mL for IL-1 β , 4 pg/mL for IL-6.

2.9. Lung histological assay and immunohistochemistry

Mouse lung was removed, following with the fixation in 10% formalin solution for 24 h at room temperature and embedded in paraffin for histopathology slice preparation. Some moderate samples were sectioned into 10- μ m slices, separated in pieces stained with hematoxylin and eosin (H&E) and Masson's trichrome (MT), and observed using the DM4000B microscope (Leica Microsystems GmbH, Wetzlar, Germany).

The sections of lung tissue were deparaffinized, rehydrated, subjected to antigen retrieval, incubated with 0.3% hydrogen peroxide and blocked by appropriate normal serum. Next, The sections were incubated with monoclonal antibodies: anti-interleukin-4 (anti-IL-4, CAS: BA0980-1, 1: 200, boser, MA, USA), anti-interleukin-17A (anti-IL-17A, CAS: A0688, 1: 1000, ABChonal, MA, USA), and anti-transforming growth factor beta-1 (anti-TGF- β , CAS: GB11179, 1: 200, Servicebio, China). Then antibody binding was measured by biotinylated immunoglobulins and avidin-biotin peroxidase complex and the reaction products were viewed by DAB complexes. In addition, The negative control was got by

omitting the primary antibody. Finally, all sections were counterstained with hematoxylin, dehydrated and mounted in DPX (Sigma Aldrich). The images were subsequently recorded using a microscope and was analyzed using an Image Pro Plus 6.0 (Media Cybernetics, USA).

2.10. Lung function measurement

The lung function in mice was measure by the AniRes2005 lung function system (Bestlab, version 2.0, China) within 24 h of the last atomization. In the process of AHR measurement, the inspiratory resistance (Ri), expiratory resistance (Re) and lung dynamic compliance (Cldyn) were recorded following the increasing concentration of methacholine for latter analysis. The R-area of Ri and Re, the area between the peak value and baseline during a 250-s period and the lowest value of Cldyn could represent the lung function in mice.

2.11. Real-time PCR analysis

0.1 g lung tissue was separated from the lung of each mouse and preserved in frozen pipe for real-time PCR analysis

After collection of lung tissues, total RNA was extracted from lung tissues and immediately performed reverse transcription. Then the mRNA levels of T-bet, GATA-3, Foxp3 and ROR γ t were quantified using qPCR (BIO-RAD CFX96, USA). The primer sequences were as follows: T-bet, 5'-TTCCCATTCCTGCTTCAC-3' and 5'-CCACATCCACAAACATCCTG-3'; GATA-3, 5'-GGAACTCCGT-CAGGGCTA-3' and 5'-AGAGATCCGTGCAGCAGAG-3'; Foxp3, 5'-CCTGCCTTGGTACATTCGTG-3' and 5'-TGTTGTGGGTGAGTGCTTGTG-3'; ROR γ t, 5'-TGAGGCCATTGATATGTGG-3' and 5'-CTTCCAT-TGCTCCTGCTTC-3'; GAPDH, 5'-TGTGTCCGTCTGGATCTGA-3' and 5'-CCTGCTTCACCACCTTCTTGAT-3'.

2.12. Statistical analysis

Experimental data were showed as mean $\pm$ SEM. Statistical graphs were generated using GraphPad Prism 5.0 (San Diego, CA, USA) and data analyses were carried out using SPSS 13.0. The significance of differences among groups was assessed by one-way ANOVA analysis by a Tukey test. A $p < 0.05$ was regarded as significant and $p < 0.01$ was regarded as extremely significant.

3. Results

3.1. Characteristics and analysis of nAl₂O₃

γ -nAl₂O₃ has a large specific surface, strong heat resistance, good formability, strong surface acidity and superior metals dispersion, and could be uniformly dispersed in deionized water after stirring (Alade and RahmanSaleh, 2019; Saleh Tawfik et al., 2019; Saleh Tawfik and Islam, 2018). As shown in Fig. 1A, the scanning electron microscope showed that the γ -nAl₂O₃ was aggregated into flocculent shape. Fig. 1B showed that the particle size was consistent with that on the drug label (<50 nm), and dispersed in deionized water, indicating that the drug was qualified and could be used for experiments.

3.2. nAl₂O₃ increased IL-4 and IL-17A levels, and decreased IFN- γ and IL-10 levels

To assess the type of immune response in terms of cytokine levels, IFN- γ , IL-4, IL-10 and IL-17 were measured in lung. As showed in Fig. 2, it was suggested that the concentrations of IFN- γ

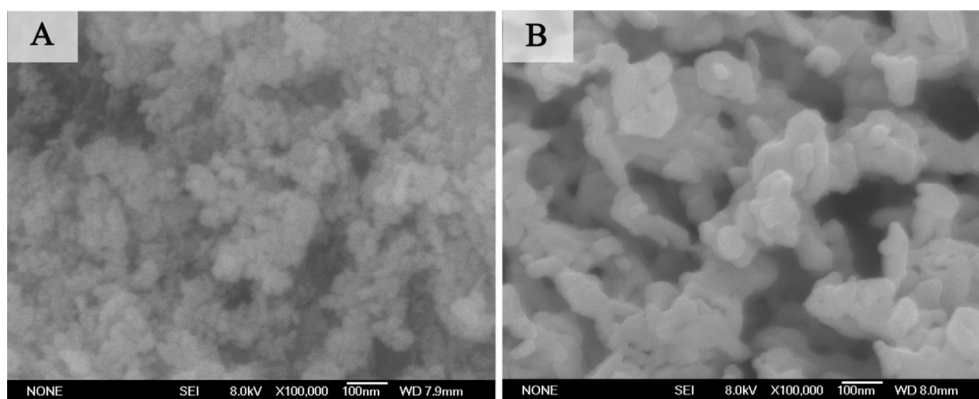


Fig. 1. Characteristics and analysis of nAl_2O_3 . (A) Scanning electron microscope image of nAl_2O_3 . (B) Scanning electron microscope image of nAl_2O_3 suspension.

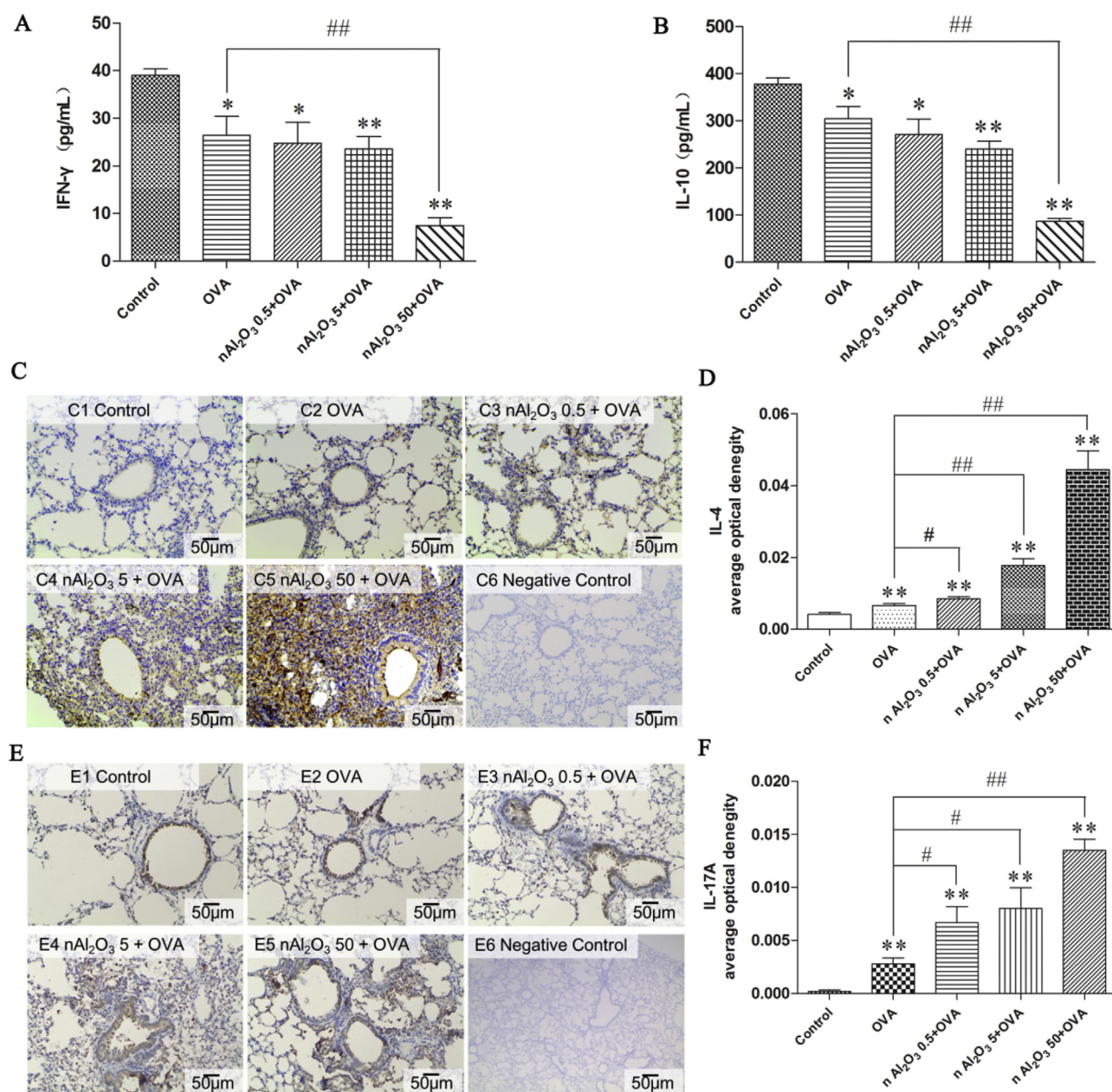


Fig. 2. Effects of nAl_2O_3 on the levels of IFN- γ , IL-4, IL-10 and IL-17A. (A) Concentrations of IFN- γ in BALF. (B) Concentrations of IL-10 in BALF. (C) Immunohistochemistry for IL-4 in lung. (D) IL-4 expression levels according to the average optical density. (E) Immunohistochemistry for IL-17A in lung. (F) IL-17A expression levels according to the average optical density. For A-F, *: $p < 0.05$, **: $p < 0.01$, compared with the control group. #: $p < 0.05$, ##: $p < 0.01$, compared with the OVA group.

and IL-10 in all OVA-sensitized nAl_2O_3 groups decreased compared with the control group ($p < 0.05$). Among nAl_2O_3 + OVA groups, 50 mg kg^{-1} nAl_2O_3 in the presence of OVA significantly decreased the level of IFN- γ and IL-10 compared with the OVA group ($p < 0.01$). On the contrary, the level of IL-4 and IL-17A in lung observed by immunohistochemical increased significantly in all OVA-sensitized nAl_2O_3 groups compared with the control group, and significantly increased in nAl_2O_3 5 + OVA and nAl_2O_3 50 + OVA groups compared with the OVA group ($p < 0.05$).

3.3. nAl_2O_3 inhibited the mRNA expression levels of T-bet/GATA-3 and Foxp3/ROR γ t

For A-F, *: $p < 0.05$, **: $p < 0.01$, compared with the control group.

To make it clear that the regulating role of type-2 and type-17 immune response in the exposure of nAl_2O_3 in the presence of OVA, real-time quantitative PCR was applied to detect the mRNA expression of T-bet, GATA-3, Foxp3 and ROR γ t, which was major transcription factors for the differentiation of Th1, Th2, Treg and Th17 cells, respectively (Fig. 3). Results showed that the expression level of GATA-3 and ROR γ t in OVA-combined with 5 mg kg^{-1} and 50 mg kg^{-1} nAl_2O_3 groups increased significantly, whereas the expression level of T-bet significantly decreased compared with the control group ($p < 0.05$). The expression level of Foxp3 did not change evidently in all OVA-sensitized groups ($p > 0.05$). It was worth mentioning that all OVA-sensitized nAl_2O_3 groups displayed markedly increased in the ratio of T-bet to GATA-3 and Foxp3 to ROR γ t compared with the control group ($p < 0.05$). Especially, 5 mg kg^{-1} and 50 mg kg^{-1} nAl_2O_3 in the presence of OVA extremely increased in the level of T-bet/GATA-3 compared with the control group ($p < 0.01$).

3.4. nAl_2O_3 enhanced serum T-IgE levels and TGF- β , IL-1 β and IL-6 in lung

For A-E, *: $p < 0.05$, **: $p < 0.01$, compared with the control group. #: $p < 0.05$, ##: $p < 0.01$, compared with the OVA group.

As showed in Fig. 4A, the concentrations of T-IgE significantly increased in all OVA-sensitized nAl_2O_3 groups compared with the control group and OVA group (Fig. 4A, $p < 0.01$). To further explore the potential mechanisms associated with nAl_2O_3 , TGF- β , IL-1 β and IL-6 levels in mice lung were measured. Results demonstrated that with the increase dose of nAl_2O_3 , there were varying degrees of increase in TGF- β , IL-1 β and IL-6 levels. The expression level of TGF- β significantly increased in all OVA-sensitized nAl_2O_3 groups compared with the control group and OVA group (Fig. 4D, E, $p < 0.05$). At the same time, it was found that nAl_2O_3 5 + OVA and nAl_2O_3 50 + OVA groups displayed significant increase in IL-1 β level compared with the control group and OVA group (Fig. 4B, $p < 0.05$). However, the exposure of nAl_2O_3 caused a significant increase in IL-6 level only when mice were treated in OVA-sensitized 50 mg kg^{-1} nAl_2O_3 compared with the control group and OVA group (Fig. 4C, $p < 0.01$).

3.5. nAl_2O_3 deteriorated effects on airway remodeling and the elimination effects of vit E

MT staining. Red arrow: bronchial remodeling, black arrow: lung tissue cell infiltration and green arrow: subepithelial collagen deposition (blue colored stain). (C) Automated Optical Inspection of MT staining.

For C1, **: $p < 0.01$, compared with the control group; ##: $p < 0.01$, compared with the OVA group. For C2, *: $p < 0.05$, compared with the nAl_2O_3 0.5 + OVA; ##: $p < 0.01$, compared with the nAl_2O_3 5 + OVA; &&: $p < 0.01$, compared with the nAl_2O_3 50 + OVA.

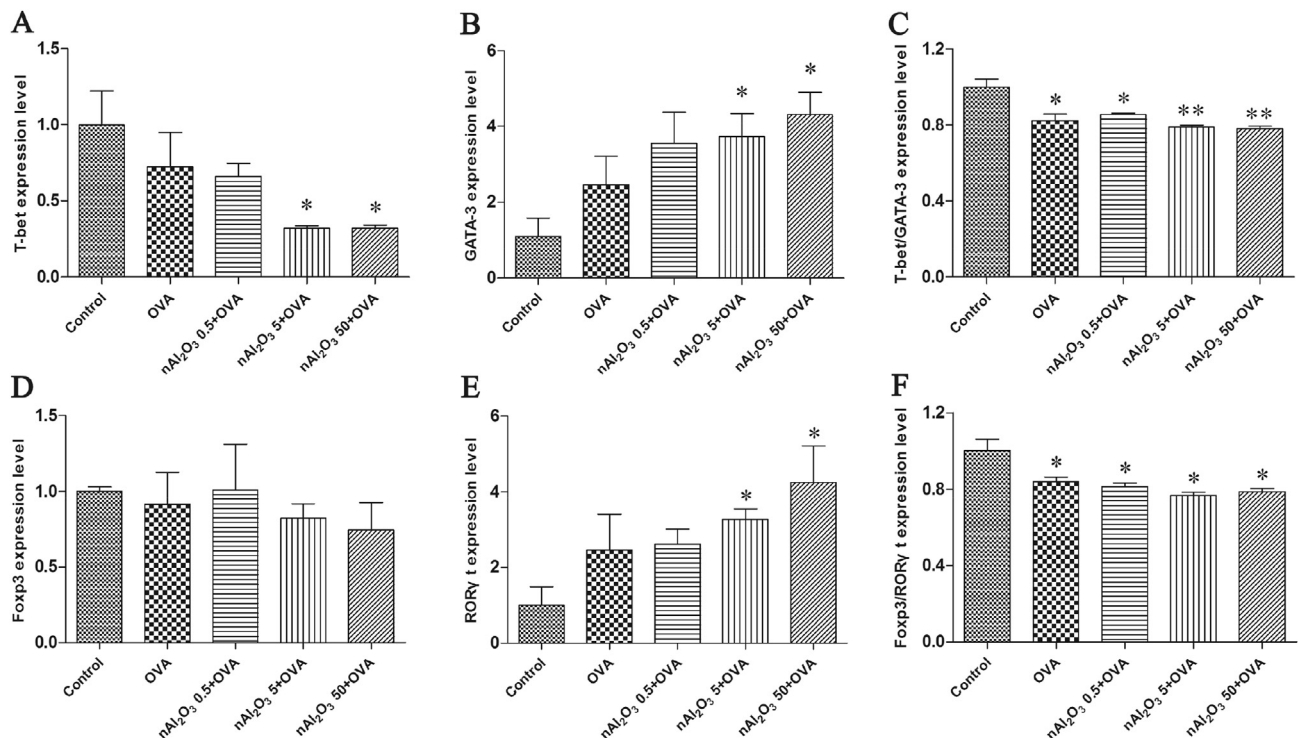


Fig. 3. Effects of nAl_2O_3 on the mRNA expression levels of T-bet/GATA-3 and Foxp3/ROR γ t in lung by real-time PCR. The mRNA expression levels of (A) T-bet, (B) GATA-3, (C) T-bet/GATA-3, (D) Foxp3, (E) ROR γ t and (F) Foxp3/ROR γ t, respectively.

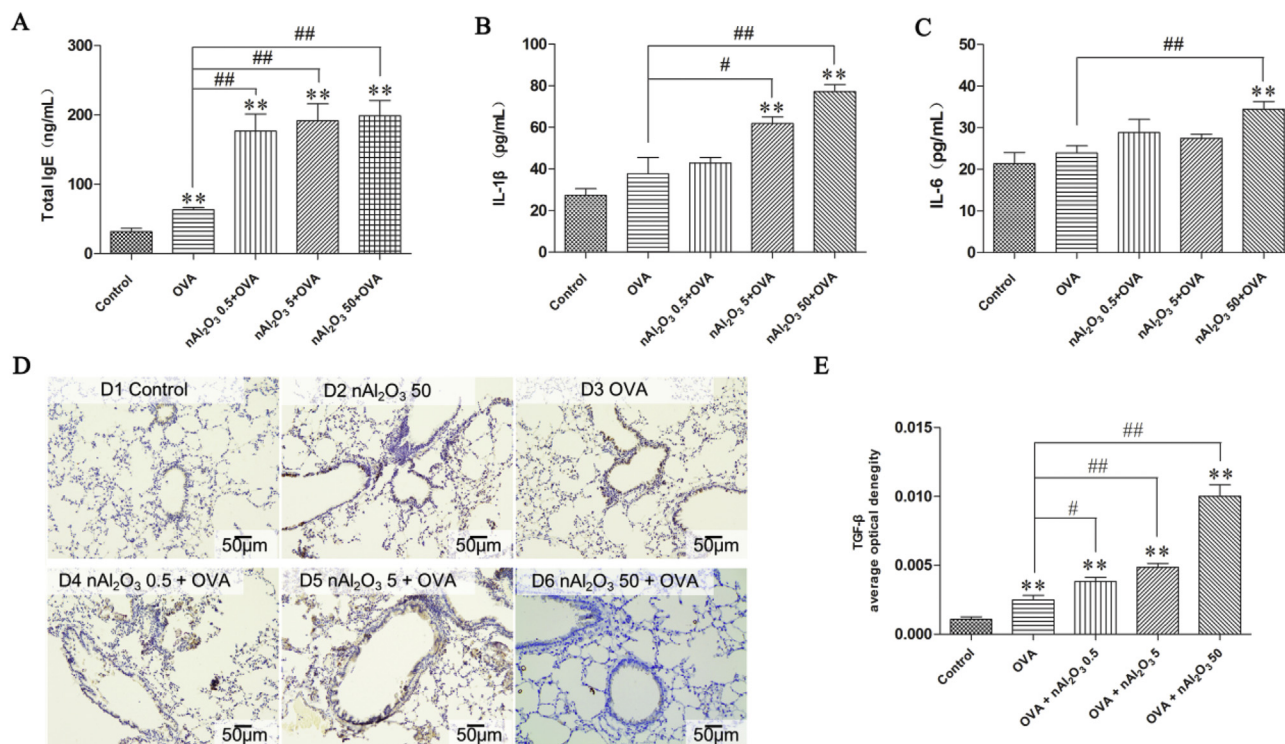


Fig. 4. Effects of nAl_2O_3 on serum T-IgE levels and TGF- β , IL-1 β and IL-6 in lung. (A) Concentrations of T-IgE. (B) Concentrations of IL-1 β . (C) Concentrations of IL-6. (D) Immunohistochemistry for TGF- β in lung. (E) TGF- β expression levels according to the average optical density.

In this study, both hematoxylin and eosin (H&E) and Masson's trichrome (MT) staining were used in that H&E staining can indirectly represent changes in airway structure of mice for visualizing airway remodeling and MT staining indirectly reveals tissue collagen fibrosis. As illustrated in Fig. 5A, B and 5C1, more serious histopathological changes in lung were observed in OVA-sensitized asthma model group compared with the control group and the exposure of nAl_2O_3 in the presence of OVA revealed evidently worsen histological changes in lung tissues compared with the OVA group. Moreover, as the concentration of nAl_2O_3 was higher, the airway wall got thicker, airway lumen was more and more shrinking, airway collagen deposition and lung tissue fibrosis as well as inflammatory cells infiltration in surrounding peribronchiolar areas were more and more serious ($p < 0.01$). In Fig. 5A, B and 5C2, Vit E could relieve deteriorated histopathological changes in nAl_2O_3 0.5 + OVA ($p < 0.05$), and significantly reduce deteriorated histopathological changes in nAl_2O_3 5 + OVA and nAl_2O_3 50 + OVA ($p < 0.01$).

For A, *: $p < 0.05$, **: $p < 0.01$, compared with the control group. #: $p < 0.05$, ###: $p < 0.01$, compared with the OVA group. For B, *: $p < 0.05$, compared with the nAl_2O_3 5 + OVA group; #: $p < 0.05$, ###: $p < 0.01$, compared with the nAl_2O_3 50 + OVA group.

As showed in Fig. 6A, 0.5 mg kg^{-1} nAl_2O_3 combined with OVA increased the number of eosinophils and lymphocytes compared with the control group ($p < 0.05$), and 5 mg kg^{-1} and 50 mg kg^{-1} nAl_2O_3 combined with OVA evidently increased the numbers of total cells, eosinophils and lymphocytes compared with the control and OVA group ($p < 0.01$). It is interesting that the number of neutrophils exceedingly increased only in nAl_2O_3 50 + OVA group compared with the control group ($p < 0.01$), increased in nAl_2O_3 50 + OVA group compared with the OVA group ($p < 0.05$). However, the degree of airway remodeling was markedly reduced in nAl_2O_3 5 + OVA + Vit E and nAl_2O_3 50 + OVA + Vit E groups (Fig. 6B). As showed in Figure 6B2 and 5B4, Vit E could relieve the number of

lymphocytes in nAl_2O_3 5 + OVA and nAl_2O_3 50 + OVA groups, and the number of neutrophils in nAl_2O_3 50 + OVA group ($p < 0.05$).

3.6. nAl_2O_3 exacerbated effects on AHR in mice and the protective effects of vit E

For A, *: $p < 0.05$, **: $p < 0.01$, compared with the control group. #: $p < 0.05$, ###: $p < 0.01$, compared with the OVA group. For C, *: $p < 0.05$, **: $p < 0.01$, compared with the nAl_2O_3 0.5 + OVA group. For D, *: $p < 0.05$, **: $p < 0.01$, compared with the nAl_2O_3 5 + OVA group. For E, *: $p < 0.05$, **: $p < 0.01$, compared with the nAl_2O_3 0.5 + OVA group.

In order to detect the lung function in mice, AHR was measured by methacholine (MCH) challenge assay and three parameters including the inspiratory resistance (Ri), expiratory resistance (Re) and lung dynamic compliance (Cldyn) were recorded after each injection of MCH. With the increasing dose of MCH, the curves of Ri and Re showed continuous upward trends. On the contrary, the Cldyn curve continued to decline. In this study, the sensitization of OVA significantly damaged lung function in mice and the exposure of nAl_2O_3 in the presence of OVA significantly increased the values of Ri and Re, and decreased the value of Cldyn compared with the control and OVA group (Fig. 7A, $p < 0.05$). Interestingly, intragastric administration of Vit E significantly recovered the values of Ri, Re and Cldyn, and sequentially relieved the damage of lung function induced by nAl_2O_3 and OVA in mice (Fig. 7C, D, E, $p < 0.05$).

3.7. nAl_2O_3 exacerbated effects on oxidative stress and the blocking effects of vit E in lung

For A and B, *: $p < 0.05$, **: $p < 0.01$, compared with the control group. #: $p < 0.05$, ###: $p < 0.01$, compared with the OVA group. For C and D, *: $p < 0.05$, **: $p < 0.01$, compared with the nAl_2O_3 0.5 + OVA group. #: $p < 0.05$, ###: $p < 0.01$, compared with the

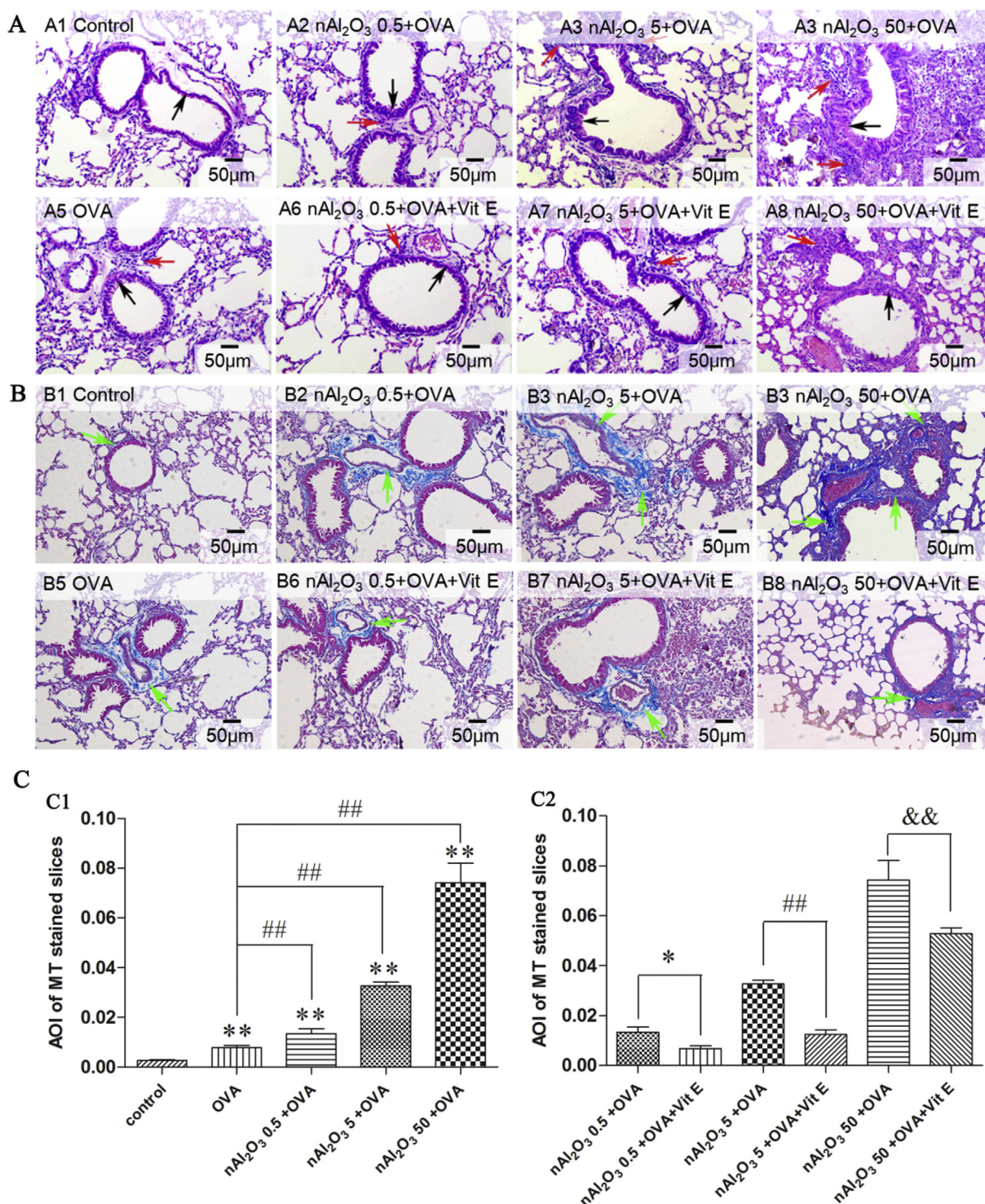


Fig. 5. Effects of nAl₂O₃ on lung histopathology and the elimination effects of Vit E. (A) H&E staining. (B).

nAl₂O₃ 5 + OVA group; &: $p < 0.05$, &&: $p < 0.01$, compared with the nAl₂O₃ 50 + OVA group.

The degree of oxidative damage was detected by ROS and GSH levels in lung of mice. As shown in Fig. 8A, the levels of ROS significantly increased in all OVA-sensitized groups compared with the control group. Meanwhile, 5 mg kg⁻¹ and 50 mg kg⁻¹ nAl₂O₃ combined with OVA evidently increased the levels of ROS compared with the OVA group ($p < 0.05$). Conversely, the contents

of GSH in all OVA-treatment groups evidently decreased compared with the control group and the exposure of 50 mg kg⁻¹ nAl₂O₃ in the presence of OVA significantly decreased GSH contents compared with the OVA group ($p < 0.05$). The addition of antioxidant Vit E significantly reduced ROS levels in combined-OVA 0.5 mg kg⁻¹, 5 mg kg⁻¹ and 50 mg kg⁻¹ nAl₂O₃ groups and increased GSH contents in nAl₂O₃ 50 + OVA group (Fig. 8C, D, $p < 0.05$).

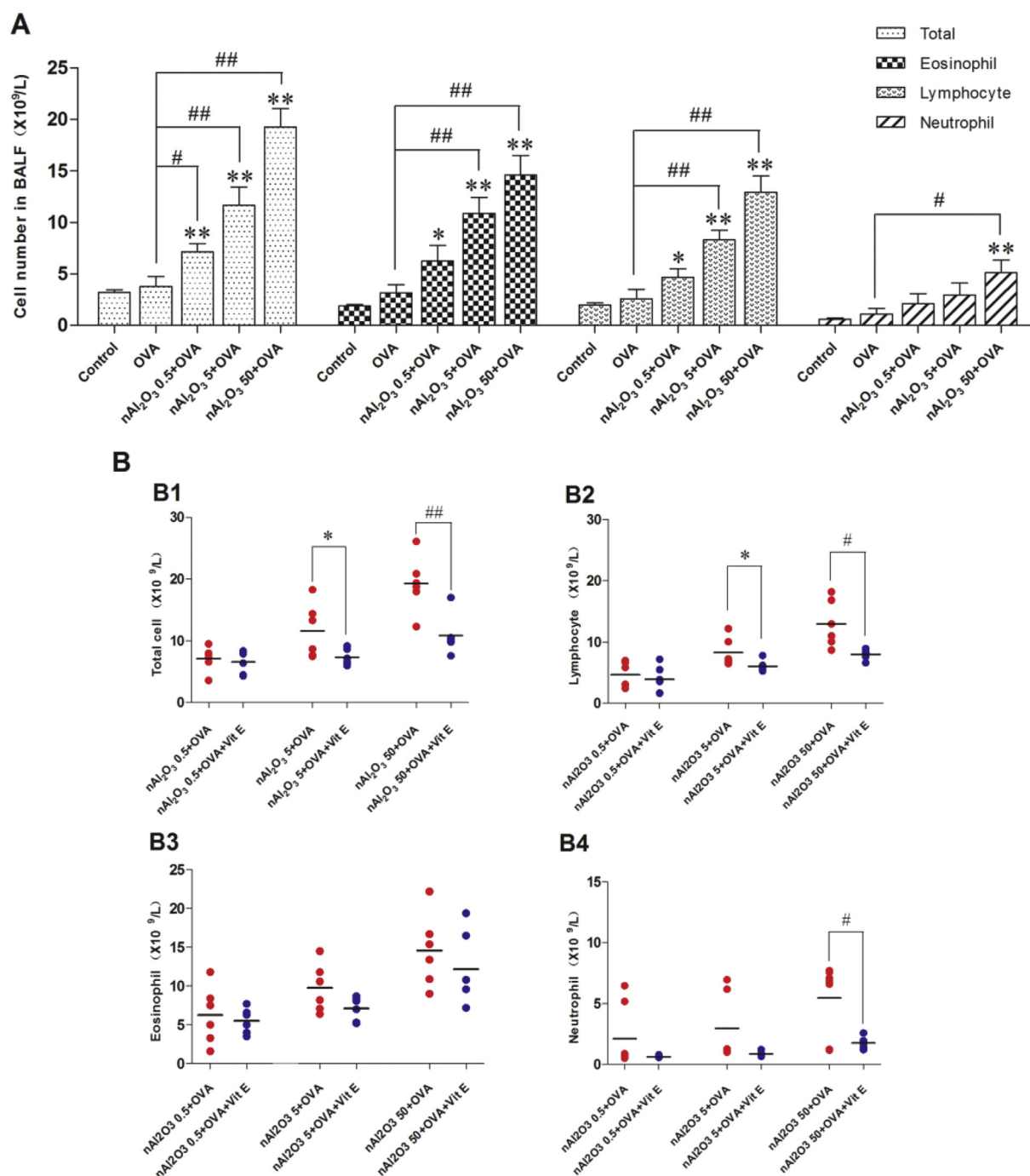


Fig. 6. Effects of nAl_2O_3 on inflammatory cell infiltration in BALF and the protective effects of Vit E. (A) Inflammatory cell infiltration. (B) The protective effects of Vit E on inflammatory cell infiltration including (B1) total cell, (B2) lymphocyte, (B3) eosinophil and (B4) neutrophil, respectively.

3.8. The treatment of vit E relieved T-IgE and cytokines levels

To explore the protective effects of Vit E, the levels of T-IgE as well as cytokines including IFN- γ , IL-4, IL-10, IL-17A, TGF- β and IL-1 β were detected. Results suggested that T-IgE levels in serum showed a decreased trend in all nAl_2O_3 + OVA groups after oral administration of Vit E (Fig. 9A, $p > 0.05$). Simultaneously, there was an increase in IFN- γ contents only in 50 mg kg^{-1} nAl_2O_3 + OVA + Vit E group compared with the nAl_2O_3 50 + OVA group, while IL-10 contents increased in all OVA-sensitized nAl_2O_3 groups with the application of Vit E (Fig. 9C, D, $p > 0.05$). On the

contrary, treatment of Vit E significantly decreased IL-4 levels in nAl_2O_3 5 + OVA and nAl_2O_3 50 + OVA groups, and evidently decreased IL-17A levels in nAl_2O_3 50 + OVA group through lung immunohistochemistry assay and analysis (Fig. 9C, E, F, G, H, $p < 0.05$). At the same time, the addition of Vit E evidently decreased TGF- β levels in all nAl_2O_3 + OVA groups and significantly decreased IL-1 β levels in nAl_2O_3 5 + OVA and nAl_2O_3 50 + OVA groups (Fig. 9B, I, J, $p < 0.05$).

For A–J, @: $p < 0.05$, compared with the nAl_2O_3 0.5 + OVA group; **: $p < 0.01$, compared with the nAl_2O_3 5 + OVA group; ##: $p < 0.01$, compared with the nAl_2O_3 50 + OVA group.

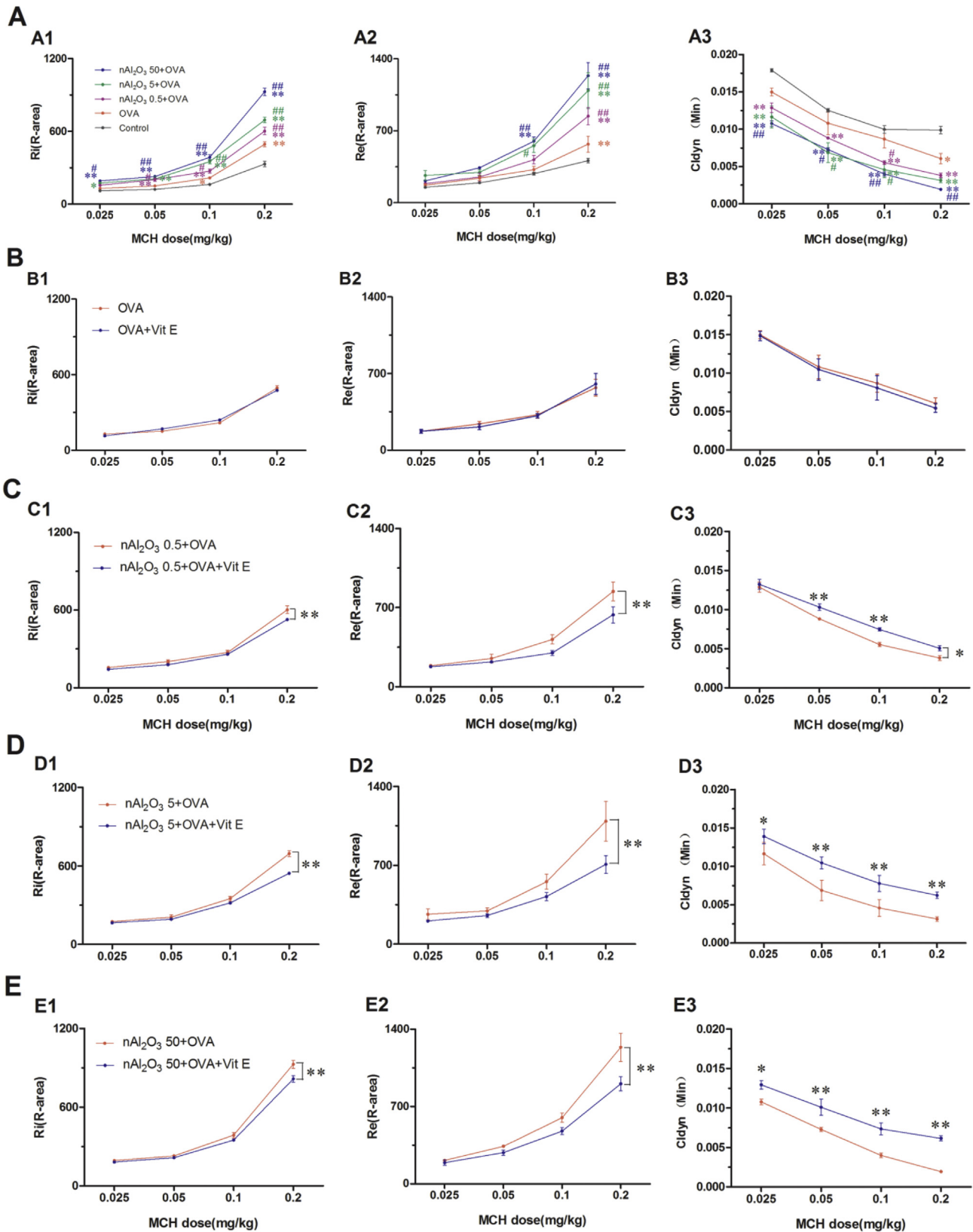


Fig. 7. Effects of nAl₂O₃ on AHR and the protective effects of Vit E in Balb/c mice. (A1–A3) Ri, Re, and Cldyn values of the control, OVA, and OVA+ nAl₂O₃ groups, respectively. (B1–B3) Ri, Re, and Cldyn values of OVA and OVA + Vit E groups, respectively. (C1–C3) Ri, Re, and Cldyn values of nAl₂O₃ 0.5 + OVA and nAl₂O₃ 0.5 + OVA+ Vit E groups, respectively. (D1–D3) Ri, Re, and Cldyn values of nAl₂O₃ 5 + OVA and nAl₂O₃ 5 + OVA+ Vit E groups, respectively. (E1–E3) Ri, Re, and Cldyn values of nAl₂O₃ 50 + OVA and nAl₂O₃ 50 + OVA+ Vit E groups, respectively.

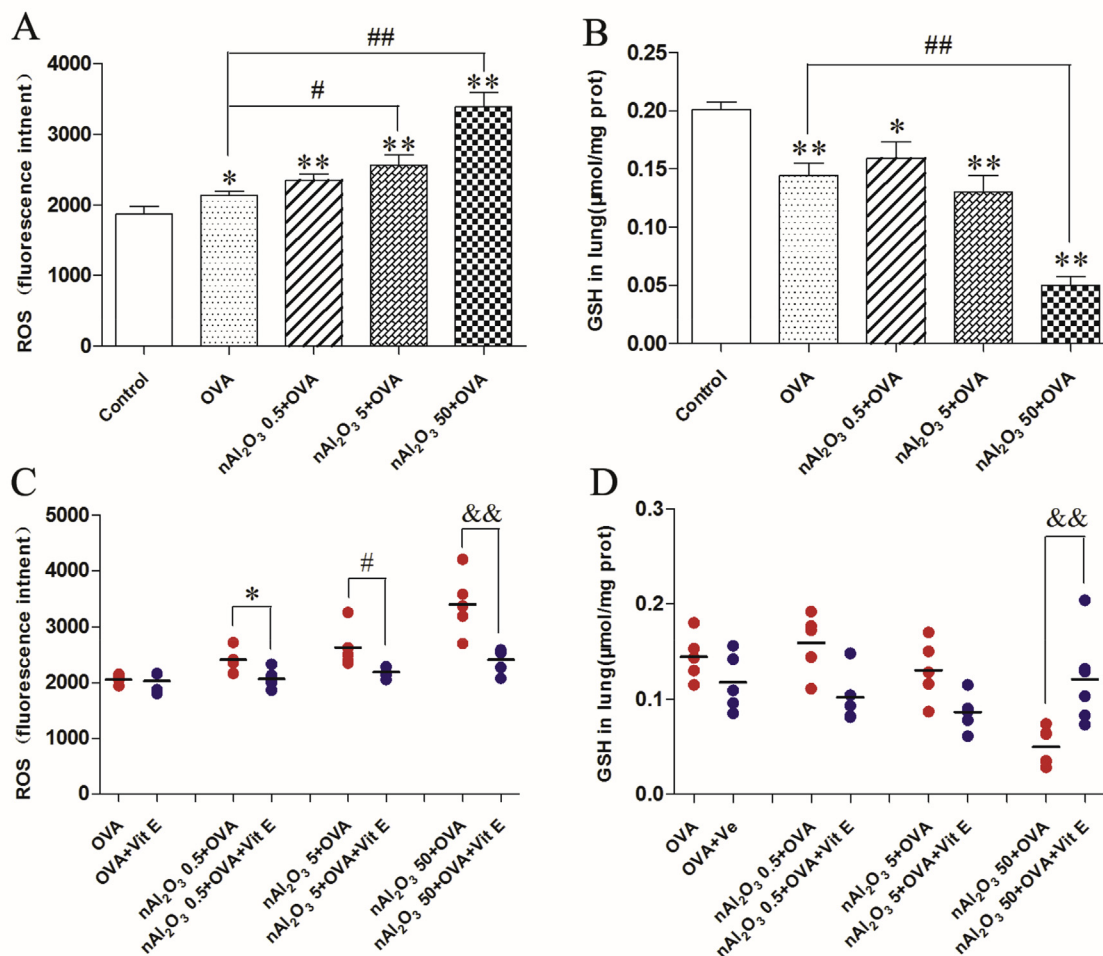


Fig. 8. Effects of nAl₂O₃ on oxidative stress in lung and the blocking effects of Vit E. (A) ROS levels. (B) Concentrations of GSH. (C) The blocking effects of Vit E on ROS levels. (D) The blocking effects of Vit E on GSH concentrations.

4. Discussion

Related studies have shown that exposure to nanomaterials such as carbon nanotubes (CNTs) and titanium dioxide nanoparticles (TiO₂-NPs) can lead to damage to lung function and deterioration of asthma (Shuai et al., 2017; Jinquan et al., 2014; Park et al., 2015; Kinaret et al., 2017; Hussain et al., 2011). Up to date, however, reports on the respiratory toxicity caused by nanomaterials is not sufficient and needs to be further explored (Meldrum et al., 2017). Meanwhile, studies have revealed that nAl₂O₃ can cause lung tissue damage *in vitro* and *in vivo* (Simondeckers et al., 2008; Lin et al., 2014). Based on these studies, the purpose of this study is to discover the relationship between nAl₂O₃ and asthma as well as the underlying molecular mechanism. At present, many animals could be used to establish the asthma model, such as rats, mice, rabbits and dogs. The establishment of asthma model is mainly divided into two stages, including sensitization and stimulation. Ovalbumin (OVA), pollen, aphid eggs and fungal spores are the most widely used inducers for the establishment of allergic asthma model. Among them, OVA is more immunogenic, easy to obtain, and cheap (Hongzhen and Cheng, 2009). Studies found that Balb/c mice are an ideal animal model of allergic asthma, and the successful establishment of animal asthma models is closely related to the concentration of OVA (Li et al., 2005; Xiaodong et al., 2012). Therefore, we established allergic asthma model with intraperitoneal injections for 3 times

and continuous stimulation for 7 days of OVA in Balb/c mice (Wei et al., 2018). The symptoms of most animal asthma models are similar to that of humans, including AHR (airway hyper-responsiveness), increased airway mucus and other inflammatory response (Hongzhen and Cheng, 2009). T-IgE levels are specific markers for allergic asthma and play an important role in clinical diagnosis (Sears et al., 1991b). In this study, the significant increases in T-IgE and AHR levels as well as lung remodeling and inflammation response in the OVA group demonstrated successful establishment of the asthma model sensitized by OVA. Simultaneously, exposure to nAl₂O₃ further increased the levels of T-IgE and AHR, which aggravated the allergic reaction of asthma.

Airway remodeling is generally considered to be a key feature of chronic airway inflammation, hence the observation on lung histopathology in Balb/c mice is essential. Our study found that with the dose increase of nAl₂O₃, the degree also gradually increased for the airway remodeling and inflammatory cell infiltration in OVA-induced asthma mice, making lung airway inflammation aggravated in mice. AHR is a critical hallmark of lung disease such as asthma, and the R-areas of the expiratory and inspiratory resistances (Ri and Re) observed here provided explanation for the change in large airways, while the change of Cldyn indicates the variation in the small airways or the parenchyma, respectively (Drazen et al., 1999; Qiao et al., 2009; Shah et al., 2015b). In this study, the significant increases of Ri and Re, and the evident decrease of Cldyn in the OVA group demonstrated reduced lung

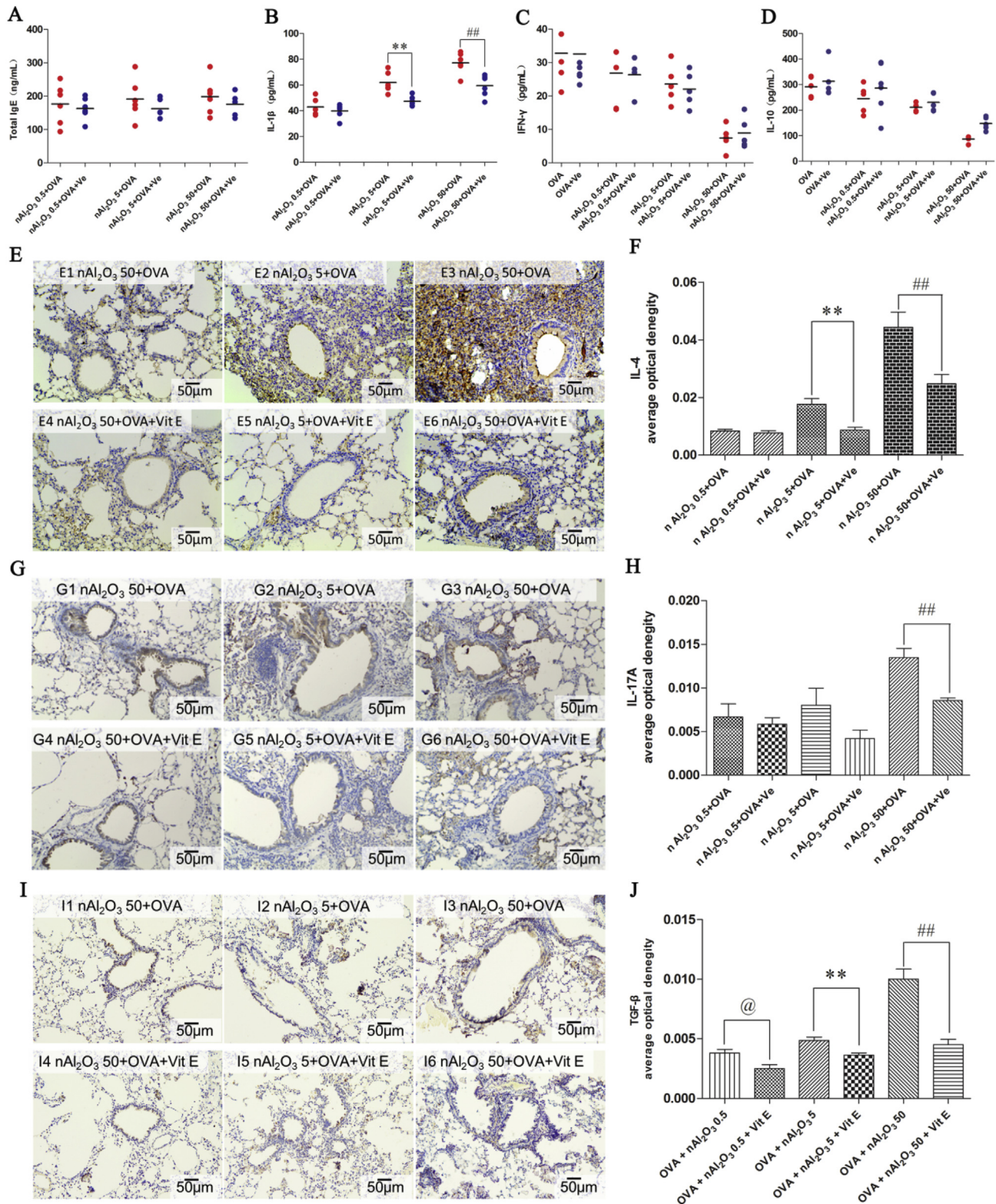


Fig. 9. The elimination effects of Vit E on T-IgE and cytokines levels. (A) T-IgE on serum, (B) IL-1 β in lung, (C) IFN- γ in BALF, (D) IL-10 in BALF, (E) Immunohistochemistry for IL-4 in lung, (F) IL-4 expression levels according to the average optical density, (G) Immunohistochemistry for IL-17A in lung, (H) IL-17A expression levels according to the average optical density, (I) Immunohistochemistry for TGF- β in lung, (J) TGF- β expression levels according to the average optical density.

function in mice, and exposure to nAl₂O₃ further enhanced respiratory resistance and decreased lung compliance, resulting in further deterioration of lung function in mice.

Related studies have displayed that nAl₂O₃ can produce reactive

oxygen species (ROS) both *in vivo* and *in vitro*, leading to the emergence of oxidative stress (Lin et al., 2014; Lushchak, 2014). Oxidative stress is defined as the imbalance between the production and elimination of ROS (Cho and Moon, 2010). In this study, it

was found that the levels of oxidative stress in mice lung tissue gradually elevated with the dose increase of nAl_2O_3 . Vit E is an important antioxidant, which blocks the free radical chain reaction and reduces oxidative damage by reducing ROS levels (Korotkova et al., 2004; Jiyan et al., 2007; Frossi et al., 2008). Here Vit E was used as a blocker of oxidative stress for mice. It was found that Vit E at a dose of $100 \text{ mg kg}^{-1} \cdot \text{day}^{-1}$ could reduce the degree of airway remodeling, fibrosis and inflammation in mice lung tissue by relieving oxidative stress induced by nAl_2O_3 , thereby reversing AHR, and finally effectively antagonizing the respiratory toxicity of nAl_2O_3 .

Oxidative stress is inextricably linked to the Th2-type immune response (King et al., 2006a; Rijt et al., 2017; King et al., 2006b; Peterson et al., 1998; Kuo et al., 2001). It is widely accepted that the Th2-type immune response plays a crucial role in the pathogenesis of allergic asthma (Ano et al., 2013). Moreover, recent studies have shown that Th17 immune response is also closely related to asthma (Poynter, 2016; Martin et al., 2014). Transcription factors, namely T-bet, GATA-3, Foxp3, and ROR γ t, are crucial for the differentiation of naive CD4^+ T cells into Th1, Th2, Treg and Th17 cells, respectively (Ivanov et al., 2006). This study demonstrated that nAl_2O_3 could stimulate the overexpression of GATA-3 and ROR γ t mRNA levels, and decreased the expression of T-bet and Foxp3, and thus promoted the differentiation of Th2 and Th17 cells while inhibited the differentiation of Th1 and Treg cells. IL-4 is the main cytokine released by Th2 type cells in allergic asthma, while interferon- γ (IFN- γ), the main cytokine released by Th1 type cells, has a down-regulating effect on Th2 type immune response (Ano et al., 2013). On the other hand, IL-17A and IL-10 are the major cytokines released by Th17 cells and Treg cells, respectively (Afzali et al., 2007). The results in this study showed that exposure to nAl_2O_3 could result in a decrease in IFN- γ , IL-10 levels and an increase in IL-4 and IL-17A levels. Since that IL-4 can promote the differentiation of Th2 cells, and TGF- β , IL-1 β and IL-6 together can promote the differentiation of Th17 cells, both contribute to the regulation of the immune response for asthma (Sears et al., 1991a; Newcomb and Peebles, 2013), the increase in levels of IL-6, TGF- β and IL-1 β found in lung tissue in nAl_2O_3 exposed group from this study, indicated that intratracheal instillation of nAl_2O_3 could promote the differentiation of naive CD4^+ T cells into Th2 type and Th17 type cells, while inhibit the differentiation of Th1 type and Treg type cells. In both mRNA and protein levels, this study has demonstrated that nAl_2O_3 could stimulate the overexpression of GATA-3 and ROR γ t, resulting in massive differentiation of Th2 and Th17 cells as well as large production of their corresponding cytokines, which ultimately exacerbated the imbalance of Th1/Th2 and Treg/Th17 immune response.

nAl_2O_3 can aggravate the inflammation of allergic asthma in a dose-dependent manner by affecting the infiltration of multiple inflammatory cells. Based on well-known facts that both eosinophils recruited by cytokines such as IL-4 generated by Th2 cells, and neutrophils recruited by cytokines produced by Th17 cells can aggravate the airway inflammation (Jinfang et al., 2006; Liang et al., 2008), significant increase in the number of neutrophils in 50 mg kg^{-1} nAl_2O_3 exposed group, as well as significant increase in the number of eosinophils in 0.5 mg kg^{-1} nAl_2O_3 . As a result, it was found that exposure by intratracheal instillation to a certain dose of nAl_2O_3 could lead to the occurrence of oxidative stress and subsequent inflammatory response, which activated the differentiation of both Th2 type and Th17 type cells, following by an exacerbation of the immune imbalance in lung of asthma mice, with the symptoms such as elevated AHR, airway remodeling, increased inflammatory cytokines, and inflammatory cell infiltration in lungs worsened allergic asthma.

5. Conclusions

In this study, elevated serum T-IgE levels suggested the successful establishment of asthma model for the purpose of investigating the relationship between nAl_2O_3 and allergic asthma as well as its underlying mechanism. The study suggested that nAl_2O_3 exposed by intratracheal instillation aggravated asthma in mice with a dose-response manner. Results showed that exposure of nAl_2O_3 deteriorated AHR, airway remodeling and inflammation response, leading to lung function damage in mice. Exposure of nAl_2O_3 increased ROS levels and decreased GSH levels and Vit E could alleviate asthma-like symptoms through blocking oxidative stress. The study demonstrated that imbalance of Th1/Th2 and Treg/Th17 immune responses might simultaneously play a significant role in nAl_2O_3 -induced aggravated asthma at mRNA and protein levels. In addition, nAl_2O_3 could increase T-IgE levels and eosinophil infiltration induced by Th2-type immune response, neutrophil infiltration recruited by Th17-type immune response, aggravating asthma. The finding might provide a certain reference for the way of safer application for nAl_2O_3 and therapeutic targets of allergic asthma.

Conflicts of interest

The authors report no conflict of interest.

Acknowledgments

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