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# Nonylphenol can aggravate allergic rhinitis in a murine model by regulating important Th cell subtypes and their associated cytokines☆

Yun-xiu Wang[a], Zhao-wei Gu[b], Zhi-wei Cao[b], Li-ying Hao[c,*]

[a] Department of Medical Insurance, China Medical University Affiliated Shengjing Hospital, Shenyang City 110004, Liaoning Province, China
[b] Department of Otorhinolaryngology, China Medical University affiliated Shengjing Hospital, Shenyang City 110004, Liaoning Province, China
[c] Department of Pharmaceutical Toxicology, School of Pharmacy, China Medical University, Shenyang City 110122, Liaoning Province, China

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ABSTRACT

Nonylphenol (NP) is a widely distributed, toxic endocrine-disrupting chemical exhibiting estrogenic activity. However, its effect on allergic rhinitis (AR) remains unclear. In this study, the effects of NP on a murine model of AR were investigated. Mice were divided into ovalbumin (OVA), NP, and control groups. OVA was used for sensitization and challenge. Mice in the NP group were administered NP during the sensitization period. Allergic nasal symptoms and eosinophil counts in nasal mucosa were measured. Serum levels of OVA-specific IgE were determined by enzyme-linked immunosorbent assay. The mRNA levels of transcription factors of Th cells were determined with real-time polymerase chain reaction. Th cell subtypes and Treg numbers were counted with the aid of multi-color flow cytometry. Cytokine concentrations in nasal mucosa were determined using the cytometric bead array method. Subcutaneous injection of NP into mice exhibiting AR enhanced not only the nasal allergic symptoms, but also eosinophil infiltration and OVA-specific IgE. Moreover, NP upregulated IL-4, IL-5, IL-13, IL-9, IL-6 and IL-17, and downregulated IL-10, in the AR mouse model; IFN-γ and IL-23 were not affected. Transcription factors and Th cell percentages were evaluated to determine whether NP regulates Th cell subtypes in an AR mouse model. GATA3, PU.1, and RORγt levels were significantly increased, but FoxP3 and Helios were decreased. In addition, Th2, Th9, and Th17 subtype percentages significantly increased, and Treg cell percentages decreased, in NP administration groups; the percentage of Th1 subtypes was not affected. NP enhanced allergic inflammation in the AR mouse model through upregulation of Th2, Th9, and Th17 responses and negative regulation of Treg responses. These results suggest that NP may be trigger AR.

## 1. Introduction

Allergic rhinitis (AR) affects approximately 500 million people globally and is characterized by itching, sneezing, watery discharge, and congestion. AR patients may suffer from sleep disorders, emotional problems, or experience injuries while engaging in activities and routine social functions, and their quality of life is severely impaired. The prevalence of AR has increased at an alarming rate in recent years in both adults and children [1,2], and there is high risk of developing asthma with AR [3,4].

Many types of inflammatory cells including mast cells, $CD4^+$ T cells, B cells, macrophages, and eosinophils, infiltrate the nasal mucosa of AR patients [5]. The increased levels of Th2 transcription factors, and the rise in the $GATA^+$/T-$bet^+$ T cell ratio, support the traditional hypothesis that a Th2 imbalance plays an important role in the development of AR [6]. Recent studies found that Th9 and Th17 cells, and their associated cytokines, are also responsible for the pathogenesis of AR [7–13]. In addition, regulatory T (Treg) cells may be unable to inhibit Th2 responses in allergic individuals [14].

Endocrine-disrupting chemicals, a heterogeneous group of contaminants present in the environment and in food, may interfere with the endocrine and reproductive system of animals or human beings, even at low doses [15]. Nonylphenol (NP) is commonly used in detergents, emulsifiers, and solvents, and can thus accumulate in the environment, causing significant pollution [16,17]. In addition, NP has been shown to exhibit weak estrogenic activity [18], and, because of its lipophilic nature and long half-life, can bioaccumulate in vivo [19,20].

Accumulation of NP is known to affect the reproductive and endocrine systems in offspring rats [21]. In addition, NP has been shown to cause oxidative stress in the pancreas and impair liver glucose

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* Corresponding author at: No.77 Puhe Road, Shenyang North New Area, Shenyang, Liaoning Province, China.
*E-mail address:* hao_liying@126.com (L.-y. Hao).

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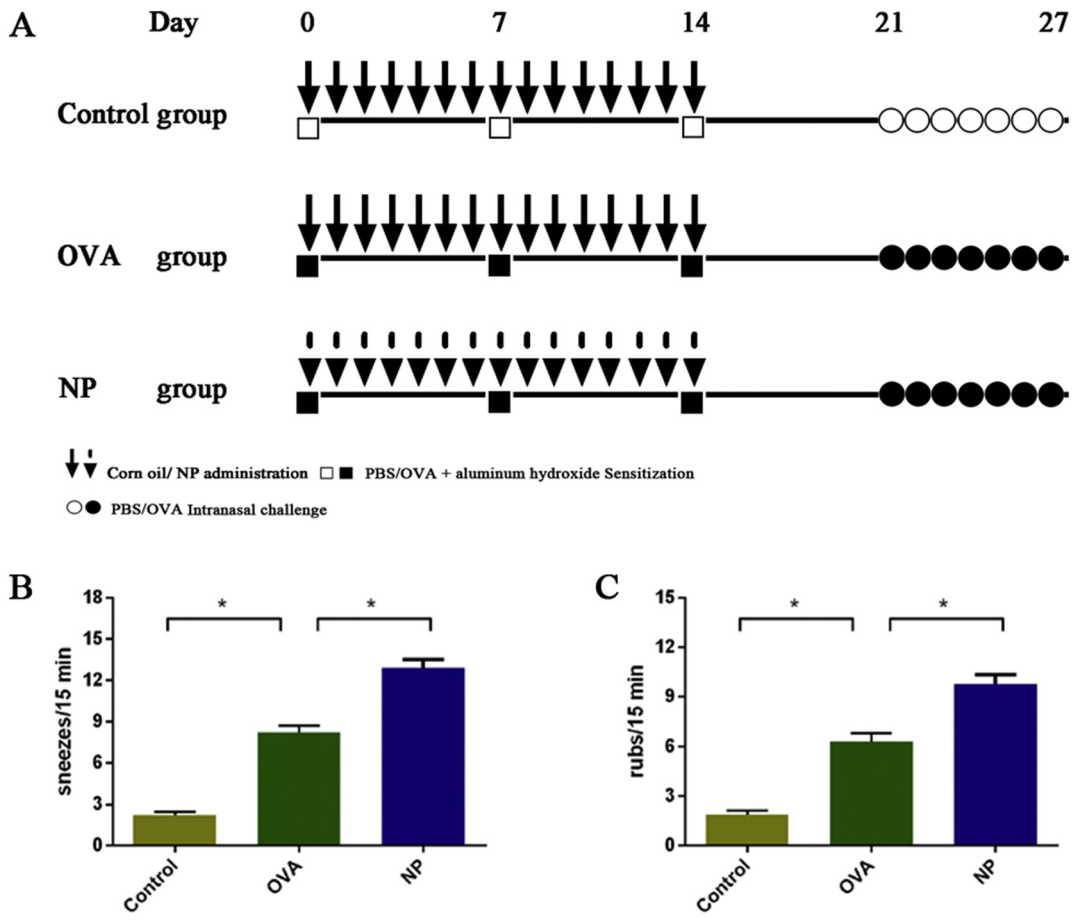


**Fig. 1.** Experimental protocol of the allergic rhinitis (AR) model in the present study (A). Briefly, on days 0, 7, and 14, the test animals (OVA and NP groups) were sensitized with 100 μg amounts of OVA and 2 mg amounts of aluminum hydroxide in 100 μL PBS via intraperitoneal injection (PBS only was injected into the controls). During the challenge, the mice in the OVA and NP groups received intranasal OVA for 7 consecutive days, i.e., from day 21 to 27, while the control group received PBS only. The NP group mice were subjected to subcutaneous injection of NP in corn oil from day 0 to day 14, while the OVA and control groups received only corn oil. The (B) sneezes and (C) nasal rubs were counted for 15 min after the final intranasal introduction of OVA (n = 10 in each group, *P < 0.05).

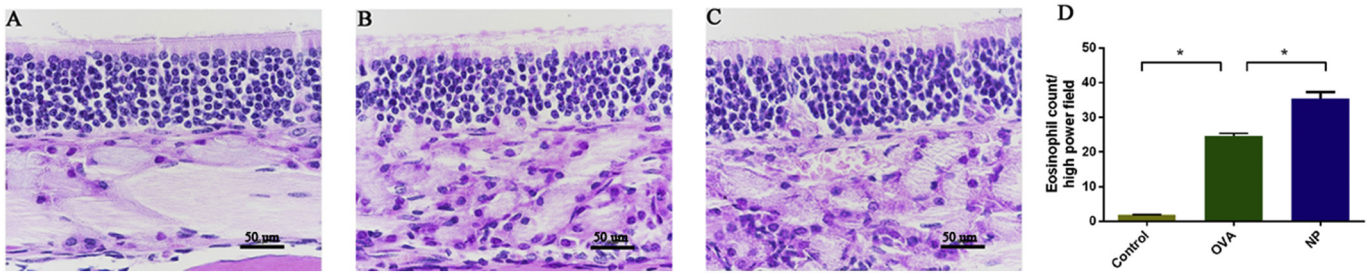


**Fig. 2.** Nasal mucosa histopathological results, eosinophil infiltration in the three groups (original magnification ×400). (A) Control, (B) OVA, (C) NP groups, (D) Eosinophil counts in nasal mucosa (n = 5 in each group, *P < 0.05).

metabolism [22], as well as having injurious effects on neurons. This is supported by the effect of NP on memory in mice, and inhibition of the ability to learn a novel task [23–25].

Recently, many studies have focused on effects of AR on the immune system, which plays a negative role in allergic disease such as asthma [26]. It remains unclear whether the endocrine disruptor NP plays a role in the occurrence of AR. In this report, we evaluated the potential role of NP in the pathogenesis of AR and explored the effect of NP on immune inflammation in an ovalbumin (OVA)-induced AR mouse model.

## 2. Materials and methods

### 2.1. AR murine model induced by OVA

Female BALB/c mice (eight week of age) which did not suffer from any murine specific pathogenic infections were purchased from experimental research center in Shengjing hospital affiliated to China Medical University (CMU). All animals exposed to an alternating light and dark cycles of 12 h can get complete access to diet and water. They did not receive any OVA in their food. Every experimental methodology was sanctioned from the Ethics Committee of Shengjing hospital affiliated to CMU. All reared animals were segregated into three cohorts with 10 mice, a control group, an OVA group, and an NP group. On

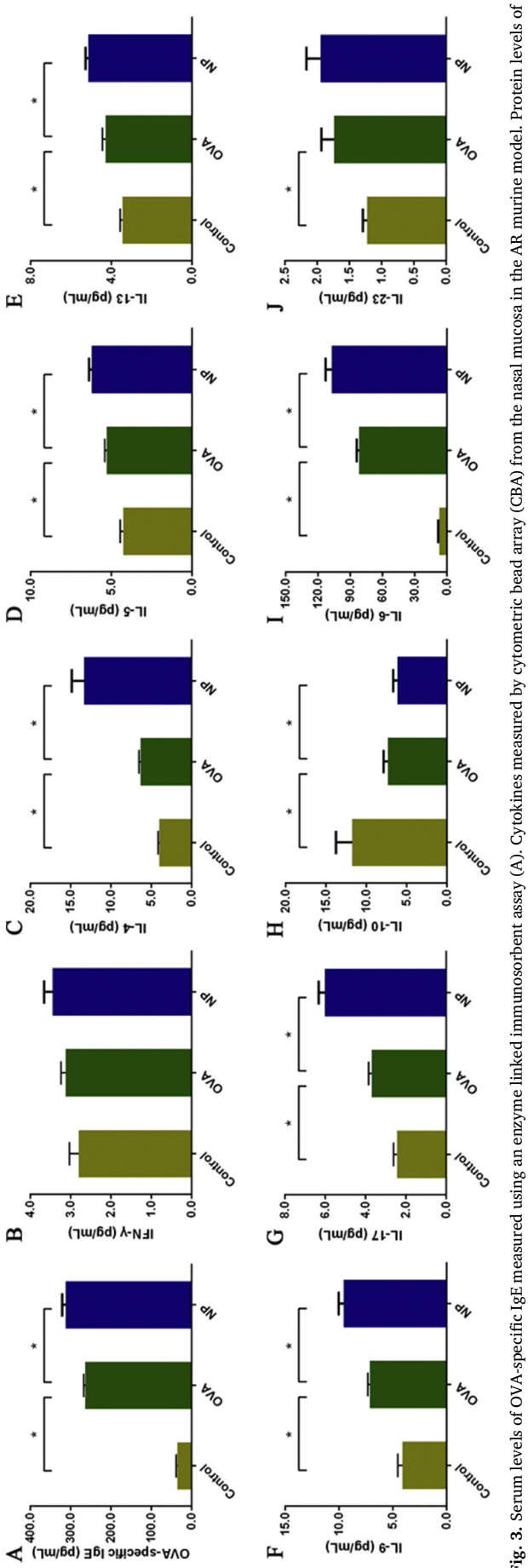


**Fig. 3.** Serum levels of OVA-specific IgE measured using an enzyme linked immunosorbent assay (A). Cytokines measured by cytometric bead array (CBA) from the nasal mucosa in the AR murine model. Protein levels of IFN-γ, IL-4, 5, 13, 9, 17, 10, 6, and 23 were analyzed by CBA (B-J, respectively) (n = 5 in each group, *P < 0.05).

days 0, 7, and 14, the test animals (OVA and NP groups) were sensitized with 100 μg OVA (Sigma-Aldrich, USA), 2 mg aluminum hydroxide in 100 μL PBS through an intraperitoneal injection. From day 21 to day 27, they were followed by daily introduction of 5 mg/mL OVA in 20 μL PBS through the nasal route. The control group was exposed to PBS.

NP group mice were also subjected to subcutaneous injection of 0.5 mg/kg/day of NP (Sigma-Aldrich) in corn oil from day 0 to 14, while the OVA and the control group received only corn oil (Fig. 1A).

### 2.2. Assessment of AR symptoms

The sneezes and nasal rubbing motions post final administration of intranasal OVA were recorded by four blinded observers. This observation was carried out on 27th day, during the tenure of 15-min post intranasal introduction of OVA. The average of the counts was then analyzed statistically.

### 2.3. Histopathology

All animals were euthanized two hours post last OVA challenge on day 27.5 animals from each group were beheaded and then put these heads into 4% paraformaldehyde. Later, 10% ethylenediaminetetraacetic acid was used for sample decalcification for 28 days. Paraffin embedded samples were sectioned with thickness of 4 μm. Hematoxylin and eosin was used to stain each section to visualize eosinophils. Blindfolded enumeration was carried out by two observers and data was analyzed for statistical significance.

### 2.4. Isolation of nasal mucosa and spleen cells

The nasal mucosae and spleens were collected from five animals of each group, crushed in RPMI medium in a glass grinder, and filtered through a cell strainer. After centrifugation, cells in splenic pellets were analyzed in terms of Th cell subset and Treg cellular proportions. Supernatants from nasal mucosae were transferred to new EP tubes and underwent cytometric bead array (CBA), while the pellets underwent polymerase chain reaction (PCR).

### 2.5. Measurement of serum OVA-specific IgE

Mouse sera were thawed from −80 °C prior to analysis. OVA-specific IgE levels were determined using an enzyme-linked immunosorbent assay (ELISA) in accordance with the manufacturer's instructions. The ELISA kit was purchased from Biolegend (San Diego, CA, USA).

### 2.6. Flow cytometry and cytokine measurements

Single-cell suspensions were treated with phorbol myristate acetate, ionomycin, and GolgiStop at 37 °C for 6 h. Prior to surface staining, cells were treated with APC-Cy7-CD3 and FITC-CD4 for 30 min. To detect intracellular cytokines, cells were treated with APC-IFN-γ, PE-Cy7-IL-17A, PE-IL-4, PerCP-Cy5,5-IL-9, PE-Foxp3, and APC-Helios after fixation and permeabilization. All data were collected on a FACS Aria III flow cytometer. The concentrations of IFN-γ, IL-4, IL-5, IL-13, IL-9, IL-17A, IL-10, IL-6, and IL-23 in supernatants were analyzed with the aid of the CBA Flex Set (BD Biosciences).

### 2.7. Real-time PCR

Total RNAs of nasal mucosa were extracted into the TRIzol reagent (Invitrogen) and cDNAs were synthesized with the aid of a PrimeScript RT kit (Takara). The β-actin-encoding gene served as a control. The target genes encoded T-bet, GATA-3, PU1, RORγt, Foxp3, and Helios. The Roche LightCycler 480 II System was used for real-time PCR analysis employing the cycle threshold ($2^{-\Delta\Delta CT}$) method.

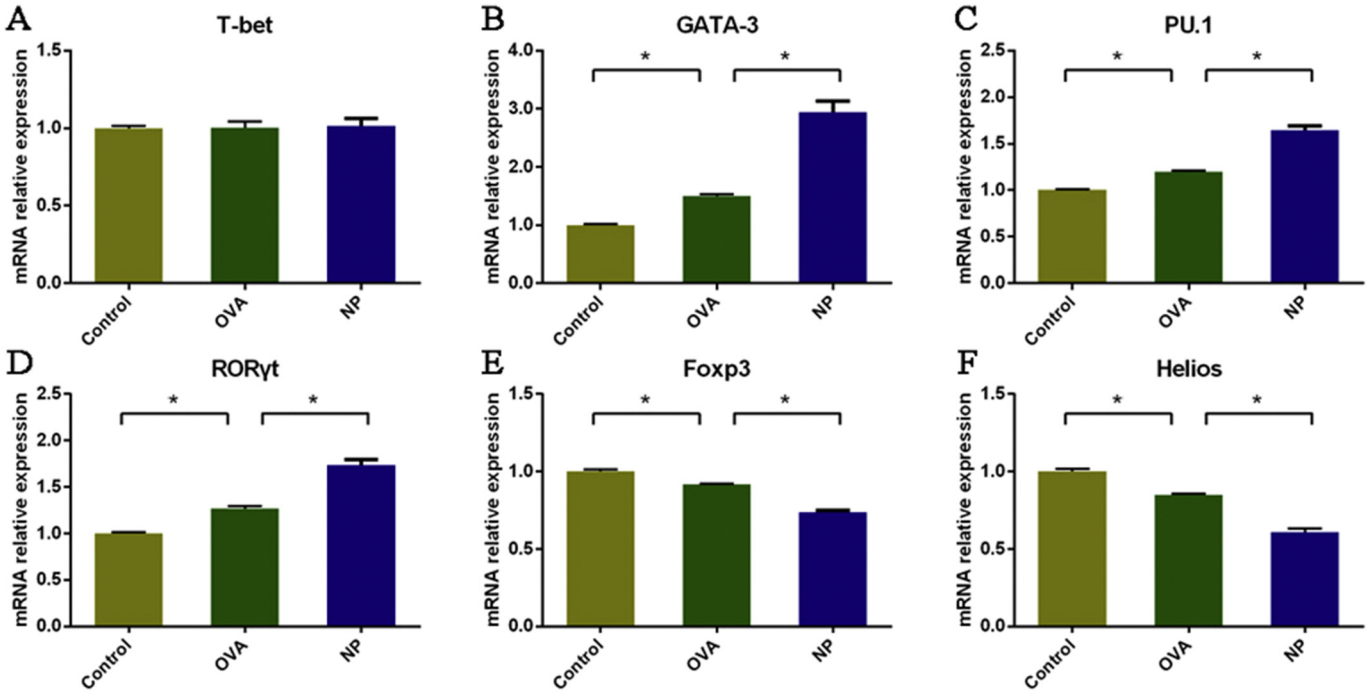


**Fig. 4.** Transcription factors measured by real-time polymerase chain reaction from nasal mucosa of the AR murine model. The levels of mRNAs encoding of T-bet, GATA-3, PU.1, RORγt, Foxp3, and Helios were analyzed using real-time PCR (A-F, respectively) (n = 5 in each group, *P < 0.05).

### 2.8. Statistical analysis

We used means ± SEM to present the data. SPSS software (IBM, USA) was used for statistical analyses. Different groups comparisons were compared by Kruskal-Wallis, followed Mann-Whitney test. GraphPad Prism software was used to prepare the graphs. A P-value < 0.05 was considered statistically significant.

## 3. Results

### 3.1. NP enhanced nasal allergy symptoms in the AR mouse model

Sneezing and nasal rubbing are the principal symptoms of AR; thus, we determined if NP increased these symptoms. Mice of the OVA group sneezed significantly more frequently (8.2 ± 1.62 sneezes/15 min) than mice in the control group (2.2 ± 0.79 sneezes/15 min, P < 0.05). Mice in the NP group sneezed significantly more frequently than those in the OVA group (12.9 ± 2.02 vs. 8.20 ± 1.62 sneezes/15 min, P < 0.05) (Fig. 1B). Nasal rubbing in the OVA group mice was more frequent (6.3 ± 1.64 rubs/15 min) than the control group (1.9 ± 0.74 rubs/15 min, P < 0.05). Nasal rubbing was more common after NP administration than OVA administration alone (9.8 ± 1.81 vs. 6.3 ± 1.64 rubs/15 min, P < 0.05) (Fig. 1C). These results indicate that NP administration can increase AR symptoms.

### 3.2. Greater eosinophil infiltration in nasal mucosa after administration of NP

Eosinophils play key roles in AR inflammation [27], we explored whether NP administration plays a role in eosinophil infiltration (Fig. 2A–C). Significantly greater eosinophil infiltration was evident in the OVA group (24.65 ± 1.52 cells/high-power field (HPF) than the control group (1.8 ± 0.48 cells/HPF, P < 0.05). Upon NP administration, eosinophilic infiltration increased significantly compared to OVA-induced mice (35.45 ± 4.24 vs. 24.65 ± 1.52 cells/HPF, p < 0.05) (Fig. 2D). These results indicate that NP administration increased eosinophil infiltration.

### 3.3. NP administration significantly increased serum levels of OVA-specific IgE

As AR is associated with an IgE-mediated immune response to environmental allergens, we determined if NP affected OVA-specific IgE. As shown in Fig. 3A, OVA-specific IgE significantly increased in the NP compared to the OVA and control groups (312.86 ± 18.76 pg/mL in NP group, 263.82 ± 9.57 pg/mL in OVA group, and 35.75 ± 5.47 pg/mL in control group, respectively; P < 0.05). Thus, NP increased serum levels of OVA-specific IgE.

### 3.4. NP administration enhanced Th2 responses but not Th1 responses

As an imbalance in the Th1/Th2 response ratio plays important role in AR inflammation, we explored whether NP administration affected this imbalance. As shown in Fig. 3C–E, IL-4, 5, and 13 increased significantly in NP group compared to the OVA and control groups (13.33 ± 3.44, 6.22 ± 0.36, and 5.14 ± 0.33 pg/mL in NP group, 6.35 ± 0.42, 5.28 ± 0.29, and 4.23 ± 0.34 pg/mL in OVA group, and 3.99 ± 0.32 pg/mL, 4.27 ± 0.44 pg/mL, and 3.45 ± 0.26 pg/mL in control group, respectively, P < 0.05). The IFN-γ did not obviously change in any groups (3.46 ± 0.45 pg/mL in NP group, 3.12 ± 0.27 pg/mL in OVA group, and 2.81 ± 0.51 pg/mL in control group, P > 0.05) (Fig. 3B). The level of GATA3, a Th2 transcription factor, markedly increased in NP group compared to the OVA and control groups (2.94 ± 0.42 in NP group, 1.5 ± 0.06 in OVA group, and 1.0 ± 0.03 in control group; P < 0.05) (Fig. 4B). T-bet, the transcription factor of Th1, did not show a difference among the groups (1.01 ± 0.11 in NP group, 1.0 ± 0.09 in OVA group, and 1.0 ± 0.03 in control group, P > 0.05) (Fig. 4A). Also, the Th2 cell percentage increased significantly in NP group compared to the OVA and control groups (4.91 ± 0.56% in NP group, 3.27 ± 0.21% in OVA group, and 1.86 ± 0.24 in control group, P < 0.05) (Fig. 5Cb). However, the Th1 cell proportions did not change significantly in the three groups (0.79 ± 0.11% in NP group, 0.76 ± 0.11% in OVA group, and 0.69 ± 0.11 in control group, P > 0.05) (Fig. 5Ca). These results support an increase in the Th2/Th1 imbalance during the Th2 response

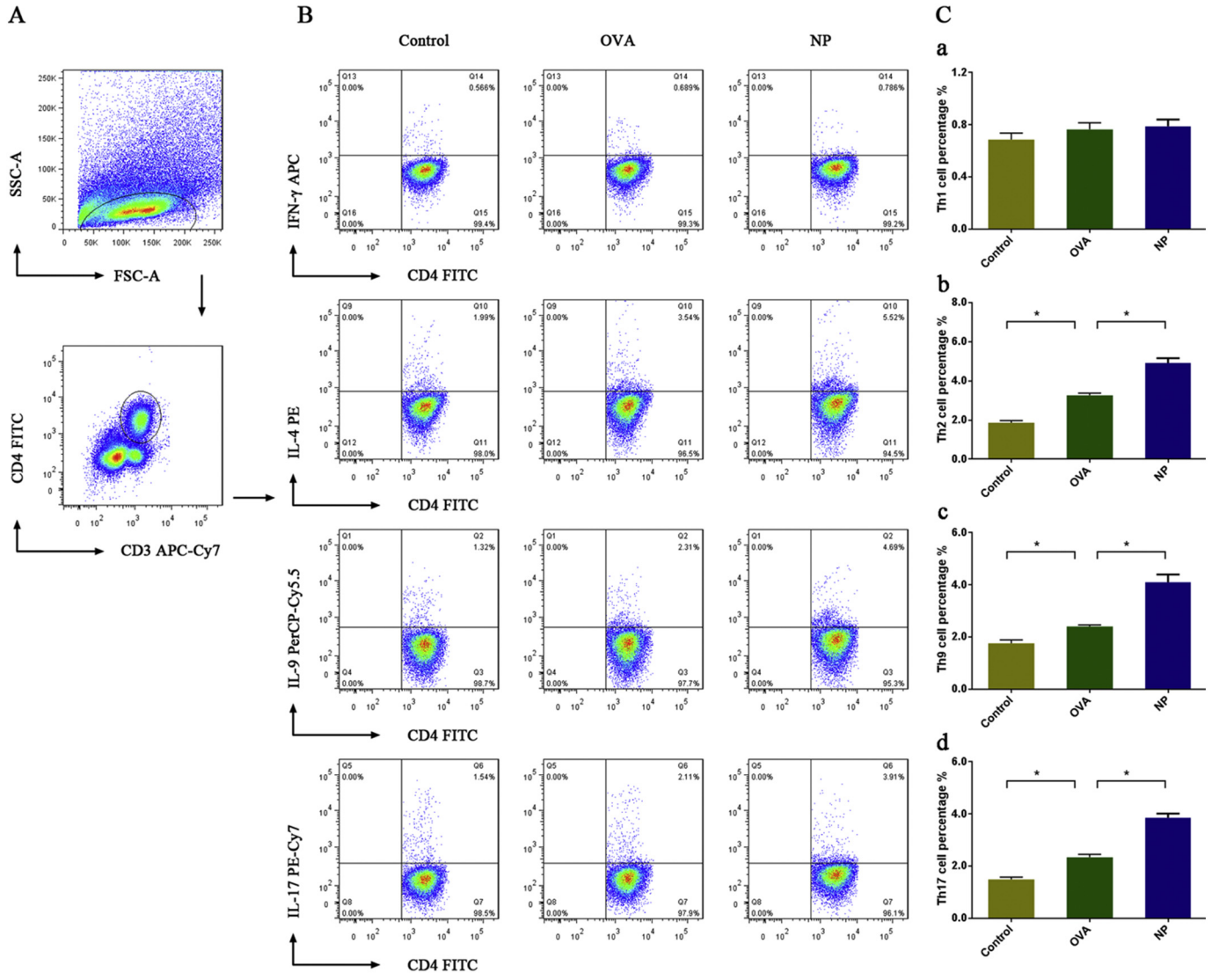


**Fig. 5.** Th cell subsets of the AR murine model as revealed by flow cytometry. $CD3^+CD4^+$ T cell subgroups (A). Representative staining of Th1, Th2, Th9, and Th17 cells of each group (B). Statistics (C). (n = 5 in each group, *P < 0.05).

(cytokines, transcription factors, and cell percentages), while Th1 was not affected by NP administration.

### 3.5. Th9 and Th17 responses were upregulated by the administration of NP

Recent studies have shown that Th9 and Th17 cells, and their associated cytokines, are responsible for the pathogenesis of AR [7,8,11,13]; thus, we evaluated the effects of NP on Th9 and Th17 cells in the AR model. As shown in Fig. 3F, G, and I, the IL-9, IL-17, and IL-6 levels increased significantly in NP group compared to the OVA and control groups (9.57 ± 1.03, 6.03 ± 0.69, and 107.25 ± 5.61 pg/mL in NP group, 7.13 ± 0.39, 3.7 ± 0.33, and 81.86 ± 2.18 pg/mL in OVA group, and 4.1 ± 0.99, 2.45 ± 0.36, and 7.37 ± 1.08 pg/mL in control group, respectively, P < 0.05). The IL-23 level increased significantly in both the NP and OVA groups compared to the control group, (1.95 ± 0.22 pg/mL in NP group, 1.74 ± 0.20 pg/mL in OVA group, and 1.23 ± 0.06 pg/mL in control group, P < 0.05), but the OVA and NP group values did not differ significantly (P > 0.05; Fig. 3J). In addition, PU.1 (transcription factor of Th9) and RORγt (transcription factor of Th17) increased in NP group compared to the OVA and control groups (1.64 ± 0.10 and 1.74 ± 0.13 in NP group, 1.2 ± 0.03 and 1.27 ± 0.06 in OVA group, and 1.0 ± 0.03 and 1.0 ± 0.03 in control group, p < 0.05; Fig. 4C, D). Th9 cell percentages increased in NP group compared to the OVA and control groups (4.1 ± 0.67%, 2.41 ± 0.13%, and 1.76 ± 0.29%, respectively, P < 0.05) (Fig. 5Cc), as did the Th17 cell percentages (3.85 ± 0.36%, 2.35 ± 0.23%, and 1.49 ± 0.21%, respectively, P < 0.05) (Fig. 5C, D). These results show that NP administration affects AR by upregulating Th9 and Th17 responses and their associated cytokines.

### 3.6. NP administration can negatively regulate Treg responses

In AR, Tregs play important roles in preventing immune activation and inhibiting inflammatory lesions [28]. CD25 and FoxP3 levels are commonly used to evaluate Treg status, but recent studies found that co-expression of Foxp3 and Helios is characteristic of a key functional phase of Treg cells [29]. As shown in Fig. 6, the percentages of $CD4^+Helios^+Foxp3^+$ T cells decreased in NP group compared to the OVA and control groups (10.1 ± 0.43%, 12.68 ± 0.68%, and 15.08 ± 1.03, respectively, P < 0.05). Meanwhile, the expression of Foxp3 and Helios (transcription factors of Tregs) at the mRNA level decreased in the NP group compared to the OVA and control groups (0.74 ± 0.03 and 0.61 ± 0.05 in NP group, 0.92 ± 0.01 and 0.85 ± 0.02 in OVA group, and 1.0 ± 0.03 and 1.0 ± 0.02 in control

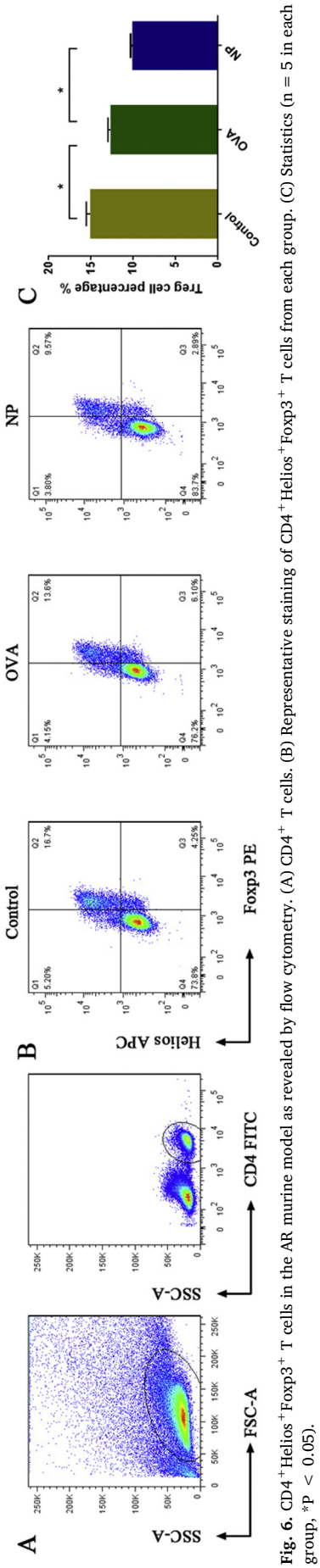


**Fig. 6.** $CD4^+Helios^+Foxp3^+$ T cells in the AR murine model as revealed by flow cytometry. (A) $CD4^+$ T cells. (B) Representative staining of $CD4^+Helios^+Foxp3^+$ T cells from each group. (C) Statistics (n = 5 in each group, *P < 0.05).

group, respectively, $p < 0.05$) (Fig. 4E,F). The level of IL-10, the major cytokine of Tregs, decreased significantly at the protein level in the NP group compared to the OVA and control groups (6.14 ± 0.52, 7.35 ± 0.50, and 11.79 ± 1.97 pg/mL, respectively, $P < 0.05$) (Fig. 3H). Our results regarding Treg cell percentages, transcription factors, and cytokines indicate that NP administration significantly inhibits Treg responses. Thus, in the AR mouse model, Treg cells may be unable to inhibit the Th2 inflammatory responses.

## 4. Discussion

In recent years, the incidence of AR has gradually increased, in line with the increasing proportion of children of preschool age [1]. However, whether there is an external factor involved in the occurrence of this disease is unclear. Intake of NP may occur by drinking water, eating contaminated food, inhaling air, absorption via the skin, or even through transmission to the fetus and infants by absorption via the placenta and breastfeeding, respectively [30,31]. Recent studies have shown its effect on allergic disease, and that it can aggravate asthma in a mouse model [26]. Therefore, we hypothesized that NP may be a potential risk factor for the increase in AR and its effects. There is no specific study in the literature regarding the association between NP and AR inflammation, although limited studies exist on other allergic diseases [26].

In this study, we found for the first time that NP administration can affect symptoms and immune inflammation in an OVA-induced AR murine model; our model provided evidence of a potential link between NP administration and AR inflammation. Here, we show that NP can aggravate AR immune inflammation.

Eosinophil infiltration is a hallmark of AR mucosa inflammation [27]. NP exposure increases the number of eosinophils in allergic airway inflammation [26]. After NP administration in our experiment, eosinophil infiltration and symptoms increased significantly compared with OVA-induced mice. In addition, NP increased the serum levels of OVA-specific IgE. Therefore, it is possible that NP aggravates type I allergic disease in mice.

As NP is lipophilic and thus tends to bioaccumulate in adipose tissue, it is very likely that NP regulates immune cells in vivo, especially after long-term exposure [26]. AR is characterized by active $CD4^+$ T subsets that synthesize certain immunomodulators [28,32]. To investigate the effect of NP on $CD4^+$T subsets, we measured the levels of Th cells and Tregs in the AR murine model.

The traditional view is that imbalance between Th1 and Th2 leads to development of AR [6]. In the present study, IL-4, -5, and -13levels; the GATA3 mRNA level in nasal mucosa, and the splenic Th2 cell proportions were all significantly increased in the AR murine model. These results suggest that the Th2 response was upregulated in AR, and this was exacerbated by NP administration. Consistent with previous studies, exposure to NP enhanced Th2-mediated lung inflammation [26]. In contrast, there was no significant change in the Th1 response (IFN-γprotein, T-bet mRNA, and the Th1 cell percentage). Thus, NP administration can enhance Th2 responses, but not Th1 responses.

In recent years, Th9 and Th17 have been implicated in the pathogenesis of AR; their specific transcription factors are PU.1 and RORγt, respectively, and their main secretory factors are IL-9 and IL-17, respectively [2,33,34]. Consistent with previous studies [35], we found that the Th9 response (IL-9, PU.1 mRNA, Th9 cell percentage) and Th17 response (IL-17, RORγt mRNA, Th17 cell percentage) were upregulated in AR condition in the present study. In the case of exposure to NP, this increasing trend was further highlighted. In terms of the Th17 response, IL-23 and IL-6 can enhance the amplitude and duration of phospho-STAT3 activity [36], which may regulate Th17 cells development [37] and maintain cytokine expression during the Th17 response [38]. Consistent with previous reports [27,39], the IL-6 and IL-23 levels increased significantly when AR was induced. After NP administration, IL-6 protein increased significantly, while IL-23 protein was also

increased, although not significantly. NP administration enhanced Th9 and Th17 responses, which are major contributors to AR inflammation.

Unlike other effector $CD4^+$ T cells, Tregs secrete IL-10 or TGF-β1 that inhibit the functions of Th1, Th2, and Th17 cells; Foxp3 is the relevant transcription factor [28]. Previous studies found that Treg responses decreased in AR subjects [8,40,41]. Recent studies have found that co-expression of Foxp3 and Helios represents a key functional phase of Treg cells, and Helios is now considered a marker for Treg cell activation [29]. We also found that Treg responses (IL-10, Foxp3 mRNA, $CD4^+Helios^+Foxp3^+$ T cell percentage) were downregulated in AR. Treg responses were further downregulated on administration of NP. Therefore, it was evident that NP could negatively regulate Treg responses.

There were several limitations to our research. First, the number of mice in each group was relatively small. Nevertheless, there were numerous significant differences among the groups. Second, we chose splenocytes instead of nasal mucosal cells for flow cytometry, mainly because mice have too few mucous membranes to meet the requirements for analysis.

In conclusion, taken together and based on the collective results, this study demonstrated that NP can enhance AR inflammation and symptoms through dysregulation of Th2, Th9, Th17, and Treg responses in an AR mouse model. These findings not only help in understanding the etiology and mechanism of AR but also provide concrete evidence that NP can aggravate AR.

## Authors' contributions

YW, ZG and LH conceived and designed the experiments. ZG and YW performed the experiments. ZC and LH analyzed the data. ZG and YW wrote the paper.

Authors read and approved the final manuscript.

## Declarations of interest

None.

## Acknowledgments

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