

Semaphorin 3A inhibits allergic inflammation by regulating immune responses in a mouse model of allergic rhinitis

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Background: It has been reported that semaphorin 3A (sema3A) could improve allergic symptoms in allergic rhinitis (AR) mice. However, the immunomodulatory roles of sema3A in AR remain unclear. This study was performed to determine the immunoregulatory effects of sema3A on airway inflammation in an AR mice model.

Methods: First, sema3A expression was measured in the serum of AR patients and also in a mice model. Then, nasal symptoms, ovalbumin (OVA)-specific immunoglobulin E (IgE) production, cytokine levels, and histologic structure were analyzed in OVA-sensitized mice, sema3A mice, mice given saline, and controls. The percentages of CD4⁺IL-4⁺IFN- γ ⁻ Th2 cells, CD4⁺IFN- γ ⁺IL-4⁻ Th1 cells, CD4⁺IL-17⁺ Th17 cells, and CD4⁺CD25⁺Foxp3⁺ Treg cells in the spleen were also analyzed.

Results: Serum sema3A levels in both AR patients and OVA-sensitized mice decreased significantly compared with controls. The intranasal administration of sema3A reduced allergic symptom scores, eosinophil infiltration, and OVA-specific IgE production in OVA-sensitized mice. In

addition, levels of IL-4 and IL-17 as well as percentages of CD4⁺IL-17⁺Th17 cells were suppressed by sema3A administration. Levels of IFN- γ and IL-10 and ratios of CD4⁺IFN- γ ⁺IL-4⁻Th1/CD4⁺IL-4⁺IFN- γ ⁻Th2 cells, as well as percentages of CD4⁺CD25⁺Foxp3⁺ Treg cells, were increased by administration of sema3A.

Conclusion: Our results demonstrate that sema3A suppressed allergic inflammation in AR via inhibition of Th2/Th17 responses and enhancement of Th1/Treg responses. © 2018 ARS-AAOA, LLC.

Key Words:

allergic reactions; immune system; T lymphocytes; regulatory; nasal mucosa; cytokines

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Allergic rhinitis (AR) is a common chronic inflammatory disease that affects 10%-40% of the population worldwide.^{1,2} It severely affects patients' quality of life and school/work performance, and creates great financial burden.³ The imbalance of T helper 1 (Th1)/Th2 cells plays a key role in the pathogenesis of AR. Recently, the imbalance of T-regulatory (Treg)/Th17 cells was also found

to contribute to the pathogenesis of AR.⁴ Treg cells expressing the forkhead transcription factor (Foxp3) inhibit the antigen-specific T-cell response through contact-dependent suppression or release of anti-inflammatory cytokines such as interleukin (IL)-10.⁵ Th17 cells expressing retinoic acid receptor-related orphan receptor-gamma-t (ROR γ t) enhances allergic inflammation by producing cytokines such as IL-17, tumor necrosis factor-alpha (TNF- α), and IL-6.⁶

Semaphorins were originally identified as axonal guidance molecules involved in the formation of cerebral cortex, hippocampus, and the spinal cord as well as in the modulation of peripheral nerves.⁷ A growing body of data shows that this family also play critical roles in tumor progression,⁸ angiogenesis,⁹ and immune responses.¹⁰ Semaphorin 3A (sema3A), a secreted member of semaphorins family, is a neurologic molecular that inhibits the intraepidermal extension of peripheral nerve.¹¹ One animal experiment¹² showed that sema3A had lower expression in the nasal mucosa of AR, and intranasal administration of sema3A could alleviate allergic symptoms

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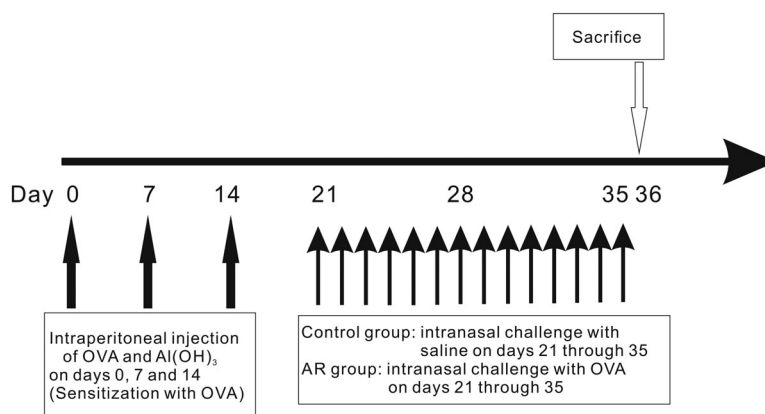


FIGURE 1. Protocol for AR mice model. Mice in the AR group were sensitized with 100 μ g OVA plus 1 mg Al(OH)₃ on days 0, 7, and 14. On days 21–35, the mice were intranasally challenged on a daily basis with 40 μ L saline containing 400 μ g of OVA (20 μ L per nostril). The control group received saline sensitization and challenge instead of OVA. AR = allergic rhinitis; OVA = ovalbumin.

by inhibiting the innervation of C fibers. Recently, it was found that sema3A is also a potent immunoregulator in the immune system.¹³ For instance, sema3A was found to suppress CpG oligodeoxynucleotides (ODN-CpG)–induced toll-like receptor (TLR)–9 expression in memory B cells *in vitro*.¹⁴ Another study reported that sema3A was expressed by activated T cells and inhibited T-cell proliferation.¹⁵ However, the immunomodulatory roles of sema3A in AR have not been investigated. Therefore, in this study, we evaluated the release of Th1-, Th2-, Treg-, and Th17-cell-associated cytokines, proportions of Th17, and Treg cells in sema3A-administered AR mice to determine the regulatory role of sema3A in T-cell-mediated immune responses in AR.

Patients and methods

Subjects and samples

Both AR patients and healthy volunteers who visited the Department of Otorhinolaryngology–Head and Neck Surgery, Renmin Hospital of Wuhan University (Wuhan, China), between June 2017 and September 2017, were recruited. The AR patients were diagnosed according to the Allergic Rhinitis and its Impact on Asthma (ARIA) guidelines.¹ All AR patients enrolled had common symptoms including nasal itching, sneezing, rhinorrhea, and nasal congestion, and were positive for common aeroallergens. Patients who had used nasal or oral corticosteroids, decongestants, or antihistamines within the previous 4 weeks were excluded from this study. We also excluded patients who had a history of allergen-specific immunotherapy or conditions that may influence sema3A level, such as asthma, diabetes, systemic lupus erythematosus (SLE), etc. All healthy volunteers had normal physical findings and a negative specific immunoglobulin E (IgE) test. Two milliliters of venous blood collected from all subjects was centrifuged for 10 minutes at 400g to separate the serum. The serum samples were then stored at

–80°C until enzyme-linked immunosorbent assay (ELISA) evaluation. The study protocol was approved by the ethics committee of Renmin Hospital of Wuhan University and informed consent was obtained from each participating subject.

Animals

Six-week-old female BALB/c mice, obtained from the Wuhan Institute of Biological Products Co, Ltd, were housed in a special pathogen-free biohazard containment facility at the Animal Experiment Center of Renmin Hospital of Wuhan University in a 12-hour light/dark cycle with free access to food and water. This study was approved by the institutional animal care and use committee of Renmin Hospital of Wuhan University (WDRM-20170616).

Establishment of AR mice model

An AR model was induced by sensitization according to methods described elsewhere.¹⁶ Briefly, on days 0, 7, and 14, mice were sensitized by intraperitoneal injection of 300 μ L saline containing 25 μ g of ovalbumin (OVA; Sigma, St Louis, MO) and 1 mg of Al(OH)₃. After primary sensitization, mice were intranasally challenged with 100 μ g of OVA diluted in 40 μ L saline for 14 consecutive days, from days 21–35. The control group received saline sensitization and challenge instead of OVA (Figure 1).

Sema3A administration

As shown in Figure 2, sema3A group mice were intranasally administered with 20 μ L saline containing 0.12 μ g recombinant mouse semaphorin 3A Fc chimera (R&D Systems, Minneapolis, MN) 1 hour before intranasal challenge with OVA for 14 consecutive days, from days 21 to 35. The AR+saline group mice were pretreated with intranasal administration of 20 μ L saline 1 hour before intranasal challenge with OVA for 14 consecutive days, from days 21–35.

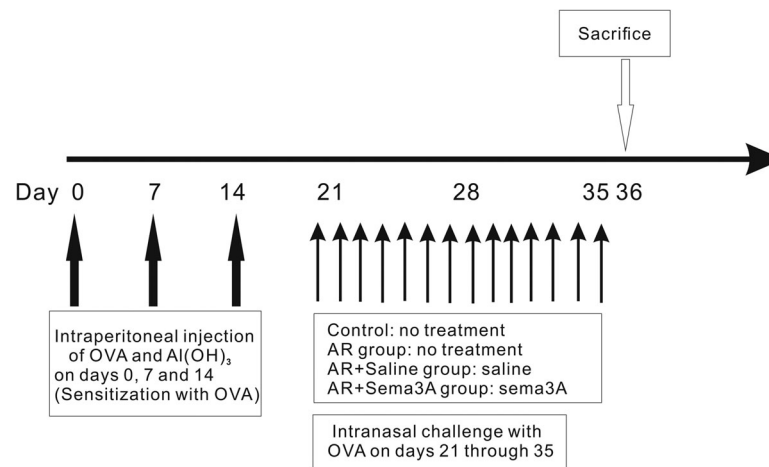


FIGURE 2. Protocol of AR and sema3A administration mouse model. Mice in the AR group, AR+saline group and AR+sema3A group were sensitized with 100 μ g of OVA plus 1 mg of Al(OH)₃ on days 0, 7, and 14. On days 21–35, the mice were intranasally challenged on a daily basis with 40 μ L saline containing 400 μ g OVA (20 μ L per nostril). In addition, before 1 hour of intranasal challenge on days 21–35, the AR+saline group was pretreated with 20 μ L saline, and the AR+sema3A group intranasally administered 0.12 μ g sema3A diluted in 20 μ L saline. AR = allergic rhinitis; OVA = ovalbumin; sema3A = semaphorin 3A.

Evaluation of allergic symptoms induced after the allergen challenge

On day 35, during the 10-minute period after last administration of intranasal OVA, the numbers of nasal rubbing motions were recorded by 3 observers blinded to the experimental groups.

Measurement of sema3A

Measurements of sema3A serum levels in both human and mice were conducted using ELISA kits (CSB-E15913h and CSB-CSB-EL020980MO; Cusabio, Wuhan, China), according to the manufacturer's instructions. The limits of detection were 0.039 ng/mL for sema3A in humans and 3.9 pg/mL for sema3A in mice.

Collection of nasal lavage fluid

Twenty-four hours after the final OVA challenge, mice were euthanized and nasal lavage fluid (NFL) was collected by flushing 1 mL of sterile saline into the tracheal opening in the direction of the posterior choanae. The NFL was then centrifuged at 250g for 15 minutes at 4°C. The supernatants were stored at -80°C for OVA-specific IgE and cytokine measurement.

Histopathology of nasal mucosa

Nasal tissues were removed from the mice and fixed in 4% paraformaldehyde for 24 hours at room temperature. The fixed tissues were next embedded in paraffin after being submerged in 10% ethylene-diamine tetraacetic acid (EDTA) standard solution for 14 days at room temperature. The specimens were embedded in paraffin wax and sectioned coronally at a thickness of 5 μ m. The sections were stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and Masson, respectively. Eosinophil (EOS) numbers in the nasal lamina propria were calculated in 5

randomly selected fields under high magnification ($\times 400$), and EOS frequencies were assessed as cells per square millimeter. PAS staining was performed to estimate the degree of goblet cell hyperplasia in the epithelium of the nasal mucus. The total number of PAS-stained cells in the epithelium was counted by 2 observers blinded to the previous treatments. Masson staining was performed to evaluate the degree of collagen deposition in the nasal mucosa, according to the manufacturer's instructions. The collagen fibers appeared blue, the nuclei were black, and the rest of the tissue stained red. Five fields of vision were chosen for observation under the microscope ($\times 400$) in each section. Image-Pro Plus 6.0 was used to semiquantitatively determine the percentage of collagen fibers throughout the total area.

Measurement of OVA-specific immunoglobulin E

Blood samples were collected from the angular vein of mice at the time of death. The sera were harvested from blood samples by centrifugation for 20 minutes at 250g at 4°C and stored at -80°C until use. The levels of OVA-specific IgE in serum and NFL were measured by using a mouse OVA-specific IgE ELISA kit (Cayman, Chemical Company, Ann Arbor, MI, USA). All procedures were performed according to the manufacturer's instructions.

Isolation of lymphoid cells

Spleens and nasal mucosa were separated into single cells using 200- μ m strainers under sterile conditions. Lymphoid cells were then isolated from the single cells of spleens and nasal mucosa by Ficoll-Hypaque density gradient centrifugation using a lymphocyte separation kit (TBD Science, Tianjin, China) according to the manufacturer's instructions. Cells were then cultured in RPMI-1640 medium (HyClone, Logan, UT) supplemented with 10% fetal bovine serum (Gibco Laboratories, Gaithersburg, MD),

2 mmol/L L-glutamine (Sigma-Aldrich, St Louis, MO), 1% nonessential amino acids (Gibco), 1 mmol/L sodium pyruvate (Sigma-Aldrich), 100 U/mL penicillin, and 100 μ g/mL streptomycin (Sigma-Aldrich) at 37°C in a humidified atmosphere of 5% CO₂.

Measurement of cytokine concentrations

A total of 4×10^6 lymphoid cells from spleens were cultured for 48 hours in the presence of lipopolysaccharides (2 μ g/mL) and concanavalin A (10 μ g/mL) for evaluation of IL-4 and IL-17. For evaluation of interferon-gamma (IFN- γ) and IL-10, 4×10^6 lymphoid cells from spleens were stimulated with lipopolysaccharides (2 μ g/mL) for 24 hours. Culture supernatants were then collected for cytokine detection. Cytokine levels, including IL-4, IFN- γ , IL-10, and IL-17 in the serum, NFL, and lymphoid cell culture supernatants were quantified by using ELISA kits (R&D Systems) according to the manufacturer's instructions. Absorbance values in each well were measured at 450 nm using an ELISA reader (Molecular Devices, Sunnyvale, CA, USA).

Flow cytometry analysis of Th1, Th2, Th17, and Treg cell proportions

To analyze the percentages of Th1, Th2, and Th17 cells, the lymphoid cells from spleens and nasal mucosa were stimulated with 2 μ L/mL of leukocyte activation cocktail, with BD GolgiPlug (BD Biosciences, San Jose, CA) for the final 6 hours of culture. For cell-surface staining, the cells were stained with fluorescein isothiocyanate (FITC)-labeled anti-CD4 antibody (BD Biosciences). The cells were then incubated with phycoerythrin (PE)-labeled anti-IL-17 antibody (BD Biosciences), antigen-presenting cell (APC)-labeled anti-IFN- γ antibody (BD Biosciences), or PE-labeled anti-IL-4 antibody (BD Biosciences) after being fixed and permeabilized using a fix/permeabilization solution (BD Biosciences) according to the manufacturer's instructions. For Treg-cell staining, unstimulated lymphoid cells were stained with FITC-labeled anti-CD4 (BD Biosciences) antibody and PE-labeled anti-CD25 antibody (BD Biosciences). Subsequently, the cells were fixed, permeabilized, and further stained with APC-labeled anti-Foxp3 antibody (BD Biosciences). Flow cytometric data were acquired using a flow cytometer (BD FACSCanto II; BD Biosciences) and analyzed using FlowJo software version 7.6 (Tree Star, Inc, San Carlos, CA).

Statistical analysis

Results are expressed as mean $\pm$ standard error of the mean (SEM). Differences between 2 groups were determined using unpaired two-tailed *t* tests. Differences among groups were compared using one-way analysis of variance (ANOVA) followed by Tukey's test. All data analyses were performed with GraphPad Prism version 6 (GraphPad, Inc, La Jolla, CA). $p < 0.05$ was considered statistically significant.

TABLE 1. Characteristics of AR patients and healthy controls

	AR	Control	<i>p</i> value
No.	20	20	>0.05
Sex (F/M)	11/9	8/12	>0.05
Age (years)	30.250 $\pm$ 3.631	27.00 $\pm$ 2.025	>0.05
IgE (IU/mL)	>0.35	0	<0.05
Itching score	3.470 $\pm$ 0.570	0	<0.05
Sneezing score	3.870 $\pm$ 0.6143	0	<0.05
Rhinorrhea score	3.935 $\pm$ 0.626	0	<0.05
Nasal congestion score	3.310 $\pm$ 0.659	0	<0.05

AR = allergic rhinosinusitis; IgE = immunoglobulin E.

Results

Sema3A expression in serum

To confirm sema3A expression in AR, we first measured serum sema3A in both AR patients and in mice. A total of 20 AR patients and 20 healthy controls were enrolled in this study. The general characteristics of enrolled subjects are shown in Table 1. There were no significant differences in age and sex between the 2 groups ($p > 0.05$). As shown in Figure 3A, the serum sema3A levels in AR patients were significantly lower compared with healthy controls ($p < 0.05$). We also measured serum sema3A levels in the AR mice. The results show that the serum sema3A levels in the AR group were significantly lower compared with control mice ($p < 0.05$) Figure 3B.

Effect of sema3A on nasal symptoms in AR mice

To assess the anti-inflammatory effect of sema3A in AR mice model, we observed the nasal symptoms in all mice. As shown in Figure 4, the frequency of nasal rubbing in the AR group was significantly higher compared with the control group ($p < 0.05$), whereas it was lower in the sema3A group when compared with the AR group ($p < 0.05$). No significant differences were observed between the AR group and the saline group.

Effect of sema3A on histology of nasal mucosa

To determine whether sema3A could suppress the allergic responses in AR, the pathologic changes in nasal mucosa were analyzed. As shown in Figure 5, the nasal mucosa in the control mice showed a normal structure with little EOS infiltration. Compared with the control group, the AR group had significantly more severe EOS infiltration in the nasal mucosa ($p < 0.05$). The numbers of EOS and level of goblet cell hyperplasia in the nasal mucosa were lower in sema3A group compared with the AR group ($p < 0.05$). No significant differences were observed for EOS infiltration between the saline group and AR group. Increased collagenous fibers were observed in the AR mice ($p < 0.05$),

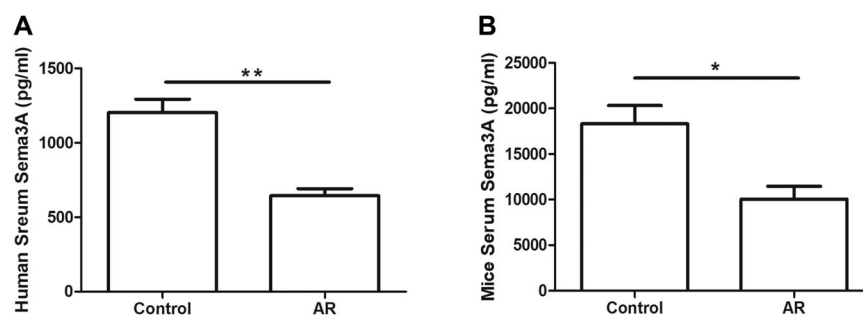


FIGURE 3. Serum sema3A concentrations in human and mice. (A) Concentrations of serum sema3A in AR patients and healthy controls measured by ELISA. (B) Concentrations of serum sema3A in AR mice and controls measured by ELISA ($n = 6$ per group). * $p < 0.05$. ELISA = enzyme-linked immunoassay; sema3A = semaphorin 3A.

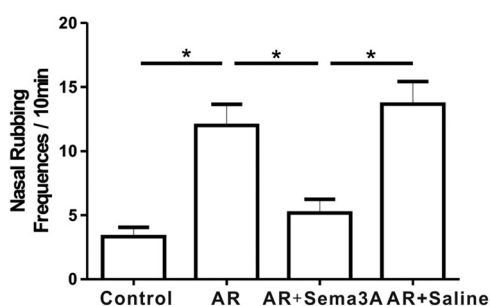


FIGURE 4. Frequencies of nasal rubbing during the 10 minutes after last OVA challenge. Allergic responses were monitored on day 35. AR mice exhibited significantly higher allergic symptom scores than control mice. Data represent the mean $\pm$ SEM ($n = 6$ per group). * $p < 0.05$. AR = allergic rhinitis; OVA = ovalbumin.

whereas sema3A administration significantly decreased expression of nasal fibrosis in the nasal mucosa ($p < 0.05$).

Effect of sema3A on OVA-specific IgE production in serum and NFL

To analyze the inhibitory effect of sema3A on AR, we detected the levels of OVA-specific IgE in both serum and NFL in all groups. As shown in Figure 6, expression of OVA-specific IgE in serum was significantly increased in the AR group compared with the control group ($p < 0.05$), whereas it was significantly decreased in sema3A group compared with the AR group ($p < 0.05$). Levels of OVA-specific IgE in the NFL of the AR group, however, showed no significant difference compared with the sema3A group ($p > 0.05$).

Effect of sema3A on cytokine expression in serum, NFL and spleen

We then investigated the effect of sema3A on AR by measuring the cytokine levels of IL-4, IFN- γ , IL-10, and IL-17 in serum, NFL, and splenocyte culture supernatants. We found that IL-4 and IL-17 levels were significantly higher in the AR group compared with the control group in serum, NFL, and splenocyte culture supernatants. In contrast, IL-4 and IL-17 levels in the sema3A group were significantly lower compared with the AR group in serum, NFL, and

splenocyte culture supernatants ($p < 0.05$). Levels of IFN- γ and IL-10 in the AR group were significantly lower compared with the control group in serum, NFL, and splenocyte culture supernatants ($p < 0.05$). In contrast, IFN- γ and IL-10 levels in the sema3A group were significantly higher compared with the AR group in serum, NFL, and splenocyte culture supernatants ($p < 0.05$). No significant difference was observed between the AR and saline groups in the production of IL-4, IFN- γ , IL-10, and IL-17 in serum, NFL, and splenocyte culture supernatants ($p > 0.05$).

Effect of sema3A on Th1, Th2, Th17, and Treg cells proportions

We further investigated the effect of sema3A on the frequency of Th1 and Th2 cells in both nasal mucosa and spleen. We regarded CD4⁺IFN- γ ⁺IL-4⁻ T cells and CD4⁺IL-4⁺IFN- γ ⁻ T cells as Th1 and Th2 cells, respectively, in our analysis. As shown in Figure 7, the ratios of CD4⁺IFN- γ ⁺ Th1/CD4⁺IL-4⁺ Th2 cells in both nasal mucosa and spleens were significantly decreased in the AR group compared with controls, whereas the ratios were significantly increased in the sema3A group compared with the AR group ($p < 0.05$). The proportions of Th17 cells and Treg cells in the spleen were also analyzed and the results show that the frequency of CD4⁺IL-17⁺ Th17 cells in the AR group increased significantly compared with the control group ($p < 0.05$), but decreased significantly in the sema3A group compared with the AR group ($p < 0.05$). There were no significant differences in frequency of CD4⁺IL-17⁺ Th17 cells between the AR and saline groups. The frequency of CD4⁺CD25⁺Foxp3⁺ Treg cells was markedly reduced in the AR group compared with controls ($p < 0.05$). Owing to sema3A, the percentage of CD4⁺CD25⁺Foxp3⁺ Treg cells in the sema3A group was significantly higher than that in the AR group ($p < 0.05$). No significant differences were observed for frequency of CD4⁺CD25⁺Foxp3⁺ Treg cells between the AR and saline groups.

Discussion

The role of sema3A in immune disorders has been investigated widely, but little is known about the

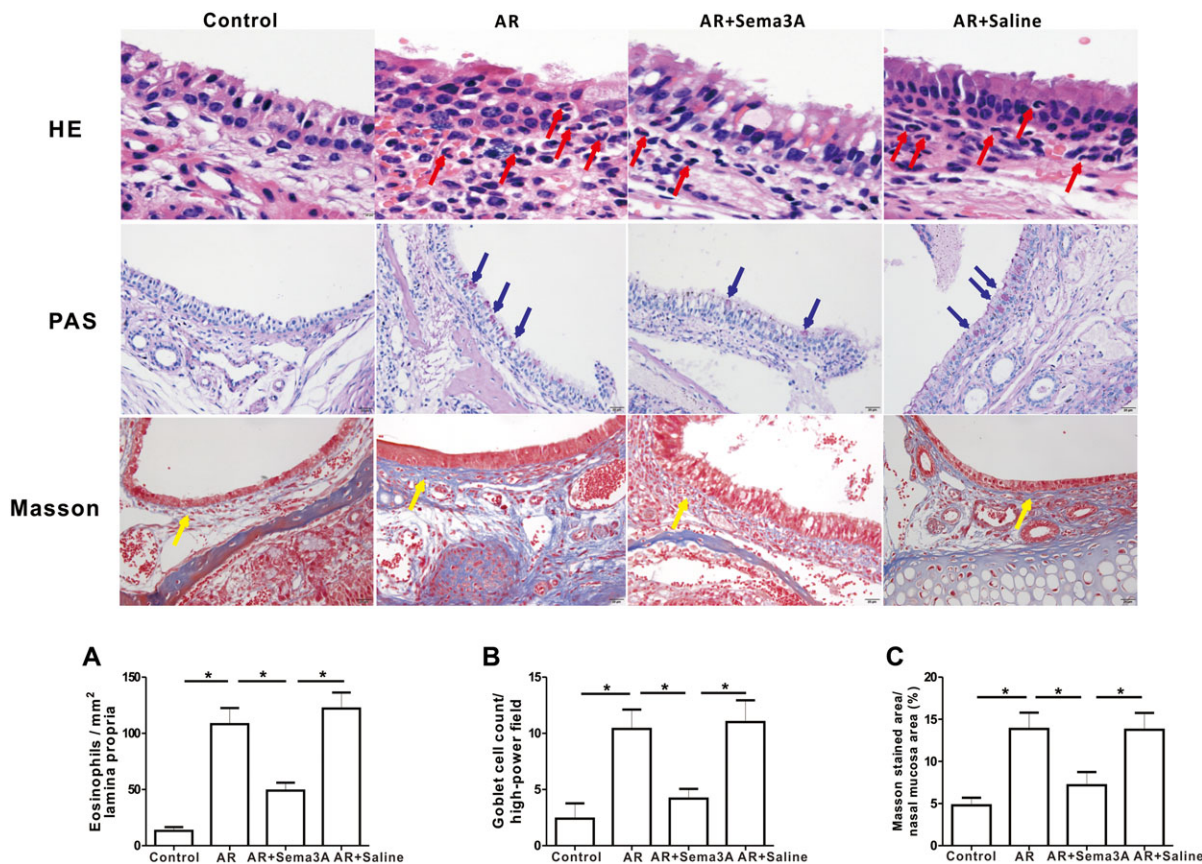


FIGURE 5. Histopathologic changes of the nasal mucosa as detected by H&E, PAS, and Masson staining (original magnification $\times 400$). The cytoplasm of EOS in the nasal lamina propria were stained red by H&E. Red arrows indicate EOS. The goblet cells in the nasal epithelial cells were stained red by PAS. Blue arrows indicate positive goblet cells. Collagenous fibers in the nasal mucosa were stained blue by Masson staining. Yellow arrows indicate collagenous fibers. (A) Statistical analysis for EOS infiltration of the nasal mucosa. (B) Statistical analysis of goblet cells in the nasal epithelium. (C) Statistical analysis for collagenous fibers in the nasal mucosa. Data are presented as mean $\pm$ SEM ($n = 6$ per group). * $p < 0.05$. Bar = $20 \mu\text{m}$. EOS = eosinophils; H&E = hematoxylin-eosin; PAS = periodic acid-Schiff; SEM = standard error of the mean.

immunoregulatory effect of sema3A on AR. In the present study, we have revealed, for the first time, that serum sema3A had lower expression in both AR patients and mice. Furthermore, we found that sema3A inhibited OVA-induced inflammation via regulating Th1-, Th2-, Th17-, and Treg-cell-mediated allergic responses. These findings indicate that sema3A may play a major immunoregulatory role in the pathophysiology of AR.

A previous study¹⁷ demonstrated that sema3A administration suppressed EOS infiltration and the production of serum total IgE and Th2-related cytokines in the conjunctiva of experimental allergic conjunctivitis mice. Further, an *in vivo* study¹⁸ showed that sema3A administration markedly inhibited CD4⁺ T-cell infiltration and IL-4 secretion in the dermis of atopic dermatitis. We hypothesized that sema3A, in addition to its anti-innervation function in sensory fibers, could exert an anti-inflammatory effect in allergic responses, including AR. In line with our hypothesis, the results show that sema3A administration significantly alleviated the allergic symptom of AR mice and inhibited EOS infiltration and goblet cell hyperplasia in the nasal mucosa. Moreover, increased ratios of Th1/Th2 cells, decreased expression of IL-4, as well as increased ex-

pression of IFN- γ were observed in sema3A-administered mice when compared with AR mice. These results indicate that sema3A alleviated allergic inflammation in AR. The findings also suggest that sema3A could exert an anti-inflammatory effect by modulating Th1/Th2 imbalance in AR. It is known that AR is characterized by increased numbers of Th2 cells and Th2-type cytokines and decreased numbers of Th1 cells and Th1-type cytokines. Moreover, Th2-related cytokines, IL-4 and IL-13, are responsible for IgE production, whereas Th1-related cytokines are associated with suppression of IgE synthesis.^{19,20} Therefore, the reduced OVA-specific IgE levels in sema3A mice may be associated with decreased Th2 cytokines and increased Th1 cytokines. Some studies have demonstrated that sema3A treatment suppresses the release of IFN- γ and IL-4. For instance, Tanaka et al¹⁷ found that sema3A treatment suppressed serum levels of IFN- γ , and Catalano et al²¹ reported that IFN- γ production was inhibited after treatment with sema3A in CD3- and CD28-stimulated primary human CD4⁺ T cells. However, our results show that sema3A significantly enhanced expression of IFN- γ in the spleen of AR mice. We speculate that the difference in inflammation types as well as the dose and timing of the administration

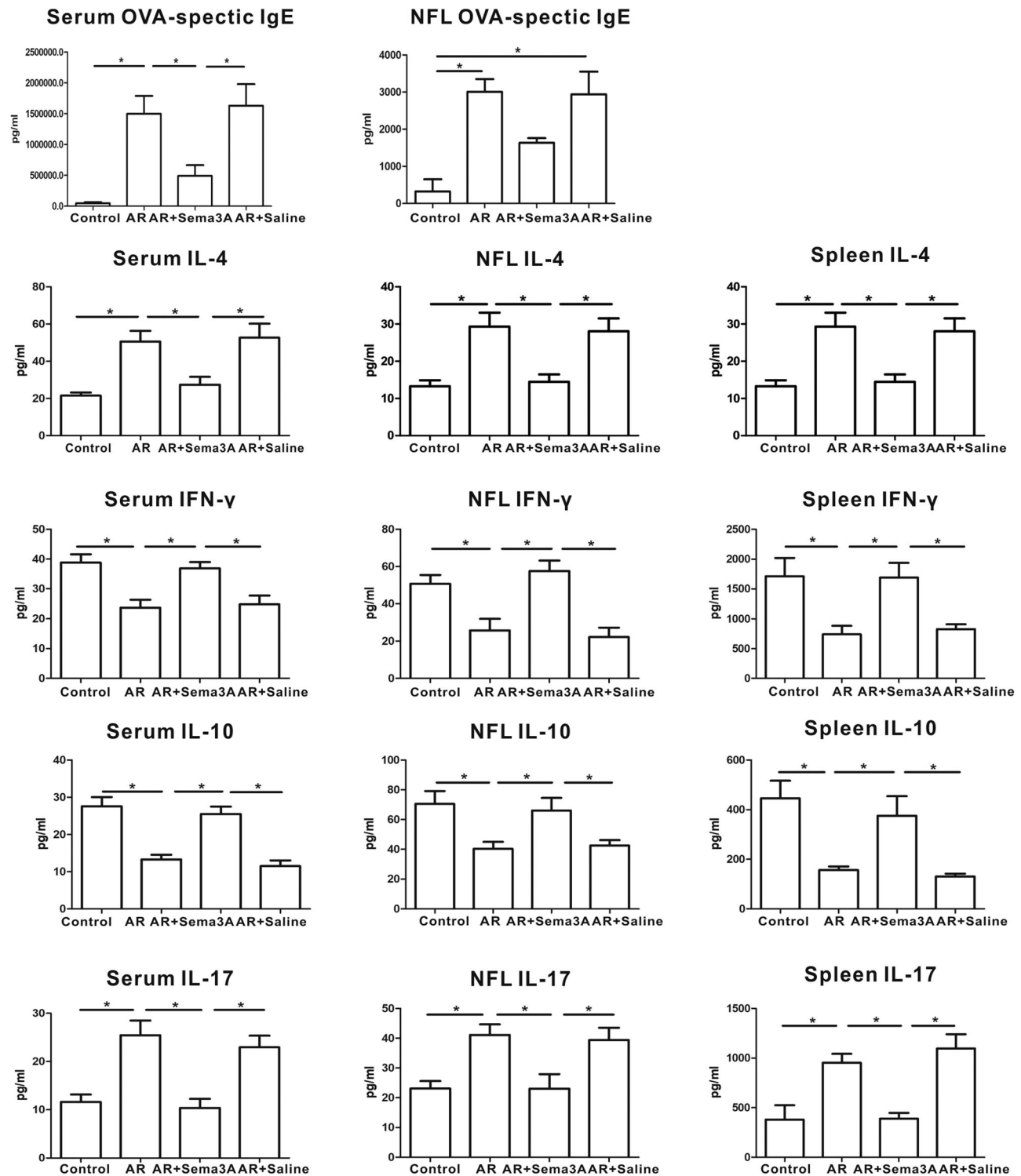


FIGURE 6. OVA-specific IgE and cytokines concentrations in serum, NFL and spleen. OVA-specific IgE levels in serum and NFL were determined by ELISA. IL-4, IFN- γ , IL-10, and IL-17 levels in serum, NFL, and splenocyte culture supernatants also determined by ELISA. Splenocytes were stimulated in vitro with lipopolysaccharides (2 μ g/mL) and concanavalin A (10 μ g/mL) for 48 hours (for IL-4 and IL-17 detection). Splenocytes were stimulated in vitro with lipopolysaccharides (2 μ g/mL) for 24 hours (for IFN- γ and IL-10 detection). Data represent the mean $\pm$ SEM (n = 6 per group). Data are representative of 3 experiments. *p < 0.05. ELISA = enzyme-linked immunosorbent assay; IgE = immunoglobulin E; IL = interleukin; INF = interferon; NFL = nasal lavage fluid; OVA = ovalbumin.

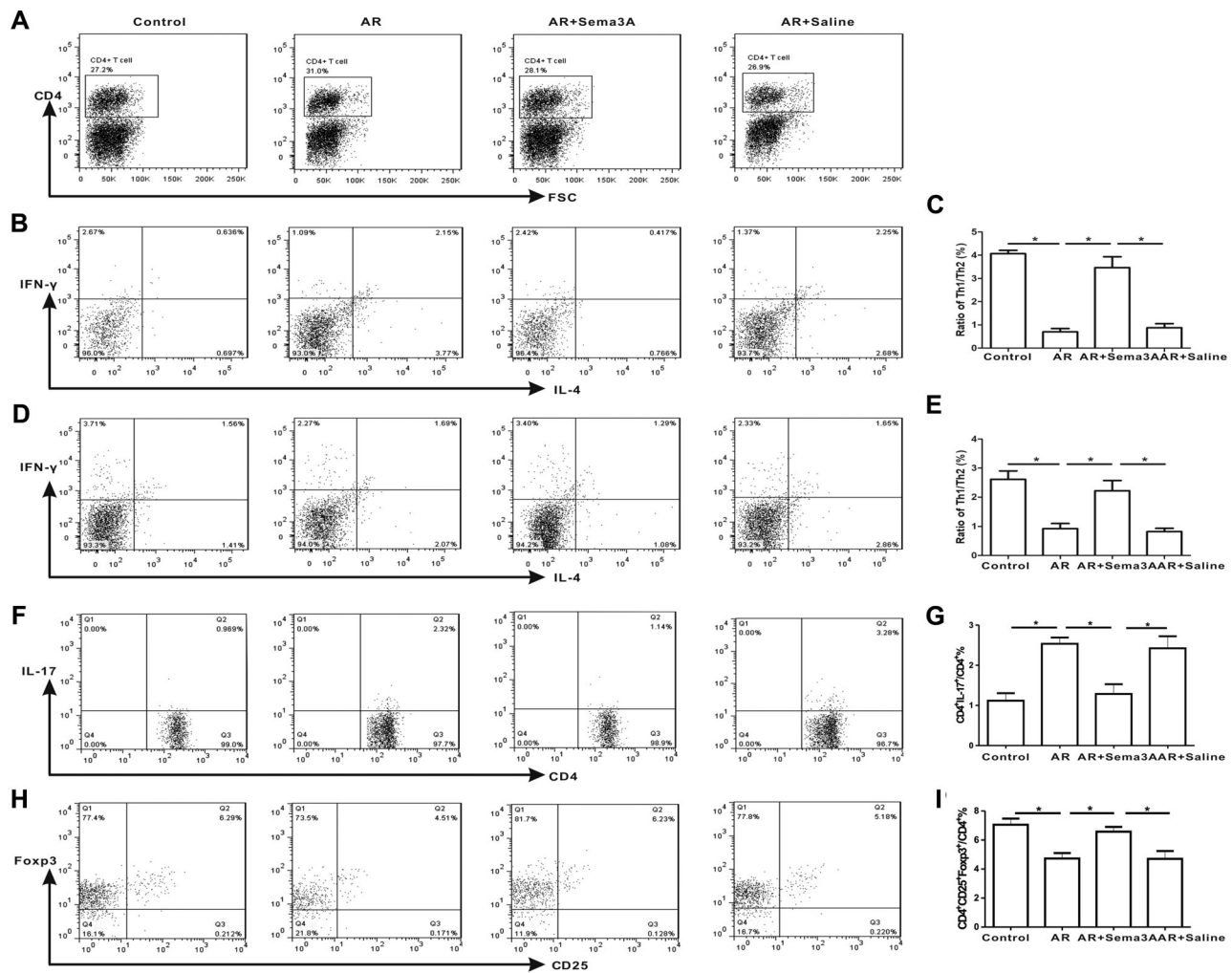


FIGURE 7. Flow cytometric analysis of a T-lymphocyte subset in the spleen. (A) Representative flow cytometry figures of CD4⁺ T cells in the spleen. (B) Representative flow cytometry figures of CD4⁺IFN- γ ⁺ Th1 cells and CD4⁺IL-4⁺ Th2 cells in the nasal mucosa. (C) Ratios of CD4⁺IFN- γ ⁺ Th1/CD4⁺IL-4⁺ Th2 cells in the nasal mucosa among the 4 groups. (D) Representative flow cytometry figures of CD4⁺IFN- γ ⁺ Th1 cells and CD4⁺IL-4⁺ Th2 cells in the spleen. (E) Ratios of CD4⁺IFN- γ ⁺ Th1/CD4⁺IL-4⁺ Th2 cells in the spleen among the 4 groups. (F) Representative flow cytometry figures of CD4⁺IL-17⁺ T cells in the spleen. (G) Percentages of CD4⁺IL-17⁺ T cells in the spleen among the 4 groups. (H) Representative flow cytometry figures of CD4⁺CD25⁺Foxp3⁺ T cells in the spleen. (I) Percentages of CD4⁺CD25⁺Foxp3⁺ T cells in the spleen among the 4 groups. Data present as the mean $\pm$ SEM ($n = 6$ per group). * $p < 0.05$. IL = interleukin; INF = interferon; NFL = nasal lavage fluid; OVA = ovalbumin; Th = T helper.

of sema3A may have contributed to this discrepancy. Apart from the Th1/Th2 imbalance, Treg/Th17 imbalance is also involved in the pathogenesis of AR.⁶ In the present study, we have shown that sema3A administration enhanced production of Treg cells and inhibited production of Th17 cells in AR mice, suggesting that it plays an important role in AR by regulating the Treg/Th17 imbalance.

It remains unclear how sema3A regulates Treg and Th17 responses in AR. According to the existing literature, sema3A may act directly on T cells and inhibit T-cell proliferation and cytokine production. Both sema3A and its receptor neuropilin-1 (NRP1) were expressed in Treg cells and they may have a regulatory effect on both Treg and Th17 cells.^{22–24} One clinical study²⁴ demonstrated that sema3A had lower expression on Treg cells in asthma patients and coculture of sema3A with Treg cells could increase the regulatory properties of these cells. Lepelletier

et al¹⁵ demonstrated that sema3A exerts its inhibitory function in T cells by regulating actin polymerization, affecting T-cell receptor (TCR) polarization and interfering with early TCR signal transduction events, such as ZAP-70 and focal adhesion kinase (FAK) phosphorylation. In another study, Catalano et al²⁵ suggested that sema3A inhibits T-cell proliferation by blockade of the Ras/mitogen-activated protein kinase (MAPK) signaling pathway.

B cells have generally been described as a positive regulator in immune responses by secreting allergen-specific IgE antibodies under pro-Th2 inflammatory conditions. Aside from their IgE antibody secretion capacities, B cells were also shown to exert suppressive regulation in immune responses, including AR, by secreting inhibitory cytokines.^{26,27} Recent studies suggested a relationship between sema3A and B cells. One clinical research study²⁸ investigated the role of sema3A in the pathogenesis of SLE

and found that expression of sema3A on B cells was decreased in SLE patients and the addition of sema3A to B-cell cocultures significantly enhanced the regulatory function of the B cells. Thus, it was speculated that sema3A could have an immunoregulatory effect on B cells in AR. However, further studies are needed to confirm the effect of sema3A on B-cell responses in AR.

Evidence suggests that neuroimmune interaction plays a role in the pathogenesis of AR.²⁹ Neuroimmune interaction is a bidirectional pathway of intersystem communication in that neuropeptides secreted by C-fiber neurons can alter the proliferation of immune cells, while cytokines produced by immune cells in turn could modify neuronal activation. Thus, it can be speculated that sema3A may also modulate T-cell-mediated inflammation indirectly by neuroimmune interactions. Nasal administration of sema3A inhibits nerve-fiber innervation in nasal mucosa and results in lower expression of neuropeptides. The secreted neuropeptides subsequently regulate T-cell-mediated reactions such as Th1/Th2 and Treg/Th17 responses. In the future, we intend to investigate the exact mechanisms of sema3A alleviating allergic inflammation through neuroimmune interactions.

Airway remodeling has been considered a significant feature of asthma. However, only a few studies have investigated remodeling in the nasal mucosa of AR, and conflicting findings have been observed. Eifan et al³⁰ found no evidence of remodeling in patients with AR. However, another study³¹ suggested that remodeling also occurs in the nasal mucosa of AR patients, although the remodeling in AR is less extensive than that seen in asthma. Movassagh et al³² reported that sema3A and its receptor, Nrp1, are involved in the regulation of airway smooth

muscle mass hyperplasia, which is a hallmark of airway remodeling in asthma and chronic obstructive pulmonary disease. In this study, we have shown that expression of collagenous fibers was increased in AR mice, whereas sema3A administration significantly decreased expression of nasal fibrosis in the nasal mucosa. Our data further suggest that sema3A has a regulatory effect on the generation of collagenous fibers in the AR mice model.

Previous evidence combined with our results suggest that sema3A may inhibit allergic responses in AR by directly regulating T-cell proliferation. It was speculated that sema3A acted directly on T cells, enhanced the production of Th1 and Treg cells, and inhibited production of Th1 and Th17 cells. On the other hand, sema3A may inhibit allergic responses in AR by indirectly regulating neuroimmune interaction. Sema3A inhibited nerve fiber innervation in nasal mucosa and resulted in decreased expression of neuropeptides, which was followed by regulated T-cell-mediated reactions, including Th1/Th2 and Treg/Th17 responses.

There are some limitations to our study. We did not use a sema3A agonist or sema3A mutant mice model to further confirm the hypothesis in the mouse model of AR. Also, we only performed a preliminary study of the effect of sema3A on T-cell responses. Further studies are needed to explore the mechanisms by which sema3A indirectly regulates allergic responses in AR.

In conclusion, our results suggest that sema3A could suppress allergic responses in AR and that sema3A may exert its antiallergic effect directly and indirectly by inhibiting Th2/Th17 responses and enhancing Th1/Treg responses. These results suggest that sema3A plays an essential immunoregulatory role in AR.

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