IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

Sympathetic Nerves Positively Regulate Eosinophil-Driven Allergic Conjunctivitis via α 1-Adrenergic Receptor Signaling

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Eosinophils are a major cause of tissue injury in allergic conjunctivitis. The biological nature of eosinophils in the conjunctiva and the mechanisms that control eosinophils' responses in allergic conjunctivitis are currently not completely understood. This study reports that conjunctival eosinophils comprise two populations—Siglec-F^{int} and Siglec-F^{hi}—in different life stages. Siglec-F^{int} eosinophils partly expressed CD34 and were in the immature (or steady) state. Siglec-F^{hi} eosinophils did not express CD34, sharply increased in number after short ragweed (SRW) pollen challenge, and were in the mature (or activated) state. Moreover, chemical sympathectomy by 6-hydroxydopamine reduced the recruitment and activation of eosinophils, whereas the activation of the sympathetic nerve system (SNS) with restraint stress accelerated the recruitment and activation of eosinophils in SRW-induced conjunctivitis. It was also found that two eosinophil populations expressed alpha-1a-adrenergic receptors (α 1a-ARs); in SRW-induced conjunctivitis, treatment with an α 1a-AR antagonist decreased eosinophil responses, whereas treatment with an α 1a-AR agonist aggravated eosinophil responses. Thus, eosinophil responses in conjunctivitis are regulated by the SNS via α 1a-AR signaling. SNS inputs or α 1a-AR function may be potential targets for the treatment of allergic conjunctivitis. (*Am J Pathol* 2020, 190: 1298–1308; <https://doi.org/10.1016/j.ajpath.2020.02.004>)

Allergies are a major public health problem with a high socioeconomic impact.^{1,2} Approximately half of all allergic patients experience allergies on the ocular surface.^{3,4} Allergic conjunctivitis is a common inflammatory disease of the eyes, which can be classified as either mild transient inflammation (seasonal conjunctivitis) or severe chronic inflammation (vernal keratoconjunctivitis).^{5,6} The responses of allergic conjunctivitis include two phases: the early IgE-dependent response after a 15-minute exposure to the allergen, which results in itchiness, eyelid swelling, conjunctival edema, and mucus accumulation, and the late T helper 2 (Th2)-type inflammatory cytokine-dependent response after 12–24 hours of exposure to the allergen,^{7–9} which involves the recruitment of eosinophils.

The infiltration of eosinophils is a major cause of tissue injury and remodeling in allergic diseases.¹⁰

Eosinophils are granulocytic leukocytes that contain a bilobed nucleus and are differentiated from CD34⁺IL-5R α ⁺ eosinophil progenitors (EoPs).¹¹ These progenitors derive from hematopoietic stem cells in bone marrow and differentiate into mature eosinophils when they are exposed to

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IL-3, granulocyte-macrophage colony stimulating factor (GM-CSF), and IL-5.¹² It was traditionally believed that EoPs in bone marrow completely differentiate into mature eosinophils before migrating into peripheral tissues¹³; however, recent research has indicated that some CD34⁺CCR3⁺ EoPs reside in lung tissues, and these cells rapidly increase and show significant proliferative capacity when stimulated by allergens.¹⁴ Moreover, eosinophils in lung tissues have been confirmed to belong to a heterogeneous population when exposed to allergens; they were classified into Siglec-F⁺Gr-1^{hi} and Siglec-F⁺Gr-1⁻.¹⁵ Although the roles of eosinophils have been addressed in the ocular allergy model,^{16–19} the subsets of eosinophils in conjunctiva have not been well understood.

Eosinophilia is a hallmark of allergic diseases, such as asthma, rhinitis, and allergic dermatitis.²⁰ A clear understanding of the mechanisms that control the recruitment of eosinophils in allergic diseases will help us locate potential targets for the therapy of eosinophilia-related allergic diseases. An increasing number of studies have confirmed that the sympathetic nerve system (SNS) plays a crucial role in regulating many immune cells.^{21–23} The SNS was also reported to participate in allergic diseases.²⁴ Eosinophils have been described as being in close proximity to the SNS,²⁵ and the adrenaline— α/β -adrenergic receptor (AR) axis controls eosinophil responses.²⁶ The expression of ARs in conjunctival eosinophils and the relationship between the SNS and eosinophil responses in allergic conjunctivitis remain unclear.

In this study, we identified and analyzed the heterogeneity of conjunctival eosinophils through immunostaining and flow cytometry. By using flow cell sorting and quantitative PCR (qPCR) analysis, the expression levels of ARs in conjunctival eosinophils were measured. With short ragweed (SRW) pollen-induced allergic conjunctivitis, chemical sympathectomy, restraint stress, and antagonist or agonist treatment, the changes in each eosinophil population and the relationship between the SNS and the eosinophil responses in allergic conjunctivitis were investigated.

Materials and Methods

Animals

Wild-type albino laboratory BALB/c specific pathogen-free mice aged 7 to 8 weeks that had no eye diseases were purchased from the Medical Experimental Animal Center (Guangdong, China). All animal procedures were approved by the Jinan University Laboratory Animal Committee on Animal Welfare. All of the animals were treated in conformity with the Association for Research in Vision and Ophthalmology's Statement for the Use of Animals in Ophthalmology and Vision Research and the guidelines of the Animal Experimental Committee at Jinan University. The animals were anesthetized by inhalation of 2% isoflurane and euthanized with an overdose of carbon dioxide and cervical dislocation.

Induction of Allergic Conjunctivitis

The procedures were performed as previously described with modification from footpad injection to intraperitoneal injection for allergen sensitization.^{7,27,28} Briefly, BALB/c mice were immunized with 50 μ g of SRW pollen (Greer Lab, Lenoir, NC) in 5 mg of Imject Alum (Pierce Biotechnology, Rockford, IL) by intraperitoneal injection on day 0. All of the mouse eyes were topically treated with 0.5 mg SRW in 10 μ L of phosphate-buffered saline (PBS) by eye drops daily, from days 6 to 12.

Clinical Scoring of Allergic Conjunctivitis

Mice were examined clinically for signs of immediate hypersensitivity 20 minutes after each topical SRW challenge and for late-phase reaction 6 hours after each topical SRW challenge, as described previously.^{29,30} Lid swelling, tearing, conjunctival chemosis, and conjunctival redness, were each scored on a scale of 1 to 3, and the scores were summed.⁹

Immunostaining

The entire ocular surface, including the cornea and the conjunctiva up to the mucocutaneous junction, was dissected, as described previously.³¹ Briefly, the entire eye was dissected together with the eyelid and fixed in paraformaldehyde for 1 hour. Next, under the dissecting microscope, the tissues were clipped to remove muscle and other accessory tissues so that only the conjunctival tissues remained. The conjunctival tissues were then blocked in 2% bovine serum albumin for 15 minutes, followed by permeabilization in 0.2% Triton X-100 for 15 minutes at room temperature. Subsequently, the conjunctival tissues were incubated with phycoerythrin (PE)-conjugated anti-mouse Siglec-F (552126; BD Biosciences, San Jose, CA), Alexa Fluor 488-conjugated anti-mouse CD125 antibody (558533; BD Biosciences), or fluorescein isothiocyanate-conjugated anti-mouse CCR3 antibody (144510; BioLegend, San Diego, CA) at 4°C overnight. The stained conjunctival tissues were washed three times with PBS and placed on glass slides to flatten them. A mounting medium containing 1 μ mol/L DAPI (28718-90-3; Sigma-Aldrich, St. Louis, MO) was put on the conjunctival tissues. Images of the conjunctiva were analyzed using a DeltaVision microscopy imaging system (Applied Precision, Issaquah, WA).

Chemical Sympathectomy

First, 6-hydroxydopamine (6-OHDA) (H2380; Sigma-Aldrich) was freshly prepared for each experiment and was given to mice by intraperitoneal injection (200 mg/kg).^{32,33} After 2 weeks, immunostaining of the SNS was performed to confirm to ablation of the SNS after a 10-day 6-OHDA treatment, as previously described.³³ Once

ablation of the SNS was successful, the induction of allergic conjunctivitis was performed as described above. After the induction of allergic conjunctivitis, conjunctival tissues were obtained to conduct further analysis ([Supplemental Figure S1](#)).

Restraint Stress Model

The restraint stress model was used to activate the SNS, as described previously.^{34–36} Briefly, mice were confined in ventilated 50-mL conical tubes for a period of 12 hours during their active nocturnal cycle. Restraint started at 6:00 PM (dark onset) and ended at 6:00 AM (light onset). The restraint was initiated 3 days before SRW-induced conjunctivitis and was also performed each day during the period of SRW challenge. Control mice were free to roam in their cages but without food and water during the treatment period. The plasma norepinephrine was then determined with an enzyme-linked immunosorbent assay kit (HEA907Ge; Cloud-Clone Corp, Wuhan, China) to validate the activation of SNS after 3 days of restraint treatment.

RNA-Seq Analysis

RNA-sequencing (RNA-seq) analysis was performed as described previously by the authors' group.³⁷ Briefly, conjunctival tissues were cut into pieces and smashed with a TissueRuptor (Qiagen, Hilden, Germany) in Buffer RZ (RK145; Tiangen, Beijing, China). Total RNA of the conjunctival tissues was obtained using an RNAsimple Total RNA Kit (DP419; Tiangen) and sent to BGI (Guangdong, China) for mRNA sequencing analysis. For the differentially expressed genes, a volcano plot was created using Excel 2010 software (Microsoft, Redmond, WA). These genes, which are used for plotting heat maps, were sorted from the differentially expressed genes with the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway. Molecular network analysis results for RNA-seq data were visualized with Cytoscape software version 3.7.2 (Free Software Foundation, Boston, MA; <https://cytoscape.org/download.html>).

Pharmacologic Agonism and Antagonism

To observe the roles of α 1a-AR in eosinophil responses, the α 1a-AR agonist A 61603 hydrobromide (5 μ L, 2.5 μ mol/L; 1052; Tocris Bioscience, Abingdon, UK) and the antagonist tamsulosin (TAM) hydrochloride (5 μ L, 20 μ mol/L; 3050/10; Tocris) were administered by subconjunctival injection.

Flow Cytometric Analysis

Conjunctival tissues were cut into pieces and digested in 0.4% collagenase type I (C0130; Sigma-Aldrich) for 30 minutes. After washing with PBS, the digested suspension

was passed through a 75- μ m filter to obtain single cells. These single conjunctival cells were blocked in Flow Cytometry Staining Buffer (00-4222; eBioscience, San Diego, CA), which contains anti-CD16/32 antibody (1:100; 14-0161-85; eBioscience), at room temperature for 10 minutes and were then incubated in a mixture of the following antibodies (1:100) at room temperature for 30 minutes: allophycocyanin-conjugated anti-mouse CD45 antibody (559864; BD Biosciences), PE-conjugated anti-mouse Siglec-F antibody (552126; BD Biosciences), Brilliant Violet 421-conjugated anti-mouse CD64 antibody (139309; BioLegend), and either Alexa Fluor 488-conjugated anti-mouse CD125 antibody (558533; BD Biosciences) or fluorescein isothiocyanate-conjugated anti-mouse CCR3 antibody (144510; BioLegend), or fluorescein isothiocyanate-conjugated anti-mouse CD34 antibody (553733; BD Biosciences). After washing with PBS, the stained cells were analyzed with a BD FACSCanto.

Transcript Amplification from Conjunctival Eosinophils

Digested conjunctival cells using collagenase type I were incubated in a 1:100 mixture of the following antibodies at room temperature for 30 minutes: allophycocyanin-conjugated anti-mouse CD45 antibody, PE-conjugated anti-mouse Siglec-F antibody, and Brilliant Violet 421-conjugated anti-mouse CD64 antibody. With the flow cell sorter, Siglec-F^{hi}CD64[−] and Siglec-F^{int}CD64[−] cells were sorted. The transcripts of these cells were amplified using a REPLI-g WTA Single Cell Kit (150063; Qiagen), as previously described.^{38–40}

qPCR

mRNA levels were analyzed by qPCR. Conjunctival tissues were cut into pieces and homogenized with a TissueRuptor (Qiagen) in Buffer RZ (RK145; Tiangen, Beijing). Total RNA of these tissues was extracted using an RNAsimple Total RNA Kit (DP419; Tiangen). cDNA from the conjunctival tissues was obtained from the extracted total RNA with the ReverTra Ace qPCR RT Kit (FSQ-101; Toyobo, Osaka, Japan). The expression level of the target genes of the conjunctival tissues and the amplified transcripts of the conjunctival eosinophils were evaluated using THUNDERBIRD SYBR qPCR Mix (QPS-201; Toyobo). The qPCR primers that were used in this study are listed in [Table 1](#).

Statistical Analysis

The results are presented as means $\pm$ SD. For comparisons between groups, one-way analysis of variance was performed, followed by Tukey's HSD test. Statistical significance was set at $P < 0.05$.

Results

Conjunctiva Contain Distinct Eosinophil Populations

The cell surface markers Siglec-F, CCR3, and IL-5Ra (CD125) are generally used to identify eosinophils in mice.^{15,41} Immunostaining analysis revealed that many Siglec-F⁺ cells exist in mouse conjunctiva, and these cells were CCR3⁺ and CD125⁺ (Figure 1A). In the lung tissues, some Siglec-F⁺ cells were identified as macrophages.⁴¹ Therefore, the authors used CD64, which is a specific marker of macrophages,⁴² to exclude the macrophages in the CD45⁺Siglec-F⁺ conjunctival cells by flow cytometry. However, all CD45⁺Siglec-F⁺ cells in the conjunctiva were CD64⁻. Some researchers have revealed that the expression levels of Siglec-F can be used to discriminate the maturation or activation of eosinophils.^{43–45} In the present study, Siglec-F⁺ conjunctival cells were classified into two populations: the Siglec-F^{int} and Siglec-F^{hi} cells. Further analysis indicated that Siglec-F^{int} and Siglec-F^{hi} conjunctival eosinophils have distinct phenotypes; Siglec-F^{int} eosinophils were CD45^{int} CD125^{lo} CD34^{+/-} (10.9% CD34⁺), and Siglec-F^{hi} eosinophils were CD45^{hi} CD125^{int} CD34⁻ (Figure 1B).

Allergic Conjunctivitis Is Associated with High Numbers of Siglec-F^{hi} Eosinophils

To investigate changes in the two eosinophil populations in allergic conjunctivitis and their contribution to it, SRW pollen was used to establish an allergic conjunctivitis model. After stimulation with SRW, BALB/c mice showed typical signs of allergic conjunctivitis, including eyelid swelling, tearing, conjunctival chemosis, conjunctival redness (Figure 2A), frequent scratching of the eyelids, and high clinical scores (Figure 2B). This was followed by a Th2 inflammatory response in the conjunctival tissues. The gene expression levels of Il4, Il5, and Il13 in the conjunctiva were markedly increased after six repeated SRW challenges (Figure 2C). In this study's immunostaining and flow cytometry analysis, an increase of eosinophils was also clearly observed within the conjunctival tissues in SRW-treated mice. The percentage of Siglec-F⁺ cells within the conjunctival cells showed a approximately sevenfold increase, whereas within the CD45⁺ cells, a approximately fourfold increase was seen in SRW-treated mice compared with saline-treated control mice. In control mice, the proportion of Siglec-F^{hi} eosinophils within the conjunctival cells was almost equivalent to that of the Siglec-F^{int} eosinophils, and the eosinophil ratio (Siglec-F^{hi}/Siglec-F^{int}) was about one. After the induction of conjunctivitis with repeated SRW challenges, the Siglec-F^{hi} eosinophils increased, and the eosinophil ratio (Siglec-F^{hi}/Siglec-F^{int}) increased to approximately 3.5 (Figure 2, D–F).

Table 1 PCR Primers Used in This Study

Gene name	Primer pair
<i>Il4</i>	Forward 5'-AGCTAGTTGTCATCCTGCTC-3' Reverse 5'-TGTGACCTCGTTCAAAATGC-3'
<i>Il5</i>	Forward 5'-GGGGTACTGTGGAAATGCTA-3' Reverse 5'-TTGTGGGGTTTTCATCTG-3'
<i>Il13</i>	Forward 5'-GCAACATCACACAAGACCAG-3' Reverse 5'-AGGCCATGCAATATCCTCTG-3'
<i>Adra1a</i>	Forward 5'-GTGGAGTTATTGGATCGCTG-3' Reverse 5'-CTCCAAAGACCAAGACCCAG-3'
<i>Adra1b</i>	Forward 5'-AGGGAAAGAAGAGTATTTGGG-3' Reverse 5'-TTGTGCTTTCTGATCCAAAC-3'
<i>Adra1d</i>	Forward 5'-TTTTACTCCTTACTGCCACC-3' Reverse 5'-CAGGGCAAAAACAACAGATT-3'
<i>Adra2a</i>	Forward 5'-CTGCTTTGACATTTCTCTGAC-3' Reverse 5'-CTTGGTCCATCGTGTAATA-3'
<i>Adra2b</i>	Forward 5'-TCACTAAGGTCCTGATCACT-3' Reverse 5'-AAACACACAGGAAGGGATT-3'
<i>Adra2c</i>	Forward 5'-GTTCCCCTTCTCTTCAGCT-3' Reverse 5'-CCTTCTCCTCCTTCGAAAG-3'
<i>Adrb1</i>	Forward 5'-TTACTCAAGACCGAAAGCAG-3' Reverse 5'-CCATACTAAGCCACACTCTC-3'
<i>Adrb2</i>	Forward 5'-CTCCTTTTTGCCTATCCAGA-3' Reverse 5'-GCACGTAGAAAGACACAATC-3'
<i>Adrb3</i>	Forward 5'-TTCCGTCGTCTTCTGTGTAGC-3' Reverse 5'-ACCTTCATAGCCATCAAACCTGT-3'
<i>Gapdh</i>	Forward 5'-CAAGGACACTGAGCAAGAG-3' Reverse 5'-TGCAGCGAAGTTTATTGATG-3'

Sympathetic Nerves Regulate Inflammation and Eosinophil Responses in Allergic Conjunctivitis

A previous study showed the SNS was associated with allergy and modified the immune system in favor of Th2 action.²⁴ However, the specific roles of the SNS in allergic conjunctivitis are unclear. To investigate whether the SNS influences inflammation and eosinophil activation in allergic conjunctivitis, a chemical sympathectomy (adrenergic denervation) was performed with 6-OHDA, and the changes in the clinical scores, conjunctival gene transcription, and eosinophil responses after SRW challenge were observed. The clinical scores of 6-OHDA-treated mice were significantly lower than those of saline-treated control mice 20 minutes and 6 hours after SRW challenge at challenge day 6 and 7 (Figure 3A). RNA-seq results revealed that the conjunctival gene transcription had changed, with 259 genes down-regulated and 46 genes up-regulated in 6-OHDA-treated mice after SRW challenge, compared with control mice after SRW challenge (Figure 3B). The expression of genes that are involved in the immune system process and the NF-kappa B and chemokine signaling pathways had all decreased in the conjunctival tissues of 6-OHDA-treated mice (Figure 3, C, D, and E). By Cytoscape network analysis, those genes involved in the regulation of Th2 cell differentiation, the type 2 response, and eosinophil chemotaxis were all down-regulated in conjunctival tissues

Figure 1 Identification of eosinophils in conjunctiva. **A:** Immunostaining of the intact conjunctiva with anti-mouse Siglec-F, anti-mouse CCR3, and anti-mouse CD125 antibodies. **B:** Analysis of the expression of CD45, CCR3, CD125, and CD34 molecules in each eosinophil population. **Top row:** P1 represents the population of the intact cells after exclusion of debris; P2, single cells; P3, CD45 positive cell population; P4, cell population that highly expressed Siglec-F antigen; P5, cell population that intermediately expressed Siglec-F antigen. **Bottom row:** Expression of CD45, CCR3, CD125, and CD34 antigens in Siglec-F (hi) eosinophils and Siglec-F (int) from P4 and P5 cell population are shown, respectively. Original magnification, $\times 400$ (A). FSC-A, forward scatter-area; FSC-H, forward scatter-height; SSC-A, side scatter-area.

of 6-OHDA-treated mice after SRW challenge (Figure 3, F and G). The flow cytometry results showed that the proportion of eosinophils in conjunctival cells and CD45⁺ leukocytes had both decreased, and the eosinophil ratio

(Siglec-F^{hi}/Siglec-F^{int}) had decreased from approximately 2.3 to approximately 0.8 (Figure 3H). These RNA-seq and flow cytometry results indicate that SNS is important for Th2 inflammatory and eosinophil responses in the allergic

Figure 2 The responses of eosinophils in SRW-induced allergic conjunctivitis. **A:** Changes in the appearance of the ocular surface at 6 hours after the last SRW challenge. **B:** Investigation of clinical scores 20 minutes and 6 hours after each SRW challenge. **C:** Alteration in the gene expression of Th2-type cytokines *Il4*, *Il5*, and *Il13* in conjunctival tissues at 12 hours after the last SRW challenge. **D:** Immunostaining of conjunctival tissues from saline-treated control mice and SRW-treated mice at 12 hours after the last SRW challenge with PE-conjugated anti-mouse Siglec-F antibody. **E and F:** Analysis of the changes in the eosinophil number at 12 hours after the last SRW challenge. Data are expressed as means $\pm$ SD. $n = 6$ mice at each time point (**B**); $n = 3$ independent experiments (**C**, **E**, and **F**); $n = 3$ mice per experiment (**C**); $n = 5$ mice per experiment (**E** and **F**). * $P < 0.05$, ** $P < 0.01$. Original magnification, $\times 40$ (**D**). PE, phycoerythrin; SRW, short ragweed; SSC-A, side scatter-area.

Figure 3 The effects of SNS in conjunctival gene transcription and eosinophil responses in conjunctivitis. **A:** Clinical scores in 6-OHDA-treated and saline-treated control mice 20 minutes and 6 hours after SRW challenges. **B:** Volcano plot of differentially expressed genes (DEGs) in allergic conjunctival tissues after 6-OHDA treatment. The numbers in parentheses indicate the number of genes that have been up-regulated (Up) and down-regulated (Down), respectively. **C–E:** Heat maps of the top 20 DEGs that are involved in immune system processes (**C**), the nuclear factor (NF)-kappa B signaling pathway (**D**), and the chemokine signaling pathway (**E**). **F** and **G:** Cytoscape network analysis of transcripts involved in T helper type 2 (Th2) cell differentiation, the type 2 response, and eosinophil chemotaxis (down-regulated genes are shown in gray). **H:** Changes in eosinophils in the allergic conjunctiva after 6-OHDA treatment. **I:** Changes in norepinephrine plasma level after restraint stress treatment. **J:** Investigation of clinical scores in restraint stress-treated and control mice after SRW challenge. **K:** The changes in eosinophils in the conjunctiva after restraint stress treatment. **B–H** and **K:** All conjunctival tissues in SRW-treated mice were collected at 12 hours after the last SRW challenge. Data are expressed as means $\pm$ SD. $n = 6$ mice at each time point (**A** and **J**); $n = 3$ independent experiments (**H** and **K**); $n = 5$ mice per experiment (**H** and **K**); $n = 6$ mice (**I**). * $P < 0.05$ versus saline+SRW; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, and $^{\dagger\dagger\dagger}P < 0.001$; $^{\ddagger}P < 0.05$ versus SRW. 6-OHDA, 6-hydroxydopamine; SNS, sympathetic nerve system; SRW, short ragweed.

Figure 4 Changes in clinical scores and eosinophil responses after treatment with α 1a-AR antagonists and agonists. **A:** Investigation of the expression of *Adra1a* gene in conjunctival eosinophils (Eo) with quantitative PCR. **B:** Investigation of clinical scores in TAM-treated and control (saline-treated) mice after SRW challenges 20 minutes and 6 hours at each challenge day. **C:** Changes of eosinophils in the conjunctiva after TAM treatment. **D:** Investigation of clinical scores in 6-OHDA treated mice 20 minutes and 6 hours after each challenge in A 61603 hydrobromide-treated or saline-treated mice ($n = 6$ mice at each time point). **E:** Changes in eosinophils in conjunctiva after A 61603 hydrobromide treatment. Data are expressed as means $\pm$ SD. $n = 3$ independent experiments (**A**, **C**, and **E**); $n = 3$ mice per experiment (**A** and **K**); $n = 6$ mice at each time point (**B**); $n = 5$ mice per experiment (**C** and **E**). * $P < 0.05$, ** $P < 0.01$; $^{\dagger}P < 0.05$ versus saline+SRW. 6-OHDA, 6-hydroxydopamine; AR, adrenergic receptor; SRW, short ragweed; TAM, tamsulosin hydrochloride.

conjunctivitis. Moreover, when using restraint stress to treat mice for 3 days, the norepinephrine plasma levels significantly increased (Figure 3I), indicated that sympathetic nerves were activated. The clinical scores of restraint stress-treated mice increased and were significantly higher than those of control mice after SRW challenge 20 minutes and 6 hours on day 7 (Figure 3J). In addition, restraint stress can accelerate eosinophil responses; the proportion of eosinophils in the conjunctival cells and CD45⁺ leukocytes, and the eosinophil ratio (Siglec-F^{hi}/Siglec-F^{int}) of the restraint stress-treated group were both higher than those of the control group after SRW challenge (Figure 3K). These results reveal that the SNS controls both the inflammatory and eosinophil responses in conjunctivitis.

Sympathetic Nerves Regulate Eosinophil Responses in Conjunctivitis through the Alpha-1a Adrenergic Receptors

The activation of SNS usually regulates immune cells through the AR signaling pathway. The ARs have both α and β subtypes. By qPCR analysis, eosinophils were confirmed to predominantly express the α 1a-AR gene *Adra1a* (Figure 4A), but the expression level of other adrenergic receptor subtypes such as alpha-adrenergic

receptors (*Adra1b*, *Adra1d*, *Adra2a*, *Adra2b* and *Adra2c*) and beta-adrenergic receptors (*Adrb1*, *Adrb2*, and *Adrb3*) were undetectable. This suggests that the SNS may mainly regulate eosinophils through α 1a-ARs. To verify this hypothesis, the authors used the α 1a-AR antagonist TAM to block the function of this receptor. The results revealed that the clinical scores were significantly decreased (Figure 4B), and the eosinophil response was attenuated; the percentage of eosinophils within conjunctival cells and CD45⁺ leukocytes, and the eosinophil ratio (Siglec-F^{hi}/Siglec-F^{int}) in TAM-treated mice were all lower than those in control mice after SRW challenge (Figure 4C). Finally, after treatment with the α 1a-AR agonist A 61603 hydrobromide, the clinical scores and the conjunctival eosinophil responses had both significantly increased in 6-OHDA-treated mice (Figure 4, D and E).

Discussion

This study shows that conjunctival eosinophils can be classified into two populations with distinct phenotypes: Siglec-F^{int} and Siglec-F^{hi}. SRW-induced allergic conjunctivitis was mainly associated with an increase in Siglec-F^{hi} eosinophils. Moreover, the authors confirmed that infiltration of these

eosinophils in SRW-induced allergic conjunctivitis was regulated by the SNS through $\alpha 1a$ -AR signaling.

Eosinophils are granulocytic leukocytes that arise from bone marrow granulocyte–macrophage progenitors through an intermediate—EoPs. These cells are rare in the blood, but they are more abundant in the peripheral tissues.⁴⁶ Until recently, eosinophils were believed to reach maturation in bone marrow before being released into the blood as end-stage granulocytes. However, a recent study reported that CD34⁺ EoPs reside in peripheral tissues and show local proliferative capacity when stimulated by allergens.¹⁴ Here, the Siglec-F^{int} and Siglec-F^{hi} eosinophil populations were found to be present in the normal conjunctiva. They differ in the expression of some surface antigens, in particular CD34, which is the hematopoietic progenitor marker. Siglec-F^{int} eosinophils had approximately 10% CD34⁺ cells, whereas Siglec-F^{hi} eosinophils had none. This suggests that Siglec-F^{int} eosinophils in conjunctiva may be in the immature stage. Previous studies identified Siglec-F^{int} cells as immature (or resting) eosinophils that settled in the lymphoid organs and uninfamed tissues, whereas Siglec-F^{hi} cells were distinguished as mature (or activated) eosinophils, which are usually located in the extralymphoid tissues and inflamed tissues.^{44,45} In this study, the number of Siglec-F^{int} eosinophils was similar to the number of Siglec-F^{hi} eosinophils in the conjunctiva in a steady state. After repeated SRW challenges, the percentage of Siglec-F^{hi} eosinophils increased, and the eosinophil ratio (Siglec-F^{hi}/Siglec-F^{int}) increased from approximately 1 to approximately 3.5. Thus, the Siglec-F^{hi} conjunctival eosinophils can be considered mature or activated eosinophils, which are usually located in inflamed tissues. In addition, the high number of Siglec-F^{hi} eosinophils may be considered as a piece of evidence that the tissues were in an inflamed condition.

Traditionally, eosinophils within bone marrow and blood are attracted to inflammatory sites by the Th2 cytokines IL-4, IL-5, and IL-13 after exposure to allergens.⁴⁷ Apart from the traditional signaling pathway, which controls the activation and recruitment of eosinophils to allergic tissues, whether there are other signaling pathways that regulate the responses of eosinophils in conjunctivitis is unclear. Recently, many studies have reported that the SNS controls the responses of immune cells.^{21–23} One novel finding focused on the group 2 innate lymphoid cells (ILC2s). These cells were confirmed to be adjacent to the SNS and to express $\beta 2$ -ARs. The $\beta 2$ -AR signaling pathway negatively regulates ILC2 responses, and deficiency in $\beta 2$ -ARs increases ILC2 responses and type 2 inflammation in the lung and intestinal tissues.⁴⁸ In the current study, eosinophils were confirmed to express $\alpha 1a$ -ARs. This suggests that eosinophils may also exert their functions through the adrenergic signaling pathway. As expected, the activation and migration of eosinophils were inhibited by chemical sympathectomy with 6-OHDA treatment and were augmented by the activation of the SNS with restraint stress treatment in SRW-treated mice. Furthermore, treatment with an $\alpha 1a$ -AR antagonist inhibited and treatment with an

$\alpha 1a$ -AR agonist promoted eosinophil responses in SRW-induced conjunctivitis. These results indicate that the conjunctival SNS promotes eosinophil responses through $\alpha 1a$ -ARs and that this signaling pathway is different from the $\beta 2$ -AR signaling pathway in the intestinal and lung tissues, which negatively regulate ILC2 responses and type 2 inflammation. The results also revealed that the SNS may have distinct effects on the target cells via different AR signaling.

Although this study highlights the important role of the activation of $\alpha 1a$ -AR in murine allergic conjunctivitis, one should consider the complexity of the mechanism underlying allergic conjunctivitis. Many immune cells participate in allergic conjunctivitis.⁴⁹ These cells also express different AR subtypes, including $\alpha 1$ (A, B, and C), $\alpha 2$ (A, B, and D), and β (1 to 3)-type ARs, and these may also be modulated by the SNS.^{50,51} For instance, mast cells,⁵² macrophages,⁵³ B cells,^{49,54} and neutrophils^{53,54} participate in allergic conjunctivitis and express β subtype ARs. Interestingly, infiltrated $\gamma\delta$ T cells in the cornea after corneal abrasion also express $\alpha 1a$ -AR receptors.⁵⁵ Another study confirmed that $\gamma\delta$ T cells are involved in the occurrence and development of allergic conjunctivitis by regulating Th2 cytokine expression.⁵⁶ Thus, both the mechanism and the role of other immune cells that are mediated by the SNS in allergic conjunctivitis also require further investigation.

Collectively, this study indicates that there are two populations of eosinophils in the conjunctiva: Siglec-F^{int} and Siglec-F^{hi}. The two populations represent the different life stages of eosinophils: the immature (or resting) stage and the mature (or activated) stage. The recruitment and activation of eosinophils in conjunctivitis are regulated by the SNS via $\alpha 1a$ -ARs. These findings improve our understanding of the specific roles of the SNS in the regulation of eosinophil responses, and they provide new potential therapeutic targets for allergic conjunctivitis.

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Author Contributions

Z.L. and J.L. conceived and designed the study; S.H. analyzed immunostaining and performed qPCR; F.L., M.W., J.H., R.Y., and X.C. helped with treatment of the animals, including conjunctivitis induced and drug treatment; J.L. performed flow cytometry analysis; Y.X., T.F., and Y.W. conducted the statistical analysis; J.L. drafted the first version of the paper; Z.L. critically reviewed and revised the paper for intellectual content; all authors critiqued the manuscript and approved its submission.

Supplemental Data

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