

Role of FcγRI in Antigen-Dependent Eosinophil Activation in Patients With Allergic Rhinitis

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Abstract

Background: The eosinophil (Eo) activation is a crucial factor evoking allergic rhinitis (AR) attacks; factors; the mechanism of triggering Eo activation remains to be further investigated. The interaction of antigen (Ag) and antibody plays a critical role in evoking allergy attacks. This study aims to elucidate the role of FcγRI, the high affinity receptor of IgG, in the Ag-mediated Eo activation.

Methods: Nasal lavage fluids (NLF) were collected from AR patients and healthy control (HC) subjects. Eos were isolated by flow cytometry cell sorting and analyzed by pertinent immunological approaches.

Results: Eos composed more than 60% of the cellular components in AR NLF. Exposure to specific Ags (sAgs) in the culture triggered Eos to release inflammatory mediators. High levels of FcγRI were detected on the surface of AR NLF Eos. Exposure to lipopolysaccharide markedly increased the FcγRI expression in naive Eos, which could be bound by Ag-specific IgG (sIgG) to form complexes on the surface of Eos; this made Eos at the sensitized status. Eos bore with the sIgG/FcγRI complexes could be activated upon exposure to sIgG in the culture; these Eos can be designated as Ag-specific Eos. Passive transfer of Ag-specific Eos resulted in profound AR response in mice upon sAg challenge. Depletion of FcγRI on Eos efficiently abolished AR response in mice.

Conclusions: AR Eos express high levels FcγRI, that can be bound by sIgG to make Eos sensitized. Re-exposure to specific Ags can activate the sensitized Eos.

Keywords

eosinophil, allergy, rhinitis, IgG, receptor

Introduction

Eosinophils (Eos) are one fraction of white blood cells in the body and occupy 1-3% white blood cells. The number of Eos may be increased in the blood or local tissues in parasite infection or allergy attacks or some chronic inflammation such as Eosinophilic esophagitis.¹ Eos contain many inflammatory mediators, such as major basic protein (MBP), Eo cation protein (ECP), Eo-derived neurotoxin (EDN) and Eo peroxidase (EPO). While it is well understood that Eos release inflammatory mediators to induce inflammation in the local tissues upon activation,¹ the mechanism by which Eos are activated remains to be further investigated.

It is a common phenomenon that the local tissues with allergic diseases are over populated by Eos.²

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Abundant Eos can be found in nasal or bronchoalveolar lavage fluids of subjects with allergic rhinitis or asthma.² Airway allergy is an adverse response to the airborne antigens (Ag) such as house dust mites (HDM), by the immune system in the airway tissues. After inhaling into the airway, Ags can be captured by Ag presenting cells, such as dendritic cells (DC). DCs present the Ag information to CD4⁺ T cells, and finally the Ag-specific antibodies, IgE or IgG, are produced by plasma cells. It is well known that IgE can bind to the high affinity IgE receptors on the surface of mast cells to induce mast cells to release allergic mediators to induce the allergy attacks.³ However, the mechanism by which Eos are activated in allergy attacks is incompletely understood yet.⁴ Because Eos do not express or express very low levels of the IgE receptors, it is presumable that Eos do not directly respond to IgE.⁴

It is suggested that Eos can be activated by multiple factors. There are many proteins on the surface of Eos, which are assumed associating with Eo activation, such as CD11b, CD44, CD54, CD69, CD162, L-selectin, cytokine receptors and Fc receptors; however, the Eo activating mechanism remains elusive.⁵ It has been known that Eos express IgG receptors.^{6,7} Thus, IgG can ligate IgG receptors to activate Eos. Three types of IgG receptors have been recognized. FcγRI/CD64, FcγRII/CD32, and FcγRIII/CD16. FcγRI, FcγRIIa and FcγRIII are activating Fc receptors, while FcγRIIb has inhibitory functions, upon interacting with immune complexes.⁸ Whether Ag-specific IgG bind the IgG receptors to induce Eo activation has not been investigated.

Therefore, we hypothesize that Ag-specific IgG (sIgG) bind to the IgG receptors on the surface of Eos to make Eos sensitized. Re-exposure to specific Ags (sAgs) activates sensitized Eos; the Eos then release inflammatory mediators and induces inflammatory responses in the local tissues. In this study, we collected nasal lavage fluids (NLF) from AR patients. Eos in the NLF were analyzed by immunological analysis approaches. A fraction of Ag-specific Eos was characterized. An AR animal study about IgG-related Eo activation was also carried out to expand the findings from AR patient study.

Materials and Methods

Reagents

shRNA kits of FcγRI and FcγRII, antibodies of FcγRI (clone: X-22, H-5), IgG (clone: 3E8, D-1) and CD11b (Alexa Fluor[®] 488, clone: 44) were purchased from Santa Cruz Biotech (Santa Cruz, CA). Anti-siglec 8 (clone: 7C9, PE) and anti-siglec F (S17007L; PE) antibodies was purchased from BioLegend (San Diego, CA).

Anti-mouse Ki67 (SP6) antibody was purchased from abcam (Cambridge, MA). LPS, ELISA kits of IgE, mite specific IgG, LPS, MBP and ECP were purchased from Dakewu BioMart (Beijing, China). ELISA kits of mouse MBP and ECP were purchased from Biocompare (South San Francisco, CA). Dust mite extract was purchased from Wowu Biotech (Huangzhou, China). Materials and reagents for RT-qPCR, Western blotting, immunoprecipitation (IP) and CM-Dil cell tracker were purchased from Invitrogen (Carlsbad, CA).

Human Subjects

Perennial AR patients (all patients were sensitized to house dust mites, HDM) at remission period and health control (HC) subjects were recruited in the Second Hospital of Shanxi Medical University (Taiyuan, China). The diagnosis of AR was carried out by our doctors following published procedures.⁹ All AR patients had perennial AR history at least two years, DME skin prick test positive (the response at least 3 mm greater than the negative control¹⁰), serum DME specific IgE positive (greater than 0.35 kU/l), did not use any anti-allergy therapies at least 2 weeks. The human subject demographic data are presented in Table 1. AR patients with any of the following conditions were excluded: Cancer, autoimmune diseases, severe organ diseases, under immunotherapy or immunosuppressive therapy for any reasons. An informed written consent was obtained from each human subject. The experimental procedures were approved by the Ethical Committee at Shanxi Medical University.

Collection of Nasal Lavage Fluids

Human subjects were seated upright. Saline (5 ml per nostril; pre-warmed to 37°C containing 0.02% ethylenedinitrilotetraacetic acid, EDTA) was used to irrigate the nasal cavity and recovered to be NLF; the irritation was repeated 3 times. The NLF was centrifuged at 1,000 g for 5 min. The supernatant was collected for further analysis. The cell pellets were resuspended in fresh pre-warmed culture medium and filtered through a filter (70 μm) to remove non-cell substances such as non-resolved mucus. The samples were centrifuged at 1,000 g for 5 min; cell pellets were resuspended in culture medium for further experiments.

Eo Isolation

Eos were isolated from blood mononuclear cells or NLF by flow cytometry cell sorting (FCCS) with CD11b and siglec 8 (for human Eos) or siglec F (for mice) as the cell markers. The purity of isolated Eos was assessed by flow cytometry. If the purity was less than 90%, FCCS was repeated with the cells.

Table 1. Demographic Data of Human Subjects.

	AR	HC
Male/Female	10/10	10/10
Age	31.5 ± 6.8	30.8 ± 7.5
Body weight	58.5 ± 11.6	61.2 ± 8.8
DME skin test positive	20 (100%)	0
DME specific IgE positive	20 (100%)	0
Blood Eo counts	4.85 ± 1.23	1.56 ± 0.54*
Serum total IgE (KU/L)	401.65 ± 158.64	95.68 ± 65.21*
Serum total IgG (KU/L)	664.25 ± 185.26	102.58 ± 78.54*
WBC, × 10 ⁹ /L	5.58 ± 1.54	5.21 ± 1.14
Hb, g/L	124.62 ± 18.75	118.76 ± 19.78
HCT	37.44 ± 5.35	36.32 ± 4.17
PLT, × 10 ⁹ /L	277.45 ± 115.01	263.67 ± 36.37
CRP, mg/L	17.28 ± 4.67	12.16 ± 6.87
ESR, mm/H	21.51 ± 11.81	20.04 ± 12.04
ALB, g/L	36.58 ± 2.29	38.24 ± 3.26

*p < 0.01, compared to the AR group.

Assessment of Specific IgE and sIgG in AR Patient NLF

Referring to published procedures,^{11,12} the NLF samples were tested for IgE and IgG antibodies against the house dust extracts (DME) (ImmunoCAP: Thermo Fisher Scientific, Uppsala, Sweden; a detection range was 0.35 to 100 kU/L). NLF with a specific IgE or IgG levels of greater than 85 kU/L were diluted to 1:10.

Immunoprecipitation

The preclearing procedures were omitted because one of the target proteins was IgG. Proteins were extracted from Eos collected from relevant experiments and incubated with antibodies of interest (or isotype IgG; used as negative control Abs) overnight to form immune complexes (Anti-FcγRI and IgG Abs were used to precipitate the FcγRI and IgG complexes; anti-FcγRI, IgG and DME Abs were used to precipitate the FcγRI, IgG and DME complexes). The samples were then incubated with protein G agarose beads for 2 h to precipitate the immune complexes. Proteins on the beads were eluted with an eluting buffer and analyzed by Western blotting with FcγRI as the target.

Gene Silence

Expression of FcγRI or FcγRII in human Eos was knocked down by RNA interference (RNAi) approach using commercial reagent kits following manufacturer's instructions. Cells were electroporated using a Bio-Rad Gene Pulse set at 950 μF and 280 V. The effects of RNAi were assessed by Western blotting and flow cytometry, respectively, 48 h after the transformation. Eo viability after RNAi was greater than 99% as assessed by flow cytometry (Figure S1 in Supplemental Materials).

Mice

BALB/c mice were purchased from Beijing Experimental Animal Center (Beijing, China) and maintained in the SPF animal facility of Shanxi Medical University. Mice could freely access water and food. The animal experimental procedures were approved by the Animal Ethic Committee at Shanxi Medical University.

AR Mouse Model Development and Assessment of AR Response

The group size of mice was determined by power analysis. Mice were immunized by subcutaneously at the back skin with DME (0.1 mg/mouse) mixed in 0.1 ml alum on day 0 and day 7, respectively. The mice were challenged with DME (50 μl per nostril of 10 mg/ml DME) daily from day 14 to day 21. Mice were challenged with high concentration of DME (50 μl per nostril of 50 mg/ml DME. Bovine serum albumin (BSA) was used as control Ag) on day 22. Sneezing and nasal scratching (nasal itch) were counted for each mouse within 30 min after the DME challenge. NLF was collected from each mouse using in total 0.5 ml pre-warmed saline. Mice were sacrificed 2 h after the challenge. Blood samples were collected from each mouse by the eyeball-pulling approach under general anesthetization. The serum was isolated from blood samples and analyzed by ELISA to determine the levels of specific IgE (sIgE) and specific IgG (sIgG). NLF was centrifuged at 5,000 g; the supernatant was collected and analyzed by ELISA to determine MBP and ECP levels; the pellets were resuspended in PBS and analyzed by flow cytometry to determine the Eo frequency and expression of FcγRI on the Eo surface.

Isolation Eos From the Mouse Airway Tissues

The airway tissues, including the mouse nasal tissues and lung tissues, were collected immediately upon the sacrifice. Tissues were cut into small pieces (2 × 2 × 2 mm), incubated with liberaseTM (100 mg/ml, Sigma Aldrich) and DNaseI (10 mg/ml, Sigma Aldrich) for 30 min at 37°C. Single cells were filtered through a mesh (70 μm) and collected by centrifugation at 1,000 g for 5 min. Eos were purified by flow cytometry cell sorting (FCCS) with CD11b and siglec F as the cell markers. The purity of isolated Eos was about 96% as assessed by flow cytometry (Figure S2(A)). Dead cells in isolated Eos were less than 1% (Figure S2(B)). As assessed by exposing to specific Ags in the culture, about 80% Eos responded to DME as the Ki67 expression was increased in Eos, which did not enhance in Eos treated with BSA (Figure S2(C)); these Eos can be mite-specific Eos.

Adoptive Eo Transplantation

Eos were isolated from AR mice or naive mice as described above. Balb/c mice were transplanted with AR Eos or naive Eos at 10^6 Eos per mouse through tail vein injection. The transplantation was repeated once 3 days later. To assess if transplanted Eos reach the airway tissues and their functional status, in separate experiments, AR Eos were labeled with CM-Dil fluorescence dye and transplanted into naive mice. After challenging with specific Ag (DME), NLF was collected; cellular elements in NLF were prepared and analyzed by flow cytometry. The results showed abundant CM-Dil-labeled Eos were detected in NLF upon challenging with specific Ag (Figure S3).

Blood Sample Collection

Blood samples were collected from human subjects (20 ml per person) through ulnar vein puncture. The serum was isolated and analyzed by ELISA. Mononuclear cells were isolated from the blood samples by the Percoll gradient density centrifugation.

Cell Culture

Cells were cultured in RPMI1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 2 mM L-glutamine. Cell viability was greater than 99% as assessed by Trypan blue exclusion assay.

Flow Cytometry

In the surface staining, cells were stained with fluorescence conjugated antibodies of interest (see Reagent section and figure legends for detail) or isotype IgG on ice for 30 min. The cells were washed with phosphate-buffered saline (PBS) 3 times and analyzed with a flow cytometer (FACSCanto II; BD Bioscience). In the intracellular staining, cells were fixed with 1% paraformaldehyde (containing 0.1% Triton X-100 to increase the cell membrane permeability) for 1 h. The cells were then processed with the procedures described in the surface staining. The data were analyzed with software FlowJo (Tree Star Inc., Ashland, OR). The data obtained from isotype IgG staining were used as gating references.

Preparation of Protein Extracts

Cells were lysed by incubation with a lysis buffer (10 mM HEPES; 1.5 mM $MgCl_2$; 10 mM KCl; 0.5 mM DTT; 1 mM EDTA; 0.05% NP40) at 4°C for 30 min. The lysates were centrifuged at 13,000 g for 10 min. Supernatant was collected and used as cytosolic proteins. The pellets were resuspended in a nuclear lysis buffer (5 mM HEPES; 1.5 mM $MgCl_2SO_4$; 4.6 M NaCl;

0.2 mM EDTA; 0.5 mM DTT; 26% glycerol) and incubated for 30 min at 4°C. The lysates were centrifuged at 13,000 g for 10 min. Supernatant was collected and used as nuclear extracts. The proteins were quantified with the BCA method. The procedures were carried out at 4°C.

Western Blotting

Proteins were fractioned by SDS-PAGE and transferred onto a PVDF membrane. After blocking with 5% skim milk for 30 min, the membrane was incubated with antibodies of Fc γ RI or Fc γ RII (diluted at 1:500) overnight, washed with TBST (Tris-buffered saline containing 0.1% Tween 20) 3 times, incubated with peroxidase-labeled second antibodies at room temperature for 2 h. Immune complexes on the membrane were developed with the enhanced chemiluminescence. The results were photographed in an imaging device (UVP, Cambridge, UK).

Real-Time Quantitative RT-PCR

Cells were harvested from pertinent experiments. Total RNA was extracted from the cells with TRIzol reagents. The RNA was converted to cDNA with a reverse transcription kit (Invitrogen) following the manufacturer's instruction. The samples were processed in a quantitative polymerase chain reaction (qPCR) device (CFX96 Touch Real-Time PCR Detection System) with the SYBR Green Master Mix in the presence of primers of Fc γ RI (agatttcactgctccacca and accgtggaagcacagatata) and Fc γ RII (ctcaggcatgggaagcatc and ttgctgccatgtagaagga). Since only a small number of Eos was isolated from each sample, nested qPCR was performed. The data were processed with the $2^{-\Delta\Delta C_t}$ method and presented as relative quantification (RQ).

Assessment of Nasal Epithelial Barrier Function of AR Mice

To determine the nasal epithelial barrier function of AR mice, FITC-dextran (MW = 40 kDa) was added to the Ag solution used in the last Ag-challenge at a final concentration of 50 mg/ml. Mice were sacrificed 2 h after the challenge. Blood samples were collected as described above. The FITC-dextran levels in the serum was determined with a fluorescent spectrometer and calculated with the standard curve. The results were used as an indicator of nasal epithelial barrier function.

Enzyme-Linked Immunosorbent Assay

MBP and ECP levels in mouse NLF were determined by ELISA with commercial reagent kits following the manufacturer's instructions. To determine sIgE and sIgG, microplates were coated with DME (20 μ g/ml) overnight

at 4°C. The serum (1:10 diluted) was then added to the plates and incubated for 2 h at room temperature. The plates were washed with phosphate-buffered saline containing 0.05% Tween 20 (PBST) 3 times and incubated with biotinylated rabbit anti-mouse IgE (1:5000) or biotinylated rabbit anti-mouse IgG (1:5000) for 2 h at room temperature; washed with PBST 3 times; incubated streptavidin-HRP for 30 min; washed plates with PBST 3 times; added 0.1 ml TMB substrate solution to each well and incubated for 30 min; added 0.1 ml HRP stop solution to each well and read the plates with a microplate reader (Bio-Tek Instruments, Winooski, VT, USA) at 450 nm.

Statistics

The data are presented as mean $\pm$ SEM. The difference between two groups was determined by Student *t* test or Mann-Whitney test. ANOVA + Dunnett's test or Bonferroni test. $P < 0.05$ was considered statistical significance.

Some experimental procedures are presented in Supplemental Materials.

Results

Eo Mediator Levels Are Correlated With sIgG Levels in NLF of AR Patients

We collected NLF from AR patients and HC subjects. The NLF was analyzed by ELISA. The results showed that both HDM-specific IgG (sIgG) and sIgE were detected in AR NLF samples that were significantly more than that in HC subjects (Figure 1(A) and (B)). MBP and ECP, the major Eo mediators, were also detected in the NLF, that were significantly lower in HC subjects than that of AR patients (Figure 1(C) and (D)). A positive correlation was identified between sIgG and MBP or ECP in NLF (Figure 1(E) and (F)). No correlation was identified between sIgE and NLF MBP or ECP (Figure 1(G) and (H)). The results suggest that the sIgG may be associated with Eo activities in AR patients.

sAgS Activate AR Eos

The data of Figure 1 suggest that sAg may be able to activate AR Eos, in which sIgG may play a critical role. To test this, we collected NLF from AR patients (sensitized to HDM). Eos were isolated from NLF by flow cytometry cell sorting (FCCS) (Figure 2(A), Table S1 in Supplementary Materials). We found that AR Eos expressed higher Fc γ RI (the high affinity receptor of IgG) levels than that in HC Eos (Figure 2(B) and (C)). Fc γ RII expression was detectable in the Eos, but at very

low levels in both groups (Figure 2(D) and (E)). The Fc ϵ RI expression in Eos was below detectable levels in both groups (not shown). Exposure of Eos to sAg (dust mite extracts, DME) in the culture, but not bovine serum albumin (BSA, an irrelevant Ag), markedly induced Eos to release mediators (a sign of Eo activation), including MBP and ECP (Figure 2(F) and (G)). Depletion of Fc γ RI, but not Fc γ RII, in Eos (Figure S1) abolished the DME-induced Eo activation (Figure 2(F) and (G)). The results indicate that AR Eos express high levels of Fc γ RI; exposure to sAg can activate AR Eos. These Eos can be designated Ag-specific Eos (sEos).

We further assessed the Fc γ RI expression in peripheral Eos. The results showed that the Fc γ RI expression was detectable in peripheral Eos in both AR patients and HC subjects. The Fc γ RI expression in AR Eos was slightly higher than that in HC Eos ($p = 0.1075$) (Figure 2(B) and (C)). The expression of Fc γ RI in AR peripheral Eos was also significantly lower than that in AR NLF Eos ($p < 0.0001$). Since exposure to LPS can up regulate the Fc γ RI expression,^{13,14} we assessed LPS levels in NLF. High LPS levels were detected in AR NLF, that was markedly lower in HC NLF (Figure S4 (A)). Therefore, we isolated naïve Eos from HC subjects and cultured in the presence of LPS for 48 h. Indeed, the Fc γ RI expression was increased in Eos by LPS in a dose-dependent manner (Figure S4(B) to (D)). The results demonstrate that AR nasal cavity detains more LPS that may be the factors causing Eos to express more Fc γ RI.

sIgG Binds the Fc γ RI on the Surface of Eos to Make the Eos Sensitized

We next analyzed protein extracts of AR Eos by immunoprecipitation (IP). A complex of Fc γ RI/sIgG was detected (Figure 3(A)). The results suggest that sAgS may bind this complex to activate AR Eos. To this end, HDM-AR Eos were purified from NLF and exposed to DME in the culture for 3 h. The Eos were then collected and analyzed by IP. A triple complex of Fc γ RI/sIgG/DME was detected in AR NLF Eo-protein extracts (Figure 3(B)). In addition, Eos were isolated from HC blood samples. The IgG-bearing Eos were selected out by FCCS; the remained Eos were used as naïve Eos. Naïve Eos were exposed to sIgG in the culture for 3 h. A complex of Fc γ RI/sIgG was found in the Eos (Figure 3(C)). These Fc γ RI/sIgG complex-bearing Eos were exposed to DME in the culture for 3 h. A triple complex of Fc γ RI/sIgG/DME was detected in the Eos (Figure 3(D)). MBP and ECP, the canonical inflammatory mediators of Eos, were detected in the culture supernatant (Figure 3(E) and (F)). The data demonstrate that sIgG binds to Fc γ RI to form a complex on Eos to

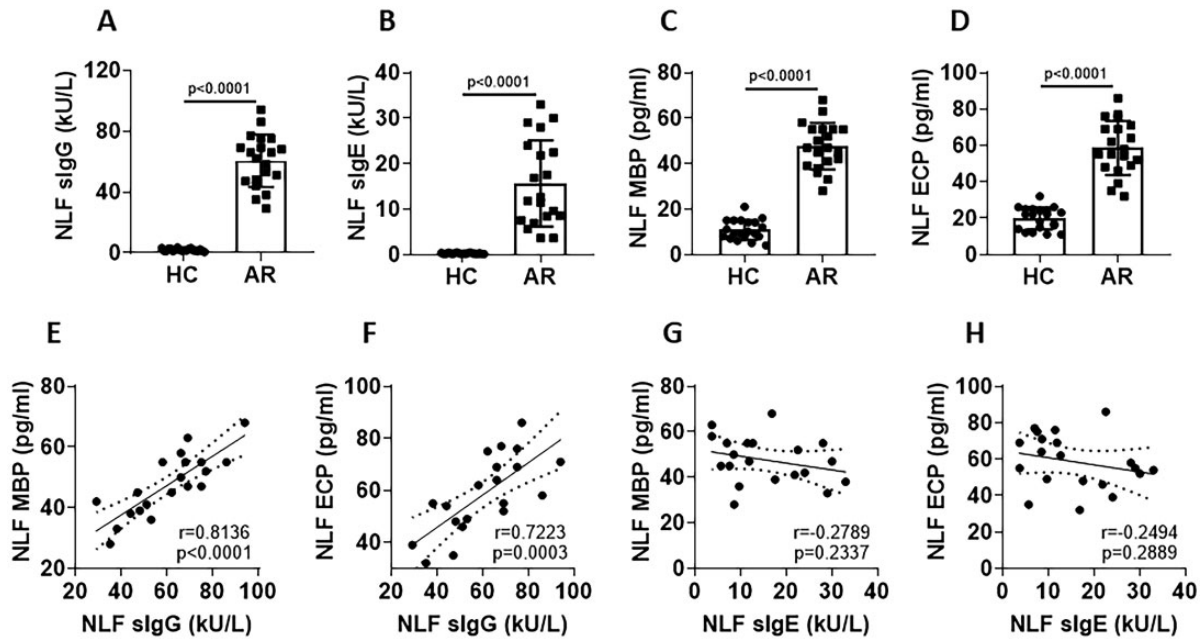


Figure 1. Positive correlation between sIgG and Eo mediators in NLF. Nasal lavage fluids (NLF) were collected from healthy control (HC) subjects ($n = 20$) and allergic rhinitis (AR) patients ($n = 20$). NLF samples were analyzed by ImmunoCap (A and B) or ELISA (C and D) individually in triplicate. A–D, bars indicate levels of sIgG (A), sIgE (B), MBP (C) and ECP (D) in NLF. E and F, positive correlation between sIgG and MBP (E) or ECP (F). G and H, no correlation was detected between sIgE and MBP (G) or ECP (H). Each dot in bars of A–D presents data obtained from one human subject. Statistics: The Mann-Whitney test (A–D) and Pearson correlation assay (E–H).

make the Eos sensitized. Re-exposure to sAg triggers sensitized Eos to release mediators.

Sensitization Induces sEos in the Mouse Airways

To further verify the role of sEos in the AR pathogenesis, an AR mouse model was developed to expand the findings from the AR patient study. Besides the AR-like symptoms (Figure 4(A) to (D)), we also found that the CD11b⁺ siglec F⁺ Eo frequency was higher in AR mouse NLF than that collected from naive control (NC) mice (Figure 4(E) and (F)). FcγRI was detected on the surface of the Eos that was significantly higher in the AR group than that in the NC group (Figure 4(G)). After nasal challenge with sAgs, Eo-derived mediators, including MBP and ECP, were detected in the NLF, that were significantly higher in AR mice than that in NC mice (Figure 4(H) to (I)). The results demonstrate that sensitization can induce sEos in the nasal tissues.

Adoptive Transfer of sEos Induces AR-Like Inflammation in Mice

From the AR mouse airway tissues, we isolated Eos by FCCS. Naive Balb/c mice were adoptively transferred with AR mouse Eos or naive mouse Eos on day 1 and day 4, respectively. The mice were challenged with nasal drops containing sAgs on day 7. AR-like response was

induced in the mice, including sneezing, nasal itch, high frequency of Eos, MBP and ECP were detected in BALF. Mice received naive Eos (nEos) were not induced the AR symptoms. In addition, in separate experiments, mice received sEos or nEos were also treated with LPS-containing nasal drops daily for one week. The results showed that the LPS-treatment markedly strengthened the AR-like response in mice received sEo transfer, but not in mice received nEo transfer (Figure 5). The results demonstrate that sEos play a critical role in AR attacks.

Inhibition of FcγRI in Eos Suppresses AR Attacks in Mice

Following published procedures,¹⁶ an AR mouse model was developed. As shown by Figure 6, in response to sAg challenge, sensitized wild type (WT) mice showed sneezing, nasal itch, high levels of MBP, ECP and Eo counts in the NLF, increase in airway epithelial barrier permeability and high serum sIgE and sIgG levels. To test the role of Eo expressing FcγRI in the induction of AR attacks, AR mice carrying FcγRI-deficient Eos were developed (Figure S5). The mice were sensitized and challenged with DME. The results showed that the depletion of FcγRI in Eos abolished the AR attacks (Figure 6(A) to (D)), inhibited sAg-induced Eo infiltration into NLF (Figure 6(E) and (F)), alleviated the sAg-induced airway epithelial barrier hyper permeability

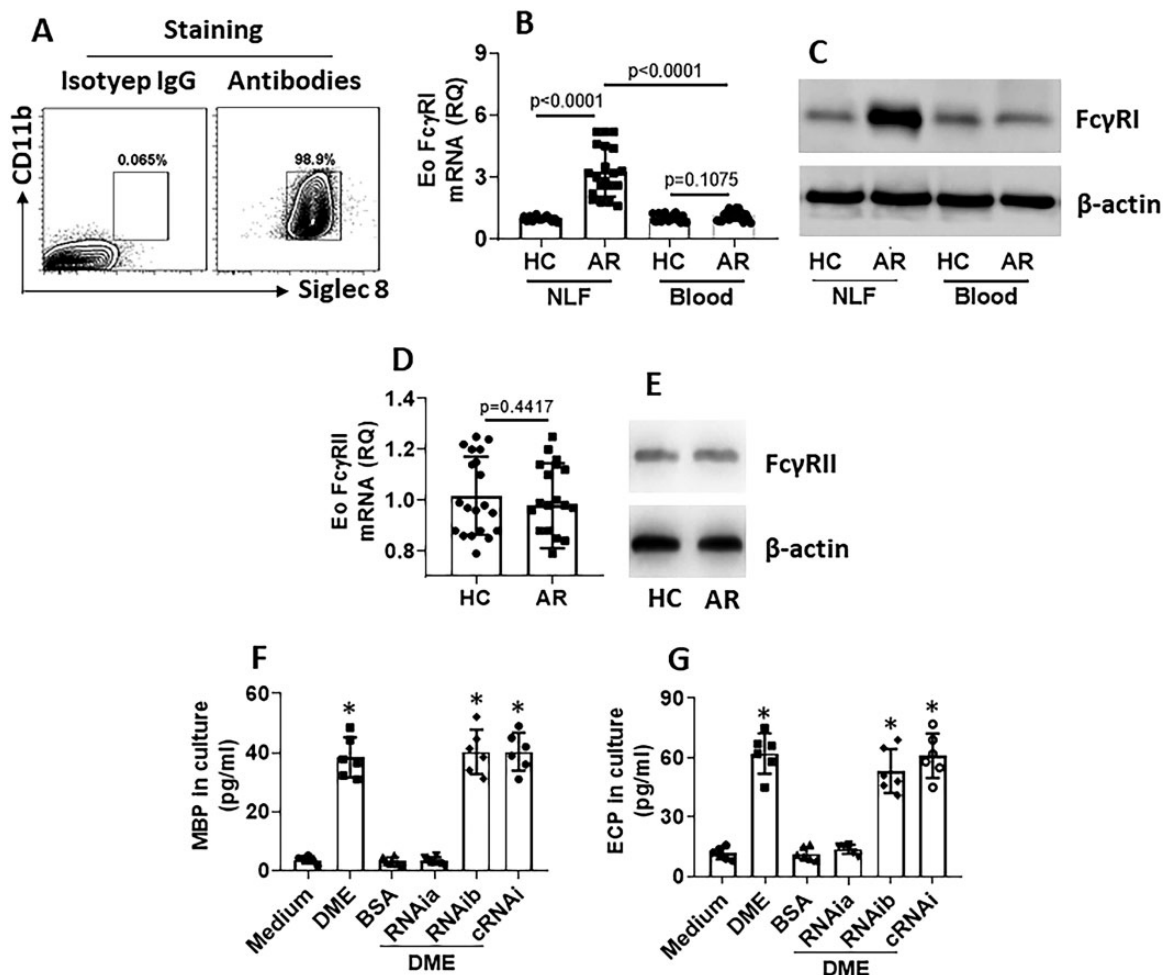


Figure 2. FcγRI mediates sAg-activated AR Eos. Eos were purified from NLF or blood cells collected from HC subjects and AR patients (sensitized to HDM) ($n = 20$) and HC subjects ($n = 20$); panel A shows cell purity of isolated Eos. B-E, bars indicate the mRNA levels of FcγRI (B) and FcγRII (D). Immunoblots show protein levels of FcγRI (C) and FcγRII (E). E-F, Eos were cultured in the presence of dust mite extracts (DME, 5 μg/ml) overnight. Supernatant was collected at the end of culture and analyzed by ELISA. Bars indicate the levels of MBP (F) and ECP (G) in the culture. RNAiA: Eos were treated with FcγRI RNAi. RNAiB: Eos were treated with FcγRII RNAi. cRNAi: Eos were treated with control RNAi. Statistics: The Mann-Whitney test (B and D) and ANOVA followed by the Dunnett's test (F and G). In A and C, each dot present data obtained from one human sample. In F and G, each dot present data obtained from one experiment.

(Figure 6(G)), but did not alter the serum levels of sIgE and sIgG (Figure 6(H) and (I)). The results emphasize the crucial role of Eos expressing FcγRI in the initiation of AR attacks.

Discussion

A fraction of Eos in AR patients was identified as Ag-specific Eos (sEos) in the present study. The sEos express the FcγRI at high levels. sIgG binds to FcγRI to make the Eos sensitized. Re-exposure to sAgs activates sEos. The activated Eos release allergic mediators, such as MBP and ECP, the representative inflammatory mediators of Eos, that are well known playing an important role in Eo-related inflammation.¹⁷ Depletion of FcγRI

expression in Eos can abolish AR attacks. The data demonstrate that sEos are the critical effector cells in the initiation of AR attacks.

The data show that the major cellular components in AR patient NLF are Eos. This is in line with published data. Han et al reported that blood Eo counts were positively correlated with the AR severity.¹⁸ Wang et al reported that Eos were the dominant cell counts in AR patients.¹⁹ We also observed high levels of the Eo-derived mediators, e.g., MBP and ECP, in AR NLF. The data indicate that Eos in NLF have been activated. A similar phenomenon was also observed by others. Choi reported that to evaluate Eo-derived mediators, e.g., EPO, was reflected Eo counts.² Rasheed et al reported that AR patients had high serum MBP

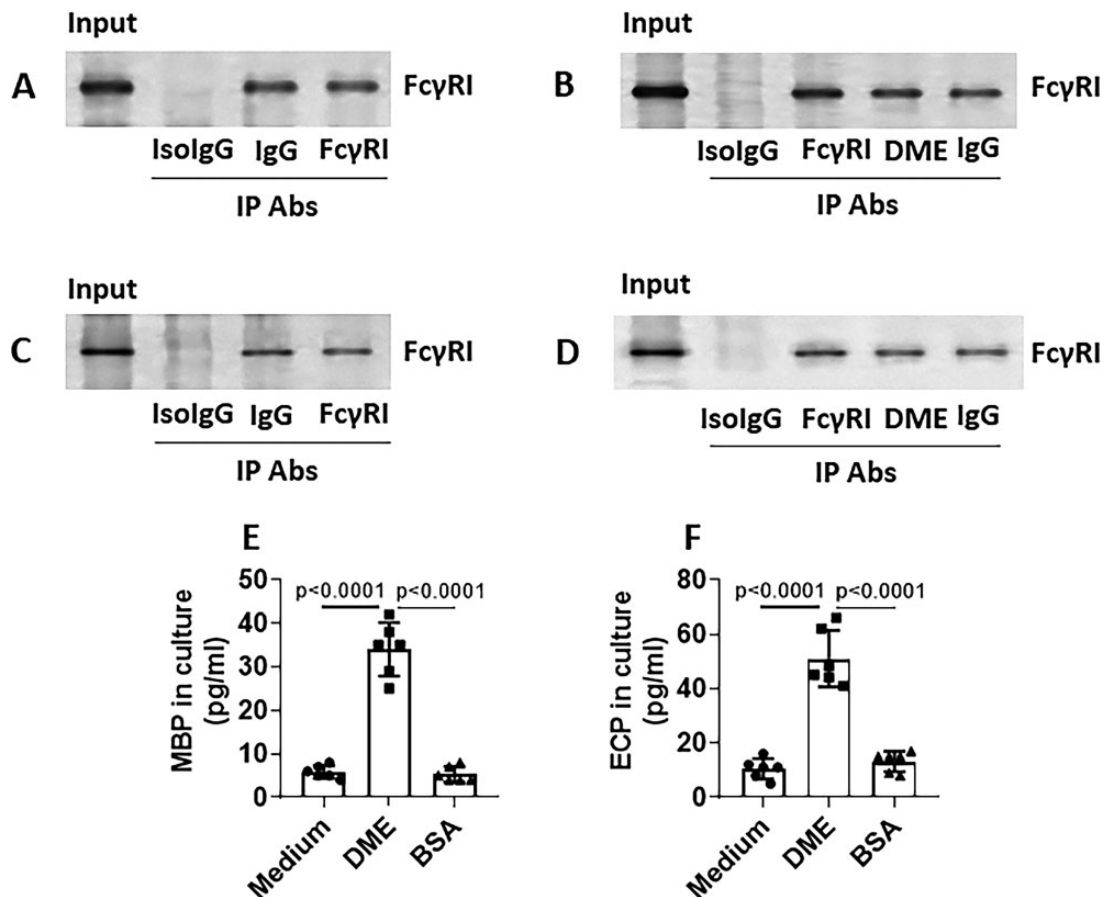


Figure 3. sAgs bind FcγRI/sIgG complexes to induce Eo activation. A, HDM-AR Eos were purified from NLF and exposed to DME (5 μg/ml) in the culture in a microplate (1000 cells/well in 0.2 ml medium) for 3 h. The Eos were then collected and analyzed by IP. The immunoblots show a complex of IgG/FcγRI in Eos isolated from AR NLF. B, A triple complex of IgG/FcγRI/DME in AR NLF-isolated Eos after exposing to DME in the culture for 3 h. C and D, Naive Eos were exposed to LPS in the culture for 48 h to increase the FcγRI expression, then, exposed to sIgG (C) or both sIgG and DME in the culture for 3 h. C, a complex of sIgG/FcγRI in the Eos. D, A triple complex of IgG/FcγRI/DME in the Eos. E and F, naive Eos were exposed to sIgG for 3 h first, then exposed to DME (5 μg/ml) in the culture overnight. Supernatant was collected at the end of culture and analyzed by ELISA. The bar graphs show levels of MBP (E) and ECP (F) in the culture supernatant of panel D experiments. A–D, The data are from one experiment that represent 6 independent experiments. E and F, The data of bars are presented as mean ± SEM. Each dot presents data obtained from one experiment. Statistics: ANOVA followed by the Bonferroni test.

levels.²⁰ High serum MBP levels were also found in allergic asthma patients.²¹ Thus, it is a consensus that Eo infiltration and activation play a critical role in eliciting AR.²²

The data show that NLF Eos of AR subjects express high levels of FcγRI. The FcγRI is one of the IgG receptors. Ligation of the FcγRI or FcγRIII can trigger the cell activation, while ligation of FcγRII may suppress the cell's activities.⁸ The FcγRI is mainly expressed by lymphocytes, including CD4⁺ T cells, CD8⁺ T cells and NK cells. Our data show that the ligation of the FcγRI also activates Eos. An early report indicated that human blood eosinophils express FcγRII, but not FcγRI or FcγRIII.²³ Others also reported that ligation of FcγRII, the inhibitory IgG receptor, could activate

Eos.⁸ Our data show that the FcγRI expression in blood Eos is detectable, but significantly lower than that in NLF Eos. Such a difference between our data and previous reports^{8,23} may be because NLF contains high amounts of LPS. The present data show that LPS can up regulate the FcγRI expression in Eos. Previous studies also found that LPS can up regulate FcγRI expression in neutrophils through activating Toll like receptor (TLR)4.^{24,25} As Eos also express TLR4 as well as several other TLRs,²⁶ it is reasonable that exposure to LPS can up regulate FcγRI expression in Eos. This was demonstrated by the present data. By exposing Eos to LPS in the culture, the FcγRI expression in Eos was markedly up regulated in a dose-dependent manner.

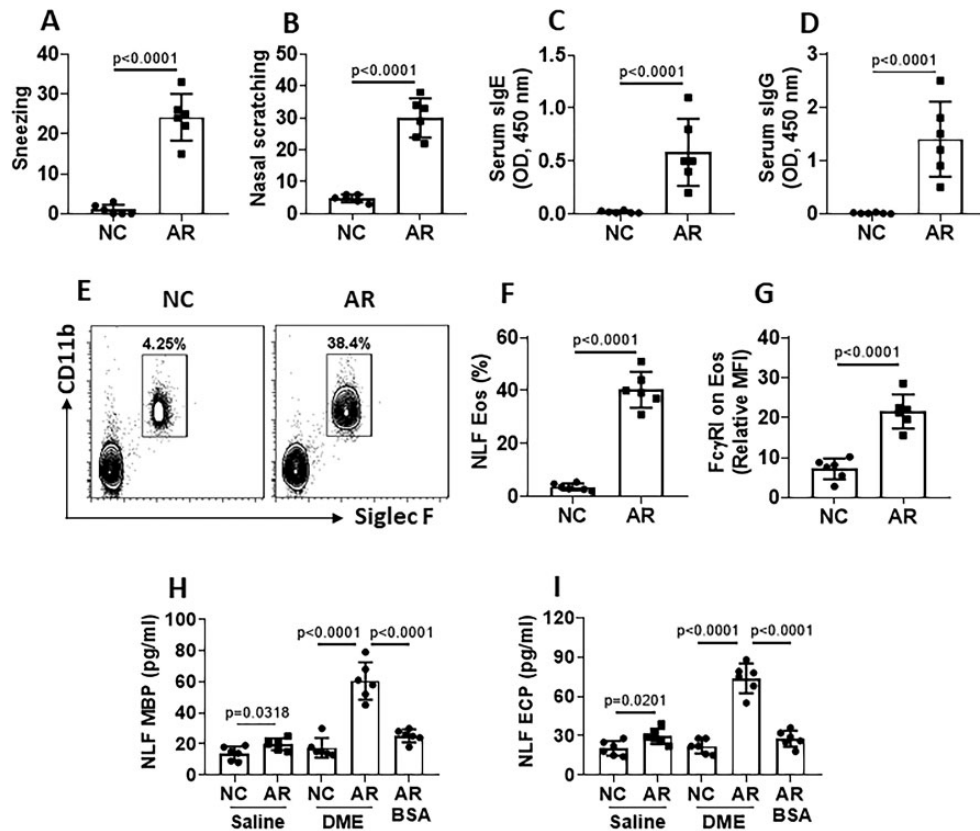


Figure 4. Sensitization induces sEos in the mouse airways. A mouse AR model was developed by sensitizing to HDM with each group consisting of 6 mice. A–D, Bars show AR response in mice, including sneezing (A), nasal itch (B), serum sIgE (C) and serum sIgG (D). E, Gated dot plots show Eo frequency in NLF. F, summarized data of Eo in NLF. E, FcγRI staining on NLF Eo surface. H and I, NLF MBP (H) and ECP (I) levels. The data of bars are presented as mean ± SEM. Each dot presents data obtained from one mouse. Statistics: A–D, F and G, Student t test; H and I, ANOVA followed by the Bonferroni test. The data of E are from one experiment that represent 6 independent experiments. NC: naïve control mice; DME: dust mite extracts; BSA: bovine serum albumin.

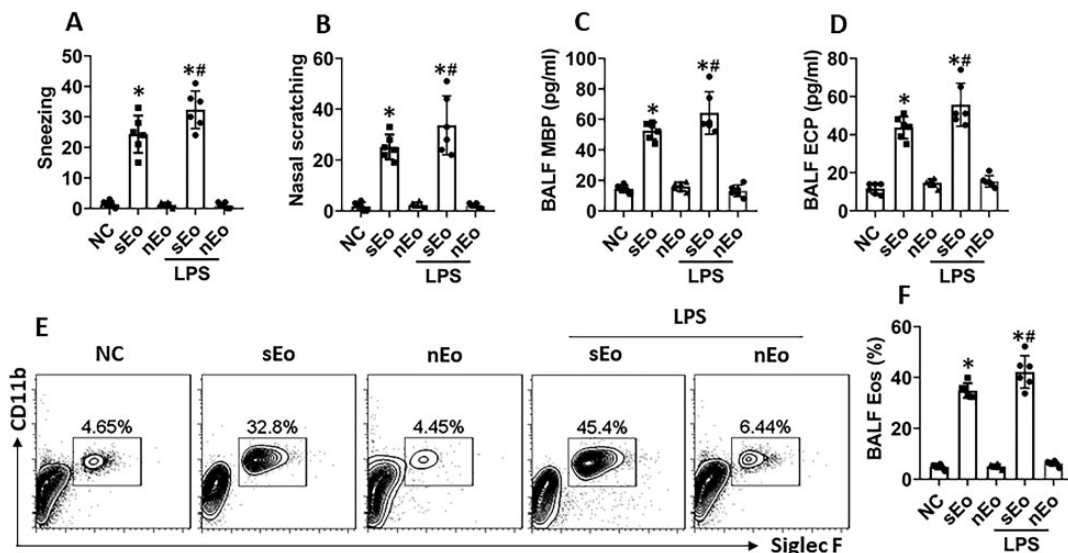


Figure 5. Adoptive transfer with sEos induces AR-like symptoms in mice. Naïve mice (6 mice per group) were transferred with Ag-specific Eos (sEos) or naïve Eos (nEos) with or without LPS-containing nasal drops (0.1 mg LPS/ml; 50 µl per nostril daily) and received nasal challenge with sAg-containing nasal drops. A and B, AR symptoms of mice recorded during 30 min interval after nasal sAg challenge. C and D, MBP and ECP levels in BALF collected from mice at 30 min after nasal sAg challenge. E, Gated dot plots show Eo frequency in BALF. F, Summarized Eos in BALF. The data of bars are presented as mean ± SEM. Each dot presents data obtained from one mouse. Statistics: ANOVA followed by the Bonferroni test.

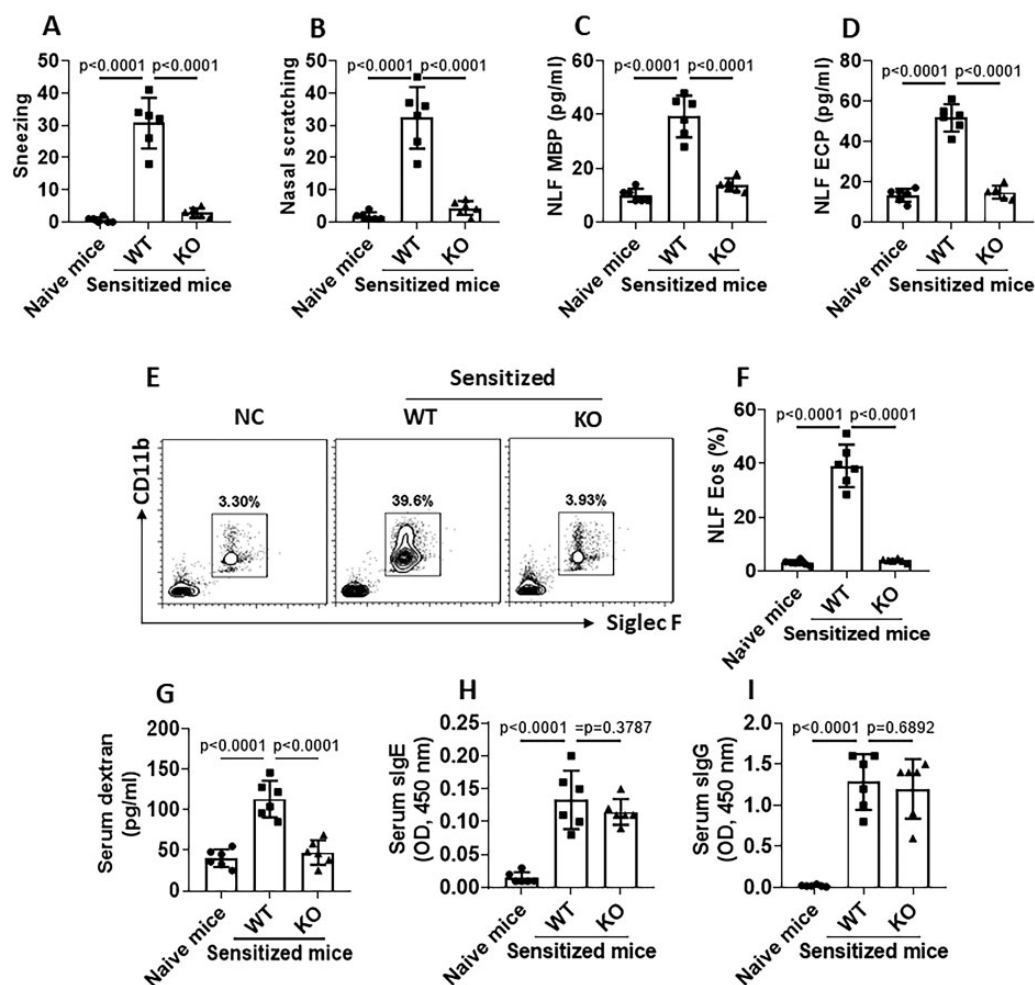


Figure 6. Depletion of Eo Fc γ RI expression inhibits experimental AR. An AR model was developed with KO mice (this mouse strain carries Fc γ RI-deficient Eos) and wild type (WT) mice that were sensitized to DME. A and B, bars show AR symptoms. C and D, Bars show MBP and ECP levels in NLF. E and F, Gated dot plots show Eo frequency in NLF; bars show summarized Eo frequency in NLF. G–I, Serum levels of sIgE, sIgG and FITC-dextran (a tracer for airway epithelial barrier permeability). The data of bars are presented as mean $\pm$ SEM. Each dot presents data obtained from one mouse. Statistics: ANOVA followed by the Bonferroni test. Each group consists of 6 mice.

It is well known that Eos are one of the major effector cells in allergic diseases, especially in airway allergy.² High Eo frequency was found in BALF and NLF in patients with allergic asthma or allergic rhinitis as reported previously.² Our data are in line with previous studies by showing profound infiltration of Eos in NLF. Yet, how Eos are activated by sAgs has not been determined. It is known that the sIgE amount is increased in subjects with allergic diseases.²² IgE can bind the high affinity receptors on mast cells to make mast cells sensitized. Re-exposure to sAgs triggers the sensitized mast cells to release allergic mediators to initiate allergy attacks.²² However, it is not the same case in Eos. Eos do not express the IgE high affinity receptors.²⁷ Although Eos express CD23, the low affinity IgE receptor, that is not different between allergic patients and healthy controls.²⁸ Thus, it is not possible that IgE can

activate Eos.²⁷ Previous reports indicated that IFN- γ could induce Eos to release RANTES; the authors suggested that exposure to IFN- γ could result in Eo's piecemeal degranulation.²⁹ Exposure to IFN- γ also increase the Fc γ RI expression in blood Eos.³⁰ The present data indicate that LPS can increase the Fc γ RI expression in Eos. We found most Eos isolated from AR NLF expressed high levels of Fc γ RI, that could be activated by sAgs; depletion of Fc γ RI abolished the sAg-induced Eo activation. We also found high sIgG levels in the serum as well as in the NLF. This meets the requirement for the Eo activation. The reasoning is verified by the data obtained from *in vitro* experiments. By exposing naive Eos to sIgG in the culture, sIgG binds the Fc γ RI to form a complex on the surface of Eos. This complex can be recognized by sAg that triggers the Eo activation. Furthermore, by adoptive transferring with sEos

induced AR response in recipient mice as shown by the present data.

As mentioned above, we found that NLF contained LPS; its amounts were higher in AR patients than that in healthy subjects. To our knowledge, this is the first report to investigate the association between nasal LPS levels and the Eo activation. Previous studies indicate that LPS can augment nasal cytokine release in AR³¹ and can facilitate AR development.³² LPS also can facilitate B cells to produce IL-10, that is beneficial to immune tolerance in the local tissues, but the IL-10 production can be suppressed by Bcl2 like protein-12 in the nasal mucosa.³³ LPS increases CCR3 expression to recruit Eos,³⁴ that implicates that sinusitis-derived LPS³⁵ may recruit Eos to the nasal tissues. Cumulative studies show that chronic sinusitis is accompanied with AR,³⁵ in which sinusitis-derived LPS may play a role in the AR development through inducing the FcγRI expression in Eos.

It is reported that depletion of Eos can effectively alleviate allergic asthma,³⁶ which pinpoints the importance of Eos in inducing allergy attacks. Our data are consistent with the reports.³⁶ The data show an alternative approach to suppress the AR response by regulating Eos' activities. By depleting the FcγRI on Eos efficiently abolished experimental AR response. The data suggest that regulating sEos' activities can be a novel remedy to alleviate or inhibit AR response. The data also implicate that the antigen-specific Eo activities may be required in mast cell activation since mast cell activation plays a critical role in AR attacks, this needs to be further investigated.

In summary, the present study has elucidated a mechanism by which Eos can be activated under an allergic environment. AR patient Eos express the FcγRI, that can be bound by sIgG, and further be recognized by sAgs. These Eos can be designated the Ag-specific Eos (sEos) and can be activated upon exposure to sAgs.

Author Contributions

YFA, LMS, JMX, HYH, GY, JQL and ZQL performed experiments, analyzed data and reviewed the manuscript. PCY and CQZ organized the study and supervised experiments. PCY designed the project and wrote the paper.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by grants from the

Shenzhen Science, Technology and Innovation Committee (KQJSCX20180328095619081, KQTD20170331145453160), Guangdong Provincial Key Laboratory of Regional Immunity and Diseases (2019B030301009), Shenzhen Nanshan District Pioneer Group Research Funds (No. LHTD20180007), and the National Natural Science Foundation of China (81870706, 31570932, 81700888, 81701589).

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Supplemental Material

Supplemental material for this article is available online.

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