Osteopontin mediated eosinophils activation by group II innate lymphoid cells

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ABSTRACT

Background: Osteopontin (OPN) can regulate Th2 inflammation in allergic rhinitis (AR). A recent study suggested that group II innate lymphoid cells (ILC2s) were very important for airway inflammation. But the role of OPN in ILC2s regulation is not explored.

Methods: Purified ILC2s were stimulated by human recombinant OPN. The expression of GATA3 and RORα was assayed using real-time polymerase chain reaction (PCR) and enzyme linked immunosorbent assay. MiR-181a was transfected into eosinophils to test the OPN production. The protein concentrations of interleukin (IL)-5 and IL-13 were examined using ELISA. Purified eosinophils and ILC2s were cocultured and stimulated by OPN and the activation of eosinophils was detected by ELISA.

Results: After OPN stimulation, the ILC2s proliferation, the mRNA levels of GATA3 and RORα, the protein of GATA3, RORα, IL-5 and IL-13 expression were up-regulated significantly in a dose dependent manner. Eosinophils cultured alone transfected with miR-181a mimics produced less OPN protein compared with eosinophils transfected with miR-control, whereas OPN production was significantly promoted when miR-181a inhibitor was transfected. In the eosinophils and ILC2s coculture system, eosinophil cationic protein (ECP) production induced by OPN or IL-33 were significantly higher than ECP production in eosinophils culture system. OPN presented similar potency with IL-33 in the activation of eosinophils. When anti-IL-5 antibody was added, the production of ECP was significantly inhibited.

Conclusions: Our data for the first time provided new evidence that OPN played important roles in innate immunity of AR by regulation of ILC2s and the interaction between ILC2s and eosinophils.

Keywords: Osteopontin, Allergic rhinitis, Group II innate Lymphoid cells, Eosinophils
INTRODUCTION

Allergic rhinitis (AR) is featured as allergen specific IgE induced type II inflammatory reaction. Eosinophils produce a marked effect in the pathogenesis of AR through producing various substances including major basic proteins, eosinophil cationic protein (ECP), etc. The maintaining and function of eosinophils depend on type 2 cytokines from Th2 or group II innate lymphoid cells (ILC2s). ILC2s are mainly distributed in lymphoid tissue, intestine, lung or skin. After stimulated by epithelial-derived IL-25, IL-33 or TSLP, allergen activated ILC2s can produce type 2 cytokines to amplify airway inflammation. A previous study had reported that ILC2s were important for maintaining eosinophils.

Osteopontin (OPN), a glycoprotein, is expressed by epithelium, macrophages, T cells, fibroblasts or eosinophils. A previous study had confirmed that OPN played important roles in IgE-mediated inflammation in allergic disease. Eosinophils also express OPN after granulocyte-macrophage colony stimulating factor (GM-CSF) or IL-5 stimulation. Then, OPN contributes to the migration and chemotaxis of eosinophils as well as eosinophil induced angiogenesis. Our previous study also confirmed that OPN overexpression in AR contributes to allergic inflammation by promoting eosinophils migration and activation.

miRNAs are small noncoding RNAs functioned as translational repressors by binding to the 3’ untranslated region of the target genes. miRNAs are widely distributed in various cells and body fluid and play important roles in a wide range of biological functions. Accumulating evidence suggested that miRNAs were involved in OPN expression, such as miR-4262 and miR-181a. Our previous data also found that OPN and miR-181a expression in AR were negatively correlated. However, no study reported whether OPN had effect on ILC2s.

We aimed to investigate: 1) the regulation of OPN on ILC2s, 2) the regulation of mir-181a in the production of OPN by eosinophils, and 3) the effect of OPN on the interaction between ILC2s and eosinophils in the present study.

MATERIALS AND METHODS

ILC2s sorting

We recruited ten AR patients and 10 controls (Han nationality) from our department from January 2021 to July 2021. Patients did not use systemic or topical corticosteroids at least 2 weeks before the study. The research obtained approval from local ethics committee boards and the informed consent was signed by the subjects. Allergic symptoms such as sneezing, rhinorrhea, itchy nose, and nasal block were scored as follows: 0, no symptom; 1 point, mild symptoms; 2 point, moderate symptom, 3 point, severe symptom. The nasal total symptom score (TNSS) was calculated accordingly. Specific IgE (Phadia, Sweden) levels (mites, pets, molds, cockroach, etc) were determined to confirm the allergen type. The AR was diagnosed by ear, nose, and throat (ENT) doctor according to positive allergen test and allergic symptoms. Fasting venous blood was obtained between 6:00 and 8:00 a.m. at clinics.

Peripheral blood mononuclear cells (2 × 10^6/ml) from venous blood samples of subjects (Table 1) were purified using density-gradient centrifugation and stained by FITC lineage cocktail, FceRI, CD45, CRTH2, and CD127. Lin^- CD45^+ CRTH2^+ CD127^+ lymphocytes were defined as ILC2s and sorted by MoFlo XDP cell sorter as described previously. The cells were incubated in RPMI-1640 supplemented with IL-25 (10 ng/ml), IL-33 (10 ng/ml), TSLP (10 ng/ml) and IL-2 (50 ng/ml) (R&D system). For stimulation, 50-100 ng/ml OPN, 100 ng/ml anti-OPN or PBS was added (R&D system). All the antibodies were bought from eBioscience. The ILC2s’ proliferation was determined by incorporation of tritiated thymidine.

| Groups          | AR    | Control |
|-----------------|-------|---------|
| Number          | 10    | 10      |
| Sex (Male: Female) | 5:5  | 9:6     |
| Age (years)     | 28.1 ± 11.3 | 25.6 ± 10.8 |
| TNSS score      | 8.1 ± 2.5 | -       |

Table 1. Demographic characteristic of AR patients and controls.
Eosinophils isolation

Human eosinophils from the venous blood of 10 AR patients confirmed by allergen test and allergic symptoms were prepared using micromagnetic beads (Miltenyi Biotec, Germany) as described previously. The purity and viability of eosinophils were verified using Gimesa and trypan blue staining. The eosinophils and ILC2s were co-cultured with the ratio of 1:1, for 3 days with or without IL-33, OPN, anti-IL-5 or GM-CSF (all the concentration was 100 ng/mL, R&D system).

Cell transfection

1 μM miRNA control, mimics or inhibitors was transfected using Lipofectamine 2000 (Invitrogen) according to protocols.

Enzyme-linked immunosorbent assay (ELISA)

The protein expression of IL-5, IL-13, OPN (R&D systems), GATA3 (Abcam), and RORα (Cloud clone) were determined by ELISA kits according to instructions. ECP level was detected by Unicap system.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using TRizol reagent (Life Technologies). The cDNA was amplified with primers for GATA3 and RORα. Beta-actin cDNA probe was selected as control. The data were calculated by 2-△△Ct method.

Statistical analysis

Comparisons between groups were determined using Kruskal–Wallis test and Bonferroni’s test for multiple comparisons or Mann-Whitney U test by statistical software (SPSS version 16.0). P value less than 0.05 was defined as significant difference.

RESULTS

Regulation of ILC2s by osteopontin

The demographics of study subjects were shown in Table 1. Subjects from 2 groups had comparable age, sex ratio, and nationality (Han). After OPN stimulation, the ILC2s’ proliferation and the mRNA levels of GATA3 and RORα were up-regulated significantly as a dose dependent manner (Fig. 1A–C). The protein expression of GATA3, RORα, IL-5 and IL-13 by ILC2s were also up-regulated significantly (Fig. 1D–G). However, those above effects were blocked when anti-OPN was added (Fig. 1). Moreover, ILC2s from healthy control showed similar trend with those from AR subjects in all experiments, but the ILC2s from AR were more active than those from controls (Fig. 1).

MiR-181a regulates the production of osteopontin by eosinophils

When eosinophils were cultured alone, GM-CSF stimulated the expression of miR-181a by eosinophils (Fig. 2A). Eosinophils cultured alone transfected with miR-181a mimics produced less OPN protein compared with miR-control, whereas OPN production was significantly promoted when miR-181a inhibitor was transfected (Fig. 2B). We also found that eosinophils from healthy control showed similar trend with those from AR subjects in all experiments, but the miR-181a and OPN expression by AR were up-regulated compared with controls (Fig. 2).

Osteopontin promotes eosinophil proliferation and activation

When eosinophils were cultured alone, OPN presented similar potency with IL-33 in the promotion of eosinophils proliferation. Eosinophil proliferation from healthy control was lower compared with eosinophils from AR (Fig. 3A).

In the eosinophils and ILC2s coculture system, ECP production induced by OPN or IL-33 was significantly higher than ECP production in eosinophils culture system (Fig. 3B). OPN presented similar potency with IL-33 in the activation of eosinophils (Fig. 3). When anti-IL-5 antibody was added, the production of ECP was significantly inhibited (Fig. 3B).

DISCUSSION

Elevated expression of OPN can be found in IgE-mediated inflammation, included asthma, AR, or allergic conjunctivitis. We found that the serum OPN expression was up-regulated in AR and its correlation with Th2 inflammation in previous study. ILC2s were a group of lymphocytes characterized by lacking B and T cell markers. ILC2s
Fig. 1 OPN regulated proliferation and function of ILC2s. A. The proliferation rate of ILC2s after stimulation by OPN. B-C. The mRNA levels of GATA3 and RORα by ILC2s after stimulation by OPN. D-G. The protein expression of GATA3, RORα, IL-5 and IL-13 by ILC2s after stimulation by OPN. * Compared with PBS group, \( P < 0.05 \); # Compared with 100 ng/mL OPN group, \( P < 0.05 \). OPN, osteopontin; ILC2s, group II innate lymphoid cells; cpm, counts per minute. Three independent tests were performed in every experiment. IL-25 (10 ng/ml), IL-33 (10 ng/mL), TSLP (10 ng/mL) and IL-2 (50 ng/ml) were added in all groups. Black bar for data from AR patients, grey bar for data from controls. All comparisons between AR and controls under the same stimulators had significant difference (\( P < 0.05 \)).

Fig. 2 MiR-181a regulates the production of OPN by eosinophils. A. The relative expression of miR-181a by eosinophils. B. OPN protein expression by eosinophils when transfected with miRNA control (1 μM), miR-181a mimics (1 μM) or miR-181a inhibitor (1 μM). * Compared with PBS or miR-control group, \( P < 0.05 \); # Compared with other three groups, \( P < 0.05 \). OPN, osteopontin; GM-CSF, Granulocyte-macrophage colony stimulating factor (100 ng/ml). Three independent tests were performed in every experiment. Black bar for data from AR patients, grey bar for data from controls. All comparisons between AR and controls under the same stimulators had significant difference (\( P < 0.05 \)).
contribute to both induction of type 2 inflammation and development of Th2 cells.\textsuperscript{4} The number of ILC2s in the peripheral blood of asthmatic or AR subjects was higher compared with controls.\textsuperscript{19,20} Here we for the first time found that OPN is involved in innate immunity by regulating the proliferation and function of ILC2s directly. Previous study reported that ILC2s were important for maintaining eosinophils.\textsuperscript{5} For example, Molofsky showed that the number of eosinophils is very low in RAG-2-deficient mice due to lack of ILC2s, suggesting that the development of eosinophil is dependent on ILC2s.\textsuperscript{21} Previous reports had demonstrated that IL-33 promoted eosinophil-mediated airway inflammation in an IL-5-dependent manner by activating ILC2s.\textsuperscript{22} Consistently, our results also showed that OPN presented similar potency with IL-33 in the activation of eosinophils by ILC2s through induction of IL-5.

MicroRNAs (miRNAs), a family of small non-coding RNAs, regulate the function of cellular responses by inhibiting mRNA translation or degradation of mRNA targets so as to affect gene expression.\textsuperscript{23} Accumulating evidence have demonstrated that miRNAs can change OPN expression. For example, miR-4262 targeted the 3’-UTR of OPN to affect its translation in osteosarcoma.\textsuperscript{12} The miR-181a was found to decrease OPN expression in hepatocellular cancer cell.\textsuperscript{13} Our results found that eosinophils transfected with miR-181a produced less OPN compared with controls. Similarly, our previous study also suggested a negative relation between OPN and miR-181a.\textsuperscript{14}

Taken together, our results described the interaction between ILC2s and eosinophils mediated by OPN in allergic inflammation (Fig. 4). On the one hand, OPN secreted from eosinophils promoted the proliferation and function of ILC2s. On the other hand, increased IL-5 secretion from ILC2s induced by OPN activated eosinophils to produce ECP. Moreover, miR-181a can inhibit OPN production by eosinophils. In summary, our data for the first time provide new evidence that OPN plays important roles in innate immunity of AR by regulation of ILC2s and the interaction between ILC2s and eosinophils.

**Fig. 3 ECP expression by eosinophils under different stimulators.** A. Percentage of viable eosinophils after stimulation by various stimulators. * Compared with IL-33 or OPN group, $P < 0.05$. OPN, osteopontin. ECP, eosinophil cationic protein. Three independent tests were performed for every experiment. IL-25 (10 ng/ml), IL-33 (10 ng/ml), TSLP (10 ng/ml) and IL-2 (50 ng/ml) were added in all groups. Black bar for data from AR patients, grey bar for data from controls. All comparisons between AR and controls under the same stimulators had significant difference ($P < 0.05$).
Abbreviations
AR, Allergic rhinitis; ILC2s, Group II innate lymphoid cells; OPN, Osteopontin; TNSS, total nasal symptom score; ELISA, Enzyme-linked immunosorbent assay; GATA3, GATA Binding Protein 3; RORα, Retinoic Acid Related-Orphan Receptor.

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Data availability statement
All data generated or analyzed during this study are included in this published article and its additional files.

Authors’ contributions
Study design: Wenlong Liu, Qingxiang Zeng; experiment: Qingxiang Zeng, Yinhui Zeng, Lifeng Zhou; data collected and analysis: Wenlong Liu, Yinhui Zeng, Luo Xi; manuscript drafting: Wenlong Liu and Qingxiang Zeng.

Ethical statement
All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from all patients for being included in the study. The name of the Institutional Review Board (IRB) that reviewed and approved the study is IRB of Guangzhou women and children’s hospital and the IRB case number for the study is 261A01.

Consent for publication
The authors provide their consent for the publication of the study results.

Declaration of competing interest
The authors declare that they have no conflicts of interest.

Acknowledgement
Not applicable.

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