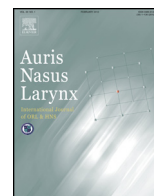




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CD4⁺ T cells induce productions of IL-5 and IL-13 through MHCII on ILC2s in a murine model of allergic rhinitis

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ABSTRACT

Objective: CD4⁺ T cells play an important role not only in the induction of allergy but also in allergic inflammation. Group 2 innate lymphoid cells (ILC2s) also mediate type 2 immune responses in allergic rhinitis (AR). However, the relationships between CD4⁺ T cells and ILC2s in allergic condition are currently not well defined. The study aimed to evaluate the potential influences of CD4⁺ T cells on ILC2s in the murine model of AR.

Methods: A murine model of AR was established using ovalbumin (OVA), and OVA-induced ILC2s were sorted and purified from the mouse nasal-associated lymphoid tissue (NALT), and cultured in vitro. Then, the expression of major histocompatibility complex class II (MHCII) on ILC2s was examined. CD4⁺ T cells were separated from AR mice peripheral blood mononuclear cells (PBMCs). After that, productions of IL-5 and IL-13 on ILC2s cultures were assessed when CD4⁺ T cells or plus anti-MHCII antibody or anti-CD4 antibody were administered into the cultures. Finally, we adoptively transferred ILC2s alone or ILC2s plus anti-MHCII antibody to the murine model of AR to investigate their roles in the nasal allergic inflammation.

Results: We showed that ILC2s could be induced by OVA in the mouse NALT. The number and percentage of ILC2s in AR mice were increased. MHCII was expressed on ILC2s, and its protein and mRNA were all enhanced in allergic condition. IL-5 and IL-13 proteins and mRNAs were elevated after CD4⁺ T cells administration, and were reduced after these cells plus anti-MHCII antibody or anti-CD4 antibody application. Numbers of sneezing and nasal rubbing as well as counts of eosinophils in nasal lavage fluid (NLF) were all enhanced after the adoptive transfer of ILC2s when compared to AR mice. IL-5 and IL-13 in the NLF of allergic mice were also increased in comparison with AR group. However, above parameters were all decreased after the transfer of ILC2s plus anti-MHCII antibody versus AR mice or ILC2s-treated ones.

Conclusion: These findings show that CD4⁺ T cells induce productions of IL-5 and IL-13 through MHCII on ILC2s in AR mice models.

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1. Introduction

Allergic rhinitis (AR) occurs as a consequence of abnormal immunologic responses to inhaled allergens, and is related to the

nasal mucosal inflammation driven by CD4⁺ type 2 helper T (Th2) cells [1]. The activation of CD4⁺ Th2 cells characterizes the type 2 adaptive immune response by producing type 2 cytokines such as interleukin (IL)-4, IL-5, IL-9 and IL-13 and inducing B-cell class-switching to immunoglobulin E (IgE) [2]. Although Th2 cells are an important source of type 2 cytokines, it is becoming increasingly clear that group 2 innate lymphoid cells (ILC2s) provide another more source of IL-5 and IL-13 [3], and accelerate allergic inflammation in AR mice models [4].

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IgE is produced as a result of complex interactions between B cells, T cells, mast cells and basophils and involves various cytokines like IL-4, IL-13 and IL-18. When the specific allergen is taken up by antigen-presenting cells (APCs), it is processed into a small peptide that binds to the specific major histocompatibility complex class II (MHCII) molecules. The MHCII-peptide complex is then expressed on the APCs surface, where it is recognized by the relevant receptor and other costimulatory molecules in Th0 cells, resulting in differentiation into CD4⁺ Th2 cells that synthesize and produce type 2 cytokines. Activation of antigen-specific Th2 by the MHCII-peptide complex stimulates receptors on B cells, which causes B cells differentiation into antibody-producing plasma cells through B-cell costimulatory molecules. Then, IL-4 induces antibody class switching to IgE isotypes that specifically recognize the peptide linked to the MHCII. As a result, antigen-specific IgE is synthesized and secreted from plasma cells. The specific IgE binds to high-affinity IgE receptors such as FcεRI on mast cells and basophils [5] and initiates the early phase of allergic inflammation in nasal mucosa [6].

As mentioned above, MHCII molecule is located to APCs such as dendritic cells (DCs), macrophages and B cells, and plays an essential role in the initiation and development of AR. It has been reported that ILC2s also express MHCII, however, the key function of this molecule in this cell type remains to be fully elucidated [7,8]. Recently, studies have shown that ILC2s and T cells cooperate through MHCII-dependent activation to potentiate the type 2 response against worms [9], and the cooperation also promotes DCs migration to the draining lymph nodes to stimulate Th2 polarization [10].

In this current study, we aimed to verify the expression of MHCII on ILC2s, and to evaluate the potential influences of CD4⁺ T cells on ILC2s in vitro and in vivo. We established a murine model of AR using ovalbumin (OVA), and separated ILC2s from the mouse nasal-associated lymphoid tissue (NALT), and cultured in vitro. We also separated CD4⁺ T cells from AR mice peripheral blood mononuclear cells (PBMCs), and administered CD4⁺ T cells or CD4⁺ T cells plus anti-MHCII antibody or CD4⁺ T cells plus anti-CD4 antibody into the cultures to evaluate productions of IL-5 and IL-13. Finally, we adoptively transferred NALT-derived ILC2s alone or ILC2s plus anti-MHCII antibody to the murine model of AR to investigate their roles in the nasal allergic inflammation.

2. Materials and methods

2.1. Animals

Female BALB/c mice (6–8 weeks old) were purchased from the Chinese Academy of Sciences Shanghai Laboratory Animal Center. These mice were maintained in horizontal laminar flow cabinets and provided sterile food and water in a specific pathogen-free facility. Animal studies were approved by the Institutional Animal Care and Use Committee of Fudan University (ethic number: 201808001Z). The mice were randomly divided into 4 experimental groups (n = 6 for each group).

2.2. Mice models

According to published procedures [11], mice were administered 0.5 mg/ml of OVA (Sigma-Aldrich, St. Louis, Missouri, USA) and 20 mg/ml of aluminium hydroxide (Sinopharm Chemical Reagent Co. Ltd., Shanghai, China) in normal saline at a dosage of 0.2 ml per mouse by intraperitoneal injection. The sensitization was repeated 3 times at weekly intervals (days 1, 8, and 15). Thereafter, mice were challenged by daily instillation of OVA solution droplet (40 mg/ml in normal saline) into the nostrils (0.02 ml per mouse) using a micropipette on days 22 to 29 (Fig. 1). As a negative control, a group of mice received the challenge treatment of normal saline alone (Normal group). Two groups of allergic mice (ILC2s group: treatment of allergic mice with ILC2s, ILC2s + anti-MHCII group: treatment of allergic mice with ILC2s + anti-MHCII antibody) received the adoptive transfer of ILC2s cultured in vitro alone or plus anti-MHCII antibody intravenously in the tail vein on challenging days, respectively, whereas another group of allergic mice was not treated with ILC2s (AR group). Nasal symptoms were evaluated by counting numbers of sneezes and nasal rubs during 10 min immediately after the last OVA intranasal provocation on day 29.

2.3. Sample preparation

After mice were killed, the fore teeth were cut off. The lower jaw and cheek muscles were removed, and NALT was exposed by carefully peeling away the plate. NALT was localized on the posterior part of the plate, and teased out with syringe needles in ice-cold RPMI 1640 culture medium supplemented with 10% FBS. Then the cells were passed through a 70 μm Falcon cell strainer. Red blood cells were lysed in Tris-buffered ammonium chloride solution (0.83% NH₄Cl and 20 mM Tris/Cl).

2.4. Flow cytometry analysis

NALT cell suspensions were centrifuged for 10 min at 200 × g at 4 °C, and cells were harvested for flow cytometry analysis. Gating strategy for ILC2s was based on CD3CD4CD8CD19CD11bCD11cFceR1 (lineage)[−] inducible costimulator (ICOS)⁺ cell surface expressions [12]. The cells were washed with 200 μl flow cytometry medium [FCM, 1% BSA and 0.1% sodium azide in phosphate-buffered saline (PBS)] and incubated with 50 μl rabbit serum for 5 min at room temperature to block the Fcγ receptor. The cells then were washed three times with FCM, and stained with 50 μl of monoclonal antibodies against the following surface molecules at 4 °C for 30 min at 1 × 10⁸ cells/ml: CD3, CD4, CD8, CD11b, CD11c, CD19, FcεR1, MHCII; all antibodies purchased from MyBioSource, Inc., San Diego, CA, USA or BioLegend, San Diego, CA, USA and conjugated to fluorescein isothiocyanate, and ICOS monoclonal antibody (purchased from MyBioSource, Inc., San Diego, CA, USA and conjugated to Phycoerythrin). They were washed again and incubated with 50 μl secondary antibody (biotin-conjugated rabbit anti-mouse IgG; Dako Denmark A/S, Glostrup, Denmark) followed by 50 μl R-phycoerythrin-conjugated streptavidin (RPE-streptavidin; Dako Denmark A/S, Glostrup, Denmark). After staining, cells were resuspended in staining buffer containing

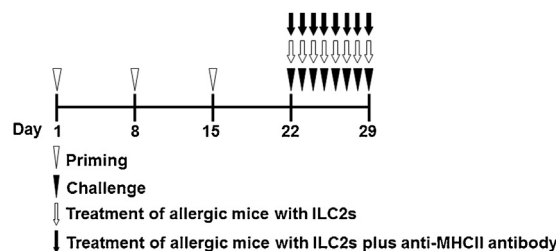


Fig. 1. Study protocols. A murine model of allergic rhinitis was prepared by intraperitoneal immunization of ovalbumin on days 1, 8, and 15 (priming) followed by daily intranasal ovalbumin challenge on days 22 to 29. A group of mice received the challenge treatment of normal saline alone (Normal group). Two groups of allergic mice (ILC2s group: treatment of allergic mice with ILC2s, ILC2s + anti-MHCII group: treatment of allergic mice with ILC2s + anti-MHCII antibody) received the adoptive transfer of ILC2s cultured in vitro alone or plus anti-MHCII antibody intravenously in the tail vein on challenging days, respectively, whereas another group of allergic mice was not treated with ILC2s (AR group). ILC2s, group 2 innate lymphoid cells. MHCII, major histocompatibility complex class II.

4', 6-Diamidino-2-phenylindole dihydrochloride (DAPI) for exclusion of dead cells. Controls for single color analysis were carried out by incubating cells with a primary antibody of the same isotype, but of irrelevant specificity at matched concentrations followed by secondary reagents, as described above. The samples were collected on an LSR II flow cytometer equipped with FACSDIVA software (BD Biosciences, Mountain View, CA) and analyzed with FlowJo version 10 (Tree Star, Inc., Ashland, OR). Set-up of the instrument was carried out visually and selection of the isotype control antibody was according to the primary antibodies' host species, isotype, and conjugation format.

2.5. Immunocytochemistry and confocal microscopy

ILC2s from AR mice were seeded on glass coverslips and grown for 48 h. Adhering cells were washed twice with PBS and fixed with 4% paraformaldehyde for 5 min at room temperature, then, rinsed three times with PBS containing 0.2% Triton X-100 (PBS-T), and blocked with 3% bovine serum albumin (in PBS) for 30 min. Samples were then incubated overnight at room temperature with primary antibodies against MHCII. After rinsing with PBS (3×5 min), samples were incubated 1 h at room temperature with fluorescein isothiocyanate-conjugated secondary antibodies in the darkness. Coverslips were then washed twice with PBS-T before nucleus staining with 10 mg/ml DAPI for 5 min at room temperature. Finally, the coverslips were washed three times by PBS-T and twice by PBS before mounting with Vectashield (Vector Laboratories, Burlingame, CA, USA). As negative controls, cell samples were incubated either with the secondary antibody alone or with the primary antibody preadsorbed with a specific blocking peptide (1:5 w/w). Images were obtained with a Leica TCS SP5 Confocal Laser Scanning Microscope by the Leica confocal software (LCS) 2.61 (Leica Microsystems, Wetzlar, Germany). Images were analyzed by LCS Lite (Leica) software. To enable comparison, all images were acquired using the same parameters of laser power and photomultiplier sensitivity. Images shown are representative of at least three separate experiments in each condition, and were processed with identical values for contrast and brightness.

2.6. In vitro ILC2s and CD4⁺ T cells cocultures

ILC2s were sorted and purified as CD3CD4CD8CD19CD11bCD11cFceR1⁺ (lineage)[−]ICOS⁺

cells at a maximum concentration of 6×10^7 cells/ml. They were cultured for 6 days in RPMI 1640 containing 10% FCS, 1% penicillin/streptomycin, 0.1% β -mercaptoethanol, 10 ng/ml of mouse recombinant (rm) IL-7 (BioLegend, San Diego, CA, USA), and 10 ng/ml of mouse rmIL-33 (BioLegend, San Diego, CA, USA).

Mice PBMCs were obtained from AR mice, and CD4⁺ T cells were separated at a concentration of 5×10^5 cells/ml using EasySepTM Mouse CD4⁺ T Cell Isolation Kit (STEMCELL Technologies, Vancouver, BC, Canada). These cells were cultured for 6 days in RPMI 1640 containing 10% FBS, 1% penicillin/streptomycin, 50 μ M β -mercaptoethanol, 100 U/ml of mouse rmIL-2 (BioLegend, San Diego, CA, USA).

ILC2s from AR mice were incubated with CD4⁺ T cells alone or plus 1000 ng/ml anti-MHCII antibody or plus 1000 ng/ml anti-CD4 antibody in the cultures. The cultures were kept for 3 days. The cell supernatants were used to measure IL-5 and IL-13 production by enzyme-linked immunosorbent assay (ELISA), and the cell lysates were used to assess the level of messenger (m) RNAs of IL-5 and IL-13 by real-time reverse transcription–polymerase chain reaction (RT-PCR). Then, ILC2s alone or these cells after treatment with anti-MHCII antibody were resuspended in normal saline, and were adoptively transferred at 100 μ l per mouse (6×10^6 cells/mouse) intravenously in the tail vein on challenging days.

2.7. Nasal lavage fluid (NLF)

After exsanguinations of the mice, one blunted 18-gauge needle was pointed toward the heads of a portion of mice for NLF. One injection of 2000 μ l normal saline was performed and the fluid was collected with a tube under both nares of the nose for NLF. A portion of NLF was centrifuged for 10 min at 150 g at 4 °C. The supernatants were stored at -70 °C for IL-5 and IL-13 assays. Another portion of NLF was collected for detection of eosinophils, and differential cell counts on 150 cells were performed on cytospins (Cytospin 4 Shandon Ltd., Runcorn, UK) stained with Giemsa [13].

2.8. ELISA analysis

IL-5 in the NLF and the culture was measured using a 96-well microplate coated with 1 μ g/ml of IL-5 rabbit antibody. The microplate was incubated in 3% bovine serum albumin at 37 °C for 1 h. Samples at 1:10 dilution were then added followed by incubation at 37 °C for 1 h. After washing, anti-IL-5 antibody

(MyBioSource, Inc., San Diego, CA, USA) that had been biotinylated using a biotinylation kit (American Qualex International Inc., San Clemente, California, USA) was then added at 1 µg/ml and allowed to incubate at 25 °C for 1 h. After washing, 1.5 µg/ml of streptavidin peroxidase was added followed by incubation at 25 °C for 1 h. After washing, tetramethylbenzidine (TMB) substrate (12.5 ml of citric-phosphate buffer, 200 µl of TMB stock solution [6 mg/ml in dimethyl sulfoxide], 100 µl of 1% hydrogen peroxide; Fluka Chemical Co., Ronkonkoma, New York, USA) was added to produce a color reaction. The reaction was terminated by the addition of 6 N sulfuric acid. Optical density was determined at 450 nm using a microplate reader (Microplate Reader MTP-32; Corona Electric, Ibaraki, Japan). Concentrations of IL-13 in the NLF and MHCII and IL-13 in the culture was evaluated using corresponding ELISA kits (all purchased from MyBioSource, Inc., San Diego, CA, USA).

2.9. Real-time RT-PCR

Total RNA of a portion of samples was extracted with Trizol (Invitrogen, Carlsbad, CA, USA) and treated with RNase-free DNase. For reverse transcription, 2 µg of above RNA was reversely transcribed with random hexamers (Invitrogen, Carlsbad, CA, USA) and cDNA was amplified according to the manufacturer's instructions. Primers were designed using Primer Express software (ABI) from sequence available in Genbank and were synthesized (Geneland Biotech, Shanghai, China). Real-time RT-PCR was performed to detect mRNAs of MHCII, IL-5 and IL-13. MHCII primers were: forward primer 5'-aagaaggagactgtctggatgc-3' and reverse primer 5'-TGAATGATGAAGATGGTGCCC-3'. IL-5 primers were: forward primer 5'-TCACCGAGCTCTGTTGACAA-3' and reverse primer 5'-CCACACTTCTCTTTTGGCG-3'. IL-13 primers were: forward primer 5'-AGACCAGACTCCCCTGTGCA-3' and reverse primer 5'-TGGGTCCTGTAGATGGCATTG-3'. GAPDH mRNA was also examined to control the sample-to-sample variation in RNA isolation and integrity by using a pair of primers: forward primer 5'-ACCACAGTCCATGCCATCAC-3' and reverse primer 5'-TCCACACCCTGTTGCTGTA-3'. After initial denaturation at 95 °C for 10 min, the amplification profile was 15 s of denaturation at 95 °C, 1 min of annealing and extension at 60 °C for 45 cycles. Negative control RT reaction mixtures contained no reverse transcriptase and no cDNA in the PCR amplification mixtures. For measurement 2 µl of diluted cDNA was amplified in a total reaction volume of 20 µl by using an 7500 real-time PCR System (Applied Biosystems, Foster City, CA, USA) with 20 × SYBR Green mixture (Invitrogen, Carlsbad, CA, USA). Specificity of PCR products was evaluated by melting curve analysis and by size in agarose gels. Using three dilutions of cDNA, linearity of PCR amplification was controlled. Evaluation of data was performed using the Δ CT method with GAPDH as internal standard.

2.10. Statistical analysis

Statistical analysis was performed using a commercially available statistical software prism 6.0 (GraphPad Software

Inc., San Diego, CA, USA). Data were analyzed with the unpaired Student *t* test ± Welch's correction or one-way ANOVA followed by Tukey's multiple comparison test, and presented as mean ± SEM (standard error of the mean). Significance of a difference was accepted at the 5% level of confidence. *p* < 0.05 was considered statistically significant.

3. Results

3.1. ILC2s arise in the NALT of AR mice models

Mice were sensitized and challenged with OVA to evaluate ILC2s in the NALT of AR mice models. These cells were sorted and purified as lineage[−]ICOS⁺ cells in the NALT of mice [12], and were increased in AR mice (Fig. 2A and B). Comparisons of ILC2s number and percentage indicated that their total number and percentage were all enhanced in the NALT in response to OVA treatment compared with those seen in normal mice (Fig. 2C and D).

3.2. MHCII is expressed on the NALT-derived ILC2s

To evaluate the expression of MHCII molecule, ILC2s were further defined as ICOS⁺MHCII⁺ in normal and AR mice through flow cytometry (Fig. 3A and B). And we also performed confocal immunofluorescence microscopy. As shown in Fig. 3, we detected MHCII staining in the plasma membrane of ILC2s (Fig. 3C–E). In addition, we also examined expressions of MHCII protein and mRNA in the cultures by ELISA and real-time RT-PCR. The results demonstrated that MHCII protein and mRNA were all elevated on ILC2s from AR mice in comparison with normal ones. The data indicate that MHCII expression is upregulated on ILC2s during allergic inflammation.

3.3. Type 2 cytokines are induced by CD4⁺ T cells from ILC2s in the cultures

To investigate the influence of CD4⁺ T cells on the cellular function of ILC2s from AR mice, we sought to examine IL-5 and IL-13 concentrations in the culture. We found that IL-5 and IL-13 productions were all upregulated in the supernatants with CD4⁺ T cells treatment in comparison with the unstimulated control, and the elevation of IL-13 was much higher (Fig. 4A and C). As for their mRNAs, productions of these two cytokines were also increased after CD4⁺ T cells alone administration (Fig. 4B and D). In order to further analyze the induction of type 2 cytokines by CD4⁺ T cells, we first administered anti-MHCII antibody in the culture to block this molecule on ILC2s, and then applied CD4⁺ T cells. Cell supernatants were also analyzed by ELISA to quantify cytokine levels. We showed that the levels of IL-5 and IL-13 were all downregulated compared to those after the only CD4⁺ T cells treatment whether in protein or mRNA expressions (Fig. 4A–D). Additionally, we administered anti-CD4 antibody in the culture to block CD4 molecule in CD4⁺ T cells, and then applied CD4⁺ T cells. We found that the contents of IL-5 and IL-13 were also decreased compared to those after the only CD4⁺ T cells treatment in protein or mRNA

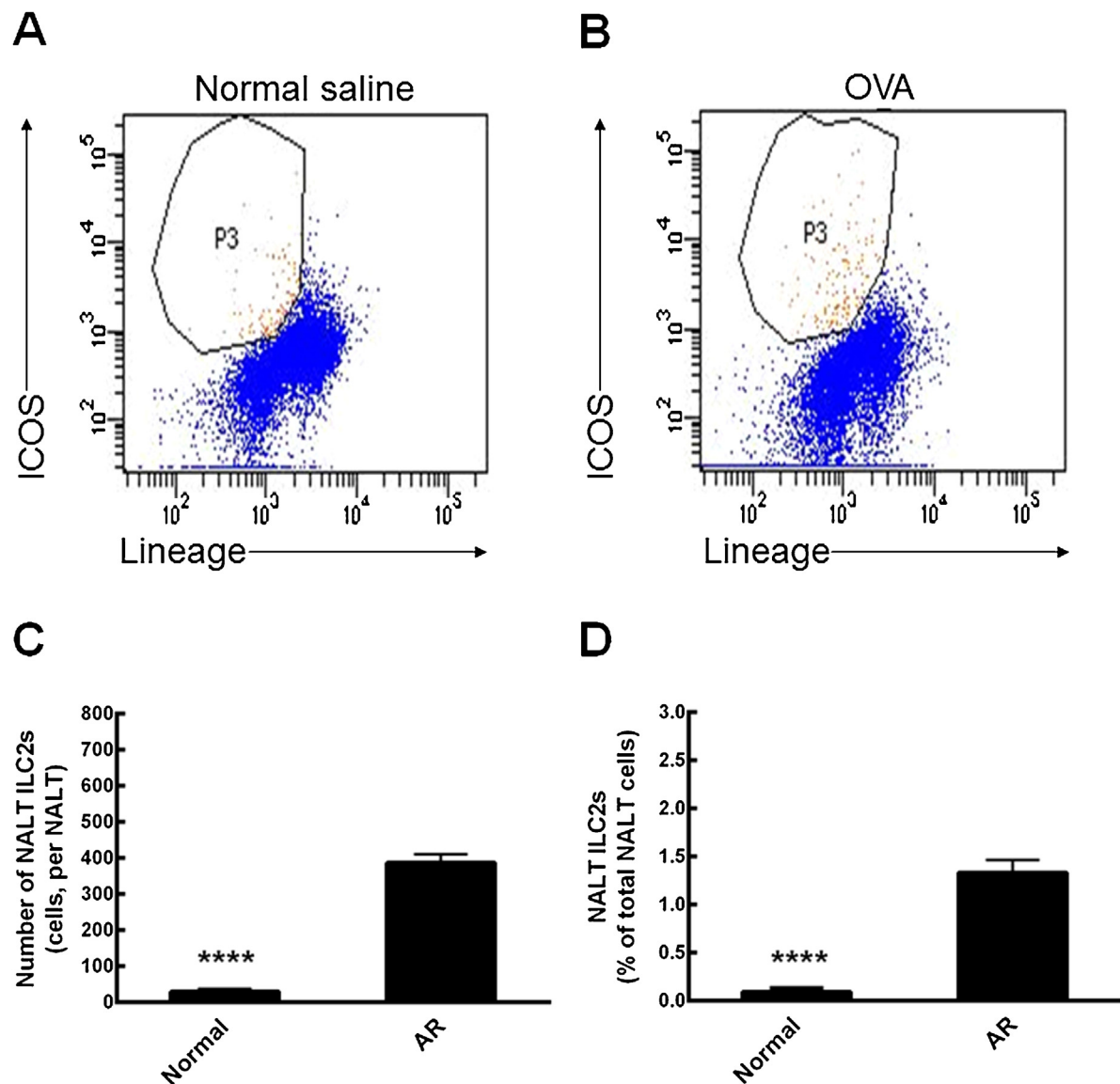


Fig. 2. ILC2s derived from nasal-associated lymphoid tissue (NALT) in a murine model of allergic rhinitis. (A) ILC2s were identified as CD3CD4CD8CD19CD11bCD11cFceR1 (lineage)[−]inducible costimulator (ICOS)⁺ cells in normal mice. (B) ILC2s were identified as CD3CD4CD8CD19CD11bCD11cFceR1 (lineage)[−]ICOS⁺ cells in allergic mice. (C) Number of the NALT-derived ILC2s. (D) Percentage of the NALT-derived ILC2s. Each value represents the mean (SEM) of 6 mice in each group. *****p* < 0.0001 vs control group. Normal, normal group. AR, AR group. OVA, ovalbumin. AR, allergic rhinitis. ILC2s, group 2 innate lymphoid cells.

(Fig. 4A–D). The data demonstrate that CD4⁺ T cells influence productions of IL-5 and IL-13 by ILC2s through MHCII protein expression on this cell type.

3.4. Allergic inflammation is aggravated by ILC2s in a murine model of AR

To examine the effects of OVA-induced allergic inflammation, sneezing and nasal rubbing were counted after the last allergen challenge. AR mice showed increases in numbers of sneezing and nasal rubbing compared with those of normal mice that had received only normal saline challenge (Fig. 5A and B). The number of infiltrating eosinophils and expressions of cytokine IL-5 and IL-13 in the NLF of AR mice also displayed increases compared to those in normal mice (Fig. 5C–E). The results clearly indicated that the murine model of AR

was established successfully. The adoptive transfer of OVA-induced ILC2s intravenously in the tail vein increased numbers of sneezing, nasal rubbing, and eosinophils, and concentrations of IL-5 and IL-13 in the NLF of AR mice (Fig. 5A–E). However, all above parameters were all reduced after the intervention of MHCII on ILC2s by anti-MHCII antibody administration in comparison with those after treatment of ILC2s alone (Fig. 5A–E). However, there were statistical differences between AR group and ILC2s plus anti-MHCII antibody treatment group in numbers of sneezing, nasal rubbing and eosinophils, and expressing levels of IL-5 and IL-13. These parameters were increased even the intervention of MHCII protein on ILC2s compared to AR mice. These findings demonstrate that ILC2s infiltrating in nasal mucosal of AR mice exacerbate allergic inflammation through the induction of IL-5 and IL-13 partly by cooperation of CD4⁺ T cells and ILC2s.

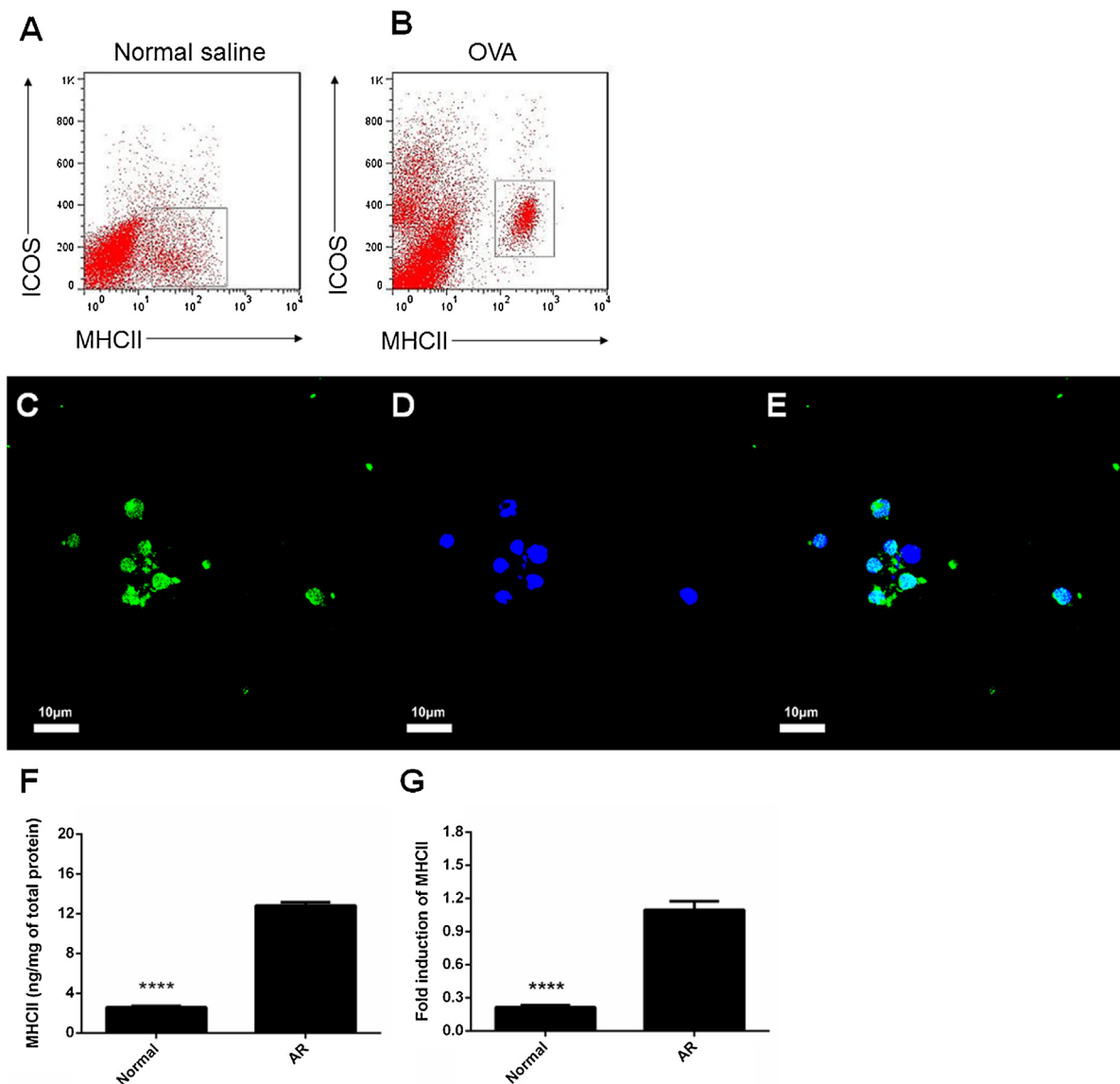


Fig. 3. The expression of MHCII on ILC2s. (A) ILC2s were further defined as ICOS⁺MHCII⁺ in normal mice. (B) ILC2s were further defined as ICOS⁺MHCII⁺ in allergic mice. (C) Confocal immunofluorescence microscopic analysis of MHCII on ILC2s (fluorescein isothiocyanate). (D) Confocal immunofluorescence microscopic analysis of MHCII on ILC2s [4',6-Diamidino-2-phenylindole dihydrochloride (DAPI)]. (E) Confocal immunofluorescence microscopic analysis of Orai1 on ILC2s (merge). (F) MHCII protein expression on ILC2s. (G) MHCII mRNA expression on ILC2s. Scale bars, 10 μm. Original magnification, C, D, E, 400 ×. Each value represents the mean (SEM) of 6 mice in each group. Normal, normal group. AR, AR group. OVA, ovalbumin. AR, allergic rhinitis. MHCII, major histocompatibility complex class II. *****p* < 0.0001 vs AR.

4. Discussion

The immunologic causes of AR underline the essential roles of both adaptive and innate immune systems. In recent years, appreciation of the role of nasal innate immunity has grown, and increasing evidence suggests that the pathogenesis of AR is partially mediated by the innate immune system [14].

ILC2s have been identified as novel IL-13-producing innate system cells which are identified as lineage (lymphocyte, macrophage, DC, basophil, eosinophil, mast cell, and natural killer cell) negative and ICOS positive [7]. These cells also express both the IL-25 receptor (IL-17RB) and the IL-33 receptor (T1/ST2) [8]. Similar innate Th2 cells, termed natural helper cells, have also been reported elsewhere [15,16]. ILC2s are induced to secrete IL-5 and IL-13 by both IL-25 and IL-33 after helminth

infection in the gut [7,8]. In addition, these cells have been reported to be an essential source of IL-13 for helminth expulsion, and can migrate into the small intestine, possibly because of their expression of the CC chemokine receptor (CCR) 9. It has been reported that ILC2s are induced by OVA as part of the allergic inflammatory lung response, and are the major source of non-T cell-derived IL-13 in the lung [12]. However, the contribution of ILC2s to AR is currently not well defined. One of our previous studies demonstrated that ILC2s might migrate into nasal mucosal of AR mice through their expression of CCR9 and the production of CC chemokine ligand 25 by nasal mucosa, and aggravated allergic inflammation through the induction of IL-5 and IL-13 by IL-25 produced from nasal epithelial cells [4]. Notwithstanding, the relationships between CD4⁺ T cells and ILC2s in allergic condition remain to be fully elucidated.

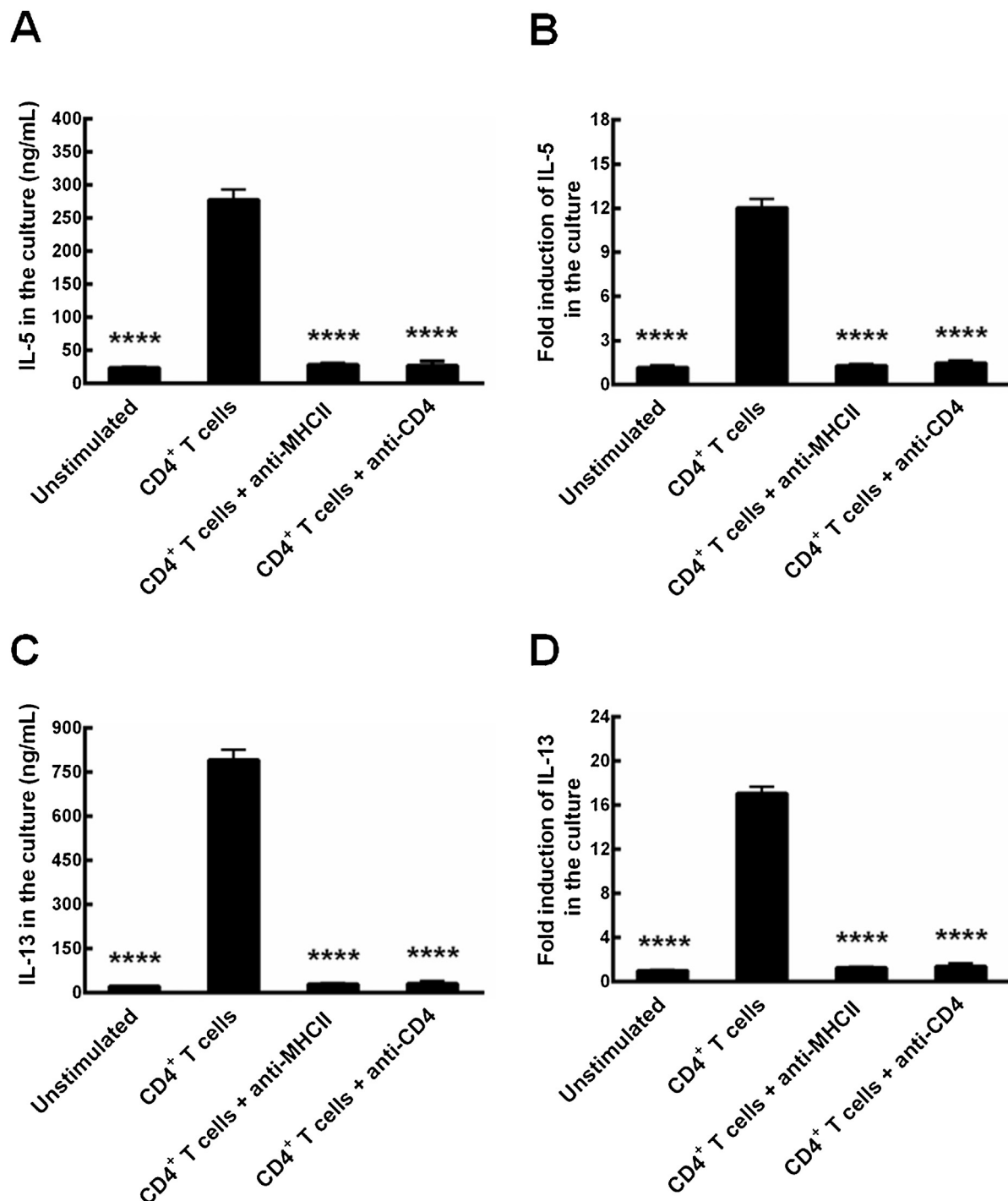


Fig. 4. Responses of the nasal-associated lymphoid tissue (NALT)-derived ILC2s to CD4⁺ T cells in the culture. (A) IL-5 in the culture. (B) IL-5 mRNA in the culture. (C) IL-13 in the culture. (D) IL-13 mRNA in the culture. Each value represents the mean (SEM) of 6 mice in each group. Unstimulated, unstimulated samples. CD4⁺ T cells, CD4⁺ T cells-treated samples. CD4⁺ T cells + anti-MHCII, CD4⁺ T cells + anti-MHCII antibody-treated samples. CD4⁺ T cells + anti-CD4, CD4⁺ T cells + anti-CD4 antibody-treated samples. MHCII, major histocompatibility complex class II. *****p* < 0.0001 vs CD4⁺ T cells.

Recently, studies have begun to explore the cooperative interplay between innate and adaptive immunity in the intestine [17,18]. ILC2s increase rapidly during helminth challenge, preceding the expansion of the adaptive Th2 response, which raises the question of whether ILC2s contribute to the initiation, polarization, or potentiation of adaptive immunity [9]. However, few reports showed the relationships between innate and adaptive type 2 response. MHCII may become the key target of

investigation on the cooperation of CD4⁺ T cells and ILC2s. MHCII is expressed on ILC2s [7] and ILC3s [18]. MHCII on ILC3s, in the absence of the costimulatory molecules CD80 and CD86, resulted in ILC3-mediated suppression of intestinal immune reactions against commensal bacteria [18]. However, the role of MHCII on ILC2s remains to be completely characterized. It has been reported that the ILC2 and T cell crosstalk leads to their mutual maintenance, expansion and

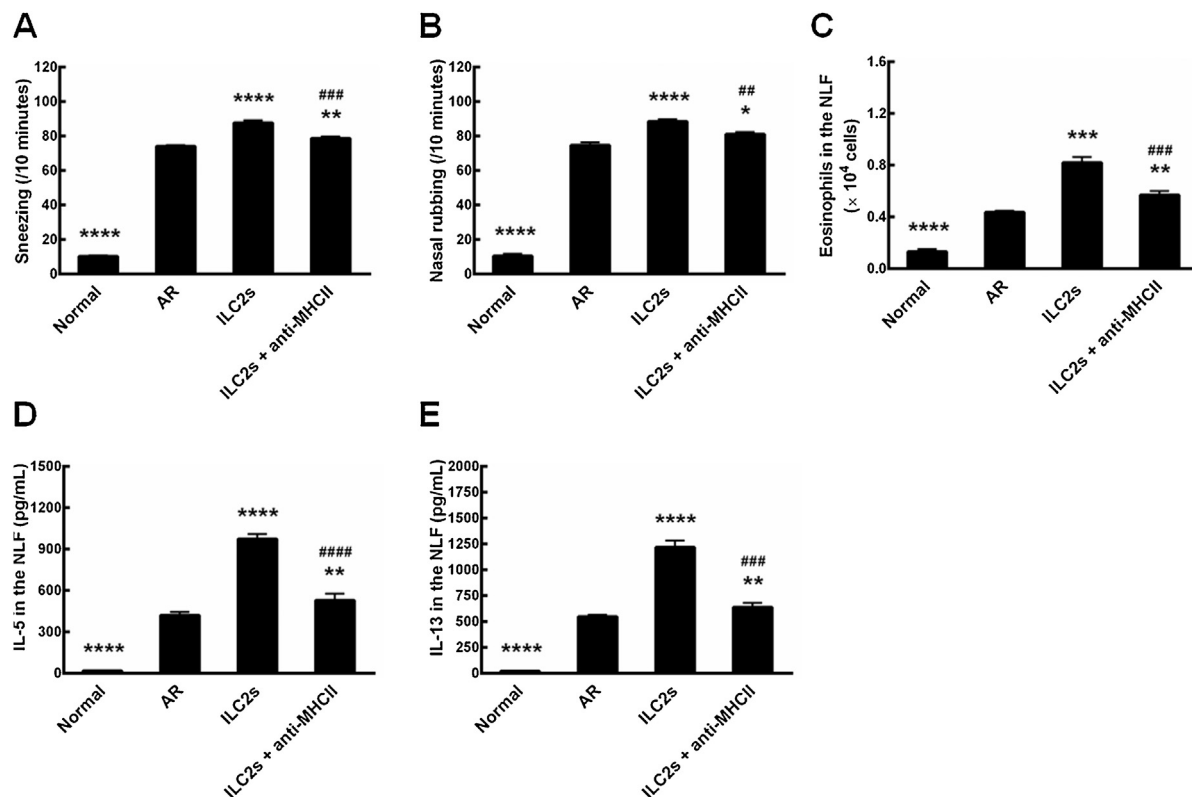


Fig. 5. Exacerbation of allergic inflammation by the ovalbumin (OVA)-induced ILC2s in a murine model of allergic rhinitis. (A) Number of sneezing. (B) Number of nasal rubbing. (C) Number of eosinophils in nasal lavage fluid (NLF). (D) Interleukin (IL)-5 in the NLF. (E) IL-13 in the NLF. Each value represents the mean (SEM) of 6 mice in each group. * $p < 0.05$ vs AR group. ** $p < 0.01$ vs AR group. *** $p < 0.001$ vs AR group. **** $p < 0.0001$ vs AR group. Normal, normal group. AR, AR group. ILC2s, ILC2s group. ILC2s + anti-MHCII, ILC2s + anti-MHCII group. AR, allergic rhinitis. ILC2S, group 2 innate lymphoid cells. MHCII, major histocompatibility complex class II.

cytokine production during the transition to adaptive CD4⁺ T cell-mediated immunity in parasitic helminth expulsion. Furthermore, this interaction appears to augment DCs-induced T cell activation and identifies a definite pathway in the regulation of type-2 immunity [9]. However, to our knowledge, there are no relevant studies on the relationships between CD4⁺ T cells and ILC2s in allergic diseases.

ILC2s such as nuocytes represent important new innate cells that produce IL-5 and IL-13 in response to IL-25 and IL-33 which are produced by airway epithelial cells [19,20]. The induction and proliferation of ILC2s has been demonstrated to be necessary for initiating type 2 immunity during worm infection [7], and has been shown to directly cause airway hyperreactivity in the absence of IL-13-producing Th2 cells during allergic lung inflammation [12]. Increased numbers of ILC2s were observed in polyp tissues from CRSwNP patients compared with nasal mucosa from CRSsNP patients or healthy controls [21–25]. However, ILC2s alone cannot induce the nasal symptoms of AR including tissue eosinophilia [26], which can be induced solely by activating ILC2s in lungs [27]. Actually, the roles of ILC2s in the mechanisms of AR is poorly understood.

In our study, we established a murine model of AR, and isolated ILC2s from NALT of normal and AR mice. The data demonstrated that ILC2s number and percentage were upregulated in NALT of mice during nasal allergic inflammation. The result indicates that allergen can induce expansion and

activation of ILC2s just like helminth. To confirm the expression of MHCII molecule on ILC2s, we used flow cytometry analysis to further define the cells as ICOS⁺MHCII⁺, and also qualitatively identified its expression through immunocytochemistry and confocal microscopy. Then, we assessed its protein and mRNA expressions using ELISA and real-time RT-PCR quantitatively. We also showed the enhancement of MHCII in AR mice compared to normal ones. To investigate the impact of CD4⁺ T cells on the cellular function of ILC2s, we examined type 2 cytokine IL-5 and IL-13 concentrations in the culture. We found that productions of IL-5 and IL-13 proteins and mRNAs were upregulated after CD4⁺ T cells coculture with ILC2s in comparison with the unstimulated sample. However, the expressing levels were all downregulated after combined anti-MHCII antibody or anti-CD4 antibody treatment. The results demonstrate that CD4⁺ T cells elevate productions of IL-5 and IL-13 by ILC2s through the pathway of MHCII and CD4 molecules coadjuvant interplay.

In the present study, the data indicated that the adoptive transfer of NALT-derived ILC2s into the murine AR model exacerbated the allergic inflammation. Numbers of sneezing, nasal rubbing and invading eosinophils, and expressing levels of IL-5 and IL-13 were all enhanced significantly when compared to those of AR group. However, the above parameters were reduced when anti-MHCII antibody was applied to the adoptively transferred ILC2s. ILC2s and CD4⁺ T cells all increase in nasal mucosa of AR mice [4,28], MHCII-mediated

dialog between ILC2s and CD4⁺ T cells potentiated type 2 immunologic responses and promoted allergic inflammation in this research. When the MHCII molecule on ILC2s was blocked, the cooperation between these two cell types was interrupted. Then, the allergic reactions in nasal mucosa were alleviated correspondingly. These findings illustrated that the OVA-induced ILC2s aggravate allergic condition through secretions of IL-5 and IL-13 cytokines induced partly by CD4⁺ T cells, which is as a result of the interplay between MHCII and CD4 molecules. Unfortunately, we did not investigate the influence of ILC2s on the cellular function of CD4⁺ T cells in this study.

In summary, the present study demonstrated that MHCII was expressed on ILC2s, and was increased in allergic condition. Productions of IL-5 and IL-13 were elevated after CD4⁺ T cells administration. Numbers of sneezing, nasal rubbing and NLF eosinophils, as well as IL-5 and IL-13 in NLF, were all enhanced after ILC2s treatment. However, after the intervention of MHCII on ILC2s, the allergic inflammation was alleviated. These findings show that CD4⁺ T cells induce productions of IL-5 and IL-13 through MHCII on ILC2s in AR mice models. It should be emphasized that MHCII on ILC2s might be a new potential target in the future AR therapy

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