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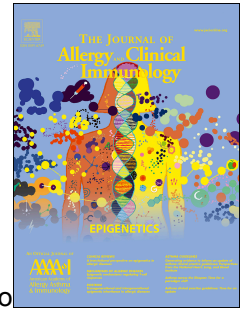
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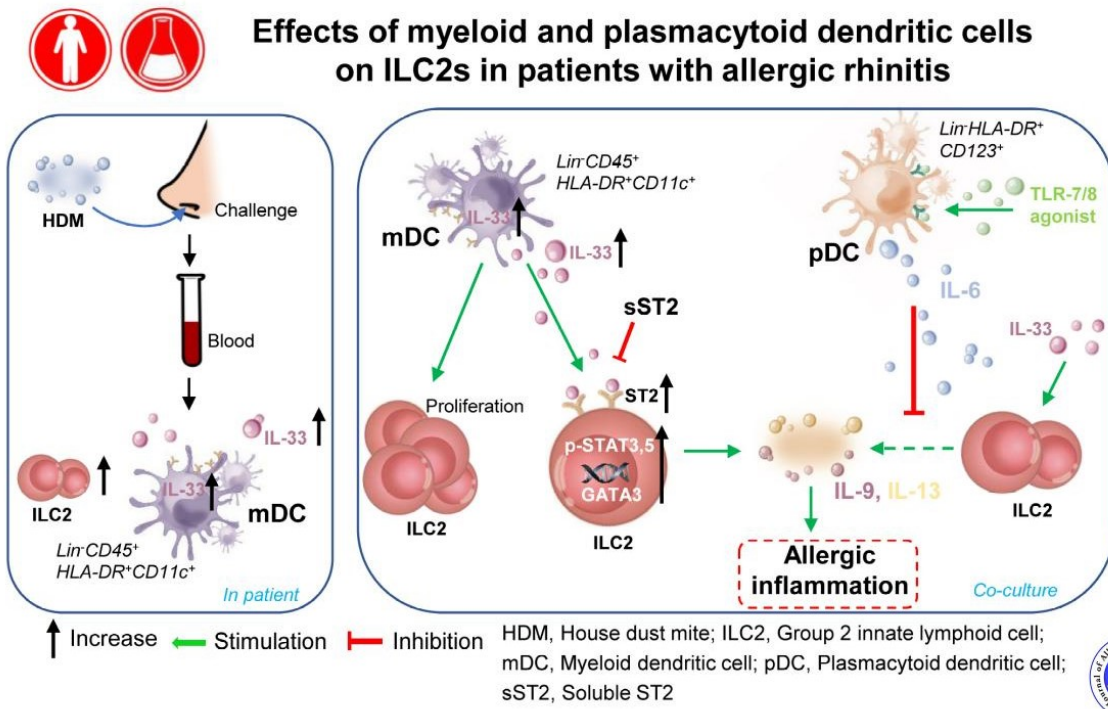
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in patients with allergic rhinitis**

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28

29 **Disclose of potential conflict of interest**

30 The authors declared no conflict of interest.

31 **Abstract**

32 **Background:** Group 2 innate lymphoid cells (ILC2s) were reported to serve a critical
33 role in allergic diseases. Myeloid dendritic cells (mDCs) and plasmacytoid DCs
34 (pDCs) play significant roles in allergic immune response. However, effects of DCs
35 on ILC2s in allergic diseases, especially for patients with allergic rhinitis (AR),
36 remain unclear.

37 **Objective:** We aimed to address the roles of mDCs and pDCs in regulating ILC2
38 function in AR.

39 **Methods:** mDCs and pDCs were co-cultured with human PBMCs isolated from
40 patients with AR or ILC2s to measure soluble or intracellular Th2 cytokines,
41 transcription factors, signaling pathways in ILC2s, and the following mechanisms
42 were further investigated. The levels of peripheral IL-33⁺mDCs, pDCs and ILC2s
43 were studied in patients under an inhaled allergen challenge.

44 **Results:** We confirmed the presence of ILC2s, mDCs and pDCs in the nasal mucosa
45 of patients of AR. Both allogenic and autologous mDCs were found to activate ILC2s
46 from patients with AR to produce Th2 cytokines, and increase the levels of GATA-3
47 and STAT signaling pathways, in which IL-33-producing mDCs exerted the major
48 role by binding on ST2 on ILC2s. We further identified high levels of IL-33⁺mDCs
49 and ILC2s in patients with AR under antigen challenge. Activated pDCs inhibited the
50 cytokine production of ILC2s isolated from patients with AR by secretion of IL-6.

51 **Conclusions:** mDCs promote ILC2 function by the IL-33/ST2 pathway, and
52 activation of pDCs suppresses ILC2 function through IL-6 in patients with AR. Our
53 findings provide new understanding of the interplay between DCs and ILC2s in
54 allergic diseases.

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Key messages

- mDCs activate the ILC2s through the IL-33/ST2 pathway.
- Inhaled allergen significantly increased the levels of IL-33⁺mDC and ILC2s in patients with allergic rhinitis.
- Activation of pDCs suppresses the ILC2 function through IL-6.

Capsule summary

mDCs and pDCs can regulate ILC2 function in allergic rhinitis. Our findings offer a novel understanding of the interplay between DCs and ILC2s in the pathology of allergic diseases.

Key words: *Group 2 innate lymphoid cells, myeloid dendritic cells, plasmacytoid dendritic cells, allergic rhinitis, interleukin-33, ST2, interleukin-6*

Abbreviations used

ANOVA: Analysis of variance

AR: Allergic rhinitis

CFSE: Carboxyfluorescein diacetate succinimidyl diester

DCs: Dendritic cells

Der p1: Dermatophagoides pteronyssinus

GATA3: GATA binding protein 3

GM-CSF: Granulocyte-macrophage colony-stimulating factor

HC: Healthy control

- 79 HDM: House dust mite
- 80 ILCs: Innate lymphoid cells
- 81 ILC2s: Group 2 innate lymphoid cells
- 82 IFN: Interferon
- 83 IL: Interleukin
- 84 IL-1R1: IL-1 receptor like-1 component
- 85 IL-6R: IL-6 receptor
- 86 Lin⁻: Lineage-negative
- 87 LPS: lipopolysaccharide
- 88 mDCs: Myeloid dendritic cells
- 89 mo-DCs: Monocyte derived dendritic cells
- 90 PBMCs: Peripheral blood mononuclear cells
- 91 pDCs: Plasmacytoid dendritic cells
- 92 PD-1: Programmed cell death 1
- 93 PD-L1: PD-1 ligand 1
- 94 PMA : Phorbol 12-myristate 13-acetate
- 95 sST2: Soluble ST2
- 96 Th2 cells: Type 2 helper T cells
- 97 TLR: Toll-like receptor
- 98 TSLP: Thymic stromal lymphopoietin
- 99 VAS: Visual analogue scale

INTRODUCTION

Allergic rhinitis (AR) is now recognized as an inflammatory disorder of the nasal mucosa brought about by a dysregulated immune response to inhaled antigens,¹ affecting 400 million people worldwide and which is accompanied by substantial adverse effects on quality of life and health-care expenditures.² There clearly is an unmet clinical need for more effective therapies for AR. The immunopathology underlying AR represents the outcome of a Type 2 helper T (Th2)-skewed immune responses, and several cells including dendritic cells (DCs) and the epithelial cells are involved in this response. It is now established that CD4⁺ Th2 cells are the major source of production of Th2 cytokines and the principal drivers of the deleterious immune response in the pathogenesis of AR.³

Group 2 innate lymphoid cells (ILC2s) were recently reported to play a critical role in the pathogenesis of allergic disease, and sometime play more important role than Th2 cells.⁴⁻⁹ ILC2s exert these effects by producing large quantities of interleukin (IL)-5, IL-9, and IL-13 and some amount of IL-4 in response to IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) produced by epithelial cells.¹⁰⁻¹² We previously reported that there were high levels of ILC2s in the blood of patients with AR and asthma.^{9, 13, 14} Because the biology of the Th cell and innate lymphoid cell (ILC2) subset is greatly shared, there is an analogy between adaptive T cells and their ILC counterparts.^{15, 16} ILC2s, for examples, express high levels of GATA binding protein 3 (GATA3), which also guides the differentiation of Th2 cells.¹⁷ Since Th2 cell polarization is also driven by DCs, the most important antigen-presenting cells in response to the stimulation of allergen, it is equally compelling to know whether ILC2s can also be regulated by DCs.

In humans, circulating DCs are subdivided into two major subsets that include

CD11c⁺ myeloid dendritic cells (mDCs) and CD123⁺ plasmacytoid dendritic cells (pDCs).^{18, 19} It is well established that mDC function is crucial to the etiology of allergic diseases by inducing Th2 immunity to inhaled allergens.²⁰ Conversely, pDCs are thought to attenuate the magnitude of the allergic response in atopic dermatitis, AR, or asthma.^{21, 22} Interestingly, cytokines secreted by DCs play a significant role in promoting ILC function and polarization. ILC3s produce IL-17 and IL-22 in response to DC-derived IL-23 and IL-1 β , which promotes innate immunity to fungi and extracellular bacteria.²³ Furthermore, CD103⁺ DCs were thought to drive ILC1 to ILC3 conversion.²⁴ Together, these data identify DCs as the bridge between innate and adaptive immune systems. mDCs are thought to produce IL-33, which is one of the most important cytokines to activate ILC2s.²⁵ The pathogenic role of the IL-33/ST2 axis in autoimmune and inflammatory disorders have been recently highlighted.²⁶⁻²⁸ Whether DCs have the potential to regulate the function of ILC2s in the pathogenesis of AR is unclear. There are no reports on effects of mDCs on ILC2s. Only one group reported that the activation of pDCs alleviates airway inflammation by suppressing ILC2 function and survival in a mouse model of asthma.²⁹ They also identified that the supernatant of human activated pDCs significantly suppress IL-5 and IL-13 production by ILC2s.²⁹ However, they did not investigate the effects of pDCs on ILC2s by directly culturing them together, especially not for the allergic diseases in human.

In this study, we investigated the effects of mDCs and pDCs on ILC2 function in patients with AR. A major effort of this study was directed to differentiating the relative roles of mDCs and pDCs on ILC2 function and identifying the mechanisms in these sequences. A particular focus of the study examined the expression of IL-33 in mDCs from AR patients after the antigen stimulation.

150

151 **METHODS**

152 The detailed methods are presented in the Methods section in this article's Online
153 Repository at www.jacionline.org.

154

155 **Subjects**

156 A total of 48 subjects with AR and 7 healthy subjects were enrolled. The inclusion and
157 exclusion criteria are presented in the Methods section in this article's Online
158 Repository at www.jacionline.org. Baseline characteristics and clinical parameters are
159 given in Table 1, and the numbers about the subjects for each experiment were
160 indicated in Table E1. The study was approved by the Ethics Committee of The First
161 Affiliated Hospital, Sun Yat-sen University, China and informed consent was obtained
162 from all subjects.

163

164 **Allergen challenge**

165 Seven patients with AR and 7 healthy subjects were sequentially challenged with an
166 inhaled saline-control (as basal control) and allergen of house dust mite (HDM)
167 extract (ALK, Horsholm, Denmark). A working concentration of HDM was
168 administered using paper disks placed in the inferior turbinate. Peripheral blood was
169 collected for the examination of IL-33⁺mDCs or ILC2s at 0.5 h after the challenge of
170 saline and allergen.

171 The peripheral blood before challenge was also used to isolate CD14⁺
172 monocytes for generation of mDCs, and mDCs were then co-cultured with autologous

peripheral blood mononuclear cells (PBMCs) which were collected 1 week later to identify the effects of mDCs on autologous ILC2s.

Generation of monocyte-derived DCs *in vitro*

The generation of monocyte derived DCs (mo-DCs) from monocytes was performed as described in our previous report.³⁰ The detailed methods are presented in the Methods section in this article's Online Repository at www.jacionline.org.

Statistical analyses

Differences between two groups were analyzed by the paired or unpaired t test for the data with normal distribution, and the Wilcoxon matched-pairs signed-rank test or Mann-Whitney *U* test was performed to compare the data with abnormal distribution. Three or more groups were compared using one-way analysis of variance (ANOVA) with Bonferroni *post hoc* test or Kruskal-Wallis test for repeated measurements. Correlations were analyzed with Spearman rank test. $P < .05$ were considered statistically significant. Analyses were performed using GraphPad Prism 6.0 software (GraphPad Software, La Jolla, CA, USA).

RESULTS

The expression of mDCs, pDCs, and ILC2s in the nasal mucosa of patients with AR

Previous studies mostly used flow cytometric approaches to identify ILC2s in the blood or peripheral tissues. Our aim was to provide an *in situ* presence of ILC2s, mDCs, and pDCs in the nasal mucosa of patients with AR by immunofluorescence

staining. Herein, we defined ILC2s as Lin⁻GATA3⁺ cells as done in previous study.³¹
 We observed the presence of ILC2s (Fig 1, A), CD11c⁺mDCs (Fig 1, B), and
 CD123⁺pDCs (Fig 1, C) in the nasal mucosa of patients with AR.

mDCs activated both allogeneic and autologous ILC2s in patients with AR

Mo-DCs were induced from CD14⁺ monocytes obtained from buffy coat *in vitro* as
 our previous report (Fig 2, A),³⁰ and here defined as mDCs as in previous studies.²⁴
 The PBMCs isolated from patients with AR were co-cultured with mDCs, and the
 effects of mDCs on ILC2 function by flow cytometry. Human blood ILC2s were
 defined as Lin⁻CD127⁺CRTH2⁺ as done in previous studies (Fig 2, B).^{9, 12} The
 administration of mDCs significantly upregulated the levels of IL-13⁺ILC2s ($P <$
 0.001) and IL-9⁺ILC2s ($P < 0.05$), which is similar to but with some less effects
 induced by the stimulation of IL-33 together with IL-2 (Fig 2, C and D). No
 difference was found for IL-5⁺ILC2 levels after the treatment of mDCs (data not
 shown). These results suggest that mDCs activated the allogeneic ILC2s by inducing
 the Th2 cytokine production in patients with AR.

We next investigated the effects of mDCs on the transcription factor and signaling
 pathways of ILC2s. GATA3 has been reported to be the key regulator of Th2 cytokine
 production profile of ILC2s.¹⁷ We found a significant increase in GATA3⁺ILC2 levels
 for PBMCs isolated from patients with AR after co-culturing with mDCs (Fig 2, E
 and F). In addition, the administration of mDCs significantly upregulated the levels of
 p-STAT3⁺ILC2s and p-STAT5⁺ILC2s but not p-STAT6⁺ILC2s (Fig 2, G).

To eliminate the possible disturbances of mixed lymphocyte reaction (MLR)
 under the allogeneic stimulation, we conducted the co-culture experiments using
 mDCs and their autologous PBMCs isolated from the same patients with AR or

healthy controls (HC). We found that the treatment of autologous mDCs increased the levels of IL-13⁺ILC2s in PBMCs obtained from the patients with AR and from healthy subjects ($P < 0.05$ or 0.01 , Fig 3, A). Similarly, higher levels of IL-9⁺ILC2s were also found for patients with AR but without statistical significance ($P = 0.0625$, Fig 3, B). There were higher levels of IL-13⁺ILC2s and IL-9⁺ILC2s in patients with AR compared to healthy controls after mDC treatment ($P < 0.05$ or 0.001), suggesting a higher responsiveness of ILC2s to mDCs in patients with AR compared to healthy subjects. Similarly, the levels of ILC2s were also significantly increased after the treatment of autologous mDCs for PBMCs from patients with AR ($P < 0.01$) but not healthy subjects (Fig 3, C), suggesting that mDCs might be involved in the proliferation of ILC2s. These findings suggest that autologous mDCs activate ILC2 function, which was consistent with the results in the allogeneic culture system above.

Next, we further confirmed our findings using freshly sorted ILC2s from the buffy coat preparations of human volunteers. Similarly, we observed that ILC2s produced higher levels of IL-9 and IL-13 after treatment with allogeneic mDCs ($P < 0.001$, Fig 4, A). Since the percentage of ILC2s were up-regulated after the treatment of mDCs (Fig 3, C), here we examined the effects of mDCs on ILC2 proliferation using carboxyfluorescein diacetate succinimidyl diester (CFSE) staining, and found that mDCs significantly promoted ILC2 proliferation ($P < 0.001$, Fig 4, B and C).

We also investigated the effects of mDCs on the surface markers of ILC2s (Fig E1). We found that mDCs upregulated TSLP receptor (TSLPR) expression ($P < 0.0001$), downregulated CD127 expression ($P < 0.01$) and had no effects on the IL-17RB and CRTH2.

Collectively, using autologous and allogeneous culture systems, we demonstrated that mDCs promoted ILC2 function especially for patients with AR.

mDCs activated the function of ILC2s by the IL-33/ST2 pathway

Previous studies demonstrated that ILC2s produce a remarkable level of Th2 cytokine in response to epithelial cytokines, especially IL-33.¹⁰ Recently, pleiotropic functions of IL-33 have emerged, with several reports supporting their role in ILC2s by binding to their receptor, ST2.^{32, 33} To determine the potential mechanism underlying the effects of mDCs on ILC2s, we first examined the expression of ST2 on circulating ILC2s in healthy subjects and patients with AR. Higher levels of ST2⁺ILC2s were found in patients with AR compared to healthy controls ($P < 0.05$, Fig 5, A). Next, we found that the administration of mDCs significantly up-regulated the ST2⁺ILC2 levels in PBMCs from patients with AR ($P < 0.05$, Fig 5, B), and there was higher intracellular IL-33 in Lin⁻CD45⁺HLA-DR⁺CD11c⁺ mDCs after the co-cultured with their autologous PBMCs ($P < 0.05$, Fig 5, C and D). The isotype was used as a specific control staining of IL-33 (Fig E2). We next investigated the IL-33 mRNA in mDCs using RT-qPCR. Because it is difficult to separate the PBMCs with DCs after co-culture, we then examined the IL-33 mRNA in mature DCs treated with lipopolysaccharide (LPS) for 2 days from day 5 or iDCs without the treatment of LPS. IL-33 mRNA in mDCs was found to be expressed at a 30-fold higher level than those in untreated DCs (iDC-D7) and further increased in mDCs generated from patients with AR ($P < 0.05$, Fig 5, E), which suggesting that mDCs from patients with AR acquired a stronger effects on ILC2s. Indeed, the techniques of flow cytometry and RT-qPCR could only examine the expression of the full-length IL-33 precursor. We next identified a higher level of IL-33 protein in the supernatants of mDCs than those

in iDC-D7 ($P < 0.05$, Fig 5, *F*). More importantly, a remarkable increase of IL-33 protein with 26-fold were observed after mDCs were co-cultured with sorted ILC2s ($P < 0.05$, Fig 5, *F*). These data suggest that mDCs may promote ILC2 activation via producing IL-33.

We further examined the role of IL-33/ST2 pathway in the effects of mDCs on ILC2s by blocking IL-33 function using soluble ST2 (sST2), a decoy receptor of IL-33. Strikingly, the enhanced expression of intracellular IL-13 and IL-9 in ILC2s for PBMCs from AR induced by mDCs was significantly blocked by the treatment of sST2 ($P < 0.05$ or 0.01 , Fig 5, *G* and *H*). More importantly, using sorted ILC2s, we further found that the treatment of sST2 significantly reversed the upregulation of mDCs on the production of IL-9 and IL-13 by sorted ILC2s ($P < 0.05$ or 0.01 , Fig 5, *I*). These findings suggest that mDCs activate the function of ILC2s by the IL-33/ST2 pathway, and the effects can be reversed by sST2.

High expression of IL-33 in mDCs after the challenge of inhaled allergen in patients with AR

The above findings indicate that the IL-33/ST2 axis plays an important role in the crosstalk between mDCs and ILC2s in AR. To further verify this hypothesis, we examined the levels of IL-33⁺mDCs and ILC2s in patients with AR and healthy subjects at 0.5 h after challenge with an inhaled saline (for one side of inferior nasal turbinate, to eliminate the possible disturbances of non-allergen factors as basal line), and then allergen of HDM (for another side of inferior nasal turbinate of same subject). After allergen challenge, IL-33⁺mDCs markedly increased in patients with AR ($P < 0.05$), but not in healthy controls, and with higher levels in patients with AR compared to healthy subjects ($P < 0.05$, Fig 6, *A* and *B*). Similarly, allergen inhalation

significantly increased the blood ILC2 percentages in patients with AR compared to saline challenge ($P < 0.05$, Fig 6, C). We also investigated the levels of plasma IL-33 in patients with AR and healthy subjects after allergen inhalation but did not find any differences (Fig 6, D). Additionally, patients with AR exhibited a higher VAS score after allergen challenge compared to saline challenge ($P < 0.05$, Fig 6, E). We also investigated the correlations between IL-33⁺mDCs and ILC2s or VAS scores (Fig 6, F), and between ILC2s and VAS scores in patients with AR after allergen challenge (Fig E3). However, no significant correlations were observed. In contrast to mDCs, pDCs have been known to exhibit significant roles in controlling and suppressing the Th2 immune response in allergic diseases.^{21, 22} Here we did not observe any difference in the Lin⁻HLA⁻DR⁺CD123⁺pDCs levels in the blood after allergen inhalation from patients with AR (Fig 6, G). However, there was a positive correlation between the ratio of IL-33⁺mDCs/pDCs and VAS score with a certain trend toward statistical significance ($P = 0.1192$, Fig 6, H). It suggests the possible involvement of the balance of these two subsets of DCs in the pathology of AR. Taken together, these data demonstrate increased levels of IL-33⁺mDC and ILC2s after allergen inhalation in patients of AR, further indicating an important role of mDCs on ILC2s in the pathogenesis of allergic diseases.

pDCs suppressed the cytokine production of ILC2s isolated from patients with AR

We supposed that pDCs play the role of suppressing ILC2 function in allergic diseases. pDCs are principally characterized by the expression of Toll-like receptor (TLR)-7 and -9, and they were functional after TLR ligand stimulation.³⁴ Here, human pDCs were sorted from the PBMCs from the buffy coat of human volunteers as

Lin⁻HLA⁻DR⁺CD123⁺ by flow cytometry as shown in Fig 7, A. Purified pDCs were activated with R848, the TLR7/8 agonist, for 2 days. PBMCs isolated from patients with AR, which received the stimulation of IL-33 plus IL-2, were further co-cultured with pDCs for an additional 3 days. The percentages of IL-13⁺ILC2s increased under the stimulation of IL-33 plus IL-2 ($P < 0.001$), but was significantly reversed by resting pDCs ($P < 0.05$, Fig 7, B and C). Moreover, pDCs activated by R848 exhibited more inhibitive effects on IL-13⁺ILC2s ($P < 0.01$). To further confirm the above findings, we reconduted the experiment using sorted ILC2s which were from same donor as pDCs. Activated pDCs significantly inhibited IL-13 and IL-9 production from autologous sorted ILC2s ($P < 0.05$ or 0.01 , Fig 7, D). Additionally, both resting pDCs and activated DCs suppressed the production of IL-5 from activated ILC2s ($P < 0.01$, Fig E4).

To further investigate the potential mechanism of the effects of pDCs on ILC2s, we measured the cytokines of IL-6, IL-10, and IFN- α produced by pDCs. We found that activated pDCs produced higher levels of IL-6, more than 10-fold, than resting pDCs (Fig 7, E), suggesting an important role of IL-6 in the effects of activated pDCs on ILC2s. We only observed little increase for IFN- α in the activated pDCs (Fig E4), and IL-10 levels were not detected. We further observed a slight increase but without significant difference for the IL-6 receptor (IL-6R) expression in ILC2s after IL-33 stimulation (Fig E5). Next, we addressed the role of IL-6 in the effect of pDCs to inhibit ILC2 function using exogenous recombinant human IL-6 and IL-6-neutralizing antibodies. Sorted ILC2s were stimulated with IL-2 and IL-33 in the presence of IL-6 for 3 days and co-cultured with their autologous pDCs. As expected, the administration of IL-6 inhibited the production of IL-13 ($P = 0.0625$) and IL-9 by

activated ILC2s ($P < 0.01$, Fig 7, *F*). More importantly, the treatment of neutralizing anti-IL-6 led to a significant reversal in levels of IL-9 and IL-13 produced from sorted ILC2s compared to activated pDCs ($P < 0.05$, Fig 7, *G*).

Additionally, previous studies showed that pDCs promote the generation of FOXP3⁺Treg cells through programmed cell death 1 (PD-1)/PD-1 ligand 1 (PD-L1) axis in human subjects.³⁵ Using flow cytometry, we found high levels of PD-L1 in pDCs but with no change after their activation (Fig E4). Using autologous ILC2s, the treatment of PD-L1 inhibitor significantly reversed the suppression of IL-9 ($P < 0.05$) but not IL-13 and IL-5 production by both resting and activated pDCs (Fig E4), suggesting that PD-1/PD-L1 axis might be partially involved in the suppressive effects of pDCs on IL-9 production by ILC2.

DISCUSSION

In this study, we investigated the interplay between DCs and ILC2s in patients with AR. We identified that mDCs activated human ILC2 function through the IL-33/ST2 pathway, and promoted the proliferation of ILC2s. By contrast, activated pDCs suppressed the cytokine production of ILC2s through IL-6. This study sheds new light on the immune regulation of mDCs and pDCs and mechanisms underlying the regulation of ILC2s in patients with allergic diseases.

ILC2s were reported to be involved in the allergic inflammation by producing large amounts of type 2 cytokines under the stimulation of epithelium-derived cytokines. We and other groups reported the increased peripheral ILC2s in AR patients during the grass pollen season³⁶ after a challenge with cat antigen,³⁷ or in HDM-sensitized patients with AR.^{13, 38} Importantly, we reported that peripheral ILC2 levels in patients with AR correlated positively with the severity of the clinical VAS

score, and stimulation with epithelium-derived cytokines or specific antigen induced significantly greater production of Th2 cytokines in PBMCs isolated from patients with AR¹³ or those with asthma and AR.^{9, 14} However, previous studies mostly used flow cytometry to identify ILC2s in the blood, sputum or sometimes in the tissues. Until now, only two groups have provided an *in situ* characterization of ILC2s in the skin of the patients with systemic sclerosis³⁹ and atopic dermatitis and psoriasis.³¹ In our study, we provided the evidence of ILC2s *in situ* in the nasal mucosa of patients with AR by immunofluorescence staining. To our knowledge, it was the first report demonstrating the *in situ* presence of ILC2s in the human airway mucosa of allergic patients. We also confirmed the presence of mDCs and pDCs in the nasal mucosa of patients with AR, suggesting the possibility of the interplay between mDCs or pDCs with ILC2s in the nasal mucosa.

mDCs are reported to be responsible for the initiation and progression of allergic inflammation.²⁰ Recently, it was demonstrated that human CD14⁺DCs in Crohn's ileum induced differentiation of ILC1s to ILC3s, which gives evidence that mDCs can act on ILCs.²⁴ Thus far, no study examined the effects of mDCs on ILC2s both in animals or in human. In our study, we induced mature mo-DCs from human CD14⁺ monocytes. Since it is technically difficult to isolate mDCs from humans, and many of previous reports generally studied mDC-mediated immune responses using mo-DCs,^{35, 40, 41} we defined mo-DCs as mDCs in our study. We found that PBMCs isolated from subjects with AR exhibited higher IL-9⁺ILC2 and IL-13⁺ILC2 levels in response to allogenic mDCs *in vitro*. Considering the T cells response to mDCs, we confirmed the above results using isolated ILC2s, and further identified that mDCs promoted ILC2 proliferation. To exclude the effects caused by MLR under the allogeneic stimulation, we repeated the co-culture experiments using mDCs and their

autologous PBMCs. Similarly, the treatment of autologous mDCs significantly increased the levels of IL-13⁺ILC2s and IL-9⁺ILC2s in PBMCs obtained from the patients with AR, which further provided strong evidence that mDCs promote ILC2 function in patients with AR. Additionally, we identified that mDCs upregulated the expression of GATA3 in ILC2s, a key transcription factor for ILC2 development, function, and activation.¹⁷ We previously reported that signaling pathways of p-STAT3, p-STAT5, and p-STAT6 were involved in the activation of ILC2s.⁹ Here, we also found that mDCs increased the levels of p-STAT3 and p-STAT5 in ILC2s. Furthermore, using CFSE and flow cytometry, we identified that mDCs promoted ILC2 proliferation. Taken together, our data provide strong evidence that mDCs promote the activation and proliferation of ILC2s, especially in allergic diseases. This suggests that mDCs can be a bridge to link the innate immune system with the adaptive immune system.

DCs were reported to promote the polarization between ILC1s and ILC3s²⁴. In our study, we found that there were higher levels of IFN- γ in the supernatant after PBMCs from AR with the treatment of mDCs (data not shown). We also found an increased percentage of ILC1s and IFN- γ ⁺ILC1s in PBMCs from buffy coat after co-cultured with allogeneic mDCs (data not shown), suggesting the effects of mDCs to promote ILC1 function. Furthermore, LPS was reported to promote iDCs to differentiate to more like mDC1s which mostly promote Th1 response on T cells. Similarly, we observed an increased expression of CD141 on mDCs (mDC1s) and some decreased expression of CD1c⁺ on mDCs (mDC2s) under the treatment of LPS. Meanwhile, we also identified that mDCs in our study promote PBMCs to produce

more Th1 cytokine IFN- γ . Previous studies reported that mDCs treated with LPS exhibited strong effects not only to promote Th1 response on T cells, but also to promote some Th2 response⁴²⁻⁴⁴. These and our findings suggest that mDCs may promote the polarization of both Th1 (Th1 cells or ILC1s) and Th2 (Th2 cells or ILC2s) responses, indicating even both stimulation but with some balance between Th1 response and Th2 response, or some stronger in different environments. Our study focused on the effects of DCs on ILC2s, and the effects of mDCs on ILC1s or the effects of the subtypes of mDCs on ILC2s should be further investigated in the future.

We next identified that IL-33/ST2 axis is the possible mechanistic pathway directing the effects of mDCs on ILC2s. We found that after co-cultured, mDCs expressed more IL-33 both in the levels of mRNA and protein, and ILC2s expressed more ST2. More importantly, soluble ST2 significantly blocked the effects of mDCs on the production of Th2 cytokines by ILC2s using PBMCs from AR and sorted ILC2s. The above findings suggest that mDCs act on ILC2s *via* the IL-33/ST2 axis. Then how about the levels of IL-33⁺mDCs in the patients of AR? We next found that allergen inhalation upregulated the peripheral IL-33⁺mDCs and ILC2s levels in HDM-positive patients with AR but not in the healthy subjects, further suggesting the important role of IL-33⁺mDCs and ILC2s especially under the condition of allergen stimulation. We observed the increase of VAS scores in patients with AR after the allergen inhalation. Unfortunately, we did not observe significant correlation between IL-33⁺mDCs with ILC2s levels or VAS scores. However, a positive correlation was found between the ratio of IL-33⁺mDCs/pDCs and VAS score, suggesting the possible involvement of the correlation in IL-33⁺mDCs/pDCs/ILC2s in the pathology of

allergic rhinitis. Of course, several reasons may result in the no correlations between IL-33⁺mDCs and ILC2s or VAS scores in our study, such as a small sample size of patients or the variation of intracellular cytokine evaluation under flow cytometry. Generally, our findings shed a light on the possible pathogenesis in AR ruled by the interplay of IL-33⁺mDCs and ILC2s.

It was previously reported that pDCs exert protective effects by preventing allergen induced airway inflammation.²² In our study, using PBMCs from patients with AR or sorted ILC2s, we identified that activated pDCs inhibited the cytokine production of ILC2s from patients with AR by producing high levels of IL-6, and neutralizing IL-6 can reverse the above effects of pDCs, suggesting activated pDCs inhibit ILC2 function via producing IL-6. One recent study reported that pDCs had suppressive effects on ILC2s in murine models of allergic asthma.²⁹ The authors also identified that the supernatant of human activated pDCs significantly suppressed IL-5 and IL-13 production by ILC2s. However, they did not investigate the effects of pDCs on ILC2s by directly culturing them together, especially not for the allergic diseases in humans. In addition, we only observed minimal increase for IFN- α after the activation of pDCs, suggesting that IFN- α may not be the main mechanism for the effects of pDCs on ILC2s under conditions of our study. Moreover, using PD-L1 inhibitor, we identified that PD-1/PD-L1 axis might be partially involved in the suppressive effects of pDCs on IL-9 production by ILC2. We think that the results of our study are the first to demonstrate the effects of mDCs and pDCs on ILC2s especially in AR patients.

We acknowledge that there are some limitations of this study. Although we showed that mDCs can activate ILC2 function and pDCs can contribute to inhibit

ILC2 function, these were performed by *in vitro* experiments. Detailed roles of mDCs and pDCs on ILC2s in the pathogenesis of allergic airway inflammation should be further determined *in vivo* using the tissues from the patients and/or animal models. Additionally, we did not include more experiments about the subtypes of mDCs (mDC1s/mDC2s), the effects of mDC1s/mDC2s on ILC2s, and the effects of mDCs on Th1 response including ILC1s in ILC2 systems.

In conclusion, we have shown that mDCs activate ILC2 function for patients with AR via IL-33/ST2 axis. We observed the increase of IL-33⁺mDCs and ILC2s in the blood of patients with AR after an allergen challenge. Activated pDCs downregulate the cytokine production of ILC2s through IL-6 production. Additionally, we provided evidence of the presence of mDCs, pDCs, and ILC2s in the nasal mucosa of patients with AR. Our findings provide a new understanding of the effects of mDCs and pDCs on ILC2s and ILC2-mediated allergic pathogenesis.

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Table 1 The characteristics of the subjects' demographics, IgE and VAS involved in the study.

Characteristic	Healthy subjects	Allergic rhinitis	
	Challenge	Challenge	Blood collection directly
No. of patients	7	7	41
Age (y)	24.57 ± 0.69	28.86 ± 1.08	29.20 ± 1.65
Gender, female/male	5/2	2/5	24/17
SPT , positive/subjects tested	0 (0%)	7 (100%)	41 (100%)
tIgE concentration (IU/mL)	60.11 ± 16.77	171.9 ± 71.16	112.4 ± 9.53**
sIgE concentration (IU/mL)		53.40 ± 26.40***	44.26 ± 10.73****
Der p/Der f	< 0.35	< 0.35	0.63 ± 0.26
House dust	< 0.35	< 0.35	< 0.35
Trees	< 0.35	< 0.35	< 0.35
Artemisia /ragweed	< 0.35	< 0.35	< 0.35
Molds	< 0.35	< 0.35	< 0.35
Hair or dander of pets	< 0.35	< 0.35	< 0.35
	< 0.35	< 0.35	< 0.35
	< 0.35	< 0.35	< 0.35
VAS	0	10.71 ± 3.93*	24.29 ± 1.64****

Data are presented as mean ± SEM. Mann-Whitney *U* tests were used to test for significant differences between patient groups. Statistically significant differences were defined as p values **P* < .05, ** *P* < .01, *** *P* < .001, **** *P* < .0001 compared to healthy subjects. *sIgE*, specific immunoglobulin E; *SPT*, skin prick tests; *tIgE*, total immunoglobulin E; *VAS*, visual analogue scale.

Figure Legends

FIG 1. The expression of ILC2s, mDCs, and pDCs in the nasal mucosa of patients with allergic rhinitis. ILC2s (A), mDCs (B), and pDCs (C) were stained by immunofluorescence. ILC2s were designated as Lin⁻GATA⁺ cells, mDCs as CD11⁺ cells, and pDCs as CD123⁺ cells. ILC2, group 2 innate lymphoid cell; mDC, myeloid dendritic cell; pDC: plasmacytoid DC.

FIG 2. mDCs promote the function of allogeneic ILC2s in patients with allergic rhinitis. A, The induction of monocyte-derived dendritic cells (moDCs) and here as mDCs. PBMCs from patients with AR were co-cultured with allogeneic mDCs (B-G). B, Gating strategy of human ILC2s with Lin⁻CRTH2⁺CD127⁺. Intracellular IL-13 (C, n = 9) and IL-9 (D, n = 3) levels, expression of GATA3 (E-F, n = 3), and the levels of p-STAT3, p-STAT5, and p-STAT6 (G, n = 4) in ILC2s were analyzed by flow cytometry. Data are shown as mean ± SEM. **P* < .05, ***P* < .01, ****P* < .001 and *****P* < .0001. AR: allergic rhinitis; GM-CSF: granulocyte-macrophage colony-stimulating factor; ILC2, group 2 innate lymphoid cell; mDC, myeloid dendritic cell; MFI: geometric mean fluorescence intensity; PBMC, peripheral blood mononuclear cell.

FIG 3. mDCs promote the function of autologous ILC2s in patients with allergic rhinitis. PBMCs isolated from healthy control or patients with AR (n = 5) were co-cultured with their autologous mDCs. Intracellular IL-13 (A) and IL-9 (B) levels in ILC2s were determined by flow cytometry. C, The percentage of ILC2s in PBMCs. Data are shown as mean ± SEM. **P* < 0.05, ***P* < 0.01 and *****P* < 0.0001. AR,

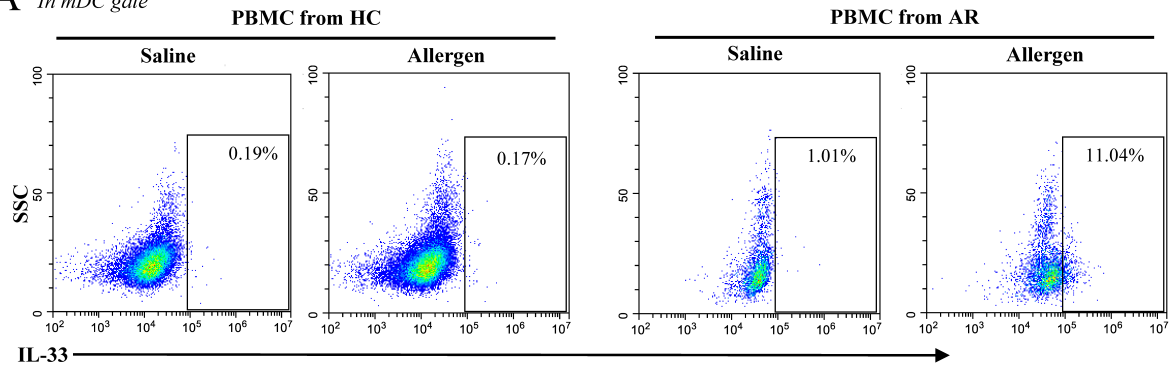
Allergic rhinitis; HC, Healthy control; ILC2, group 2 innate lymphoid cell; mDC, myeloid dendritic cell; PBMC, peripheral blood mononuclear cell.

FIG 4. mDCs promote the function and cell proliferation of sorted ILC2s. Sorted ILC2s from the buffy coat of human volunteers were cultured with allogeneic mDCs for 3 days. **A**, The levels of IL-13 and IL-9 in the supernatants (n = 7). **B-C**, Proliferation of CFSE-labeled ILC2s. Data are shown as mean $\pm$ SEM (n = 3 - 6). *** $P < .001$. CFSE: carboxyfluorescein diacetate succinimidyl diester; ILC2, group 2 innate lymphoid cell; mDC, myeloid dendritic cell.

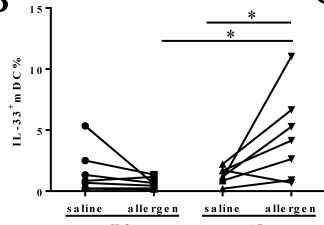
FIG 5. mDCs activate the ILC2 function via IL-33/ST2 pathway. **A**, The levels of ST2⁺ILC2s in the blood of healthy controls (n = 3) and patients with AR (n = 5). mDCs were co-cultured with allogeneic (B) or autologous (C, D) PBMCs from patients with AR. **B**, ST2⁺ILC2s determined by flow cytometry (n = 4). **C-D**, IL-33⁺mDC levels (n = 4). **E**, iDCs were stimulated with (mDCs) or without lipopolysaccharide (iDC-D7) for an additional 2 days, and the levels of IL-33 mRNA were examined by RT-qPCR (n = 5). **F**, The levels of IL-33 in the supernatants of iDC-D7, mDCs and mDCs co-cultured with allogeneic sorted ILC2s (n = 4). PBMCs from patients with AR (G-H) or sorted ILC2s (I) were pretreated with sST2 and co-cultured with allogeneic mDCs. **G-H**, Intracellular IL-9 and IL-13 levels in ILC2s (n = 6 - 8 each). **I**, The levels of IL-9 and IL-13 in the supernatants (n = 5). Data are shown as mean $\pm$ SEM. * $P < 0.05$ and ** $P < 0.01$. AR: allergic rhinitis; ILC2, group 2 innate lymphoid cell; mDC, myeloid dendritic cell; PBMC, peripheral blood mononuclear cell; sST2: soluble ST2.

FIG 6. High levels of IL-33⁺mDCs after a challenge of inhaled allergen in patients with allergic rhinitis. Healthy subjects and patients with AR were sequentially challenged with an inhaled saline-control and then allergen (n = 7 for each group), and the blood was collected at 0.5 h. The levels of IL-33⁺mDCs (**A-B**), ILC2s (**C**), the level of IL-33 in the serum (**D**) and pDCs with Lin⁻HLA⁻DR⁺CD123⁺ (**G**). **E**, The VAS scores. The correlation between the levels of IL-33⁺mDCs with ILC2s or VAS scores (**F**), and IL-33⁺mDCs%/pDC% with VAS scores (**H**) in patients with AR after allergen challenge. **P* < .05. AR, Allergic rhinitis; HC, Healthy control; mDC, myeloid dendritic cell; PBMC, peripheral blood mononuclear cell; pDCs: plasmacytoid DC; VAS, Visual analog scale.

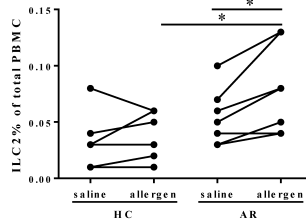
FIG 7. pDCs suppress the function of ILC2s isolated from patients with AR. A, The progress of the gating of pDCs with Lin⁻HLA⁻DR⁺CD123⁺. PBMCs from patients with AR (**B-C**) or autologous sorted ILC2s (**D, F-G**) stimulated with IL-2 and IL-33 were co-cultured with pDCs stimulated with R848 for an additional 3 days. **B-C**, Intracellular IL-13 in ILC2s (n = 5). **D**, The levels of IL-13 and IL-9 in the supernatant (n = 5 - 7 each). **E**, The levels of IL-6 in the supernatants of pDCs (n = 3). **F-G**, The level of IL-13 and IL-9 in the supernatants of sorted ILC2s (n = 5). Data are shown as mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001. AR, allergic rhinitis; FACS, fluorescence-activated cell sorting. ILC2, group 2 innate lymphoid cell; pDCs: plasmacytoid DC.

A *In mDC gate*

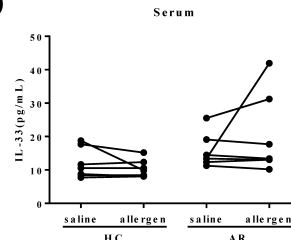
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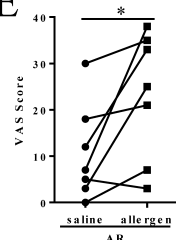
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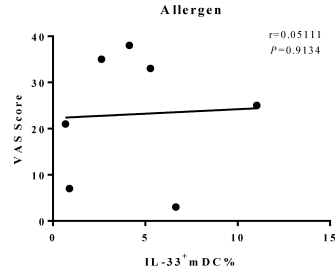
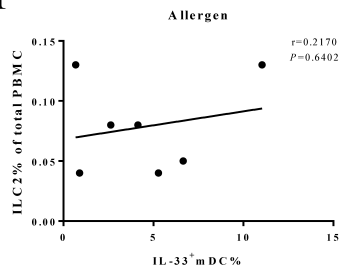
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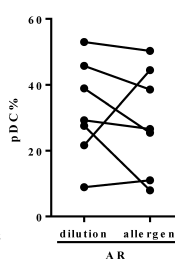
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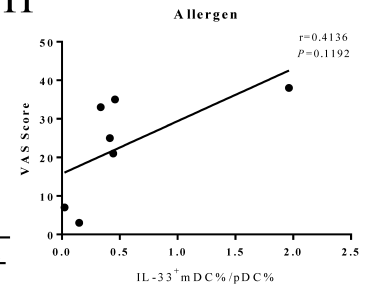
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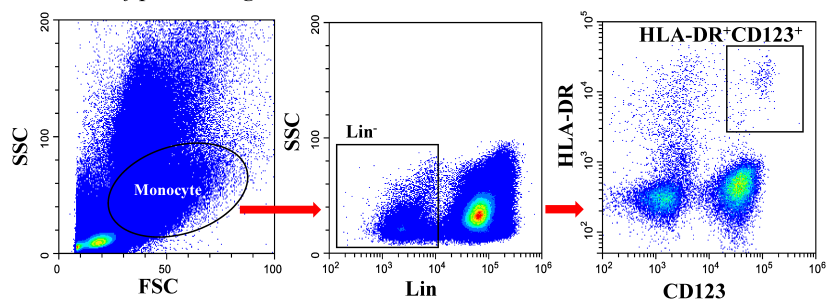
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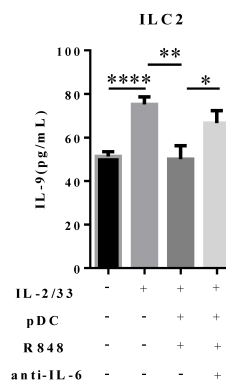
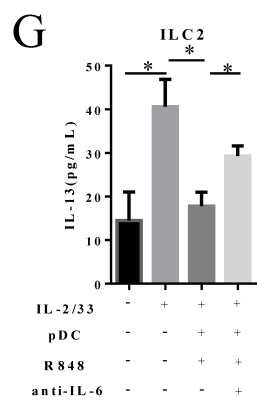
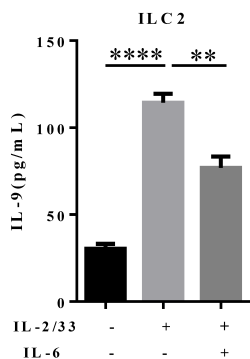
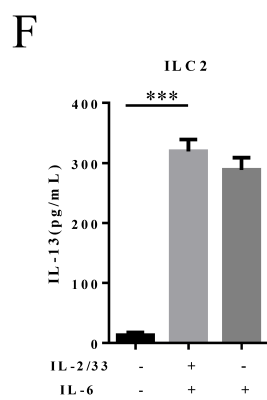
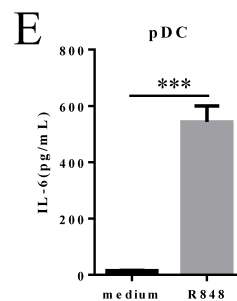
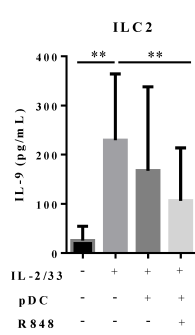
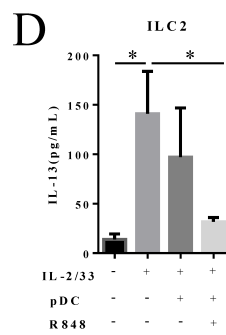
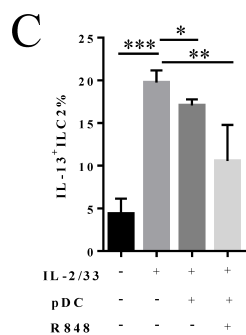
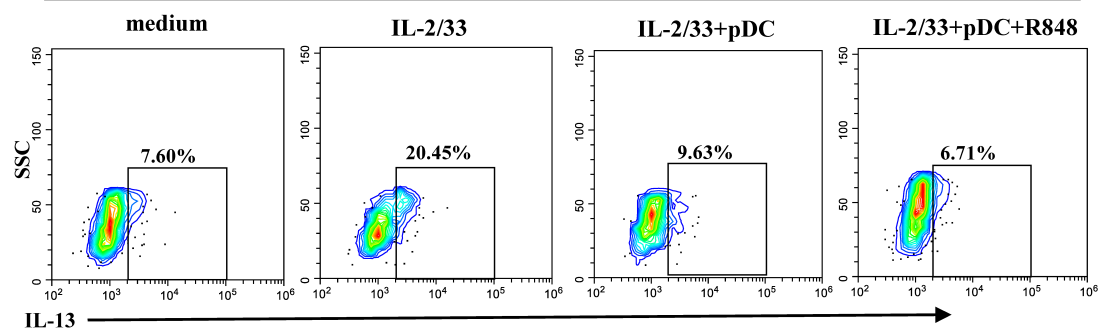


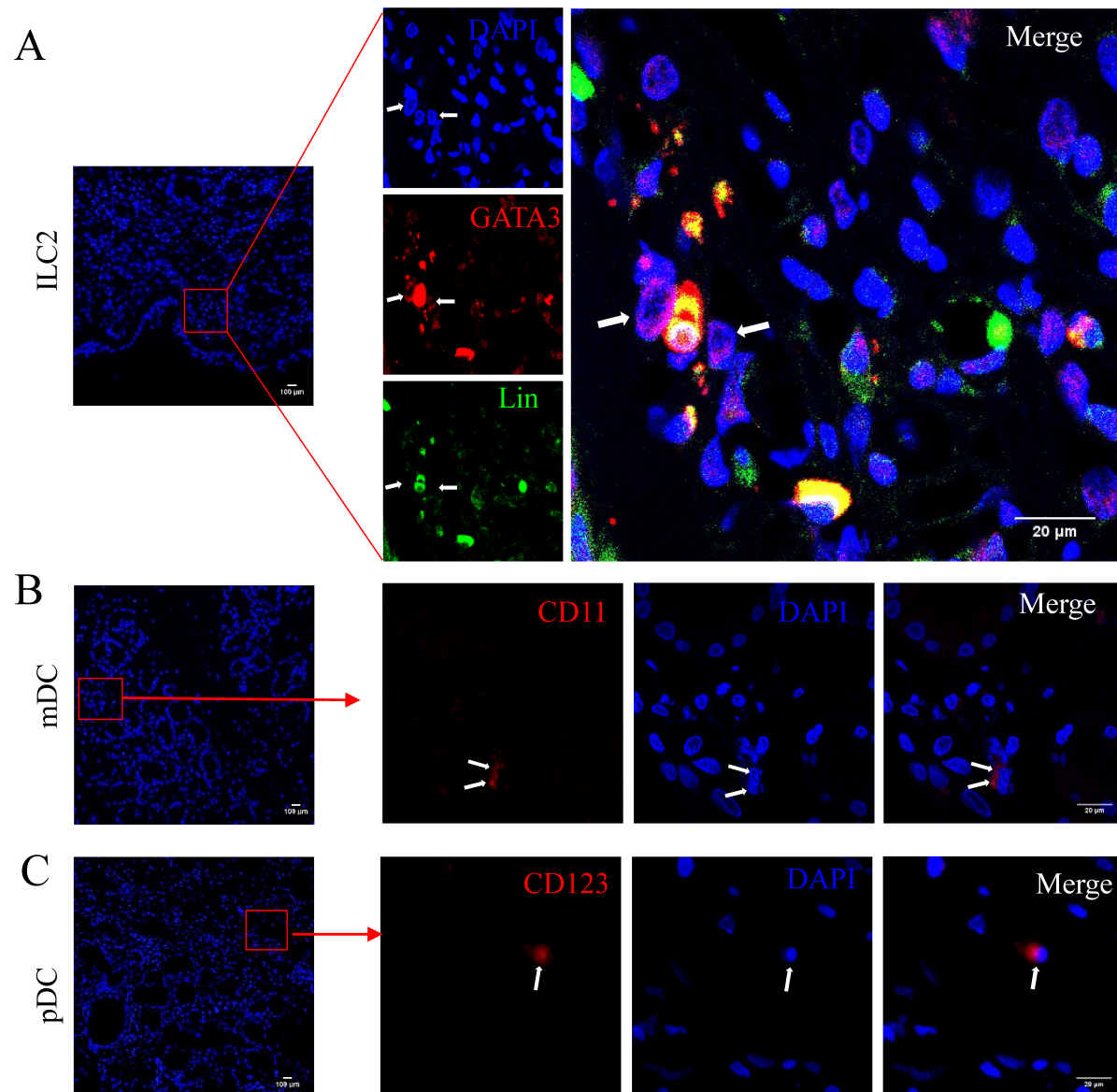
A The isolation of pDC using FACS

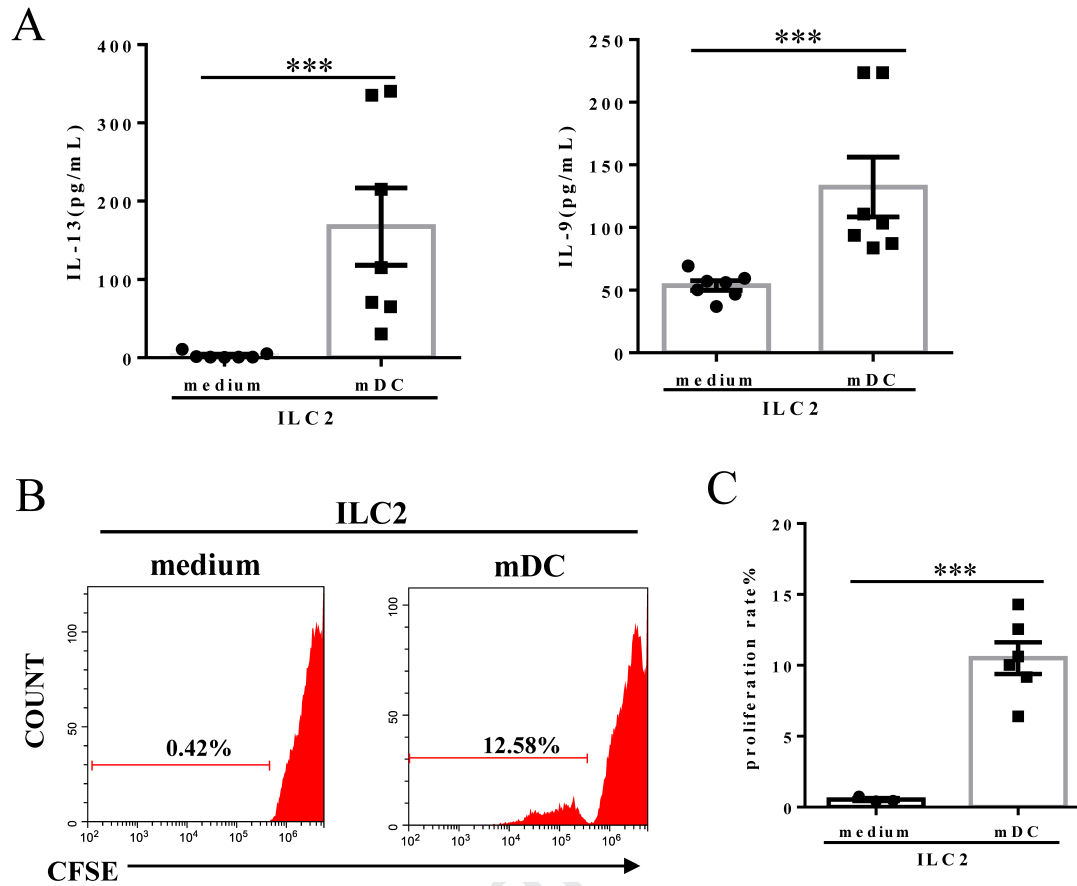


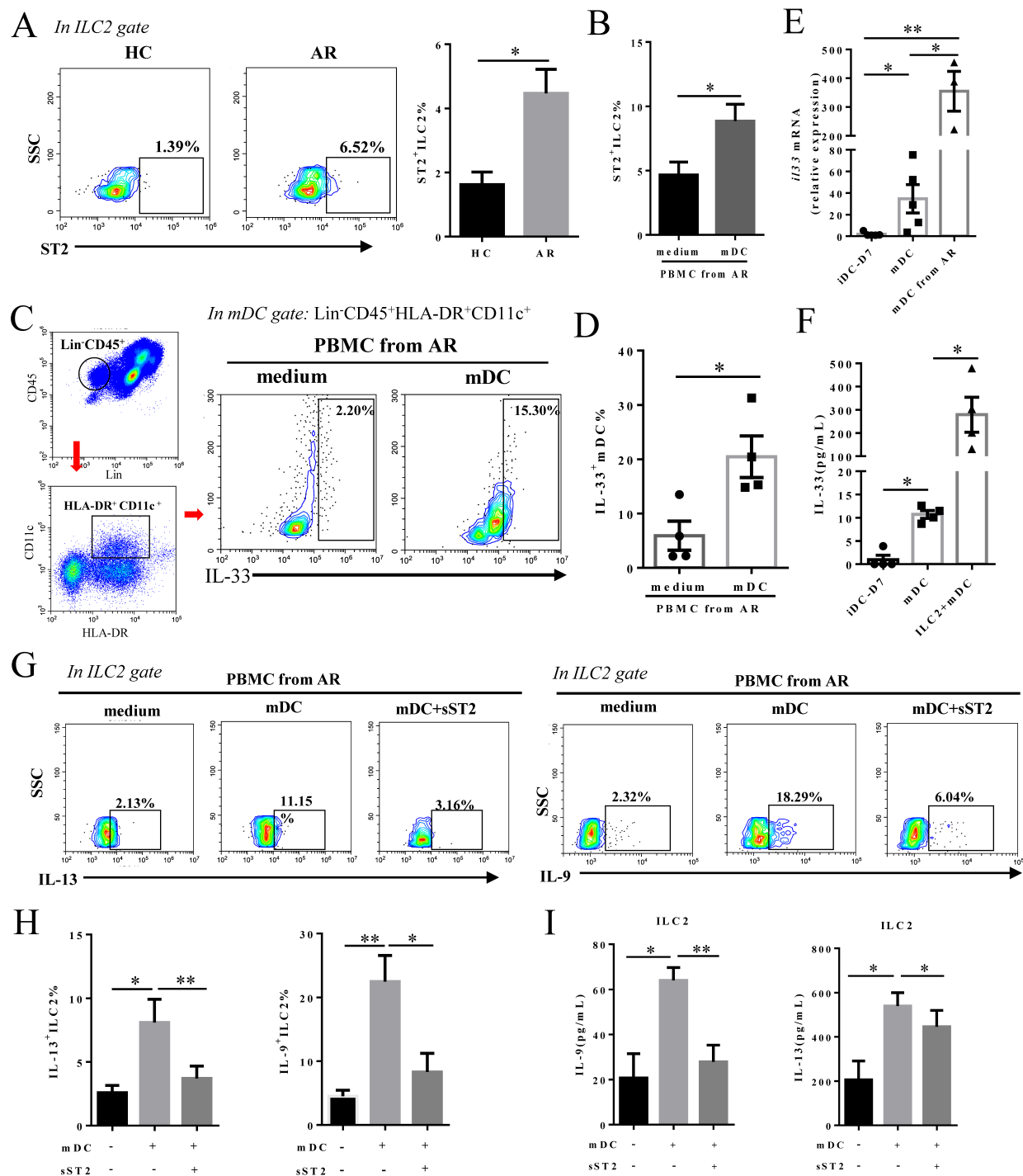
B In ILC2 gate

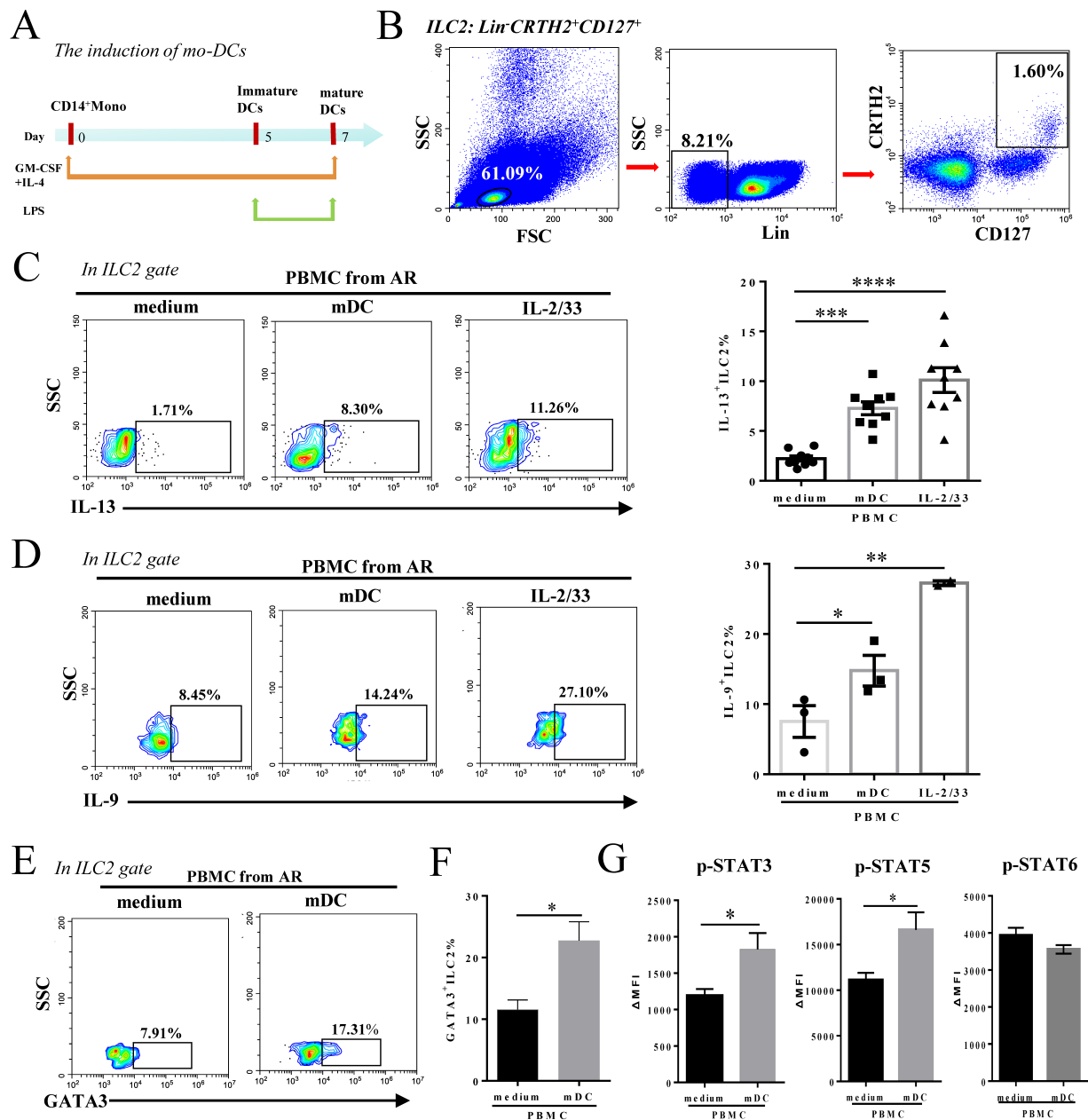
PBMC from AR











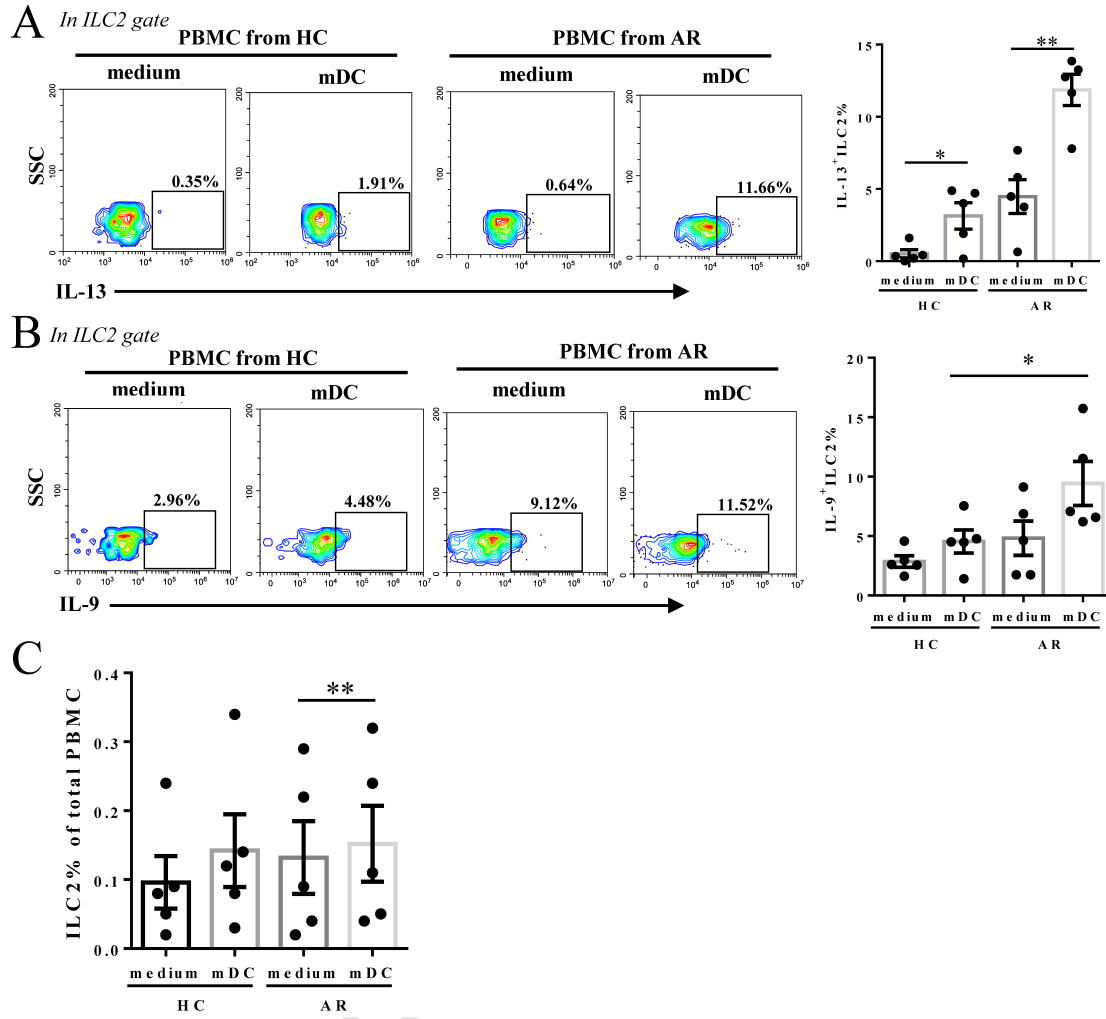


Table E1 Numbers of the patients with AR and healthy subjects used in this study

		Inhalation challenge		PBMC+mDC							PBMC+pDC		Total	
				Autologous		Allogeneic								
		IL-33 ⁺ mDC /pDC/ILC2	ST2 ⁺ ILC2	IL-9/13 ⁺ ILC2	IL-33 ⁺ mDC	IL-13 ⁺ ILC2	IL-9 ⁺ ILC2	GATA3 ⁺ ILC2	STAT pathway	ST2 ⁺ ILC2	IL-9/13 ⁺ ILC2	IL-13 ⁺ ILC2	IL-6R ⁺ ILC2	
Healthy subjects		7*	3*	5*										7
Patients	with AR	7 [€]	5 [€]	5 [€]	4 [€]	9	3	3	4	4	8	5	5	48

* and [€]: The subjects were shared. The serum from the subjects was used for cytokine evaluation. AR: allergic rhinitis; ILC2s: group 2 innate lymphoid cells; IL-6R: IL-6 receptor; mDCs: myeloid dendritic cells; PBMCs: peripheral blood mononuclear cells; pDCs: plasmacytoid dendritic cells.

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SUPPLEMENTARY METHODS

Subjects

A total of 48 subjects with allergic rhinitis (AR) and 7 healthy subject (healthy controls, HC) were enrolled (Table 1). The number about the subjects for each experiments were indicated in Table E1. The inclusion criteria for patients with AR were established according to the criteria of the Initiative on Allergic Rhinitis and its Impact on Asthma (ARIA): (1) history of nasal symptoms by nose itching, obstruction, sneezing, and rhinorrhea; (2) positive specific immunoglobulin E (sIgE)¹. All patients with AR were positive for Der p/Der f on the basis of the positive skin prick test (SPT) result and specific IgE tests (sIgE) to Der p/Der f (> 0.35 IU/mL, Pharmacia CAP system, Pharmacia Diagnostics, Uppsala, Sweden). Seven healthy nonatopic controls (with both a negative skin prick test and a specific IgE test) were also enrolled. The subjects were excluded if pregnant, current smokers, or ex-smokers with more than 10 pack-years. No subject used oral or nasal glucocorticoids or other treatment (e.g., antihistamines or immunotherapy) for 6 weeks before the study. The visual analog scale (VAS) scores were evaluated for the symptoms of the subjects. The study was approved by the Ethics Committee of The First Affiliated Hospital, Sun Yat-sen University, China. Human blood buffy coats from “anonymous donors” were from Guangzhou Blood Center; exemption of written informed consent was approved.

Allergen challenge

A total of 7 patients with house dust mite (HDM)-induced AR and 7 healthy subjects underwent a nasal allergen challenge using HDM extract (ALK, Horsholm,

Denmark). The saline solution has been administered as the basal control to eliminate the effects caused by a nonspecific reaction to the physical stimulation itself. In details, a saline paper disk was placed on the anterior end of left inferior turbinate for 5 min. Peripheral blood was collected for the examination of IL-33⁺ myeloid DCs (mDCs) or group 2 innate lymphoid cells (ILC2s) at the 0.5 h after the saline challenge. At 5 min later, a working concentration of allergen was administered by using paper disks placed on the anterior end of right inferior turbinate for 5 min, and the titration of allergen was determined by the results of SPT of each individual. After 30 min, peripheral blood was collected for the IL-33⁺mDC or ILC2 evaluation. VAS scores and clinical observations were assessed at serial intervals after saline or allergen challenges in all study subjects. After the nasal allergen challenge, the subjects with clear symptoms were given histamine H1 receptor antagonist to relieve their symptoms.

The peripheral blood before challenge was also used to isolate CD14⁺ monocytes for generation of mDCs, and mDCs were then co-cultured with autologous peripheral blood mononuclear cells (PBMCs) which were collected 1 week later to identify the effects of mDCs on autologous ILC2s. The peripheral blood was also collected from these subjects before the challenge for the examination of ST2⁺ILC2s.

Isolation of peripheral blood mononuclear cells

PBMCs from healthy subjects, patients with AR, subjects accepted challenge or buffy coat from “anonymous donors” were isolated by density centrifugation with Ficoll-Paque Plus (MP Biomedicals, Santa Ana, CA, USA). Levels of IL-33⁺mDCs,

plasmacytoid dendritic cells (pDCs), ILC2s and ST2⁺ILC2s were determined by flow cytometry, or PBMCs were co-cultured with mDCs or pDCs.

Generation of monocyte-derived DCs *in vitro*

As our previous study², CD14⁺ monocytes from PBMCs in the buffy coat “anonymous donors” provided by Guangzhou Blood Center were isolated using the MACS CD14 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and were stimulated with recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF; 50 ng/mL; PeproTech Inc., Rocky Hill, NJ, USA) and IL-4 (10 ng/mL; R&D systems, Minneapolis, MN, USA) for 5 days. The cells were then stimulated with lipopolysaccharide (LPS; 100 ng/mL; Sigma-Aldrich, St Louis, MO, USA) for an additional 2 days to be mature DCs (Fig 2, A). Immature DCs (iDCs) treated with or without LPS, or mDCs from patients with AR were used for RT-qPCR of IL-33.

Isolation of ILC2s and pDCs

Because of the relatively low levels of ILC2s and pDCs in the peripheral blood and considering the ethical factors for the human beings, ILC2s and pDCs were isolated from the PBMCs obtained in human buffy coat preparations from the volunteers provided by Guangzhou Blood Center (n = 48). ILC2s were co-cultured with their autologous pDCs from the same donor. ILC2s were sorted using the ILC2 Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instruction. Briefly, Lin⁺ cells were depleted from PBMCs by using biotin antibody cocktail and antibiotin MicroBeads (Cocktail of biotin-conjugated monoclonal antibodies against CD2, CD3, CD11b, CD14, CD15, CD16, CD19, CD56, CD123, and CD235a) and LS columns (Miltenyi Biotec, Bergisch Gladbach, Germany).

Pre-enriched Lin⁻ cells were additionally labeled with CD294 (CRTH2)-PE, as a primary labeling reagent, and with anti-PE MicroBeads, as a secondary labeling reagent, and they were isolated by positive selection over MS columns as ILC2s.

For isolating pDCs, PBMCs were stained with the antibodies against human lineage markers (CD2, CD3, CD14, CD16, CD19, CD56, and CD235a; eBioscience, San Diego, CA, USA), HLA-DR (BD Pharmingen, Bergen, NJ, USA), and CD123 (eBioscience, San Diego, CA, USA). pDCs were further sorted as Lin⁻HLA-DR⁺CD123⁺ cells on a FACS Aria instrument (Beckman Coulter, Brea, CA, USA) (FIG 7A).

The purity of sorted human ILC2s and pDCs was determined to be more than 95%.

Co-culture experiments of DCs with PBMCs or ILC2s

Human mDCs and pDCs were washed, and then were co-cultured with allogeneic or autologous PBMCs isolated from patients with AR (1:10) or healthy subjects or freshly sorted ILC2s (1:20) for 3 days. In some experiments, PBMCs or ILC2s were treated with the recombinant human ST2/IL-33R Fc chimera protein (0.1 µg/mL; R&D systems, Minneapolis, MN, USA) for 30 min before the co-culture. In some experiments, PBMCs or ILC2s were stimulated with recombinant human (rh)IL-2 (20 U/mL; PeproTech Inc., Rocky Hill, NJ, USA) and rhIL-33 (10 ng/mL; PeproTech Inc., Rocky Hill, NJ, USA) as a positive control. pDCs were stimulated with R848 (the agonist of TLR7/8, 20 µg/mL; InvivoGen, Nunningen, Switzerland) for 48 h, washed and then co-cultured with PBMCs isolated from patients with AR or ILC2s stimulated with rhIL-2 and rhIL-33 for an additional 3 days. In some experiments, ILC2s were stimulated with rhIL-6 (2 µg/ml; PeproTech Inc., Rocky Hill, NJ, USA) along with

the addition of rhIL-2 and rhIL-33. pDCs stimulated with or without R848 for 48 h were washed, and co-cultured with their autologous ILC2s in the presence of anti-IL-6-neutralizing antibody (5 µg/mL; R&D systems, Minneapolis, MN, USA) or PD-L1 inhibitor (1 µM; AbMole BioScience, Houston, TX, USA) for 3 days.

The culture supernatants were collected for the analysis of the cytokine levels by ELISA, and the PBMCs were analyzed for IL-13⁺ILC2s, IL-9⁺ILC2s, ST2⁺ILC2s, GATA3⁺ILC2s, p-STAT3⁺ILC2s, p-STAT5⁺ILC2s, and p-STAT6⁺ILC2s by flow cytometry. In some experiments, the mDCs were examined for IL-33⁺mDCs.

Flow cytometry

PBMCs were stained with the following specific mAbs: FITC-conjugated lineage cocktail: anti-CD2 (RPA-2.10); anti-CD3 (OKT3); anti-CD14 (61D3); anti-CD16 (CB16); anti-CD19 (HIB19); anti-CD56 (TULY56); anti-CD235a (HIR2); PE-conjugated anti-CRTH2 (BM16); PE-Cy7-conjugated anti-CD127 (eBioRDR5; all from eBioscience, San Diego, CA, USA); polyclonal mAbs APC-conjugated anti-ST2 (R&D systems, Minneapolis, MN, USA) and PE-Cy7-conjugated anti-IL-6Rα (UV4; BioLegend, San Diego, CA, USA), for ILC2 evaluation. mDCs and pDCs were assessed using the antibodies of lineage markers, eFluor 450-conjugated anti-CD45 (2D1; eBioscience, San Diego, CA, USA), APC-H7-conjugated anti-HLA-DR (G46-6; BD Pharmingen, Bergen, NJ, USA), PE-conjugated anti-CD11c (BU15), PE-CY7-conjugated anti-CD123 (6H6), eFluor 450-conjugated anti-PD-L1 (MIH1; all from eBioscience, San Diego, CA, USA), PE-Cy7-conjugated anti-CD1c (L161) and APC-conjugated anti-CD141 (M80; both from BioLegend, San Diego, CA, USA).

For intracellular cytokine detection, PBMCs or mDCs were stimulated with the cell stimulation cocktail (plus protein transport inhibitors) consisting of phorbol

12-myristate 13-acetate (PMA), ionomycin, brefeldin A, and monensin (eBioscience, San Diego, CA, USA) for 5 h. Thereafter, the cells were fixed, permeabilized with intracellular fixation and permeabilization buffer set (eBioscience, San Diego, CA, USA), and stained for intracellular IL-13 (eFluor 450-conjugated anti-IL-13; eBio13A; eBioscience, San Diego, CA, USA), IL-9 (Alexa Fluor 647-conjugated anti-IL-9; MH9A3; BD Pharmingen, Bergen, NJ, USA), or IL-33 (PE-conjugated anti-IL-33; 390412; R&D systems, Minneapolis, MN, USA).

PBMCs were processed with the FoxP3/Transcription Factor Staining Buffer Set (eBioscience, San Diego, CA, USA) before staining with antibodies of Brilliant Violet 421-conjugated anti-GATA-3 (16E10A23; BioLegend, San Diego, CA, USA), eFluor 450-conjugated anti-p-STAT3 (LUVNKLA), APC-conjugated anti-p-STAT5 (SRBCZX), or APC-conjugated anti-p-STAT6 (CHI2S4N; all from eBioscience, San Diego, CA, USA) for the evaluation of their levels in ILC2s.

ILC2 proliferation assay

For the analysis of cell division, sorted ILC2s stained with carboxyfluorescein diacetate succinimidyl diester (CFSE; Invitrogen/Molecular Probes, Eugene, OR, USA) were co-cultured with mDCs for 3 days, and the proliferation rate was evaluated on a CytoFLEX S flow cytometer; data were analyzed with CytExpert (Beckman Coulter, Brea, CA, USA).

ELISA

The cell supernatants were analyzed by using IL-13 and IL-5 ELISA kits (Invitrogen, Waltham, MA, USA), IL-9, IL-10 and IFN- α ELISA kits (NeoBioscience, Shenzhen,

Guangdong, China), IL-6 ELISA kit (R&D systems, Minneapolis, MN, USA) and IL-33 High Sensitivity ELISA kit (Multi Sciences, Hangzhou, Zhejiang, China), according to the manufacturer's instruction.

Immunofluorescence for mDCs, pDCs, and ILC2s

Inferior turbinate mucosal samples were collected from patients with HDM-sensitive AR and nasal septal deviation undergoing septoplasty, which study was approved by The Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, and conducted with written informed consent from every patient as those in our previous study³. Immunofluorescence staining for mDCs (CD11c⁺ cells)⁴, pDCs (CD123⁺ cells),⁵ and ILC2s (Lin⁻ GATA3⁺ cells)⁶ was performed using the following antibodies of mouse anti-human CD11c (1:100), mouse antihuman CD123 (1:100), FITC-conjugated lineage cocktails (1:50; all from eBioscience, San Diego, CA, USA), and rabbit anti-human GATA3 (1:250; Abcam, Cambridge, UK). Thereafter, CF543-conjugated goat anti-rabbit (1:300; Biotium, Fremont, CA, USA), AF546-conjugated goat anti-mouse and AF488-conjugated goat anti-mouse (1:300; Invitrogen, Waltham, MA, USA) antibodies were used as secondary antibodies. Images were acquired and analyzed using the confocal microscope Zeiss LSM 710 and ZEN 2.3 lite software (Carl Zeiss Microscopy GmbH, Jena, Germany).

RT-qPCR

Total RNA was isolated from mDCs with RNAiso Plus reagent and 5X PrimeScript™ RT Master Mix kit (all from TaKaRa, Shiga, Kusatsu, Japan) was used for converting 1 µg of total RNA to first strand cDNA following the manufacturer's instructions. The

quantitative PCR of IL-33 (sense primers, 5'-ATGGTAACCCTGAGTCCTACAAA-3', and reverse primer, 5'-ATGAAACACAGTTGG AGTGCATA-3') was performed by using the FastStart Universal SYBR Green Master kit (Roche, Mannheim, Germany). β -actin (sense primers, 5'-AGAGCTACGAGCTGCCTGAC-3', and reverse primer, 5'-AGCACTGTGTTGGCGTACAG-3') was used as an endogenous reference. The PCR was performed as 10 min' initial denaturation at 95 °C, 40 cycles consisted of 10 s at 95 °C and 30 s at 60 °C carried out on the CFX96™ Real-Time PCR cycler (Bio-Rad, Hercules, CA, USA). Expression of target gene was expressed as fold increase relative to the expression of β -actin. The mean value of the replicates for each sample was calculated and expressed as cycle threshold (Ct). The amount of gene expression was then calculated as the difference (Δ Ct) between the Ct value of target gene and the Ct value of β -actin. Fold changes in target gene mRNA were determined as $2^{-\Delta\text{Ct}}$.

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FIG E legends

FIG E1. The expression of surface markers of ILC2s after treatment with mDCs.

PBMCs isolated from patients with AR were co-cultured with mDCs for 3 days. A, TSLPR⁺ILC2%; B, IL-17RB⁺ILC2%; C, CD127⁺% in Lin⁻ cells and CRTH2⁺% in Lin⁻ cells. n = 7 for each group. TSLPR: thymic stromal lymphopoietin receptor. ****P** < .01 and ******P** < .0001. AR, allergic rhinitis; ILC2, group 2 innate lymphoid cell; mDC, myeloid dendritic cell; PBMC, peripheral blood mononuclear cell.

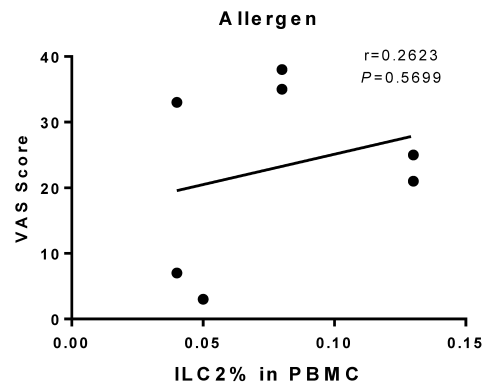
FIG E2. Specific control staining of IL-33 using isotype.

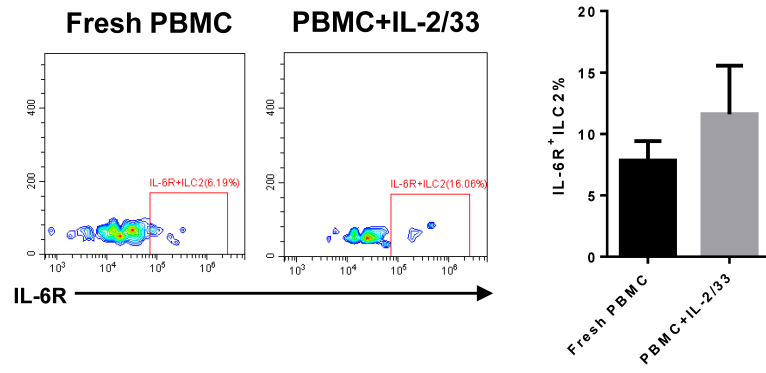
PBMCs isolated from patients with allergic rhinitis were co-cultured with autologous mDCs for 3 days. Representative histogram of IL-33 staining in mDCs, pre-gated in Lin⁻CD45⁺HLA-DR⁺CD11c⁺ cells. mDC, myeloid dendritic cell; PBMC, peripheral blood mononuclear cell.

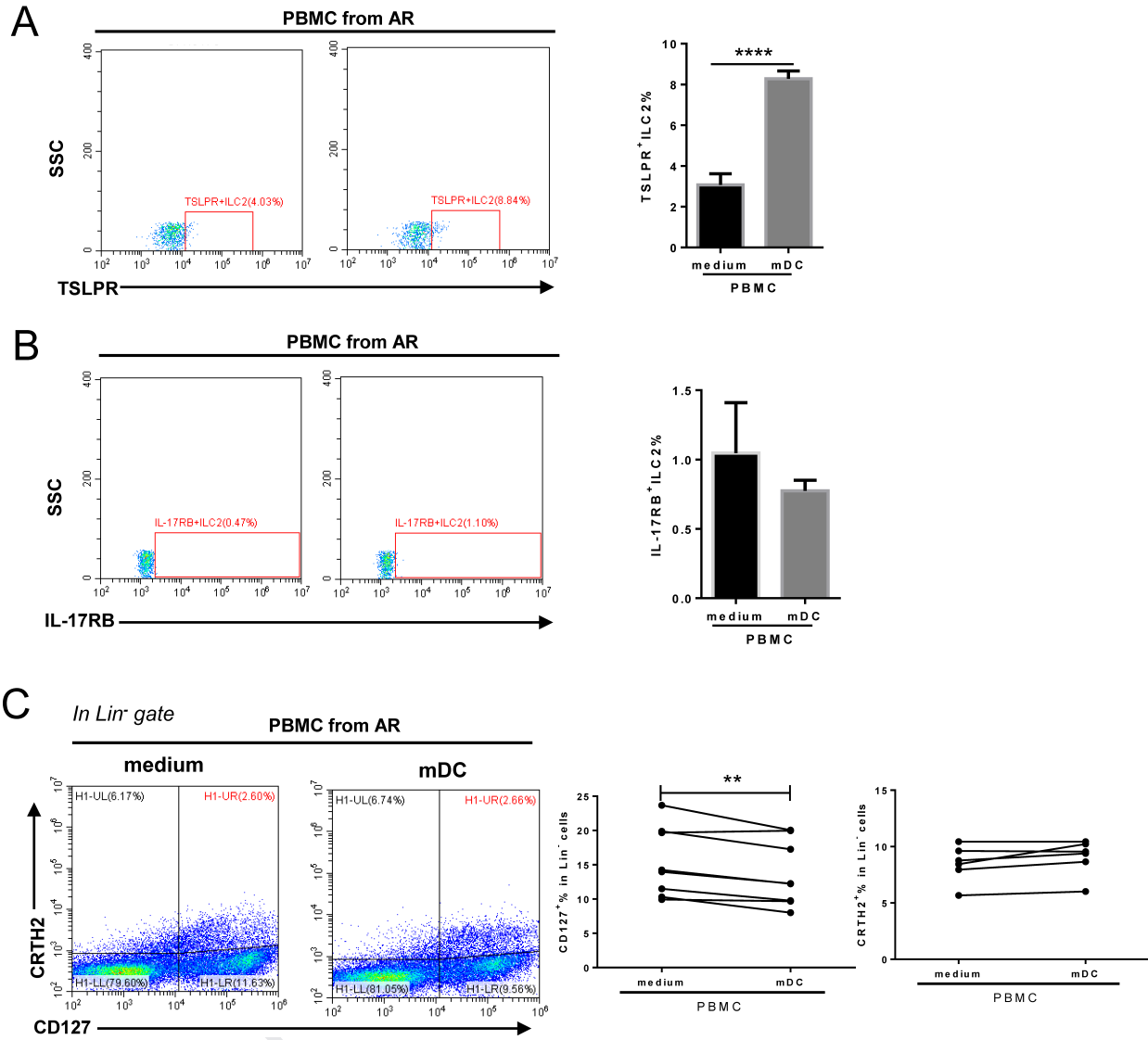
FIG E3. The correlation between ILC2 levels with VAS scores after allergen challenge. The parameters were evaluated at 0.5 h after challenge with inhaled allergen in patients with allergic rhinitis (n = 7). ILC2, group 2 innate lymphoid cell; PBMC, peripheral blood mononuclear cell; VAS, Visual analogue scale.

FIG E4. The role of PD-1/PD-L1 in the effects of pDCs on ILC2s. A, pDCs sorted from the buffy coat of human volunteers were stimulated with Toll-like receptor agonist, R848, for 48 h, and the levels of IFN- α in the supernatant were determined (n = 9). B, pDCs were stimulated with R848, and the MFI of PD-L1 in pDCs were measured by flow cytometry (n = 3). C-D, pDCs were stimulated with R848 for 48 h, washed, and then co-cultured with their autologous ILC2s with or without the presence of PD-L1 inhibitor for 3 days. The IL-13, IL-9 and IL-5 levels in the supernatant were examined (n = 4 - 7). IFN- α , interferon- α ; ILC2, group 2 innate lymphoid cell; pDCs, plasmacytoid dendritic cells; PD-L1, PD-1 ligand 1.

FIG E5. IL-6R expression in ILC2s in the patients with allergic rhinitis. PBMCs from patients with AR were stimulated with IL-33 plus IL-2, and IL-6R⁺ ILC2s were determined by flow cytometry. IL-6R⁺ cells were gated in ILC2s (n = 3 - 5). IL-6R, IL-6 receptor; ILC2, group 2 innate lymphoid cell; PBMC, peripheral blood mononuclear cell.







In gate of mDC

