Enhanced innate type 2 immune response in peripheral blood from patients with asthma

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Background: In mice, group 2 innate lymphoid cells (ILC2s) likely mediate helminth immunity, inflammation, and tissue repair and remodeling. However, the involvement of ILC2s in human diseases, such as asthma, is not well understood.
Objectives: The goals of this study were to investigate whether peripheral blood specimens can be used to monitor innate type 2 immunity in human subjects and to examine whether ILC2s are involved in human asthma.
Methods: PBMCs from subjects with allergic asthma (AA), subjects with allergic rhinitis (AR), or healthy control (HC) subjects were cultured *in vitro* with IL-25 or IL-33. Flow cytometry and cell sorting were used to identify, isolate, and quantitate ILC2s in PBMCs.
Results: Human PBMCs produced IL-5 and IL-13 when stimulated with IL-33 or IL-25 in the presence of IL-2 without antigens. In addition, IL-7 or thymic stromal lymphopoietin were able to replace IL-2. The cell population with phenotypic ILC2 characteristics, lineage$^-$CD127$^+$CRTH2$^+$ cells, responded to IL-33 and produced large quantities of IL-5 and IL-13 but undetectable levels of IL-4. PBMCs from subjects with AA produced significantly larger amounts of IL-5 and IL-13 in response to IL-25 or IL-33 than from subjects with AR or HC. The prevalence of ILC2s in blood was greater in the AA group than in the AR group or the HC group.
Conclusions: Innate type 2 immune responses are increased in asthma but not in AR, suggesting potential differences in the immunopathogenesis of these diseases. Peripheral blood is useful for evaluating innate type 2 immunity in humans. (J Allergy Clin Immunol 2014;134:671-8.)

Key words: *IL-33, IL-25, IL-5, IL-13, ILC2, innate immunity, asthma, allergy*

Abbreviations used
AA: Allergic asthma
AR: Allergic rhinitis
FITC: Fluorescein isothiocyanate
ILC2s: Group 2 innate lymphoid cells
HC: Healthy control
Lin$^-$: Lineage-negative
Lin$^+$: Lineage-positive
SNP: Single nucleotide polymorphism
TSLP: Thymic stromal lymphopoietin

Asthma is a chronic inflammatory disease of the respiratory tract that is characterized by airway inflammation, remodeling, hyperresponsiveness, and reversible obstruction. Asthma affects more than 300 million people worldwide and causes substantial medical and financial burden in the United States.[1] Both genetic and environmental factors are likely involved in asthma pathogenesis.[2] Genomewide association studies show an association between asthma and single nucleotide polymorphisms (SNPs) in several genes, including *IL1RL1*, *IL18R*, *IL33*, *TSLP*, and *RORA*.[3,4]

CD4$^+$ T_H2 cells have a significant role in asthma.[5-7] Indeed, type 2 cytokines produced by T_H2 cells, such as IL-4, IL-5, and IL-13, drive many features of asthma, including IgE class switch, airway remodeling, airway hyperresponsiveness, and mucus overproduction. In addition, type 2 cytokines recruit, maintain, and activate eosinophils.[8-10] However, the mechanisms involved in the development of antigen-specific T_H2 cells, as well as those involved in the ongoing production of type 2 cytokines in asthmatic airways, are poorly understood.

Over the past decade, rapid progress has been made in our understanding of mechanisms involved in the development of type 2 immunity. First, 3 cytokines, thymic stromal lymphopoietin (TSLP), IL-25, and IL-33, have emerged as strong candidates for the development of type 2 immunity, acting as the link(s) between innate recognition of pathogens and the resulting type 2 adaptive immune response.[11,12] Airway epithelial cells, as well as other tissues and immune cells, produce and release these cytokines on the activation of Toll-like receptors as well as via other as yet undefined mechanisms.[12-14] Second, an emerging family of innate lymphoid cells is recognized for its crucial role in mucosal immunity, tissue repair, and remodeling.[15,16] Group 2 innate lymphoid cells (ILC2s) are a newly recognized subset of the innate lymphoid cell family and have been gaining attention in several scientific fields. ILC2s are identified in mesenteric fat-associated lymphoid clusters, gut-associated lymphoid tissue, mesenteric lymph nodes, spleen, liver, and lungs of mice.[17-23] Murine ILC2s are characterized by ST2 expression (the IL-33 receptor, also known as IL1RL1), require RORα and IL-7Rα for their development,[16,24] and rapidly produce IL-5 and IL-13 in response to IL-25 or IL-33.[25]

These epithelium-derived cytokines and ILC2s are involved in several aspects of lung pathology in mouse models of airway inflammation and immunity. For example, ILC2s mediate airway

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hyperresponsiveness, airway remodeling, and homeostasis after influenza virus infection.[21,22] Intranasal IL-33 or IL-25 administration causes eosinophilic airway inflammation in mice in the absence of T cells.[23,26,27] Indeed, in the lungs of mice exposed to the protease papain or the natural aeroallergen *Alternaria alternata*, IL-33–responsive ILC2s provide the primary early cellular source of IL-5 and IL-13.[23,28] Furthermore, in a conventional ovalbumin-induced adaptive immune response model, ILC2s as well as $CD4^+$ T cells likely produce IL-13.[26] Despite increasing knowledge regarding the roles of ILC2s in mice, our knowledge is limited about this novel cell type in human diseases.

In humans, ILC2s have been shown to accumulate at the site of eosinophilic inflammation, including nasal polyps and sinus mucosa in subjects with chronic rhinosinusitis, skin lesions in subjects with atopic dermatitis, and pleural effusions from subjects with spontaneous pneumothorax.[29-32] Here, to extend this investigation and to examine whether innate type 2 immune response and/or ILC2s can be used as a biomarker of human diseases, we performed a prospective study. We addressed 2 specific questions: First, because blood specimens are more widely available than tissue specimens in clinical studies, we evaluated whether the innate type 2 response can be observed and whether ILC2s can be identified in peripheral blood. Second, we examined whether ILC2s are involved in allergic diseases in humans. We report that human PBMCs produce type 2 cytokines, IL-5 and IL-13, but not IL-4, on stimulation with IL-25 or IL-33 in the absence of antigens. We verified that $CD3^+$ cells are not required for the response and that lineage-negative (Lin^-) $CD127^+CRTH2^+$ cells in PBMCs robustly produce these cytokines. Innate type 2 responses were enhanced and ILC2s were increased in subjects with allergic asthma (AA) than in normal control subjects. Intriguingly, increased innate type 2 responses were not observed in subjects with allergic rhinitis (AR), suggesting potential differences in the immunopathogenesis of AA and AR.

METHODS

Study subjects

Peripheral blood was obtained from normal control subjects (healthy control [HC]), subjects with AR, or subjects with AA outside of the allergy season. Inclusion criteria for AA were as follows: (1) a prebronchodilator FEV_1 of more than 60% of predicted normal with a postbronchodilator increase in flow of more than 12% and/or a reduction in FEV_1 of more than 20% after 1 to 5 breaths of 25 mg/dL inhaled methacholine, (2) physician-diagnosed asthma, and (3) allergic sensitization to aeroallergens confirmed by new or existing skin testing or IgE serum immunoassays. The Mayo Clinic Institutional Review Board approved this study, and informed consent was obtained from all participants. Peripheral blood from normal individuals and allergic donors was used in the experiments shown in Figs 1 and 3. The study populations shown in Figs 2 and 4 are described in detail in the legends. (See additional descriptions in the Methods section in this article's Online Repository at www.jacionline.org.)

Reagents

Fluorescein isothiocyanate (FITC)-labeled antibodies to CD3 (SK7), CD14 (MϕP9), CD16 (NKP15), CD19 (4G7), and CD56 (NCAM16.2), peridinin chlorophyll protein complex-Cy5.5–labeled antibody to CD44 (G44-26), phycoerythrin-labeled antibody to IL-5, and AF647-labeled antibodies to CD127 (HIL-7R-M21) and CRTH2 (BM16) were purchased from BD Biosciences (San Jose, Calif). (See additional descriptions in the Methods section in this article's Online Repository.)

PBMC isolation and stimulation for cytokine production

Heparinized peripheral blood was layered over an equal volume of Histopaque 1077 (Sigma-Aldrich; St Louis, Mo) and centrifuged per the manufacturer's instructions. Mononuclear cells were collected from the interface between Histopaque and plasma. PBMCs were washed and resuspended in RPMI 1640 media (Gibco/Life Technologies; Grand Island, NY) containing 10% heat-inactivated human AB serum, penicillin (100 U/mL), and streptomycin (100 μg/mL) (Gibco/Life Technologies). Cells were cultured in 96-well round-bottom plates ($1.3\text{-}3.3 \times 10^6$ cells/mL) at 37°C with 5% CO_2 for 7 days with IL-33 or IL-25 at the indicated concentrations in the presence or absence of IL-2 (20 U/mL), IL-7 (10 ng/mL), or TSLP (10 ng/mL). IL-5, IL-13, and IFN-γ concentrations in cell-free supernatants were determined using ELISA kits as recommended by the manufacturer (Thermo Fisher Scientific; Rockford, Ill). (See additional description in the Methods section in this article's Online Repository.)

FACS sorting and human peripheral blood ILC2 culture

PBMCs were stained with antibodies to lineage markers (CD3, CD14, CD16, CD19, CD56, and FcεRIα) and antibodies to CD127 and CRTH2. After staining, cells were washed and fixed with 1% paraformaldehyde and analyzed using flow cytometry. For sorting experiments, Lin^- cells from PBMCs of subjects with AA were enriched by depleting lineage-positive (Lin^+) cells using FITC-conjugated antibodies to CD3, CD14, CD16, CD19, CD56, and FcεR1α, along with the EasySep FITC selection kit (StemCell Technologies, Vancouver, British Columbia, Canada) as per manufacturer's instructions. This enrichment process produced approximately 50% pure Lin^- cells. Lin^--enriched cells were stained with AF647-conjugated anti-CRTH2 and phycoerythrin-Cy7–conjugated anti-CD127 or appropriate isotype-matched controls and sorted using a FACSAria (BD Biosciences) into Lin^+ and Lin^- populations. Lin^- cells were further sorted into 3 populations on the basis of CRTH2 and CD127 expression, resulting in $Lin^-CD127^-CRTH2^-$, $Lin^-CD127^+CRTH2^+$, and $Lin^-CD127^+CRTH2^-$ populations. Cells were cultured at $4\text{-}10 \times 10^4$ cells/mL in 96-well tissue culture plates. (See additional descriptions in the Methods section in this article's Online Repository.)

Statistical analyses

Data are presented as the mean ± SEM for the number of subjects or experiments as indicated. Statistics were performed using paired and unpaired Student *t* tests, 1-way ANOVA, or repeated-measures ANOVA as appropriate for each set of experimental conditions. $P < .05$ was considered statistically significant.

RESULTS

IL-33 and IL-25 stimulate type 2 cytokine production by PBMCs without antigens

In vitro stimulation of murine tissue cells (adipose tissues, lung, and bone marrow) with IL-33 results in IL-5 and IL-13 production and ILC2s are predominantly responsible for the response.[17,22,23,28,33] ILC2s were also identified in human peripheral blood.[29] To determine whether human peripheral blood, in particular PBMCs, displays similar antigen-independent type 2 cytokine responses, we cultured PBMCs with IL-33 in the presence or absence of IL-2. No antigen was added to the culture. When cultured with IL-33 alone, PBMCs produced minimal amounts of IL-5 (Fig 1, *A*, left panel). IL-2 alone induced modest IL-5 production. When combined with IL-2, IL-33 induced significant IL-5 production in a concentration-dependent manner ($P < .01$). In addition, IL-33 alone induced minimal IL-13 production (Fig 1, *A*, right panel). In the presence of IL-2, IL-33 induced concentration-dependent production of IL-13.

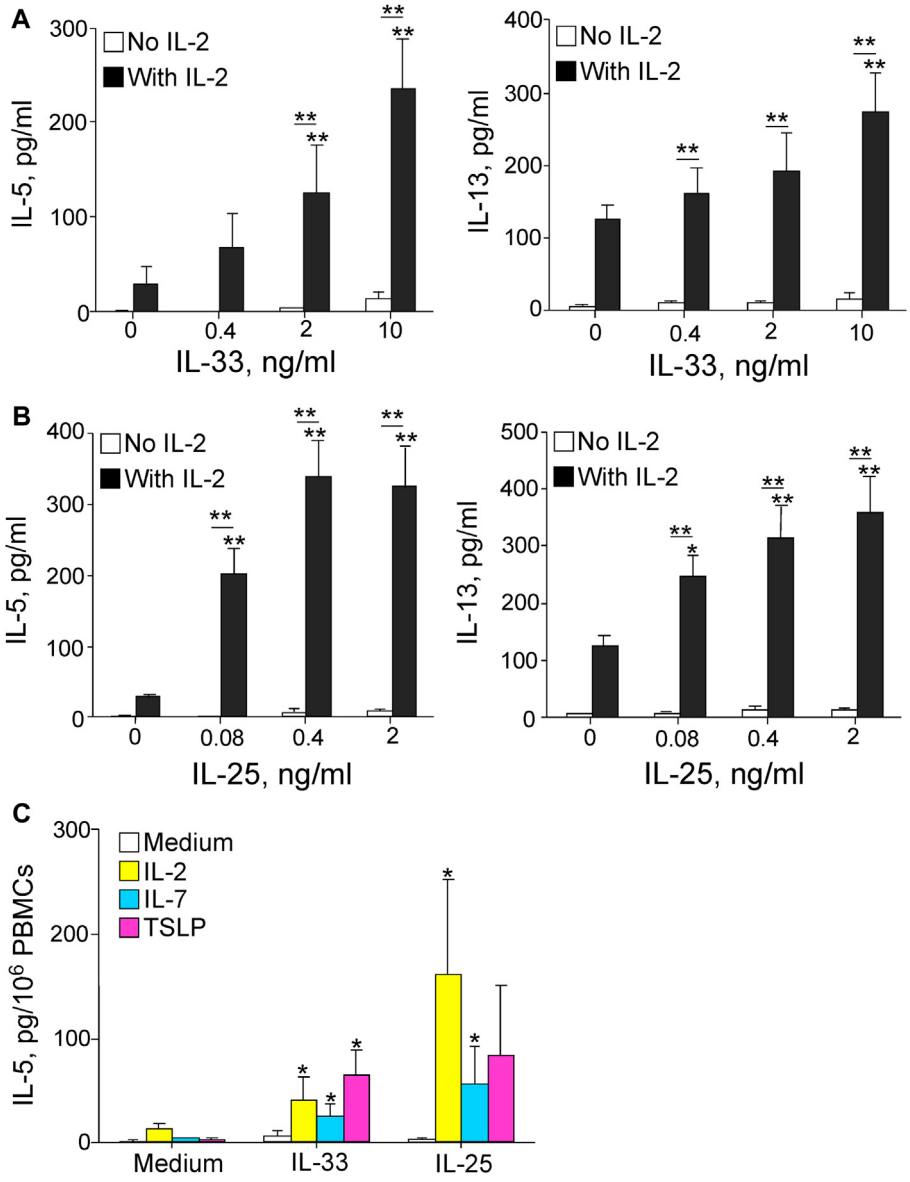

FIG 1. IL-33 or IL-25 induce antigen-independent type 2 cytokine production by human PBMCs. PBMCs were cultured without antigen for 7 days with increasing concentrations of IL-33 **(A)** or IL-25 **(B)** in the presence or absence of 20 U/mL IL-2. IL-5 and IL-13 levels in the supernatants were determined by using ELISA. Data are the mean ± SEM of 30 subjects. **P* < .05; ***P* < .01, compared with IL-2 alone or between groups as indicated by *horizontal bars*. **C,** PBMCs were cultured with medium, IL-33 alone (10 ng/mL), IL-25 alone (0.4 ng/mL), or in combination with IL-2 (20 U/mL), IL-7 (10 ng/mL), or TSLP (10 ng/mL). IL-5 levels in the supernatants were determined. Data are the mean ± SEM of 6 experiments. **P* < .05 compared with IL-33 alone or IL-25 alone.

To examine whether PBMCs can be stimulated with another pro–type 2 cytokine, we performed parallel experiments using IL-25. Similar to IL-33, IL-25 alone induced minimal production of IL-5 or IL-13 in PBMCs (Fig 1, *B*). In the presence of IL-2, IL-25 significantly induced IL-5 and IL-13 production in a concentration-dependent manner ($P < .01$). A comparison of the dose-response curves in panels A and B showed that IL-25 is approximately 25 times more potent than IL-33. Because IL-2 belongs to the common cytokine receptor γ-chain family and activates the STAT5 pathway,[34] we questioned whether other cytokines in the same family are capable of replacing IL-2 and stimulating PBMCs. Indeed, although IL-7 or TSLP alone induced minimal IL-5 or IL-13 production, they synergized with IL-33 or IL-25 and significantly enhanced IL-5 and IL-13 production by PBMCs (Fig 1, *C*; $P < .05$).

To verify that the type 2 responses we detected in PBMCs are independent of T cells, we depleted PBMCs of $CD3^+$ cells before culture using immunomagnetic beads. $CD3^+$ cell depletion did not decrease but rather enhanced PBMC IL-5 and IL-13 production when stimulated with IL-25 plus IL-2 (see Fig E1 in this article's Online Repository at www.jacionline.org).

Finally, to compare antigen- and IL-25/IL-33–dependent type 2 cytokine production, PBMCs from subjects with AA who were sensitive to house-dust mite were divided into 2 fractions: those with both Lin^+ cells and Lin^- cells and those with Lin^+ cells alone. The fraction with both Lin^+ cells and Lin^- cells clearly

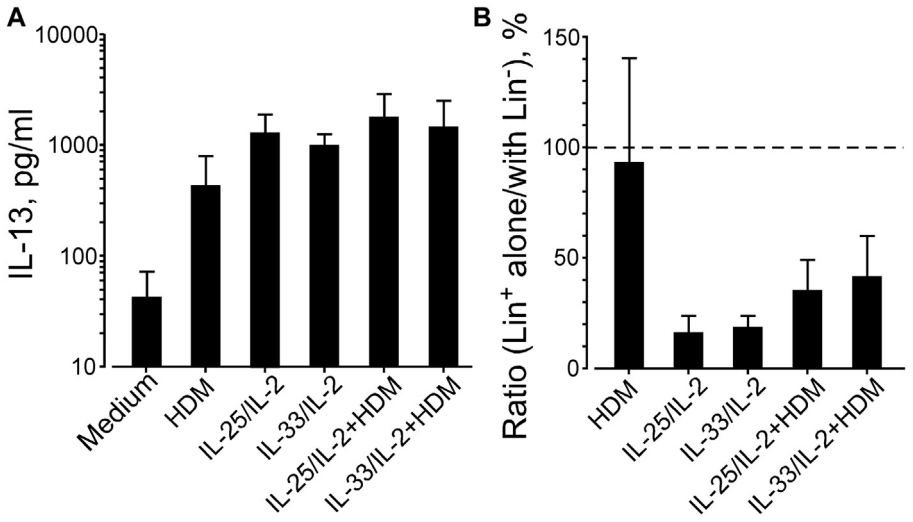

FIG 2. Lin^- cells are necessary for IL-25- and IL-33–induced IL-13 production. PBMCs from subjects with AA and skin test-positive for house-dust mite (HDM) were separated into 2 fractions, the Lin^+ with Lin^- cell fraction and the Lin^+ cell alone fraction. The Lin^+ with Lin^- cell fraction contained 82.0% Lin^+ cells and 18.0% Lin^- cells; the Lin^+ cell alone fraction contained 99.5% Lin^+ cells and 0.5% Lin^- cells. The fractions were cultured with HDM extract (25 μg/mL), IL-25 or IL-33 (10 ng/mL) plus IL-2 (20 U/mL), or combinations of the HDM extract and cytokines for 7 days. **A,** IL-13 levels in cell-free supernatants of the Lin^+ with Lin^- cell fraction were determined. **B,** IL-13 levels in cell-free supernatants of the Lin^+ cell alone fraction are presented as the ratio of the Lin^+ cell alone fraction divided by the Lin^+ with Lin^- cell fraction from the same donor. Data are the mean ± SEM of 3 subjects.

produced IL-13 when stimulated with house-dust mite extract or IL-25 or IL-33 in the presence of IL-2 (Fig 2, *A*). IL-25- and IL-33–induced IL-13 production was nearly abolished in the fraction with Lin^+ cells alone (Fig 2, *B*); in contrast, house-dust mite–induced IL-13 production was not affected in this fraction. Taken together, these findings suggest that, similar to mouse mucosal tissues, human PBMCs produce type 2 cytokines when stimulated with IL-33 or IL-25 in the presence of IL-2 and other γ-chain family cytokines and that the response is likely mediated by innate immune cells.

Functional ILC2s are present in human peripheral blood

In antigen-dependent systems, $CD4^+$ T_H2 cells are the predominant source of IL-5 and IL-13.[7] In 2010, several laboratories independently identified a murine Lin^- lymphoid cell type that responds to IL-25 and/or IL-33 and produces type 2 cytokines.[17-19] These cells were subsequently termed ILC2s by a consensus report.[25] A homologous population of innate immune cells is beginning to be identified in human mucosal organs and peripheral blood.[29-32] We hypothesized that ILC2s are the source of IL-5 and IL-13 in PBMC cultures. Therefore, we freshly isolated cells from human PBMCs using sorting with flow cytometry. Because there is no established protocol for identifying human ILC2s, we followed the strategy used by Mjosberg et al.[25,29] We used a lineage cocktail of antibodies to CD3, CD14, CD16, CD19, CD56, and FcεRIα to identify T cells, monocytes, neutrophils, B cells, natural killer cells, mast cells, and basophils. Lin^- cells, which are negative for these lineage markers, were further subdivided and sorted on the basis of CD127 and CRTH2 expression (Fig 3, *A*). Finally, the Lin^+ cell population as well as 3 populations of Lin^- cells (Fig 3, *A*, identified by red boxes 1-4) were cultured with IL-33 plus IL-2 for 5 days. The Lin^- and CD127/CRTH2 double-positive cell population (ie, $Lin^-CD127^+CRTH2^+$) produced a large quantity of IL-5 and IL-13 (Fig 3, *B*, please note the scale of the y-axis), whereas $Lin^-CD127^+CRTH2^+$ cells cultured with medium alone did not produce detectable levels of IL-5 or IL-13 (data not shown). Lin^+ cells as well as the $Lin^-CD127^+CRTH2^-$ population produced small amounts of IL-5 and IL-13, and no cytokine was detected in the $Lin^-CD127^-CRTH2^-$ population. Minimal IFN-γ was produced by Lin^+ cells but not by any of the 3 Lin^- populations. No or minimal amounts of IL-4 (<4 pg/10^6 cells) or IL-17A (107 pg/10^6 cells) were produced by $Lin^-CD127^+CRTH2^+$ cells stimulated with IL-33 plus IL-2. In addition, in a separate experiment, large quantities of IL-5 (79 ng/10^6 cells) and IL-13 (121 ng/10^6 cells) were produced by $Lin^-CD127^+CRTH2^+$ cells stimulated with IL-33 plus IL-7.

Stimulation of $Lin^-CD127^+CRTH2^+$ cells with IL-33 plus IL-2 also dramatically changed their cell morphology. Before culture, $Lin^-CD127^+CRTH2^+$ cells were approximately 10 μm in diameter with a high nucleus to cytoplasm ratio and were indistinguishable from common lymphocytes (Fig 3, *C*, left panel). Culture with IL-33 plus IL-2 caused marked increases in cellular size, the development of Golgi bodies and endoplasmic reticulum, and a decreased nucleus to cytoplasm ratio (Fig 3, *C*, right panel). In addition, cells with apparent mitotic nuclei were frequently observed. We also used intracellular cytokine staining to verify the production of IL-5 by ILC2s within the PBMCs. To circumvent technical problems associated with intracellular staining, ILC2s were gated as the $Lin^-CD127^+CD44^{hi}$ population (see Fig E2 in this article's Online Repository at www.jacionline.org). This cell population uniformly expressed the IL-33 receptor ST2 and produced IL-5 when PBMCs were stimulated with IL-33 plus IL-2. Taken together, these results indicate that within human PBMCs, the $Lin^-CD127^+CRTH2^+$ population, which is compatible with the published phenotypic

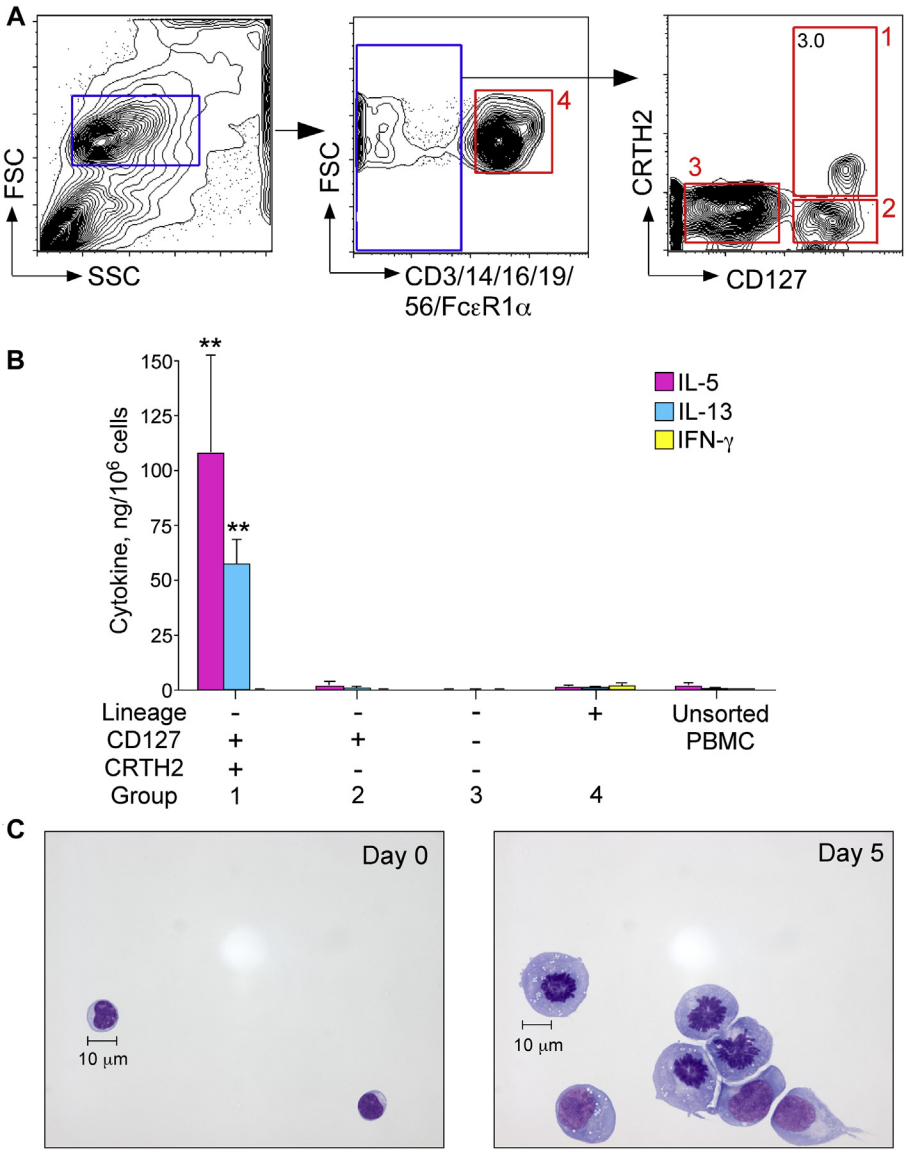

FIG 3. Circulating ILC2s in peripheral blood respond to IL-33 *ex vivo*. **A,** Gating strategy to isolate ILC2s and other Lin^- and Lin^+ cell populations. Lin^- lymphocytic cells were subdivided into 3 populations on the basis of CD127 and CRTH2 expression. *Red boxes* (labeled 1-4) indicate the sorted cells. **B,** Sorted cell populations from *A* were stimulated for 5 days with medium alone or IL-33 (10 ng/mL) plus IL-2 (20 U/mL). Levels of IL-5, IL-13, and IFN-γ in cell-free supernatants were measured using Milliplex. Data shown are the mean ± SEM of 3 independent experiments. $^{**}P < .01$ compared with medium alone. **C,** ILC2s (Box 1 in *A*) were cultured for 5 days with IL-33 plus IL-2. Cell morphology is shown before and after culture by staining the cytospin preparations with Wright Giemsa. *Scale bar* = 10 μm. *FSC,* Forward scatter; *SSC,* side scatter.

characteristics of human ILC2s, responds vigorously to IL-33 and produces large amounts of IL-5 and IL-13, but not IL-4. Other Lin^- cell populations or Lin^+ cells do not appear to have similar capabilities.

Innate type 2 responses are enhanced in the peripheral blood of patients with asthma

To investigate the utility of PBMC analyses of innate type 2 responses and to investigate the association between ILC2s and human asthma, we performed a prospective study. We examined PBMCs from subjects with AA and recruited subjects with AR and HC as disease and normal controls, respectively. These 3 groups did not differ by age or sex (Table I). However, as expected, FEV_1 was significantly lower in the AA group than in the AR group. Inhaled corticosteroid and short-acting bronchodilator use was significantly greater in the AA group than in the AR group or the HC group. No apparent differences in allergen sensitizations were observed between subjects with AA and AR.

Fresh PBMCs from donors were stimulated with IL-33 plus IL-2 or IL-25 plus IL-2 without antigens, and the concentrations of IL-5 and IL-13 in cell-free supernatants were analyzed as an indicator of the innate type 2 response. As described earlier (Fig 1), IL-2 alone induced minimal IL-5 production from PBMCs of all 3 groups (Fig 4, *A*, left panel). When stimulated with IL-33 plus IL-2, PBMCs from the HC group produced

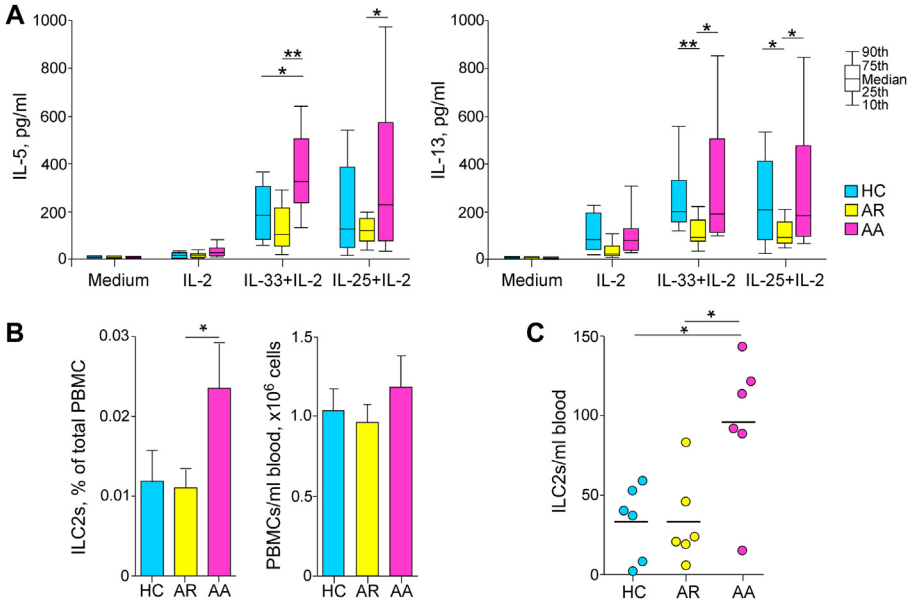

FIG 4. PBMCs from patients with AA show enhanced type 2 responses to IL-33 or IL-25. **A,** PBMCs from HC subjects, subjects with AR, or subjects with AA were cultured for 7 days with medium alone, IL-2 (20 U/mL) alone, IL-2 plus IL-33 (20 U/mL and 10 ng/mL, respectively), or IL-2 plus IL-25 (20 U/mL and 0.4 ng/mL, respectively). IL-5 *(left)* and IL-13 *(right)* levels in cell-free supernatants were determined using ELISA. Data shown are box and whisker plots (10th, 25th, 50th, 75th, and 90th percentile) of 14 or 15 subjects per group. **P* < .05; ***P* < .01, between the groups indicated by *horizontal bars*. **B,** ILC2s in PBMCs were identified and enumerated using flow cytometry as described in Fig 2, *A*. Proportion of ILC2s in PBMCs and the total number of PBMCs per milliliter of blood are shown as the mean ± SEM (n = 6 per group). **P* < .05, between the groups indicated by the *horizontal bar*. **C,** Total numbers of ILC2s per milliliter of blood were calculated. Each *dot* represents 1 patient, and *horizontal bars* indicate the mean for each group.

modest amounts of IL-5, suggesting that innate type 2 responses exist in normal individuals. Importantly, IL-5 production by PBMCs stimulated with IL-33 plus IL-2 was significantly greater in subjects with AA than in HC subjects (Fig 4, *A*; $P < .05$). In contrast, PBMCs from the AR group produced comparable amounts of IL-5 compared with PBMCs from the HC group and significantly lower amounts compared with PBMCs from the AA group (Fig 4, *A*; $P < .01$). When stimulated with IL-25 plus IL-2, PBMCs from the AA group produced significantly higher amounts of IL-5 than did PBMCs from the AR group ($P < .05$). When IL-13 levels in the same specimens were analyzed, PBMCs from the AA group produced significantly more IL-13 than did those from the AR group (Fig 4, *A*, right panel; $P < .05$). Furthermore, considerable individual variability in the PBMC responses to IL-33 plus IL-2 or IL-25 plus IL-2 was noted in the AA group.

To investigate whether the observed differences in innate type 2 responses among the groups can be explained by the numbers of ILC2s, we used flow cytometry to quantitate ILC2s in peripheral blood. Using the same gating strategy outlined in Fig 3, *A*, we identified ILC2s as $Lin^-CD127^+CRTH2^+$ cells. ILC2s generally consisted of a small fraction, approximately 0.01% to 0.03%, of total PBMCs in peripheral blood (Fig 4, *B*). The proportion of ILC2s in PBMCs (Fig 4, *B*) and the number of ILC2s in a given volume of peripheral blood (Fig 4, *C*) were significantly increased in the AA group than in the AR group or the HC group ($P < .05$). In addition, within the AA group, heterogeneity in the number of ILC2s was observed (Fig 4, *C*). There were no apparent differences in the expression levels of IL-33 receptor ST2 by ILC2s between the AA group and the HC group (see Fig E3 in this article's Online Repository at www.jacionline.org). These findings suggest that the innate type 2 immune response as well as the number of ILC2s is increased in patients with AA but not in patients with AR.

DISCUSSION

The $Lin^-CD127^+CD161^+CRTH2^+ST2^+$ cell population was initially identified in human fetal gut, adult peripheral blood, and nasal polyp tissues.[29] These cells produced IL-13 in response to IL-25, suggesting that they are the human counterpart of murine ILC2s. In this study, we verified these previous observations using cell sorting. Sorted human $Lin^-CD127^+CRTH2^+$ cells produced IL-5 and IL-13, but not IL-4 or IFN-γ on *in vitro* stimulation with IL-33 (Fig 3). They proliferated vigorously and developed prominent Golgi bodies and endoplasmic reticulum structures, consistent with their robust ability to produce cytokine proteins (Fig 3). In addition, IL-2, as well as IL-7 and TSLP, acted synergistically with IL-25 or IL-33 (Fig 1). Taken together, the phenotypic data as well as the functional and morphologic characteristics strongly suggest that the $Lin^-CD127^+CRTH2^+$ cells in human peripheral blood are ILC2s.

In the present study, we used flow cytometry and found ILC2s in the PBMCs of HC subjects as well as subjects with AA or AR. Thus, the presence of ILC2s per se is unlikely to be unique to a specific disease. Consistently, PBMCs from HC subjects responded to IL-33 or IL-25 stimulation and produced type 2 cytokines. Importantly, however, such innate type 2 responses were significantly upregulated in patients with AA. Furthermore, ILC2s were more abundant in peripheral blood from patients with

TABLE I. Study subjects

Characteristic	HC (n = 18)	AR (n = 16)	AA (n = 18)	*P* value
Age (y), mean ± SEM	43.4 ± 2.9	47.7 ± 3.0	45.6 ± 3.3	.5781
Sex, female/male	14/4	9/7	9/9	.2960
Asthma, n	0	0	18	
AR, n	0	16	18	
Chronic rhinosinusitis, n	0	0	0	
Ever smoked (%)	11.1	25.0	0	.0746
Current smoker (%)	0	12.5	0	.0963
Ever had immunotherapy (%)	0	0	5.6	.3818
FEV_1 (% predicted), mean (subjects tested)	112 (1)	105 (9)	85 (16)	.0004*
Medications, n				
ICS	0	2	10	.0087*, .0002†
LABA	0	0	2	NS
SABD	0	0	18	<.0001*†
LTM	0	0	3	NS
Skin test, positive/subjects tested	0/5	16/16	15/15	<.0001†‡
Trees (%)	0	62.5	55.6	
Grass (%)	0	50.0	38.9	
Ragweed (%)	0	81.3	72.2	
Mold (%)	0	50.0	38.9	
Dust mite (%)	0	31.3	50.0	
Pet (%)	0	31.3	61.1	
Cockroach (%)	0	6.3	16.7	

ICS, Inhaled corticosteroids; *LABA*, long-acting beta agonists; *LTM*, leukotriene modifiers; *NS*, not significant; *SABD*, short-acting bronchodilators.
*AR vs AA.
†HC vs AA.
‡HC vs AR.

AA, providing a plausible explanation for the increased cytokine production by PBMCs. However, it is also possible that ILC2s from subjects with AA are optimally primed for IL-33 or IL-25 responsiveness or enhanced for cytokine production, and our results cannot rule out this possibility. Biochemical characterization, such as expression and phosphorylation of GATA3 and p38 MAPK, may be necessary to explain the potential functional differences in ILC2s from different diseases because these molecules may provide critical regulatory signals in murine ILC2 functions.[35] Nonetheless, our study demonstrates that human fresh peripheral blood cells, namely PBMCs, can be used to monitor functional innate type 2 responses and to quantitate ILC2 numbers.

One of the major findings in this study is the clear difference in innate type 2 responses between patients with AA and patients with AR (Fig 4, *A*). Therefore, increased innate type 2 immunity and ILC2 numbers are unlikely to be markers for T_H2-type immune responses in general. We speculate that ILC2s and their products, including IL-5, IL-13, and tissue growth factors, play a more prominent role in AA than in AR. Indeed, a genomewide association study identified associations between asthma and SNPs in *IL1RL1/IL18*, *IL33*, and *RORA*,[3,4] thus implicating both ILC2s and IL-33 in the pathophysiology of asthma. *RORA* is a key transcription factor for ILC2 development.[24,36] In the same genomewide association study, associations between serum IgE levels and *FCER1A*, *IL13*, *STAT6*, and *IL4R/IL21R* SNPs were detected,[4] but no overlap was observed between the key SNPs associated with serum IgE and those associated with asthma.[37] It is also intriguing to note that ILC2s have the capacity to regulate basal eosinophilopoiesis and tissue eosinophil accumulation through constitutive and stimulated production of IL-5 in mice.[38] Thus, the immunopathogenesis of asthma and allergy is likely different, and asthma may be affected by type 2 innate immunity as well as adaptive immunity whereas allergy may be affected predominantly by type 2 adaptive immunity.

A question remains regarding why ILC2s are increased in peripheral blood from subjects with AA. ILC2s, similar to other innate lymphoid cell family members, arise from common lymphoid progenitors in the bone marrow and require the transcriptional inhibitor Id2 and transcription factors RORα and GATA3 for their development.[24,35,36,39,40] Like natural killer cells, ILC2s are recruited directly to mucosal tissues through peripheral blood circulation.[16] Systemic IL-25 or IL-33 administration into the peritoneal cavity of mice increases the number of ILC2s in the lungs.[26] Human ILC2s respond to IL-33 or IL-25[29,30] as well as lipid mediators, such as prostaglandin D_2[41] and leukotriene D_4[42] *in vitro*. Indeed, increased IL-33 protein expression is observed in respiratory mucosa of patients with severe asthma.[43] IL-33 was also readily induced by exposing airway epithelial cells from patients with nasal polyps to fungal antigens *in vitro*.[30] Thus, increased numbers of ILC2s in human peripheral blood may reflect increased production and/or release of IL-33 and other innate inflammatory mediators in respiratory mucosa.

In summary, we have described the connections between innate type 2 immune responses and AA. However, a limitation of this study is its relatively small sample size, which precludes us from performing subgroup analyses. Several questions remain to be answered. For example, do the number or activity of circulating ILC2s correlate with disease severity or exacerbation? Clearly, clinical studies with different study designs and larger sample sizes are necessary to address such questions. Nonetheless, this report demonstrates that the innate type 2 immune response and ILC2s can be monitored using PBMCs from patients with allergic diseases as well as from normal individuals. These data provide a novel research tool and evidence for its feasibility in

addressing the roles of innate type 2 immunity and ILC2s in human diseases.

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Key messages

- **Human PBMCs demonstrate innate type 2 immune responses to IL-33 and IL-25 in the presence of IL-2.**
- **Group 2 innate lymphoid cells (ILC2s) isolated from PBMCs produce large quantities of IL-5 and IL-13, but not IL-4, when stimulated with IL-33.**
- **Innate type 2 responses are enhanced and ILC2s are increased in the peripheral blood of subjects with AA but not in subjects with AR.**

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METHODS

Study subjects

Inclusion criteria for AR were as follows: (1) clinical history of seasonal allergies evidenced by nose itching, sneezing, and clear rhinorrhea and (2) no history or symptoms of chronic rhinosinusitis or asthma. HC subjects had no history of allergy, asthma, or sinus disease. Exclusion criteria for all 3 groups were as follows: (1) systemic glucocorticoids within the past 4 weeks, (2) smoking within the past 12 months or a history of more than 10 packs/y, (3) immunodeficiency or cystic fibrosis diagnosis, (4) immunotherapy for any allergens, or (5) pregnancy.

Reagents

Phycoerythrin-Cy7–labeled antibody to CD127 (R34.34) was purchased from Beckman-Coulter, Inc (Brea, Calif). FITC-labeled anti-FcεR1α (AER37) was purchased from eBioscience (San Diego, Calif). Phycoerythrin-labeled anti–ST2/IL-1R4 (97203; anti-ST2) and recombinant human IL-33, IL-25, IL-7, and TSLP were purchased from R&D Systems (Minneapolis, Minn). Recombinant human IL-2 was purchased as Proleukin (aldesleukin) from Novartis Pharmaceuticals Corporation (East Hanover, NJ). House dust mite (HDM) extract was purchased from Greer Laboratories (Lenoir, NC).

PBMC isolation and stimulation for cytokine production

In some experiments, PBMCs from subjects who had AA and were skin test-positive for HDM were mixed with FITC-conjugated antibodies to lineage markers (CD3, CD14, CD16, CD19, CD56, and FcεRIα). Using the EasySep FITC selection kit (StemCell Technologies, Vancouver, BC, Canada), magnetic separation was performed as per manufacturer's instructions. Cells that were not bound to the magnet were collected as the fraction of a mixture of Lin^- cells and Lin^+ cells. After extensive washing, the cells that were bound to the magnet were collected as the fraction of pure Lin^+ cells. The purity of these 2 PBMC fractions was determined by using flow cytometry. The Lin^+ with Lin^- cell fraction contained 82.0% Lin^+ cells and 18.0% Lin^- cells; the pure Lin^+ cell fraction contained 99.5% Lin^+ cells and 0.5% Lin^- cells (mean of 3 subjects). These 2 factions were cultured in parallel in 96-well round-bottom plates (3.1×10^6 cells/mL) for 7 days with HDM extract (25 μg/mL), IL-33 (10 ng/mL) or IL-25 (10 ng/mL) with IL-2 (20 U/mL), or combinations of HDM extract and cytokines. IL-13 concentrations in cell-free supernatants were determined by using a Milliplex magnetic bead kit (EMD Millipore Corporation, Billerica, Mass).

FACS sorting and human peripheral blood ILC2 culture

Unsorted PBMCs, Lin^+ cells, and all 3 Lin^- populations were cultured in 96-well round-bottom plates ($4\text{-}10 \times 10^5$ cells/mL) in RPMI-1640 containing 10% autologous serum and penicillin/streptomycin for 5 days at 37°C and 5% CO_2. Cells were stimulated using medium alone or a combination of IL-33 (10 ng/mL) with IL-2 (20 U/mL) or IL-7 (10 ng/mL) for 5 days. Cell-free supernatants were analyzed for IL-4, IL-5, IL-13, IL-17A, and IFN-γ using a Milliplex magnetic bead kit (EMD Millipore Corporation). In addition, cytospin preparations of unsorted and sorted cells before and after the 5-day culture period were prepared and stained with Wright's Giemsa stain.

In some experiments, PBMCs were enriched for Lin^- cells as above and cultured with 10 ng/mL IL-33 plus 20 U/mL IL-2 or with medium alone for 4 days. Brefeldin A (GolgiPlug, BD Biosciences) was added for the final 12 hours. Cells were stained with FITC-labeled antibodies to lineage markers (CD3, CD14, CD16, CD19, CD56, and FcεR1α), AF647-labeled antibody to CD127, and peridinin chlorophyll protein complex-Cy5.5–labeled antibody to CD44; fixed and permeabilized with a Cytofix/Cytoperm kit (BD Biosciences); and stained intracellularly with phycoerythrin-labeled antibody to IL-5.

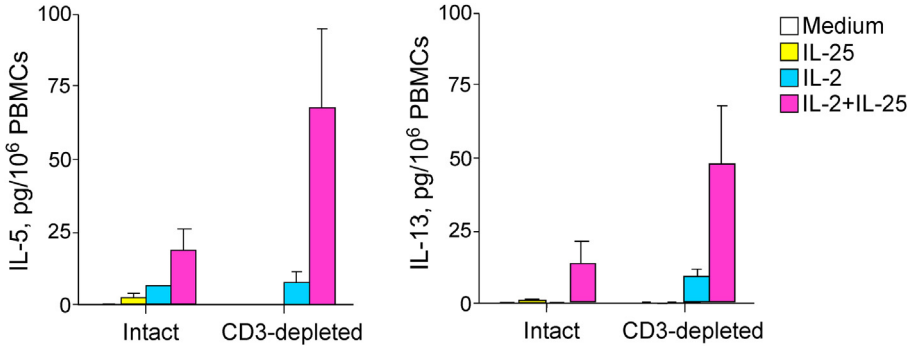

FIG E1. $CD3^+$ T cells are unnecessary for type 2 immune response to IL-25. Intact PBMCs (Intact) or PBMCs from which $CD3^+$ cells had been magnetically depleted (CD3-depleted) were cultured with medium, IL-25 (10 ng/mL) alone, IL-2 (20 U/mL) alone, or IL-2 plus IL-25 for 5 days. IL-5 and IL-13 levels in cell-free supernatants were determined using ELISA. Data are the mean ± SEM of 2 to 5 independent experiments.

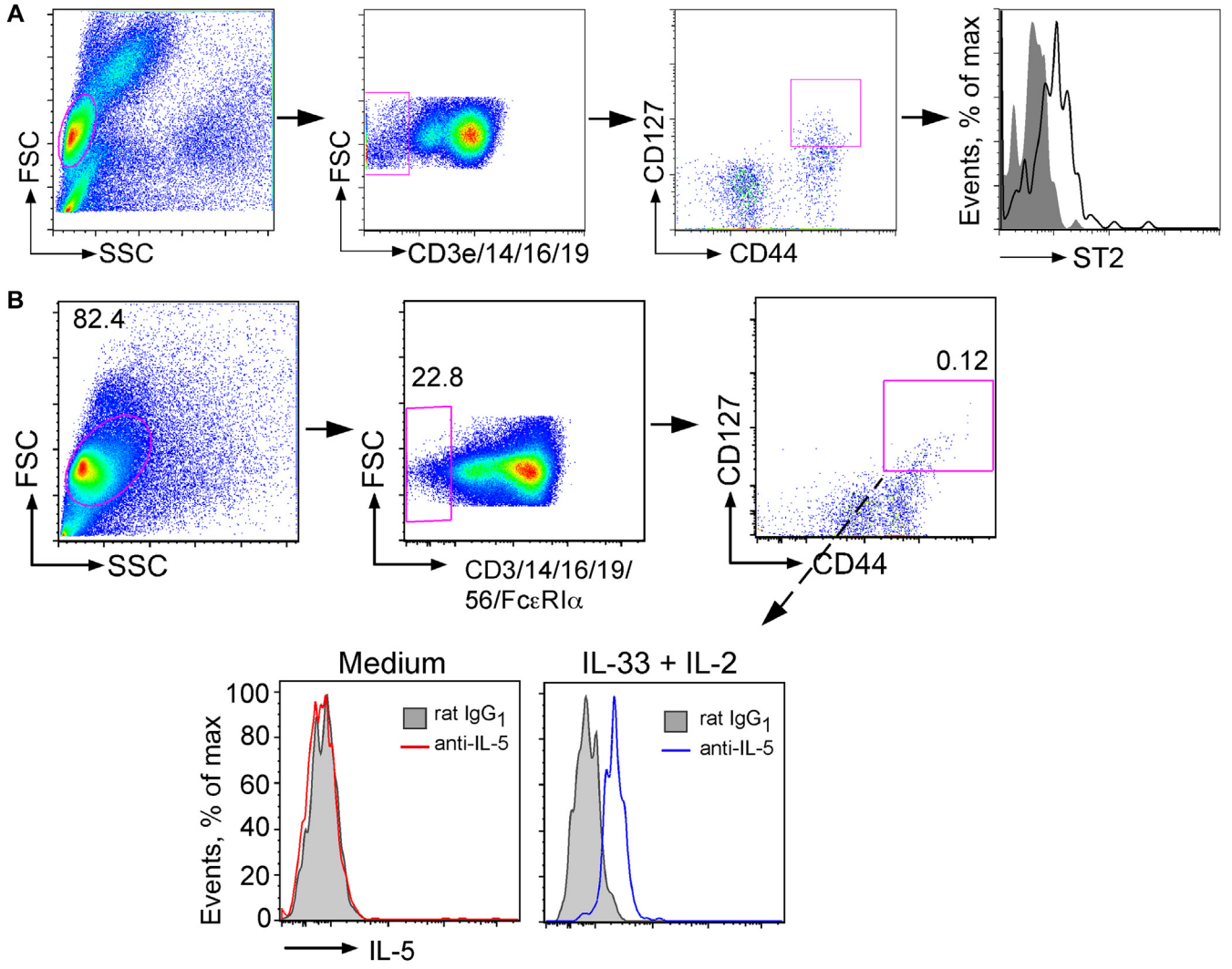

FIG E2. Blood ILC2s express the IL-33 receptor ST2 and respond to IL-33 *in vitro*. **A,** Gating strategy to identify ILC2s in PBMCs. In the histogram, anti-human ST2 staining *(black line)* and isotype control antibody staining *(filled gray)* are shown. **B,** PBMCs were cultured with medium alone or with IL-33 (10 ng/mL) plus IL-2 (20 U/mL) for 4 days. Brefeldin A was added for the last 12 hours. *Top panels*, Gating strategy for ILC2 identification. *Bottom panels*, Intracellular IL-5 staining in ILC2s cultured with medium alone *(left panel, red line)* or with IL-33 plus IL-2 *(right panel, blue line)* is shown. *Gray-filled* histograms depict isotype control staining for each culture condition. *FSC*, Forward scatter; *SSC*, side scatter.

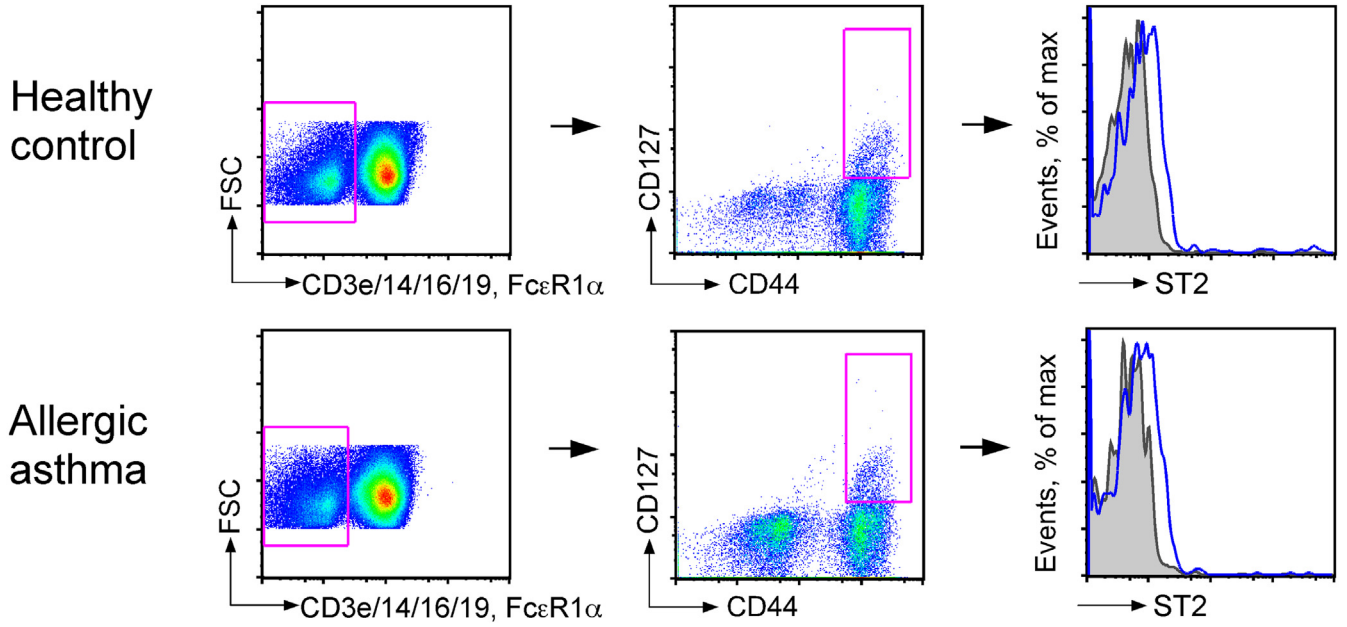

FIG E3. Blood ILC2s from HC subjects and subjects with AA express comparable levels of ST2. PBMCs from the 2 subject groups were gated as described in Fig E2, and ST2 expression was analyzed. In the histogram, anti-human ST2 staining is indicated by the *blue line* and isotype control staining is depicted by the *filled gray area*. Representative data from 3 HC subjects and 3 subjects with AA are shown.