

Increased numbers of activated group 2 innate lymphoid cells in the airways of patients with severe asthma and persistent airway eosinophilia

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Background: In patients with severe eosinophilic asthma, local maturation rather than systemic recruitment of mature cells might contribute to persistent airway eosinophilia. Group 2 innate lymphoid cells (ILC2s) are a major source of type 2 cytokines (IL-5 and IL-13) and can facilitate eosinophilic inflammatory responses in mouse models of asthma in the absence of CD4⁺ lymphocytes. This study investigated the potential role of ILC2s in driving chronic airway eosinophilia in patients with severe asthma, despite regular high-dose oral corticosteroid therapy.

Methods: In a cross-sectional study we enumerated blood and sputum ILC2s (lin⁻CD45⁺127⁺ST2⁺) and levels of intracellular IL-5 and IL-13 in patients with severe asthma (n = 25), patients with steroid-naïve mild atopic asthma (n = 19), and nonatopic

control subjects (n = 5). Results were compared with numbers of CD4⁺ lymphocytes, eosinophil lineage-committed progenitors (eosinophilopoietic progenitor cells [EoPs]), and mature eosinophils.

Results: Significantly greater numbers of total and type 2 cytokine-producing ILC2s were detected in blood and sputum of patients with severe asthma compared to mild asthmatics. In contrast, intracellular cytokine expression by CD4 cells and EoPs within the airways did not differ between the asthmatic groups. In patients with severe asthma, although sputum CD4⁺ cells were more abundant than ILC2s and EoPs, proportionally, ILC2s were the predominant source of type 2 cytokines. In addition, there were significantly greater numbers of sputum IL-5⁺IL-13⁺ ILC2s in patients with severe asthma whose airway eosinophilia was greater than 3%, despite normal blood eosinophil numbers (<300/μL).

Conclusions: Our findings suggest that ILC2s can promote the persistence of airway eosinophilia in patients with severe asthma through uncontrolled localized production of the type 2 cytokines IL-5 and IL-13, despite high-dose oral corticosteroid therapy. (*J Allergy Clin Immunol* 2015;■■■■:■■■■-■■■■.)

Key words: Group 2 innate lymphoid cells, severe asthma, eosinophilic bronchitis

Asthma is characterized by airway inflammation, reversible airway obstruction, and airway hyperresponsiveness. Eosinophilic inflammation is a major contributor to the physiologic changes and remodeling seen in asthmatic patients.^{1,2} Eosinophils are present in the bronchial mucosa and airway lumen of asthmatic patients and are increased when asthma is uncontrolled³ or severe⁴ and decreased when asthma is controlled.⁵ In patients with severe prednisone-dependent asthma with eosinophilic bronchitis, treatment strategies that aim to control airway eosinophilia are significantly more effective at decreasing asthma exacerbations compared with guideline-based clinical strategies, as well as being steroid sparing and therefore less expensive in improving asthma control.⁶⁻⁸

Airway eosinophilia in asthmatic patients can arise as a result of the recruitment of mature eosinophils from the peripheral circulation in response to locally elaborated chemoattractants, such as eotaxin,⁹ and/or localized maturation of eosinophil lineage-committed progenitors (eosinophilopoietic progenitor cells [EoPs]), which is termed *in situ* differentiation and driven by the locally elaborated eosinophilopoietic cytokine IL-5.¹⁰⁻¹³ Other type 2 cytokines, IL-13 and IL-4, have been shown to prime the migrational responsiveness of mature eosinophils and progenitors, thereby likely promoting lung homing of these cells in

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Abbreviations used

EoP: Eosinophilopoietic progenitor cell
 HPC: Hematopoietic progenitor cell
 ILC2: Group 2 innate lymphoid cell
 IL-5R: IL-5 receptor
 PE: Phycoerythrin
 TSLP: Thymic stromal lymphopoietin

asthmatic patients.¹⁴ In addition, IL-4 and IL-13 upregulate vascular cell adhesion molecule 1 expression on vascular endothelial cells and stimulate the production of eotaxin by airway smooth muscle and epithelial cells, which together enhance eosinophil recruitment to the lungs.¹⁵ Although it is evident that CD4⁺ T_H2 cells are an important source of the type 2 cytokines that drive many of the features of asthma, the upstream mechanisms involved in the development of antigen-specific T_H2 cells from naive CD4⁺ cells and the ongoing production of IL-4, IL-5, and IL-13 in asthmatic airways remain poorly understood.¹⁶⁻¹⁸

An emerging hypothesis proposes that chronic inflammatory airway diseases are driven by alterations of the mucosal interface compartment.¹⁹ Lung epithelial cells activated by proteases, viruses, or environmental pollutants produce a triad of cytokines, thymic stromal lymphopoietin (TSLP), IL-33, and IL-25, which are critical initiators of type 2 eosinophilic inflammatory events in the lung, gut, and skin.²⁰ These cytokines are present at increased levels in airways of asthmatic patients and correlate with disease severity.²¹⁻²³ The downstream targets of epithelium-derived cytokines are a novel group of cells termed group 2 innate lymphoid cells (ILC2), which, when activated, produce more canonical type 2 cytokines (IL-5 and IL-13) than CD4⁺ lymphocytes on a per-cell basis.²⁴ Genome-wide association studies show an association between asthma and single nucleotide polymorphisms in several genes, including *IL1RL1* (IL-33 receptor ST2), *IL18R*, *IL33*, *TSLP*, and retinoic acid–related orphan receptor α (*RORA*; a key transcription factor for ILC2 development) implicating ILC2s and epithelium-derived cytokines in the pathophysiology of asthma.^{25,26} This has resulted in a paradigm shift that asthma is not simply a T_H2 cell–dependent, IgE-mediated allergic inflammatory disease but also involves an innate pathway in which ILC2s provide the primary cellular source of IL-5 and IL-13, which are likely important for initiation of adaptive type 2 immune responses²⁷⁻²⁹ and regulation of the persistent airway inflammation and tissue remodeling associated with chronic asthma.^{30,31}

To date, evidence for a role for ILC2s in driving type 2 immune responses has come from murine studies with little information from patients with human inflammatory diseases and less so from asthmatic patients.^{32,33} In human subjects ILC2s have been detected in fetal and adult lung, gut, and blood.³⁴ Enhanced accumulations of ILC2s have been reported at sites of eosinophilic inflammation, including nasal polyps and sinus mucosa in patients with chronic rhinosinusitis, skin lesions in patients with atopic dermatitis, and pleural effusions from patients with spontaneous pneumothorax.³⁵⁻³⁷ Recent studies have reported that ILC2s, with a greater capacity to produce IL-5 and IL-13 on stimulation with epithelium-derived cytokines *ex vivo*, are found in the blood of allergic asthmatic patients compared with healthy control subjects.³⁸ In addition, increased numbers of ILC2s are found in the bronchoalveolar lavage fluid of patients with severe

asthma compared with those in disease-matched control subjects.³⁹ However, there is little information about the *in vivo* activation levels of ILC2s compared with other type 2 cells within the airways of patients with severe eosinophilic asthma and how this relates to levels of airway eosinophilia.

We hypothesize that ILC2 numbers are increased in the airways of asthmatic patients and that uncontrolled activation of these cells drives chronic eosinophilia of the airways. The current study enumerated (1) ILC2 numbers and *in vivo* activation levels in patients with severe asthma by using induced sputum samples to noninvasively sample the airway lumen and (2) compared these measurements with other proinflammatory cellular sources of type 2 cytokines, including CD4⁺ lymphocytes and hematopoietic progenitor cells (HPCs).

METHODS**Subjects**

The subjects (aged 19–60 years) recruited to this cross-sectional study were stable for 4 weeks before sampling and were given a diagnosis of (1) severe eosinophilic asthma or (2) mild atopic asthma. Patients with severe asthma had demonstrated exacerbation of symptoms associated with sputum eosinophilia of greater than 3% on at least 2 occasions when the maintenance dose of prednisone was reduced, confirming their prednisone dependence. All patients with severe eosinophilic asthma had prebronchodilator FEV₁ of less than 80% of predicted value, greater than 12% FEV₁ reversibility on challenge with short-acting bronchodilator, PC₂₀ of less than 8 mg/mL, an at least 6-month history of treatment with oral steroids (5–35 mg of daily prednisone or equivalent), use of inhaled corticosteroids (>880 μ g/d), and eosinophilic inflammation determined by a blood eosinophil count of 150 cells/ μ L or greater currently or less than 300 cells/ μ L within the past 12 months. Patients with mild atopic asthma had positive skin prick test responses, FEV₁ of 70% of predicted value or greater, greater than 12% FEV₁ reversibility, and PC₂₀ of 16 mg/mL or less and were steroid naive with infrequent use of inhaled β_2 -agonists. Baseline characteristics are summarized in Table E1 in this article's Online Repository at www.jacionline.org.

All subjects provided written informed consent, and all experimental procedures were reviewed and approved by the institutional research ethics board.

Sputum induction and cell isolation

Sputum was induced through inhalation of an aerosol of hypertonic saline, as previously described.⁴⁰ The sputum samples were processed by selecting the mucus plugs, mixing with 4 parts 0.1% dithiothreitol and Dulbecco PBS, and filtered through a 52- μ m nylon mesh.⁴¹ The cells in the filtrate were pelleted and resuspended in Dulbecco PBS. Cytospin preparations were prepared on glass slides and stained with Diff-Quik (American Scientific Products, McGaw Park, Ill) for differential counts expressed as percentage means of duplicate slides (400 cells counted per slide). The remaining cells were placed in aliquots for flow cytometry.

Peripheral blood collection and cell isolation

Peripheral blood from venipuncture was drawn into heparin (1000 U/mL)–containing tubes and layered on Accuprep (Accurate Chemical & Scientific, Westbury, NY) for density gradient sedimentation, and monocytes were depleted by adherence to plastic (2 hours in a 5% CO₂ atmosphere at 37°C), as previously described.⁴²

Immunofluorescence staining and flow cytometric gating strategy for type 2 immune cells

Freshly isolated blood-derived mononuclear cells and sputum-extracted cells were immediately fixed in PBS plus 1% paraformaldehyde, with no

further *ex vivo* stimulation. Cells were then immediately stained for ILC2, EoP, and CD4 enumerations with antibodies to the surface markers Lin-eFluor450, FcεRI-eFluor450, CD45-Amcyan, CD34-phycoerythrin (PE)-Cy7, ST2-PE, IL-7Rα (CD127)-PE Texas Red, CD125 (IL-5 receptor [IL-5R] α)-allophycocyanin, PE-Texas Red, and CD4-allophycocyanin-Cy7 or relevant isotype controls in PBS plus 0.1% NaN₃ (BD Biosciences, Mississauga, Ontario, Canada; eBioscience, San Diego, Calif; R&D Systems, Minneapolis, Minn). Lin cocktail antibodies to CD3, CD4, CD19, CD20, and CD56 plus FcεRI exclude T and B lymphocytes, B cells, monocytes, eosinophils, natural killer cells, and basophils. Cells were then incubated in Perm/Fix Buffer and optimal concentrations of intracellular stains for anti-IL-5-FITC and anti-IL-13-PerCP-Cy5.5 (BD Biosciences). Cells were resuspended in PBS and analyzed by using a FACS LSR II flow cytometer within 24 hours (Becton, Dickinson Instrument Systems, Franklin Lakes, NJ). After gating in the lymphomononuclear region (low side scatter/low forward scatter) and acquisition of 300,000 events, data were analyzed by using FlowJo software (TreeStar, Ashland, Ore) to detect ILC2s (lin⁻CD45⁺CD127⁺ST2⁺), as described in Fig 1. The gating strategy for detecting CD3⁺CD4⁺ lymphocytes, HPCs (CD34⁺CD45⁺), and EoPs (CD34⁺CD45⁺IL-5Rα⁺) expressing intracellular IL-5 and IL-13 is described in Figs E1 and E2, respectively, in this article's Online Repository at www.jacionline.org.

Statistical analyses

Data are expressed as means ± SEMs. Antigen-induced changes from baseline were analyzed by using paired *t* tests with GraphPad Prism 5 software (GraphPad Software, La Jolla, Calif). Priming of migration was analyzed by using ANOVA. For data not normally distributed, statistics were performed on the log-transformed data. The α value was set at .05.

RESULTS

Identification of ILC2s in induced sputum by means of flow cytometry

By using sequential gating flow cytometry, airway luminal ILC2s were detected as lin⁻FcεRI⁻CD45⁺CD127⁺ST2⁺ cells in induced sputum samples from asthmatic patients (Fig 1, A). Intracellular cytokine staining of ILC2s was performed in freshly isolated cells without any further *ex vivo* stimulation to provide *in vivo* activation levels of these cells (Fig 1, B). Similar measurements were made in blood (see Fig E3 for gating strategy in this article's Online Repository at www.jacionline.org). In accordance with previously published data, we show that the majority of ILC2s express chemoattractant receptorehomologous molecule expressed on T_H2 lymphocytes (CRTH2) expressed on T_H2 lymphocytes (81% ± 3%, n = 12) and, to a lesser extent, c-kit (26% ± 3%, n = 12; see Fig E4, A and B, in this article's Online Repository at www.jacionline.org).^{34,43} A novel finding of this study was that 68% ± 10% (n = 12) of ILC2s expressed surface IL-5Rα (CD125, see Fig E4, C). However, investigation of the functional role of this receptor on ILC2s is beyond the scope of the current study.

Enumerating ILC2s in blood and sputum from patients with severe and those with mild asthma

Baseline characteristics of patients with severe and those with mild asthma are described in Table E1. A significantly greater number of ILC2s was detected in blood and sputum samples from patients with prednisone-dependent severe eosinophilic asthma compared with those from patients with mild atopic asthma (blood: 7844 ± 2350 vs 310 ± 74 cells/mL, *P* < .001; sputum:

8715 ± 2404 vs 182 ± 44 cells/mL, *P* < .001, respectively; Fig 2). Although there was trend for 2-fold greater ILC2 numbers in sputum samples compared with blood from the same asthmatic patient, these compartmental differences were not significant when the entire data set was compared for patients with severe versus mild asthma. The rarity of ILC2s and low cell recovery from induced sputum samples precluded analyses in healthy control subjects. However, significantly lower absolute numbers of ILC2s were detected in blood from healthy nonatopic subjects compared with those in blood from patients with severe asthma (see Table E2 in this article's Online Repository at www.jacionline.org).

Significantly greater numbers of ILC2s expressing IL-5 (3523 ± 1345 vs 84 ± 28 cells/mL, *P* < .001), IL-13 (1827 ± 666 vs 21 ± 7 cells/mL, *P* < .001), and IL-5/IL-13 (965 ± 369 vs 18 ± 6 cells/mL, *P* < .01) were detected in blood from patients with severe asthma compared with blood from patients with mild asthma (Fig 3). Although there was a trend for all type 2 cytokine-expressing ILC2s to be increased in the sputum, only IL-5⁺ ILC2 levels were significantly greater in patients with severe asthma compared with those in patients with mild asthma (IL-5⁺ ILC2s: 2598 ± 641 vs 132 ± 36 cells/mL, *P* < .01; IL-13⁺ ILC2s: 4347 ± 2069 vs 74 ± 44, *P* = .07; Fig 3).

Enumerating CD4⁺ lymphocytes and EoPs in patients with severe and those with mild asthma

In contrast to ILC2s, significantly greater total numbers of CD4⁺ cells were only found in the airway lumen but not blood of patients with severe compared with mild asthma (sputum: 1.5 ± 0.6 vs 0.3 ± 0.2 × 10⁶ cells/mL, *P* < .05; blood: 2.6 ± 0.7 vs 2.86 ± 0.16 × 10⁶ cells/mL, not significant; Fig 4, A). In assessing intracellular cytokine expression in freshly isolated cells without further *ex vivo* stimulation, we found that IL-5⁺CD4⁺ cells were significantly fewer in the blood of patients with severe compared with mild asthma (blood: 0.2 ± 0.08 vs 0.4 ± 0.09 × 10⁶ cells/mL, *P* < .05), whereas there were no significant differences between the 2 subject groups in sputum (Fig 4, B). In addition, there were no significant differences between the 2 subject groups in absolute numbers of CD4⁺IL-13⁺ or IL-5⁺IL-13⁺ cells in either blood or sputum samples.

Increased numbers of EoPs (CD34⁺CD45⁺CD125⁺ cells) were detected in the blood and sputum of patients with severe asthma compared with those in patients with mild asthma (Fig 5, A), which is consistent with previous findings.^{44,45} When activation levels were compared, although absolute numbers of IL-5⁺, IL-13⁺, and IL-5⁺IL-13⁺ EoP numbers were significantly increased in the blood of patients with severe compared with those in patients with mild asthma, these findings were not reflected in sputum (Fig 5, B).

In patients with severe asthma, CD4⁺ lymphocytes were the most abundant cells in blood and sputum compared with ILC2s, HPCs, and EoPs (Fig 6, A). When absolute numbers of intracellular cytokine-positive cells were compared, CD4⁺ cells exhibited the greatest level of positive expression for IL-5 and IL-13 in the blood and IL-5 cells in the sputum compared with all other type 2 cell types (see Fig E5 in this article's Online Repository at www.jacionline.org). However, proportionally, ILC2s were the predominant source of IL-5 and IL-13 in blood and induced sputum samples from patients with severe asthma (Fig 6, B).

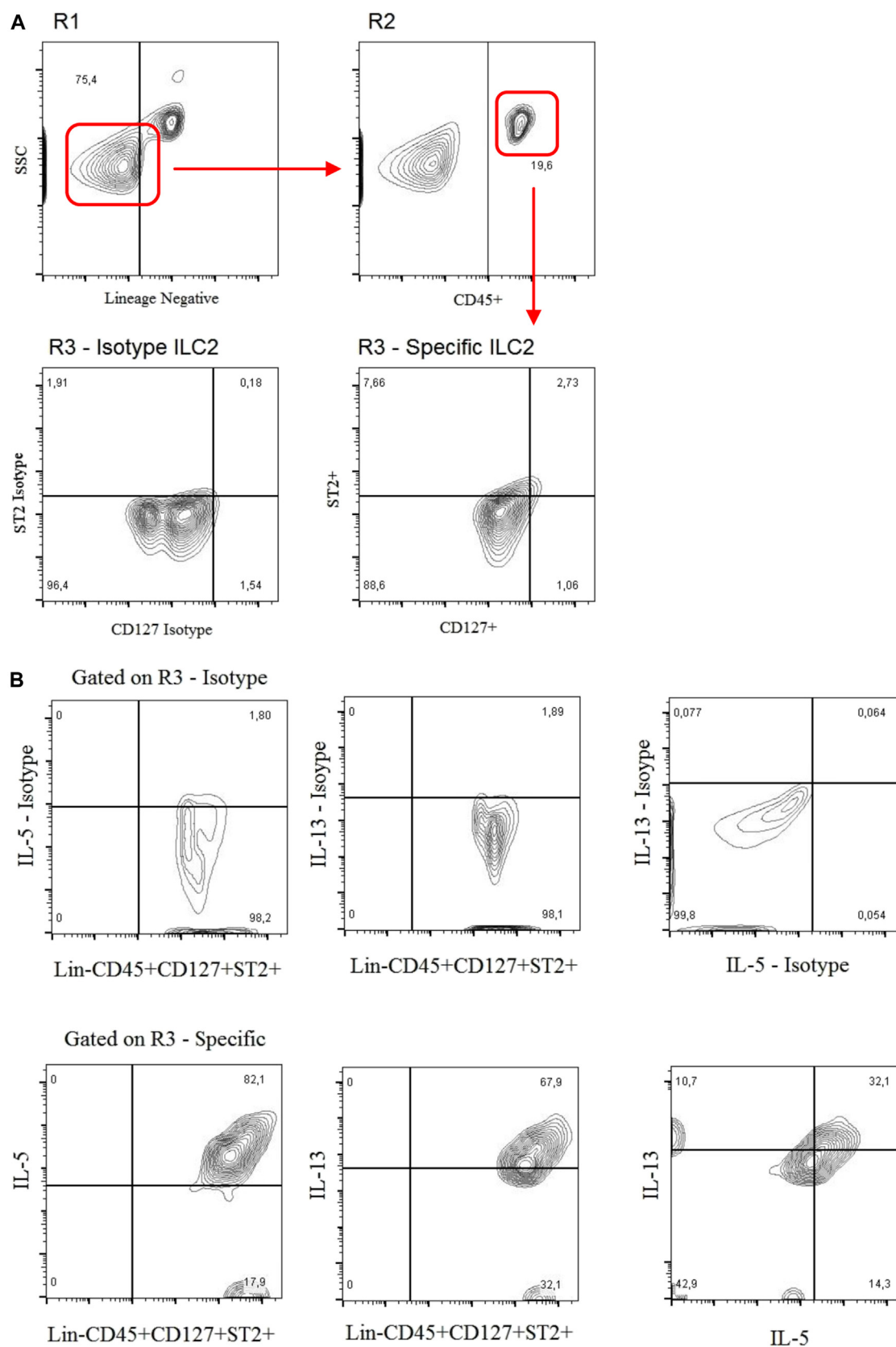


FIG 1. Multigating strategy for ILC2 enumeration from sputum. Representative plots from a patient with severe asthma show sequential gating of sputum cells to identified Lin⁺ (region R1), CD45⁺ (region R2), and CD127⁺ST2⁺ cells (region R3; **A**) and intracellular IL-5⁺, IL-13⁺, and IL-5⁺IL-13⁺ ILC2 quantification (**B**) by using a 98% confidence limit.

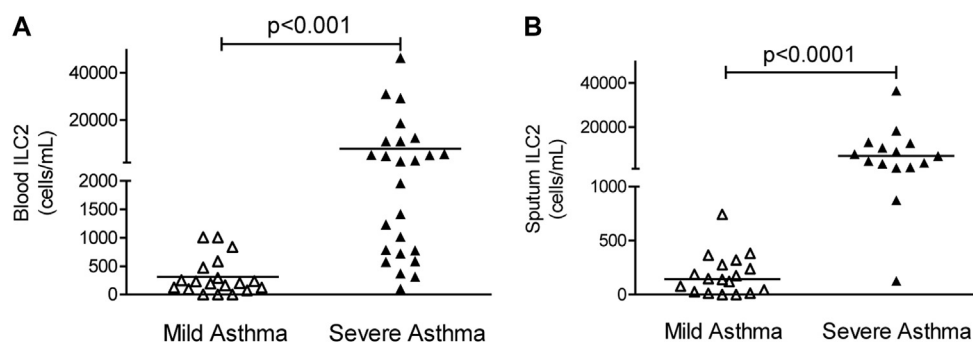


FIG 2. Absolute ILC2 numbers in asthmatic patients. **A**, Absolute numbers of ILC2s were significantly increased in the blood of patients with severe asthma compared ($n = 25$) with values in those with mild asthma ($n = 19$). **B**, Similar findings were observed in sputum samples from the same subjects (severe asthma, $n = 15$; mild asthma, $n = 18$). Absolute cell numbers enumerated by means of flow cytometry are presented as cells per milliliter, and the bars represent the median values of each data set.

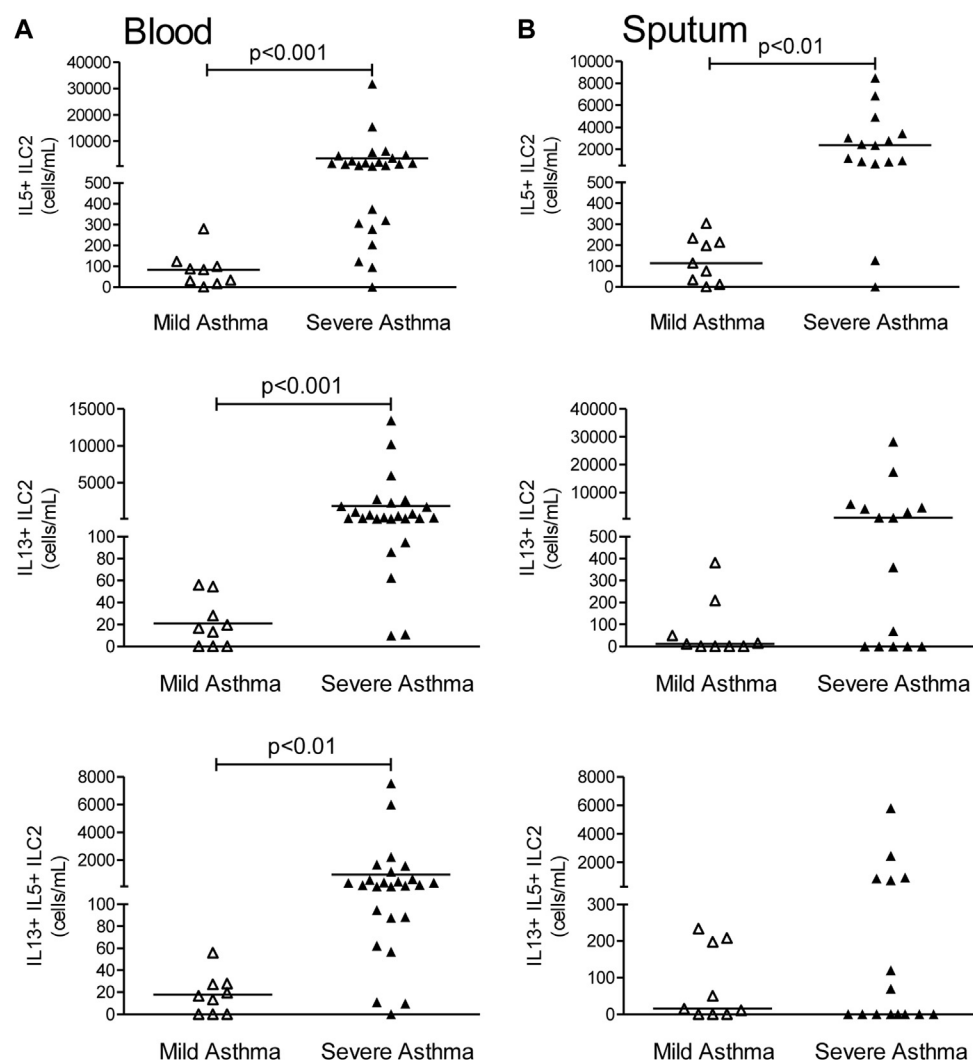


FIG 3. Levels of intracellular cytokine expression by ILC2s in blood and sputum. **A**, Absolute numbers of ILC2s expressing intracellular IL-5, IL-13, or both were significantly greater in the blood of patients with severe asthma ($n = 25$) compared with values in those with mild asthma ($n = 9$). **B**, In sputum only the absolute number of IL-5⁺ ILC2s was significantly greater in patients with severe asthma ($n = 15$) compared with those in patients with mild asthma ($n = 9$). Data are presented as cells per milliliter, and the bars represent median values of each data set.

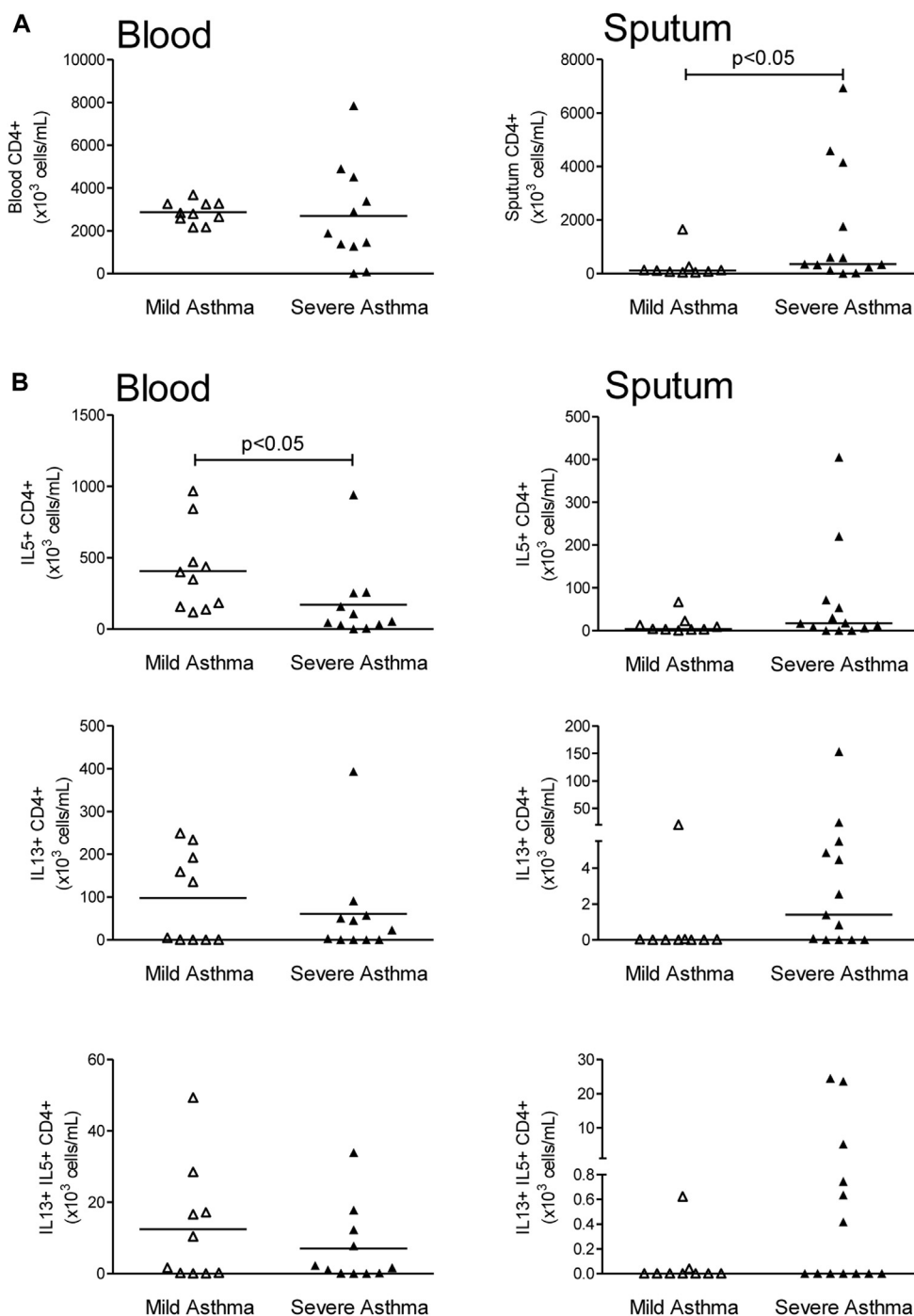


FIG 4. Comparison of absolute and intracellular cytokine expression in CD4⁺ lymphocytes. **A**, Significantly greater absolute numbers of CD4⁺ cells were detected in sputum of patients with severe asthma ($n = 13$) compared with those in patients with mild asthma ($n = 9$). **B**, No significant between-group differences were found for CD4⁺ cells expressing intracellular T_H2 cytokines, except for IL-5⁺ CD4 cells, numbers of which were lower in the blood of patients with severe asthma ($n = 11$) compared with those in patients with mild ($n = 10$) asthma. Data are presented as cells per milliliter, and the bars represent median values of each data set.

Heterogeneity of cytokine expression by sputum ILC2s from patients with severe asthma

A high degree of heterogeneity in absolute numbers and activation levels of ILC2s was observed in sputum samples from patients with severe eosinophilic asthma. It was noted that

despite all of the subjects having controlled blood eosinophil levels (<300 cells/ μ L), these subjects could be categorized as having uncontrolled or increased airway eosinophilia ($>3\%$ eosinophilia, $n = 9$) compared with those in whom airway eosinophilia was controlled (ie, $<3\%$ eosinophilia;

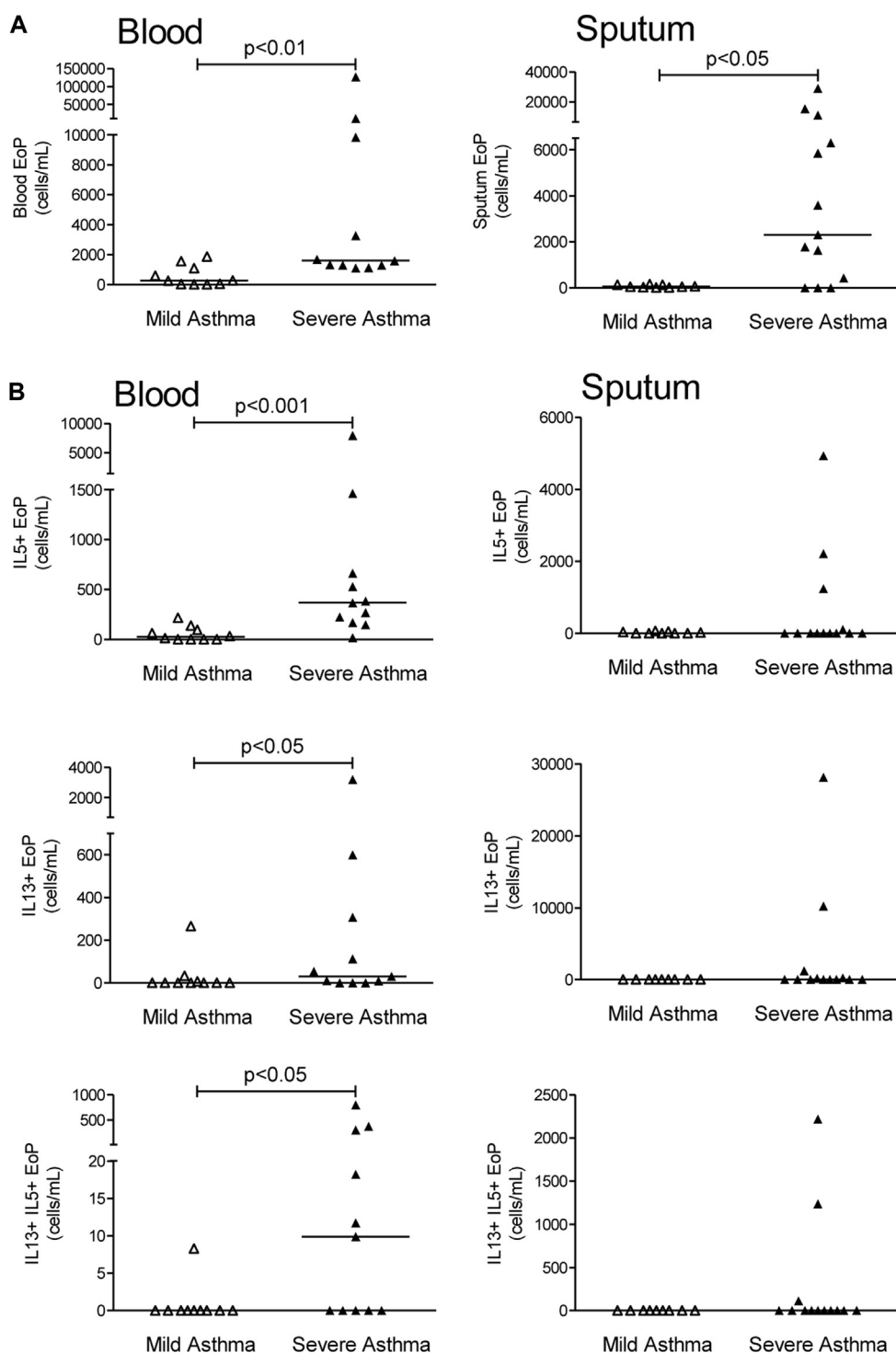


FIG 5. Comparison of absolute and intracellular cytokine expression levels of EoPs. **A**, Significantly higher CD34⁺CD45⁺IL-5R α ⁺ cell numbers were detected in blood and sputum of patients with severe asthma (n = 13) compared with value sin those with mild asthma (n = 9). **B**, Significantly greater numbers of EoPs expressing IL-5, IL-13, and both were found in the blood but not sputum of patients with severe asthma. Data are presented as cells per milliliter, and the bars represent median values of each data set.

n = 6; Fig 7, A). Based on these categorization criteria, there were significantly greater numbers of activated ILC2s (ILC2⁺IL-5⁺IL-13⁺ cells) present in induced sputum samples from the group with uncontrolled compared with controlled airway eosinophilia (Fig 7, B). Similar differences between the

groups of patients with severe asthma were not found in blood levels of ILC2s (see Fig E6 in this article's Online Repository at www.jacionline.org). In addition, there was no significant difference in the dose of oral corticosteroids that patients with controlled asthma were receiving (median, 7.5 mg/d; n = 6)

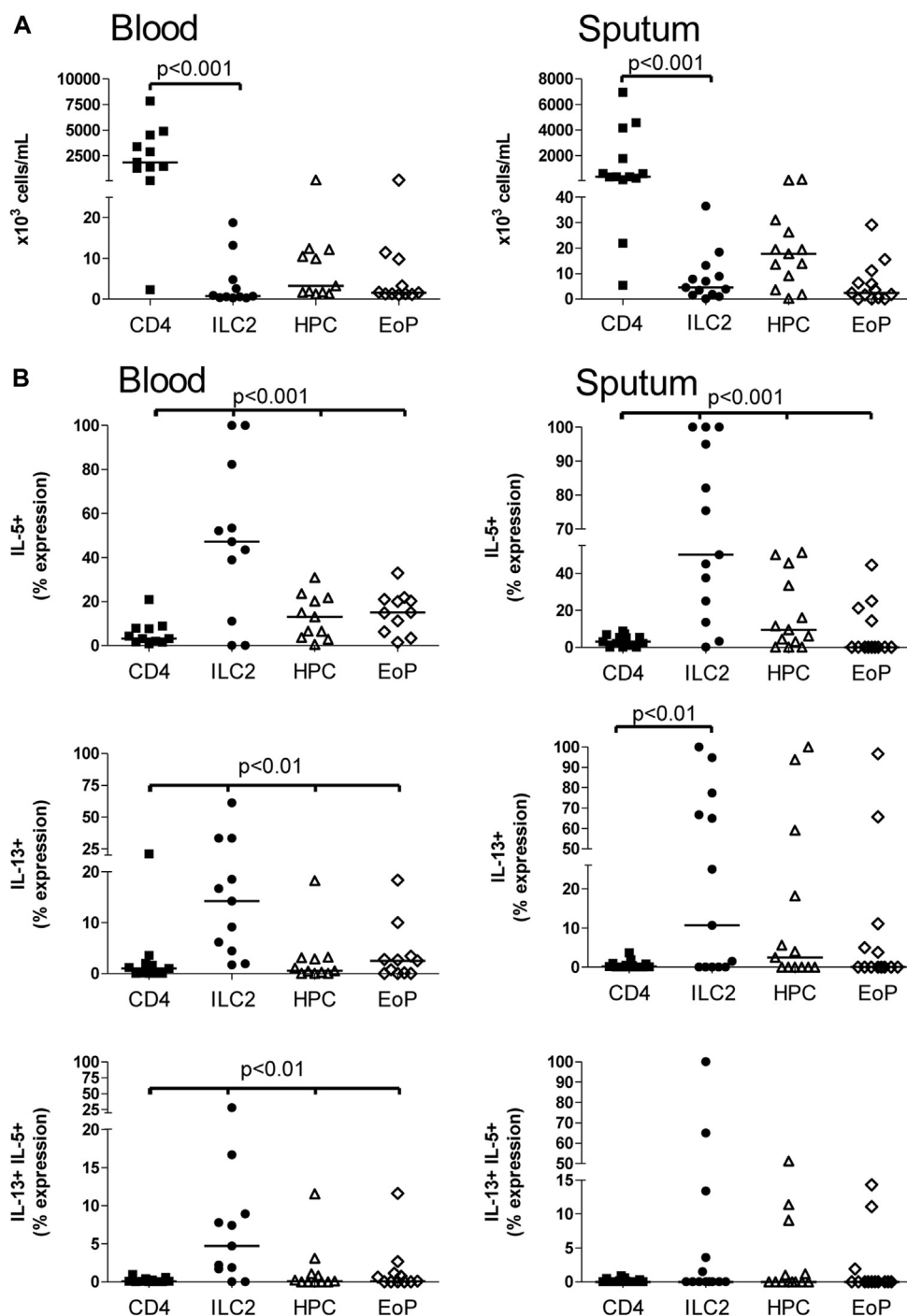


FIG 6. Absolute numbers and T_H2 cytokine expression by type 2 cells in patients with severe asthma. **A**, $CD4^+$ lymphocytes were more abundant than ILC2s, HPCs, and EoPs in both blood ($n = 11$) and sputum ($n = 13$). **B**, Proportionally, ILC2s are the predominant source of IL-5 and IL-13 in blood and sputum. The bars represent median values of each data set.

compared to those with uncontrolled asthma (median, 12.5 mg; $n = 9$) ($P = .22$).

DISCUSSION

For the first time, this study has demonstrated that airway lumen ILC2s can be enumerated in induced sputum samples by

using flow cytometry. Significantly greater numbers of total and type 2 cytokine-expressing ILC2s were found in the airways and peripheral blood of patients with prednisone-dependent severe eosinophilic asthma compared with those in asymptomatic patients with steroid-naïve mild atopic asthma. Although absolute numbers of $CD4^+$ lymphocytes are more abundant in sputum, proportionally, ILC2s are the predominant source of type 2

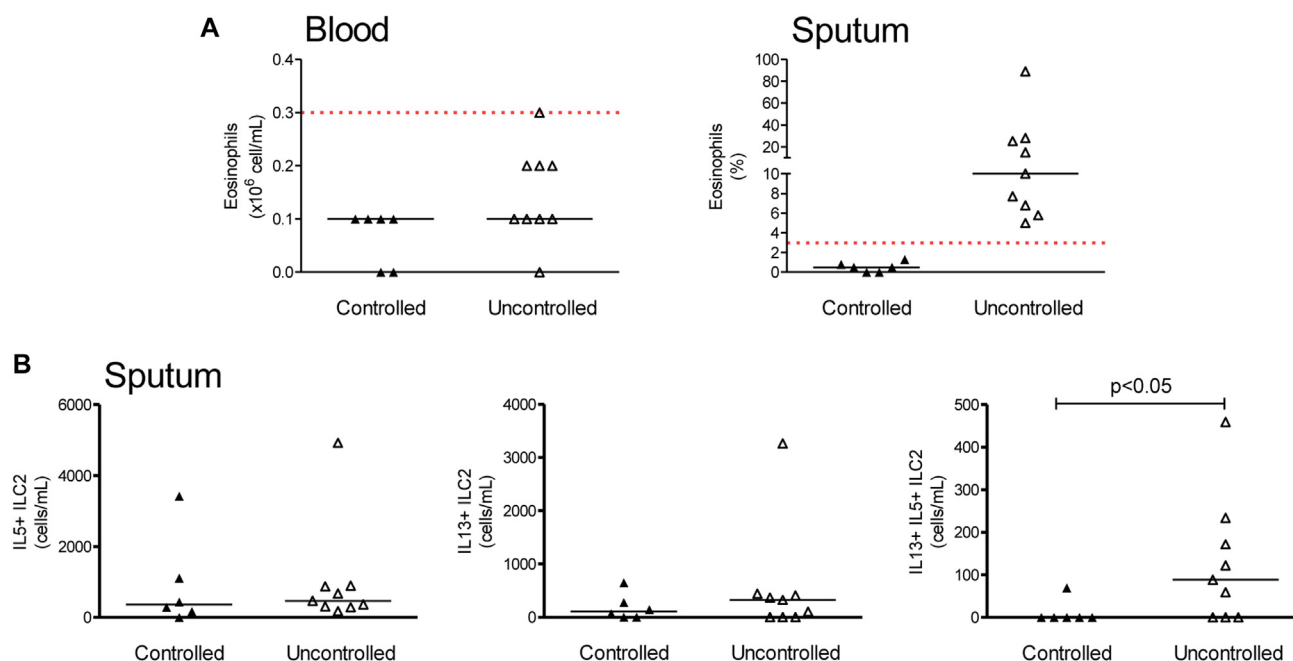


FIG 7. Heterogeneity of ILC2 activation. **A**, Patients with severe asthma, all with controlled blood eosinophil numbers of less than $0.3 \times 10^6/\text{mL}$, were grouped into those with controlled airway eosinophilia ($<3\%$ sputum eosinophils, $n = 5$) or uncontrolled airway eosinophilia ($>3\%$ sputum eosinophils, $n = 6$). **B**, Significantly higher numbers of ILC2s expressing IL-5 and IL-13 were detected in the sputum of patients with uncontrolled asthma compared with those in patients with controlled asthma.

cytokines within the airways of patients with severe asthma. In addition, there were significantly greater numbers of IL-5⁺IL-13⁺ ILC2s in the sputum but not blood of patients with severe asthma with uncontrolled airway eosinophilia despite blood eosinophil numbers being within the normal range. The findings of this study suggest that uncontrolled localized production of IL-5 and IL-13 by ILC2s might represent a steroid-insensitive population of cells that promotes the development of airway eosinophilia in patients with severe prednisone-dependent eosinophilic asthma.

The development of eosinophilic airway inflammation in asthmatic patients is associated with the ability of the bone marrow and affected tissues to support the mobilization, proliferation, and local differentiation of EoPs.^{46,47} It has been shown that numbers of IL-5-responsive EoPs are higher in the blood of patients with allergic asthma compared with healthy control subjects⁴⁴ and that these cells are increased within the airways after allergen or IL-5 challenge in patients with atopic asthma, where the increase in EoP precedes the development of airway eosinophilia.^{42,48,49} Consistent with these findings, the current study demonstrated that EoP numbers are increased in the blood and airways of patients with severe compared with mild asthma. That bone marrow-derived progenitors can differentiate locally is inferred by findings that CD34⁺ cells extracted from mucosal tissue undergo IL-5-stimulated proliferation and differentiation into eosinophil colonies *in vitro*.^{11,50} In a clinical trial, treatment with anti-IL-5 caused a significant attenuation of EoPs in the bronchial mucosa of asthmatic patients.⁵¹ This was associated with partial reduction in mature eosinophil numbers, indicating that controlling local IL-5-driven differentiation events might be an important strategy for attenuating lung eosinophilia in asthmatic patients. Similarly, treatment with inhaled antisense

oligonucleotides targeting the common β chain (βc) of the IL-3/IL-5/GM-CSF receptors and the chemokine receptor CCR3 showed a significant attenuation of allergen-induced increases in sputum EoP and mature eosinophil numbers and airway hyper-responsiveness in patients with mild asthma.⁴¹ In contrast, the inability of a potent oral CCR3 inhibitor to decrease sputum numbers of EoPs or mature eosinophils in patients with moderate asthma supports the view that local IL-5-driven *in situ* eosinophilopoietic events may be the predominant process promoting the development of airway eosinophilia in severe asthmatic patients.⁵² We have previously reported that airway structural cells, including airway smooth muscle cells, have the capacity to drive *in situ* eosinophilopoiesis through production of eosinophilopoietic cytokines, including IL-5.¹³ In the current study we show that in patients with severe asthma, numbers of IL-5⁺ ILC2s are significantly increased in the sputum of patients with severe asthma compared with numbers seen in those with mild asthma. Furthermore, there were significantly greater levels of IL-5⁺IL-13⁺ ILC2s in the sputum of patients with severe asthma with uncontrolled sputum eosinophilia, despite normal blood eosinophil numbers. This discordance of eosinophil levels might be related to the uncontrolled localized activation of ILC2s within the airways resulting in production of (1) IL-13, which can prime progenitor cell migrational responses promoting lung homing, and (2) IL-5-driven local maturational processes contributing to airway eosinophilia (Fig 8).

There is controversy within the literature over the potential steroid sensitivity of ILC2s. It has been reported that increased ILC2 numbers are found in eosinophilic nasal polyps and that these numbers correlate with tissue eosinophilia.⁵³ In addition, ILC2 levels in these steroid-naïve patients predicted responsiveness to corticosteroid therapy. On the other hand, *in vitro* studies

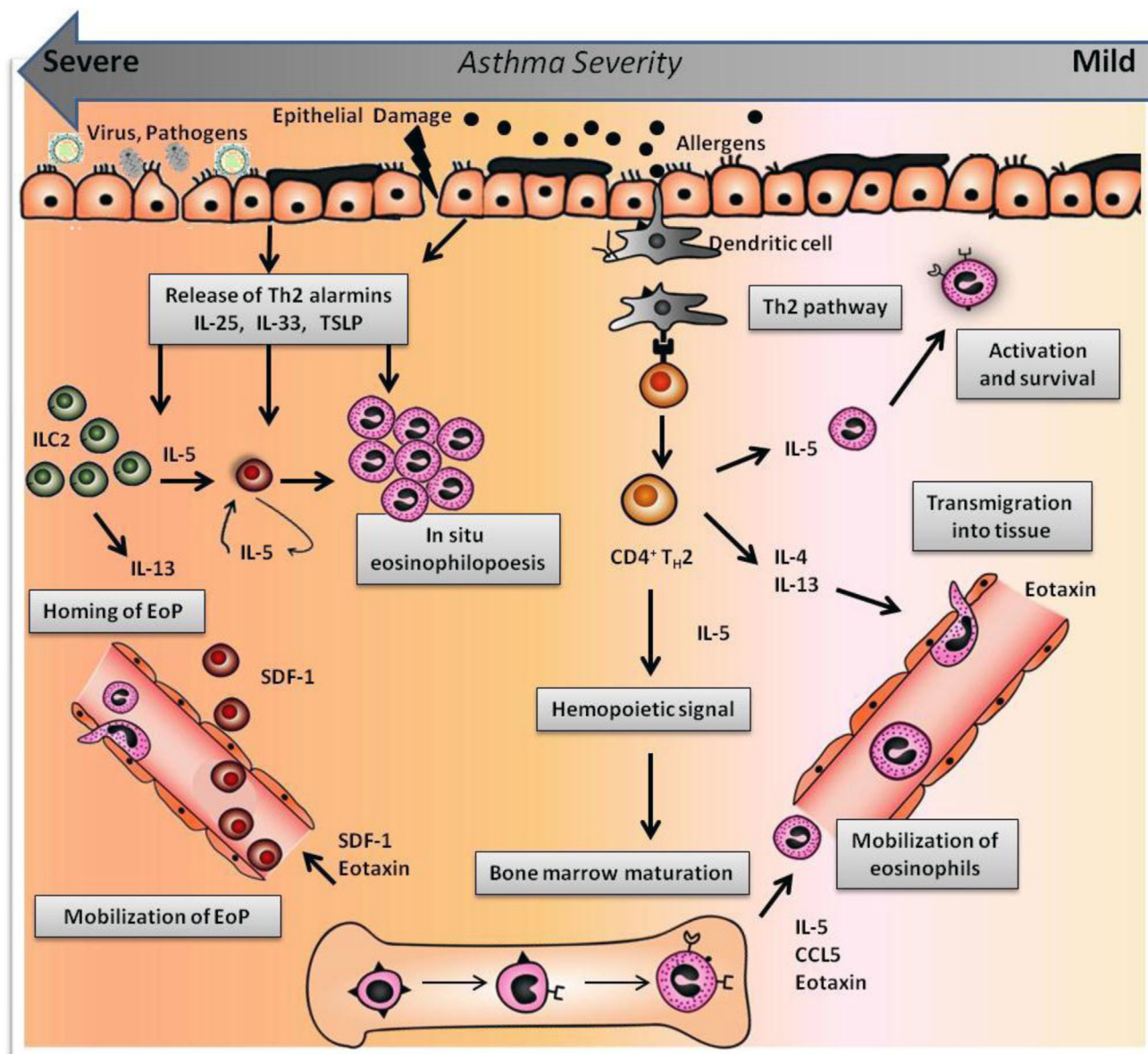


FIG 8. Diagrammatic representation of the predominant mechanisms promoting airway eosinophilia in patients with severe asthma versus those with mild asthma. We propose that in patients with prednisone-dependent severe asthma, in response to disruptive agents, epithelial cells release alarmins that activate ILC2s, which can drive *in situ* eosinophilopoiesis within the lung, likely through local production of IL-5 and IL-13. CD4 lymphocyte-mediated release of T_H2 cytokines stimulating lung homing of mature and immature eosinophils appears to be steroid sensitive and thus diminished in patients with severe asthma but not those with mild asthma, in whom we have previously shown that IL-4 and IL-13 prime migrational responses of progenitor cells and mature eosinophils to various chemoattractants. *SDF-1*, Stromal cell-derived factor 1.

show that ILC2s are steroid insensitive in the presence of TSLP.⁵⁴ This might explain why increased numbers of $CD45^+IL-13^+$ cells found to be non-T cells, mast cells, or eosinophils but considered to be nuocytes colocalized to TSLP-immunopositive regions within the bronchial mucosa of patients with prednisone dependent-severe asthma.⁵⁵ In agreement with this, the current study shows that both absolute numbers of ILC2s and levels of activation, as determined by intracellular levels of type 2 cytokines, are increased in the blood and sputum of patients with severe asthma receiving high-dose steroid therapy compared with those seen in patients with steroid-naïve mild asthma. In contrast,

as shown in Table E1, sputum and blood eosinophil levels are comparable between the 2 groups of asthmatic patients, likely because of the anti-inflammatory effects of high-dose steroid therapy. Similarly, despite the absolute numbers of $CD4^+$ lymphocytes being greater in patients with severe versus those with mild asthma, activation levels of these cells were not significantly different between the groups of asthmatic patients, suggesting that lymphocytes are responsive to steroid therapy in patients with severe asthma.

A limitation of the comparison of ILC2s and $CD4^+$ lymphocytes in this study might be that we have compared freshly

isolated cells without further *ex vivo* stimulation. Despite this, we found significant differences between patients with mild asthma and those with severe asthma for all cell types. The latter group were currently symptomatic with chronic ongoing airway inflammation, suggesting that *in vivo* chronic activational events were responsible for the observed changes in intracellular type 2 cytokine expression, despite regular oral corticosteroid therapy.

In summary, our findings suggest that mechanisms that increase ILC2 numbers and activation are resistant to steroid therapy. This is supported by the findings that numbers of ILC2s expressing both IL-5 and IL-3 are greatest in sputum samples of patients with severe asthma with increased sputum eosinophil numbers (>3% eosinophilia) despite normalized blood eosinophil numbers (<300 cells/ μ L). We propose that uncontrolled activation of ILC2s can provide a type 2 cytokine proinflammatory environment that promotes the development of uncontrolled airway eosinophilia. It has been suggested that epigenetic changes within the bronchial epithelium in patients with chronic inflammatory conditions can promote the continued production of IL-33.^{20,39} In turn, this might promote continued activation of local ILC2s, thus providing a feed-forward circuit through which progenitor cells are recruited to the airways by IL-13 and stimulated to differentiate locally into mature eosinophils by IL-5. Targeting IL-33 or ILC2s that express IL-5 (by using anti-IL-5R antibody) and/or IL-13 (by using anti-IL-13 antibody) might be beneficial in patients who have steroid-dependent asthma with persistent airway eosinophilia.

We thank the subjects for volunteering their participation in this study. We thank Rick Watson and Katherine Radford for their expert technical assistance and Dr Manali Mukherjee, PhD, for developing a diagrammatic summary of our findings in Fig 8.

Key messages

- ILC2s are detectable in the airways of patients with severe asthma.
- Absolute numbers of ILC2s and Th2 type 2 cytokine-positive ILC2s were significantly greater in the blood and sputum of patients with severe asthma compared with those in patients with mild atopic asthma and healthy control subjects.
- Although absolute numbers of CD4⁺ and EoPs were increased in the sputum of patients with severe asthma, intracellular cytokine levels were comparable with those in patients with mild asthma.
- ILC2s that coexpressed intracellular IL-5 and IL-13 were significantly more abundant in the sputum of patients with severe asthma with uncontrolled airway eosinophilia despite normal blood eosinophil levels.

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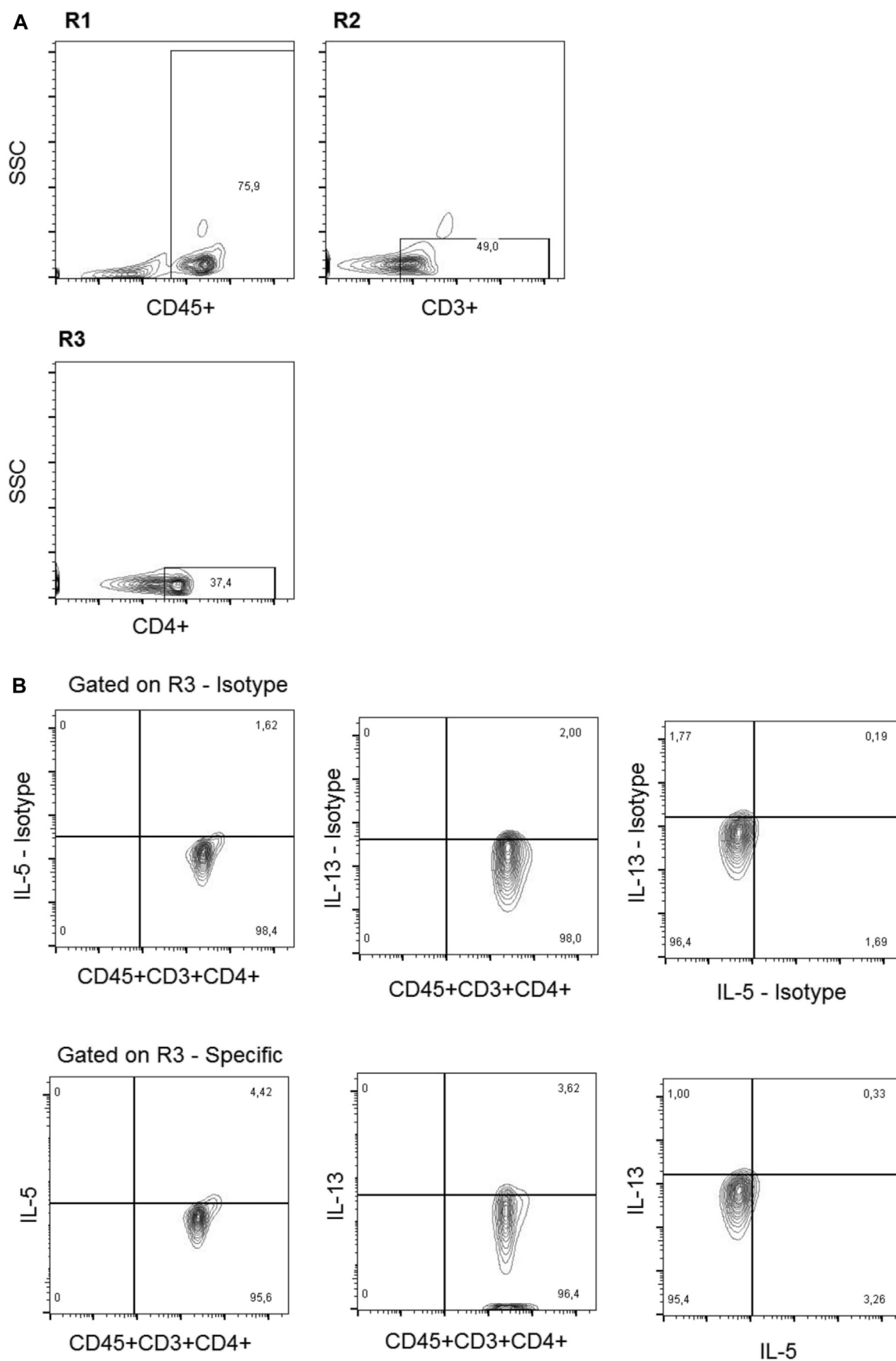


FIG E1. CD3⁺CD4⁺ lymphocyte gating strategy in sputum. **A**, identification of CD45⁺CD3⁺CD4⁺ lymphocytes by using FlowJo software. **B**, IL-5, IL-13, or both intracellular cytokine expression compared with the isotype control, as assessed with a 98% confidence limit.

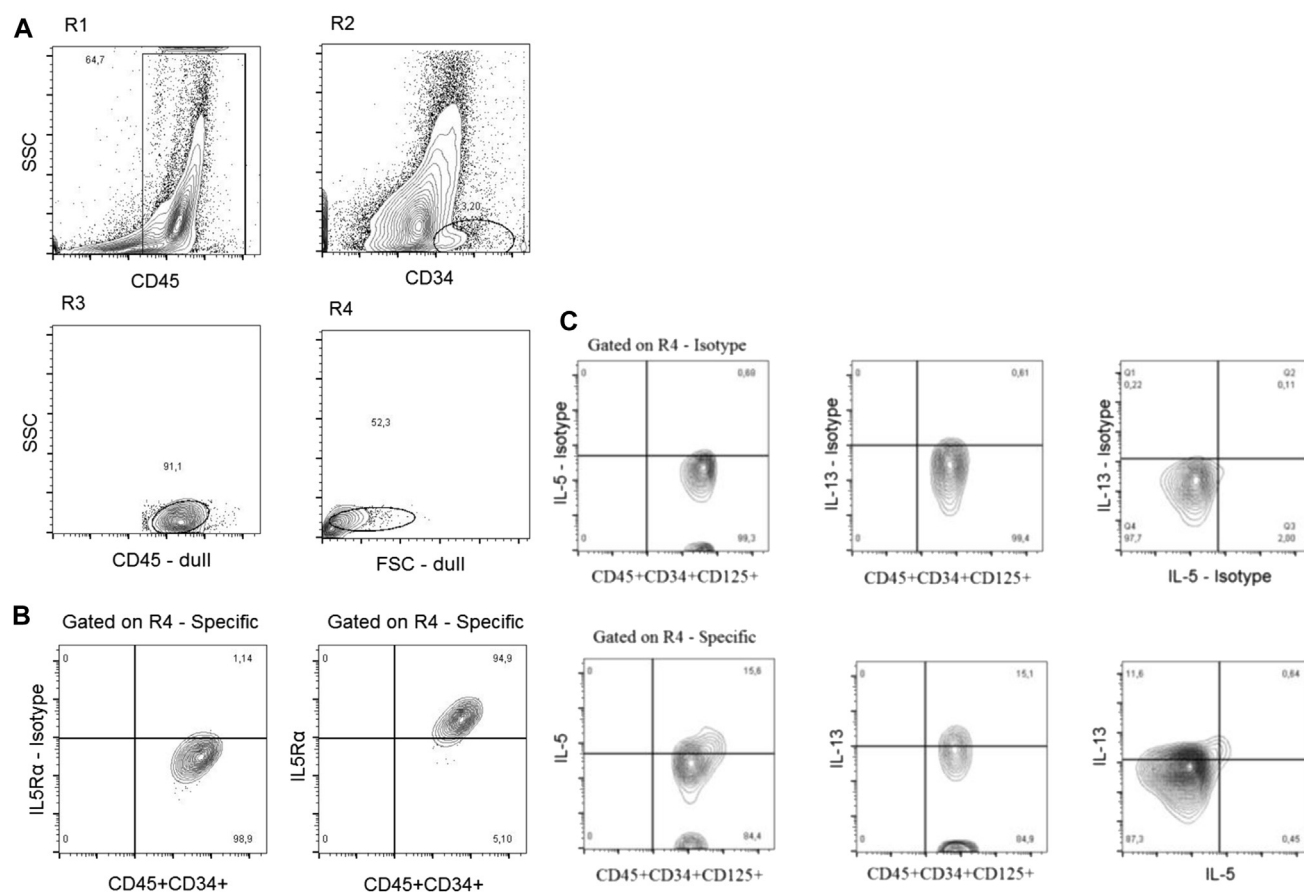


FIG E2. Sequential multigating strategy. **A**, Identification of HPCs ($CD34^+CD45^+$ cells) by using FlowJo software. Scatter plots show side scatter, and the CD45 region R1 identifies nucleated white blood cells. Region R2 identifies the $CD34^+$ marker. True $CD34^+$ cells are further isolated based on low granularity and low $CD45^+$ marker expression (region R3). HPCs are characterized by medium size (medium forward scatter [FSC]) and low granularity (low side scatter [SSC]) region R4. **B**, EoPs are identified as HPCs expressing CD125 compared with the isotype control with a 98% confidence limit. **C**, Intracellular cytokine expression of IL-5, IL-13, or both compared with isotype control assessed with a 98% confidence limit.

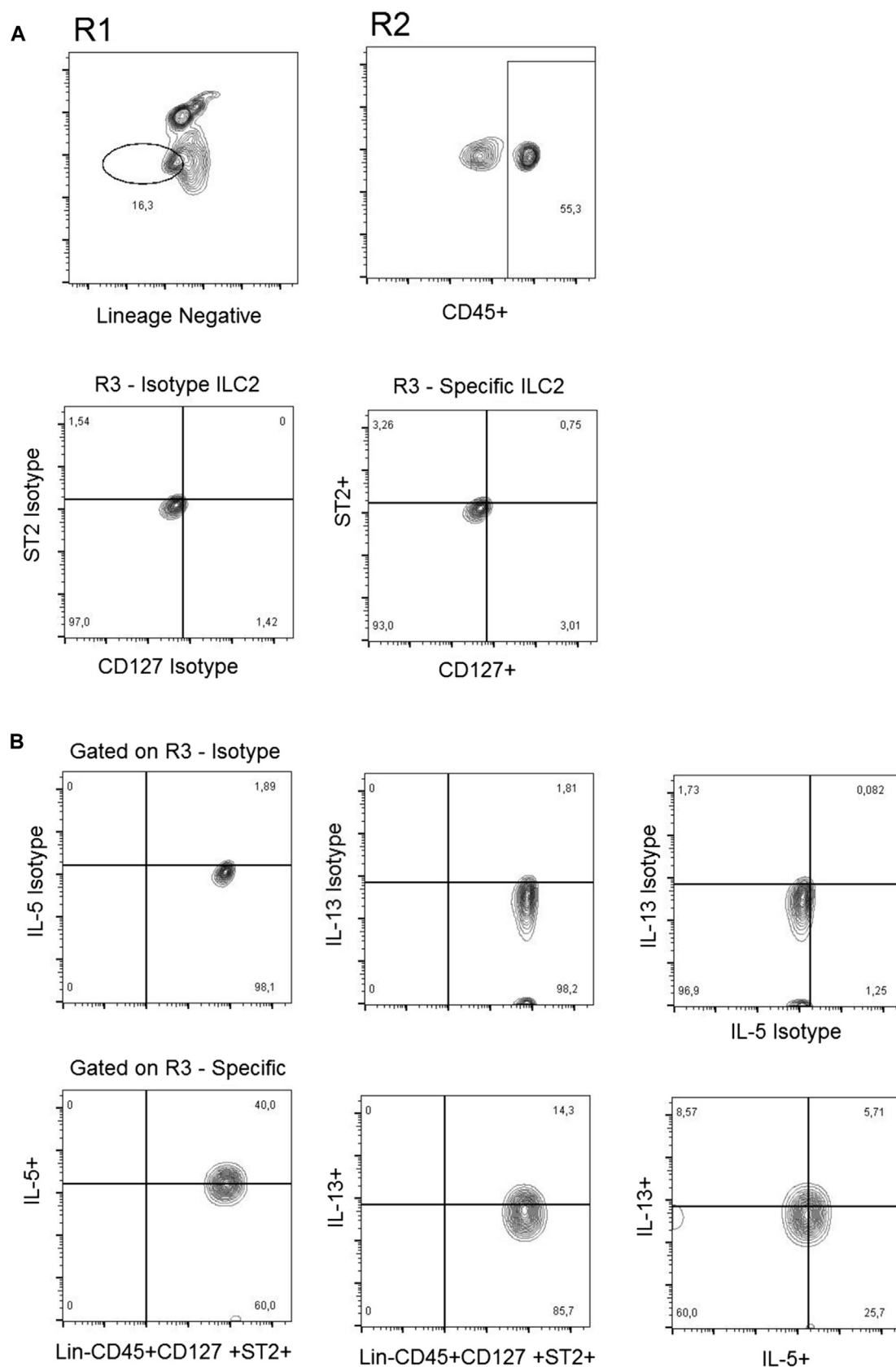


FIG E3. Multigating strategy for ILC2 enumeration from blood. Representative plots from 1 patient in whom, by means of sequential gating, blood-derived low-density mononuclear cells were identified as **(A)** Lin[−] (region R1), CD45⁺ (region R2), and CD127⁺ST2⁺ cells (region R3; **B**) and intracellular IL-5⁺, IL-13⁺, and IL-5⁺IL-13⁺ ILCs were enumerated (**B**) compared with isotype control by using a 98% confidence limit.

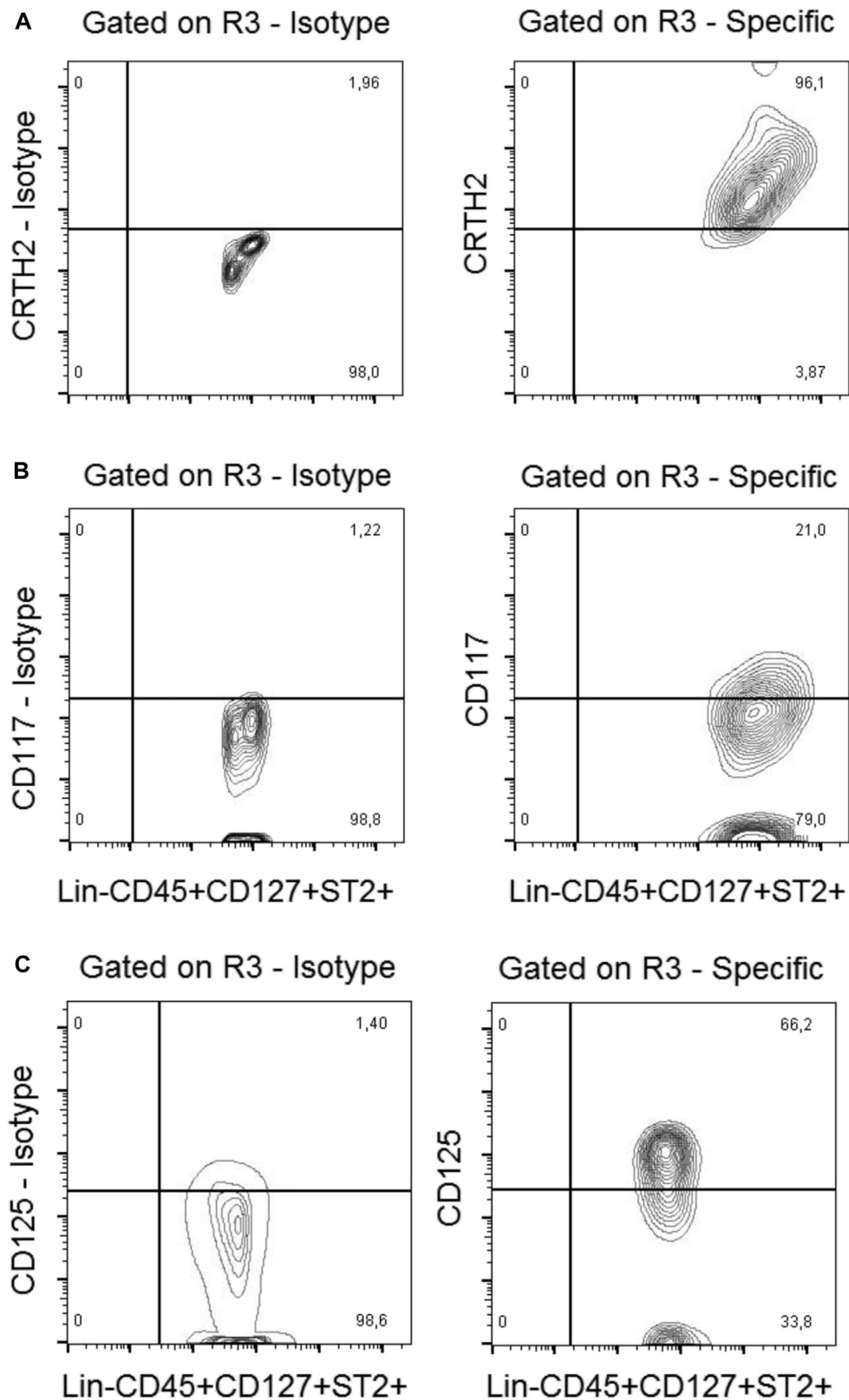


FIG E4. Multigating strategy for receptor expression on sputum ILC2s. Cells identified as Lin⁺CD45⁺CD127⁺ST2⁺ cells (as in Fig 1) coexpress chemoattractant receptor-homologous molecule expressed on T_H2 lymphocytes (*CRTH2*; **A**), c-Kit (*CD117*; **B**), and CD125 (*IL-5Rα*; **C**) compared with the isotype control. Gating was set at a 99% confidence limit.

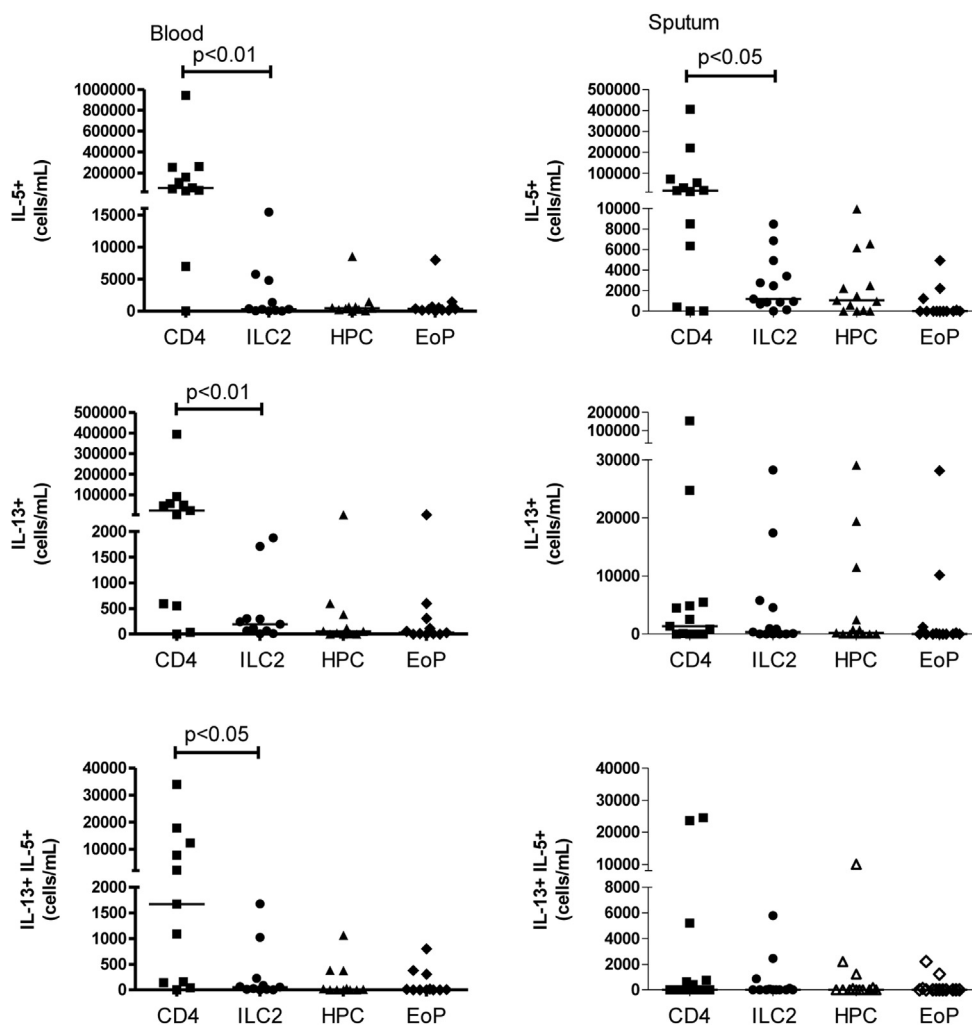


FIG E5. Comparisons of absolute numbers of type 2 cells expressing T_H2 cytokines in patients with severe asthma. Compared with ILC2s, HPCs, and EoPs, significantly greater absolute numbers of $CD4^+$ lymphocytes expressing IL-5, IL-13, or both were found in the blood and $CD4^+IL-5^+$ in the sputum of patients with severe asthma ($n = 13$). Bars represent median values of each data set.

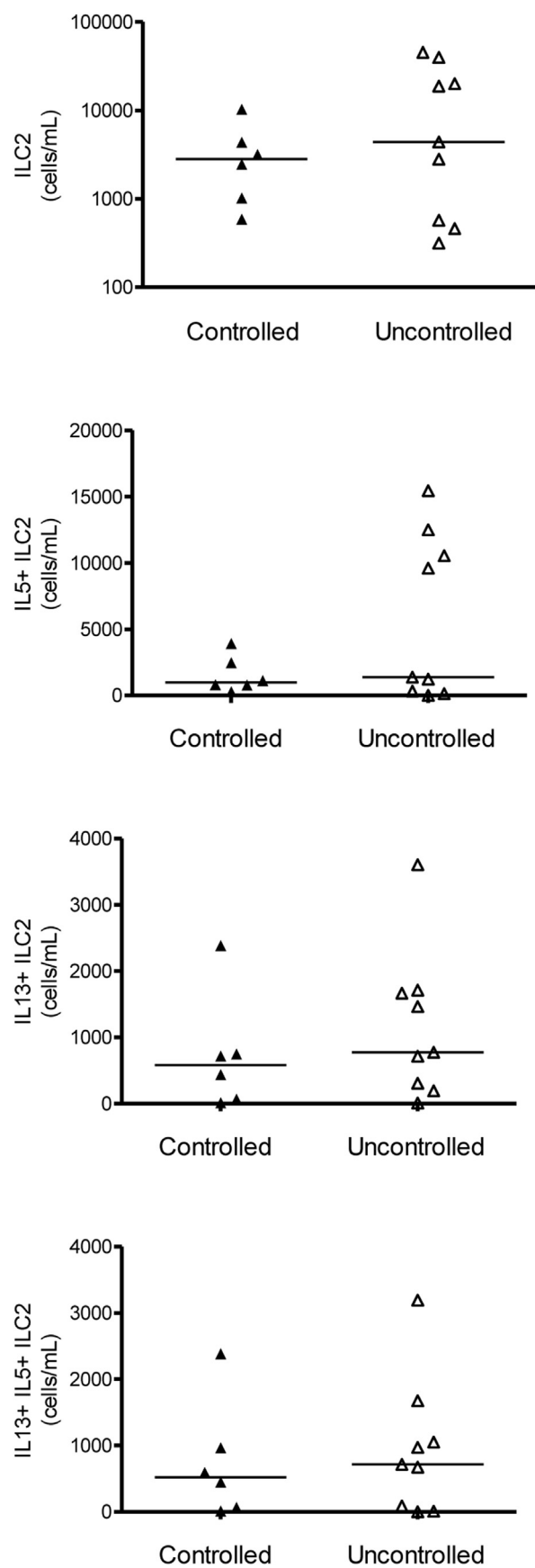


FIG E6. ILC2 levels in blood of patients with controlled and those with uncontrolled severe asthma. Patients with severe asthma, all with controlled blood eosinophils of less than $0.3 \times 10^6/\text{mL}$, were grouped into those with controlled airway eosinophilia ($<3\%$ sputum eosinophils, $n = 5$) or uncontrolled airway eosinophilia ($>3\%$ sputum eosinophils, $n = 6$). No differences were observed in blood ILC2 activation levels between these groups of patients with severe asthma.

TABLE E1. Baseline subject characteristics for type 2 cell enumerations

Characteristic	Patients with severe asthma	Patients with mild atopic asthma
No. of patients	25	19
Age (y)	49.4 ± 10.7	34.8 ± 13.4
Sex (% male)	48	37
Polyposis/CRS (no.)	15	0
Aspirin sensitivity (no.)	1	0
Smoking (exsmoker; <10 pack years [no.]	9	0
Prednisone (mg/d)		
Median	12.5	0
Minimum-Maximum	7.5-40.0	0
ICS dose* (total daily dose)		
Median	1500	0
Minimum-Maximum	1000-3250	0
Long-acting β-agonist (no. of patients)	25	0
Leukotriene receptor antagonist (no. of patients)	9	0
Blood total leukocytes (×10 ⁶ /mL)		
Median	8.9 [†]	5.6
Minimum-Maximum	5.3-14.8	3.05-8.09
Blood eosinophils (×10 ⁶ /mL)		
Median	0.4	0.34
Minimum-maximum	0.15-0.75	0.10-2.8
Sputum total leukocytes (×10 ⁶ /mL)		
Median	9.9	4.59
Minimum-maximum	1.9-1.04	0.99-9.51
Sputum eosinophils (%)		
Median	3.05	2.13
Minimum-maximum	0-89.0	0-17.6
FEV ₁ (% predicted before BD)	56.8 ± 16.3	96.2 ± 7.4
FEV ₁ (L)	1.84 ± 0.57	3.67 ± 0.74
FVC (L)	3.0 ± 0.78	4.5 ± 1.02
FEV ₁ /FVC ratio (%)	65.6 ± 12.8	89.2 ± 21.1
FEV ₁ reversibility (%)	11.9 ± 7.97	ND
Methacholine PC ₂₀	1.21 ± 0.35	3.97 ± 3.6

Unless otherwise stated, data are presented as means ± SDs.

BD, Bronchodilator; CRS, chronic rhinosinusitis; FVC, forced vital capacity; ICS, inhaled corticosteroid; ND, not done.

*ICS dose is the equivalent of inhaled fluticasone.

[†]*P* < .05 for between-group comparisons.

TABLE E2. Enumeration of blood-derived type 2 cells and T_H2 intracellular cytokine expression from healthy control subjects

Cell type	Total cells (cells/mL)	IL-5 ⁺ cells (cells/mL)	IL-13 ⁺ cells (cells/mL)	IL-5 ⁺ IL-13 ⁺ cells (cells/mL)
ILC2s	17,694 ± 1,531*	33 ± 14* (39% ± 19%)	35 ± 28* (8% ± 6%)	4 ± 4* (7% ± 7%)*
CD4 cells	2,638,725 ± 534,302	39,628 ± 4,468† (2% ± 0.2%)*†	38,991 ± 10,712 (1% ± 0.1%)	1,006 ± 129 (0.04% ± 0.01%)
HPCs	4,357 ± 1,144	78 ± 25* (3% ± 1%)*	44 ± 22 (1% ± 0.4%)	3 ± 3 (0.04% ± 0.04%)
EoPs	1,942 ± 370	50 ± 25* (3% ± 2%)*	27 ± 17 (1% ± 1%)	3 ± 3 (0.1% ± 0.1%)

Analyses of absolute and T_H2 cytokine expression by various type 2 cells was assessed in the blood of healthy nonatopic asymptomatic control subjects (n = 5). Data are presented as means ± SEMs and in parentheses as proportional expression of T_H2 cytokines for each cell population.

Cell numbers in blood from healthy subjects were significantly lower than those in blood from asthmatic patients:

**P* < .05 for comparisons with patients with severe asthma; and

†*P* < .05 for comparison with patients with mild asthma.