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$^+$127$^+$ST2$^+$) and levels of intracellular IL-5 and IL-13 in patients with severe asthma (n = 25), patients with steroid-naive mild atopic asthma (n = 19), and nonatopic control subjects (n = 5). Results were compared with numbers of CD4$^+$ 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 2016;137:75-86.)

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 uncontrolled[3] or severe[4] 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

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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

progenitors, thereby likely promoting lung homing of these cells in 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 responses[27-29] 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 FEV_1 of less than 80% of predicted value, greater than 12% FEV_1 reversibility on challenge with short-acting bronchodilator, PC_{20} 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 μg/d), and eosinophilic inflammation determined by a blood eosinophil count of 150 cells/μL or greater currently or less than 300 cells/μL within the past 12 months. Patients with mild atopic asthma had positive skin prick test responses, FEV_1 of 70% of predicted value or greater, greater than 12% FEV_1 reversibility, and PC_{20} 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-μm 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% CO_2 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

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