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Group 2 innate lymphoid cells promote airway hyperresponsiveness through production of VEGFA.

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Authors
Shen, Xiaofei
Pasha, Muhammad Asghar
Hidde, Kelsi
et al.

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To the Editor:

Asthma is a complex and heterogeneous disease characterized by chronic airway inflammation, airway hyperresponsiveness (AHR), and airway remodeling. We recently demonstrated the importance of group 2 innate lymphoid cells (ILC2s) in AHR.1 Their precise roles in asthma, and the specific effector mechanisms through which ILC2s would promote AHR, remain unclear. Recent work indicates that ILC2s may have important functions other than producing classical T helper cytokines. By reanalyzing previously published microarray data,2 we found that VEGFA was one of the highest expressed genes in activated ILC2.

Vascular endothelial growth factor A (VEGFA) (formerly VEGF) is a protein member of the platelet-derived growth factor/VEGF family. VEGFA was shown to induce eosinophilic airway inflammation, mucus metaplasia, subepithelial fibrosis, myocyte hyperplasia, dendritic cell activation, and to elicit AHR through IL-13-dependent and -independent mechanisms.3 To investigate the relevance of VEGFA expression in human ILC2s, we isolated ILC2s from the blood of healthy controls and patients with asthma and examined mRNA expression of Vegfa together with other VEGF family members (see Table E1 in this article’s Online Repository at www.jacionline.org). Human ILC2 were identified as CD45⁻Lin⁻IL-7Rα⁺CRTH2⁺ cells (Lin = lineage; see Fig E1 in this article’s Online Repository at www.jacionline.org). ILC2 in healthy controls expressed very low or undetectable levels of Vegfa mRNA (Fig 1, A). However, Vegfa mRNA expression in ILC2 of patients with asthma was significantly increased (P < .05, Mann-Whitney U test) (Fig 1, A). Vegfb and Vegfc mRNA was also detected in human ILC2s, but the expression levels of these 2 molecules were comparable between ILC2s from healthy controls and those from patients with asthma (Fig 1, A). Vegfd and Pgf mRNA was undetectable in human ILC2s from either healthy controls or patients with asthma. Together, these data indicated that Vegfa mRNA expression in ILC2s is selectively upregulated in human asthma. To study whether such differences in gene activation were associated with differential VEGFA protein expression, we sorted human ILC2s and cultured them with IL-25, IL-33, and IL-2. ILC2s from both healthy controls and patients with asthma were capable of producing VEGFA protein in vitro (Fig 1, B). However, ILC2s from patients with asthma produced higher amounts of VEGFA in response to activating cytokines (Fig 1, B). Of note, ILC2s cultured without cytokines failed to produce VEGFA, verifying that production of VEGFA is a signature of ILC2 activation (Fig E1).

To examine the in vivo relevance of ILC2 activation, we treated mice with IL-33. Mouse lung ILC2s were identified as CD45⁻Lin⁻Thy1⁺ST2⁺ cells (see Fig E2 in this article’s Online Repository at www.jacionline.org). mRNA expression of Il13 and Il5 was markedly increased in lung ILC2s as early as 12 hours after IL-33 treatment (Fig 2, A). Lung ILC2s but not T cells upregulated Vegfa mRNA expression at this time point (Fig 2, B). Using ELISA, we confirmed that activated mouse ILC2s produced VEGFA protein in the presence of IL-2 and IL-33 (Fig 2, C). Of note, ILC2s cultured with IL-2 alone, or cultured without cytokines, did not produce VEGFA (Fig 2, C). Thus, VEGFA production by ILC2s requires IL-33.

Our previous work indicated that activated ILC2s are potent inducers of AHR.1 Because high amounts of VEGFA itself can cause AHR,3 we hypothesized that at least in part, VEGFA mediates this ILC2 effect. IL-33, an epithelial-derived alarmin, is a crucial activator of ILC2s in allergic inflammation.4 We thus treated BALB/c mice with IL-33 in combination with SU1498, a specific and potent inhibitor of VEGF receptor 2 (VEGFR2).3 IL-33 treatment elicited AHR in mice within 12 hours. This effect was abolished by SU1498 (Fig 2, D).

To confirm the relevance of the IL-33/ILC2/VEGF axis, we used a model of allergic airway inflammation. Alternaria alternata is a common fungal allergen that can trigger acute and severe asthma attacks. Alternaria inhalation causes rapid and strong ILC2 activation through airway release of IL-33.5,6 We thus tested the role of VEGFA in Alternaria-induced airway responses. A single dose of Alternaria inhalation induced airway eosinophil and neutrophil infiltration within 12 hours (Fig 2, E). Inhibition of VEGF signaling by SU1498 treatment abolished eosinophil infiltration and reduced

FIG 1. ILC2 in asthma patients expressed high amounts of VEGFA. A, mRNA expression of the indicated genes in sorted peripheral blood ILC2s from healthy controls and patients with asthma. B, VEGFA concentrations in the supernatant of ILC2s isolated from the peripheral blood of healthy controls and patients with asthma and cultured with IL-2, IL-33, and IL-25 for 7 days. n = 3 independent experiments.

*P < .05, n.s., Not significant.
neutrophil infiltration (Fig 2, E). *Alternaria* inhalation also rapidly induced strong AHR within 12 hours that was significantly suppressed by SU1498, demonstrating that VEGFA is required for *Alternaria*-induced AHR (Fig 2, F). Other cells, such as mast cells and CD4+ T cells, can also produce VEGFA.7,9 To verify the importance of ILC2s in VEGFA-induced AHR, we performed adoptive transfer of these cells. We transferred ILC2s into *Rag2*−/−*Il2rg*−/− mice lacking all adaptive and innate lymphoid cells, in combination with SU1498. *Alternaria* inhalation elicited very weak AHR in *Rag2*−/−*Il2rg*−/− mice (Fig E2). Adoptive transfer of ILC2s significantly enhanced AHR that was inhibited by SU1498 (Fig 2, G). Taken together, these results indicated that in allergic airway inflammation, ILC2s are a significant source of VEGFA necessary and sufficient to elicit AHR in mice.

Activated ILC2s can also produce classical type 2 cytokines such as IL-5 and IL-13.1 Interestingly, activated ILC2s in IL-33-treated mice upregulated surface expression of VEGFR2 (Fig 2, H). Inhibition of VEGF signaling by SU1498 treatment repressed ILC2 activation as demonstrated by reduced IL-13 expression (Fig 2, I and J). Thus, ILC2 produces VEGFA in an autocrine manner. We next treated mice with anti-IL-13 neutralizing antibody to examine AHR that develops in the absence of IL-13. Neutralization of IL-13 reduced, but did not completely prevent AHR in mice that inhaled *Alternaria* extracts (Fig 2, K). Inhibition of VEGF signaling by SU1498 treatment further reduced AHR even in the absence of IL-13 (Fig 2, K). Thus, ILC2-derived VEGFA might promote AHR through both IL-13-dependent and IL-13-independent mechanisms. Although more work is needed to decipher the proinflammatory characteristics of VEGFA, our data indicate that autocrine regulation of ILC2 responses by VEGFA may underlie the remarkable capability of ILC2 to initiate and amplify allergic airway inflammation in asthma development and exacerbation.

In summary, we have identified VEGFA as a novel effector molecule produced by both human and mouse ILC2s. We show that ILC2s from patients with asthma expressed increased levels of neutrophils and VEGFA, indicating that these cells may contribute to the pathogenesis of asthma. The role of VEGFA in allergic airway inflammation may be further explored in future studies to develop new therapeutic strategies for the treatment of asthma.
of Vegfa mRNA. Activation of ILC2s by IL-33 treatment or Alternaria inhalation in mice rapidly caused AHR that was prevented by inhibition of VEGF signaling, indicating a critical role of VEGFA in ILC2-induced AHR. Inhibition of VEGF signaling also repressed the ability of ILC2 to produce IL-13, suggesting an autocrine regulatory mechanism. Presence of VEGFAhi ILC2s may denote a subgroup of severe asthma.

Xiaofei Shen, MDa,b
Muhammad Asghar Pasha, MDc
Kelsi Hidde, BSb
Adil Khan, MDp
Mingwei Liang, PhDp
Wenshuan Guan, MD, PhDa
Yitao Ding, MD, PhDd
Angela Haczku, MD, PhDd
Qi Yang, PhDp

From the Department of General Surgery, Nanjing Drum Tower Hospital, the Affiliated Hospital of Nanjing University Medical School, Nanjing, China; the Division of Allergy/Immunology, Department of Medicine, Albany Medical College, Albany, NY; and the Pulmonary, Critical Care, and Sleep Division, University of California, Davis, Calif. E-mail: haczku@ucdavis.edu. Or: yangq@mail.amc.edu.

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REFERENCES
METHODS

Study subjects and samples
Patients with diagnosis of asthma and healthy controls were recruited at the Division of Allergy and Immunology of Albany Medical Center. All patients with asthma continue to take their daily asthma medications, as prescribed. Asthma severity was classified based on the 2007 Expert Panel Guidelines for the Diagnosis and Management of Asthma.1 Informed consent was obtained from all participants. PBMCs were isolated by Ficoll gradient centrifugation. ILC2s were sorted by flow cytometric cell sorting, and mRNA was extracted. Gene expression was examined using quantitative PCR analysis as we previously described.2-6 The study was approved by the Albany Medical Center Institutional Review Board.

Mice, allergen and cytokine challenge, adoptive transfer and FlexiVent analysis
Female and male BALB/c mice between 8 and 12 weeks old were purchased from Taconic Biosciences (Hudson, NY) or the Jackson Laboratory (Bar Harbor, Me). Rag2⁻/⁻ mice were purchased from the Jackson Laboratory. Mice were housed in specific pathogen-free conditions. For IL-33 treatment, 0.8 µg recombinant mIL-33 (BioLegend, San Diego, Calif) was administered once intraperitoneally at 12 hours before FlexiVent analysis. For Alternaria challenge. 100 µg Alternate extracts (GREER, Lenoir, NC) were administered once intranasally at 12 hours before FlexiVent analysis. In some mice, SU1498 was administrated as previously described.7 Specifically, mice were injected with 10 mg/kg of SU1498 intraperitoneally at 24 hours before IL-33 treatment, or intratracheally at 24 hours before Alternaria administration. For adoptive transfer experiments, ILC2s were sorted from the lungs of recombinant mIL-33-treated BALB/c mice. Then 5 × 10⁵ cells were transferred intravenously into Rag2⁻/⁻I2rg⁻/⁻ mice at 12 hours before Alternaria administration. Airway hyperresponsiveness was measured by a FlexiVent system (SCIReq) as we previously described.5,6 All mouse procedures were approved by the Institutional Animal Care and Use Committee of Albany Medical College.

Flow cytometry and cell sorting
Human ILC2s were identified as CD45⁻Lin IL7Ra⁻CRHT2⁺ cells. Mouse lung-resident ILC2s were identified as CD45⁻Lin Thy1.2⁻ST2⁺ cells as we previously described.5,6,12 Antilineage antibodies for human ILC2s included anti-CD19 (HB19), anti-CD3 (UCHT1), anti-CD5 (L17F12), anti-CD16 (3G8), anti-CD11c (3.9), anti-CD123 (6H6), anti-CD49 (DX22), and anti-CD34 (581). Lineage antibodies for mouse ILC2s included anti-B220 (RA3-6B2), anti-CD3 (2C11), anti-TCRβ (H57), anti-TCRγδ (GL-3), anti-CD11b (M1/70), and anti-CD5 (53.7.3). Other antibodies used include anti-ST2 (D78), anti-Thy1 (53-2.1), anti-CD25 (PC61.5), anti-VEGFR2 (89B3A5), anti-IL-13 (eBio13A), anti-CRTH2 (BM16), anti-CD127 (A019D5), and anti-CD117 (104D2). Antibodies were purchased from eBioscience (San Diego, Calif), BioLegend or MD Bioproducts (Oakdale, Minn).

Flow cytometric analysis was performed on a 3-laser FACS Canto (BD Biosciences, San Jose, Calif) and cell sorting was performed on a FACSAria II (BD Biosciences).

Cell culture
Sorted human ILC2s were cultured with α-minimal essential medium containing 20% FCS with or without 10 ng/mL recombinant IL-2, IL-25, and IL-33 for 7 days. Mouse ILC2s were cultured with α-minimal essential medium containing 20% FCS with or without 10 ng/mL recombinant mouse IL-2 and IL-33 for 7 days. Cytokines were purchased from BioLegend or R&D Systems (Minneapolis, Minn).

VEGFA measurement
The concentration of VEGFA in the supernatant of cultured human ILC2s was measured by the LEGENDplex bead-based multiplex assay (BioLegend) according to the manufacturer’s protocol. The production of VEGFA by cultured mouse ILC2s was measured by standard sandwich ELISA (R&D Systems).

Assessment of cytokine production by lung-resident ILC2s
Mice were exsanguinated and lungs were perfused by injecting 10 ml PBS into the right ventricle of the heart. Lungs were cut into small fragments and digested in HBSS containing 0.1 mg/mL Liberase TL (Roche Diagnostics, Risch-Rotkreuz, Switzerland) and 10 U/mL DNase I (Roche Diagnostics). After being filtered through a 100 µm cell strainer, the cells were incubated with 1 µg/mL monensin for 2 hours. Cells were then stained for surface antibodies followed by intracellular staining of IL-13. Intracellular staining of IL-13 ILC2s was performed using a fixation/permeability kit (BD Bioscience) as we previously described.5,6

Statistical analysis
Mann-Whitney U test was used to compare the difference in mRNA expression levels of isolated blood ILC2s between patients with asthma and healthy controls as well as their production of VEGFA in vivo. Student t test was used in all other experiments. P < .05 was considered statistically significant.

REFERENCES
FIG E1. **A**, Representative flow cytometry plots of human peripheral blood ILC2s. **B**, VEGFA concentrations in the supernatant of isolated human ILC2s cultured with or without 10 ng/mL of IL-2, IL-33, and IL-25 for 7 days.
FIG E2. A, Representative flow cytometry plots of mouse lung ILC2s. B, FlexiVent analysis of BALB/c mice and Rag2−/−Il2rg−/− mice that received intranasal administration of PBS or Alternaria extracts. *P < .05 and **P < .01.
TABLE E1. Characteristics of human subjects

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F, Female; ICS, inhaled corticosteroid; LABA, long-acting beta-agonist; LTM, leukotriene modifier; M, male; SABD, short-acting bronchodilator.