Background: Asthmatic patients are highly susceptible to air pollution and in particular to the effects of ozone (O3) inhalation, but the underlying mechanisms remain unclear.

Objective: Using mouse models of O3-induced airway inflammation and airway hyperresponsiveness (AHR), we sought to investigate the role of the recently discovered group 2 innate lymphoid cells (ILC2s).

Methods: C57BL/6 and BALB/c mice were exposed to Aspergillus fumigatus, O3, or both (3 ppm for 2 hours). ILC2s were isolated by means of fluorescence-activated cell sorting and studied for Il5 and Il13 mRNA expression. ILC2s were depleted with anti-Thy1.2 mAb and replaced by means of intratracheal transfer of ex vivo expanded Thy1.2 ILC2s. Cytokine levels (ELISA and quantitative PCR), inflammatory cell profile, and AHR (flexiVent) were assessed in the mice.

Results: In addition to neutrophil influx, O3 inhalation elicited the appearance of eosinophils and IL-5 in the airways of BALB/c but not C57BL/6 mice. Although O3-induced expression of IL-33, a known activator of ILC2s, in the lung was similar between these strains, isolated pulmonary ILC2s from O3-exposed BALB/c mice had significantly greater Il5 and Il13 mRNA expression than C57BL/6 mice. This suggested that an altered ILC2 function in BALB/c mice might mediate the increased O3 responsiveness. Indeed, anti-Thy1.2 treatment abolished but ILC2s added back dramatically enhanced O3-induced AHR.

Conclusions: O3-induced activation of pulmonary ILC2s was necessary and sufficient to mediate asthma-like changes in BALB/c mice. This previously unrecognized role of ILC2s might help explain the heightened susceptibility of human asthmatic airways to O3 exposure. (J Allergy Clin Immunol 2016;137:571-8.)

Key words: Ozone, group 2 innate lymphoid cells, airway hyperresponsiveness

With the emergence of heavily polluted megacities, exposure to the common air pollutant ozone (O3) is a global respiratory health problem. O3 is an important trigger of asthma exacerbation, but the underlying pathways remain unclear. O3-induced inflammatory changes occur acutely, and clinical symptoms can last up to a week in asthmatic patients. The effects of O3 involve activation of the pulmonary epithelium and innate immune system, with release of IL-6 and IL-8 followed by influx of neutrophilic granulocytes. In both asthmatic patients and mouse models of allergic airway inflammation, O3 also elicited IL-5 release within hours of exposure. IL-5 is classically considered one of the TH2 cell–derived cytokines released together with IL-4 and is responsible for eliciting airway eosinophilia, a cardinal pathologic feature of asthma. In our previous studies IL-5 appeared without detectable levels of IL-4 in the airways within a few hours of O3 exposure, suggesting an early source of IL-5 during the inflammatory airway response distinct from Th2 cells.

Lung-resident group 2 innate lymphoid cells (ILC2s) are recently described innate lymphocytes with a capability to release IL-5 and IL-13 in response to IL-33 without antigenic stimulation and to initiate and perpetuate allergic airway inflammation. ILC2s reside in adipose tissues and at various barrier mucosal sites in the intestine and lung. During influenza virus infection, ILC2s accumulate and mediate virus-induced airway hyperresponsiveness (AHR), as well as epithelial repair. Lung ILC2s also promote acute airway eosinophilia in mice exposed to the protease papain or the fungal allergen Alternaria species. The function of ILC2s appears to be evolutionarily conserved because human ILC2s also produce Th2 cytokines in response to IL-33 and IL-25 similarly to mouse ILC2s. We hypothesized that activation of lung-resident ILC2s could contribute to the rapid inflammatory changes elicited by O3 exposure in mice.

METHODS

Mice

All experimental animals used in this study were housed under pathogen-free conditions. Experiments were performed on male mice between 8 and 12 weeks...
of age. Animals received water and food ad libitum. C57BL/6, BALB/c, and Thy 1.1 mice were purchased from the National Institutes of Health (Bethesda, Md) and the Jackson Laboratory (Bar Harbor, Me). Bicistronic IL-4 reporter (4gt) mice were a gift from Dr Kerry Campbell (Fox Chase Cancer Center, Philadelphia, Pa) and bred in house. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

O₃ exposure and allergen challenge protocols
Allergen sensitization/challenge and O₃ exposure were carried out, as previously described. Briefly, mice were sensitized with intraperitoneal injections of 20 μg of Aspergillus fumigatus mixed with alum on days 0 and 7 and received an intranasal challenge with 25 μg of A fumigatus on day 13. Mice were exposed to air or O₃ (5 ppm) or to forced air for 2 hours on day 16 and were killed 12 hours after O₃ exposure on day 17. When mice were exposed to O₃ or forced air alone, unless otherwise indicated, they were studied 12 hours after the exposure ended. This time point was selected based on pilot time-course studies to represent the height of proinflammatory changes, as seen in BALB/c mice. The chosen level of O₃ exposure in our studies was based on numerous previously published works on mice, rats, and human subjects by other laboratories and ours. The 3-ppm O₃ dose represents a level of exposure that is well tolerated by both BALB/c and C57BL/6 mice and that causes a significant airway inflammatory response. That a 3-ppm inhaled dose in rodents results in O₃ concentration in the lungs relevant to human exposure levels has been experimentally also validated by others by using oxygen-18-labeled O₃ (18O₃). For example, in a study by Hatch et al., it was demonstrated that exposure to 18O₃ (0.4 ppm for 2 hours) caused 4- to 5-fold higher 18O₃ concentrations in human subjects than in rats. Rats exposed to 2.0 ppm had still less 18O₃ in bronchoalveolar lavage (BAL) fluid than human subjects exposed to 0.4 ppm. The mechanisms underlying the species discrepancies in the recoverable O₃ levels in the lung are not entirely clear. Slade et al demonstrated that after exposure to O₃, mice react with a rapid decrease of core temperature in a strain-specific manner. The recoverable 18O₃ level in lung tissue was negatively associated with the extent of hypothermia that significantly altered O₂ consumption and pulmonary ventilation, explaining at least in part the interspecies differences seen in O₃ susceptibility.

BAL was performed and cells were assessed on cytospin preparations stained with Diff-Quik (Thermo Scientific, Fishnut, KY). Mice were compared for total and differential BAL cell counts by using a Countess Automated Cell Counter (Life Technologies, Grand Island, NY) and cytospin counts. In other experiments BAL was also processed for fluorescence-activated cell sorting (FACS) analysis. Lungs were perfused and removed for isolation of ILC2s and RNA extraction. The BAL fluid supernatant was processed for ELISA for measurement of IL-4 and IL-5 expression.

Immunoglobulin treatment, isolation of lung hematopoietic cells, and adoptive transfer
Four doses of anti-Thy1.2 mAb (30H12; BioXCell, West Lebanon, NH) and anti-CD4 mAb (GK1.5; BD Biosciences, San Jose, Calif) were administered intraperitoneally every other day (0.5 mg per mouse) to deplete ILC2s. For FACS analysis and ILC2 isolation, mice were exsanguinated, and lungs were perfused by injecting 10 mL of PBS into the right ventricle of the heart. Lungs were carefully cut into small fragments and digested in HBSS containing 0.025 mg/mL Liberase D (Roche Diagnostics, Mannheim, Germany) and 10 U/mL DNase I (Roche Diagnostics). Cells were filtered with a cell strainer. For adoptive transfer, lung ILC2s were isolated from Thy1.1 mice by means of FACS sorting and grown with IL-2, IL-7, and IL-33 for 7 days. Cells (10⁶) were intratracheally transferred into anti-Thy1.2 mAb-treated Thy1.2 mice.

Flow cytometry and cell sorting
Antibodies were purchased from eBioscience (San Diego, Calif) unless specified otherwise. The anti-basal (Lin) mixture included anti-F4/80 (MAR-1), anti-B200 (RA3-6B2), anti-CD19 (ID3), anti-Mac-1 (M1/70), anti-Gr-1 (8C5), anti-CD11c (HL3), anti-NK1.1 (PK 136), anti–Ter-119 (Ter-119), anti-CD3 (2C11), anti-CD8b (53.5.8), anti–T-cell receptor β (H57), and anti–γδ T-cell receptor (GL-3). We also used anti-Thy1.2 (53.2.1), anti–Siglec-F (E50-2440; BD Biosciences), and anti-ST2 (DJ8; DB Bioproducts, St Paul, Minn). Cell sorting was performed on a FACS Aria II (BD Biosciences), and flow cytometric analysis was performed on an LSR II Flow Cytometer (BD Biosciences).

Cytokine assessment
BAL fluid IL-4 and IL-5 expression was measured by using a standard sandwich ELISA (eBioscience), according to the manufacturer’s protocol. Total mRNA and protein were extracted from perfused lung tissue. In the O₃ time course experiments, Il3 mRNA was measured as part of an Affymetrix GeneChip Microarray (Affymetrix, Santa Clara, Calif). IL-33 protein was assessed as part of a Luminex assay (Luminex, Austin, Tex). For comparison of BALB/c and C57BL/6 lung Il3 mRNA, lung tissue was obtained and total RNA was extracted 12 hours after air or O₃ exposure, and quantitative PCR (qPCR) was performed. ILC2s and CD4 T cells were FACS sorted, total RNA was extracted, and cytokine mRNA expression was examined by using qPCR.

Lung function measurements
AHR to aerosolized acetyl-β-methylcholine chloride methacholine inhalation was assessed by using the flexiVent system (SCIREQ, Montreal, Quebec, Canada), as described previously. Briefly, lung mechanics were studied in tracheostomized mice after achievement of anesthesia by means of intraperitoneal injection of ketamine and xylazine. Mice were ventilated with a tidal volume of 8 mL/kg at a rate of 150 breaths/min and a positive end-expiratory pressure of 2 cm H₂O by using a computerized flexiVent System. After mechanical ventilation for 2 minutes, a sinusoidal 1-Hz oscillation was applied to the tracheal tube. The single-compartment model was fitted to these parameters by using multiple linear regression to calculate dynamic resistance, compliance, and tissue damping of the airway. Baseline measurements and responses to aerosolized saline were followed by measurements of responses to increasing doses of 0.625 to 25 mg/mL of aerosolized methacholine (Sigma-Aldrich, St Louis, Mo). Recorded values were averaged for each dose and used to obtain dose-response curves for each mouse.

Statistical analysis
Data were expressed as means ± SEMs. All data were analyzed with GraphPad Prism 5 software (GraphPad Software, San Diego, Calif). Multiple-group comparisons were made by using 1-way ANOVA, followed by the Bonferroni correction. Differences between groups in methacholine responsiveness were assessed by using 2-way ANOVA.

RESULTS
O₃ exposure enhanced A fumigatus–induced eosinophilia in BALB/c but not C57BL/6 mice
To model exacerbation of allergic airway changes (as seen in asthmatic patients on O₃ inhalation), we used a combined allergen sensitization/challenge and O₃ exposure protocol. In this model
mice were sensitized with intraperitoneal injections of *A fumigatus* mixed with alum on days 0 and 7 and received an intranasal challenge with *A fumigatus* on day 13. Mice were exposed to O₃ or forced air for 2 hours on day 16 and killed 12 hours later on day 17 (Fig 1, A). *A fumigatus* induced eosinophilic airway inflammation and O₃ induced airway neutrophilia, whereas the combination of these treatments elicited appearance of both eosinophils and neutrophils in the airways (Fig 1, B). Quantitation of the changes revealed that in allergen-sensitized and allergen-challenged mice, O₃ virtually doubled the numbers of eosinophils in the BAL fluid of BALB/c mice but not C57BL/6 mice (Fig 1, C and D), indicating strain differences in the airway response.

**O₃** exposure induced IL-5 release and airway eosinophilia in BALB/c but not C57BL/6 mice

BALB/c mice have increased susceptibility to Th2-mediated inflammatory conditions in comparison with C57BL/6 mice. To investigate whether BALB/c mice would respond to O₃ differently without the priming effects of allergen sensitization and challenge, we further assessed these strains exposed to O₃ alone (Fig 2, A) and found that the total (Fig 2, B) and neutrophil (Fig 2, C) cell counts in BAL fluid were increased to a lesser extent in C57BL/6 mice than in BALB/c mice in response to O₃. Furthermore, in BALB/c but not C57BL/6 mice O₃ inhalation resulted in an influx of small but significant numbers of eosinophils in the BAL fluid 12 hours later (Fig 2, D and E) together with increased IL-5 expression (Fig 2, F). IL-5 release within the first 12 hours after O₃ exposure occurred without increased numbers or activation of Th2 cells in the lung, as indicated by the lack of IL-4 release into the airways (data not shown) or activation of the *Il4* gene promoter in the BAL cells of “4get” mice (data not shown).

**O₃** exposure activates lung-resident ILC2s

IL-5 was produced by isolated lung ILC2s in response to addition of the epithelium-derived cytokine IL-33 (Fig 3, A). O₃ exposure of these mice induced *Il33* mRNA activation and increased protein expression in the lung tissue in a time-dependent manner (Fig 3, B-D; BALB/c data are shown). The kinetics or extent of IL-33 expression were comparable between the 2 mouse strains (Fig 3, D). Numbers of ILC2s remained similar between air- and O₃-exposed mice (Fig 3, E).
suggesting no ILC2 influx or proliferation within 12 hours after O3 exposure.

We then isolated ILC2s from the lungs (Fig 4, A) and studied them ex vivo. qPCR showed a significantly increased activation of Il5 and Il13 mRNA 12 hours after O3 exposure (Fig 4, B and C). This was evident in both strains. However, the effects were markedly greater in BALB/c ILC2s compared with C57BL/6 ILC2s. O3 did not induce IL-5 or IL-13 production by CD4+ Thy1+ TH cells isolated from the lungs 12 hours after exposure, supporting that ILC2s and not TH cells were the major source of these cytokines at this early time point after O3 inhalation.

ILC2s mediate O3-induced airway inflammation and AHR

To determine whether ILC2 activation in BALB/c mice is causally related to O3-induced airway inflammation, we treated mice with anti-Thy1.2 mAb, which depletes both innate lymphoid cells and T cells (Fig 5, A and B), or anti-CD4 mAb, which specifically depletes CD4+ TTh cells. Anti-Thy1.2 mAb treatment significantly reduced total cell counts and neutrophilia and abolished eosinophils in the BAL fluid of O3-exposed BALB/c mice (Fig 5, C-E). Anti-Thy1.2 treatment (Fig 5, F and G) but not anti-CD4 mAb treatment (Fig 5, G) abolished IL-5 release in BAL fluid of O3-exposed mice, confirming that innate lymphoid cells and not TTh cells were likely the major source of IL-5 12 hours after exposure.

Because anti-Thy1.2 mAb treatment depletes both ILC2s and T cells, we added back Thy1.1+ ILC2s by means of intratracheal transfer to verify the specific role of these cells in O3-induced airway inflammation (Fig 2, A and B). We transferred ILC2s that were expanded in vitro with IL-2, IL-7, and IL-33. ILC2 transfer restored total and neutrophil cell counts and significantly enhanced IL-5 levels and airway eosinophilia in response to O3 in anti-Thy1.2 mAb–treated mice (Fig 2, C-F).

To confirm whether the effects of ILC2s had any physiologic relevance, we investigated lung function of the O3-exposed mice by studying their methacholine responsiveness (Fig 5, H). Anti-Thy1.2 mAb treatment (pink squares) significantly reduced AHR after O3 inhalation. Conversely, ILC2 transfer into the anti-Thy1.2 mAb–treated mice (Fig 5, H, red squares) dramatically enhanced AHR to methacholine after O3. Notably, ILC2 transfer did not induce spontaneous AHR in air-exposed mice (Fig 5, H, white squares), suggesting that in vivo activation of these cells by O3 exposure is a requirement for the physiologic effects.

DISCUSSION

In this article we describe a novel role of innate lymphoid cells in O3-induced acute airway inflammation and AHR. We found
that O₃ exposure increased airway levels of IL-33, a potent activator of ILC2s. We showed that lung-resident ILC2s were the predominant early source of the TH2 cytokines IL-5 and IL-13 in O₃-exposed mice. Cell depletion and add-back experiments established an essential role of ILC2s in mediating O₃-induced airway inflammation and AHR. Together, these data

**FIG 3.** O₃ induced time-dependent IL-33 expression in the lung. A, Lung ILC2s isolated by means of FACS, stimulated in vitro with IL-33 for 5 days, and assessed for IL-5 and IL-13 by means of FACS. B-D, BALB/c mice exposed to O₃ for 2 hours and studied for lung IL-33 mRNA and protein expression at the indicated time points. E, Number of lung-resident ILC2s assessed by means of FACS. *P < .05, ANOVA with Bonferroni correction. Results are means ± SEMs (n = 3-5). n.s., Not significant.

**FIG 4.** O₃ activated expression of *Il5* and *Il13* mRNA was enhanced in lung-resident ILC2s of BALB/c mice. A, Experimental design. Lung ILC2s and CD4⁺ T cells were isolated 12 hours after O₃ exposure and processed for qPCR analysis. B and C, Expression of *Il5* and *Il13* mRNA of ILC2s and T cells was normalized to that of glyceraldehyde-3-phosphate dehydrogenase. Results are means ± SEMs (n = 3). **P < .01, ANOVA with Bonferroni correction. Ctrl, Control.
indicated that ILC2s are critical effector cells in O₃-induced airway inflammation in mice.

The heightened sensitivity of asthmatic patients in comparison with healthy subjects to air pollution, especially O₃ inhalation, is not well understood. In mouse models allergen-induced and O₃-induced airway changes also vary among different strains, implying genetically determined pathologies. Here we mimicked exacerbation of allergic airway changes by O₃.
Ozone (O₃) ingestion in allergen-sensitized and allergen-challenged mice. O₃ virtually doubled eosinophil numbers in the BAL fluid of BALB/c but not C57BL/6 mice, confirming strain differences in the airway response. Indeed, BALB/c mice responded to O₃ inhalation differently from C57BL/6 mice, even without the priming effects of allergen sensitization and challenge. In addition to the increased numbers of neutrophils, BALB/c mice had eosinophils, together with increased IL-5 expression, in the airways induced by O₃. Although eosinophilia can be synergistically promoted by a number of chemokines, cytokines, and growth factors (inducible by O₃), the presence of IL-5 is a prerequisite. Importantly, IL-5 expression within the first 12 hours after O₃ exposure occurred without increased numbers or activation of T₄₁₂ cells in the lung, as indicated by the lack of IL-4 release into the airways or activation of the Il4 gene promoter in the BAL cells of “4get” mice, suggesting an alternative source of IL-5.

In addition to T₄₁₂ cells, ILC2s can also produce IL-5 when stimulated by the epithelium-derived cytokine IL-33. We interestingly found that O₃ induced IL-33 expression in the lung in a time-dependent manner. This effect was comparable between BALB/c and C57BL/6 mice. The number of ILC2s did not change after O₃ exposure in either strain, suggesting that within the 12-hour time period, no ILC2 influx or proliferation took place in the lung. Notably, however, when ILC2s were isolated from the lungs, their capability to express Il5 and Il13 mRNA was significantly increased. Although both strains showed Il5 and Il13 mRNA induction, the effects were markedly greater in BALB/c ILC2s compared with C57BL/6 ILC2s. Furthermore, O₃ did not induce IL-5 or IL-13 production by CD4⁺ cells, confirming that ILC2s but not T₄₁₂ cells were the major source of these cytokines at this early time point after O₃ inhalation. These results established that in comparison with C57BL/6 mice, BALB/c mice exhibited increased airway neutrophilia, displayed evidence of eosinophil granulocytes in the airways, and expressed heightened IL-5 levels in the BAL fluid after O₃ inhalation. Such discrepancies in O₃ responsiveness were associated with a markedly amplified activation of Il5 and Il13 mRNA in pulmonary ILC2s of BALB/c mice. Thus O₃ activated BALB/c ILC2s to a significantly greater extent than C57BL/6 ILC2s, suggesting that ILC2s in these strains are intrinsically different in their function. Therefore it is possible that ILC2 functional differences are also responsible for O₃ susceptibility in asthmatic patients.

Although recent work has made significant advances in understanding the biology of innate lymphoid cells, their functional capability remains to be better appreciated. ILC2 activation can cause asthma-related features, including airway inflammation, mucus production, and AHR. To determine whether ILC2 activation in BALB/c mice is causally related to O₃-induced airway inflammation, we depleted both innate lymphoid cells and T cells or, specifically, CD4⁺ T₄₁₂ cells. Depletion of ILC2s and T cells significantly reduced total cell counts and neutrophilia and abolished the presence of eosinophils in the BAL fluid of the O₃-exposed BALB/c mice. ILC2 but not CD4 depletion abolished IL-5 release in the BAL fluid of O₃-exposed mice, confirming that ILC2s were the source of this cytokine. ILC2s add back restored total and neutrophil cell counts and significantly enhanced IL-5 and airway eosinophilia in response to O₃.

To investigate the physiologic relevance of the effects of ILC2s, we investigated the lung function of the O₃-exposed mice. Because we found that O₃ heightened IL-13 induction in BALB/c ILC2s and because IL-13 can directly induce AHR, we hypothesized that the presence of ILC2s in the lung is necessary for the O₃-induced AHR. Indeed, ILC2 depletion significantly reduced AHR after O₃ inhalation, whereas ILC2s add back dramatically enhanced AHR after O₃. Because ILC2 transfer did not induce spontaneous AHR in air-exposed mice, we propose that in vivo activation of these cells by O₃ exposure is a requirement for the physiologic effects.

Our data clearly demonstrated the effectiveness of activated ILC2s in altering lung physiology. The mechanism and significance of the direct ILC2 action on AHR in response to O₃ will need further clarification. Nonetheless, the facts that O₃-activated ILC2 outcomes were disproportionately greater on methacholine responsiveness than on airway inflammation and that ILC2s can affect AHR through IL-13 during inflammatory changes in the lung suggest a potential direct regulatory role of these cells in airway physiology.

In summary, we identified lung-resident ILC2s as the cell type responsible for airway inflammation induced by an air pollutant. Our study suggests that ILC2s might significantly contribute to the mechanisms by which air pollution induces asthma exacerbation.

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Key messages

- O₃ induced release of IL-33 in the lungs of mice.
- Increased susceptibility of BALB/c mice to O₃-induced inflammatory changes was associated with increased production of IL-5 and IL-13 by pulmonary ILC2s.
- Depletion of ILC2s suppressed, although repletion enhanced, airway inflammatory changes induced by O₃ inhalation in mice.

REFERENCES


