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Group-2 innate lymphoid cells mediate ozone induced airway inflammation and hyperresponsiveness in mice

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Abstract

Background—Patients with asthma are highly susceptible to air pollution and in particular, to the effects of ozone (O_3) inhalation, but the underlying mechanisms remain unclear.

Objective—Using mouse models of O_3-induced airway inflammation and hyperresponsiveness (AHR), we sought to investigate the role of the recently discovered group 2 innate lymphoid cells (ILC2).

Methods—C57BL/6 and Balb/c mice were exposed to *Aspergillus fumigatus* and/or O_3 (2ppm, 2h). ILC2 were isolated by FACS sorting and studied for IL-5 and IL-13 mRNA expression. ILC2 were depleted with anti-Thy1.2 mAb and replaced by intratracheal transfer of *ex vivo* expanded Thy1.1 ILC2. Cytokines (ELISA, qPCR), inflammatory cell profile and AHR (FlexiVent) were assessed in the mice.

Results—In addition to neutrophil influx, O_3 inhalation elicited the appearance of eosinophils and IL-5 in the airways of Balb/c but not C57BL/6 mice. Although O_3 induced expression of IL-33, a known activator of ILC2 in the lung was similar between these strains, isolated pulmonary ILC2 from O_3 exposed Balb/c mice had significantly greater IL-5 and IL-13 mRNA expression than those of C57BL/6 mice. This suggested that an altered ILC2 function in Balb/c mice may mediate the increased O_3 responsiveness. Indeed, anti-Thy1.2 treatment abolished, whereas ILC2 add-back dramatically enhanced O_3-induced AHR.

Conclusions—O_3-induced activation of pulmonary ILC2 was necessary and sufficient to mediate asthma-like changes in Balb/c mice. This previously unrecognized role of ILC2 may help explain the heightened susceptibility of human asthmatic airways to O_3 exposure.

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Keywords

ozone; group-2 innate lymphoid cells; airway hyperresponsiveness

INTRODUCTION

With the emergence of heavily polluted megacities, exposure to the common air pollutant ozone (O_3) is a global respiratory health problem. O_3 is an important trigger of asthma exacerbation[1–3] but the underlying pathways remain unclear. The O_3-induced inflammatory changes occur acutely and the clinical symptoms can last up to a week in asthmatic patients[4]. The O_3 effects 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 asthmatic patients[5, 6] and in mouse models of allergic airway inflammation[7] O_3 also elicited IL-5 release within hours of exposure. IL-5 is classically considered to be one of the T helper type 2 (Th2) cell derived cytokines released together with IL-4 and is responsible for eliciting airway eosinophilia, a cardinal pathologic feature of asthma[8, 9]. In our previous studies IL-5 appeared without detectable levels of IL-4 in the airways within a few hours of O_3 exposure[7], suggesting an early source of IL-5 during the inflammatory airway response, distinct from Th2 cells.

Lung resident group-2 innate lymphoid cells (ILC2)[10] 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. ILC2 reside in adipose tissues and at various barrier mucosal sites in the intestine and lung. During influenza virus infection ILC2 accumulate and mediate virus-induced AHR as well as epithelial repair[11, 12]. Lung ILC2 also promote acute airway eosinophilia in mice exposed to the protease papain or the fungal allergen *Alternaria*[13–17]. The function of ILC2 appears to be evolutionarily conserved, because human ILC2 also produce Th2 cytokines in response to IL-33 and IL-25 similarly to mouse ILC2[3, 11, 18, 19]. We hypothesized that activation of lung resident ILC2 could contribute to the rapid inflammatory changes elicited by O_3 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 Thy1.1 mice were purchased from the National Institutes of Health and the Jackson Laboratory. Bicistronic IL-4 reporter (4get) mice[20] were the gift of Dr. Kerry Campbell (Fox Chase Cancer Center, Philadelphia, PA) and were bred in house. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

O_3 exposure and allergen challenge protocols

Allergen sensitization/challenge and O_3 exposure have been carried out as before[7]. Briefly, mice were sensitized with intraperitoneal (i.p.) injections of 20 μg *Aspergillus fumigatus* (*Af*) mixed with alum on day 0 and 7 and received an intranasal challenge with 25μg of *Af* on day 13. Mice were exposed to air or O_3 (2 ppm) or to forced air for 2 hours on Day 16 and were sacrificed 12h after O_3 exposure on Day 17. When mice were exposed to O_3 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 ozone exposure in our studies was based on numerous previously published works on mice, rats and humans by other laboratories and ours. The 2ppm O_3 dose represents a level of exposure that is well tolerated by both Balb/c and C56BL/6 mice and that causes a significant airway inflammatory response. That a 2ppm inhaled dose in rodents results in O_3 concentration in the lungs relevant to human exposure levels has been experimentally also validated by others, using oxygen-18-labeled O_3 ($18O_3$). For example, in a study by Hatch et al. it was demonstrated that exposure to $18O_3$ (0.4 ppm for 2 h) caused four- to fivefold higher $18O_3$ concentrations in humans than in rats[21]. Rats exposed to 2.0 ppm, had still less $18O_3$ in BAL than humans (exposed to 0.4 ppm). The species discrepancies between the recoverable O_3 levels in the lung are not entirely clear but Slade et al. demonstrated that after exposure to O_3, mice react by a rapid decrease of core temperature, a species and strain specific characteristics[22]. The recoverable $18O_3$ in the lung tissue was negatively associated with the extent of hypothermia that significantly altered O_2 consumption and pulmonary ventilation, explaining at least partly, the interspecies differences seen in O_3 susceptibility.

BAL was performed and cells were assessed on cytospin preparations stained with DiffKwik. Mice were compared for total and differential BAL cell counts, using a Countess® Automated Cell Counter and Cytospin counts. In other experiments BAL was also processed for FACS analysis. Lungs were perfused and removed for isolation of ILC2 cells and RNA extraction. The BAL supernatant was processed for ELISA for IL-4 and IL-5 expression.

Immunoglobulin treatment, isolation of lung hematopoietic cells and adoptive transfer

To deplete ILC2 cells, 4 doses of anti-Thy1.2 mAb (30H12, West Lebanon), and anti-CD4 mAb (GK1.5, BD bioscience) were administered intraperitoneally every other day (0.5 mg/mouse). For FACS analysis and ILC2 isolation mice were exsanguinated and lungs were perfused by injecting 10 ml PBS into the right ventricle of the heart. Lungs were carefully cut into small fragments and digested in Hank's balanced salt solution containing 0.025 mg/ml Liberase D (Roche Diagnostics) and 10 U/ml DNase I (Roche Diagnostics). Cells were filtered by using a cell strainer. For adoptive transfer, lung ILC2 were isolated from Thy1.1 mice by FACS sorting, and grown with IL-2, IL-7, IL-33 for 7 days. 10^6 cells were intratracheally transferred into anti- Thy1.2 mAb treated Thy1.2 mice.

Flow Cytometry and Cell Sorting

Antibodies were purchased from eBioscience unless specified otherwise. The anti-lineage (Lin) mixture included anti-FcεR (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-TCRb (H57), and anti-gdTCR (GL-3). We also used anti-Thy1.2 (53-2.1), anti-Siglec F (E50-2440; BD Biosciences), and anti-ST2 (DJ8; MD Bioproducts). Cell sorting was performed on a FACSAria II (BD Biosciences), and flow cytometric analysis was performed on a LSR-II (BD Biosciences).

Cytokine assessment

BAL IL-4 and IL-5 expression was measured by standard sandwich ELISA (eBioscience) according to the manufacturer's protocol. Total mRNA and protein were extracted from perfused lung tissue. In the O_3 time course experiments *il33* mRNA was measured as part of an Affymetrix GeneChip® Microarray. IL-33 protein was assessed as part of a Luminex assay. For comparison of Balb/c and C57BL/6 lung *il33* mRNA, lung tissue was obtained and total RNA extracted 12 h after air or O_3 exposure and qPCR was performed. ILC2 and CD4 T cells were FACS sorted, total RNA extracted and cytokine mRNA expression was examined by qPCR.

Lung function measurements

Airway hyperresponsiveness to aerosolized acetyl-β-methylcholine chloride methacholine (MCh) inhalation was assessed using the FlexiVent system (SCIREQ, Montreal, Canada) as described before[23]. Briefly, lung mechanics were studied in tracheostomized mice under anesthesia by intra-peritoneal injection of ketamine and xylazine. Mice were ventilated with a tidal volume of 8ml/kg at a rate of 150 breaths/min and a positive end-expiratory pressure of 2cm H_2O by a computerized FlexiVent System. After mechanical ventilation for 2 min, a sinusoidal 1-Hz oscillation was applied to the tracheal tube. The single-compartment model was fitted to these parameters by 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 (0.625–25 mg/ml; Sigma-Aldrich). Recorded values were averaged for each dose and used to obtain dose-response curves for each mouse.

Statistical analysis

Data were expressed as Mean±SEM. All data were analyzed using GraphPad Prism 5 Software, (GraphPad Inc., San Diego, CA). Multiple group comparisons were made using one way ANOVA followed by the Bonferroni correction. Differences between groups in MCh responsiveness were assessed by two-way ANOVA.

RESULTS

Ozone exposure enhanced *Aspergillus fumigatus* induced eosinophilia in Balb/c, but not C57BL/6 mice

To model exacerbation of allergic airway changes (seen in asthmatic patients upon O_3 inhalation), we used a combined allergen sensitization/challenge and O_3 exposure protocol[7]. In this model mice were sensitized with intraperitoneal injections of *Aspergillus fumigatus* (*Af*) mixed with alum on day 0 and 7 and received an intranasal challenge with *Af* on day 13. Mice were exposed to O_3 or to forced air for 2 hours on Day 16 and were sacrificed 12h

later, on Day 17 (Figure 1A). *Af* induced eosinophilic airway inflammation, O_3 induced airway neutrophilia, while the combination of these treatments elicited appearance of both eosinophils and neutrophils in the airways (Figure 1B). Quantitation of the changes revealed that in allergen-sensitized and challenged mice O_3 virtually doubled the numbers of eosinophils in the BAL fluid of Balb/c mice but not in C57BL/6 mice (Figure 1C–D) indicating strain differences of the airway response.

Ozone 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[24]. To investigate whether Balb/c mice would respond to O_3 differently without the priming effects of allergen sensitization and challenge, we further assessed these strains exposed to O_3 alone (Figure 2A) and found that the total (Figure 2B) and neutrophil (Figure 2C) cell counts in the BAL were increased to a lesser extent in C57BL/6 than in Balb/c mice in response to O_3. Further, in Balb/c but not C57BL/6 mice O_3 inhalation resulted in an influx of a small but significant numbers of eosinophils in the BAL 12 h later (Figure 2D–E), together with increased IL-5 expression (Figure 2F). IL-5 release within the first 12 hours after O_3 exposure occurred without increased numbers or activation of Th2 cells in the lung as indicated by lack of IL-4 release into the airways (not shown) or activation of the IL-4 gene promoter in the BAL cells of “4get” mice (not shown).

Ozone exposure activates lung-resident ILC2

IL-5 was produced by isolated lung ILC2 in response to the addition of the epithelial derived cytokine, IL-33 (Figure 3A). O_3 exposure of the mice induced IL-33 mRNA activation and increased protein expression in the lung tissue in a time dependent manner (Figure 3B–D Balb/c data are shown). The kinetics or extent of IL-33 expression were comparable between the two mouse strains (Figure 3D). The number of ILC2 remained similar between air and O_3 exposed mice (Figure 3E) suggesting no ILC2 influx or proliferation within 12 h after O_3 exposure.

We have then isolated ILC2 from the lungs (Figure 4A) and studied them *ex vivo*. qPCR showed a significantly increased activation of IL-5 and IL-13 mRNA 12 h after O_3 exposure (Figure 4B–C). This was evident in both strains. However, the effects were markedly greater in Balb/c ILC2 compared with C57BL/6 ILC2. O_3 did not induce IL-5 or IL-13 production by $CD4^+$ $Thy1^+$ T helper cells isolated from the lungs 12 h after exposure, supporting that ILC2 but not T helper cells were the major source of these cytokines at this early time point following O_3 inhalation.

ILC2 mediate ozone-induced airway inflammation and hyperresponsiveness

To determine whether ILC2 activation in Balb/c mice is causally related to ozone induced airway inflammation, we treated mice with anti-Thy1.2 mAb (that depletes both innate lymphoid cells and T cells, Figure 5A, 5B), or anti-CD4 mAb (that specifically depletes $CD4^+$ T helper cells). Anti-Thy1.2 mAb treatment significantly reduced total cell count and neutrophilia and abolished eosinophils in the BAL fluid of the O_3 exposed Balb/c mice (Figure 5C–E). Anti-Thy1.2 treatment (Figure 5F–G), but not anti-CD4 mAb (Figure 5G),

abolished IL-5 release in the BAL of O_3 exposed mice, confirming that innate lymphoid cells but not T helper cells were likely the major source of IL-5, 12 h after exposure.

Since anti-Thy1.2 mAb treatment depletes both ILC2 and T cells, we added back Thy1.1+ ILC2 by intratracheal transfer to verify the specific role of these cells in O_3-induced airway inflammation (Figure 2A–B). We transferred ILC2 that were expanded *in vitro* with IL-2, IL-7 and IL-33. ILC2 transfer restored total and neutrophil cell count and significantly enhanced IL-5 and airway eosinophilia in response to O_3 in anti-Thy1.2 mAb treated mice (Figure 2C–F).

To confirm if the ILC2 effects had any physiological relevance we investigated the lung function of the O_3 exposed mice by studying their methacholine responsiveness (Figure H5). Anti-Thy1.2 mAb treatment (pink squares) significantly reduced AHR after O_3 inhalation. Conversely, ILC2 transfer into the anti-Thy1.2 mAb treated mice (red squares), dramatically enhanced AHR to methacholine after O_3. Notably, ILC2 transfer did not induce spontaneous AHR in air-exposed mice (white squares), suggesting that *in vivo* activation of these cells by O_3 exposure is a requirement for the physiological effects.

DISCUSSION

In this paper we described a novel role of innate lymphoid cells in ozone-induced acute airway inflammation and hyperresponsiveness. We found that ozone exposure increased airway levels of IL-33, a potent activator of ILC2. We showed that lung-resident ILC2 were the predominant early source of Th2 cytokines IL-5 and IL-13 in ozone-exposed mice. Cell depletion and add-back experiments established an essential role of ILC2 in mediating ozone-induced airway inflammation and hyperresponsiveness. Together, these data indicated that ILC2 are critical effector cells in O_3-induced airway inflammation in mice.

The heightened sensitivity of asthma patients in comparison with healthy individuals, to air pollution, especially O_3 inhalation is not well understood[5, 25–27]. In mouse models, allergen-[28, 29] and O_3-induced airway changes also vary among different strains[7, 30] implying genetically determined pathologies. Here we mimicked exacerbation of allergic airway changes by O_3 inhalation[7] in allergen-sensitized and challenged mice. O_3 virtually doubled the numbers of eosinophils in the BAL fluid of Balb/c but not in C57BL/6 mice confirming strain differences of the airway response. Indeed Balb/c mice responded to O_3 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 an increased IL-5 expression in the airways induced by O_3. Although eosinophilia may be synergistically promoted by a number of chemokines, cytokines and growth factors (inducible by O_3[7, 31, 32]), presence of IL-5 is a prerequisite. Importantly, IL-5 expression within the first 12 hours after O_3 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 or activation of the IL-4 gene promoter in the BAL cells of “4get” mice, suggesting an alternative source of IL-5.

Besides Th2 cells, ILC2 can also produce IL-5 when stimulated by the epithelial derived cytokine, IL-33[10, 12, 33]. Interestingly, we found that O_3 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 ILC2 did not change after O_3 exposure in either strains, suggesting that within the 12-hours time period, no ILC2 influx or proliferation took place in the lung. Notably however, when ILC2 were isolated from the lungs, their capability to express IL-5 and IL-13 mRNA was significantly increased. While both strains showed IL-5 and IL-13 mRNA induction, the effects were markedly greater in Balb/c ILC2 compared with C57BL/6 ILC2. Further, O_3 did not induce IL-5 or IL-13 production by $CD4^+$ cells confirming that ILC2 but not T helper cells were the major source of these cytokines at this early time point following O_3 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 O_3 inhalation. Such discrepancies in O_3 responsiveness were associated with a markedly amplified activation of IL-5 and IL-13 mRNA in pulmonary ILC2 of Balb/c mice. Thus, O_3 activated Balb/c ILC2 to a significantly greater extent than C57BL/6 ILC2 suggesting that ILC2 in these strains are intrinsically different in their function. It is possible therefore that ILC2 functional differences are also responsible to O_3 susceptibility in asthmatic patients.

Although recent work has made significant advances in understanding the biology of ILC, 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 ozone induced airway inflammation, we depleted both innate lymphoid cells and T cells or specifically, $CD4^+$ T helper cells. Depletion of ILC2 and T cells significantly reduced total cell counts and neutrophilia and abolished the presence of eosinophils in the BAL fluid of the O_3 exposed Balb/c mice. ILC2 but not CD4 depletion abolished IL-5 release in the BAL of O_3 exposed mice, confirming that the source of this cytokine were the ILC2. ILC2 add back restored total and neutrophil cell count and significantly enhanced IL-5 and airway eosinophilia in response to O_3.

To investigate the physiological relevance of the ILC2 effects we investigated the lung function of the O_3 exposed mice. Because we found that O_3 heightened IL-13 induction in Balb/c ILC2 and because IL-13 can directly induce airway hyperresponsiveness (AHR)[12, 34, 35], we hypothesized that presence of ILC2 in the lung is necessary for the O_3-induced AHR. Indeed ILC2 depletion significantly reduced AHR after O_3 inhalation while ILC2 add back dramatically enhanced AHR after O_3. As ILC2 transfer did not induce spontaneous AHR in air-exposed mice, we propose that *in vivo* activation of these cells by O_3 exposure is a requirement for the physiological effects.

Our data clearly demonstrated the effectiveness of activated ILC2 in altering lung physiology. The mechanism and significance of the direct ILC2 action on AHR in response to O_3 will need further clarification. Nonetheless the facts that the O_3 activated ILC2 outcomes were disproportionately greater on methacholine responsiveness than on airway inflammation and that ILC2 can affect AHR through IL-13 during inflammatory changes in

the lung[12, 35] suggest a potential direct regulatory role of these cells in airway physiology. In summary, we identified lung-resident ILC2 as the cell type responsible for airway inflammation induced by an air pollutant. Our study suggests that ILC2 may significantly contribute to the mechanisms by which air pollution induces asthma exacerbation.

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ABBREVIATIONS

ANOVA	analysis of variance
BAL	bronchoalveolar lavage
ELISA	enzyme linked immunosorbent assay
EP	eosinophil
i.n.	intranasal
i.p.	intraperitoneal
i.t.	intratracheal
IL	interleukin
ILC2	type 2 innate lymphoid cell
LC	lymphocyte
MP	macrophage
NP	neutrophil
O_3	ozone
ppm	parts per million
qPCR	quantitative polymerase chain reaction
Th2	T helper type 2

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KEY MESSAGES

- O_3 induced release of IL-33 in the lung of mice.
- Increased susceptibility of Balb/c mice to O_3 induced inflammatory changes was associated with elevated production IL-5 and IL-13 of by pulmonary ILC2.
- Depletion of ILC2 suppressed while repletion enhanced airway inflammatory changes induced by O_3 inhalation in mice.

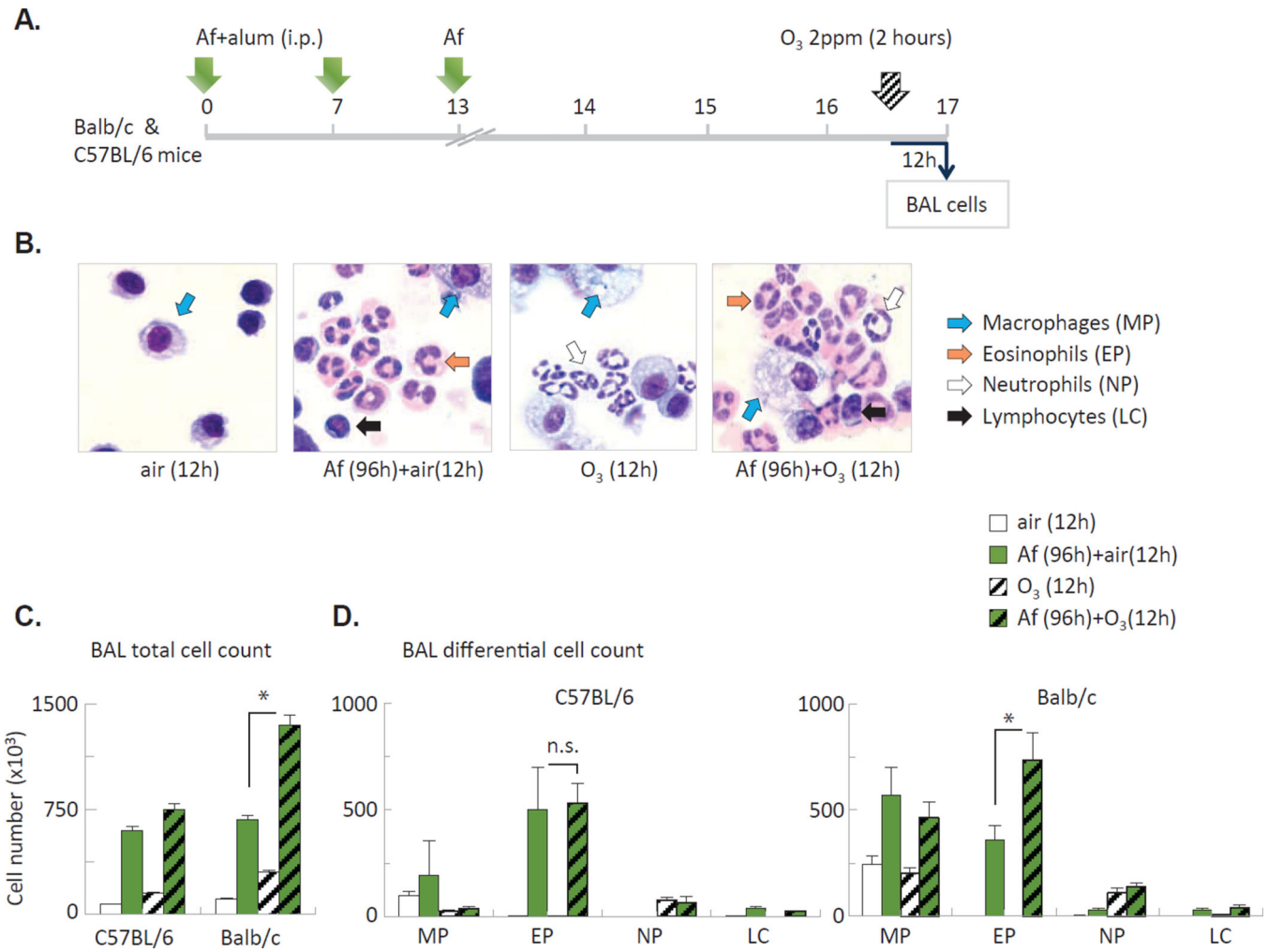

Figure 1. O_3 enhanced airway eosinophilia in allergen challenged Balb/c but not C57BL/6 mice

(A) Combined allergen and O_3 exposure protocol. **(B)** Representative cytospin preparations of BAL cells from Balb/c mice. **(C–D)** BAL total and differential cell counts from C57BL/6 and Balb/c mice. MP: macrophages; EP: eosinophils; NP: neutrophils; LC: lymphocytes. *p<0.05, Mean±SEM of n=3–9.

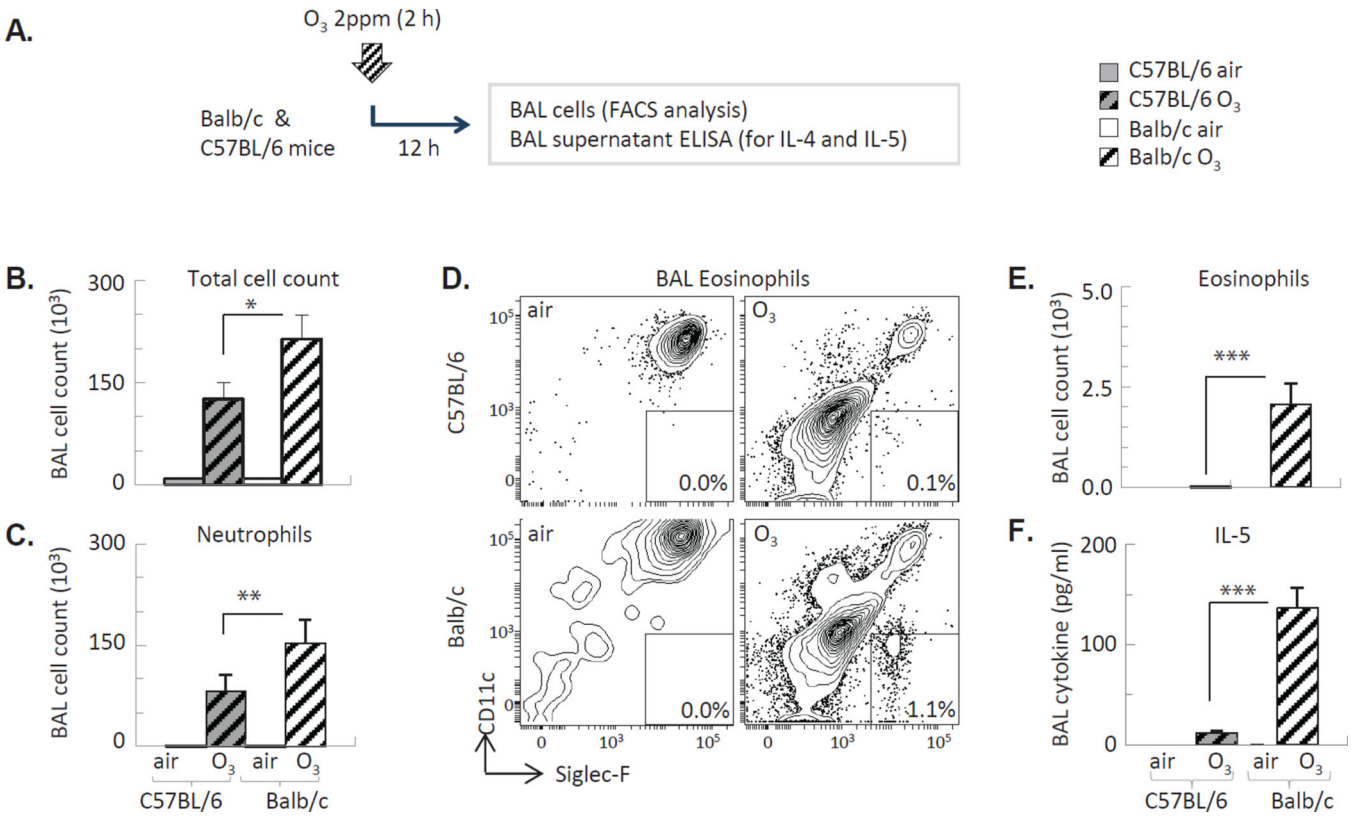

Figure 2. O_3 exposure of Balb/c mice induced eosinophil influx and IL-5 release into the airways

(A) Experimental design. (B) Total BAL cell counts (Countess®). (C, E) BAL neutrophils and eosinophils were quantified by FACS analysis. (D) Representative flow profile of Siglec F+ eonsinophils in the BAL. (F) IL-5 assessed from the BAL supernatant by ELISA *p<0.05; **p<0.01; ***p<0.001 (ANOVA with Bonferroni correction), Mean±SEM of n=3–5.

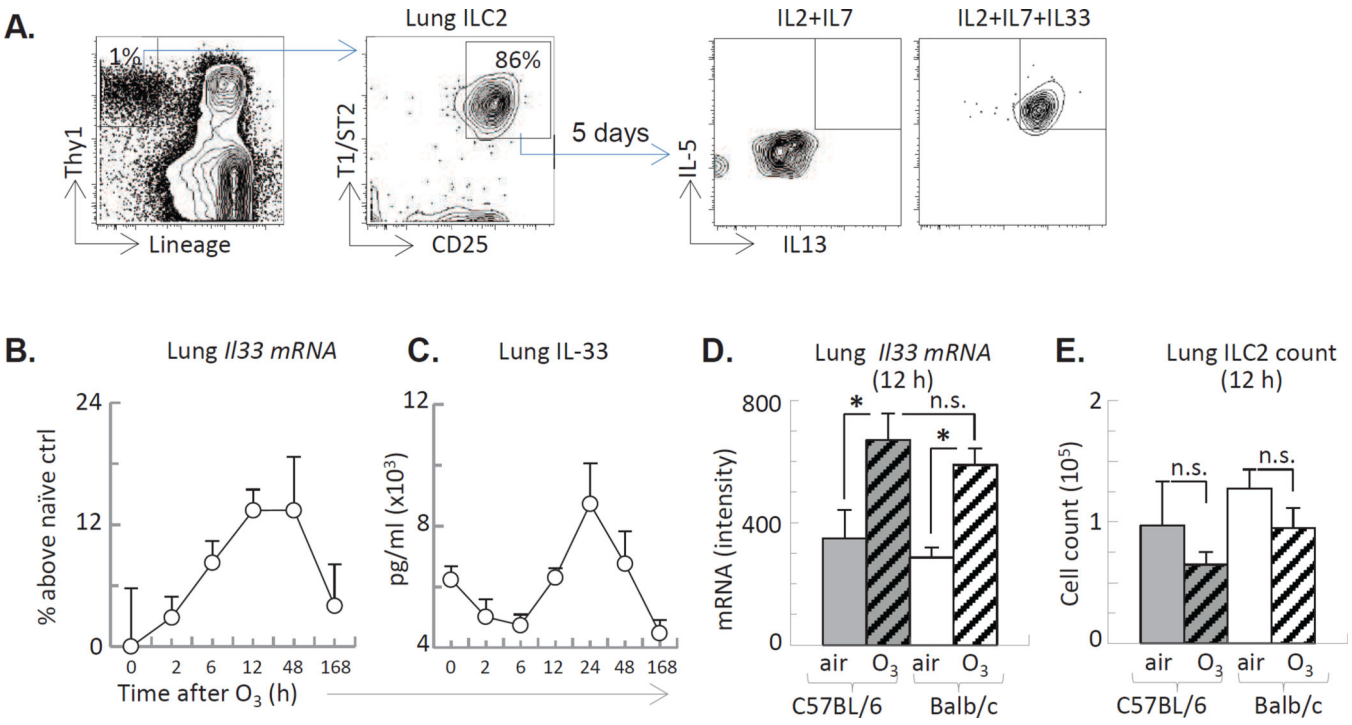

Figure 3. O_3 induced time dependent IL-33 expression in the lung

(A): Lung ILC2 isolated by FACS sorting, stimulated *in vitro* with IL-33 for 5 days and assessed for IL-5 and IL-13 by FACS. (B–D): Balb/c mice exposed to O_3 for 2 h and studied for lung IL-33 mRNA and protein expression at the indicated time points. (E): The number of lung-resident ILC2, assessed by FACS. *p<0.05; (ANOVA with Bonferroni correction), Mean±SEM of n=3–5 (n.s. not significant)

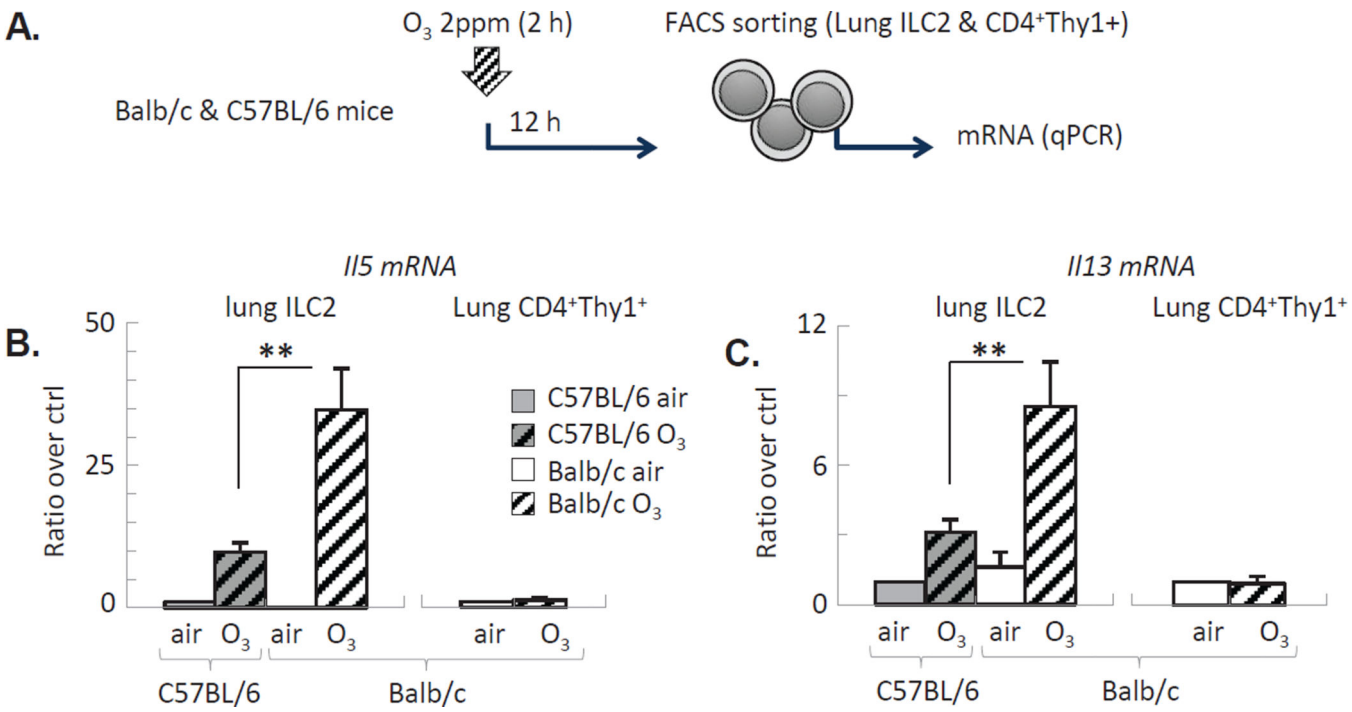

Figure 4. O_3 activated expression of IL-5 and IL-13 mRNA was enhanced in lung-resident ILC2 of Balb/c mice

(A): Experimental design: Lung ILC2 and $CD4^+$ T cells were isolated 12 h after O_3 exposure and processed for qPCR analysis. (B–C): *Il5* and *Il13* mRNA of ILC2 and T cells was normalized to GAPDH. Mean±SEM of n=3; **p<0.01; (ANOVA with Bonferroni correction).

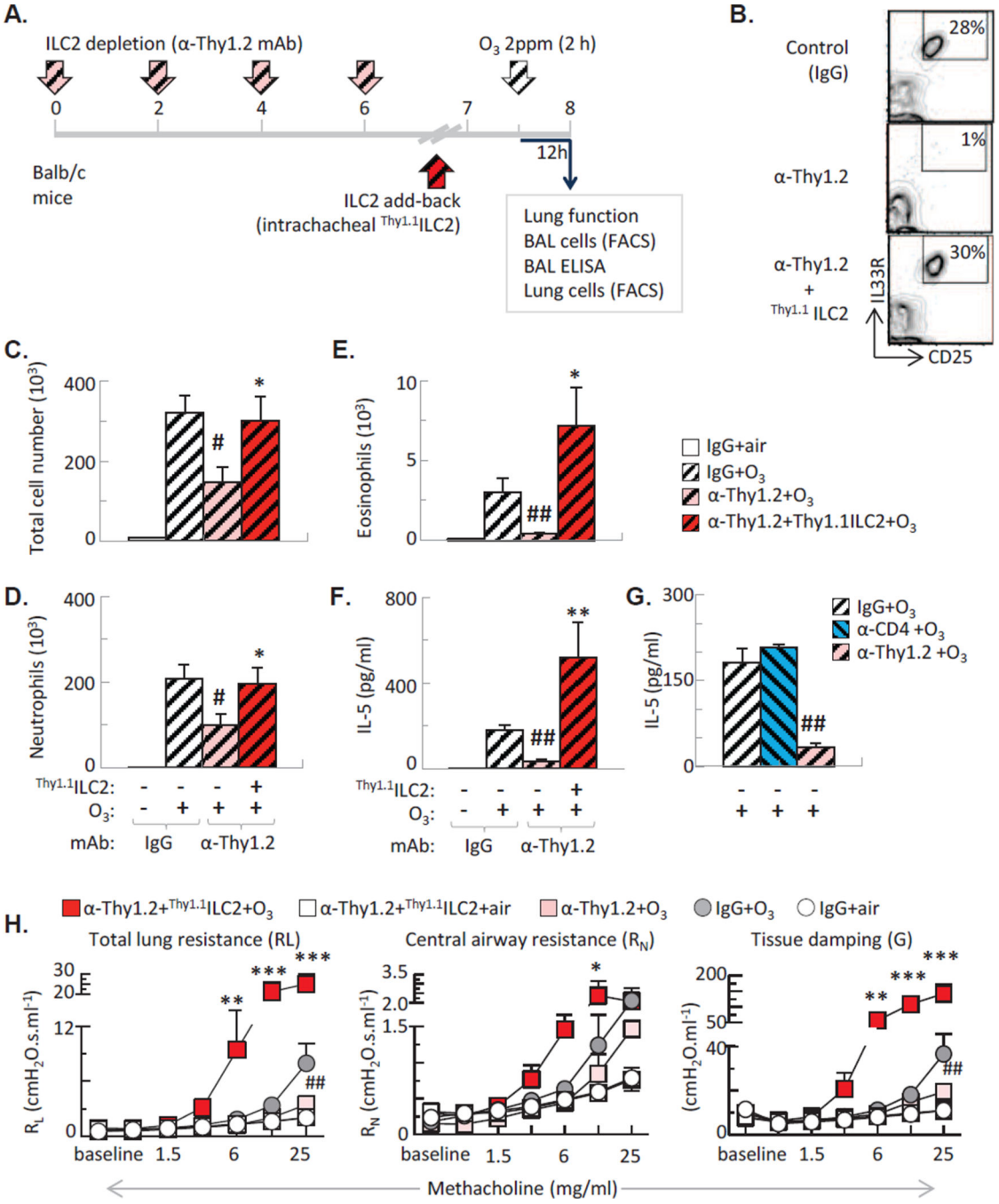

Figure 5. ILC2 was required for O_3-induced airway inflammation and AHR

(A): Experimental design: Balb/c mice received anti-thy1.2 (or control IgG) mAb. ILC2 were subsequently replaced by intratracheal transfer of Thy1.1 ILC2. **(B):** Representative flow plots indicating that the proportion of recovered ILC2 after transfer is commensurate with that of the non-depleted mice. **(C–E):** Anti-Thy1.2 mAb treatment inhibited, transfer of ILC2 increased lung total, neutrophil and eosinophil counts in O_3 exposed Balb/c mice. **(F):** antiThy1.2 but not anti-CD4 attenuated IL-5 release into the airways. ILC2 replacement significantly enhanced IL-5 in response to O_3. **(G):** Anti-Thy1.2 inhibited, ILC2

replacement significantly enhanced AHR after O_3. Mean±SEM of n=8–9; *p<0.05; **p<0.01; ***p<0.001 *vs.* α-Thy1.2 +O_3; $^{\#}$p<0.05; $^{\#\#}$p<0.01; vs. IgG+O_3 (ANOVA with Bonferroni correction).