

Allergen-induced Increases in Sputum Levels of Group 2 Innate Lymphoid Cells in Subjects with Asthma

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Abstract

Rationale: Group 2 innate lymphoid cells (ILC2), a major source of type 2 cytokines, initiate eosinophilic inflammatory responses in murine models of asthma.

Objectives: To investigate the role of ILC2 in allergen-induced airway eosinophilic responses in subjects with atopy and asthma.

Methods: Using a diluent-controlled allergen challenge crossover study, where all subjects (n = 10) developed allergen-induced early and late responses, airway eosinophilia, and increased methacholine airway responsiveness, bone marrow, blood, and sputum samples were collected before and after inhalation challenge.

Measurements and Main Results: ILC2 (lin[−] FcεRI[−] CD45⁺ CD127⁺ ST2⁺) and CD4⁺ T lymphocytes were enumerated by flow cytometry, as well as intracellular IL-5 and IL-13 expression. Steroid sensitivity of ILC2 and CD4⁺ T cells was investigated *in vitro*. A significant increase

in total, IL-5⁺, IL-13⁺, and CRTH2⁺ ILC2 was found in sputum, 24 hours after allergen, coincident with a significant decrease in blood ILC2. Total, IL-5⁺, and IL-13⁺, but not CRTH2⁺, CD4⁺ T cells significantly increased at 24 and 48 hours after allergen in sputum. In blood and bone marrow, only CD4⁺ cells demonstrated increased activation after allergen. Airway eosinophilia correlated with IL-5⁺ ILC2 at all time points and allergen-induced changes in IL-5⁺ CD4⁺ cells at 48 hours after allergen. Dexamethasone significantly attenuated IL-2- and IL-33-stimulated IL-5 and IL-13 production by both cell types.

Conclusions: Innate and adaptive immune cells are increased in the airways associated with allergic asthmatic responses. Total and type 2 cytokine-positive ILC2 are increased only within the airways, whereas CD4⁺ T lymphocytes demonstrated local and systemic increases. Steroid sensitivity of both cells may explain effectiveness of this therapy in those with mild asthma.

Keywords: innate lymphoid cells; eosinophilic inflammation; allergic asthma

Asthma is a chronic and heterogeneous disease characterized by reversible airflow obstruction, airway hyperresponsiveness, and inflammation of the airways (1). Type 2 cytokines, including IL-4, IL-5, and IL-13, drive features of eosinophilic asthma, including IgE-class switching, tissue remodeling, airway hyperresponsiveness,

and mucus hypersecretion. Type 2 cytokines recruit, maintain, and activate mature eosinophils and, in addition, promote lung homing and *in situ* differentiation of eosinophil-lineage committed progenitor cells (2–4). Although CD4⁺ T cells are an important source of type 2 cytokines, the major cellular sources

and timeline of IL-5 and IL-13 production in allergic asthmatic responses are poorly understood.

Recently identified cells, termed type 2 innate lymphoid cells (ILC2), may form a critical link between the innate and adaptive components of type 2 immunity (5–7). ILC2 are lineage-negative cells that lack antigen

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At a Glance Commentary

Scientific Knowledge on the

Subject: Group 2 innate lymphoid cells (ILC2), a major source of type 2 cytokines, initiate eosinophilic inflammatory responses in murine models of asthma in the absence of adaptive immune cells, including CD4⁺ T cells. The relative activation of ILC2 to adaptive immune cells within the airways of patients with atopy and asthma after allergen inhalation challenge has not been investigated. The objective of this study was to investigate compartmental fluctuations and activation of the role of ILC2 in allergen-induced airway eosinophilic responses.

What This Study Adds to the

Field: Relative activation of innate and adaptive immune cells within the airways in subjects with allergic asthma inflammatory responses remains poorly defined. Here, we show that both ILC2 and CD4⁺ T lymphocytes are activated in response to allergen challenge in subjects with mild asthma, but compartmental differences exist. ILC2 are increased and rapidly activated locally within the airways over a discrete time course (within 24 h after challenge). In contrast, CD4⁺ T lymphocytes are activated in both the airways and the systemic compartments, including blood and bone marrow, and this effect was detectable up to 48 hours after allergen challenge. Targeting localized activation of both ILC2 and CD4⁺ T lymphocytes may be important for optimal control of eosinophilic inflammation in the airways in asthma.

recognition receptors, and produce type 2 cytokines when activated by members of the alarmin family of cytokines IL-33, IL-25, and thymic stromal lymphopoietin (TSLP) produced by perturbed airway epithelial cells (8, 9). ILC2 are concentrated within mucosal tissues and shown to contribute to several aspects of lung pathology in mouse models of airway inflammation and immunity (10–14).

Exposure to proteases, such as papain or the natural aeroallergen, *Alternaria alternata*, show that ILC2 provide the primary early innate cellular source of IL-5 and IL-13 (15, 16) and drive eosinophilic airway inflammation in the absence of CD4⁺ T cells (15, 17–19). More recently, a study demonstrated that a cooperative interaction between ILC2 and CD4⁺ T cells is required for a maximal adaptive immune response (20), thus highlighting the importance of investigating the relative contribution of innate and adaptive immune cells in type 2 inflammatory responses.

In humans, ILC2 are found in the lung, gut, and blood (21) with enhanced accumulations at tissue sites of eosinophilic inflammation (22–24). ILC2 express the master type 2 cytokine transcription factor, GATA-3, supporting a role for rapid ILC2-derived type 2 cytokine production (25, 26). In agreement with this, increased numbers of circulating ILC2 with a greater capacity to produce IL-5 and IL-13 (27) and greater pro-type 2 transcriptome expression in ILC2 are found in subjects with atopy and asthma (28). In the airways, increased ILC2 numbers can be detected in severe asthma (29), the greatest number of IL-5⁺IL-13⁺ ILC2 present in sputum of those with severe asthma with persistent airway eosinophilia despite high-dose oral corticosteroids (30). Despite the potential

for ILC2 to contribute to eosinophilic inflammatory responses, activation of these cells within the airways in atopic asthma has not been investigated and the steroid sensitivity of these cells is not fully understood.

The role of ILC2 in the development of eosinophilic airway inflammation in those with mild asthma was investigated by enumerating total numbers and activated cells, assessed as cells immunopositive for intracellular IL-5 and/or IL-13. Induced sputum was collected to noninvasively sample the airways of subjects with asthma. Cross-compartmental comparisons were undertaken to provide a comprehensive picture of the trafficking of ILC2 and CD4⁺ T cells from the bone marrow to the lungs. In addition, we investigated the steroid sensitivity of ILC2, *in vitro*.

Methods

Subjects

Ten nonsmoking subjects with mild stable asthma were recruited for this study. Subjects (aged 19–60 yr) were not on any regular asthma treatment, infrequently used inhaled β_2 -agonists as reliever therapy, and were asymptomatic for 4 weeks before the study. Baseline subject characteristics are summarized in Table 1. All experimental procedures were reviewed and approved by

Table 1. Subject Characteristics

Sex	Age (yr)	FEV ₁ (% Predicted)	Mch PC ₂₀ (mg/ml)	Allergen	Allergen Dilution
M	53	97.6	9.56	HDMDP	1:64
M	50	98.4	3.37	Cat	1:64
M	66	95.4	9.97	Cat	1:16
M	27	112.4	4.7	HDMDP	1:128
M	23	88.8	5.65	Cat	1:512
M	21	94.3	10.25	Tree	1:16
F	21	80.7	0.35	Ragweed	1:4
F	23	80.6	7.73	HDMDP	1:128
M	35	85.1	5	Grass	1:1,024
F	21	96.1	0.32	Horse	1:256
M/F 7:3	34 ± 5.2	92.9 ± 3.0			

Definition of abbreviations: HDMDP = house dust mite protein; Mch = methacholine; PC₂₀ = provocative concentration of methacholine causing a 20% fall in FEV₁. Baseline characteristics of subjects with mild asthma (n = 10) enrolled in the study. Allergen refers to antigen (at a specified dilution) used for inhalation challenge. Nonsmoking subjects with mild stable asthma (n = 10; aged 21–66) were enrolled in the study. Subjects were skin prick–positive to common aeroallergens, FEV₁ greater than or equal to 70% predicted, and baseline methacholine PC₂₀ less than or equal to 16 mg/ml. Subjects had no other lung disease or chronic illness, lower respiratory tract infection, or worsening of asthma for 4 weeks before screening, and avoided exposure to sensitizing allergens apart from house dust mite. Subjects were not on steroid treatment, infrequently used inhaled β_2 -agonist for treatment of asthma, and refrained from β_2 -agonist and caffeinated beverages before laboratory visits. In the bottom row, age and FEV₁ data are presented as mean ± SEM.

the Hamilton Integrated Research Ethics Board and all subjects provided informed consent.

Study Design

Subjects completed a randomized, diluent-controlled, allergen-challenge crossover study (Figure 1). Subjects underwent skin prick testing, provocative concentration of methacholine causing a 20% fall in FEV₁ (PC₂₀), and a medical history during a screening period. Eligible subjects participated in two series of experiments. In series 1, baseline measurements of blood and a bone marrow aspirate were collected before challenge. On the following day, subjects were randomized to inhale diluent or allergen. Spirometry was performed hourly to 7 hours after challenge, blood was drawn 6 and 24 hours after challenge, and a bone marrow aspirate was collected 24 hours after challenge. After a washout period of 4 weeks, subjects returned for the corresponding challenge (allergen or diluent), and matching measurements of blood, bone marrow, and spirometry were repeated.

Series 2 was similar to series 1; however, bone marrow and blood sampling was replaced by methacholine PC₂₀ tests, and

sputum induction was conducted at baseline before challenge and 24 and 48 hours after challenge (Figure 1).

Methacholine Inhalation and Airway Challenges

Methacholine inhalation challenges were performed by the tidal breathing method using a Wright nebulizer as previously described (31). Asthma was required to be stable (FEV₁ within 10% of baseline) to proceed with the inhaled challenges. Allergen inhalation challenge was performed as previously described (32) and detailed in the online supplement.

Cell Collection and Isolation

Sputum was induced by hypertonic saline inhalation as previously described (33) and processed as described in the online supplement. Cytospins were prepared for differential counts and the remaining cells were aliquoted for flow cytometry. Sputum fluid phase was separated by centrifugation and stored at -70°C for ELISA.

Peripheral blood and bone marrow aspirates were collected as previously described and detailed in the online supplement (34).

Immunofluorescence Staining and Flow Cytometric Gating Strategy

Sputum-extracted cells and mononuclear cells from blood and bone marrow were stained for ILC2 and CD4 enumerations with antibodies to surface markers Lin-eFluor450, FcεR1-eFluor450, CD45-BV786, ST2-PE, CRTH2-BV421, IL7Rα (CD127)-PE-cy7, and CD4-AF700 or relevant isotype controls. Details of immunostaining are described in the online supplement and gating strategies for ILC2 and CD4⁺ T cells in Figures E1 and E2 in the online supplement, respectively.

Fluorescence-activated Cell Sorting of ILC2 and *In Vitro* Cell Culture

See the online supplement for details.

Multiplex Analysis of Cytokines in Sputum Fluid Phase

See the online supplement for details.

Statistical Analysis

Data were analyzed using GraphPad Prism 6 software (GraphPad Software Inc., La Jolla, CA). Data are expressed as mean $\pm$ SEM unless stated otherwise. For data not normally distributed, statistical analyses were performed on the log-transformed

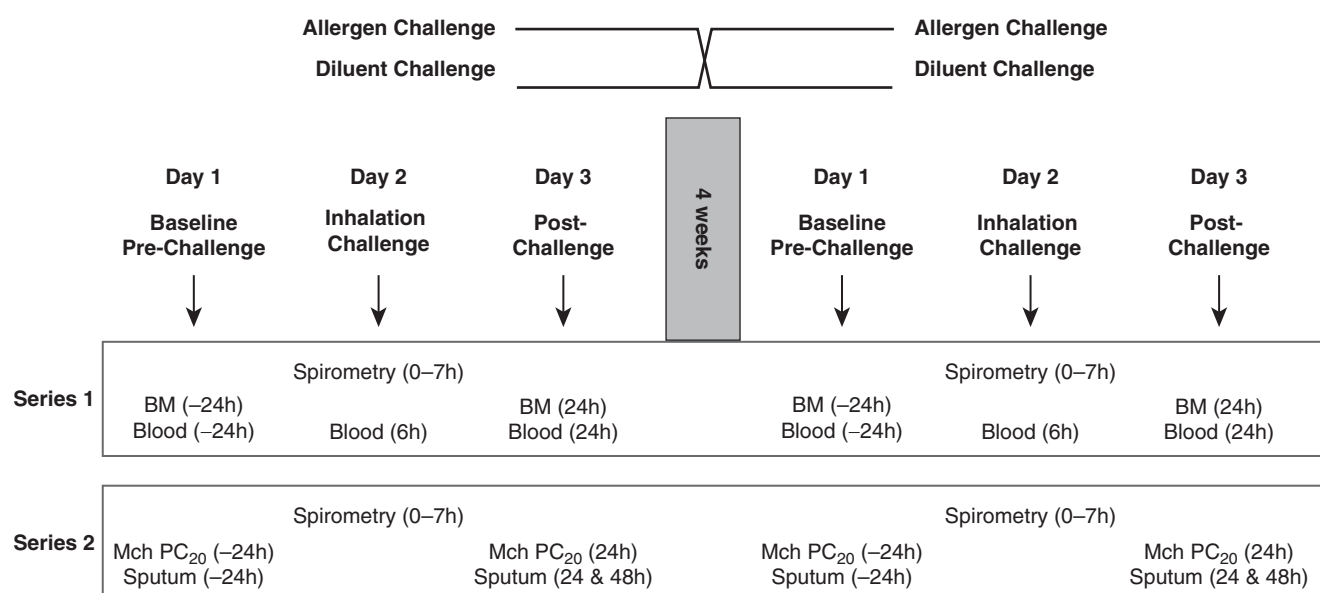


Figure 1. Diluent controlled-allergen challenge study in subjects with mild asthma. Series 1: on Day 1, baseline samples of bone marrow (BM) and blood (PB) were collected. On Day 2, subjects were randomized to inhale diluent or allergen; spirometry was performed hourly to 7 hours after challenge and PB drawn 6 hours after challenge. On Day 3, BM and PB were taken 24 hours after challenge. There was a 4-week washout period between each arm. Series 2: on Day 1, baseline measurements of Mch PC₂₀, PB, and sputum were collected. On Day 2, subjects were randomized to inhale diluent or allergen; spirometry was performed hourly to 7 hours after challenge. On Day 3, Mch PC₂₀ and sputum were collected 24 hours after challenge and again at 48 hours after challenge on Day 4. All subjects attended both series and completed the study within a year. Mch = methacholine; PC₂₀ = provocative concentration of methacholine causing a 20% fall in FEV₁.

data. Statistical analysis for the inhalation challenges was performed using two-way analysis of variance and the Bonferroni *post hoc* test. All *in vitro* experiment data were analyzed by using one-way analysis of variance with a Tukey *post hoc* test. Correlations were analyzed with Spearman rank test. Alpha was set at 0.05.

Results

Allergen-induced Airway Responses

All subjects in this study had a dual response after inhaled allergen, with a mean maximal fall in FEV₁ from baseline of $41.02 \pm 3.70\%$ during the early response ($P < 0.05$) and $23.67 \pm 4.57\%$ during the late response ($P < 0.05$) (see Figure E3). In contrast, there was no significant change in FEV₁ from baseline during the diluent challenge. There was a significant decrease in methacholine PC₂₀ 24 hours after allergen, but not diluent challenge (Table 2). In sputum, although total cell counts remained unchanged, there was a significant increase in absolute numbers and percent eosinophils when preallergen levels were compared with 24-hour and 48-hour postallergen levels, but not diluent challenge (Table 2).

Allergen-induced Changes in ILC2 and CD4⁺ Cells

Compared with preallergen levels, absolute numbers of ILC2 in sputum were significantly increased at 24 hours after

allergen challenge, returning to baseline levels by 48 hours after challenge (118 ± 30 vs. 483 ± 149 vs. 224 ± 88 cells/ml, respectively; $P < 0.01$) (Figure 2A). In addition, compared with baseline levels, there was also a significant increase in ILC2 expressing intracellular levels of IL-5 (62 ± 19 vs. 292 ± 68 cells/ml; $P < 0.01$), IL-13 (73 ± 29 vs. 316 ± 106 cells/ml; $P < 0.01$), and both IL-5 and IL-13 (32 ± 14 vs. 109 ± 83 cells/ml; $P < 0.01$) at 24 hours, but not at 48 hours after allergen (Figures 2B–2D, respectively). In contrast, diluent inhalation had no significant effect on absolute numbers of ILC2 in sputum.

Compared with preallergen levels, significantly greater numbers of CD4⁺ T cells were detected at 24 hours after allergen and persisted to 48 hours after allergen challenge (22 ± 11 vs. 108 ± 45 vs. $45 \pm 21 \times 10^3$ cells/ml, respectively; $P < 0.05$) (Figure 2E). In addition, significantly increased numbers of sputum CD4⁺ T cells expressing intracellular IL-5, IL-13, and both IL-5 and IL-13 were detected at both 24 and 48 hours after allergen but not diluent challenge (Figures 2F–2H, respectively).

Further phenotypic analyses of sputum ILC2 showed a significant increase in ILC2 numbers expressing CRTH2, a receptor for prostaglandin D₂ (PGD₂), a potent ILC2 chemoattractant (35), 24 hours after allergen but not diluent challenge (see Figure E4A). In addition, there was a significant increase

in the number of ILC2 expressing TSLPR 24 hours after allergen challenge (see Figure E4B). In contrast, allergen-induced changes in CRTH2 or TSLPR expression were not found on CD4⁺ T lymphocytes in sputum (see Figures E4C and E4D).

When ILC2 were identified as lin[−]CD45⁺CD127⁺CRTH2⁺ cells comparable fluctuation in intracellular type 2 cytokine levels were observed as described for lin[−]CD45⁺CD127⁺ST2⁺ phenotype (see Figure E5). In addition, a significant increase in ST2 expression but not TSLPR on these cells was observed in the airways 24 hours postallergen level compared with preallergen level sputum.

Absolute airway eosinophil numbers correlated with the absolute numbers of IL5⁺ ILC2, as well as CD4⁺ T lymphocytes at all time points ($r = 0.510$, $P = 0.004$ and $r = 0.724$, $P = 0.005$, respectively) (see Figure E6).

Blood and Bone Marrow: Allergen-induced Changes in ILC2 and CD4⁺ Cells

In blood, the absolute number of ILC2 was unchanged at 6 hours, but were significantly decreased at 24 hours after allergen challenge (227 ± 65 vs. 92 ± 22 cells/ml blood; $P < 0.01$) (Figure 3A). There were no significant changes in numbers of ILC2 expressing intracellular type 2 cytokines after allergen or diluent challenge in blood after allergen challenge (Figures 3B–3D). In contrast, although absolute numbers of CD4⁺ T cells in blood did not change

Table 2. Lung Function and Inflammatory Cell Details

	Allergen Challenge	Diluent Challenge
Methacholine PC ₂₀ , mg/ml		
Before challenge	3.47 (14.74)	3.67 (14.10)
24 h after challenge	1.44 (3.59) [†]	5.49 (10.31)
Total sputum cells $\times 10^6$ cells/ml		
Before challenge	5.21 $\pm$ 0.92	3.63 $\pm$ 0.74
24 h after challenge	6.95 $\pm$ 1.10	5.54 $\pm$ 0.82
48 h after challenge	6.19 $\pm$ 1.68	7.96 $\pm$ 1.57
Sputum eosinophils $\times 10^3$ cells/ml (%)		
Before challenge	42.62 $\pm$ 37.72 (3.31 $\pm$ 1.31)	10.18 $\pm$ 4.24 (1.44 $\pm$ 0.37)
24 h after challenge	187.51 $\pm$ 92.07 (13.94 $\pm$ 1.98) [†]	75.37 $\pm$ 53.20 (2.65 $\pm$ 0.84)
48 h after challenge	163.62 $\pm$ 122.61 (14.66 $\pm$ 4.18) [†]	83.44 $\pm$ 62.87 (1.59 $\pm$ 0.30)

Definition of abbreviation: PC₂₀ = provocative concentration of methacholine causing a 20% fall in FEV₁.

Airway hyperresponsiveness and airway inflammatory cell changes after inhalation challenge of subjects with mild asthma ($n = 10$). There were significant allergen-induced changes in methacholine PC₂₀ levels and sputum eosinophil numbers compared with diluent inhalation challenge. Data are presented as median and interquartile range for methacholine PC₂₀ measurements made when sputum was collected. For total and eosinophil cell counts from sputum, data are presented as mean $\pm$ SEM.

^{*} $P < 0.05$ for comparison with baseline.

[†] $P < 0.05$ for comparison with diluent.

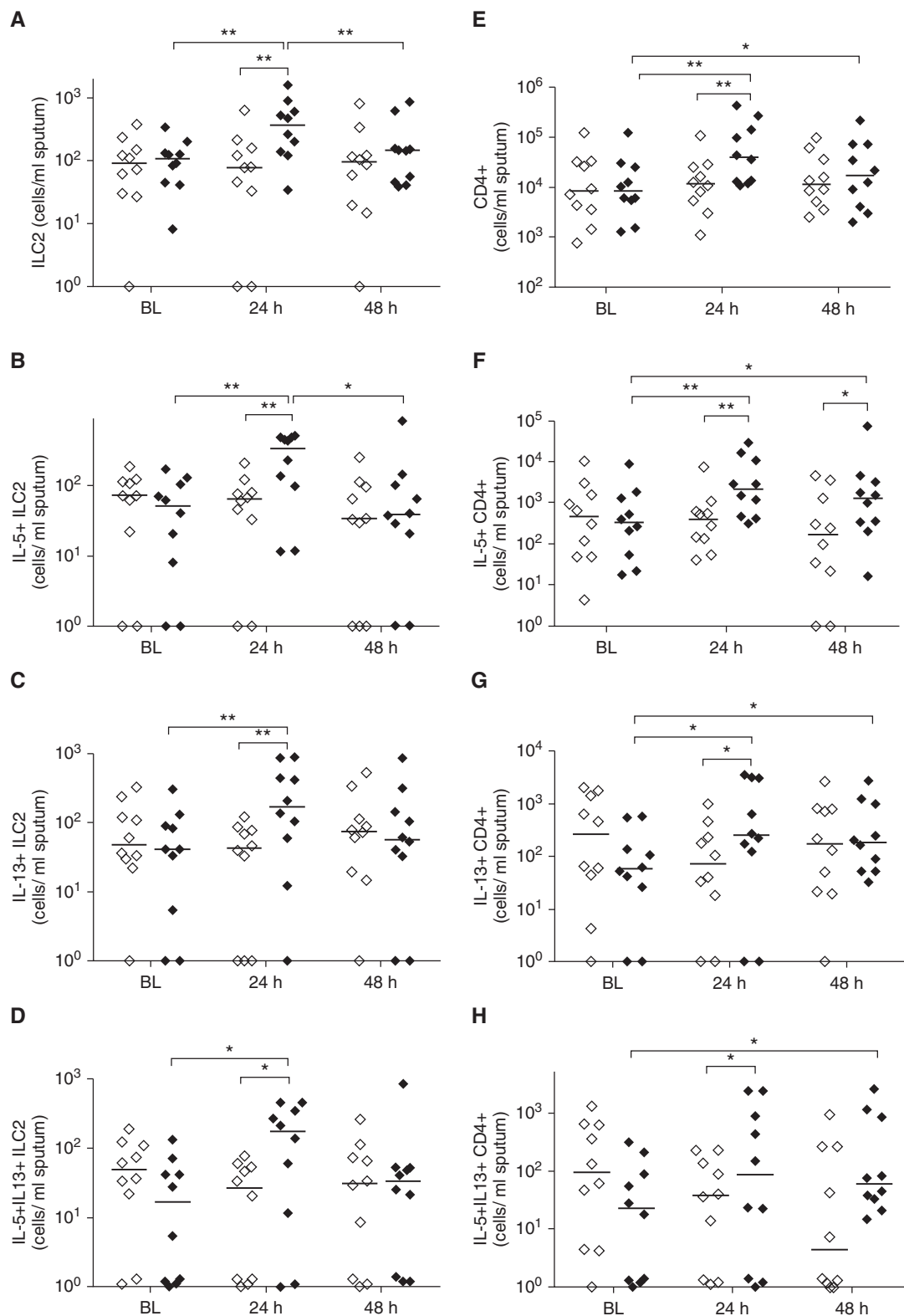


Figure 2. (A–D) Absolute ILC2 and (E–H) CD4⁺ T-lymphocyte cell numbers and intracellular cytokine expression in sputum from subjects with mild asthma after diluent or allergen challenge (open and solid symbols, respectively). After allergen but not diluent challenge, there was a significant increase in (A) absolute numbers of sputum total ILC2, (B) IL-5⁺ ILC2, (C) IL-13⁺ ILC2, and (D) IL-5⁺IL-13⁺ ILC2 in sputum 24 hours after challenge, only. For CD4⁺ T cells in sputum, allergen-induced increases were observed for (E) total and (F) IL-5⁺, (G) IL-13⁺, and (H) IL-5⁺IL-13⁺ cells at BL and 24 and 48 hours after inhalation challenge. Horizontal lines represent median values of each data set. n = 10; *P < 0.05; **P < 0.01. BL = baseline; ILC2 = type 2 innate lymphoid cells.

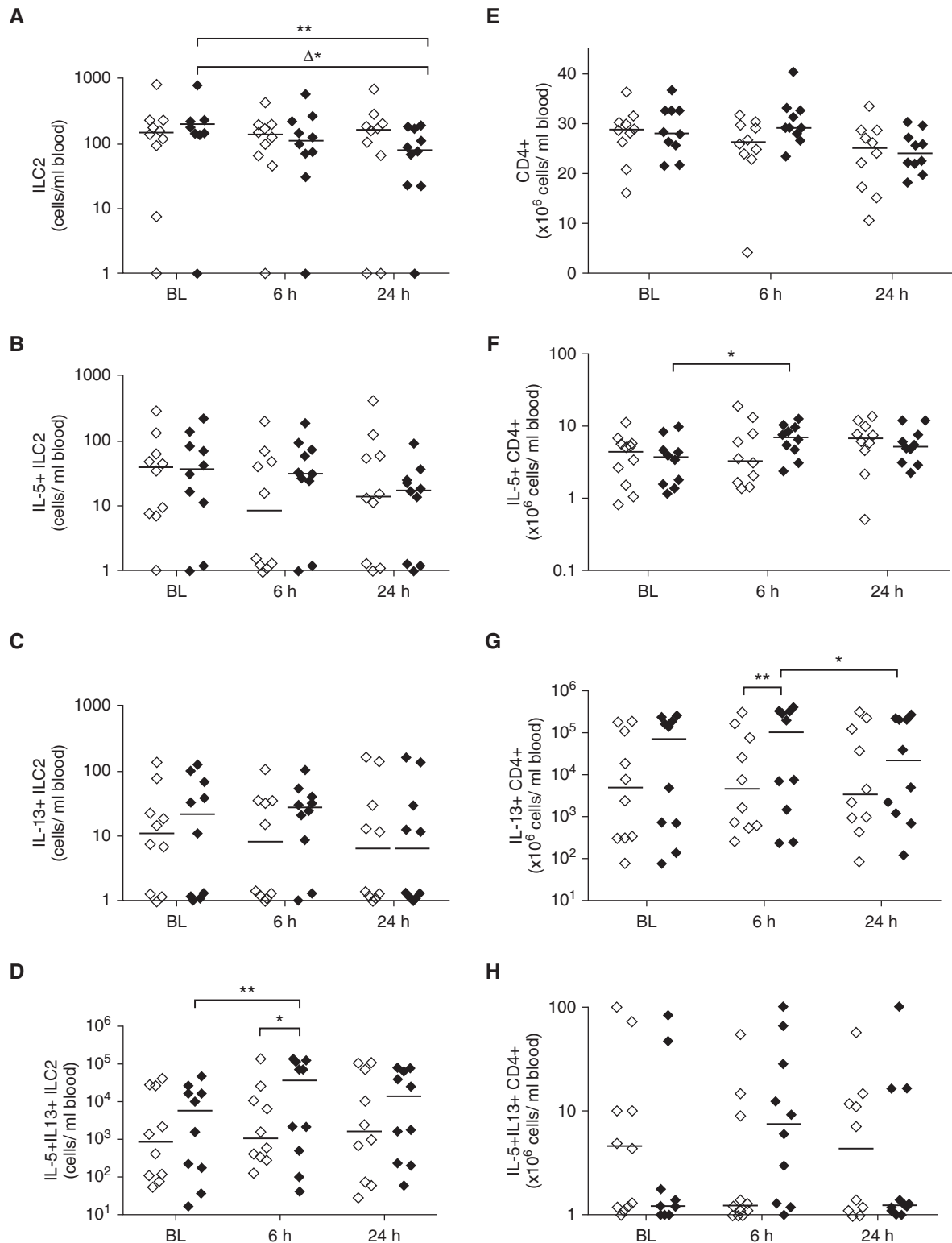


Figure 3. (A–D) Absolute ILC2 and (E–H) CD4⁺ T-lymphocyte cell numbers and intracellular cytokine expression in blood from subjects with mild asthma after diluent or allergen challenge (*open* and *solid* symbols, respectively). In blood, (A) absolute numbers of ILC2 were significantly decreased 24 hours after allergen challenge. (B–D) No significant allergen-induced changes were found for ILC2 expressing intracellular type 2 cytokines. Absolute numbers of CD4⁺ T cells in (E) blood did not change significantly after allergen challenge, but there were significant increases in type 2 expressing CD4⁺ T cells in blood (F–H) at 6 hours after allergen challenge. Horizontal lines represent median values of each data set. $n = 10$; * $P < 0.05$; ** $P < 0.01$. BL = baseline; ILC2 = type 2 innate lymphoid cells.

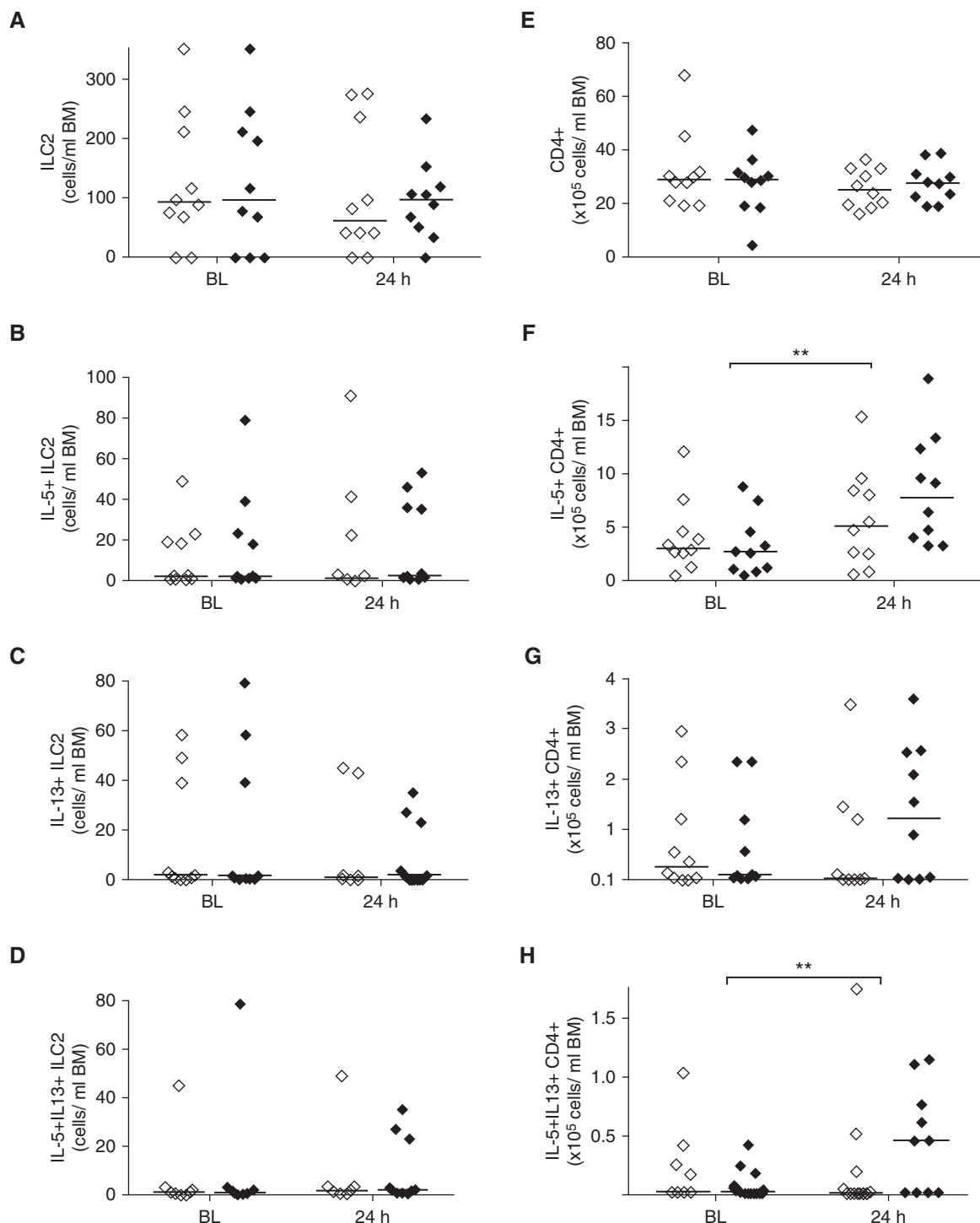


Figure 4. (A–D) Absolute ILC2 and (E–H) CD4⁺ T-lymphocyte cell numbers and intracellular cytokine expression in bone marrow (BM) from subjects with mild asthma after diluent or allergen challenge (open and solid symbols, respectively). No significant allergen-induced changes were observed in (A) absolute ILC2 or (B–D) intracellular expression of IL-5, IL-13, or both. (E) Absolute numbers of CD4⁺ T cells in BM did not change significantly after allergen challenge, but there was a significant increase in (F) IL-5⁺ and (H) IL-5⁺IL13⁺ but not (G) IL-13⁺ CD4⁺ T cells in BM 24 hours after allergen-inhalation challenge. Horizontal lines represent median values of each data set. $n = 10$; ** $P < 0.01$. BL = baseline; ILC2 = type 2 innate lymphoid cells.

significantly after allergen challenge, there was a significant increase in the number of CD4⁺ T cells expressing IL-5 at 6 hours after allergen challenge (Figures 3E and 3F). Similarly, numbers of CD4⁺ cells expressing IL-13 and both IL-5 and IL-13 were greater at 6 hours after allergen challenge compared with 6 hours after diluent challenge, but not 24 hours after allergen challenge (Figures 3G and 3H).

In bone marrow, no significant changes were observed in absolute ILC2 numbers or intracellular expression of type 2 cytokines by ILC2, 24 hours after allergen or diluent challenge (Figures 4A–4D). Similarly, there was no change in absolute numbers of CD4⁺ cells after allergen inhalation challenge in the bone marrow

(Figure 4E), although activation of CD4⁺ cells assessed as increased intracellular expression of type 2 cytokines was significantly increased at 24 hours after allergen challenge for both CD4⁺IL-5⁺ (3.4 ± 0.9 vs. $8.5 \pm 1.6 \times 10^5$ cells/ml; $P < 0.01$) and CD4⁺IL-5⁺IL-13⁺ (0.10 ± 0.04 vs. $0.46 \pm 0.14 \times 10^5$ cells/ml; $P < 0.01$) (Figures 4F–4H).

Allergen-induced Changes in Cytokine Levels

Compared with before allergen challenge, IL-5 levels in sputum supernatants were significantly increased 24 hours but not 48 hours after allergen challenge (6.8 ± 1.2 vs. 40.1 ± 17.0 pg/ml vs. 19.5 ± 5.9 pg/ml; $P < 0.05$). Although there was a trend for an increase in IL-13 levels in sputum

supernatants at 24 hours after allergen compared with preallergen levels, this was not significant (14.6 ± 2.8 vs. 22.5 ± 3.5 pg/ml). No significant changes were found in IL-33 levels in sputum supernatants at any time point (Figure 5A). Levels of IL-25 and TSLP were below the limit of detection in all samples tested (data not shown). Sputum levels of IL-5 correlated with IL-13 levels ($r = 0.568$; $P = 0.001$) and sputum eosinophil numbers at all time points ($r = 0.797$; $P < 0.001$) (Figure 5B).

IL-33 Stimulated Th2 Cytokine Generation by ILC2 and CD4⁺ Cells

A lineage cocktail of antibodies to CD2, CD3, CD14, CD16, CD19, CD56, CD235a, and FcεRI was used to identify lineage-positive cells including T cells, monocytes,

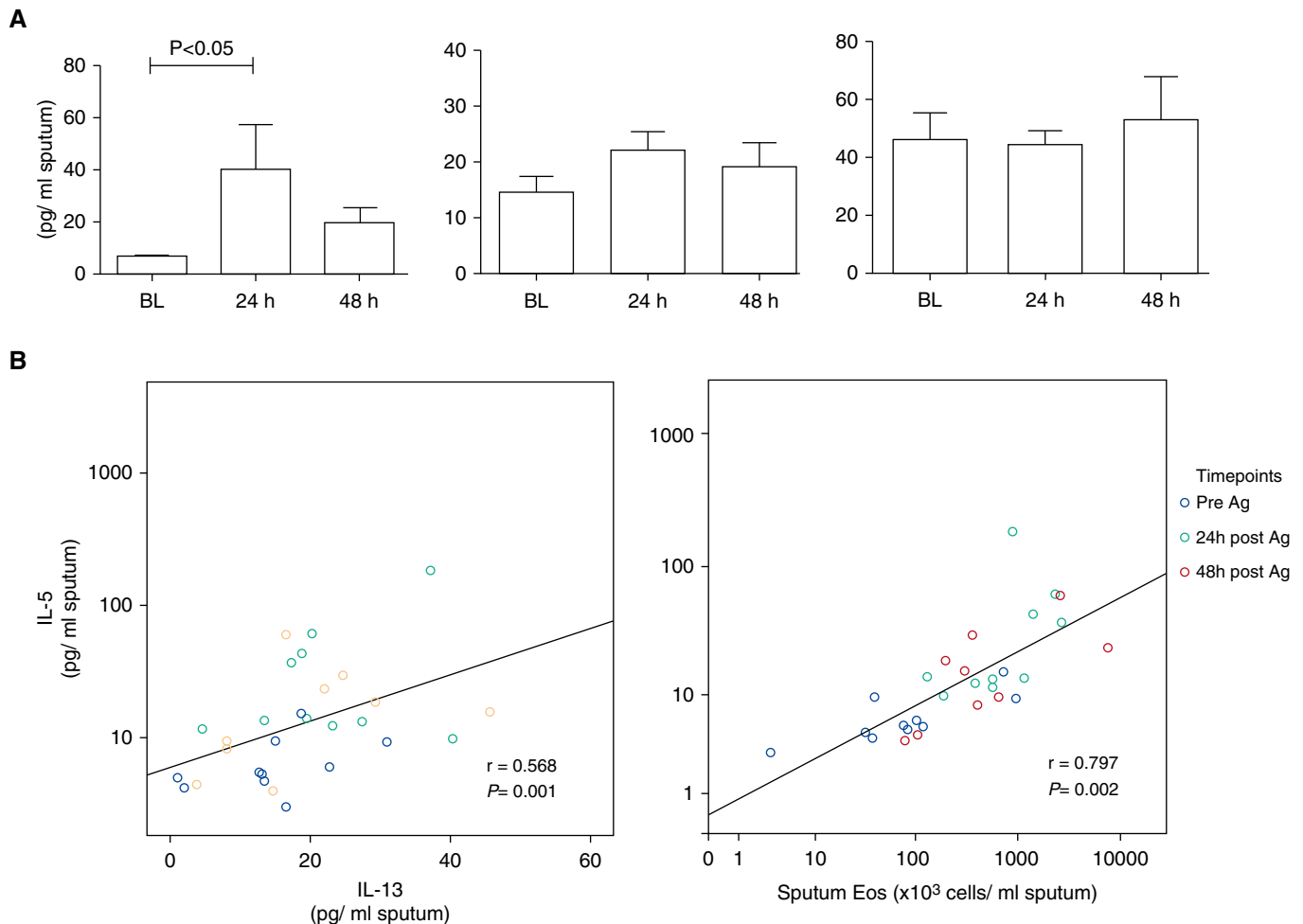


Figure 5. Allergen inhalation-induced changes in cytokine levels in sputum supernatants from subjects with mild asthma ($n = 10$). (A) IL-5 (left) was significantly increased 24 hours after allergen challenge, but no significant changes in IL-13 (middle) or IL-33 (right) were detected after allergen challenge. No changes after diluent challenge were detected. Data are presented as mean $\pm$ SEM. (B) Significant correlation was found between allergen-induced levels of IL-5 or IL-3 in sputum supernatants and absolute sputum eosinophil numbers. Data are presented as correlations with Spearman rank r values and the line of best fit. $P < 0.05$ was considered significant. Ag = allergen; BL = baseline; Eos = eosinophil.

neutrophils, B cells, natural killer cells, mast cells, and basophils. Lin⁻ cells were further subdivided and sorted on the basis of CD127 and ST2 expression (Figure 6A). To determine whether the epithelial-derived cytokines can induce IL-5 and IL-13 expression by ILC2, Lin⁻ and Lin⁺ cells were cultured with IL-33 and IL-2 for 6 days. On a per-cell basis, ILC2 (Lin⁻CD45⁺CD127⁺ST2⁺) produced a greater quantity of IL-5 and IL-13 compared with Lin⁺ cells and Lin⁻CD127⁻ST2⁻ cells ($P < 0.01$) (Figure 6B). Cell morphology of ILC2 changed after stimulation with IL-33 and IL-2. Before culture, ILC2 were approximately 10 μm in diameter with a high nucleus-to-cytoplasm ratio (Figure 6C). Culture with IL-33 and IL-2 caused marked increases in cellular size, the development of Golgi bodies and endoplasmic reticulum, and a decreased nucleus-to-cytoplasm ratio (Figure 6D).

Steroid Sensitivity of ILC2 Compared with Lin⁺CD4⁺ Lymphocytes, *In Vitro*

Coculture of purified populations of blood-derived CD4⁺ cells with cytokine stimulants IL-2 and IL-33 in the presence of dexamethasone showed a dose-dependent inhibition of IL-5 and IL-13 production (Figures 7A and 7B). In the presence of an optimal dose of dexamethasone (10^{-7} M), the sort-purified ILC2 population showed a significant reduction in IL-5 ($72.9 \pm 15.8\%$; $P < 0.05$) and IL-13 ($78.6 \pm 15.3\%$; $P < 0.05$) production (Figures 7C and 7D).

Discussion

This study investigated activation of innate and adaptive immune cells within the airways after allergen inhalation challenge in subjects with atopy and asthma. The novel findings are that after allergen inhalation challenge, ILC2 are only activated within the airways, over a discrete time course

suggestive of a mucosal role in the initiation of eosinophilic inflammation. By comparison, CD4⁺ T cells, although activated within the airways 24 hours after allergen challenge, showed sustained activation to 48 hours after challenge and displayed increased activation within the blood and bone marrow, supporting both local and systemic roles in the genesis of the type 2 immune responses.

We show for the first time that ILC2 are increased and activated in humans with asthma after allergen-inhalation challenge, maximal at 24 hours after allergen. Although ILC2 activation may peak earlier than 24 hours after allergen, this was not investigated in the current study. A previous study has reported that sputum levels of lin⁻CD34⁺ cells expressing intracellular IL-5 and IL-13 were increased at 24 hours and further at 48 hours after allergen challenge compared with preallergen challenge levels in those with mild asthma (36). Using a similar time course, this study

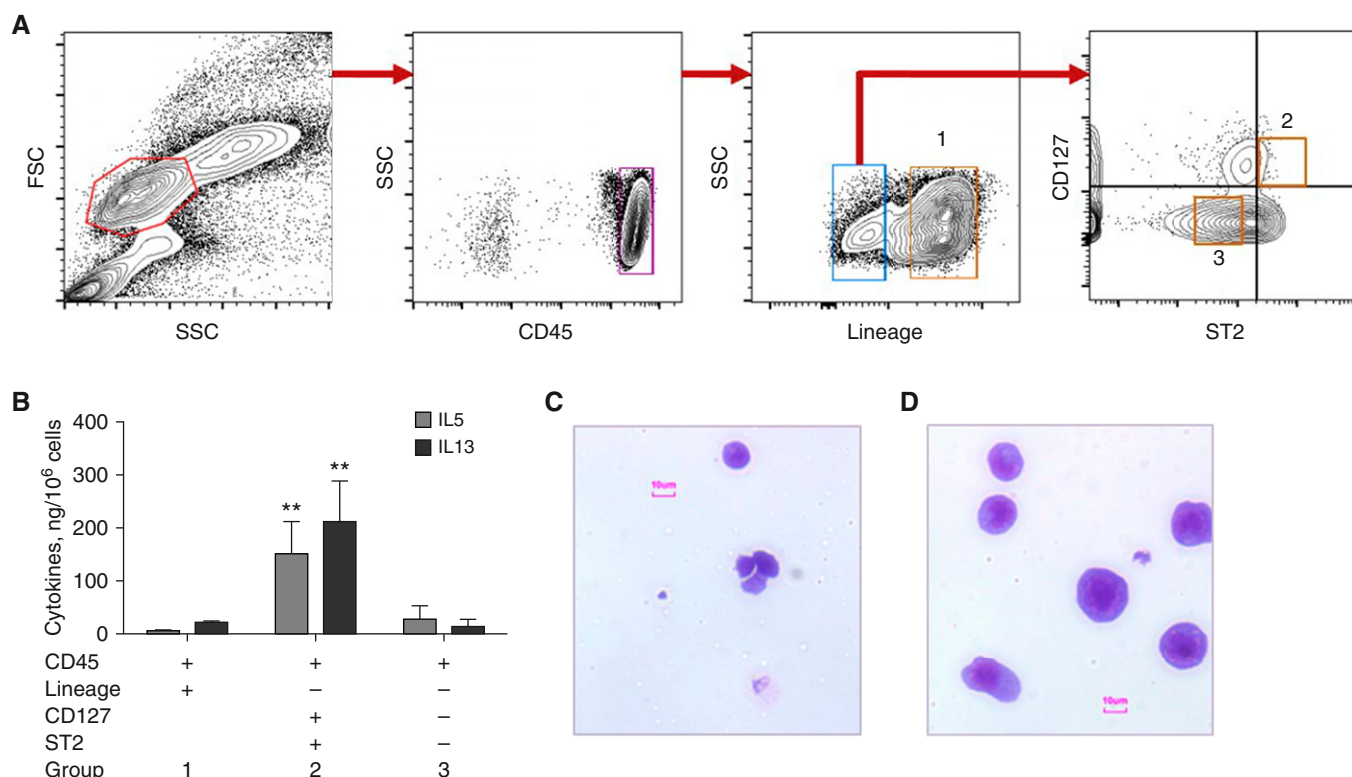


Figure 6. ILC2 produce type 2 cytokines in response to IL-33 stimulation, *in vitro*. (A) By flow cytometry, various cell populations were sort-purified from blood region 1 (CD45⁺Lin⁺ cells; mixed leukocytes), region 2 (Lin⁻CD45⁺CD127⁺ST2⁺ cells; ILC2), and region 3 (Lin⁻CD45⁺CD127⁻ST2⁻ cells; primitive cells). (B) The cell populations were stimulated for 6 days with medium alone or IL-33 (10 ng/ml) plus IL-2 (20 U/ml). Levels of IL-5 and IL-13 in cell-free supernatants were measured by multiplex bead immunoassay. Data are presented as mean $\pm$ SEM of seven independent experiments. $**P < 0.01$ compared with medium alone. ILC2 cell morphology is shown (C) before and (D) after culture by staining the cytospin preparations with hematoxylin and eosin stain. Scale bars = 10 μm . FSC = forward scatter; ILC2 = type 2 innate lymphoid cells; SSC = side scatter; ST2 = receptor for IL-33 (also known as IL-1 receptor-like 1).

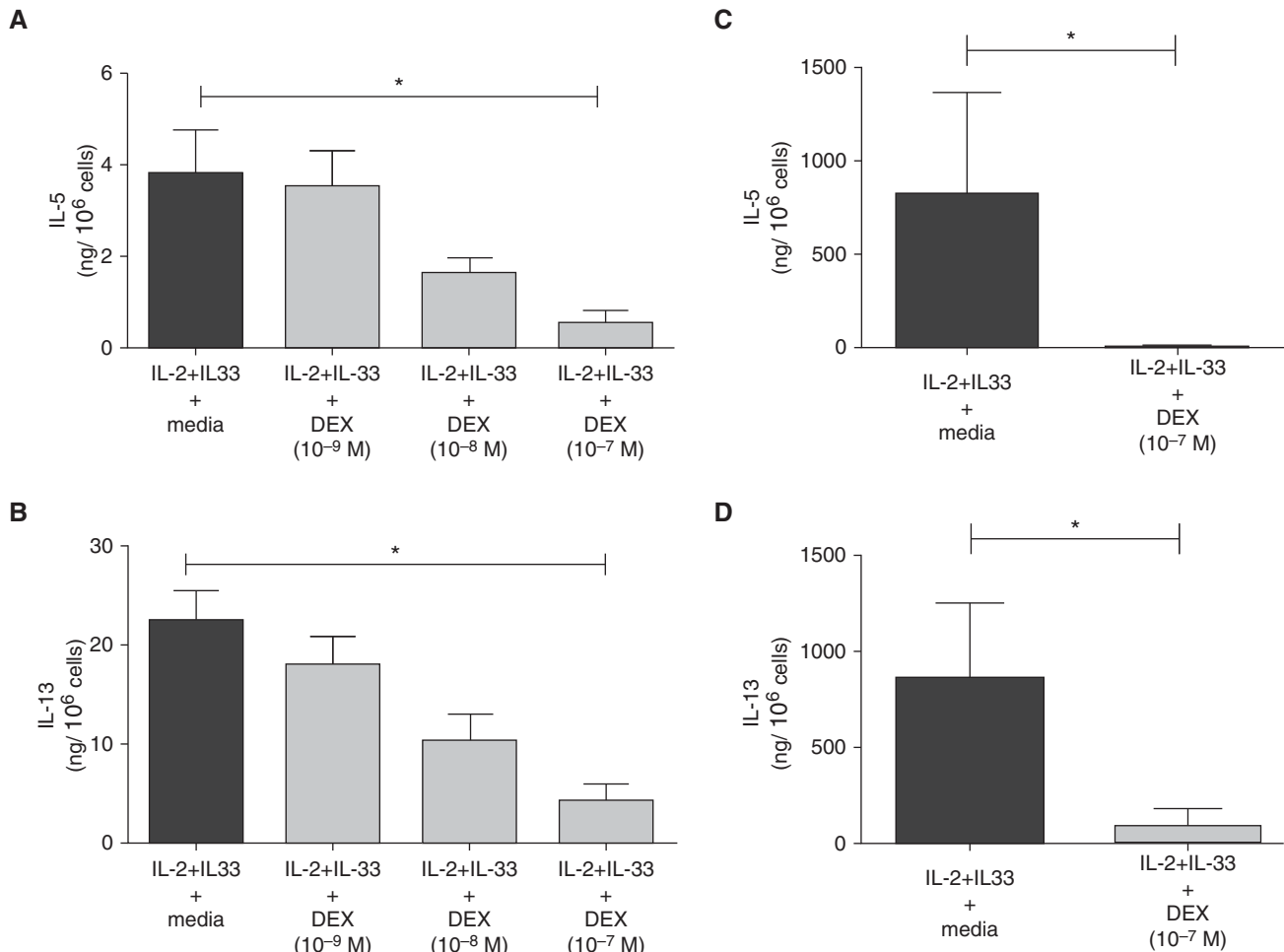


Figure 7. Steroid sensitivity of blood (A and B) CD4⁺ cells and (C and D) ILC2, *in vitro*. Sort-purified populations of CD4⁺ T cells and ILC2 from blood of subjects with mild asthma were cultured in the presence of IL-33 (10 ng/ml) and IL-2 (20 U/ml) with dexamethasone for 6 days. (A and C) IL-5 and (B and D) IL-13 in cell-free supernatants were measured using multiplex bead immunoassay. Both CD4⁺ cells and ILC2 demonstrated significant attenuation of cytokine generation in the presence of steroid. Data are presented as mean $\pm$ SEM of six independent experiments. * P < 0.05 for comparisons without drug. DEX = dexamethasone; ILC2 = type 2 innate lymphoid cells.

found that total and activated numbers of ILC2, identified as lin⁻CD45⁺CD127⁺ST2⁺ cells or lin⁻CD45⁺CD127⁺CRT2⁺ and lacking CD34 expression, peaked at 24 hours and diminished to baseline levels by 48 hours after challenge. These differences suggest that we are likely identifying different cell populations. Whereas lin⁻CD34⁺ precursor cells may act as proinflammatory cells sustaining local eosinophil differentiation and prolonged survival of mature eosinophils over a protracted time course, the current data indicate that ILC2 are a distinct cell population providing an early sentinel source of type 2 cytokines in allergic asthmatic responses. This is in agreement with mouse models of asthma where lung ILC2 expand rapidly in

number and expression of type 2 cytokines in response to allergen exposure, which then decreased in number and ceased cytokine generation (18, 37). The sensitized ILC2 remaining within the lung were shown to be reminiscent of memory cells with the capacity to promote subsequent responses to multiple allergens (37).

That ILC2 are locally activated within the mucosa is in keeping with the considerable body of evidence that purports marked and rapid local tissue activation of these cells in type 2 inflammatory responses. Furthermore, analyses of cross-compartmental trafficking of ILC2 in parabiotic mouse pairs showed that, after helminth infestation, the acute inflammatory phase was associated with

local expansion of tissue resident ILC2, whereas the chronic inflammatory phase was associated with recruitment of ILC2 from hematogenous sources but that this accounted for less than 5% of total tissue-resident ILC2 (38). In allergic rhinitis, allergen-inhalation challenge demonstrated an increase in ILC2 within the blood 4 hours after allergen (39). In contrast, we observed a significant decrease in ILC2 24 hours after allergen, supporting the view that cross-compartmental migration from the systemic circulation into the airways may occur in the allergic asthmatic responses. Difference between the two studies likely reflected in the allergen exposure model whereby the former involved multiple nasal-allergen challenges,

whereas the latter, a single allergen-inhalation challenge.

We found increased total numbers of ILC2 expressing CRTH2, the receptor for the PGD₂, a reported chemoattractant for ILC2 isolated from blood and skin (40). The PGD₂–CRTH2 axis has been shown to regulate the *in vivo* accumulation of ILC2 and the development of type 2 inflammation in the lung in mouse models (35, 41). Although CRTH2 antagonism in a broad population of patients with asthma was not effective (42), a recent clinical trial using fexupirant effectively reduced airway eosinophilia in patients with persistent moderate-to-severe asthma who have an eosinophilic phenotype of asthma (43). Whether CRTH2/PGD₂ axis is involved in modulating the ILC2 traffic to the lungs in eosinophilic inflammatory responses remains to be investigated, although the current findings are supportive of this possibility.

CD4⁺ T lymphocytes demonstrated both local mucosal and systemic activation detected at 24 hours after allergen and persisted to levels greater than baseline levels at 48 hours after allergen in the airways. This suggests that within the airways, T lymphocytes may have a prolonged time course of activation in allergic asthmatic responses, thereby promoting the persistence of the eosinophilic response. In addition, systemic activation of CD4⁺ T lymphocytes, as determined by intracellular type 2 cytokine expression, is in agreement with studies in allergic rhinitis that detected increases at 6 hours after nasal allergen challenge (44). In addition, we found increases in CD4⁺IL-5⁺T lymphocytes in the bone marrow 24 hours after allergen challenge to levels that were comparable with our previous study, which detected type 2 cytokine production by CD3⁺ cells in the bone marrow at the mRNA level (45). These findings show that unlike ILC2, activated CD4⁺ T lymphocytes are detected both locally and systemically, the latter likely playing a role in stimulating IL-5–driven eosinophilopoietic processes within the bone marrow and priming the mobilization of mature eosinophils to the peripheral circulation during asthmatic responses.

In agreement with previous reports, we showed that ILC2 from those with mild asthma cultured with epithelial-derived cytokines IL-33 and IL-2 produced high

levels of type 2 cytokines (27). Comparable numbers of CD4⁺ cells produced 100-fold lower levels of IL-5 and IL-13 than ILC2. In sputum supernatants, we detected significant allergen-induced increase in IL-5, which peaked at 24 hours after allergen. Although IL-13 levels showed a trend for increase, this was not significant after allergen challenge. The levels of IL-5 detected at all time points correlated closely with IL-13 levels and the level of sputum eosinophilia. These are in agreement with established findings that type 2 cytokines promote eosinophilic inflammation in allergic asthma. In contrast, IL-33, an epithelial-derived cytokine known to stimulate ILC2 activation, did not show any change after allergen. IL-33 is rapidly oxidized, forming a biologic block at the ST2 binding site to modulate activity of the secreted native isoform (46). This may affect the detection of the cytokines by ELISA making it difficult to relate IL-33 levels at the protein level with factors of type 2 inflammatory responses in human models of disease. Alternatively, other factors that were not measured in this study may be involved in activating ILC2 after allergen-inhalational challenge. Tumor necrosis factor–like ligand 1A produced by activated macrophages, stressed epithelial cells, and activated dendritic cells has been shown to activate human ILC2 *in vitro* through the cognate death receptor 3 (47) and may play a role in activating ILC2 after allergen-inhalation challenge.

We have previously shown that ILC2 are increased in the airways of those with severe asthma on high-dose oral corticosteroid therapy compared with those with mild asthma (29, 30). The activation of ILC2 was greatest in those with uncontrolled airway eosinophilia (<3% sputum eosinophilia) despite normalization of CD4 T-cell activation levels to those comparable with subjects with mild asthma. This suggests that ILC2 may be steroid insensitive, which we investigated in the current study. In the presence of dexamethasone, ILC2 and CD4⁺ T cells from those with mild asthma demonstrated complete attenuation of IL-33–stimulated generation of type 2 cytokines without affecting the viability of the cells, in agreement with a recent study and supporting the effectiveness of inhaled

steroids as a gold standard for treatment of exacerbations in this group of subjects with asthma. Similar findings have been reported in patients with eosinophilic nasal polyposis treated with oral prednisone (48). The increased ILC2 activation found in patients with severe asthma with eosinophilic bronchitis may result from altered steroid sensitivity of ILC2 from these patients, or, alternatively, in the presence of exogenous mediators, such as TSLP, ILC2 may become steroid resistant, as has been shown in a murine model of allergic inflammation (49).

Although TSLP “high” asthma has not been described, a study has shown increased TSLP immune-positive regions in bronchial biopsies from subjects with severe asthma that colocalized to lin[−]CD45⁺IL-13⁺ cells assumed to be ILC2 (50). Furthermore, a clinical trial using anti-TSLP showed attenuation of baseline eosinophilia and allergen-induced airway eosinophilia together with improvement in airway function in those with mild asthma indicating a putative central role for this cytokine in eosinophilic inflammation (51). The role for TSLP in mediating steroid insensitivity in airway ILC2 from those with severe asthma remains to be investigated.

In conclusion, this study shows for the first time that ILC2 are activated locally within the airway after allergen inhalation challenge in subjects with atopy and asthma and may provide the an additional source of type 2 cytokines together with CD4⁺ T lymphocytes in the development airway eosinophilia in response to allergen challenge in subjects with mild asthma. Understanding factors that activate ILC2 activation may be an important therapeutic target for the treatment of subjects with difficult to control eosinophilic asthma. ■

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