

Activation of group 2 innate lymphoid cells after allergen challenge in asthmatic patients



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Background: Group 2 innate lymphoid cells (ILC2s) are effective producers of IL-5 and IL-13 during allergic inflammation and bridge the innate and adaptive immune responses. ILC2 numbers are increased in asthmatic patients compared with healthy control subjects. Thus far, human data describing their phenotype during acute allergic inflammation in the lung are incomplete.

Objectives: This study aims to characterize and compare blood- and lung-derived ILC2s before and after segmental allergen challenge in patients with mild-to-moderate asthma with high blood eosinophil counts (≥ 300 cells/ μ L).

Methods: ILC2s were isolated from blood and bronchoalveolar lavage (BAL) fluid before and after segmental allergen challenge. Cells were sorted by means of flow cytometry, cultured and analyzed for cytokine release or migration, and sequenced for RNA expression.

Results: ILC2s were nearly absent in the alveolar space under baseline conditions, but numbers increased significantly after allergen challenge ($P < .05$), whereas at the same time, ILC2 numbers in blood were reduced ($P < .05$). Prostaglandin D₂ and CXCL12 levels in BAL fluid correlated with decreased ILC2 numbers in blood ($P = .004$, respective $P = .024$). After allergen challenge, several genes promoting type 2 inflammation were expressed at greater levels in BAL fluid compared with blood ILC2s, whereas blood ILC2s remain unactivated.

Conclusion: ILC2s accumulate at the site of allergic inflammation and are recruited from the blood. Their transcriptional and functional activation pattern promotes type 2 inflammation. (J Allergy Clin Immunol 2019;144:61-9.)

Key words: Group 2 innate lymphoid cells, allergic asthma, allergen challenge, migration, gene expression

The discovery of innate lymphocyte cells as the innate counterpart of adaptive T lymphocytes has extended our understanding of the interaction of innate and adaptive immunity.¹ In patients with atopic diseases, such as asthma or rhinitis, the role of group 2 innate lymphoid cells (ILC2s) has drawn a lot of interest² because these cells are capable of linking the innate immune response to type 2 adaptive immunity mediating allergic responses. Even though they are rare in numbers and lack antigen-specific receptors, ILC2s appear to be remarkably potent cells in activating type 2 inflammation. They reside in mucosal tissues and directly sense alarmins (IL-33, IL-25, and thymic stromal lymphopoietin [TSLP]) released from the epithelium after encountering allergens, viruses, or worm parasites.³ In turn, ILC2s release significant amounts of IL-5 and IL-13 and have been identified as the initial source for recruitment of eosinophils to the lung.⁴ The role of ILC2s in allergic inflammation is evolving to be more complex, beyond releasing cytokines. They interact with T cells through expression of MHC class II complexes and OX40 ligand,^{5,6} enhance B-cell proliferation and immunoglobulin production,⁷ and potentiate memory T_H2 cell responses through modulating dendritic cells,⁸ and inducible T-cell costimulator (ICOS) signaling is essential for ILC2 function mediating airway hyperactivity.⁹

In asthmatic patients numbers of ILC2s are increased in peripheral blood and bronchoalveolar lavage (BAL) fluid compared with those in healthy control subjects.^{10,11} Furthermore, increased numbers of ILC2s with greater intracellular IL-5 expression have been reported in sputum of patients with severe asthma when compared with those with mild asthma.¹² Numbers of circulating ILC2s increased in patients with grass allergy and asthma during the pollen season,¹³ and ILC2s were also considered a potential biomarker for eosinophilic asthma.¹⁴ ILC2s remain in a nonactivated state under steady-state conditions. However, after nasal or inhaled allergen challenge, ILC2s infiltrate the site of inflammation, with increased amounts of intracellular IL-5.^{15,16} These studies reveal a clear link between the presence of ILC2s and type 2 inflammation in human subjects, but their numbers and phenotype during homeostasis and episodes of acute inflammation in the lung have not been elucidated in detail.

In the present study we aimed to further characterize ILC2s during allergic inflammation by analyzing ILC2s from blood and lung tissue before and after segmental allergen challenge in asthmatic patients with high blood eosinophil counts (≥ 300 cells/ μ L), representing a so-called type 2 phenotype.

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Abbreviations used

AHR: Aryl hydrocarbon receptor gene
 BAL: Bronchoalveolar lavage
 CRTH2: Chemoattractant receptor-homologous molecule expressed on T_H2 cells
 DEG: Differentially expressed gene
 FDR: False discovery rate
 FITC: Fluorescein isothiocyanate
 ILC2: Group 2 innate lymphoid cell
 ILC3: Group 3 innate lymphoid cell
 IRF4: Interferon regulatory factor 4 gene
 PGD₂: Prostaglandin D₂
 RORC: RAR-related orphan receptor γ gene
 TF: Transcription factor
 TSLP: Thymic stromal lymphopoietin

METHODS**Clinical study design and study population**

Patients with physician-diagnosed mild intermittent asthma controlled by as-needed short-acting β_2 -agonists only according to Global Initiative for Asthma guidelines were enrolled in the study. FEV₁ had to be 80% or greater of the predicted normal value after a washout of at least 6 hours for short-acting β_2 -agonists. All subjects had positive skin prick test responses for at least 1 respective aeroallergen used for bronchial challenge. Subjects had peripheral blood eosinophil counts of 300 cells/ μ L or greater and were nonsmokers. Peripheral blood (approximately 200 mL) was taken before the first bronchoscopy and 24 hours after allergen challenge before the second bronchoscopy. Sputum was induced at screening by using increasing concentrations of hypertonic saline, as described in detail previously.¹⁷

For bronchoscopy, patients underwent segmental allergen challenge with seasonal allergens (grass mix or *Dermatophagoides pteronyssinus*; Leti Pharma GmbH, Ismaning, Germany). The general technique of bronchoscopy with segmental allergen challenge has been described in detail.^{18,19} During a first bronchoscopy, 2 lung segments were challenged with allergen, and 1 lung segment received saline as a control. The dose of instilled allergen was determined by means of skin prick testing, choosing the allergen concentration that elicited a 3-mm wheal reaction (0.6–1.3 histamine equivalent prick, Table I). Bronchoscopy with BAL was performed 24 hours later.

The clinical study was performed in compliance with the Declaration of Helsinki. The Ethics Committee of Hannover Medical School approved the study (reference no. 7055). All study subjects provided written consent after being fully informed.

Sample preparation

Processing of BAL fluid was done, as previously described.¹⁸ Supernatants were placed in aliquots and directly frozen for cytokine measurement, whereas cell pellets were immediately used for differential cell counts and ILC2 isolation. Serum samples were obtained after 30 minutes of clotting at room temperature after centrifugation (2000g for 10 minutes) and stored frozen at -80°C .

ILC2 isolation

ILC2s were isolated from peripheral blood and BAL fluid and defined as Lin[−] (lineage: CD1a, CD3, CD4, CD14, CD16, CD19, CD34, CD56, CD94, CD123, CD336, CD303, Fc ϵ RI α , T-cell receptor [TCR] $\alpha\beta$, and TCR $\gamma\delta$), CD127⁺, CD161⁺, chemoattractant receptor-homologous molecule expressed on T_H2 cells (CRTH2)⁺, and c-Kit^{+/−}. For more details, see the Methods section in this article's Online Repository at www.jacionline.org.

In vitro culture

ILC2s were cultured in Yssel medium (Gemini Bio, Sacramento, Calif) with 1% autologous human serum at a concentration of 2×10^4 cells/mL in 96-well round-bottom plates (2000 cells/well) and stimulated with IL-2

TABLE I. Patients' characteristics

	Asthmatic patients
No.	7
Sex (male/female)	6/1
Age (y)	24 (20–43)
FEV ₁ (% predicted)	91.6 (88.5–105.5)
FEV ₁ /FVC ratio (%)	76.0 (74.9–85.2)
Allergen (grass mix/HDM)	1/6
Instilled allergen dose (HEP)	0.6 (0.6–1.3)
IgE (U/mL)	144 (68–309)
Blood eosinophils (cells/ μ L)	300 (300–400)
Sputum eosinophils (%)	1.5 (1–4.5)
Sputum eosinophils ($\times 10^6$ /g)	0.04 (0.02–0.14)

Values are presented as medians (interquartile ranges).

FVC, Forced vital capacity; HDM, house dust mite; HEP, histamine equivalent prick.

(1 ng/mL; PeproTech, Rocky Hill, NJ), IL-25, IL-33, and TSLP (50 ng/mL each; R&D Systems, Minneapolis, Minn) for 3 days at 37°C. Supernatants were taken for mediator analysis.

Analyte measurement

Mediators in BAL fluid, serum, and cell-culture supernatants were measured by using human U-PLEX Kits (Meso Scale Discovery, Rockville, Md), according to the manufacturer's protocol. Prostaglandin D₂ (PGD₂) levels were measured by means of ELISA (R&D Systems).

Migration assay

Peripheral blood from healthy donors was obtained in accordance with ethical principles according to the Declaration of Helsinki and the AstraZeneca policy on Bioethics and Human Biological Samples. ILC2s were isolated by means of flow cytometric sorting, as described above, and expanded on 3000 OP9 cells for 14 days in the presence of 5 ng/mL IL-2, 5 ng/mL IL-7, 10 ng/mL IL-25, and 10 ng/mL IL-33 (all from R&D Systems) in 96-well plates. After expansion, 0.5×10^5 ILC2s were placed in 75- μ L X-VIVO 15 medium (Lonza, Basel, Switzerland) in the upper chamber of the transwells (Corning, Corning, NY), and 235 μ L of medium with 2000 ng/mL CCL17, 500 ng/mL CCL22, 100 ng/mL CXCL12 (all from R&D Systems), and 100 nmol/L PGD₂ (Sigma, St Louis, Mo) was added into the lower chamber of the transwell plate. Migrated cells were recovered from the lower chamber. Cells were incubated at 37°C for 105 minutes and quantified by using the CellTiter-Fluor Cell Viability Assay (Promega, Madison, Wis).

Next-generation RNA sequencing

For RNA sequencing, 1×10^4 ILC2s were collected in 350 μ L of RLT buffer (Qiagen, Hilden, Germany) from blood and BAL fluid after allergen challenge. Isolated RNA from ILC2s was used as input to create mRNA libraries by using the QIAseq FX Single Cell RNA Library Kit (Qiagen) with a purified RNA protocol together with a poly-A protocol by using dual indexing. The input amount was 0.4 ng of total RNA, and 700 ng of DNA from the MDA reaction was used as input for fragmentation (15 minutes with an enhancer). Library size was validated on the Fragment Analyzer platform (AATI, Ankeny, Iowa) by using a standard sensitivity next-generation sequencing fragment analysis kit, and the concentration was determined by using the QIAseq Library Quant Assay Kit (Qiagen). Sample libraries in equimolar concentrations were pooled, diluted, and denatured according to Illumina guidelines (Illumina, San Diego, Calif). Sequencing was performed with a High Output 1 $\times$ 76 bp Kit on an Illumina NextSeq500. Analysis of data was carried out as described in the Methods section in this article's Online Repository.

Statistics

Statistical analysis was performed by using linear mixed-effect models, Wilcoxon signed-rank tests, and Student *t* tests (paired and unpaired). Contrast conducted for least squares means with multiple comparisons adjustment

TABLE II. Bronchoalveolar lavage data

Segment	Recovery (mL)	Macrophages (%)	Monocytes (%)	Eosinophils (%)	Neutrophils (%)	Lymphocytes (%)
Baseline	99.0 (80.4-107)	90.5 (73-93.5)	0 (0-0.75)	1 (0.3-1.3)	0.8 (0.5-2.5)	5.8 (3-22)
Saline	100.8 (82-107.2)	69.8 (0.5-86.0)‡	0.8 (0.3-2.0)	0.8 (0-2.0)§	8.5 (5.8-18.5)	3 (2.0-6.0)
Allergen (A ₁ + A ₂)	146 (107-201.8)	6.8 (2.3-8.8)*	1.3 (0.3-8.0)	81.3 (69.3-87.3)†	5.5 (4.0-14.3)†	2.3 (1.8-6.8)

Bronchoalveolar lavage data from segments at baseline and following challenge with saline and allergen (pooled A₁ + A₂). Median and interquartile ranges are given. Least squares mean comparisons (*t* tests) are based on a linear mixed model with Tukey multiple comparison adjustment. **P* < .0001, †*P* < .00001, allergen compared to baseline, ‡*P* < .0001, §*P* < .00001 allergen compared to saline, and ||*P* < .05 saline compared to baseline.

using the Tukey method for tests based on the linear mixed-effect models while using Holm multiple comparison adjustment for comparisons based on Wilcoxon signed-rank tests and Student *t* tests. We used 2-sided tests at an α significance level of 5%.

RESULTS

Segmental allergen provocation induced strong type 2 inflammation

To study pulmonary ILC2s during allergic inflammation, we performed a model of segmental allergen challenge in patients with mild-to-moderate asthma. Patients' characteristics are shown in Table I. Two lung segments were allergen challenged, and samples were pooled to increase the ILC2 yield. The inflammatory response in baseline, saline-challenged, and allergen-challenged segments is displayed as differential cell count (as a percentage) in Table II, as well as in absolute cell numbers (see Table E1 in this article's Online Repository at www.jacionline.org).

Allergen challenge led to a pronounced type 2 inflammation in the lung characterized by a significant increase in numbers of total leukocytes (*P* < .05; Fig 1, A), with eosinophils accounting for an average of 80% of total cells (*P* < .00001; Fig 1, B). Levels of TSLP (*P* < .05), but not IL-5, were significantly increased in BAL fluid after allergen challenge (Fig 1, C and D); however, levels of both cytokines were increased in blood serum after allergen challenge (*P* < .05; Fig 1, E and F).

ILC2 counts decreased in blood and increased in lung tissue after allergen challenge

Human ILC2s were sorted by means of fluorescence-activated cell sorting from peripheral blood and BAL fluid (see Fig E1 in this article's Online Repository at www.jacionline.org). Peripheral blood ILC2 numbers decreased in 6 of 7 subjects after allergen challenge (*P* = .047; Fig 2, A). At the same time, ILC2 numbers increased remarkably after allergen challenge in BAL fluid when compared with baseline and saline conditions (*P* < .05; Fig 2, B). No correlation was observed between eosinophil numbers in sputum or BAL fluid and ILC2 numbers in BAL fluid or blood (see Fig E2 in this article's Online Repository at www.jacionline.org). ILC2s from BAL fluid after allergen challenge were highly activated because they spontaneously released IL-5 and IL-13 compared with blood ILC2s, which did not release these cytokines on *in vitro* culture (Fig 2, C). Additional stimulation with IL-2, IL-25, IL-33, and TSLP tended to further increase cytokine release in all assays; however, these changes only reached statistical significance for blood ILC2s (*P* < .05). Cytokine release of blood and BAL fluid ILC2s was similar after *in vitro* stimulation. Expression of the ILC2 surface proteins CCR4, CD25, CD117, CD127, and

CRTH2 between blood ILC2s isolated at baseline and after allergen challenge was similar (Fig 3, A-F, and see Fig E3 in this article's Online Repository at www.jacionline.org).

ILC2s migrated toward chemokine gradients

Depletion of blood ILC2s after allergen challenge led us to explore which mechanisms regulate their recruitment to the lung. Protein expression of CCR4 was decreased in BAL fluid compared with that in blood-derived ILC2s (*P* < .05; Fig 3, A). Transcripts of *CCR4* showed a reverse pattern, with greater expression in BAL fluid ILC2s (*P* < .05; Fig 3, B). At the same time, its ligands, CCL17 (Fig 3, C) and CCL22 (Fig 3, D), showed a trend toward increased levels in BAL fluid after allergen challenge (*P* = .07). Protein expression for the PGD₂ receptor was not changed (Fig 3, E), whereas its transcript levels (*PTGDR2*) were lower in BAL fluid ILC2s (*P* < .05; Fig 3, F). PGD₂ levels in BAL fluid were not increased significantly 24 hours after allergen provocation (Fig 3, G), but levels correlated inversely with blood ILC2 numbers at the same time (*R* = -0.847, *P* = .0035; Fig 3, H), which was not observed for CCL17 and CCL22. Transcript levels for the chemokine receptor *CXCR4* were greater in BAL fluid ILC2s (*P* < .001; Fig 3, I), and levels of its ligand, CXCL12, were increased in BAL fluid after allergen challenge (*P* < .05; Fig 3, J). CXCL12 levels in BAL fluid correlated with the decrease in ILC2 numbers in blood after allergen challenge (*R* = -0.672, *P* = .024; Fig 3, K).

Migration assays with blood-derived ILC2s from healthy subjects were performed to assess the role of CCL17, CCL22, PGD₂, and CXCL12 in inducing migration *in vitro*. Migration was induced by CCL17 and CCL22 (*P* < .05), as well as through PGD₂ (*P* < .01) and CXCL12 (*P* < .05; Fig 3, L).

BAL fluid ILC2s are transcriptionally distinct from blood ILC2s

To characterize the transcriptional changes induced by allergen challenge, we sequenced RNA transcripts from blood ILC2s at baseline and after allergen challenge and from BAL fluid ILC2s after allergen challenge (*n* = 4). Principal component analysis indicated that allergen-induced local lung inflammation did not alter the gene expression pattern in blood ILC2s because these samples clustered together in contrast to BAL fluid-derived ILC2s, which clustered separately (Fig 4, A). A total of 112 differentially expressed genes (DEGs) with a false discovery rate (FDR) of less than 0.05 were detected, and only 18 DEGs were more than 2-fold regulated ($|\log_2\text{FC}| > 1$) between blood ILC2s isolated at baseline and after allergen challenge (Fig 4, B). The top 10 DEGs that were most upregulated or downregulated are shown in Table E2 in this article's Online

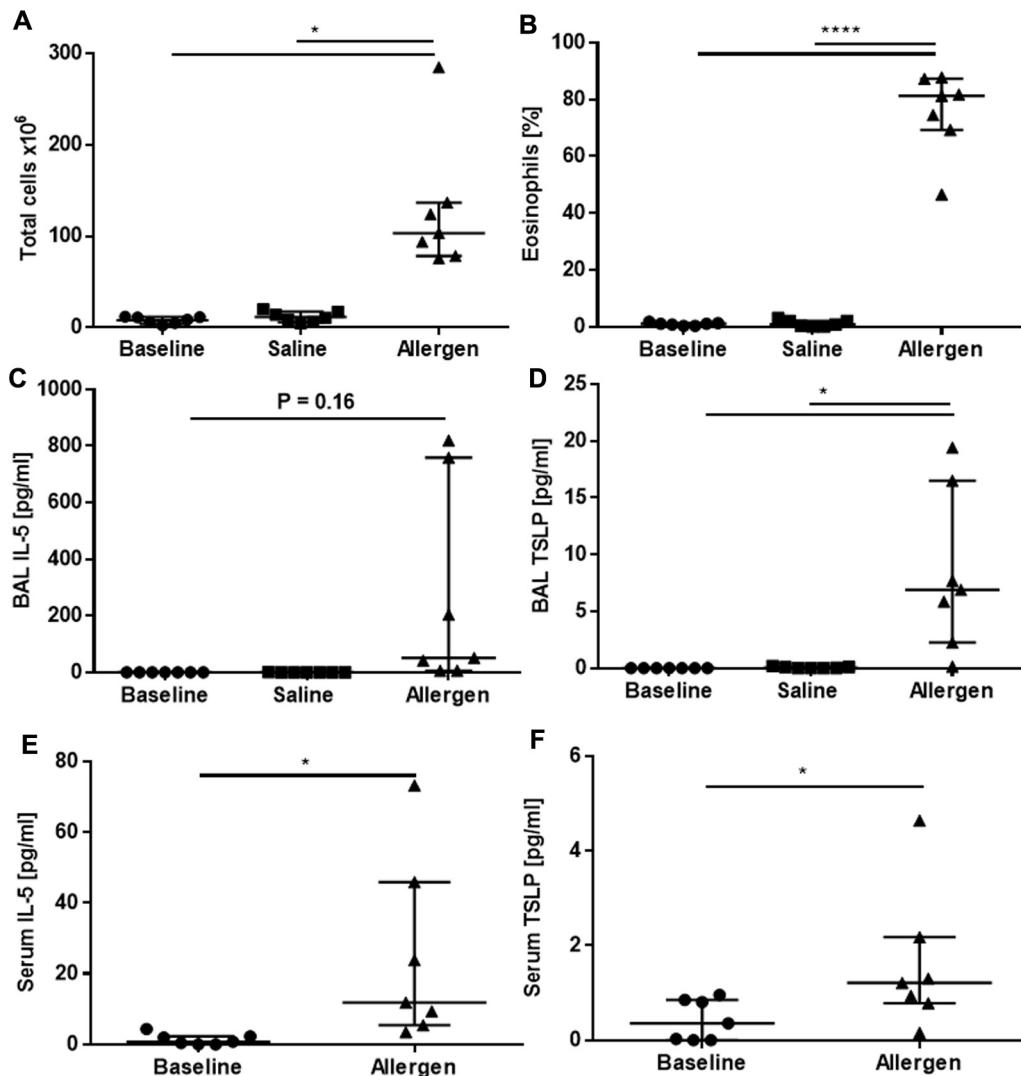


FIG 1. Pulmonary inflammation after segmental allergen challenge in asthmatic patients. Absolute cell numbers in BAL fluid (A), percentage of BAL fluid eosinophils (B), and IL-5 (C) and TSLP protein levels in BAL fluid (D) and serum (E and F) for IL-5 and TSLP, respectively are shown. Medians and interquartile ranges are shown (n = 7). Data were analyzed by using least squares means comparisons (t tests) based on a linear mixed model with Tukey multiple comparison adjustment. * $P < .05$ and **** $P < .00001$ (Fig 1, A-D) or Wilcoxon t test (Fig 1, E and F).

Repository at www.jacionline.org. In total, 1270 DEGs were detected between blood and BAL fluid ILC2s after allergen challenge, with 1009 DEGs more than 2-fold regulated (Fig 4, C). Transcripts for *IL13*, the IL-33 receptor (*IL1RL1*), and the leukemia inhibitory factor (*LIF*), a member of the IL-6 signaling family, were the most upregulated genes, whereas the genes coding for FRAS1 related extracellular matrix 3 protein (*FREM3*) and the proline-rich region 7 protein (*PRR7*) were the most downregulated ones (see Table E3 in this article's Online Repository at www.jacionline.org). The pluripotent cytokine *IL32* showed the greatest transcripts in both BAL fluid and blood ILC2s. BAL fluid-derived ILC2s had significantly greater expression of *IL13*, as well as amphiregulin (*AREG*) and vascular endothelial growth factor A (*VEGFA*), whereas transcripts for *IL17*, *IL22*, or *IFNG* were not detectable at all (Fig 4, D). Although gene expression of *IL1RL1* and its coreceptors *IL1RAP*, *IL17RB*, and *IL2AR* were markedly greater, expression of

CRTH2, the TSLP receptor (*CRLF2*), and its coreceptor, *IL7R*, were expressed at lower levels in BAL fluid ILC2s. The major ILC2 transcription factors (TFs) *GATA3* and RAR-related orphan receptor α (*RORA*) were constantly highly expressed in blood and BAL fluid ILC2s, whereas other TFs, such as aryl hydrocarbon receptor (*AHR*), interferon regulatory factor 4 (*IRF4*), the peroxisome proliferator activated receptor γ (*PPARG*), and fos-related antigen 1 (*FOSL1*), showed greater expression in BAL fluid ILC2s (Fig 4, E). The major TFs for group 1 innate lymphoid cells and group 3 innate lymphoid cells (ILC3s), T-box transcription factor 21 (*TBX21*) and forkhead box P3 (*FOXP3*), were found to be expressed at lower levels. Of note, RAR-related orphan receptor γ (*RORC*) expression was lower in BAL fluid compared with blood ILC2s. Molecules being important for interorgan trafficking of ILC2s from the gut to the lung, such as sphingosine 1-phosphate receptor (*S1PR1*)²⁰ and the β_2 -integrin CD18 (*ITGB2*)²¹ were highly expressed but did

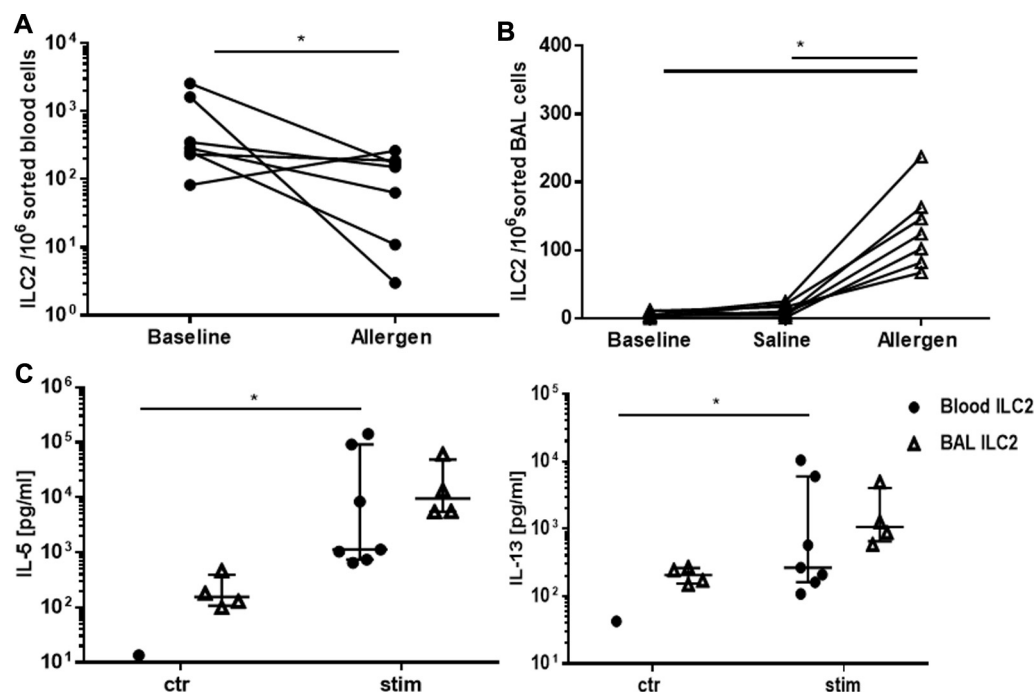


FIG 2. ILC2 numbers in peripheral blood and BAL fluid. ILC2 counts in blood before and 24 hours after allergen provocation (**A**). ILC2 counts in BAL fluid (**B**) at baseline and 24 hours after saline or allergen provocation. **C**, Cytokine release (IL-5 left panel, IL-13 right panel) from blood ILC2s before allergen challenge (circles) and BAL fluid ILC2s after allergen provocation (triangles) after 3 days in culture. Stimulation was with either IL-2 (ctr) or IL-2, IL-25, IL-33, and TSLP (stim). In Fig 2, C, medians and interquartile ranges are shown (n = 4-7). Data were analyzed by using the Wilcoxon signed-rank test (Fig 2, A), with *P* values being Holm adjusted (Fig 2, B and C). **P* < .05.

not differ between BAL fluid and blood ILC2s (Fig 4, F). Expression of the lymphocyte homing receptor CD62L (*SELL*) was lower in BAL fluid ILC2s, whereas the intracellular adhesion molecule (*ICAM1*) was expressed more. Furthermore, transcripts for the activation markers *CD69*, *ICOS*, and *CD137* (*TNFRS9*), as well as genes encoding for the MHC class II molecule (*HLA-DRA* and *HLA-DBR1*) were more highly expressed in BAL fluid ILC2s.

To bring our sequencing data into a broader functional context, we compared them with 2 ILC2-related asthma maps from MetaCore (Clarivate Analytics, Philadelphia, Pa), which were combined into a single ILC2 asthma master map. When analyzing transcriptional profiles for the ILC2 populations from blood at baseline and after allergen challenge with ILC2s from BAL fluid, the latter revealed a significant enrichment of genes associated with an activated ILC2 phenotype in asthmatic patients (*P* < .05; Fig 5, A). Network analysis for gene signatures increased in BAL fluid versus blood ILC2s after allergen challenge revealed a great overlap of genes associated with migration but also activation and differentiation of T_H2- and T_H9-related pathways and CCR4-induced chemotaxis, which reflects our findings of activated BAL fluid ILC2s promoting type 2 inflammation (Fig 5, B).

DISCUSSION

This study demonstrated a significant increase in ILC2 numbers in the lung accompanied by a decrease in blood ILC2 numbers in asthmatic patients after allergen challenge. Our data support earlier reports^{15,16} suggesting that blood ILC2s might

form the major source of tissue-infiltrating ILC2s in the context of allergen challenge. Notably, the increase in numbers of ILC2s was specifically directed toward the inflamed segments because we could not detect an increase in the saline segment. BAL fluid ILC2s showed an activated transcriptional and functional profile compared with blood ILC2s after allergen challenge, whereas blood ILC2s did not differ in terms of transcripts and proteins expressed before and after allergen challenge. This indicates that ILC2s are predominantly activated locally on allergen challenge in the lung, which is concordant with Chen et al.¹⁵ The increase in IL-5 and TSLP levels in blood after allergen challenge indicated a spillover from the lung to the systemic circulation, without subsequent activation of ILC2s in the blood.

Because ILC2s mediate immediate and very potent responses, our data indicate that their activation pattern is strictly regulated to the site of inflammation. The mechanism driving the increase in ILC2 numbers in the lung during allergic inflammation is not fully understood. A parabiosis mouse model revealed local proliferation of ILC2s in the lung in response to IL-33 instillation.²² In contrast, a recent study showed that ILC2 traffic between organs was mediated through sphingosine-1-phosphate-mediated migration.²⁰ In addition, lipid mediators are known to be potent inducers of ILC2 migration.²³ Because of the decrease in numbers of blood ILC2s after allergen challenge, it is tempting to speculate that these cells traffic to the lung in response to inflammation.

The inflammatory milieu during allergic airway inflammation is very complex, and we could show that in addition to prostaglandins, CLL17, CLL22, and CXCL12 also induce

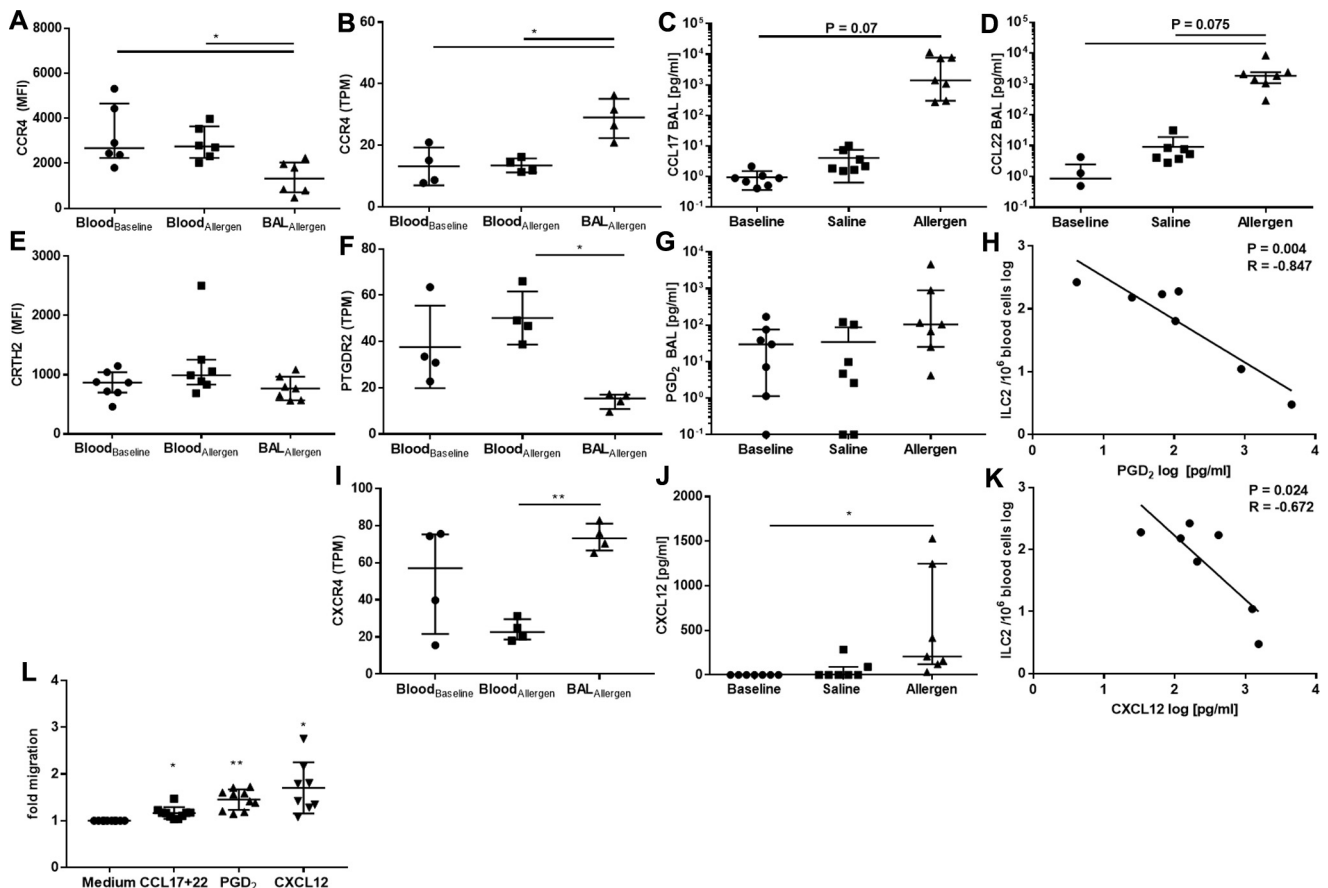


FIG 3. CCL17, CCL22, and PGD₂ induce ILC2 migration. **A** and **B**, CCR4 receptor protein expression on blood- and BAL fluid-derived ILC2s (Fig 3, A) and gene expression (Fig 3, B). **C–G**, CCR4 ligand protein levels of BAL fluid CCL17 (Fig 3, C) and CCL22 (Fig 3, D), CRTH2 protein (Fig 3, E) and gene (*PTGDR2*) expression levels on blood and BAL fluid ILC2s (Fig 3, F), and PGD₂ levels in BAL fluid (Fig 3, G). **H**, Correlation of PGD₂ levels in BAL fluid with blood ILC2 numbers 24 hours after allergen provocation. **I** and **J**, CXCR4 gene expression (Fig 3, I) and CXCL12 protein levels (Fig 3, J) in BAL fluid. **K**, Correlation of CXCL12 levels in BAL fluid with blood ILC2 numbers 24 hours after allergen provocation. **L**, Migration of blood-derived ILC2s *in vitro* toward CCL17 plus CCL22, PGD₂, or CXCL12. Fig 3, B, F, and I, n = 4; Fig 3, L, n = 8–10. Medians and interquartile ranges (Fig 3, A–G, I, and J) and means and SDs (Fig 3, L) are shown. Least squares means comparisons (*t* tests) based on linear mixed models with Tukey multiple comparison adjustment (Fig 3, A–G, I, and J) and standard *t* tests with multiple comparison adjustment by using the Holm method (Fig 3, L) are shown. **P* < .05, ***P* < .001, and ****P* < .0001.

migration. Downregulation of CCR4 on BAL fluid ILC2s could be due to receptor internalization, because it has been reported for CCR4 on T cells after CCR4 activation by its ligand.²⁴ Interestingly, the CXCR4–CXCL12 axis seems also capable of inducing ILC2 migration, as was previously described for T cells.²⁵ Pulmonary CXCL12 levels correlated negatively with blood ILC2 numbers after allergen challenge, as well as CXCL12-induced ILC2 migration *in vitro*. Lipid mediators are released within minutes after allergen contact and thus might be the source for initial recruitment of ILC2s, whereas CCL17, CCL22, and CXCL12 step in later.

When Björklund et al²⁶ performed single-cell sequencing from innate lymphoid cells isolated from human tonsils, they could not identify different ILC2 subpopulations under steady-state conditions. In mice during cytokine- or helminth-induced inflammation, inflammatory ILC2 (iILC2s),²⁷ as well as IL-10 producing ILC2²⁸ and IL-17 producing ILC2,²⁹ have been described with altered functionality and plasticity. Our transcriptomic data from BAL fluid ILC2s do not point

specifically to the development of innate lymphoid cell subpopulations because expression of the main TFs *GATA3* and *RORA* remain unchanged after allergen challenge.

The increased expression of *AHR* in BAL fluid ILC2s is of note because it is an important TF for the development of ILC3s and together with *RORC* induces IL-17 and IL-22 expression.³⁰ *AHR* expression is directly repressed by Bcl11b, which contributes to silencing of ILC3 genes in ILC2s.³¹ Expression of *BCL11B* is reduced on BAL fluid ILC2s and thus might relate to the increased *AHR* expression we see in parallel in these cells. However, changes in TFs are not accompanied by increased *IL17* or *IL22* expression, as reported for ST2⁺ lung ILC2s when stimulated with IL-33 and leukotrienes.²⁹ Notch signaling has also been described to elicit ILC2 plasticity, converting natural ILC2 to iILC2s by promoting *RORC* expression and IL-17 production.³²

Although these data do not support ILC2 plasticity in allergic inflammation, it should be emphasized that our analysis of total BAL fluid-derived CHRT2⁺ ILC2s is not sensitive enough to

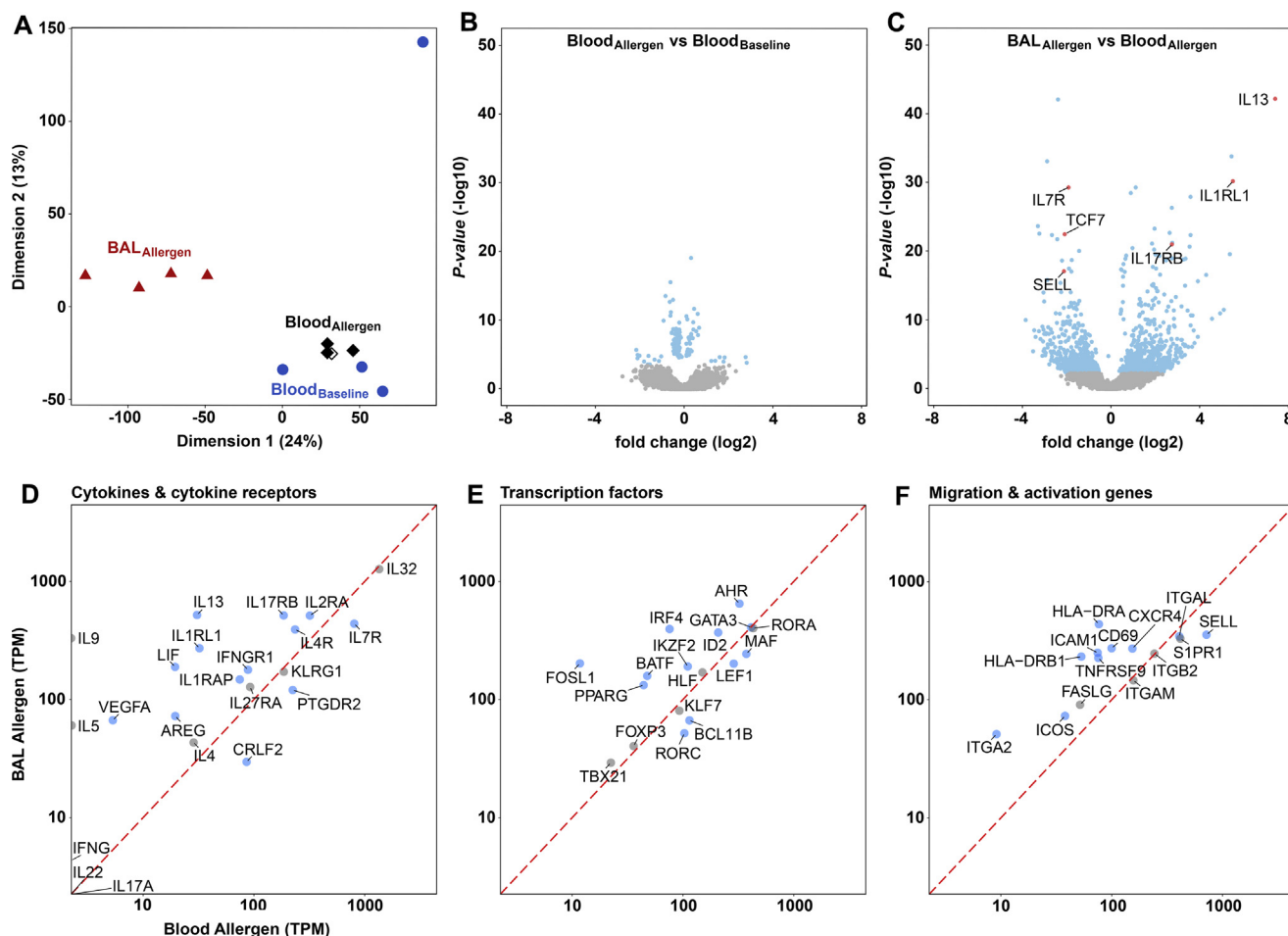


FIG 4. RNA sequencing data. **A**, Principal component analysis of RNA sequencing data from ILC2s isolated from BAL fluid and blood after allergen challenge and blood ILC2s at baseline. **B**, Volcano plot comparison of whole-transcriptome gene expression of ILC2s from blood at baseline and after allergen challenge. **C**, Volcano plot comparison between BAL fluid and blood ILC2s after allergen challenge. **D-F**, DEGs between ILC2s from BAL fluid and blood after allergen challenge as transcripts per million reads (TPM): cytokine and cytokine receptors (Fig 4, **D**), TFs (Fig 4, **E**), and genes involved in migration and activation (Fig 4, **F**). Significantly changed genes ($P < .05$) are indicated as blue dots, and nonsignificant changes are indicated as gray dots ($n = 4$).

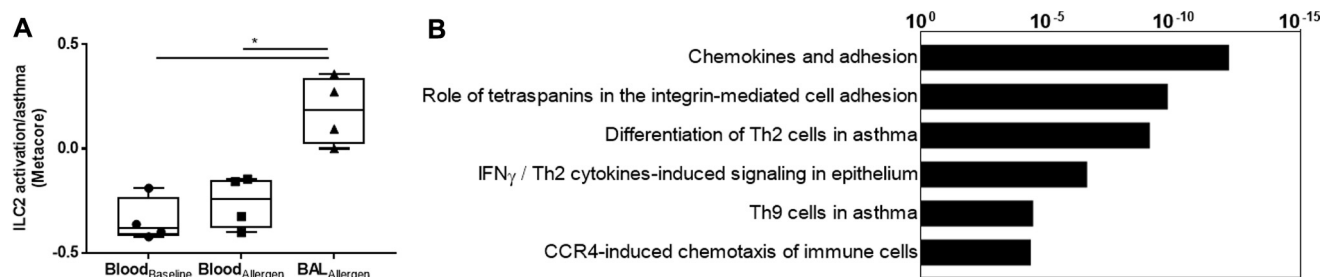


FIG 5. Database analysis of ILC2 gene signature. **A**, Gene signature variant (MetaCore) analysis of blood ILC2s at baseline and after allergen challenge and ILC2s from BAL fluid after allergen challenge. **B**, Network analysis for gene signatures increased in BAL fluid versus blood ILC2s (FDR < 0.05). Least squares means comparisons (t tests) based on linear mixed models with Tukey multiple comparison adjustment ($n = 4$) are shown. * $P < .05$.

identify potential minor subsets, some of which might have lost CHRT2 expression. Thus a single-cell RNA sequencing approach is warranted. Interestingly, *IL9* and its master TF *IRF4* were expressed at high levels in ILC2s from BAL fluid

compared with blood. Previous data from mice show that IL-33 and TSLP trigger *IRF4* expression, leading to autocrine IL-9 release in ILC2, which in turn augments their IL-5 and IL-13 release.³³ The importance of ILC2-derived IL-9 has been reported

in the resolution of rheumatoid arthritis but needs to be further evaluated in the context of type 2 inflammation.³⁴

In addition to molecules involved in promoting type 2 inflammation, BAL fluid ILC2s expressed transcripts of tissue-repairing proteins. Monticelli et al³⁵ showed that the release of amphiregulin by BAL fluid ILC2s is critical to restore epithelial integrity and tissue remodeling after influenza infection. Epithelial damage is also a central feature in asthma, and thus increased *AREG* expression in lung-derived ILC2s could contribute to tissue repair, remodeling, or both. In addition, *LIF* was highly expressed in BAL fluid–derived ILC2s, which has been shown to protect the lungs from injury during bacterial and viral infections, dampening inflammatory cytokine release from epithelial cells.^{36,37} This could result in an additional ILC2-derived factor that promotes epithelial repair mechanisms and restores epithelial integrity after allergic airway inflammation.

In contrast to other studies in asthmatic patients looking at ILC2s in BAL fluid^{10,23} or sputum,¹² we found very few numbers of ILC2s in baseline BAL fluid, which could be due to different sorting strategies. Because lymphocytes generally enter the alveolar space only under inflammatory conditions,¹⁸ low numbers are not surprising at baseline because all asthmatic patients included in the study had very mild disease at baseline and were without inflammatory symptoms in the lung at baseline lavage. Furthermore, in contrast to previous studies,^{14,15} we did not observe any correlation between eosinophil counts in sputum and ILC2 counts in blood or BAL fluid, which might be due to our low patient numbers and inclusion of only patients with mild-to-moderate asthma.

Although this study uses a powerful model of pulmonary allergen challenge in human subjects together with in-depth characterization of ILC2 attraction and activation, it has limitations. First, selected patients had only very mild intermittent allergic asthma, a distinct phenotype that has been demonstrated to be safe for experimental bronchoscopies with segmental allergen challenge. Although patients were selected to be type 2 prone with higher numbers of peripheral eosinophils, activation of ILC2s might be different in more severe stages and in nonallergic conditions of asthma. Second, the study design only allowed us to capture allergen-driven changes at one selected time point (24 hours), which limits our temporal knowledge gain.

Taken together, we show that ILC2 numbers increase in BAL fluid of asthmatic patients during allergic airway inflammation paralleled with a decrease in blood. Their transcriptomic profile and cytokine release indicate local activation at the site of inflammation, where they trigger further ongoing type 2 inflammation in the lung.

Clinical implications: These findings strengthen the implication of pulmonary ILC2s promoting type 2 inflammation during allergic airway inflammation in human subjects.

REFERENCES

- Artis D, Spits H. The biology of innate lymphoid cells. *Nature* 2015;517:293.
- Mjösberg JM, Trifari S, Crellin NK, Peters CP, van Drunen CM, Piet B, et al. Human IL-25- and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CCR2 and CD161. *Nat Immunol* 2011;12:1055.
- Mjösberg J, Spits H. Human innate lymphoid cells. *J Allergy Clin Immunol* 2016;138:1265-76.
- Cosmi L, Annunziato F. Group 2 innate lymphoid cells are the earliest recruiters of eosinophils in lungs of patients with allergic asthma. *Am J Respir Crit Care Med* 2017;196:666-8.
- Oliphant Christopher J, Hwang You Y, Walker Jennifer A, Salimi M, Wong See H, Brewer James M, et al. MHCII-mediated dialog between group 2 innate lymphoid cells and CD4(+) T cells potentiates type 2 immunity and promotes parasitic helminth expulsion. *Immunity* 2014;41:283-95.
- Drake LY, Iijima K, Kita H. Group 2 innate lymphoid cells and CD4(+) T cells cooperate to mediate type 2 immune response in mice. *Allergy* 2014;69:1300-7.
- Drake LY, Iijima K, Bartemes K, Kita H. Group 2 innate lymphoid cells promote an early antibody response to a respiratory antigen in mice. *J Immunol* 2016;197:1335-42.
- Halim TYF, Hwang YY, Scanlon ST, Zaghoulani H, Garbi N, Fallon PG, et al. Group 2 innate lymphoid cells license dendritic cells to potentiate memory T helper 2 cell responses. *Nat Immunol* 2016;17:57-64.
- Maazi H, Patel N, Sankaranarayanan I, Suzuki Y, Rigas D, Soroosh P, et al. ICOS:ICOS-ligand interaction is required for type 2 innate lymphoid cell function, homeostasis, and induction of airway hyperreactivity. *Immunity* 2015;42:538-51.
- Christianson CA, Goplen NP, Zafar I, Irvin C, Good JT, Rollins DR, et al. The persistence of asthma requires multiple feedback circuits involving ILC2 and IL33. *J Allergy Clin Immunol* 2015;136:59-68.e14.
- Bartemes K, Kephart G, Fox SJ, Kita H. Enhanced innate type 2 immune response in peripheral blood from patients with asthma. *J Allergy Clin Immunol* 2014;134:671-8.e4.
- Smith SG, Chen R, Kjarsgaard M, Huang C, Oliveria JP, O'Byrne PM, et al. Increased numbers of activated group 2 innate lymphoid cells in the airways of patients with severe asthma and persistent airway eosinophilia. *J Allergy Clin Immunol* 2016;137:75-86.e8.
- Lombardi V, Beuraud C, Neukirch C, Moussu H, Morizur L, Horiot S, et al. Circulating innate lymphoid cells are differentially regulated in allergic and nonallergic subjects. *J Allergy Clin Immunol* 2016;138:305-8.
- Liu T, Wu J, Zhao J, Wang J, Zhang Y, Liu L, et al. Type 2 innate lymphoid cells: a novel biomarker of eosinophilic airway inflammation in patients with mild to moderate asthma. *Respir Med* 2015;109:1391-6.
- Chen R, Smith SG, Salter B, El-Gammal A, Oliveria JP, Obminski C, et al. Allergen-induced increases in sputum levels of group 2 innate lymphoid cells in subjects with asthma. *Am J Respir Crit Care Med* 2017;196:700-12.
- Eastman JJ, Cavagnero KJ, Deconde AS, Kim AS, Karta MR, Broide DH, et al. Group 2 innate lymphoid cells are recruited to the nasal mucosa in patients with aspirin-exacerbated respiratory disease. *J Allergy Clin Immunol* 2017;140:101-8.e3.
- Janssen O, Schaumann F, Holz O, Lavae-Mokhtari B, Welker L, Winkler C, et al. Low-dose endotoxin inhalation in healthy volunteers—a challenge model for early clinical drug development. *BMC Pulm Med* 2013;13:19.
- Erpenbeck VJ, Hohlfeld JM, Volkmann B, Hagenberg A, Geldmacher H, Braun A, et al. Segmental allergen challenge in patients with atopic asthma leads to increased IL-9 expression in bronchoalveolar lavage fluid lymphocytes. *J Allergy Clin Immunol* 2003;111:1319-27.
- Atochina-Vasserman EN, Winkler C, Abramova H, Schaumann F, Krug N, Gow AJ, et al. Segmental allergen challenge alters multimeric structure and function of surfactant protein D in humans. *Am J Respir Crit Care Med* 2011;183:856-64.
- Huang Y, Mao K, Chen X, Sun MA, Kawabe T, Li W, et al. SIP-dependent interorgan trafficking of group 2 innate lymphoid cells supports host defense. *Science* 2018;359:114-9.
- Karta MR, Rosenthal PS, Beppu A, Vuong CY, Miller M, Das S, et al. β 2 integrins rather than β 1 integrins mediate Alternaria-induced group 2 innate lymphoid cell trafficking to the lung. *J Allergy Clin Immunol* 2018;141:329-38.e12.
- Moro K, Kabata H, Tanabe M, Koga S, Takeno N, Mochizuki M, et al. Interferon and IL-27 antagonize the function of group 2 innate lymphoid cells and type 2 innate immune responses. *Nat Immunol* 2015;17:76.
- Liu S, Verma M, Michalec L, Liu W, Sripada A, Rollins D, et al. Steroid resistance of airway type 2 innate lymphoid cells from patients with severe asthma: the role of thymic stromal lymphopoietin. *J Allergy Clin Immunol* 2018;141:257-68.e6.
- Mariani M, Lang R, Binda E, Panina-Bordignon P, D'Ambrosio D. Dominance of CCL22 over CCL17 in induction of chemokine receptor CCR4 desensitization and internalization on human Th2 cells. *Eur J Immunol* 2004;34:231-40.
- Ottoson NC, Pribila JT, Chan ASH, Shimizu Y. Cutting edge: T cell migration regulated by CXCR4 chemokine receptor signaling to ZAP-70 tyrosine kinase. *J Immunol* 2001;167:1857-61.

26. Björklund ÅK, Forkel M, Picelli S, Konya V, Theorell J, Friberg D, et al. The heterogeneity of human CD127+ innate lymphoid cells revealed by single-cell RNA sequencing. *Nat Immunol* 2016;17:451.
27. Huang Y, Guo L, Qiu J, Chen X, Hu-Li J, Siebenlist U, et al. IL-25-responsive, lineage-negative KLRG1(hi) cells are multipotential 'inflammatory' type 2 innate lymphoid cells. *Nat Immunol* 2015;16:161-9.
28. Seehus CR, Kadavallore A, Torre Bdl, Yeckes AR, Wang Y, Tang J, et al. Alternative activation generates IL-10 producing type 2 innate lymphoid cells. *Nat Commun* 2017;8:1900.
29. Cai T, Qiu J, Ji Y, Li W, Ding Z, Suo C, et al. IL-17-producing ST2+ group 2 innate lymphoid cells play a pathogenic role in lung inflammation. *J Allergy Clin Immunol* 2019;143:229-44.e9.
30. Li S, Bostick JW, Zhou L. Regulation of innate lymphoid cells by aryl hydrocarbon receptor. *Front Immunol* 2017;8:1909.
31. Califano D, Cho JJ, Uddin MN, Lorentsen KJ, Yang Q, Bhandoola A, et al. Transcription factor Bcl11b controls identity and function of mature innate lymphoid cells type II. *Immunity* 2015;43:354-68.
32. Zhang K, Xu X, Pasha MA, Siebel CW, Costello A, Haczku A, et al. Cutting edge: notch signaling promotes the plasticity of group-2 innate lymphoid cells. *J Immunol* 2017;198:1798-803.
33. Mohapatra A, Van Dyken SJ, Schneider C, Nussbaum JC, Liang HE, Locksley RM. Group 2 innate lymphoid cells utilize the IRF4-IL-9 module to coordinate epithelial cell maintenance of lung homeostasis. *Mucosal Immunol* 2015;9:275.
34. Rauber S, Luber M, Weber S, Maul L, Soare A, Wohlfahrt T, et al. Resolution of inflammation by interleukin-9-producing type 2 innate lymphoid cells. *Nat Med* 2017;23:938-44.
35. Monticelli LA, Sonnenberg GF, Abt MC, Alenghat T, Ziegler CGK, Doering TA, et al. Innate lymphoid cells promote lung tissue homeostasis following acute-influenza virus infection. *Nat Immunol* 2011;12:1045-54.
36. Quinton LJ, Mizgerd JP, Hilliard KL, Jones MR, Kwon CY, Allen E. Leukemia inhibitory factor signaling is required for lung protection during pneumonia. *J Immunol* 2012;188:6300-8.
37. Foronjy RF, Dabo AJ, Cummins N, Geraghty P. Leukemia inhibitory factor protects the lung during respiratory syncytial viral infection. *BMC Immunol* 2014;15:41.

METHODS

ILC2 isolation

Peripheral blood PBMCs were isolated with Lymphoprep and SepMate-50 tubes (STEMCELL Technologies, Vancouver, British Columbia, Canada), according to the manufacturer's protocol. T cells, B cells, and monocytes were depleted by using fluorescein isothiocyanate (FITC)-conjugated anti-CD3 (OKT3), anti-CD14 (HCD14), anti-CD19 (HIB19; all from BioLegend, San Diego Calif), and anti-FITC MicroBeads, (Miltenyi Biotec, Mönchengladbach, Germany). Depletion was performed with LD columns on a QuadroMACS Separator, according to the manufacturer's protocol (Miltenyi Biotec). Alternatively, the Lineage Cell Depletion Kit, human (Miltenyi Biotec), was used for predepletion of PBMCs. For cell sorting of human ILC2s from BAL fluid, the same protocol was applied without any predepletion step.

The remaining cells were counted and Live-Dead stained with Zombie Red (BioLegend), followed by antibody staining for subsequent sorting: FITC anti-CD1a (HI149), anti-CD16 (3G8), anti-CD56 (HCD56), anti-CD94 (DX22), anti-CD123 (6H6), anti-FcεRIα (AER-37), anti-TCRαβ (IP26; all from BioLegend), anti-TCRγδ (B1), anti-CD34 (581), anti-CD336 (P44-8; BD Biosciences, Franklin Lakes, NJ), anti-CD303 (AC144; from Miltenyi Biotec), phycoerythrin anti-CCR4 (L291H4), BV421 anti-CRTH2 (BM16), BV510 anti-CD161 (HP-3G10), BV711 anti-c-Kit (104D2; from BioLegend), peridinin-chlorophyll-protein complex anti-CD45 (2D1; BD Biosciences), and phycoerythrin-Cy7 anti-CD127 (A64618; Beckman Coulter, Brea Calif). FACSaria Fusion (BD Bioscience) was used for sorting, and flow cytometric data were analyzed by using FlowJo software (TreeStar, Ashland, Ore).

RNA sequencing analysis

RNA-seq fastq files were processed by using bcbio (version 0.9.7),^{E1} in which reads were mapped to the human genome build hg38 (GRCh38.78) by using hisat2 (version 2.0.2-beta),^{E2} yielding between 17.2 and 22.7 million

mapped reads per sample. Gene-level quantifications, counts, and transcript per million values were generated with feature counts (version 1.4.4)^{E3} and sailfish (version 0.9.0),^{E4} respectively, all within bcbio. All analyses were performed with R software (version 3.4.0, www.r-project.org). Differential gene expression was assessed with DESeq2 (version 1.14.1)^{E5} by using raw counts as input. Genes were considered significantly differentially expressed if they had FDRs of less than 0.05. Plots were created by using ggplot2 (version 2.2.1). Gene set enrichment analysis was done with Pathway Studio (version 11.4.0.11; Elsevier, Amsterdam, The Netherlands). An ILC2-relevant gene set was generated by taking the union of genes from 2 ILC2 pathways in Metabase (version 6.28.68602): "Role of type 2 innate lymphoid cells in asthma" (60 entities) and "Role of type 2 innate Lymphoid cells in airway allergic inflammation and tissue repair" (57 entities). Enrichment of the gene signature was then evaluated by using Gene Set Variation Analysis in R software (version 3.3.3).^{E6}

REFERENCES

- E1. Bcbio-nextgen. Validated, community developed variant calling, RNA-seq and small RNA analysis. Available at: <https://github.com/chapmanb/bcbio-nextgen>. Accessed 2017.
- E2. Kim D, Langmead B, Salzberg SL. HISAT: a fast spliced aligner with low memory requirements. *Nat Methods* 2015;12:357.
- E3. Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* 2014;30:923-30.
- E4. Patro R, Mount SM, Kingsford C. Sailfish enables alignment-free isoform quantification from RNA-seq reads using lightweight algorithms. *Nat Biotechnol* 2014;32:462.
- E5. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014;15:550.
- E6. Hänzelmann S, Castelo R, Guinney J. GSEA: gene set variation analysis for microarray and RNA-Seq data. *BMC Bioinformatics* 2013;14:7.

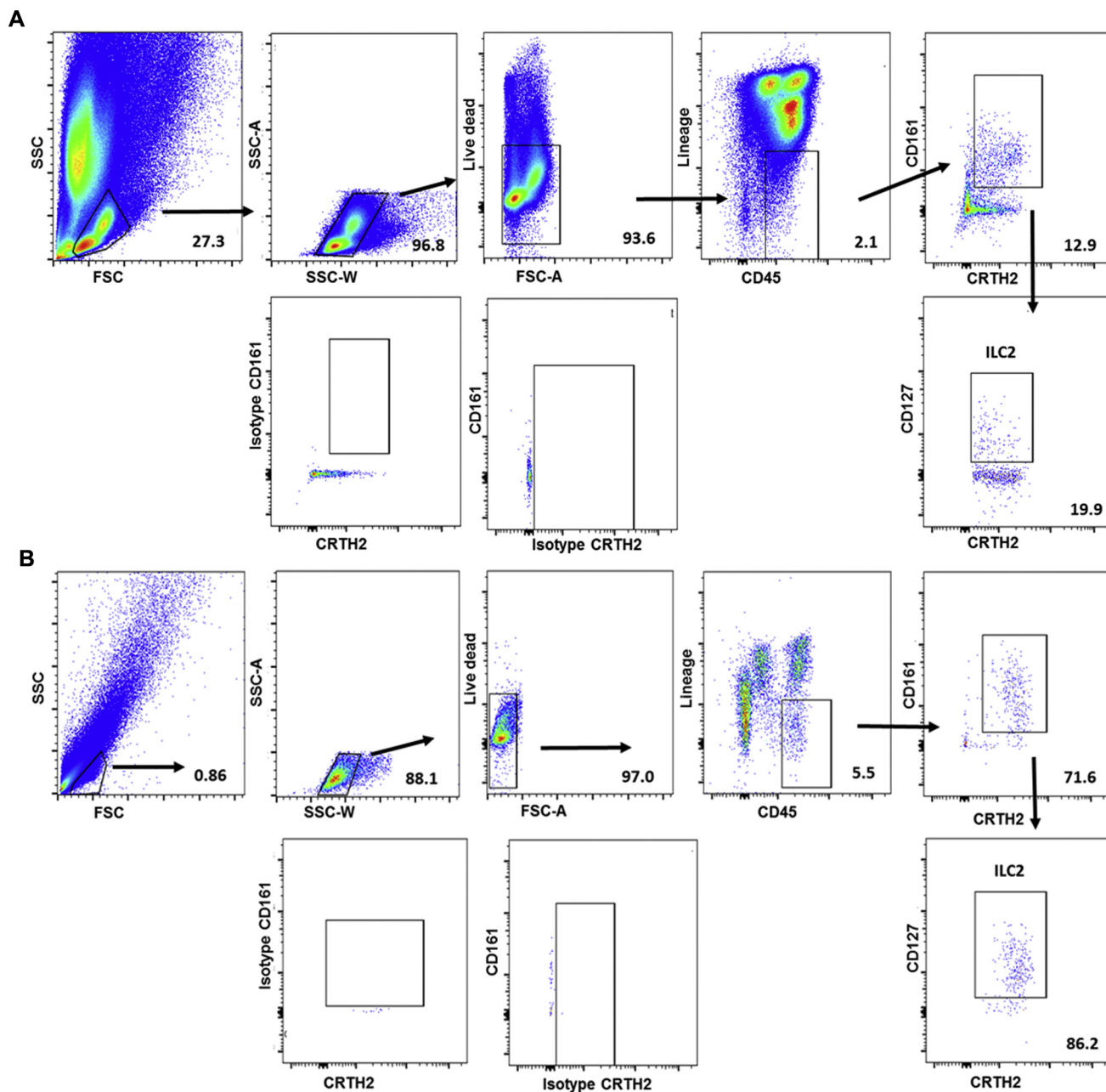


FIG E1. Gating strategy for ILC2s from BAL fluid (**A**) and blood (**B**) of 1 subject. Blood cells were predepleted of lineage-positive cells before sorting. *FSC*, Forward scatter; *SSC*, side scatter.

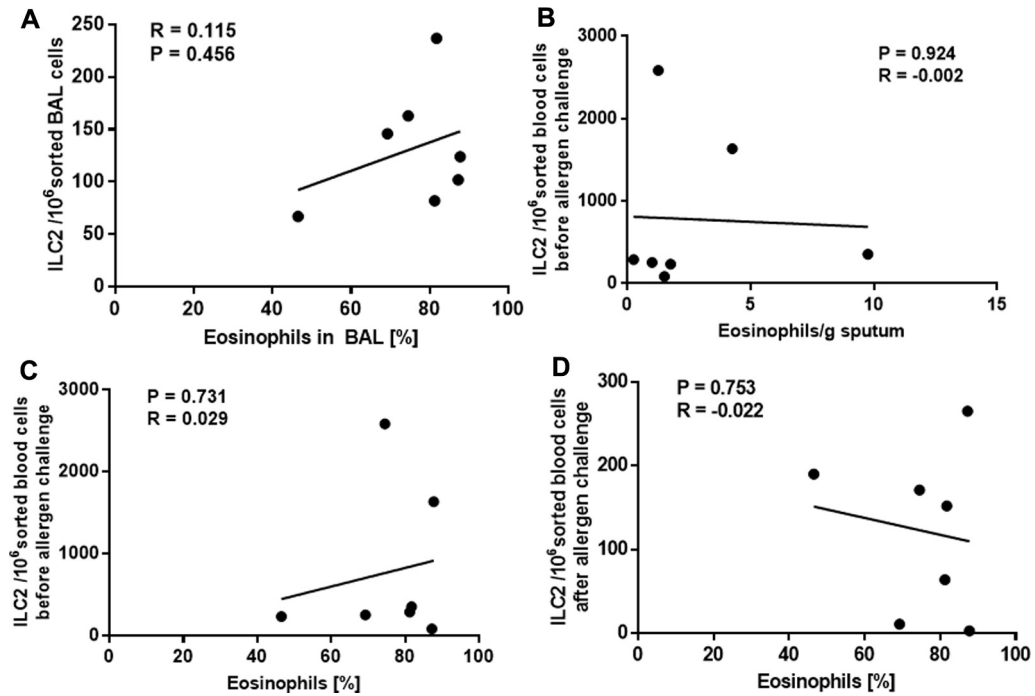


FIG E2. Correlation between ILC2 numbers in BAL fluid and percentages of BAL fluid eosinophils (A), ILC2 numbers in blood and eosinophils per gram of sputum at baseline and after allergen challenge (B), ILC2 numbers in blood before allergen challenge and percentages of BAL fluid eosinophils after challenge (C), and ILC2s in blood and percentages of BAL fluid eosinophils after allergen challenge (D; $n = 7$).

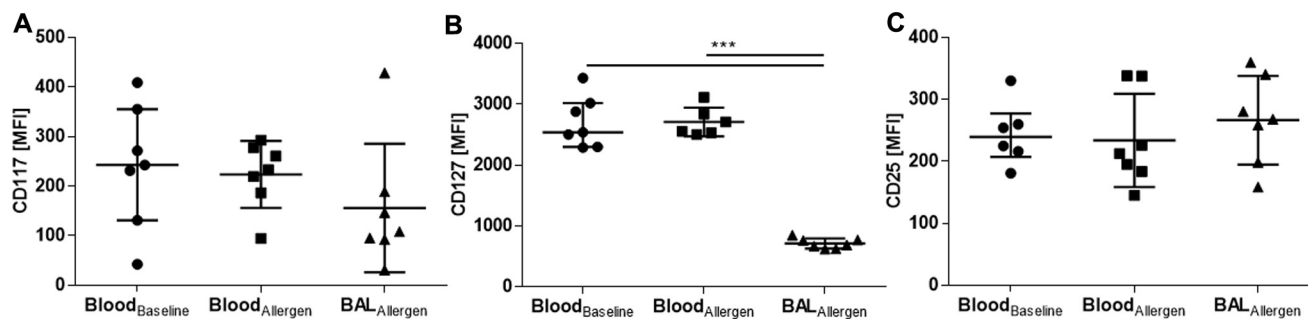


FIG E3. Surface marker expression of blood- and BAL fluid-derived ILC2s at baseline and after allergen provocation for CD117 (A), CD127 (B), and CD25 (C; n = 6-7). *** $P < .0001$, paired t test with multiple comparison adjustment by using the Holm method.

TABLE E1. Absolute cell numbers in BAL fluid

Segment	Macrophages (× 10 ⁶)	Monocytes (× 10 ⁶)	Eosinophils (× 10 ⁶)	Neutrophils (× 10 ⁶)	Lymphocytes (× 10 ⁶)
Baseline	7.4 (3.4-10.7)	0 (0-0.1)	0.1 (0.03-0.1)*	0.1 (0.03-0.3)†	0.6 (0.2-0.7)*
Saline	7.7 (4.1-12.4)	0 (0-0.7)	0.1 (0.1-0.3)*	0.4 (0.2-0.7)*	1.0 (0.4-1.2)
Allergen (A ₁ + A ₂)	5.3 (2.5-24.9)	1.0 (0.3-7.5)	83.9 (52.6-111.8)	11.2 (5.5-14.7)	2.9 (1.6-8.7)

Absolute cell numbers in BAL fluid, in the baseline segment, and after challenge with saline and allergen in 2 segments (pooled A₁ and A₂). Medians and interquartile ranges are shown (n = 7). Least squares mean comparisons (*t* tests) based on a linear mixed model with Tukey multiple comparison adjustment: **P* < .05 and †*P* < .001 compared with allergen.

TABLE E2. Highest and lowest fold expression of genes in blood ILC2s after allergen provocation versus blood ILC2s at baseline

Blood _{Allergen} :Blood _{Baseline} , 10 most downregulated genes				Blood _{Allergen} :Blood _{Baseline} , 10 most upregulated genes			
Gene	Log ₂ fold change	P value, adjusted	Gene name	Gene	Log ₂ fold change	P value, adjusted	Gene name
ENSG00000168970	−2.122	6.16E-04	<i>JMJD7-PLA2G4B</i>	ENSG00000004939	2.856	2.94E-02	<i>SLC4A1</i>
ENSG00000115459	−2.068	6.99E-03	<i>ELMOD3</i>	ENSG00000181227	2.808	4.87E-03	<i>RP4-682C21,2</i>
ENSG00000101246	−2.063	2.54E-03	<i>ARFRP1</i>	ENSG00000149260	2.066	7.11E-02	<i>CAPN5</i>
ENSG00000237499	−1.977	3.23E-03	<i>RP11-356I2,4</i>	ENSG00000103257	1.862	5.41E-03	<i>SLC7A5</i>
ENSG00000168405	−1.602	7.68E-02	<i>CMAHP</i>	ENSG00000174851	1.813	2.09E-02	<i>YIF1A</i>
ENSG00000179091	−1.512	4.99E-02	<i>CYC1</i>	ENSG00000123472	1.639	4.87E-03	<i>ATPAF1</i>
ENSG00000128016	−1.001	3.97E-03	<i>ZFP36</i>	ENSG00000274270	1.621	1.00E+00	<i>RP11-34F20,7</i>
ENSG00000274258	−0.894	1.97E-07	<i>MIR6728</i>	ENSG00000090776	1.616	1.35E-01	<i>EFNB1</i>
ENSG00000229950	−0.806	2.05E-10	<i>TFAP2A-AS1</i>	ENSG00000066654	1.439	1.58E-02	<i>THUMPDI</i>
ENSG00000150510	−0.485	1.14E-04	<i>FAM124A</i>	ENSG00000157557	1.260	1.65E-02	<i>ETS2</i>

TABLE E3. Highest and lowest fold expression of genes in BAL fluid versus blood ILC2s after allergen provocation

BAL _{Allergen} :Blood _{Allergen} , 10 most downregulated genes				BAL _{Allergen} :Blood _{Allergen} , 10 most upregulated genes			
Gene	Log ₂ fold change	P value, adjusted	Gene name	Gene	Log ₂ fold change	P value, adjusted	Gene name
ENSG00000183090	−3.815	9.6E-09	<i>FREM3</i>	ENSG00000169194	7.443	4.29E-39	<i>IL13</i>
ENSG00000131188	−3.481	1.2E-04	<i>PRR7</i>	ENSG00000115602	5.535	1.40E-27	<i>IL1RL1</i>
ENSG00000263264	−3.431	2.2E-07	<i>CTB-133G6,1</i>	ENSG00000128342	5.473	5.64E-31	<i>LIF</i>
ENSG00000168329	−3.368	1.7E-06	<i>CX3CR1</i>	ENSG00000122877	5.398	1.12E-17	<i>EGR2</i>
ENSG00000196187	−3.264	2.2E-21	<i>TMEM63A</i>	ENSG00000175592	5.125	3.84E-10	<i>FOSL1</i>
ENSG00000238121	−3.218	2.9E-07	<i>LINC00426</i>	ENSG00000134247	4.959	1.18E-09	<i>PTGFRN</i>
ENSG00000132359	−3.200	2.1E-20	<i>RAP1GAP2</i>	ENSG00000158714	4.591	6.56E-09	<i>SLAMF8</i>
ENSG00000134775	−3.147	3.2E-06	<i>FHOD3</i>	ENSG00000137265	4.329	6.43E-15	<i>IRF4</i>
ENSG00000272980	−3.061	4.6E-04	<i>RP11-517H2,6</i>	ENSG00000124762	3.955	4.95E-14	<i>CDKN1A</i>
ENSG00000172020	−3.061	7.1E-08	<i>GAP43</i>	ENSG00000100453	3.952	1.63E-06	<i>GZMB</i>