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Lipoxin A₄ regulates natural killer cell and type 2 innate lymphoid cell activation in asthma

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Abstract

Asthma is a prevalent disease of chronic inflammation in which endogenous counter-regulatory signaling pathways are dysregulated. Recent evidence suggests that innate lymphoid cells (ILCs), including natural killer (NK) cells and type 2 innate lymphoid cells (ILC2), can participate in the regulation of allergic airways responses, in particular airway mucosal inflammation. Here, we have identified both NK cells and ILC2 in human lung and peripheral blood in healthy and asthmatic subjects. NK cells were highly activated in severe asthma, linked to eosinophilia and interacted with autologous eosinophils to promote their apoptosis. ILC2 generated antigen-independent IL-13 in response to the mast cell product prostaglandin D₂ (PGD₂) alone and in a synergistic manner with the airway epithelial cytokines IL-25 and IL-33. Both NK cells and ILC2 expressed the pro-resolving ALX/FPR2 receptors. Lipoxin A₄, a natural pro-resolving ligand for ALX/FPR2 receptors, significantly increased NK cell mediated eosinophil apoptosis and decreased IL-13 release by ILC2. Together, these findings indicate that ILCs are targets for lipoxin A₄ to decrease airway inflammation and mediate the catabasis of eosinophilic inflammation. Because lipoxin A₄ generation is decreased in severe asthma, these findings also implicate unrestrained ILC activation in asthma pathobiology.

Introduction

Asthma is characterized by chronic airway inflammation with mucosal infiltration of eosinophils, T lymphocytes, mast cells and release of pro-inflammatory cytokines and lipid mediators (1). In health, the resolution of inflammation is now appreciated to involve active biochemical programs that enable inflamed tissues to return to homeostasis (2). Counter-regulatory lipid mediators are rapidly generated from essential fatty acids during inflammation to promote resolution. Lipoxins are the lead members of this new class of pro-

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Author contributions: C.B. designed the study, performed experiments, collected and analyzed data, and wrote the manuscript; M.C. provided conceptual advice and edited the manuscript, S.D., S.K., M.E.W. and E.I. provided samples from asthma patients and edited the manuscript; X.L. and M.P. performed experiments, collected and analyzed data, and edited the manuscript; B.D.L. designed the study, performed experiments, analyzed data, and wrote the manuscript.

Competing interest:

BDL is a co-inventor on patents related to lipoxin A₄ and asthma that have been licensed by the Brigham and Women's Hospital (BWH) for clinical development and he receives a share of licensing income through BWH. None of the other authors declare any financial competing interests.

resolving mediators (3) with cell type specific actions that include inhibition of neutrophil activation and promotion of macrophage engulfment of apoptotic neutrophils for the resolution of acute tissue inflammation. Lipoxins are generated in asthma (4), and defects in the production of pro-resolving mediators have been associated with chronic inflammatory diseases, including severe asthma (4, 5).

Cellular targets for lipoxins to regulate asthmatic airway responses remain to be determined. Innate lymphoid cells (ILCs) serve protective roles in immune responses (6). Natural Killer (NK) cells are members of the ILC family that serve essential roles in host defense (7), including cytokine secretion, contact-dependent cell-cell signaling and direct killing of other immune cells. NK cells display functional diversity and both disease-controlling and disease-promoting roles have been implicated for NK cells in chronic inflammatory disease (reviewed in (8)). Potential roles for NK cells in asthma and allergic diseases are undefined; however, recent evidence in model systems suggests that NK cells can participate in the down-regulation of allergic airways responses, in particular airway mucosal inflammation (9).

In addition to NK cells, the ILC family also includes type 2 innate lymphoid cells (ILC2), which have been implicated in allergic responses (6). In an antigen-independent manner, ILC2 can generate the cytokines IL-5 and IL-13 that were previously linked to Th2 lymphocytes. ILC2 were recently identified in humans as a population of Lin⁻CD127⁺CD161⁺ ILCs, which also express the chemoattractant receptor-homologous molecule expressed on Th2 lymphocytes (CRTH2) (10). Several studies in murine models of lung disease have demonstrated a role for ILC2s in the development of airway inflammation (11, 12).

Here, we have identified both NK cells and ILC2 in human lung and peripheral blood from healthy and asthmatic subjects. NK cells were highly activated in severe asthma, linked to eosinophilia and interacted with autologous eosinophils to promote their apoptosis. ILC2 generated IL-13 in response to the mast cell product prostaglandin D₂ (PGD₂) alone and in a synergistic manner with the airway epithelial cytokines IL-25 and IL-33. In addition, both NK cells and ILC2 expressed pro-resolving receptors. A natural pro-resolving mediator lipoxin A₄ increased NK cell mediated eosinophil clearance and decreased IL-13 release by ILC2. Together, these findings establish two new cellular targets for pro-resolving mediators and assign critical roles to innate immune lymphoid cells in asthma pathobiology.

Results

Severe asthmatic subjects have lower lung function and more symptoms despite increased use of corticosteroids

Subject characteristics are described in Table 1. The Asthma Control Questionnaire (ACQ) score was higher and lung function (i.e., FEV1) was lower in the subjects with severe asthma compared with mild asthma. None of the subjects with severe asthma were taking oral corticosteroids. Most of the asthmatics were on daily inhaled corticosteroids, and the total daily dose was higher in severe asthma (Table 1). All the patients with severe asthma were treated with long acting β_2 -agonists, 18% with leukotriene antagonists and 18% with omalizumab.

Natural Killer cells are activated in asthma

Natural Killer cells (NK cells) were identified as a lymphoid morphology cell population that expressed NKp46 but not CD3 (Fig. 1A). Relative to healthy subjects, total NK cell numbers in peripheral blood were decreased in mild and severe asthma (Fig. 1A). In contrast, the number of peripheral T lymphocytes (CD3⁺), and their CD4⁺ and CD8⁺ subsets

were similar in healthy subjects and mild and severe asthma (Fig. S1). NK cells were further subdivided based on their surface expression of CD56 and CD16 (Fig. 1B). The numbers of CD56^{dim} NK cells were decreased in both mild and severe asthma (Fig. 1B), while the numbers of CD56^{bright}CD16^{neg} and CD56^{bright}CD16^{pos} NK cells were similar in all cohorts (Fig. S2). To determine the proportion of CD56^{dim} and CD56^{bright} NK cells at sites of inflammation, NK cell subsets were enumerated in BALF samples from subjects with severe asthma. When compared to peripheral blood samples from the same subjects, the CD56^{bright} subset was highly enriched in BALF samples (Fig. 1C). The percentage of CD3⁺CD4⁺ and CD3⁺CD8⁺ cells was similar in PBMC and BALF samples (Fig. S3).

Peripheral blood NK cells were highly activated in severe asthma relative to cells from mild asthmatics or healthy subjects (Fig. 1D and 1E). In severe asthma, the NK cell activation marker CD69 was markedly increased (Fig. 1D), in particular in the CD56^{dim} subset (Fig. 1D). The NK cell activating receptor NKG2D, which specifically interacts with several stress-induced self-ligands (13), was also selectively increased in severe asthma (Fig. 1E). There was a similar increase in the expression of this receptor in severe asthma in both CD56^{dim} and CD56^{bright} NK subsets (Fig. S4).

Activated NK cells interact with eosinophils

Severe asthmatics had a higher peripheral blood total white blood cell count than mild asthmatics and healthy subjects ($P < 0.05$, one-way ANOVA) (Fig. S5A) with increased numbers of circulating eosinophils ($P < 0.05$, one-way ANOVA) (Fig. S5B). In severe asthmatics, there was a positive relationship between the total number of peripheral blood NK cells and eosinophils (Fig. 2A). In addition, NK cell surface expression of CD69 and NKG2D were also positively correlated to peripheral blood eosinophil count (Fig. 2B and 2C).

To determine the impact of NK cells on granulocyte function, freshly isolated NK cells were incubated (4h, 37°C) with autologous granulocytes. Cytospins of these incubations revealed clear evidence of granulocyte cell death (cell shrinkage and nuclear condensation) that was more prominent in the presence of NK cells (Fig. 2D). Quantitative analyses by flow cytometry indicated that NK cells triggered apoptosis of both eosinophils (Fig. 2E) and neutrophils (Fig. 2F). During co-incubations, NK cells from healthy and mild asthmatic subjects led to similar increases in both eosinophil and neutrophil apoptosis. In sharp contrast, NK cells from severe asthmatic subjects had a significantly reduced capacity to augment eosinophil apoptosis (Fig. 2E) despite the increase in NK cell expression of CD69 and NKG2D (Fig. 1). This impairment in severe asthma NK cell responses was selective for eosinophils, as NK cell mediated neutrophil apoptosis was similar for all three cohorts (Fig. 2F). Of interest, NK cells that were incubated with eosinophils and neutrophils did not express surface CD107, which was distinct from similar incubations with the K562 myeloid leukemia cells (Fig. S6).

NK cells express pro-resolving receptors and respond to lipoxin A₄

With this evidence that NK cells display the pro-resolving capacity to inactivate granulocytes by inducing apoptosis (Fig. 2), their expression of specific receptors for pro-resolving mediators was next determined. Circulating NK cells expressed ALX/FPR2 (Fig. 3A), a receptor for Lipoxin A₄ (14). Both CD56^{dim} and CD56^{bright} NK cell subsets expressed ALX/FPR2 at similar levels (Fig. S7). Surface expression of this receptor was increased in severe asthma (Fig. 3A). Peripheral blood NK cells also expressed CMKLR1, a receptor for Resolvin E1 (15). Distinct from ALX/FPR2, the cell surface expression of CMKLR1 receptors was similar in all subjects (Fig. 3B).

To determine if lipoxin A₄ could influence NK cell mediated granulocyte apoptosis, NK cells or granulocytes were selectively exposed to lipoxin A₄ (100 nM) prior to cell-cell interactions during their co-incubations. When NK cells were exposed (15 min) to lipoxin A₄, NK cell induced apoptosis of both eosinophils and neutrophils was significantly increased (Fig. 3C and 3D). This effect was not observed if the granulocytes were exposed to lipoxin A₄ prior to their co-incubations with NK cells (Fig. 3C and 3D). Lipoxin A₄'s actions were inhibited in part by concomitant exposure to the ALX/FPR2 receptor antagonist WRW4 (Fig. 3C and 3D).

Type 2 Innate Lymphoid Cells are present in peripheral blood of healthy and asthmatic subjects and express pro-resolving receptors

In addition to NK cells, other ILC family members, including type 2 innate lymphoid cells (ILC2) are present in human peripheral blood (10). To determine potential roles for these cells in asthma pathobiology, samples of human peripheral blood from healthy and asthmatic subjects were analyzed by flow cytometry for the presence of helper ILCs (Fig. 4). Peripheral blood mononuclear cells were first depleted of most T cells (with anti-CD3), B cells (with anti-CD19) and monocytes (with anti-CD14). After gating on lineage negative (Lin⁻) cells, a population of CD127⁺ cells distinct from CD56-expressing NK cells was identified (Fig. 4A). In the Lin⁻CD127⁺ population, we were able to further distinguish subsets of CD117⁺ and CD117⁻ cells (Fig. 4A) that expressed the NK cell receptors CD161, NKG2D (Fig. 4B) and NKp46 (Fig. 4C). A subpopulation of Lin⁻CD127⁺ cells were identified as ILC2 by the expression of CRTH2 receptors (Fig. 4D and 4E), which ranged between 1.78 – 27.9% in healthy subjects, 1.08 – 24.2% in mild asthmatics and 1.08 – 17.8% in severe asthmatics (Fig. 4E). These Type 2 ILCs (Lin⁻CD127⁺CRTH2⁺ cells) also expressed both ALX/FPR2 and CMKLR1 receptors (Fig 4F).

IL-13 generation by ILC2 is induced by PGD₂ and inhibited by Lipoxin A₄

Distinct from NK cells, Lin⁻CD127⁺ circulating cells stimulated with PMA plus a divalent cation ionophore (A23187) produced bioactive quantities of IL-13 that were in the pg range (8.68 +/- 3.58 (SEM) pg IL-13/1000 cells) (Fig. 5A). IL-13 generation by helper ILCs is linked to ILC2 that express CRTH2 receptors (10). To determine the impact of PGD₂, a ligand for CRTH2 (16), Lin⁻CD127⁺ circulating cells were incubated in the presence of PGD₂ (100 nM) alone or in combination with select cytokines that can induce IL-13 release by ILC2 (10). Relative to control incubations with only vehicle, PGD₂ significantly induced IL-13 production by 72.6% (Mean), which was quantitatively similar to the increase in IL-13 levels with exposure to IL-25 and IL-33 (54.6%, Mean). Of note, when ILCs were exposed to PGD₂ in combination with IL-25 and IL-33, there was a synergistic increase in IL-13 generation by 500.7% (Mean) (3.9 +/- 2.4 (SEM) pg IL-13/1000 cells) (Fig. 5B).

In addition to CRTH2, ILC2 expressed ALX/FPR2 receptors (Fig. 4F), so the impact of lipoxin A₄ on IL-13 generation was next determined. The PGD₂ and cytokine induced IL-13 production was significantly inhibited by equimolar amounts of lipoxin A₄ ($P < 0.05$) (Fig. 5B). IL-13 generation triggered by PGD₂ alone was decreased 42.5 +/- 3.9% by lipoxin A₄ (Mean +/- SEM) and IL-13 levels after PGD₂, IL-25 and IL-33 were decreased 42.7 +/- 4.4% by lipoxin A₄ (Mean +/- SEM), suggesting a common mechanism for ILC2 regulation.

PGD₂ can activate cells via either DP1 or DP2 (CRTH2) receptors, so Lin⁻CD127⁺ cells were exposed to PGD₂ in the presence of either a DP1 receptor antagonist (BWA868C) or DP2 receptor antagonist (BAYu3405). Both antagonists partially inhibited IL-13 release by the ILCs (Fig. 5C); however, only inhibition with the DP2 antagonist reached statistical significance. To further explore PGD₂ receptor-mediated ILC generation of IL-13, ILCs

were exposed to DP1 and DP2 selective agonists. Relative to control incubations with only IL-2, IL-25 and IL-33, the DP2 agonist (15R-methyl-PGD₂) but not the DP1 agonist (BW245C) led to significant increases in IL-13 release (Fig. 5C). Together, these findings suggest a dominant, but not exclusive role for the DP2 receptor in PGD₂-mediated ILC2 activation. Of note, regulation of PGD₂'s actions by LXA₄ was significantly inhibited (30%) by an ALX/FPR2 receptor antagonist WRW4 (Fig. 5D). Together, the marked increase in IL-13 release with PGD₂ and its regulation by lipoxin A₄ indicate that ILC2 functional responses are selectively regulated by lipid mediators.

Innate Lymphoid Cells are present in human lung

To identify ILCs and related cells in lung tissue, samples of perfused human lung from a previously healthy individual were fixed and stained for immunofluorescence microscopy. Because unique surface markers for each of these cell types were not available, a combination surface markers was used to identify and distinguish helper ILCs (cKit⁺, CD161⁺, tryptase⁻), NK cells (cKit⁻CD161⁺tryptase⁻) and mast cells (cKit⁺, CD161⁻, tryptase⁺) (see Methods). NK cells were also positively identified by perforin. Sensitive and specific antibody for CRTH2 was not available for immunohistochemistry, so the ILC2 subset was not targeted in these experiments. Cells with the distinct immunofluorescence staining characteristics of helper ILCs and NK cells were present near medium and small airways (Fig. 6, Fig. S8). In addition, ILCs were in proximity of tissue mast cells in these anatomic locations (Fig. S8).

Discussion

While evidence from murine model systems has uncovered critical roles for ILCs (NK cells and ILC2) in regulating allergic airway inflammation (9, 12, 17–19), only limited translational findings are available in human asthma. Here, total circulating NK cells were decreased in asthma. Relative to healthy subjects, the decrements were most significant in the CD56^{dim} subset, which displays potent cytotoxic actions (7). In severe asthma, peripheral blood NK cells had significantly increased expression of CD69 and NKG2D, especially in CD56^{dim} NK cells, indicating that the cells were activated. BALF NK cell subsets were in sharp contrast to the distribution of circulating NK cells and displayed a further decrease in the CD56^{dim} subset. There is no prior information on NK cells in severe asthma, but asthmatics in general have increased *in vitro* NK cell activity (20, 21) that can be reduced by fluticasone propionate (22); however, severe asthma is defined, in part, as a corticosteroid refractory condition (23). Despite the use of high dose inhaled corticosteroids, samples from our subjects with severe asthma had circulating NK cells that were significantly activated. While peripheral blood NK cells were decreased here in asthma relative to healthy subjects, these findings do not preclude a substantial increase in NK cells during asthma exacerbations, which are often caused by viral infection. NK cells play important roles in anti-viral host defense (7) and NK cells transiently increase in PBMCs from asthmatic children during acute exacerbations (24). Together, these findings indicate that asthmatics, in particular severe asthmatics, have a relative decrease in the numbers of peripheral blood and lung cytotoxic NK cells (ie, CD56^{dim}) and severe asthma is distinguished from milder asthma and health by increased NK cell activation (i.e., CD69 and NKG2D expression). This suggests a tissue specific and dynamic regulation of NK cell phenotype that is unrestrained in severe asthma.

While the peripheral blood numbers of CD56^{dim} NK cells were significantly decreased in asthma relative to healthy subjects, their numbers were still significantly greater than CD56^{bright} NK cells. In contrast to the relative abundance of CD56^{dim} NK cells in peripheral blood, the majority of NK cells found in peripheral lymphoid tissue are CD56^{bright} (25). Here, paired analyses of severe asthma NK cell subsets in peripheral blood and BALF also

uncovered a marked switch in CD56 subsets with increased CD56^{bright} and decreased CD56^{dim} NK cells in the airway relative to the circulation. There are biological implications to this distribution, because, in general, the functional responses of these NK cell subsets are distinct - the CD56^{dim} subset exhibits potent cytotoxicity and the CD56^{bright} subset secretes large amounts of cytokines and chemokines but displays limited cytotoxic capacity (7). Affected joints from patients with rheumatoid arthritis also contain an enriched population of CD56^{bright} NK cells (26, 27) with an activated phenotype (CD69⁺) (28), suggesting a common mechanism for NK cell tissue recruitment in chronic inflammation. Of interest, NK cells from healthy subjects obtained from BAL or lung tissue are profoundly impaired in their cytotoxic capacity (29, 30), likely reflecting an enriched CD56^{bright} population of NK cells. Moreover, patients with sarcoidosis have increased CD56^{bright} NK cells in their BALF without differences in peripheral blood (31). Accumulation of CD56^{bright} NK cells to the lung or at sites of inflammation may result from selective recruitment, as CD56^{bright} NK cells and CD56^{dim} NK cells differ in their pattern of expression of chemokine receptors and adhesion molecules (32, 33). Evidence from rodent models of allergic asthma support an important role for selective NK cell homing to the lung and associated lymph nodes (9, 34). NK cell cytotoxicity is critical for the timely clearance of antigen-specific T cells in the lung (9), so decreased CD56^{dim} NK cells would predispose to chronic airway inflammation.

Most human asthma phenotypes are characterized by increased eosinophils in peripheral blood, lung tissue and bone marrow and peripheral blood eosinophilia generally correlates with disease severity (35). Preclinical evidence links NK cells to eosinophilia. NK cell depletion in mice leads to persistent allergic airway inflammation with significantly increased numbers of antigen-specific T cells and eosinophils in BALF after allergen challenge (9) and cytokine-primed murine NK cells can suppress airway hyper-responsiveness and eosinophilia (36). When activated, eosinophils secrete an array of cytotoxic cationic proteins that can cause significant lung tissue damage, so induction of eosinophil apoptosis is crucial to avoid inadvertent tissue injury. Because granulocyte apoptosis can accelerate resolution of tissue inflammation (37), induction of eosinophil apoptosis would likely participate in the timely resolution of airway inflammation in controlled asthmatic responses. NK cells appear to be linked to eosinophil clearance, as peripheral eosinophilia in asthmatic subjects correlated with NK cell numbers and activation (ie, CD69, NKG2D). Similar to neutrophils (38), when NK cells from healthy subjects or mild asthmatic subjects were incubated *ex vivo* with autologous eosinophils, there was a significant increase in eosinophil apoptosis. Of interest, eosinophil apoptosis induced by severe asthma NK cells was significantly decreased despite their increased expression of CD69 and NKG2D. The lysosomal-associated membrane protein-1 (LAMP-1 or CD107a), a degranulation marker, was not upregulated on NK cells during co-incubations with granulocytes, suggesting that the NK cells triggered granulocyte apoptosis in a manner distinct from cytotoxicity. For neutrophils, NK cells utilize a caspase-dependant mechanism that is mediated by the activating NK cell receptor NKp46 and Fas pathway (38), and a similar mechanism is likely for eosinophils. Of note, a positive association between NK cells and eosinophils has also been observed in BALF from patients with eosinophilic pneumonias (39).

In addition to NK cells, ILC2s are innate lymphocytes that have been linked in murine studies to asthma pathobiology. Amongst the larger family of ILCs, ILC2 share a lymphoid morphology, lack specific antigen receptors and depend on the transcriptional repressor Id2 and the common γ -chain of the IL-2-receptor (6). In addition to their roles in protective immunity to helminths (6), ILC2s constitute a significant and early source of type 2 cytokines in several murine models of experimental asthma and are instrumental in mediating inflammatory aspects of airway hyperresponsiveness (12, 17–19). ILC2s can serve as an innate source of IL-13 that directly induces airway hyperresponsiveness in the

absence of IL-13-producing CD4⁺ T cells (17). This ILC2 subset has been recently identified in humans in the gut, lung and blood of healthy individuals and was strikingly enriched in nasal polyps from patients with chronic rhinosinusitis, a typical type 2 mediated disease (10). Here, healthy and asthmatic subjects had CRTH2⁺ ILC2 in their peripheral blood, and cells with the staining characteristics of ILCs were also identified in human lung near medium and small airways in close proximity to cells with the staining pattern for NK cells and mast cells, suggesting that these cells interact *in vivo*. When activated by PMA and A23187, peripheral blood ILC2, but not NK cells, generated measurable levels of the type 2 cytokine IL-13. Located in the lung near mast cells and airway epithelial cells, the ILC2 released significant amounts of IL-13 in response to receptor-mediated, soluble stimuli from these cell types, namely PGD₂ (mast cells) and IL-25 and IL-33 (airway epithelia). ILC2 express CRTH2 (DP2) and PGD₂'s activation of IL-13 release by ILC2 was predominantly mediated via this receptor. As antigen independent sources of IL-13 in response to environmental cues from activated cells in the human airway, ILC2 are well poised to participate in the genesis of asthmatic responses, including eosinophilic inflammation and airway hyperresponsiveness.

Allergic airway responses, including eosinophilic inflammation, airway hyperresponsiveness and mucus metaplasia, are regulated by select members of a new genus of anti-inflammatory and pro-resolving mediators, including lipoxin A₄ and resolvin E1 (14, 40). Lipoxin A₄ is enzymatically derived from arachidonic acid and serves as an agonist at ALX/FPR2 receptors to mediate cell type-specific actions in acute inflammatory responses, including inhibition of neutrophil activation, promotion of macrophage engulfment of apoptotic neutrophils and regulation of epithelial cell cytokine release (41–44). Resolvin E1 is enzymatically derived from the omega-3 fatty acid eicosapentaenoic acid and serves as a pro-resolving agonist at CMKLR1 receptors (15), including for the resolution of airway inflammation in a murine model of allergic asthma (40). CMKLR1 also serves as a receptor for chemerin, a chemotactic protein found in inflammatory exudates (45) and implicated in the colocalization of NK cells and DC subsets in pathologic peripheral tissues (46). Lipoxin A₄ is present in asthmatic lung (47), and when administered to asthmatic subjects via nebulization, lipoxin A₄ prevents LTC₄-mediated bronchoconstriction (48). In addition, treatment of the allergic condition infantile eczema with topical 15(R/S)-methyl-lipoxin A₄ significantly decreases eczema severity and duration and improved patients' quality of life, with similar efficacy as topical corticosteroids (49). Bioactive lipoxin A₄ stable analogs have been prepared that in experimental asthma, block both airway hyper-responsiveness and allergic inflammation (14). Eosinophil trafficking and tissue accumulation are markedly inhibited by lipoxins (14, 50, 51), and lipoxin A₄ decreases NK cell cytotoxicity *in vitro* (52, 53). Severe asthma is characterized by a defect in lipoxin biosynthesis (4, 5), suggesting that the chronic inflammatory airway responses in asthma may stem, in part, from inadequate counter-regulation. NK cells and ILC2 expressed the pro-resolving receptors ALX/FPR2 and CMKLR1, and NK cells from subjects with severe asthma had increased ALX/FPR2 expression. In nanomolar concentrations and in an ALX/FPR2-dependent manner, lipoxin A₄ decreased release of IL-13 by ILC2 and increased NK cell mediated eosinophil apoptosis. These properties of lipoxin A₄ are consistent with potent anti-inflammatory (ILC2) and pro-resolving (NK cell) actions on ILCs and highlight a putative mechanism for the pathogenesis of severe asthma that links defective lipoxin A₄ generation to the increased eosinophilia and chronic airway inflammation that characterize the disease.

The peripheral blood characteristics of ILCs (NK cells, ILC2s) identified here may serve as useful biomarkers of asthma control and severity; however, there are potential limitations to note. Because type 2 ILCs are a rare cell population, an enrichment step was required that limited their accurate enumeration and characterization and restricted the cell numbers available for functional assay. While type 2 ILCs were detectable in peripheral blood and

lung tissue, they were not found in aliquots of BALF and it was not feasible to obtain lung samples from asthmatic subjects. NK cells were more abundant, but the pool of peripheral blood NK cells is heterogeneous and dynamically regulated by several environmental triggers, most notably viral infection. In addition, in this mechanistic study there was in depth analysis of a relatively small sample size. Because of asthma's syndromic nature with individual variation in severity and medication use, the differences identified in ILCs between severe and mild asthma will require validation in larger clinical cohorts.

In conclusion, NK cells and ILC2 were present in human peripheral blood and lung from healthy and asthmatic subjects. In subjects with severe asthma, the circulating NK cells were highly activated and associated with peripheral eosinophilia. During coinfections simulating cell-cell interactions in inflamed tissue, NK cells interacted with autologous eosinophils and neutrophils to promote their apoptosis. In addition to the airway epithelial cytokines IL-25 and IL-33, PGD₂ induced the release of IL-13 by CRTH2⁺ILC2. Both NK cells and ILC2 expressed ALX/FPR2 receptors, which were increased in severe asthma, and the natural ALX/FPR2 ligand lipoxin A₄ displayed potent anti-inflammatory and pro-resolving actions; decreasing PGD₂ and cytokine mediated IL-13 release by ILC2 and increasing NK cell mediated eosinophil apoptosis. Together, these findings assign critical roles to ILCs in asthma pathobiology and identify new cellular targets and mechanisms for endogenous counter-regulatory mediators. The anti-inflammatory and pro-resolving actions for lipoxin A₄ also suggest a potential new therapeutic strategy in asthma and allergic diseases that emphasizes this natural resolution pathway and ILCs.

Material and Methods

Patients and samples

After approval by the Partners Healthcare institutional review board and written informed consent was obtained, subjects with severe or mild asthma were recruited by the Brigham and Women's Hospital Asthma Research Center using criteria defined by the Severe Asthma Research Program (23). Healthy control subjects were in good health, denied a history of atopy, asthma or other chronic medical illness and had taken no prescription or over-the-counter medications within two weeks prior to enrollment. Heparinized peripheral whole blood was collected by venipuncture from severe asthmatics (n=11), mild asthmatics (n=11) and healthy subjects (n=12). Paired samples of peripheral blood and bronchoalveolar lavage fluid (BALF) were obtained from 5 severe asthmatics. Peripheral blood mononuclear cells (PBMC) and granulocytes were isolated by density-gradient centrifugation over Histopaque 1077 (Sigma Aldrich) and Histopaque 1119 (Sigma Aldrich). Residual erythrocytes were lysed by incubating the cell pellet with a small volume of distilled water for 20 s followed by quenching with a large volume of isotonic PBS (as in (54)).

Media and reagents

RPMI-1640 (Lonza) supplemented with 2 mM L-glutamine, and 10% heat-inactivated fetal calf serum (FCS) (all from Gibco BRL) was used as media for NK cell culture with granulocytes (4h, 37°C, 5% CO₂). For longer (24h) incubations, this medium was supplemented with 100 IU/mL penicillin and 100 µg/ml streptomycin. Lipoxin A₄, PGD₂, 15(R)-methyl-PGD₂, BW 245C, BW A868C and BAY-u3405 were purchased from Cayman Chemical. WRW4 was purchased from Tocris Bioscience. Recombinant human (rh) IL-2, rhIL-25 and rhIL-33 were purchased from R&D Systems. Phorbol 12-myristate 13-acetate (PMA) and the divalent cation ionophore A23187 were obtained from Sigma.

Flow cytometry analysis and sorting

The following antibodies to human proteins were used: anti-CD3 (SK7), anti-CD56 (CMSSB), anti-CD16 (CB16), anti-CD69 (FN50), anti-CD161 (HP-3G10), anti-CD11c (3.9), anti-human-CD123 (6H6), anti-CD19 (HIB19), anti-CD14 (61D3), anti-alpha beta TCR (IP26), anti-gamma delta TCR (B1.1), mouse IgG1 kappa isotype control (P3.6.2.8.1) (all from eBioscience; anti-CD34 (581), Annexin V and 7 AAD (from BD Pharmingen; anti-CD335 (NKp46) (9E2), anti)NKG2D (1D11), anti-CD117 (104D2), anti-CD127 (A019D5), anti-CD294 (CRTH2) (BM16), mouse IgG1 kappa isotype control (MOPC 21), Rat IgG2a kappa isotype control (RTK2758) (from Biolegend); anti-ChemR23 (84939), anti-FPRL1 (304405), mouse IgG3 isotype control (133319) and mouse IgG2b isotype control (133303) (from R & D Systems).

For analyses of cell phenotype, data were acquired on a Canto II flow cytometer (Becton Dickinson Bioscience) and were analyzed with FlowJo software (Tree Star). NK cells were identified in a PBMC suspension or in BALF samples as a cell population with a lymphoid morphology based on forward and side scatter characteristics expressing NKp46 and lacking CD3. 20,000 events of NK cells were acquired and results were expressed as a percentage of the total lymphocyte population. If required, cells were preincubated with blocking mAb (Purified Human IgG, R & D Systems) before specific staining. For ILC phenotyping, PBMCs were first depleted of most T and B lymphocytes and monocytes by labeling with FITC conjugated anti-CD3, anti-CD14 and anti-CD19 (eBioscience) plus anti-FITC magnetic beads (Easy Sep, StemCell Technology) and further identified among a cell population with a lymphoid morphology based on forward and side scatter characteristics. For compensation control, BD CompBeads (BD Pharmingen) were used and appropriate isotype negative controls were used to define background staining.

For flow cytometry sorting, peripheral blood mononuclear cells were depleted of most of T cells, B cells and monocytes by labeling with FITC conjugated anti-CD3, anti-CD14 and anti-CD19 (eBioscience) plus anti-FITC magnetic beads (Easy Sep, StemCell Technology). Remaining cells were labeled with FITC-conjugated anti-CD3, TCR- α , TCR- β , CD14, CD19, CD16, CD123, CD11c, CD34, CD56 and CD127. Lin⁻(CD3⁻TCR- α ⁻TCR- β ⁻CD14⁻CD19⁻CD16⁻CD123⁻CD11c⁻CD34⁻)CD127⁺ ILCs, Lin⁻CD56^{bright} NK cells and Lin⁻CD56^{dim} NK cells were sorted with an Aria SORP flow cytometer (Becton Dickinson Bioscience) to a purity of \geq 96%.

NK cells and granulocyte incubations

Peripheral blood NK cells were isolated from PBMCs using a NK cell isolation kit (EasySep, Stem Cell Technologies). Briefly, non-NK cells (i.e., T cells, B cells, stem cells, DCs, monocytes, granulocytes, and erythroid cells) were magnetically labeled and depleted using a magnet. The purity of isolated NK cells was assessed by flow cytometric analysis of cells stained against CD3 and NKp46. The purity of isolated NK cells was $>$ 96%.

Autologous human granulocytes were freshly isolated from peripheral whole blood, resuspended in RPMI 1640 medium, supplemented with 10% heat inactivated FCS, 2 mM L-glutamine and incubated in the absence or presence of NK cells (1:5 ratio of granulocytes:NK cells) (4h, 37°C, 5% CO₂) and assayed by FACS using Annexin V and 7-AAD (BD Pharmingen) to identify and distinguish cells undergoing apoptosis from necrosis. Neutrophils and eosinophils were gated based on FSC and SSC characteristics, and eosinophils (CD16⁻) were also distinguished from neutrophils (CD16⁺) by immunostaining. In some experiments, cells were selectively exposed (15 min, 37°C) to lipoxin A₄ (100 nM) or vehicle (0.1% ethanol) prior to co-incubation. To assess ALX/FPR2-mediated signaling,

some NK cells were exposed to the receptor antagonist WRW4 (230 nM, IC₅₀) prior to lipoxin A₄ (100 nM).

ILC cultures and IL-13 release

In some subjects, Lin⁻CD127⁺, CD56^{bright} NK cells and CD56^{dim} NK cells were isolated from whole blood by flow cytometry (see above) and cultured for 24h (37°C, 5% CO₂) in the presence of vehicle (0.1% ethanol) or soluble stimuli, including PMA (50 ng/ml) and calcium ionophore A23187 (500 ng/ml) (PMA/A2), IL-2 alone (2 ng/ml) (IL-2), IL-2 (2 ng/ml), IL-25 (50 ng/ml) and IL-33 (50 ng/ml) (IL-2/IL-25/IL-33), IL-2 (2 ng/ml) and PGD₂ (100 nM) (IL2/PGD₂), or IL-2 (2 ng/ml), IL-25 (50 ng/ml), IL-33 (50 ng/ml) and PGD₂ (100 nM) (IL-2/IL-25/IL-33/PGD₂). In select experiments, pharmacological agents were used to investigate receptor dependency, including BWA868C (5 nM, DP1 IC₅₀), BAY-u3405 (100 nM, CRTH2 (DP2) IC₅₀), BW245C (2.5 nM, DP1 EC₅₀), 15(R)-methyl-PGD₂ (2.5 nM, DP2 EC₅₀) and WRW4 (230 nM, ALX/FPR2 IC₅₀). IL-13 was measured in the collected cell culture supernatants by ELISA (eBioscience).

Immunofluorescent identification of ILCs and mast cells in human lung

Healthy human lungs that were not used for transplantation, and had their vasculature perfused to remove the blood, were fixed in 10% buffered formalin and paraffin embedded for immunofluorescence staining. For ckit/CD161 double staining, the 5 μm sections were incubated with a ckit antibody (1:100, Anti-human CD117 polyclonal rabbit IgG, Dako cat#A4502) and a CD161 antibody (1:10, anti-human CD161 monoclonal IgG, Abcam cat#ab23624) at 4°C overnight, followed by incubation with secondary antibodies of rabbit IgG conjugated with FITC (1:50) and mouse IgG conjugated with TRITC (1:30), respectively, at 37°C for 1h. For ckit/CD161/perforin triple staining, after the ckit/CD161 staining was complete, the sections were incubated with a perforin antibody (1:20, Anti-human perforin monoclonal IgG, eBioscience cat#14-9994-80) at 37°C for 2h, following by incubation with secondary anti-mouse antibody conjugated with far-red (1:30 for 1h). For ckit/tryptase staining, after the ckit staining was complete, the sections were incubated with goat anti-human tryptase antibody (1:30, Santa Cruz sc-17039), followed by incubation with an anti-goat IgG conjugated with far-red (1:30) for 1h. Stained slides were photographed by immunofluorescence microscope or confocal microscopy.

Statistical analysis

Data are shown as mean ± SEM. The statistical significance of differences was assessed by two-tailed Student's *t*-test or one-way analysis of variance (ANOVA) followed by post-hoc tests to determine the levels of significance for each group. Correlations were evaluated by Spearman or Pearson's correlation coefficient (*r*). A *p*-value <0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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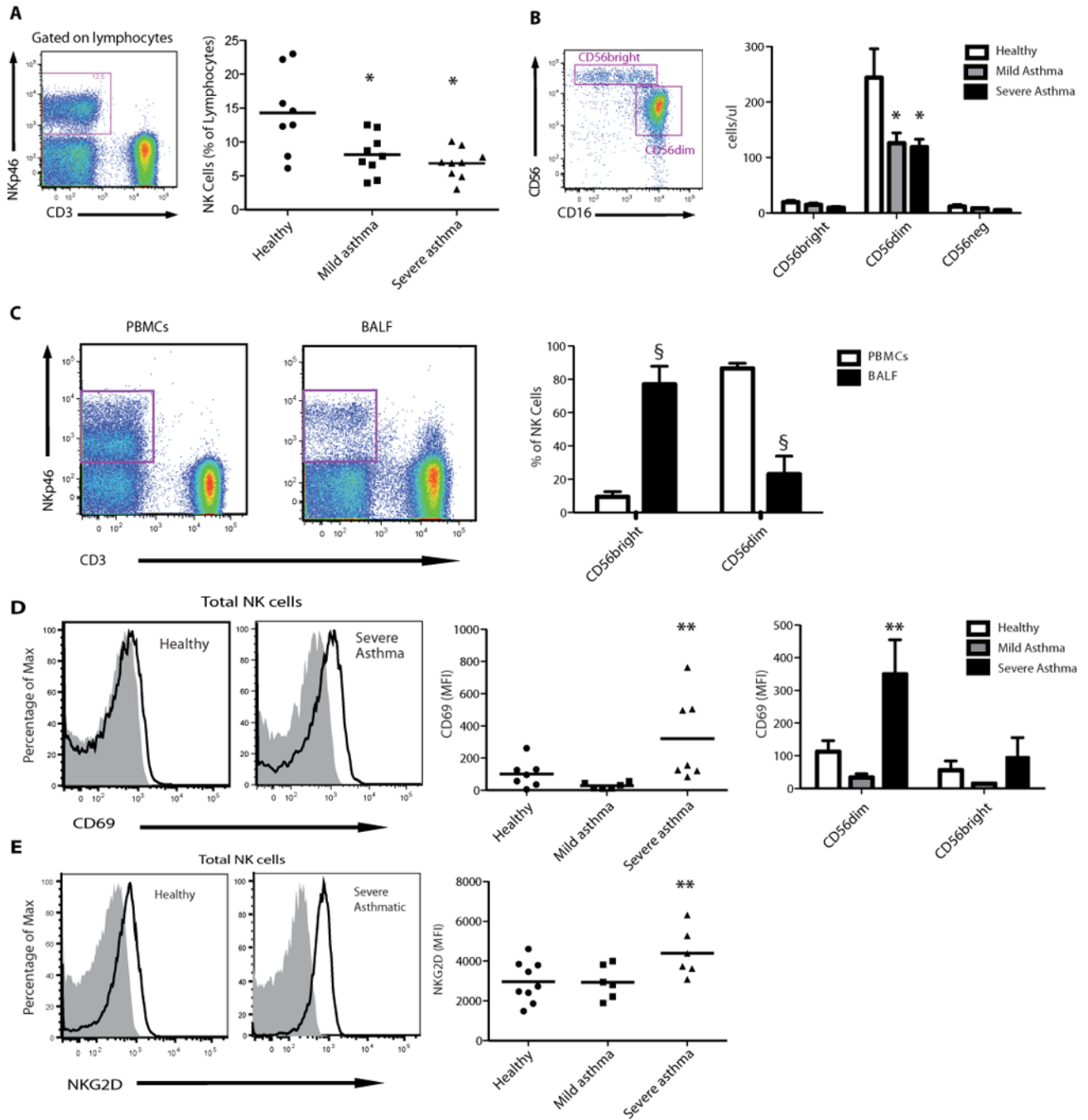


Fig. 1. NK cells are activated in asthma

Peripheral venous blood was obtained from healthy subjects and individuals with mild and severe asthma. (A) NK cells were identified as a Nkp46⁺CD3⁻ cell population in a lymphocyte morphology gate based on SSC and FSC characteristics (violet box, left panel) and NK cells as a percentage of total lymphocytes were enumerated (right panel), bar indicates mean value. (B) NK cells were further phenotyped by flow cytometry analysis of CD56 expression for CD56^{bright} and CD56^{dim} subsets (violet boxes, left panel) in samples from healthy and asthmatic subjects (right panel). (C) Paired samples of BALF and blood were obtained on a subset of severe asthmatics. Representative FACS plots show an

enriched NKp46^{bright} cell population in BALF compared to PBMCs (left panel). BALFs were also enriched for CD56^{bright} subset of NK cells (n=5) (right panel). **(D)** Representative FACS histograms showing CD69 expression in peripheral blood NK cells (left panel) that was upregulated in severe asthma (middle panel), in particular in the CD56^{dim} subset (right panel). **(E)** Representative FACS histograms showing NKG2D expression in peripheral NK cells (left panel) that was upregulated in severe asthma (right panel). Values are expressed as mean \pm s.e.m.; *, $P < 0.05$, compared with healthy subjects (one-way ANOVA); §, $P < 0.0001$, compared with PBMCs (one-way ANOVA); **, $P < 0.05$, compared with healthy and mild asthmatic subjects (one-way ANOVA).

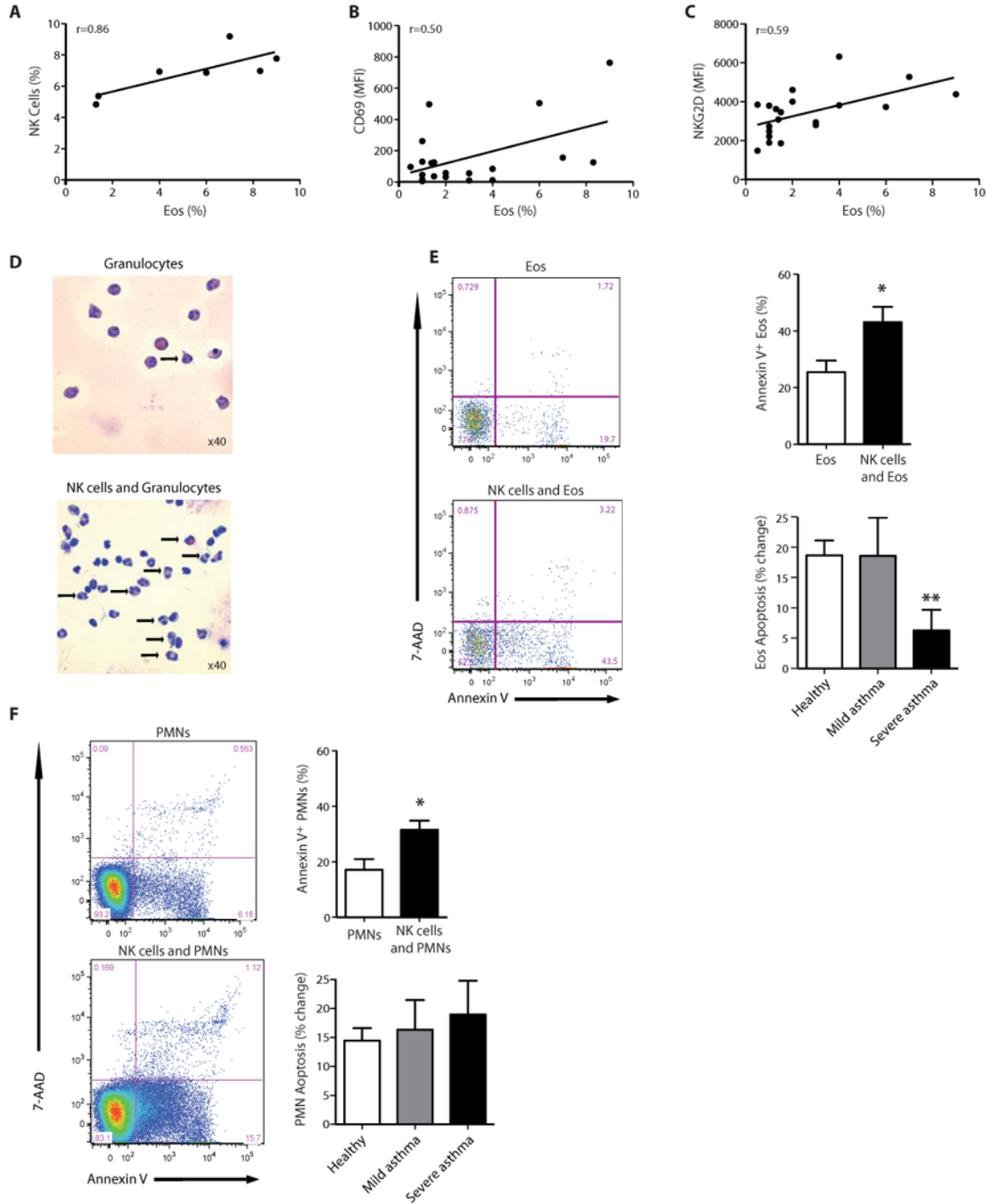


Fig. 2. NK cells interact with eosinophils

(A–C) The percentage of peripheral blood eosinophils in severe asthma was correlated to (A) the percentage of peripheral blood NK cells; Spearman $r=0.86$, $P < 0.05$. (B) NK cell CD69 expression; Pearson $r=0.50$, $P < 0.05$, and (C) NK cell NKG2D expression, Spearman $r=0.59$, $P < 0.01$. (D–F) Incubation (4h, 37°C) of autologous peripheral blood granulocytes with NK cells (1:5 ratio) triggered increased granulocyte apoptosis compared to granulocytes incubated alone by (D) morphological appearance on cytospin (arrows, apoptotic granulocytes) and (E,F) by FACS criteria (Annexin V⁺7-AAD⁻) for (E) eosinophils (Eos) and (F) neutrophils (PMN) in samples of peripheral blood from healthy

subjects and individuals with mild or severe asthma. Results are expressed as mean \pm SEM.; n=5 individual healthy donors, n=5 mild asthmatic donors, n=5 severe asthmatic donors; *, $P < 0.05$, compared with granulocytes alone and **, $P < 0.05$ compared with healthy donors (Student's *t*-test).

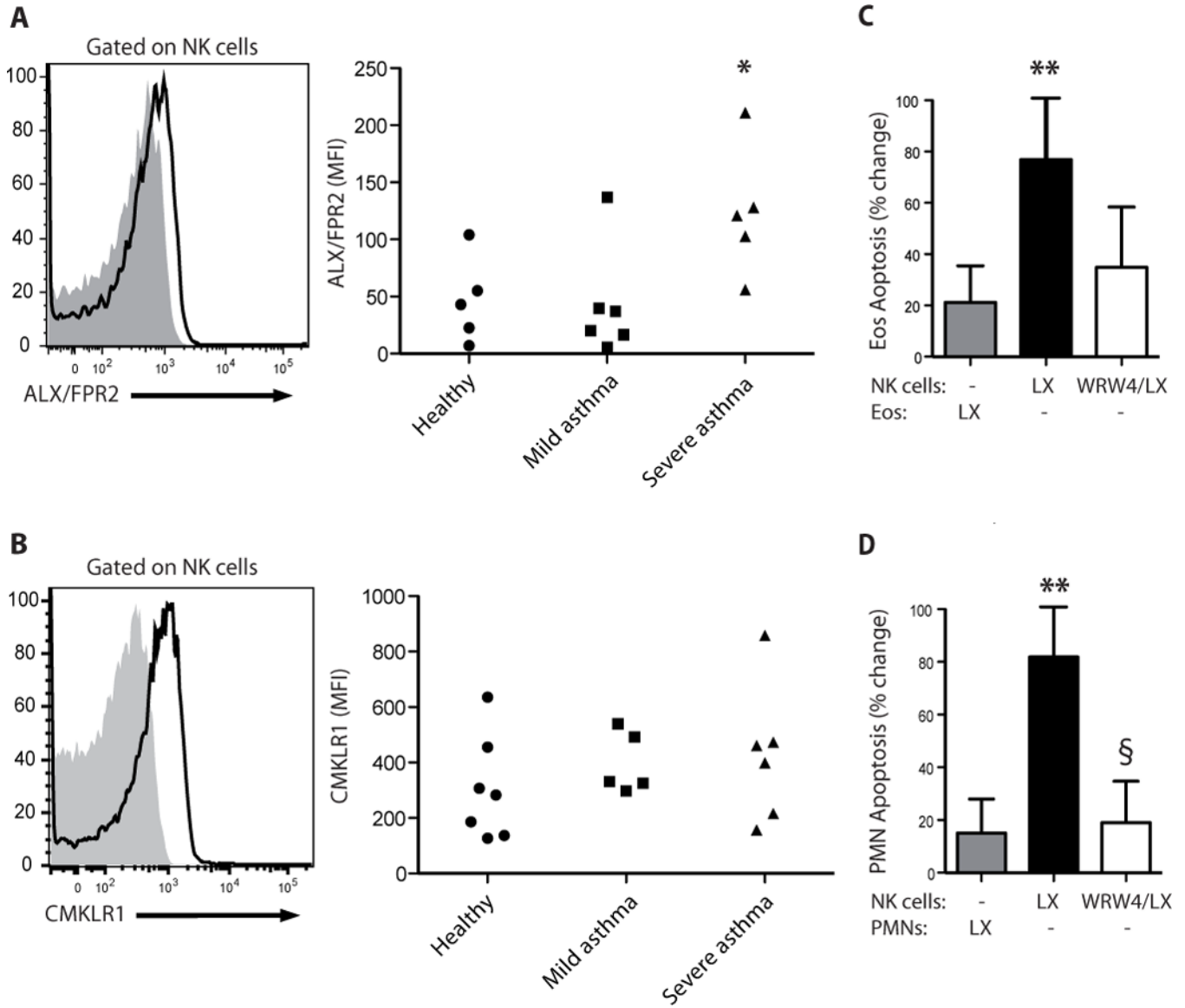


Fig. 3. NK cells express pro-resolving receptors and respond to LXA₄
(A) Flow cytometry histograms for anti-ALX (FPRL1) Ab (white) and isotype control (gray) for peripheral blood NK cells (left panel) and MFI for ALX expression on peripheral NK cells in healthy subjects and mild and severe asthmatic subjects (right panel). **(B)** Flow cytometry histograms for anti-CMKLR1 Ab (white) and isotype control (gray) for peripheral blood NK cells (left panel) and MFI for CMKLR1 expression on peripheral NK cells in healthy subjects and mild and severe asthmatic subjects (right panel). *, $P < 0.05$, compared with healthy and mild asthmatic subjects (one-way ANOVA). **(C, D)** Autologous granulocytes and NK cells from healthy subjects were selectively exposed to lipoxin A₄ (LX, 100 nM, 15 min, 37°C) in the absence or presence of the ALX/FPR2 antagonist (WRW4, 230nM) prior to co-incubation and the percent change in apoptosis was determined for **(C)** eosinophils (Eos) and **(D)** neutrophils (PMN) (see Methods). Results are expressed as mean \pm SEM; n 4; **, $P < 0.05$, compared with control (one-way ANOVA); §, $P < 0.05$, compared with NK(LX).

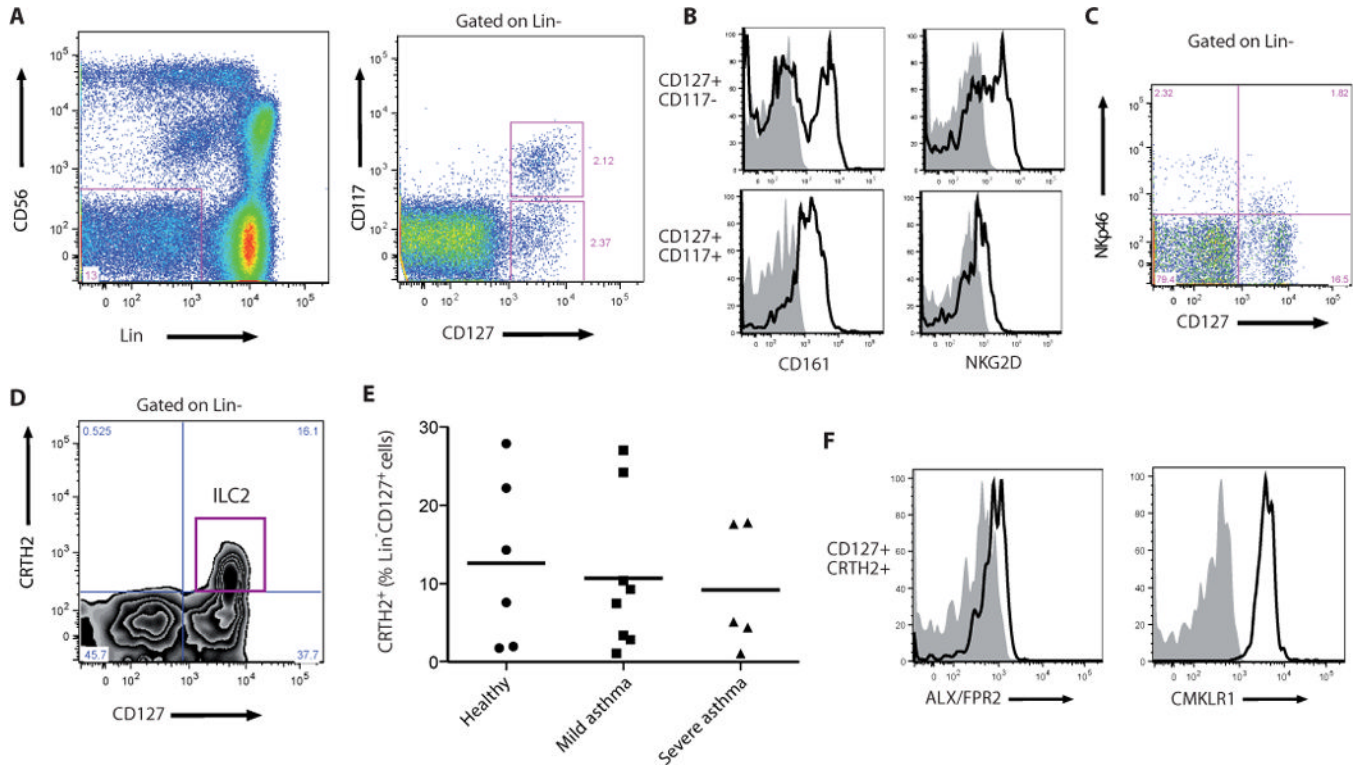


Fig. 4. ILC2 are present in peripheral blood of healthy and asthmatic subjects and express pro-resolving receptors

(A–D) Flow cytometry gating strategy for the identification of helper ILC and the ILC2 subset. PBMCs were depleted of most T and B lymphocytes and monocytes, and a lymphoid morphology cell population was selected based on their forward and side scatter characteristics. (A) Flow cytometry analysis showing the presence of a Lin⁻ cell population (violet box, CD3⁻TCR⁻TCR⁻CD19⁻CD14⁻CD16⁻CD34⁻CD123⁻CD11c⁻) different from NK cells (Lin⁻CD56⁺), left panel; identification of a Lin⁻CD127⁺CD117^{+/-} population, right panel. (B) Lin⁻CD127⁺CD117^{+/-} expressed NK cell receptors, including CD161, left panel; NKG2D, right panel, and (C) NKp46. (D) ILC2 were identified as a subpopulation of Lin⁻CD127⁺ cells that also expressed CRTH2. (E) The number of Lin⁻CD127⁺CRTH2⁺ ILC2 cells was determined in healthy and mild and severe asthmatic subjects. (F) ILC2 also expressed the pro-resolving receptors ALX (left panel) and CMKLR1 (right panel) on their surface (see Methods).

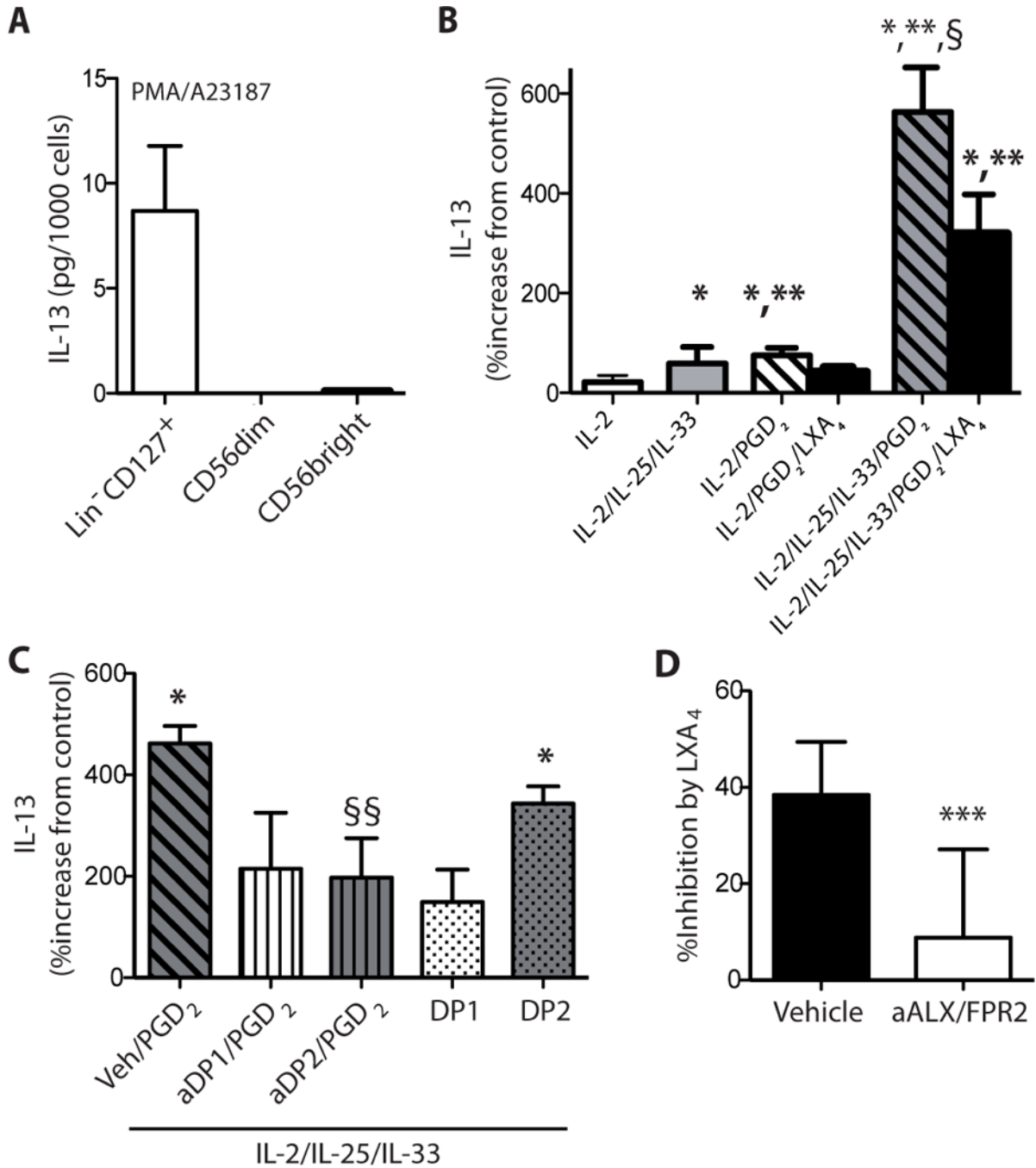


Fig. 5. IL-13 production by peripheral blood ILC2 is enhanced by PGD₂ and inhibited by lipoxin A₄

IL-13 was quantitated by ELISA in the cell-free supernatants from incubations with (A) Lin⁻CD127⁺ cells, CD56^{bright} NK cells and CD56^{dim} NK cells that were sorted by flow cytometry and cultured for 24h alone (Control) or with PMA (50 ng/ml) and A23187 (500 ng/ml). Results are expressed per 1,000 cells. (B) In separate incubations, IL-13 release was determined for Lin⁻CD127⁺ cells that were incubated (15 min, 37°C) with lipoxin A₄ (100 nM) or vehicle (0.1% ethanol) prior to (24h, 37°C) media alone, IL-2 (2 ng/ml) (IL-2), IL-2 (2 ng/ml), IL-25 (50 ng/ml) and IL-33 (50 ng/ml) (IL-2/IL-25/IL-33), IL-2 (2 ng/ml) and PGD₂ (100 nM) (IL2/PGD₂), or IL-2 (2 ng/ml), IL-25 (50 ng/ml), IL-33 (50 ng/ml) and

PGD₂ (100 nM) (IL-2/IL-25/IL-33/PGD₂). (C) To assess PGD₂ receptor signaling, ILCs were incubated with IL-2/IL-25/IL-33/PGD₂ in the presence of a DP1 receptor antagonist (BWA868C, aDP1) or DP2 receptor antagonist (BAYu3405, aDP2). Some cells were exposed to IL-2/IL-25/IL-33 in conjunction with a selective DP1 receptor agonist (BW245C, DP1) or DP2 receptor agonist (15(R)-methyl-PGD₂, DP2) without PGD₂. (D) To determine the role of ALX/FPR2 signaling, cells were exposed (15 min, 37°C) to the receptor antagonist WRW4 (aALX/FPR2) prior to LXA₄ and IL-2/IL-25/IL-33/PGD₂. Results are expressed as the percent increase from control (mean ± s.e.m.); n = 3 individual healthy donors; *, $P < 0.01$, compared with control; **, $P < 0.05$, compared with IL-2; §, $P < 0.05$, compared with IL-2/IL-25/IL-33, §§, $P < 0.05$, compared with IL-2/IL-25/IL-33/PGD₂; ***, $P < 0.05$, compared with vehicle.

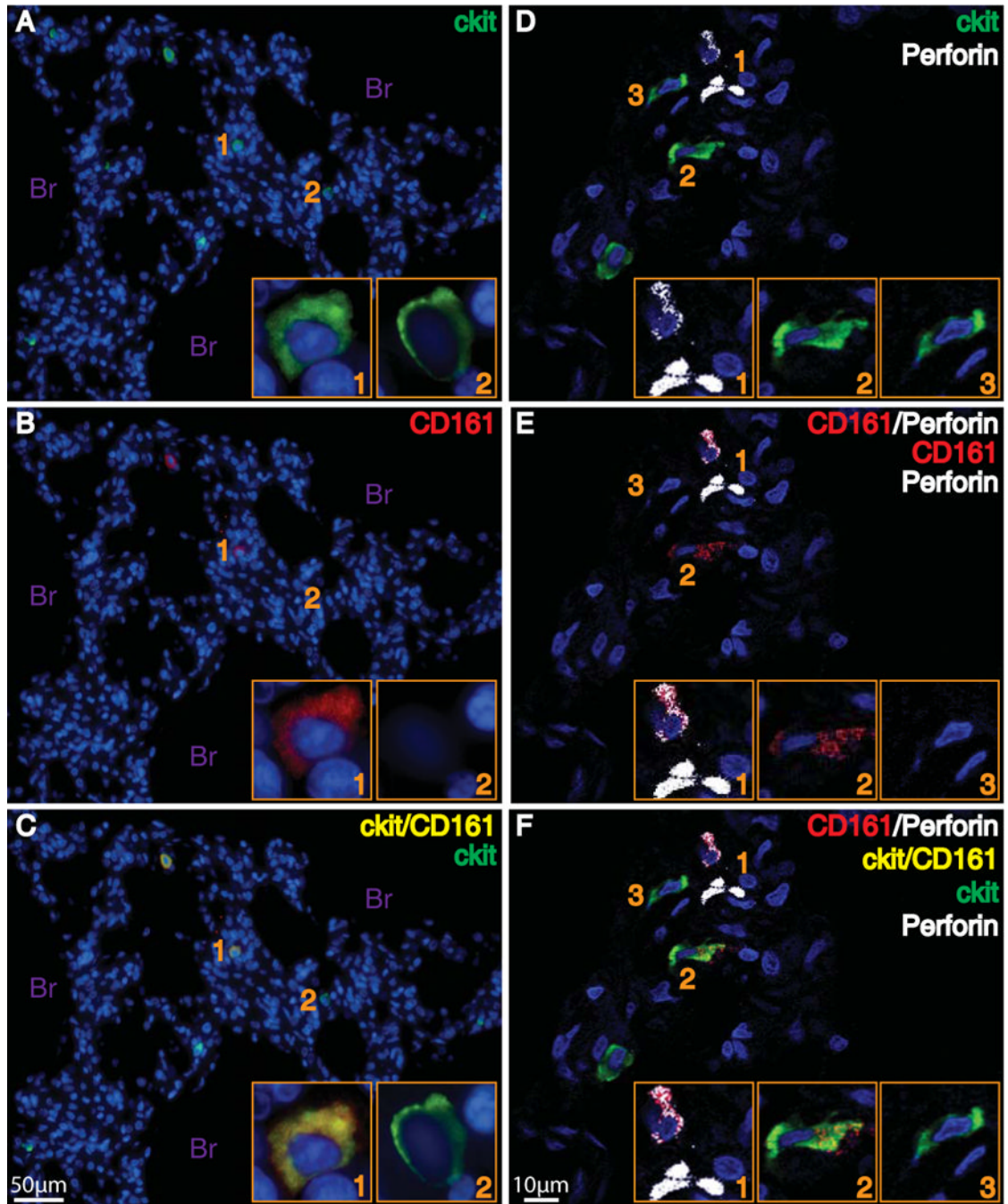


Fig. 6. Innate lymphoid cells are present in human lung

Immune cells were assessed in the distal lung using immunofluorescent staining with antibodies for cKit, CD161, and perforin to identify cells with the staining characteristics of ILC (cKit⁺, CD161⁺) and NK cells (cKit⁻, CD161⁺, perforin⁺). Cell nuclei were stained with DAPI (blue). Panels on the left show cells that stain positively for ckit (green, Panel A) and CD161 (red, Panel B). When images A and B are merged, cells stain for ckit and CD161 (yellow, Panel C) or ckit alone (green, Panel C). Light microscopy images taken with 20X objective, and 100X objective for inset. Panels on the right show cells that that stain positively for ckit (green, Panel D) or Perforin (white, Panel D). Panel E depicts cells that are positive for Perforin (white), CD161 (red), or both Perforin and CD161 (white and red).

When images D and E are merged, cells are present in Panel F that stains for Perforin and CD161 (white and red), Perforin alone (white), ckit alone (green), or ckit and CD161 (yellow). Confocal images taken with 60X objective. Inset images provide higher power views of the cells, and correspond numerically with the lower power images. Br, bronchioles

Table 1

Subject characteristics.

	Mild Asthma	Severe Asthma
No. of subjects	11	11
Clinical data		
Age (years)	30.6±10.2 (21–54)	41.5±11.8 (26–60) *
Gender (M:F)	3:8	2:9
Race (% white)	45	55
Ethnicity (% Hispanic)	9	18
Inhaled corticosteroids (Fluticasone equivalent, µg per day)	152.73±143.74 (0–360)	1085.46±303.06 (640–1760) *
Oral corticosteroids (%)	0	0
Long Acting Beta-Agonist (%)	0	100 *
Leukotriene Antagonists (%)	0	18 *
Omalizumab (%)	0	18 *
ACQ Score	1.18±0.58 (0.43–2.43)	2.52±0.64 (1.57–3.57) *
FEV1 (L)	3.25±0.39 (2.74–3.93)	2.14±0.57 (1.27–3.35) *
FEV1 (% predicted)	96.8±8.5 (82–106)	71.9±17.3 (45–101) *

Definition of abbreviations: ACQ = Asthma Control Questionnaire.

Results are expressed as mean ± SD (range)

* $P < 0.05$ when compared with subjects with mild asthma.