

ORIGINAL ARTICLE

Asthma and Lower Airway Disease

CCR10⁺ ILC2s with ILC1-like properties exhibit a protective function in severe allergic asthma

Chloé Beuraud¹ | Vincent Lombardi¹  | Sonia Luce¹ | Stéphane Horiot¹ | Emmanuel Naline² | Catherine Neukirch³ | Sabi Airouche¹ | Thibaut Perchet⁴ | Rachel Golub⁴ | Philippe Devillier²  | Sylvie Chollet-Martin⁵ | Véronique Baron-Bodo¹ | Emmanuel Nony¹ | Michel Aubier³ | Laurent Mascarell¹  | Philippe Moingeon¹

¹Research Department, Stallergenes Greer, Antony, France

²UPRES EA 220, Airway Disease Department, Foch Hospital, University Paris-Saclay, Suresnes, France

³Department of Pulmonary Medicine Bichat Hospital, Faculty of Medicine, Paris Diderot University, INSERM UMR1152, Paris, France

⁴Unit for Lymphopoiesis, Immunology Department, INSERM U1223, Institut Pasteur, Paris, France

⁵INSERM UMRS996, Paris-Sud University, Châtenay Malabry, France

Correspondence

Laurent Mascarell, Research Department, Stallergenes Greer, Antony, France.
Email: laurent.mascarell@stallergenesgreer.com

Present address

Philippe Moingeon, Research and Development, Servier, Suresnes, France.

Abstract

Background: We previously showed that patients with severe allergic asthma have high numbers of circulating ILC2s expressing CCR10.

Method: Herein, CCR10⁺ ILC2s were further analyzed in the blood of healthy individuals or patients with allergic and non-allergic asthma. Characteristics of human CCR10⁺ and CCR10⁻ ILC2s were assessed by flow cytometry as well as single-cell multiplex RT-qPCR. The role of CCR10⁺ ILC2s in asthma pathophysiology was studied in allergen-treated mice.

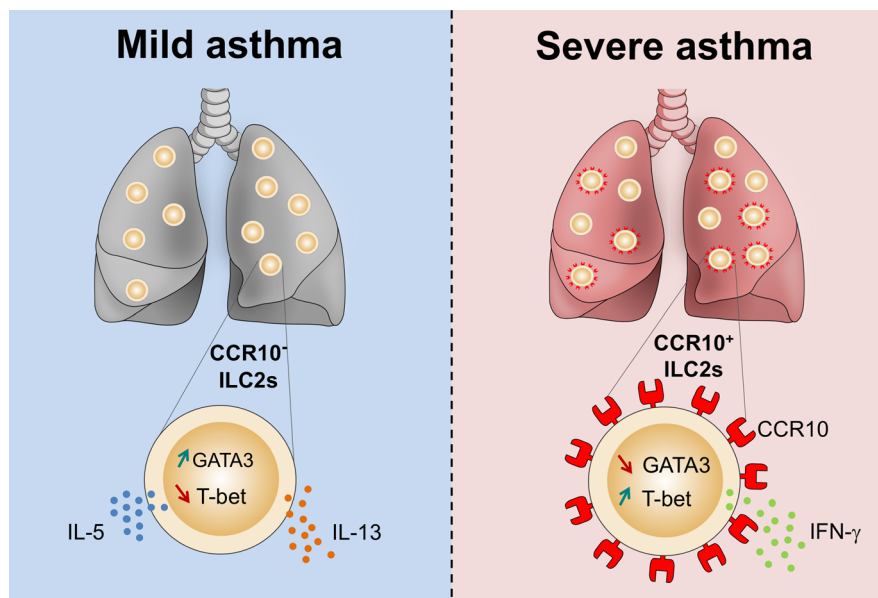
Results: When compared to healthy controls, CCR10⁺ ILC2s are enriched in the blood of both allergic and non-allergic severe asthmatic patients, and these cells are recruited to the lungs. Plasma concentrations of the CCR10 ligand CCL27 are significantly increased in severe asthmatics when compared to non-asthmatic patients. CCR10⁺ ILC2s secrete little T_H2 cytokines, but exhibit ILC1-like properties, including a capacity to produce IFN- γ . Also, single-cell analysis reveals that the CCR10⁺ ILC2 subset is enriched in cells expressing amphiregulin. CCR10⁺ ILC2 depletion, as well as blocking of IFN- γ activity, exacerbates airway hyperreactivity in allergen-challenged mice, providing evidence for a protective role of these cells in allergic inflammation.

Conclusions: Frequencies of circulating CCR10⁺ ILC2s and CCL27 plasma concentrations represent candidate markers of asthma severity. The characterization of CCR10⁺ ILC2s in human samples and in mouse asthma models suggests that these cells downregulate allergic inflammation through IFN- γ production.

1 | INTRODUCTION

Over the past few years, innate lymphoid cells (ILCs) have been identified as a new family of immune cells subdivided into three distinct populations, including ILC1s, ILC2s, and ILC3s, based on their similarities with T_H1, T_H2, and T_H17/T_H22 cells, respectively.¹ Growing evidence supports a role for ILC2s in the pathophysiology of allergic rhinitis and asthma. In this regard, we recently reported both quantitative and qualitative differences in the regulation of circulating human

ILCs between non-allergic and allergic individuals.¹ Specifically, we observed that frequencies of ILC2s expressing the CCR10 chemokine receptor are significantly increased in the blood of patients with severe allergic asthma. CCR10 has been previously shown to regulate the migration of T cells^{2,3} and ILCs⁴⁻⁶ to the skin, in both healthy subjects and patients with psoriasis or atopic dermatitis. In line with these observations, CCR10⁺ ILCs were shown to promote regulatory and effector T-cell homeostasis in the skin.⁶ As of today, however, the role of CCR10⁺ ILC2s in the pathophysiology of asthma is unknown.



GRAPHICAL ABSTRACT

In humans, CCR10 is highly expressed by blood ILC2s in severe asthmatic patients. CCR10⁺ ILC2s secrete little TH2 cytokines, but exhibit ILC1-like properties, including a capacity to produce IFN- γ . T-bet expression is increased in CCR10⁺ ILC2s when compared to CCR10⁻ ILC2s, whereas GATA3 expression is reduced.

In this context, we performed a detailed characterization of blood CCR10⁺ ILC2s in a large cohort of allergic and non-allergic asthmatic patients. Functional studies of this cell population were also performed in allergen-challenged mice. Collectively, our results establish that the frequency of CCR10⁺ ILC2s in the blood increases in relationship with asthma severity, irrespective of the allergic status of the patients and further, that these cells migrate to the lungs where they play a beneficial role in the control of allergic inflammation.

2 | METHODS

2.1 | Human samples

Asthma status was defined according to the GINA guidelines.⁷ Mild asthma status corresponds to GINA step 1 to 2 and severe asthma to GINA steps 4 to 5. The patients' allergic rhinitis status was determined according to the ARIA guidelines, and IgE sensitization was evaluated using ImmunoCAP assays for grass pollen, birch, ragweed, and house dust mite allergens (Thermo Fisher Scientific, Uppsala, Sweden). Blood samples were collected at the Bichat Claude Bernard Hospital (Paris, France) after approval of the studies protocols (study ref. #120147-30 and COBRA [Cohorte Obstruction BRonchique et Asthme] cohort⁸) by a local ethical committee. Specifically, blood from 24 non-allergic patients, including 14 patients exhibiting severe non-allergic asthma (GINA > 3), and 38 individuals with allergic rhinitis (AR) without ($n = 10$) or with mild (GINA ≤ 2 , $n = 16$) or severe asthma (GINA > 3, $n = 12$) was analyzed (Table S2). Treatment with inhaled corticosteroids was accepted for allergic patients as well as oral or inhaled corticosteroid therapies for asthmatic subjects.

Patients with immune disorders, or treated by anti-IgEs or allergen immunotherapy were excluded. Written informed consent was obtained from all participants of these studies.

Alternatively, buffy coats from allergen-sensitized donors (Etablissement Français du Sang, Rungis, France) were used for innate lymphoid cells (ILC) sorting and characterization. Allergen sensitization was determined using the Allergen kit (Beckman Coulter, Villepinte, France) as per manufacturer's instructions. Experiments with human tissues were approved by the regional independent ethics committee (Comité de Protection des Personnes Île de France VIII, Paris, France). Lung samples were obtained from 9 patients undergoing surgical resection at the Foch Hospital (Suresnes, France) for lung carcinoma who had not received preoperative anticancer chemotherapy.^{9,10} Lung lysates were obtained after digestion with collagenase D (Merck, Lyon, France) and filtration through a cell strainer prior to immediate flow cytometry analysis.

2.2 | Analysis and sorting of human ILC2s

For surface phenotyping, ILC2s were stained with anti-CD45, anti-CD127, anti-CD294 (CRTH2), anti-CCR10 antibodies and a lineage cocktail comprising anti-CD3, anti-CD11c, anti-CD14, anti-CD16, anti-CD20, anti-CD34, anti-CD56, anti-CD123, and anti-TCR α/β antibodies (Table S3 and Figure S1A). The expression of various surface markers was assessed by co-staining (see details in Table S3). The expression of GATA3, ROR γ t and T-bet transcription factors was monitored by flow cytometry using specific antibodies (Figure S1 and Table S3) and the Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's recommendations. Samples were analyzed using an Attune NxT (Thermo Fisher) apparatus, and

data interpreted using the FlowJo software (TreesStar, Ashland, OR, USA).

Lineage-positive cells were depleted using the Lineage Cell Depletion kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and an Auto-MACS Pro Separator apparatus following the manufacturer's instructions. CCR10⁺ or CCR10[−] ILC2s (Lineage[−] CD45⁺ CD127⁺ CRTH2⁺) were then sorted using a FACSaria III (BD Biosciences) apparatus (Figure S1B). To assess the cytokine production of these cells, 3000 CCR10⁺ or CCR10[−] ILCs were cultivated for 5 days in presence of IL-2 and IL-7 (20 ng/ml, R&D Systems, Lille, France). T_H2 cytokine production was measured in the culture supernatant by ELISA while IFN- γ secretion was evaluated by intracellular staining of cytokine by flow cytometry after PMA and ionomycin restimulation. Alternatively, single-cell multiplex RT-quantitative PCR (qPCR) was performed to compare the expression of genes (Table S1) between CCR10⁺ and CCR10[−] ILC2s. We used a Fluidigm BioMark microfluidics-based RT-qPCR system (Les Ulis, France) as previously described.¹¹

2.3 | Analysis of ILC2s in mice

BALB/c female mice (8-week-old) were obtained from Charles River (L'Arbresle, France). The experimental protocol was approved by Stallergenes Greer's (Antony, France) ethical committee. To analyze CCR10⁺ ILC2s in the lungs, mice were challenged with either 4 daily intranasal administrations of a birch pollen extract (Stallergenes Greer) equivalent to 250 μ g of the birch pollen major allergen Bet v 1 per dose (Figure S5). Alternatively, to induce airway hyperreactivity, mice received 6 intranasal administrations of a birch pollen extract equivalent to 500 μ g Bet v 1 per dose (Figure S5). Measurements of airway hyperreactivity were performed by whole-body plethysmography (Buxco, Winchester, UK) after exposure to increasing doses (ie, 0, 6.125, 12.5, 25, and 50 mg/mL) of methacholine (Merck).¹² For blocking antibody experiments, 50 μ g of either anti-CCL27 (clone 68623), anti-CCL28 (clone 134306), anti-IFN- γ (clone XMG1.2) antibodies, or an IgG2a control isotype (Clone 141945, all from R&D Systems) were injected intraperitoneally 2 hours before intranasal challenges. Lung homogenates were subsequently stained with anti-CCR10, anti-CD25, anti-CD45, anti-CD127, anti-ICOS, and anti-ST2 antibodies as well as a lineage cocktail comprising both anti-B220, anti-CD3e, anti-CD11b, anti-CD11c, anti-CD19, anti-CD45R, anti-Ly6C, anti-Ly6G, anti-NK1.1, and anti-Ter-119 antibodies (Table S3). IFN- γ production by mouse ILC2s was assessed with a specific antibody (Table S3) by intracellular staining using the same protocol as for human PBMCs.

2.4 | Measurement of immune mediators in the blood

CCL2 (MCP-1), CCL11 (eotaxin-1), CCL27, CCL28, and osteopontin concentrations were determined using specific ELISA (R&D Systems or R&D Systems and Novus Biologicals for CCL28). CCL28 and YKL-40 were quantified by a human magnetic Luminex screening assay (R&D Systems).

3 | RESULTS

3.1 | Frequencies of CCR10⁺ ILC2s are elevated in the blood of severe asthmatic patients, irrespective of their allergic status

To better understand the role of CCR10⁺ ILC2s in asthma, these cells were quantified in PBMCs obtained from a cohort including 24 non-allergic individuals (out of which 14 had non-allergic severe asthma [GINA>3]), as well as 38 patients with allergic rhinitis (AR) either without ($n = 10$) or with mild (GINA ≤ 3 , $n = 16$) or severe asthma (GINA>3, $n = 12$). As described earlier,^{1,13} increased frequencies of circulating ILC2s (Lineage[−] CD45⁺ CD127⁺ CRTH2⁺ cells) were observed in asthmatic patients compared to non-asthmatic subjects. Moreover, the frequencies of CCR10⁺ cells among ILC2s were dramatically augmented in blood samples from allergic patients with severe asthma, when compared to non-allergic non-asthmatic patients (with average percentages of positive cells of 24.9 and 5.4%, respectively, $P < 0.0001$) (Figure 1A,B). Surprisingly, non-allergic patients with asthma also exhibited higher frequencies of CCR10⁺ cells among blood ILC2s (average percentages of 19.5%) compared to non-asthmatic non-allergic individuals (Figure 1A,B). These results demonstrate that the frequency of CCR10⁺ ILC2s is linked to asthma severity, irrespective of the allergic status of the patients. Since CCR10 has been mainly described as a skin homing receptor,¹⁴ we then investigated whether CCR10⁺ ILC2s could also be found in human lungs. Significant numbers of ILC2s were found in healthy parts of lung parenchyma (retrieved from patients with adenocarcinoma), with up to 40% of those cells expressing CCR10 (Figure 1C). Altogether, these results suggest that CCR10⁺ ILC2s may play a role in the pathophysiology of severe asthma.

3.2 | CCL27 plasma concentrations are correlated to frequencies of circulating CCR10⁺ ILC2s and reflect asthma severity

To confirm the link between CCR10⁺ ILC2s and asthma severity, we measured plasma concentrations of CCR10 ligands, that is, CCL27 and CCL28, in the same cohort of healthy and asthmatic patients. Whereas we failed to detect any CCL28 in plasma (despite using three different commercial ELISA or Luminex assays with a detection threshold of 8 pg/mL), CCL27 levels were significantly correlated ($P = 0.0097$) to the expression of CCR10 by ILC2s (Figure 2A). Consequently, CCL27 plasma concentrations were higher in allergic patients with severe asthma, when compared to healthy individuals. Similarly, non-allergic asthmatic patients had significantly higher CCL27 levels than non-asthmatic controls (Figure 2B). In this cohort of 62 subjects, CCL27 plasma concentrations also correlated with levels of several markers associated with asthma severity,^{15–19} including eotaxin-1 ($P = 0.045$), MCP-1 ($P = 0.0067$), osteopontin ($P = 0.016$), and YKL-40 ($P = 0.0024$) (Figure 2C). Noteworthy, we did not observe significant correlations between CCL27 concentration and periostin or ECP concentrations (data not shown, ie, two other known asthma-related

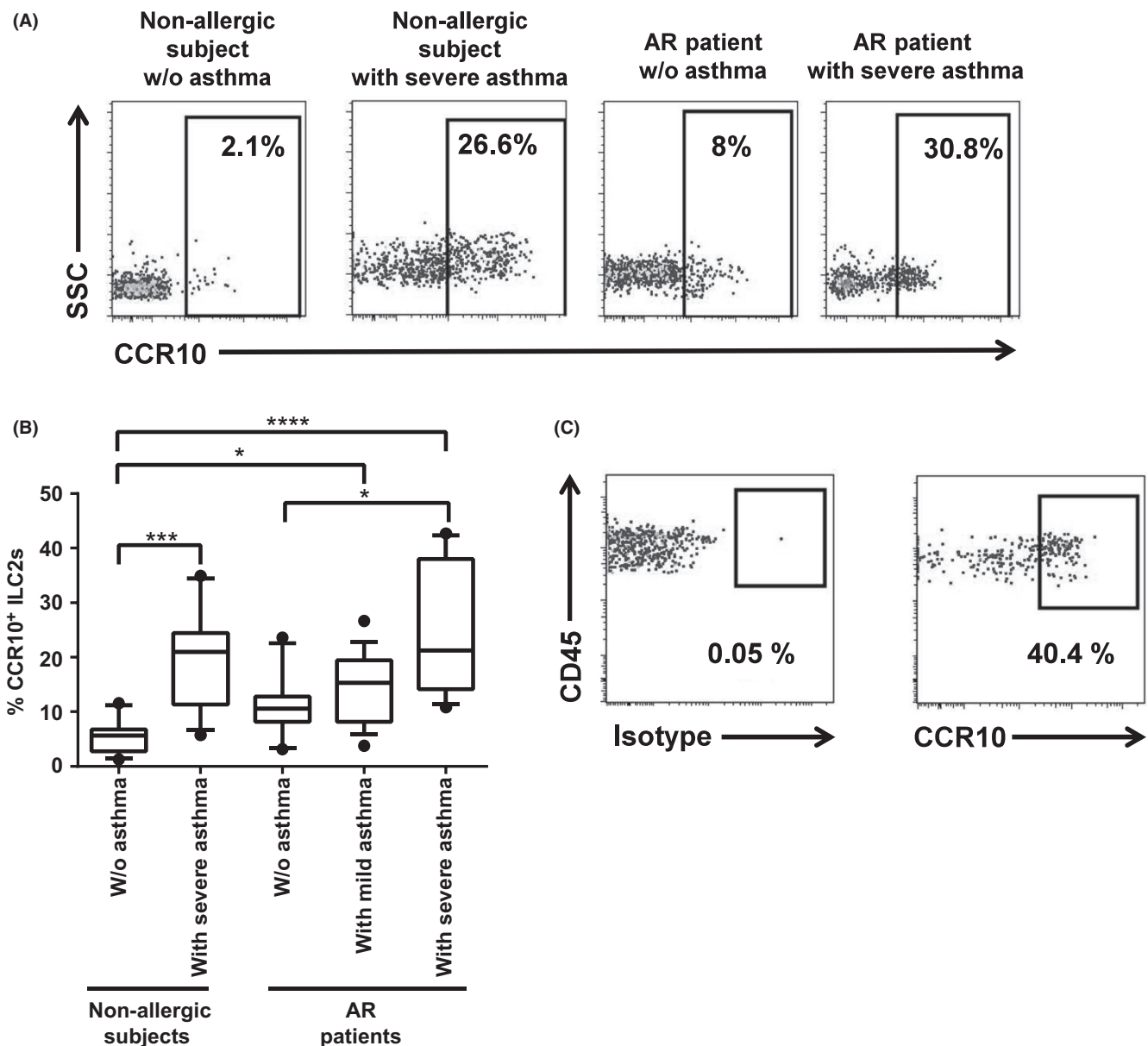


FIGURE 1 CCR10 expression by circulating ILC2s is increased in asthmatic patients in relationship with disease severity. CCR10 expression by ILC2s (Lin^- , CD45^+ , CD127^+ , CRTH2^+) was assessed by flow cytometry in PBMCs from non-allergic subjects without (w/o, $n = 10$) or with severe asthma ($n = 14$), allergic rhinitis patients without (AR, $n = 10$) or with concomitant mild ($n = 16$) or severe ($n = 12$) asthma. A, Representative dot plots of CCR10 expression by ILC2s are shown. B, Percentages of CCR10 expression by ILC2s are represented as box plots and compared using Kruskal-Wallis test and Dunn's multiple comparisons, * $P < 0.05$, *** $P < 0.001$ and **** $P < 0.0001$. C, CCR10 expression by ILC2s was assessed in lung cells from 9 patients. Representative dot plots are shown

markers²⁰). Altogether, both CCR10^+ ILC2 frequencies and CCL27 concentrations in the blood reflect asthma severity.

3.3 | Blockade of CCR10^+ ILC2 recruitment into the lungs enhances airway hyperreactivity in allergen-treated mice

We then assessed the presence of CCR10^+ ILC2s in the lungs of mice following intranasal challenges performed on 4 consecutive days with a birch pollen extract. In agreement with other studies,²¹ allergen challenges elicited a fivefold increase in total numbers of pulmonary ILC2s

(Lineage^- CD45^+ CD127^+ CD25^+ ST2^+ ICOS^+) at day 5 (Figure 3A). More than 20% of these cells in asthmatic animals express CCR10, whereas in contrast, this marker is not expressed by pulmonary ILC2s isolated from naive mice (Figure 3B,C). To investigate the potential contribution of CCR10^+ ILC2s to airway inflammation following pollen challenge, antibodies specific for CCL27 or CCL28 were administered before each of 6 birch pollen extract challenges to prevent the engagement of CCR10. Intraperitoneal injections of a combination of anti-CCL27 and anti-CCL28 antibodies completely abolished the presence of CCR10^+ ILC2s in the lungs (Figure 3D) with no significant impact on CCR10^- ILC2 frequencies (Figure S1A). Of note, frequencies of T

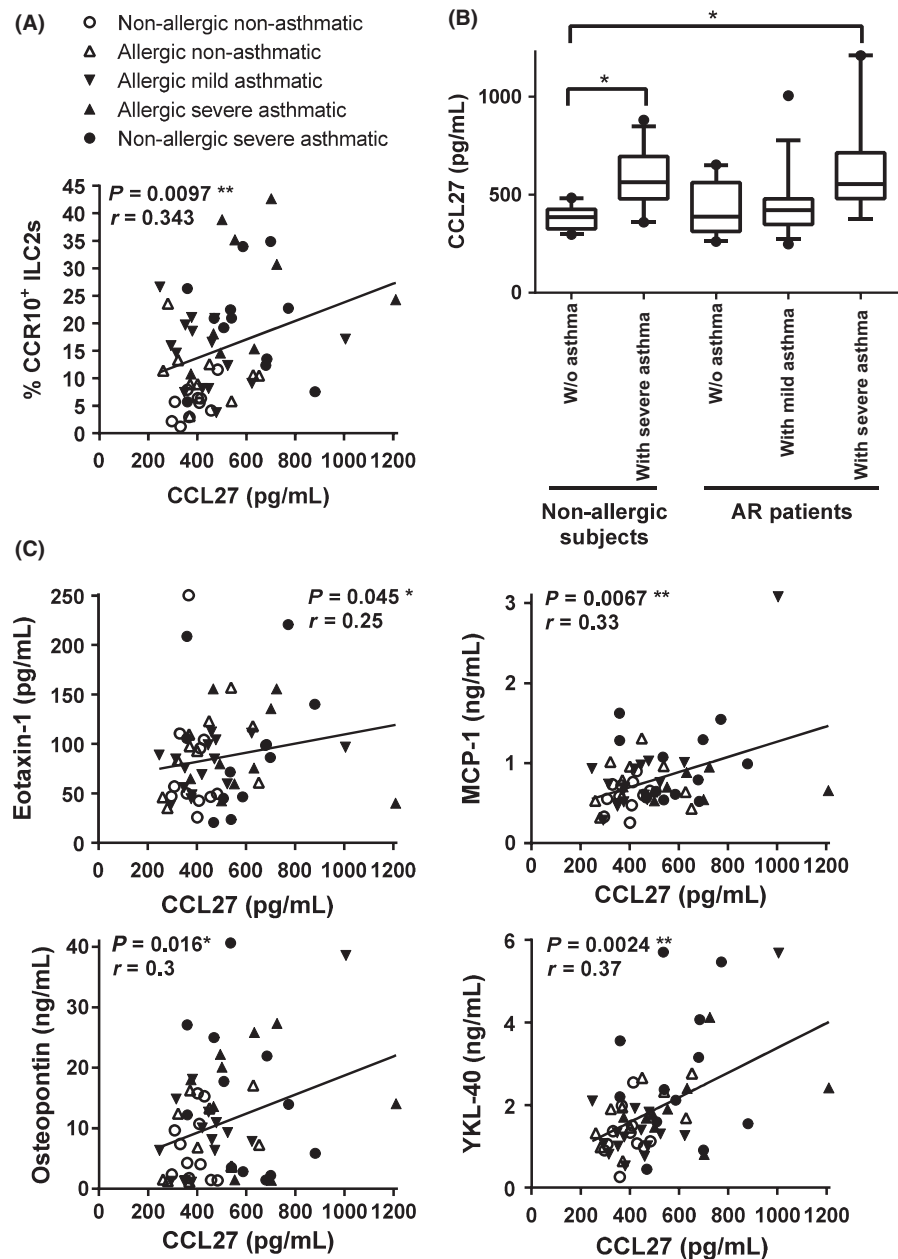


FIGURE 2 Plasma CCL27 concentrations in patients are correlated to CCR10 expression by ILC2s and asthma severity. A, Spearman correlation between CCR10 expression by blood ILC2s and plasma CCL27 concentration in 10 non-allergic subjects without asthma, 12 non-allergic subjects with severe asthma, 10 allergic patients without asthma, 15 allergic patients with mild asthma, and 9 with severe asthma. B, CCL27 plasma concentrations of the same group of patients are represented using box plots and compared with the Kruskal-Wallis test and Dunn's multiple comparison test. C, Spearman correlations between plasma CCL27 concentration and eotaxin-1, MCP-1, osteopontin, and YKL-40 concentrations in plasma samples from 56 asthmatic or non-asthmatic subjects, $*P < 0.05$, $**P < 0.01$

cells, B cells, eosinophils, neutrophils, monocytes, or macrophages were not altered in this experiment (data not shown). Intriguingly, blocking CCL27 and CCL28 in birch pollen-challenged mice resulted in a significant exacerbation of the airway hyperreactivity, when compared to mice treated with either PBS (Figure 3E) or isotype controls (Figure S1B,C). Collectively, these data suggest that CCR10⁺ ILC2s are recruited to the lungs and may help to reduce allergen-induced airway inflammation.

3.4 | CCR10⁺ ILC2s from allergen-sensitized subjects exhibit ILC1-like properties

To further investigate how CCR10⁺ ILC2s could downregulate airway inflammation, we first studied the surface expression of a panel of chemokine receptors and activation markers by these cells.

Interestingly, the expression of several markers related to cell migration was markedly different between circulating CCR10⁺ and CCR10⁻ ILC2s obtained from allergen-sensitized subjects. CCR10⁺ ILC2s express higher levels of CCR8 and CLA but exhibit a lower expression of CD49d compared to their negative counterparts (Figure 4A). Also, the expression of the inhibitory molecules CTLA-4 and PD-L1 is higher in CCR10⁺ ILC2s, while the activation markers CD25 or KLRG1 are rather down-regulated at the surface of these cells compared to CCR10⁻ ILC2s (Figure 4A). Together, these phenotypic differences reveal a low activation status for CCR10⁺ ILC2s and raise the hypothesis of potentially distinct migratory capacities for this cell population relative to CCR10⁻ ILC2s. Noteworthy, no significant differences in the expression of other markers (ie, CCR3, CCR4, CCR5, CCR6, CCR7, CXCR3, CD40, GITR, ICOS, ICOS-L, LAP, and TGF- β R) were observed between CCR10⁺ and CCR10⁻ ILC2s (Figure S2).

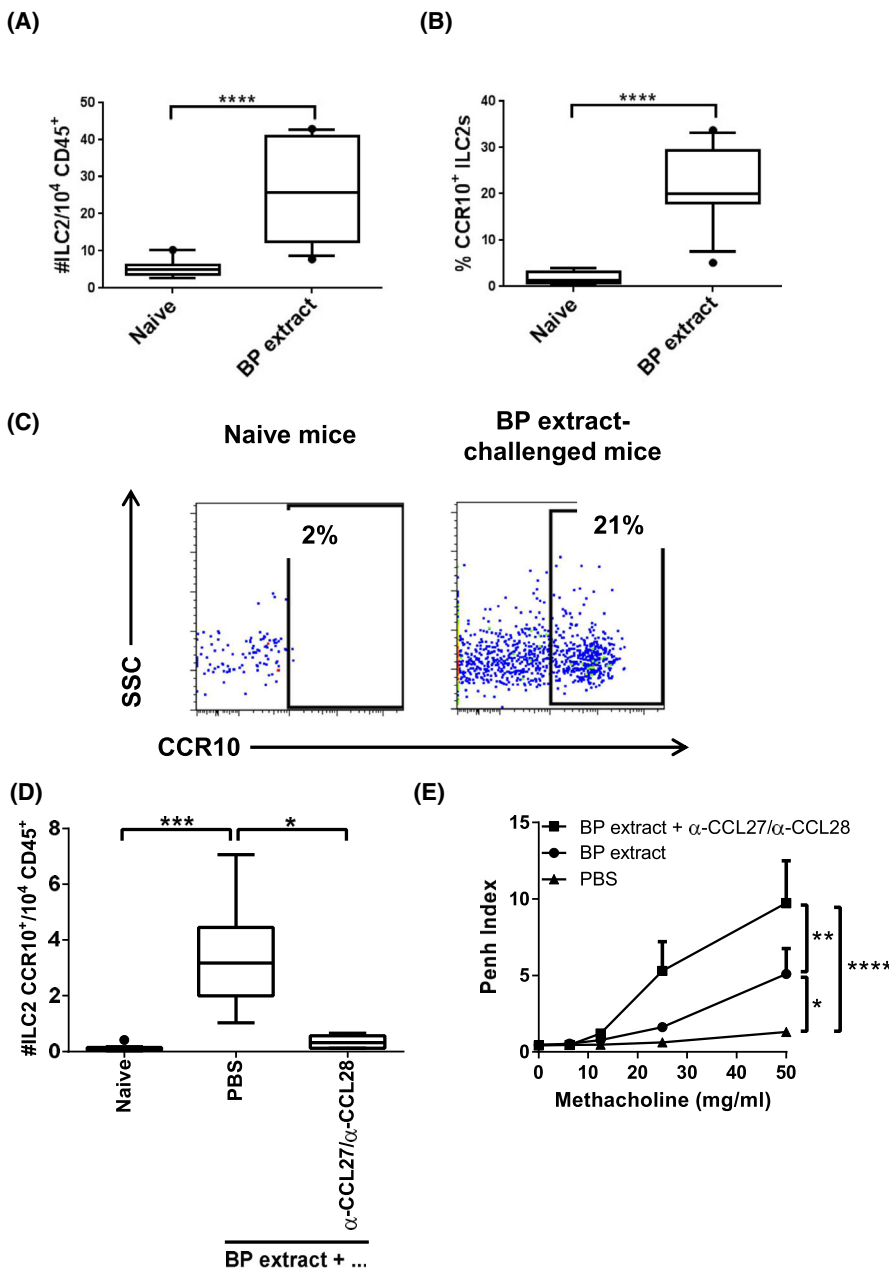


FIGURE 3 Anti-CCL27 and anti-CCL28 antibodies inhibit the recruitment of CCR10⁺ ILC2s in the lungs of allergen-challenged mice. Mice were challenged with a birch pollen (BP) extract intranasally on 4 consecutive days. (A) Frequencies of ILC2s (Lin⁻ CD45⁺ CD127⁺ ST2⁺ CD25⁺ ICOS⁺ cells) among 10⁴ CD45⁺ cells from the lungs and (B) percentages of CCR10 expression by lung ILC2s in naive and BP-challenged mice assessed by flow cytometry. Results are represented as box plots and compared using Mann-Whitney tests ($n = 9$ for naive mice and $n = 12$ for challenged ones). (C) Representative flow cytometry dot plots of CCR10 expression by ILC2s in mouse lungs. (D) Analysis by flow cytometry of CCR10⁺ ILC2s in lungs of birch pollen-challenged mice treated with anti-CCL27 and anti-CCL28 antibodies. Box plots representing CCR10⁺ ILC2 frequencies among 10⁴ CD45⁺ cells and percentages of CCR10 expression are shown. Kruskal-Wallis tests were used for statistical analyses. (E) Airway hyperreactivity was assessed in birch pollen-challenged mice treated or not with anti-CCL27 and anti-CCL28 by whole-body plethysmography in response to increasing methacholine doses and expressed as Penh index values, represented as mean $\pm$ SEM. Two-way ANOVA was used for statistical analysis ($n = 12$ per group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ [Colour figure can be viewed at wileyonlinelibrary.com]

We then evaluated the expression of the transcription factors GATA3, ROR γ t and T-bet by flow cytometry in human circulating CCR10⁺ and CCR10⁻ ILC2s. T-bet expression was increased in CCR10⁺ ILC2s when compared to CCR10⁻ ILC2s, whereas GATA3 expression was rather reduced (Figure 4B). ROR γ t expression was not significantly different between CCR10⁺ and CCR10⁻ ILC2s (Figure S4B). When assessing the production of cytokines following cell sorting (Figure S1B) and in vitro culture, we observed that CCR10⁺ ILC2s produce very little IL-5 and IL-13 compared to CCR10⁻ ILC2s (Figure 4C). Following PMA/ionomycin restimulation, the percentages of circulating ILC2s producing IFN- γ , assessed by flow cytometry, were significantly higher ($P = 0.0026$) in the CCR10⁺ subset (Figure 4D).

In further experiments, the expression of selected genes (Table S1) was analyzed by single-cell multiplex RT-qPCR in sorted CCR10⁺ and CCR10⁻ ILC2s from allergen-sensitized patients. We

confirmed that the expression of chemokine receptors was differentially regulated between the two subsets (Table 1). Specifically, while CCR3 is the most abundant chemokine receptor in both subsets, CCR6, CCR4, and CCR2 are expressed by more than half of ILC2s from the CCR10⁻ subset. In contrast, CCR2 is expressed by 38% and CCR6 and CCR4 by only one-third of CCR10⁺ ILC2s. Interestingly, cells expressing the amphiregulin (a member of the epidermal growth factor family²²) gene AREG are more frequent within the CCR10⁺ subset (Table 1). In contrast, AHR and ID2 genes (two transcriptional regulators important for the development of ILCs^{23,24}) are little expressed by CCR10⁺ ILC2s. Furthermore, the gene coding for the interferon alpha and beta receptor subunit 1 (IFNAR1) is exclusively expressed by ILC2s belonging to the CCR2⁻ subset. Multiplex RT-qPCR performed on 50 cells from each of CCR10⁺ and CCR10⁻ subsets confirmed that differences in gene expression observed at the

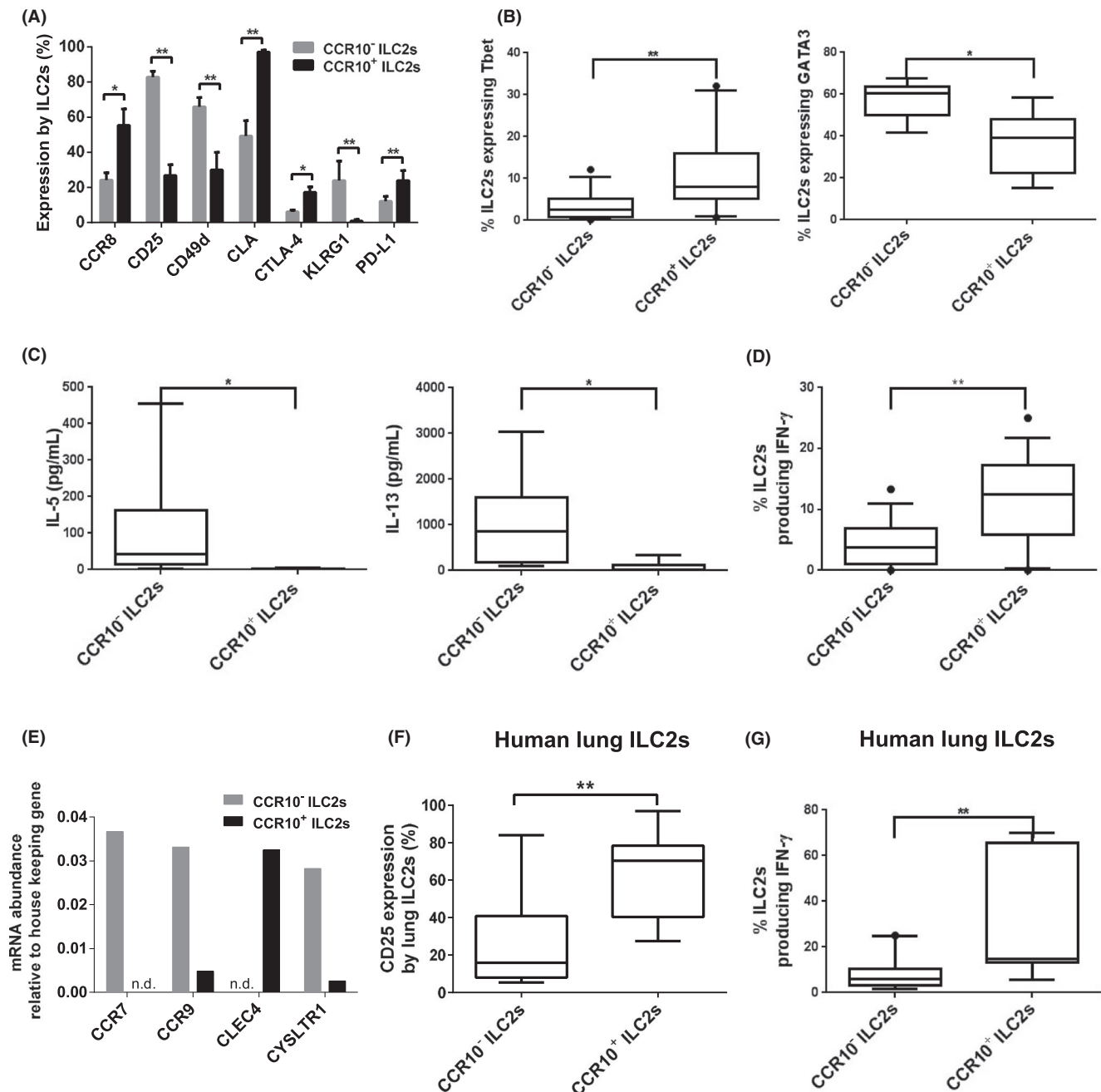


FIGURE 4 CCR10⁺ ILC2s from allergen-sensitized subjects exhibit an ILC1-like profile. (A) Expression of CCR8, CD25, CD49d, CLA, CTLA4, KLRG1, and PD-L1 on CCR10⁺ or CCR10⁻ ILC2s was assessed by flow cytometry in PBMCs from eight allergen-sensitized donors. (B) Tbet and GATA3 expression by human blood CCR10⁺ and CCR10⁻ ILC2s was determined by intranuclear staining ($n = 6$). (C) Human CCR10⁺ and CCR10⁻ ILC2s sorted from PBMCs were cultivated for 5 days with IL-2 and IL-7. IL-5 and IL-13 concentrations were assessed in culture supernatants ($n = 6$). (D) IFN- γ production by human ILC2s from PBMCs was measured by intracytoplasmic staining of cytokines following PMA/ionomycin restimulation ($n = 15$). (E) mRNA levels relative to GAPDH assessed by multiplex RT-qPCR in 50 CCR10⁻ ILC2s and 50 CCR10⁺ ILC2s from allergen-sensitized subjects (n.d.: not detected). (F) Expression of CD25 and (G) production of IFN- γ by CCR10⁺ and CCR10⁻ ILC2s from human lungs was measured by flow cytometry ($n = 9$). Data are expressed as mean $\pm$ SEM or box plots, and Wilcoxon tests were used to compare CCR10⁺ from CCR10⁻ ILC2s. * $P < 0.05$ and ** $P < 0.01$

single-cell level are representative of global subset compositions, with, for example, evidence that the C-type lectin *CLEC4* gene is not expressed by CCR10⁺ ILC2s, whereas conversely *CCR7*, *CCR9*, and *CYSLTR1* (cysteinyl leukotriene receptor 1) genes are preferentially expressed by the CCR10⁻ subset (Figure 4E). These results (Table 1

and Figure 4E) unambiguously highlight the significant phenotypic differences between CCR10⁺ and CCR10⁻ ILC2s, suggesting dramatically distinct functional properties for those two subsets.

We subsequently compared the phenotype and function of CCR10⁺ and CCR10⁻ ILC2s from human lungs using post-surgical

TABLE 1 Single-cell multiplexed RT-qPCR of CCR10⁺ and CCR10⁻ ILC2s

	CCR10 ⁻ ILC2s	CCR10 ⁺ ILC2s
AHR	78	50
AREG	11	25
CCR2	56	38
CCR3	89	63
CCR4	56	25
CCR6	67	25
ID2	44	13
IFNAR1	22	0

Gene expression was quantified by multiplex RT-qPCR in blood CCR10⁺ and CCR10⁻ ILC2s at the single-cell level using microfluidic chips. The frequency of gene expression is calculated by taking into account the expression/non-expression by individual single ILC2 from CCR10⁺ or CCR10⁻ subsets.

tissues obtained from cancer patients. CCR10⁺ ILC2s in the lungs exhibit a slightly different phenotype than the ones in the blood. Specifically, 64% of CCR10⁺ ILC2s in the lungs express the activation marker CD25, compared to only 26% of those cells in the blood (Figure 4F, to be compared to Figure 4A). Nonetheless, lung CCR10⁺ ILC2s still display ILC1-like properties with even higher levels of IFN- γ produced when compared to CCR10⁻ ILC2s (Figure 4G). Altogether, these results suggest that CCR10⁺ ILC2s exhibiting ILC1-like features are recruited to the lungs.

3.5 | IFN- γ production by CCR10⁺ ILC2s contributes to the control of airway hyperresponsiveness in allergen-challenged mice

We confirmed that, as for their human counterparts, lung CCR10⁺ ILC2s from mice challenged with a birch pollen extract produce higher levels of IFN- γ compared to the CCR10⁻ subset, following PMA and

ionomycin restimulation (Figure 5A). To determine whether the production of IFN- γ contributes to the beneficial role of CCR10⁺ ILC2s on airway hyperreactivity (Figure 3E), we blocked the biological activity of this cytokine using an anti-IFN- γ antibody administered to mice before their exposure to the birch pollen extract. The airway hyperreactivity was significantly increased in mice treated with anti-IFN- γ compared to untreated mice challenged with the birch pollen extract only (Figure 5B), confirming that IFN- γ produced by cells such as CCR10⁺ ILC2s have a beneficial effect on lung inflammation in allergen-challenged mice.

4 | DISCUSSION

The role of recently described ILCs, and more particularly of the ILC2 subset, in the pathophysiology of allergic diseases remains to be fully documented. Murine models have confirmed that ILC2s represent a major source of type 2 cytokines in the lungs^{21,25} and, as such, can promote airway inflammation and asthma.²⁶ In humans, indirect evidence supports a contribution of ILC2s to allergen-driven inflammation, with the observation that such cells are overrepresented in the blood, sputum, and BALs of asthmatic patients.^{13,27,28} However, ILC2s are also present in the blood of non-allergic individuals, although with phenotypic and functional differences from their counterparts in allergic individuals. In particular, we recently reported that blood ILC2s from allergic asthmatic patients upregulate CCR10 expression in comparison with ILC2s from healthy subjects.¹ In this context, we investigated in the present study the role of CCR10⁺ ILC2s in asthma.

Herein, we confirmed and extended our previous observation that CCR10 expression by circulating ILC2s is significantly increased in asthmatic subjects in relationship with disease severity. Surprisingly, frequencies of circulating CCR10⁺ ILC2s are also increased in non-allergic asthmatic patients, although additional investigations

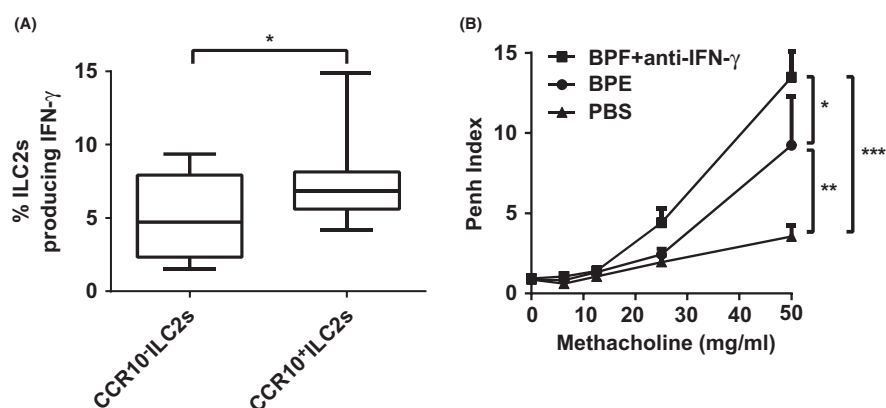


FIGURE 5 IFN- γ -producing ILC2s contribute to the control of airway hyperreactivity in allergen-challenged mice. A, Production of IFN- γ by CCR10⁺ and CCR10⁻ ILC2s from lungs of birch pollen-treated mice assessed by flow cytometry following PMA/ionomycin restimulation (n = 6). B, In response to increasing doses of methacholine, airway hyperreactivity was assessed by whole-body plethysmography in birch pollen-challenged mice treated or not with anti-IFN- γ prior to allergen challenges. Results are expressed as Penh index and represented as mean $\pm$ SEM (n = 12 per group). Wilcoxon tests or 2-way ANOVA was used for statistical analysis. *P < 0.05, **P < 0.01 and ****P < 0.0001

will be needed to assess whether these cells exhibit similar or distinct functional properties in the latter condition. We demonstrated as well that CCR10⁺ ILC2s could be recruited to the lungs, based on the observations that (a) significant numbers of these cells are found in human lungs and (b) intranasal birch pollen extract challenges in mice rapidly result in increased numbers of CCR10⁺ ILC2s in the lungs. This dramatic increase (up to 50-fold, Figure 3B) of CCR10⁺ ILC2 numbers in the lungs of animals challenged with a birch pollen extract on 4 consecutive days suggests that these cells are mostly recruited from the blood. Our gene expression analyses confirmed that CCR10⁺ and CCR10⁻ ILC2s exhibit different chemokine receptors. However, in light of the complete abrogation of ILC2 recruitment in mice by anti-CCL27 and anti-CCL28 antibodies, we conclude that CCR10 plays a major role in the migration of ILC2s to the lungs in response to these two chemokines. Surprisingly, inhibiting the recruitment of CCR10⁺ ILC2s into the lungs results in a higher airway hyperreactivity, leading to the hypothesis that CCR10⁺ ILC2s mediate a beneficial role in downregulating airway inflammation.

Although the pro-inflammatory function of ILC2s is well documented, there is growing evidence that such cells can mediate as well a positive role in health and disease, for example, by contributing to helminth clearance,²⁹ maintaining mucosal integrity³⁰ or promoting adipose tissue browning.³¹ To further understand the functional properties of CCR10⁺ ILC2s, we studied these cells in both the blood and lungs of patients. Blood CCR10⁺ ILC2 cells are less activated and express higher levels of co-inhibitory molecules (ie, CTLA-4 and PD-L1) than their CCR10⁻ counterparts. Interestingly, skin CCR10⁺ ILCs were also shown by others to express PD-L1 and to promote Treg induction and homeostasis.⁶ Consistent with this, our single-cell multiplex RT-qPCR analysis revealed that the CCR10⁺ ILC2 subset is enriched in cells expressing the amphiregulin gene, a protein able to promote tissue repair and regulatory T-cell function.²² The single-cell multiplex RT-qPCR also revealed that the *IFNAR1* gene, which encodes a subunit of a receptor for type I interferons, is exclusively expressed by the CCR10⁻ subset. Thus, in light of two recent reports that type I interferons regulate cytokine production by mouse and human ILC2s^{32,33} differences in response to type I interferons could possibly lead to distinct functional programs in CCR10⁻ and CCR10⁺ ILC2s.

Although the existence of a regulatory subset of ILCs (ILCregs) secreting IL-10 has been recently reported,^{34,35} we failed to detect any significant IL-10 production by circulating CCR10⁺ ILC2s (data not shown). The latter rather exhibit ILC1-like properties, including a high T-bet expression, a capacity to produce IFN- γ , but only very little T_H2 cytokines. These results are in agreement with the observation made by others that lung ILC2s expressing T-bet are present in the airways in a murine model of eosinophilic airway inflammation induced by IL-33.³⁶ Using antibody inhibition, we confirmed in our murine model that IFN- γ directly contributes to the control of lung inflammation in allergen-challenged mice. Although CCR10⁺ ILC2s may not be the only source of IFN- γ in this model, our allergic asthma protocol relying upon 6 daily administrations of birch pollen

extract likely involves predominantly ILCs. Noteworthy, we did not observe any impact of CCR10⁺ ILC2 recruitment on eosinophil and neutrophil infiltrates, nor on T_H2 cytokine production. Thus, the exact mechanism of action by which CCR10⁺ ILC2s reduce allergic inflammation remains to be clarified. Nonetheless, the hypothesis that T-bet⁺ IFN- γ -producing-CCR10⁺ ILC2s have a protective role in asthma is consistent with the known antagonistic effect of IFN- γ against allergic inflammation^{37,38} as well as with recent evidence that lung inflammation is exacerbated in T-bet^{-/-} mice.³⁶ Several reports have documented the plasticity of ILC2s, based on their capacity to acquire an ILC1³⁹⁻⁴¹ or ILC3⁴² phenotype. Given their T-bet expression and low expression of GATA3 and ROR γ t, CCR10⁺ ILC2s could represent a subset of ILC2s prone to differentiate into ILC1s. The presence of higher numbers of such CCR10⁺ ILC2s in the blood of severe asthmatic individuals raises the possibility that these cells are expanded as a counter-regulatory mechanism in the context of severe inflammation.

We also established that plasma concentrations of the CCR10 ligand, CCL27, are closely related to the expression of CCR10 by blood ILC2s, thus representing a candidate marker of asthma severity. In support of this, CCL27 concentrations in the blood are correlated as well to various markers of T_H2 response (Eotaxin-1)⁴³ and/or severe asthma (YKL-40).¹⁹ Together with previous reports documenting that CCL27 blood levels are higher in patients with either poorly controlled asthma,²⁰ psoriasis, or atopic dermatitis,⁴⁴⁻⁴⁷ this suggests that this chemokine is upregulated in association with various chronic inflammatory conditions.

Overall, the present study suggests a beneficial role of ILC1-like CCR10⁺ ILC2s in controlling allergic asthma inflammation. In addition, frequencies of circulating CCR10⁺ ILC2s as well as CCL27 plasma levels appear to represent new candidate biomarkers for asthma severity, to be validated in larger cohorts of patients. Future studies should assess as to whether CCL27, alone or in combination with other markers, can improve the stratification and treatment of severe asthmatic patients. We propose that therapies aiming at recruiting CCR10⁺ ILC2s in the lungs should be considered in the management of severe inflammatory conditions of the airways.

CONFLICT OF INTEREST

Several co-authors (CB, EN, LM, PM, SA, SH, SL, VB-B, VL) are or have been employees at Stallergenes Greer. Other authors have no conflict of interest in relation to this study.

AUTHORS' CONTRIBUTION

CB and VL designed and performed experiments and contributed to the preparation of the manuscript; SL, SH, SA, and TP performed experiments; ENa, CN, PD, SCM, and MA screened the patients and provided clinical samples; VB-B, ENo, LM, and PM contributed to the design of the study and to the preparation of the manuscript.

ORCID

Vincent Lombardi  <https://orcid.org/0000-0002-5748-9383>

Philippe Devillier  <https://orcid.org/0000-0001-6054-1886>

Laurent Mascarell  <https://orcid.org/0000-0001-7199-0345>

REFERENCES

- Lombardi V, Beuraud C, Neukirch C, et al. Circulating innate lymphoid cells are differentially regulated in allergic and nonallergic subjects. *J Allergy Clin Immunol*. 2016;138:305-308.
- Morales J, Homey B, Vicari AP, et al. CTACK, a skin-associated chemokine that preferentially attracts skin-homing memory T cells. *Proc Natl Acad Sci USA*. 1999;96:14470-14475.
- Homey B, Alenius H, Muller A, et al. CCL27-CCR10 interactions regulate T cell-mediated skin inflammation. *Nat Med*. 2002;8:157-165.
- Salimi M, Barlow JL, Saunders SP, et al. A role for IL-25 and IL-33-driven type-2 innate lymphoid cells in atopic dermatitis. *J Exp Med*. 2013;210:2939-2950.
- Teunissen MB, Munneke JM, Bernink JH, et al. Composition of innate lymphoid cell subsets in the human skin: enrichment of NCR (+) ILC3 in lesional skin and blood of psoriasis patients. *J Invest Dermatol*. 2014;134:2351-2360.
- Yang J, Hu S, Zhao L, Kaplan DH, Perdew GH, Xiong N. Selective programming of CCR10(+) innate lymphoid cells in skin-draining lymph nodes for cutaneous homeostatic regulation. *Nat Immunol*. 2016;17:48-56.
- Bateman ED, Hurd SS, Barnes PJ, et al. Global strategy for asthma management and prevention: GINA executive summary. *Eur Respir J*. 2008;31:143-178.
- Pretolani M, Soussan D, Poirier I, et al. Clinical and biological characteristics of the French COBRA cohort of adult subjects with asthma. *Eur Respir J*. 2017;50:1700019.
- Buenestado A, Chaumais MC, Grassin-Delye S, et al. Roflumilast inhibits lipopolysaccharide-induced tumor necrosis factor- α and chemokine production by human lung parenchyma. *PLoS One*. 2013;8:e74640.
- Wex E, Kollak I, Duechs MJ, Naline E, Wollin L, Devillier P. The long-acting β_2 -adrenoceptor agonist olodaterol attenuates pulmonary inflammation. *Br J Pharmacol*. 2015;172:3537-3547.
- Perchet T, Chea S, Hasan M, Cumano A, Golub R. Single-cell gene expression using multiplex RT-qPCR to characterize heterogeneity of rare lymphoid populations. *J Vis Exp*. 2017;(119). <https://doi.org/10.3791/54858>
- Razafindratsita A, Saint-Lu N, Mascarell L, et al. Improvement of sublingual immunotherapy efficacy with a mucoadhesive allergen formulation. *J Allergy Clin Immunol*. 2007;120:278-285.
- Bartemes KR, Kephart GM, Fox SJ, Kita H. Enhanced innate type 2 immune response in peripheral blood from patients with asthma. *J Allergy Clin Immunol*. 2014;134:671-678 e674.
- Griffith JW, Sokol CL, Luster AD. Chemokines and chemokine receptors: positioning cells for host defense and immunity. *Annu Rev Immunol*. 2014;32:659-702.
- Kim MA, Shin YS, le Pham D, Park HS. Adult asthma biomarkers. *Curr Opin Allergy Clin Immunol*. 2014;14:49-54.
- Teran LM. CCL chemokines and asthma. *Immunol Today*. 2000;21:235-242.
- Jahnz-Ro yk K, Plusa T, Mierzejewska J. Eotaxin in serum of patients with asthma or chronic obstructive pulmonary disease: relationship with eosinophil cationic protein and lung function. *Mediators Inflamm*. 2000;4:175-179.
- Szalai C, Kozma GT, Nagy A, et al. Polymorphism in the gene regulatory region of MCP-1 is associated with asthma susceptibility and severity. *J Allergy Clin Immunol*. 2001;108:375-381.
- Letuve S, Kozhich A, Arouche N, et al. YKL-40 is elevated in patients with chronic obstructive pulmonary disease and activates alveolar macrophages. *J Immunol*. 2008;181:5167-5173.
- Meyer N, Nuss SJ, Rothe T, Siebenhuner A, Akdis CA, Menz G. Differential serum protein markers and the clinical severity of asthma. *J Asthma Allergy*. 2014;7:67-75.
- Klein Wolterink RG, Kleinjan A, van Nimwegen M, et al. Pulmonary innate lymphoid cells are major producers of IL-5 and IL-13 in murine models of allergic asthma. *Eur J Immunol*. 2012;42:1106-1116.
- Zaiss DMW, Gause WC, Osborne LC, Artis D. Emerging functions of amphiregulin in orchestrating immunity, inflammation, and tissue repair. *Immunity*. 2015;42:216-226.
- Qiu J, Heller JJ, Guo X, et al. The aryl hydrocarbon receptor regulates gut immunity through modulation of innate lymphoid cells. *Immunity*. 2012;36:92-104.
- Walker JA, McKenzie AN. Development and function of group 2 innate lymphoid cells. *Curr Opin Immunol*. 2013;25:148-155.
- Bartemes KR, Iijima K, Kobayashi T, Kephart GM, McKenzie AN, Kita H. IL-33-responsive lineage-CD25⁺CD44(hi) lymphoid cells mediate innate type 2 immunity and allergic inflammation in the lungs. *J Immunol*. 2012;188:1503-1513.
- Mjosberg J, Bernink J, Golebski K, et al. The transcription factor GATA3 is essential for the function of human type 2 innate lymphoid cells. *Immunity*. 2012;37:649-659.
- Christianson CA, Goplen NP, Zafar I, et al. Persistence of asthma requires multiple feedback circuits involving type 2 innate lymphoid cells and IL-33. *J Allergy Clin Immunol*. 2015;136:59-68.
- Nagakumar P, Denney L, Fleming L, Bush A, Lloyd CM, Saglani S. Type 2 innate lymphoid cells in induced sputum from children with severe asthma. *J Allergy Clin Immunol*. 2016;137:624-626.
- Oliphant CJ, Hwang YY, Walker JA, 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;41:283-295.
- Monticelli LA, Sonnenberg GF, Abt MC, et al. Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nat Immunol*. 2011;12:1045-1054.
- Brestoff JR, Kim BS, Saenz SA, et al. Group 2 innate lymphoid cells promote beiging of white adipose tissue and limit obesity. *Nature*. 2015;519(7542):242-246.
- Duerr CU, McCarthy CD, Mindt BC, et al. Type I interferon restricts type 2 immunopathology through the regulation of group 2 innate lymphoid cells. *Nat Immunol*. 2016;17:65-75.
- Moro K, Kabata H, Tanabe M, et al. Interferon and IL-27 antagonize the function of group 2 innate lymphoid cells and type 2 innate immune responses. *Nat Immunol*. 2016;17:76-86.
- Wang S, Xia P, Chen Y, et al. Regulatory innate lymphoid cells control innate intestinal inflammation. *Cell*. 2017;171:201-216.
- Kim HS, Jang JH, Lee MB, et al. A novel IL-10-producing innate lymphoid cells (ILC10) in a contact hypersensitivity mouse model. *BMB Rep*. 2016;49:293-296.
- Matsuki A, Takatori H, Makita S, et al. T-bet inhibits innate lymphoid cell-mediated eosinophilic airway inflammation by suppressing IL-9 production. *J Allergy Clin Immunol*. 2016;139:1355-1367.
- Iwamoto I, Nakajima H, Endo H, Yoshida S. Interferon γ regulates antigen-induced eosinophil recruitment into the mouse airways by inhibiting the infiltration of CD4⁺ T cells. *J Exp Med*. 1993;177:573-576.
- Leonard C, Tormey V, Burke C, Poulter LW. Allergen-induced cytokine production in atopic disease and its relationship to disease severity. *Am J Respir Cell Mol Biol*. 1997;17:368-375.

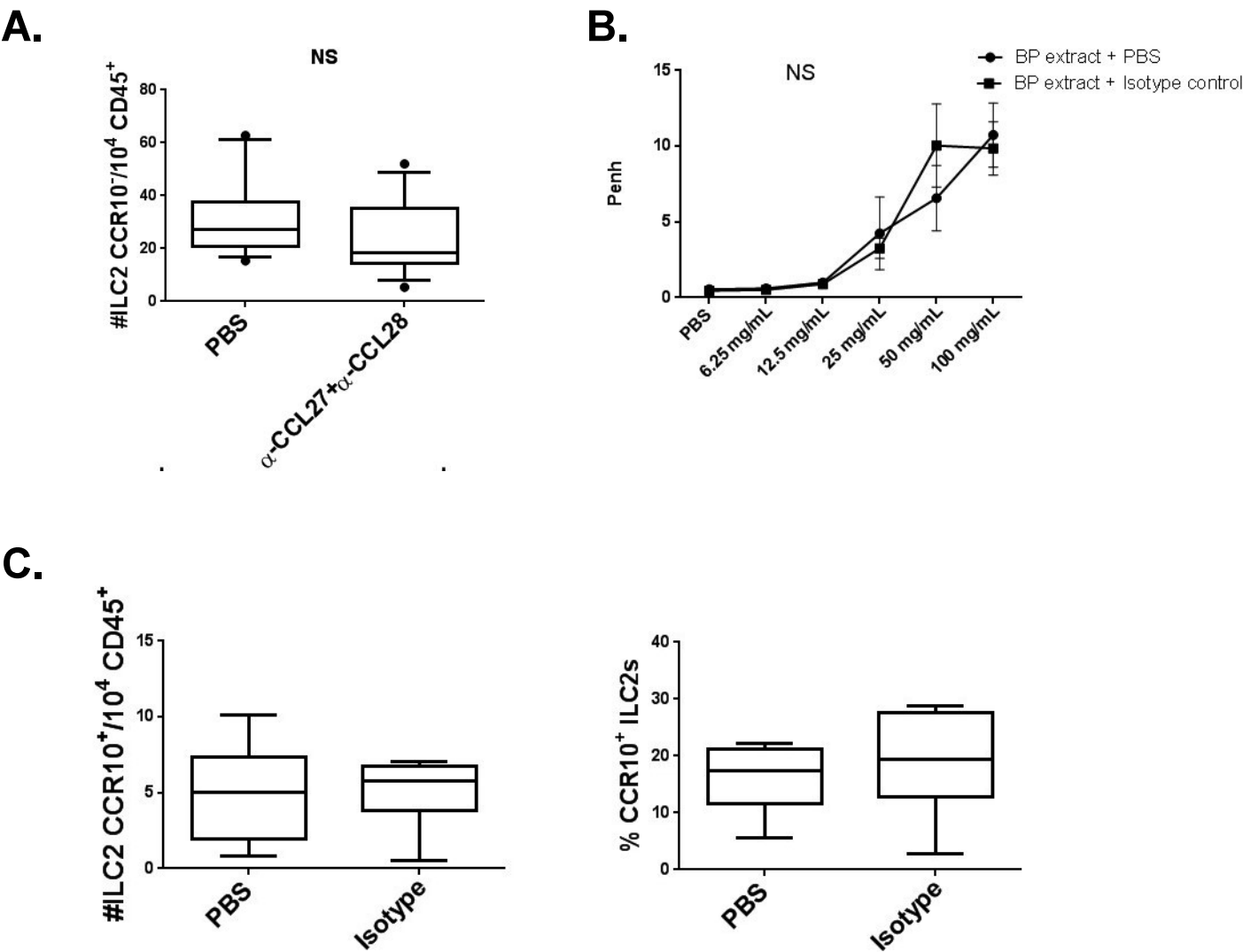
39. Silver JS, Kearley J, Copenhaver AM, et al. Inflammatory triggers associated with exacerbations of COPD orchestrate plasticity of group 2 innate lymphoid cells in the lungs. *Nat Immunol*. 2016;17:626-635.
40. Ohne Y, Silver JS, Thompson-Snipes L, et al. IL-1 is a critical regulator of group 2 innate lymphoid cell function and plasticity. *Nat Immunol*. 2016;17:646-655.
41. Bal SM, Bernink JH, Nagasawa M, et al. IL-1beta, IL-4 and IL-12 control the fate of group 2 innate lymphoid cells in human airway inflammation in the lungs. *Nat Immunol*. 2016;17:636-645.
42. Huang Y, Guo L, Qiu J, et al. IL-25-responsive, lineage-negative KLRG1 cells are multipotential 'inflammatory' type 2 innate lymphoid cells. *Nat Immunol*. 2014;16:161-169.
43. Wu D, Zhou J, Bi H, et al. CCL11 as a potential diagnostic marker for asthma? *J Asthma*. 2014;51:847-854.
44. Moed H, Boorsma DM, Tensen CP, et al. Increased CCL27-CCR10 expression in allergic contact dermatitis: implications for local skin memory. *J Pathol*. 2004;204:39-46.
45. Kakinuma T, Saeki H, Tsunemi Y, et al. Increased serum cutaneous T cell-attracting chemokine (CCL27) levels in patients with atopic dermatitis and psoriasis vulgaris. *J Allergy Clin Immunol*. 2003;111:592-597.
46. Gudjonsson JE, Ding J, Johnston A, et al. Assessment of the psoriatic transcriptome in a large sample: additional regulated genes and comparisons with in vitro models. *J Invest Dermatol*. 2010;130:1829-1840.
47. Riis JL, Johansen C, Vestergaard C, Bech R, Kragballe K, Iversen L. Kinetics and differential expression of the skin-related chemokines CCL27 and CCL17 in psoriasis, atopic dermatitis and allergic contact dermatitis. *Exp Dermatol*. 2011;20:789-794.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Beuraud C, Lombardi V, Luce S, et al. CCR10⁺ ILC2s with ILC1-like properties exhibit a protective function in severe allergic asthma. *Allergy*. 2019;74:933-943. <https://doi.org/10.1111/all.13679>

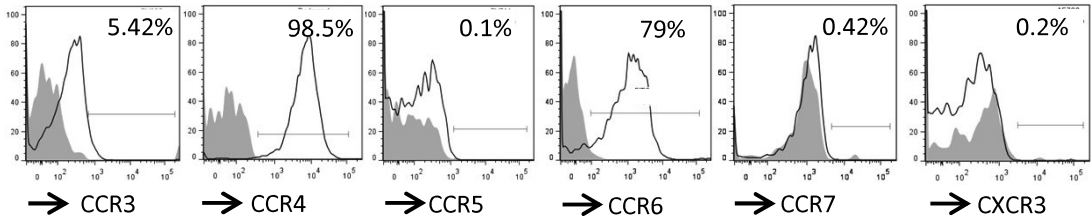
Supplementary Figure 1.



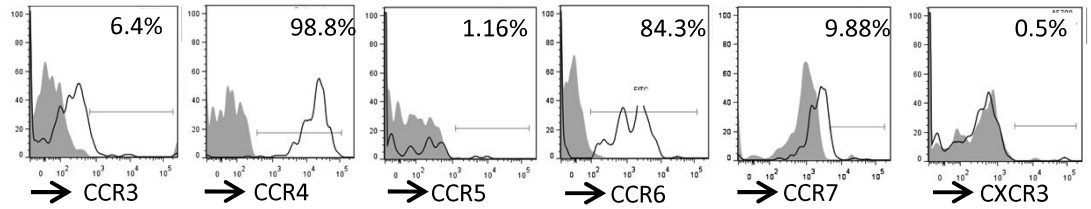
Supplementary Figure 2.

Isotype control
Surface marker antibody

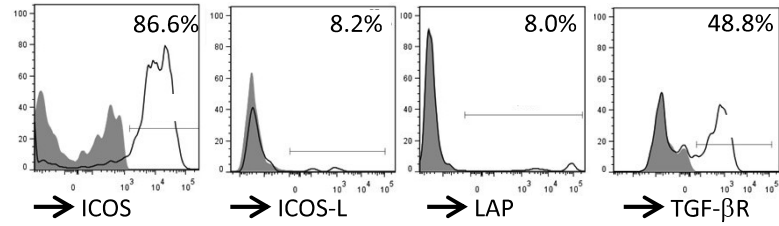
CCR10⁻ ILC2



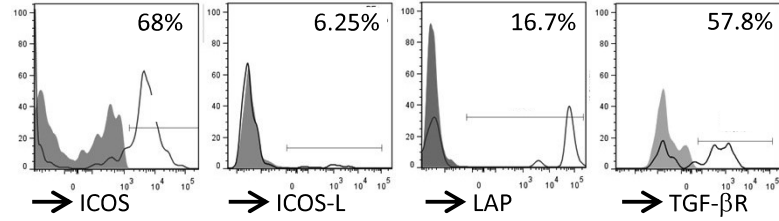
CCR10⁺ ILC2



CCR10⁻ ILC2

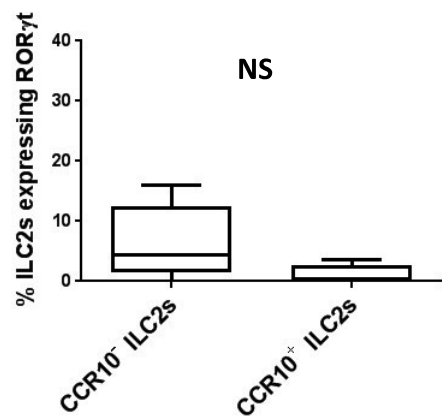


CCR10⁺ ILC2

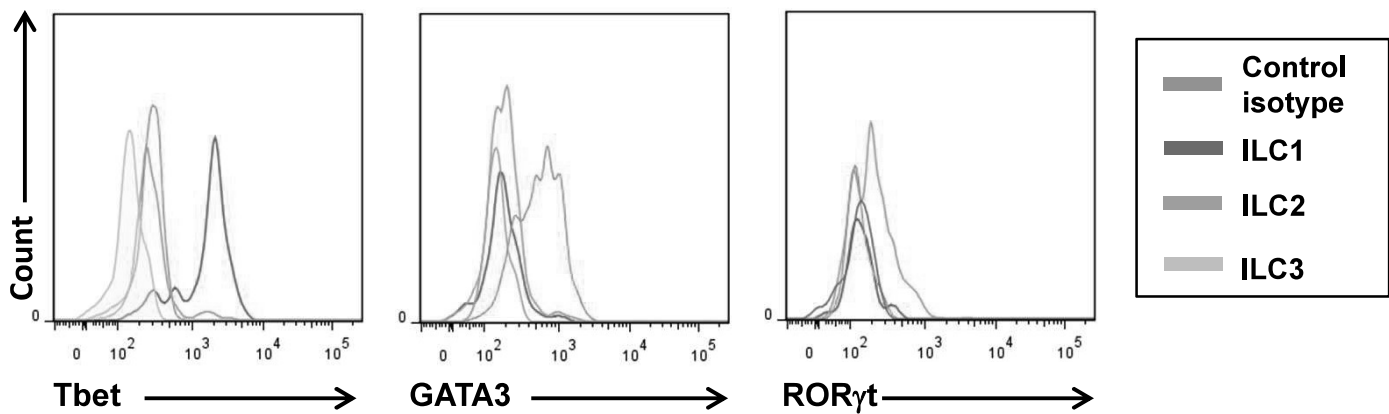


Supplementary Figure 3.

A.

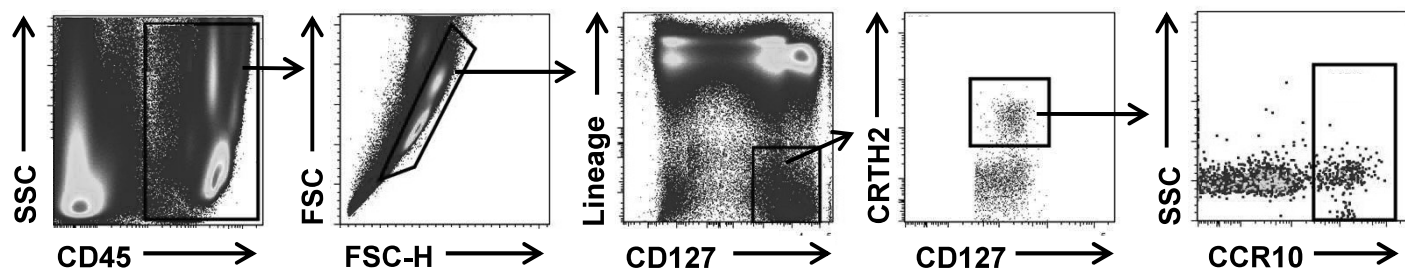


B.

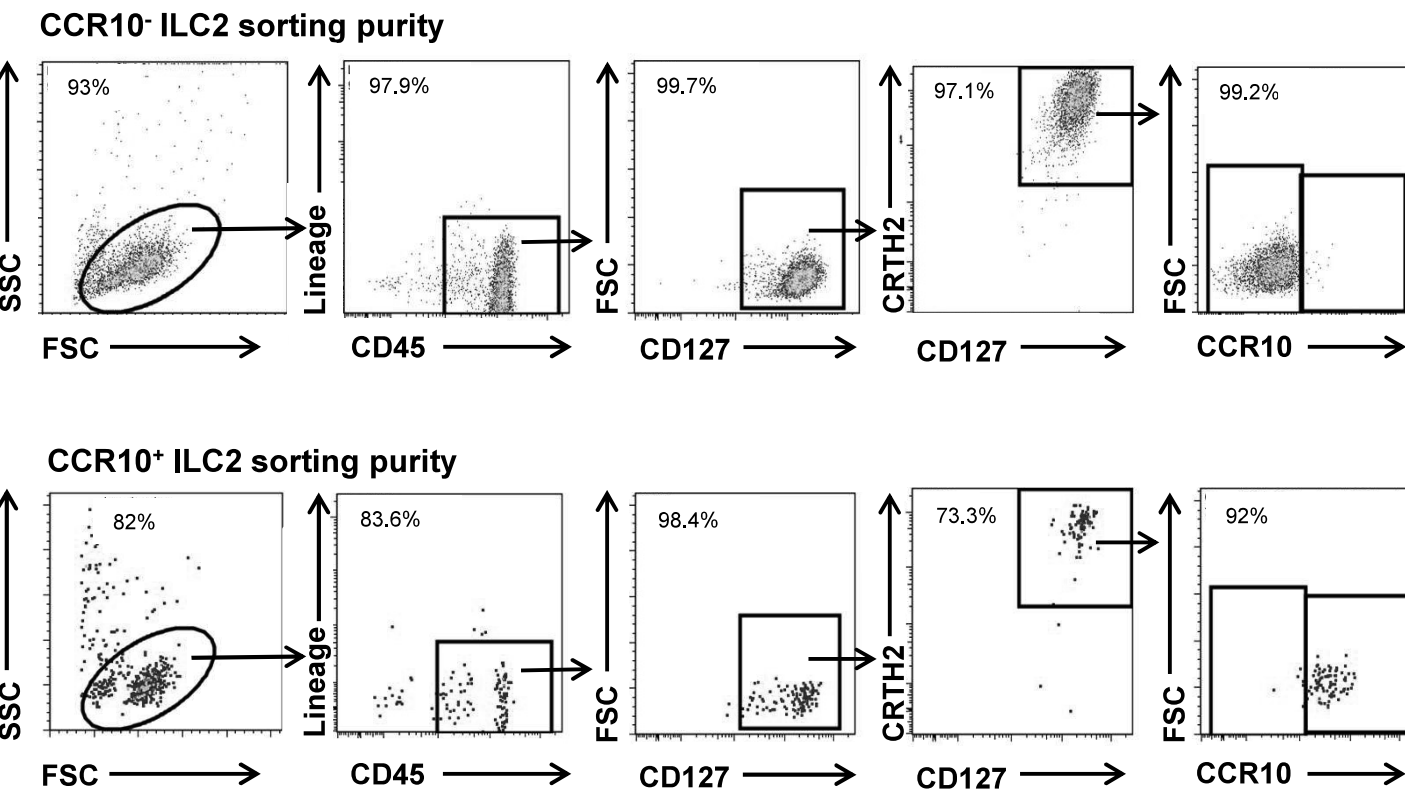


Supplementary Figure 4.

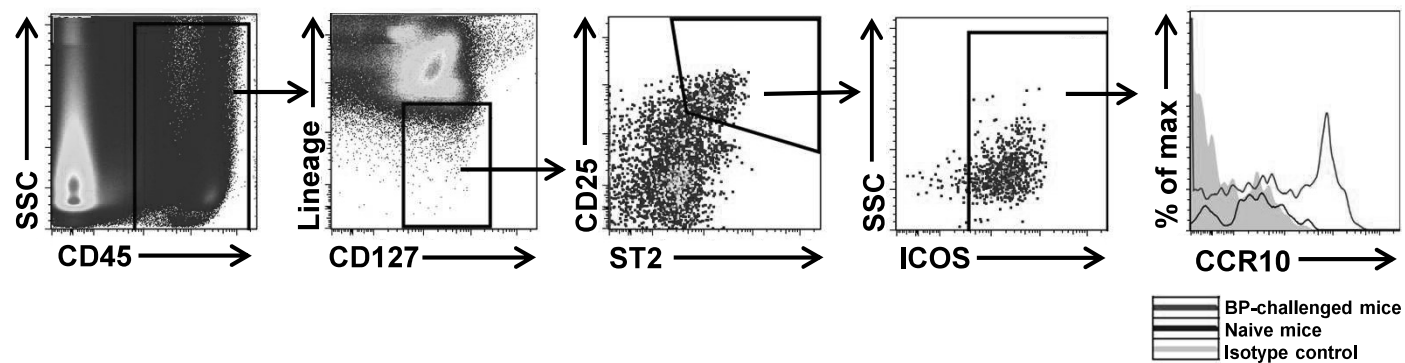
A.



B.

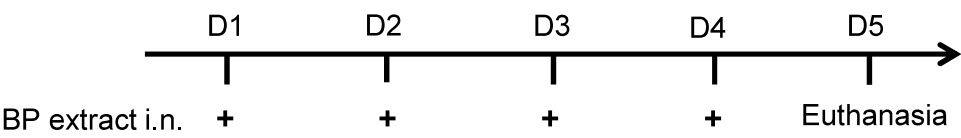


C.

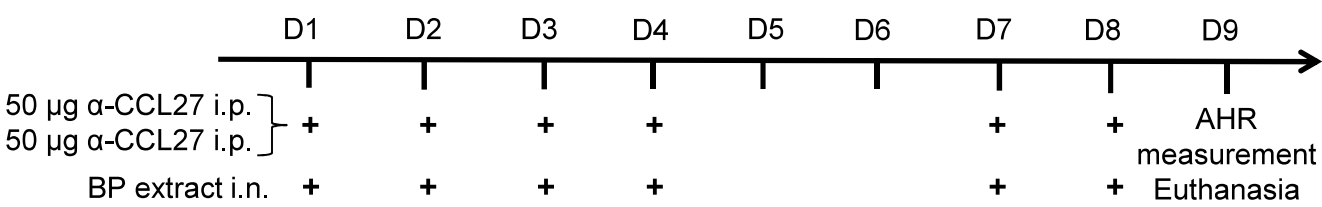


Supplementary Figure 5.

A.



B.



Supplementary Table 1.

Gene	Primer reference
ACTB	Hs99999903_m1
AHR	Hs00169233_m1
AREG	Hs00950669_m1
ARG1	Hs00163660_m1
CCL11	Hs00237013_m1
CCL2	Hs00234140_m1
CCL27	Hs00171157_m1
CCL28	Hs00219797_m1
CCR1	Hs00928897_s1
CCR10	Hs00706455_s1
CCR2	Hs00704702_s1
CCR3	Hs00266213_s1
CCR4	Hs00747615_s1
CCR5	Hs99999149_s1
CCR6	Hs01890706_s1
CCR7	Hs01013469_m1
CCR8	Hs00174764_m1
CCR9	Hs01890924_s1
CD1D	Hs00939886_g1
CD27	Hs00609654_g1
CD86	Hs01567026_m1
CLA	Hs04276253_m1
CLEC4A	Hs01087625_m1
CRTH2	Hs01867513_s1
CTLA4	Hs00175480_m1
CYSLTR1	Hs00272624_s1
DR3	Hs00600930_g1
F2RL1	Hs00608346_m1
FCGR3A	Hs02388314_m1
FOS	Hs04194186_s1
FOSB	Hs00171851_m1
FOXP3	Hs01085834_m1
GAPDH	Hs99999905_m1
GATA3	Hs00231122_m1
GILZ	Hs00608272_m1
GMCSF	Hs00929873_m1
HLADRB1	Hs04192464_mH
HPRT1	Hs99999909_m1
ICOS	Hs00359999_m1
ICOSL	Hs00323621_m1
ID2	Hs04187239_m1
IFNAR1	Hs01066116_m1
IFNG	Hs00989291_m1
IFNGR1	Hs00988304_m1
IL10	Hs00961622_m1
IL10R	Hs00155485_m1
IL12A	Hs01073447_m1

Gene	Primer reference
IL12RB1	Hs01106578_m1
IL13	Hs00174379_m1
IL17A	Hs00174383_m1
IL17F	Hs01028648_m1
IL17RB	Hs00218889_m1
IL18R1	Hs00977691_m1
IL1B	Hs01555410_m1
IL1R1	Hs00991010_m1
IL1RL1	Hs00249384_m1
IL2	Hs00174114_m1
IL21	Hs00222327_m1
IL22	Hs01574154_m1
IL25	Hs03044841_m1
IL27RA	Hs00945029_m1
IL2RA	Hs00907777_m1
IL3	Hs00174117_m1
IL33	Hs00369211_m1
IL4	Hs00174122_m1
IL5	Hs01548712_g1
IL6	Hs00174131_m1
IL7R	Hs00902334_m1
IL8	Hs00174103_m1
IL9	Hs00174125_m1
IL9R	Hs01108522_m1
JUN	Hs01103582_s1
KLRG1	Hs00195153_m1
LIF	Hs01055668_m1
LTA	Hs04188773_g1
NCAM1	Hs00941830_m1
NCR1	Hs00183118_m1
NCR2	Hs00183113_m1
NKG7	Hs01120688_g1
OX40L	Hs01911853_s1
PD1	Hs01550088_m1
PDL1	Hs00204257_m1
PDL2	Hs00228839_m1
RORC	Hs01076112_m1
RUNX3	Hs01091094_m1
SOCS1	Hs00705164_s1
TBX21	Hs00894392_m1
TGFB1	Hs00998133_m1
TGFBR2	Hs00234253_m1
TLR4	Hs00152939_m1
TNFA	Hs00174128_m1
TSLP	Hs00263639_m1
TSLPR	Hs00845692_m1

Supplementary Table 2.

		#	Female / Male	Age	% of uncontrolled asthma	FEV1 %	Total IgE (UI/mL)	Eosinophils count (cells/ μ l)
Non-allergic subjects	w/o asthma	10	5/5	28	N/A	107,2	67,7	N/A
	with severe asthma	14	8/6	54	57,1%	75,1	269,9	320,7
Allergic rhinoconjunctivitis patients	patients w/o asthma	10	3/7	28	N/A	107,3	181,8	N/A
	with mild asthma	16	7/9	55	37,5%	97,9	261,2	N/A
	with severe asthma	12	6/6	49	91,7%	64,3	437,6	220

Supplementary Table 3.

Anti-human antibodies

Specificity	Fluorochrome	Clone	Vendor
CCR3	BV605	5E8	BD horizon
CCR4	Pe-Cy7	161	BD horizon
CCR4	Pe-Dazzle	L29H4	Biolegend
CCR5	BV711	2D7	BD horizon
CCR6	BB515	11A9	BD horizon
CCR7	BV786	3D12	BD horizon
CCR8	PE	226368	Biolegend
CCR10	APC	314305	Bio-Techne
CD3	Viogreen	BW264/56	Miltenyi
CD11c	BV510	B-ly6	BD horizon
CD14	Viogreen	TÜK4	Miltenyi
CD16	Viogreen	VEP13	Miltenyi
CD20	Viogreen	LT20	Miltenyi
CD25	PE	3G10	Miltenyi
CD27	PE	L128	BD horizon
CD34	BV510	581	Biolegend
CD45	FITC	5B1	Miltenyi
CD49d	BB515	9F10	BD horizon
CD56	APC-H7	CMSSB	eBioscience
CD117	BV421	YB5.B8	BD horizon
CD123	BV510	9F5	BD horizon
CD127	PE	MB15-18C9	Miltenyi
CD127	PE-vio770	MB15-18C9	Miltenyi
CLA	PE	HECA452	BD pharma
CRTH2	PerCP-Cy5.5	BM16	BD horizon
CXCR3	AF700	2C6	BD horizon
GATA3	PE	TWAJ	eBioscience
HLA-DR	PE	LN3	eBioscience
ICOS	PE	C398.4A	Biolegend
ICOSL	PE	9F.8A4	Biolegend
IFN- γ	FITC	B27	BD pharma
KI-67	PerCP-Cy5.5	5B6	BD pharma
KLRG1	FITC	REA261	Miltenyi
LAP	PE	TW42F8	BD horizon
ROR- γ t	PE	Q21-559	BD pharma
Tbet	PE	ebio4B10	eBioscience
TCRab	BV510	T10B9.1A-31	BD horizon
TGFBRII	AF700	FAB2411N	Bio-Techne

Anti-mouse antibodies

Specificity	Fluorochrome	Clone	Vendor
CCR3	FITC	83101	Bio-Techne
CCR10	PE	248918	Bio-Techne
CD3e	BV450	500A3	BD horizon
CD4	Pe-Cy7	GK1.5	eBioscience
CD8	APC	53.6.7	eBioscience
CD11b	BV450	M1/70	BD horizon
CD11b	PerCP-Cy5.5	M1/70	BD horizon
CD11c	BV421	HL3	BD horizon
CD19	BV421	1D3	BD horizon
CD19	APC-Cy7	1D3	BD horizon
CD25	BV510	PC61	BD horizon
CD45	APC-Cy7	30F11	BD pharma
CD45	V500	30F11	BD pharma
CD45R.B220	BV421	RA3.56B2	BD horizon
CD127	Pe-Cy7	ebioSB/199	eBioscience
GR1	Pe-Cy7	RB6-8C5	BD pharma
ICOS	BB515	7E.17G9	BD horizon
IFN- γ	APC	XMG1.3	eBioscience
Ly6G and C	BV421	RB68C5	BD horizon
MHC Class II	FITC	M5/114.15.2	eBioscience
NK1.1	BV450	PK136	BD horizon
ST2	PerCP-efluor710	PMST2.2	eBioscience
Ter 119	BV421	Ter119	BD horizon