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Dual role for CXCR3 and CCR5 in asthmatic Type 1 inflammation

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Abstract

Background: Many severe asthma (SA) patients fail to respond to Type 2 inflammation targeted therapies. We previously identified a cohort of SA subjects expressing Type 1 inflammation manifesting with IFN-γ expression with variable Type 2 responses.

Objective: We investigated the role of the chemotactic receptors CXCR3 and CCR5 in establishing Type 1 inflammation in SA.

Methods: Bronchoalveolar lavage microarray data from the Severe Asthma Research Program (SARP I/II) were analyzed for pathway expression and paired with clinical parameters. Wild Type, *Cxcr3*$^{-/-}$ and *Ccr5*$^{-/-}$ mice were exposed to a Type 1^{High} SA model with analysis of whole lung gene expression and histology. Wild type and *Cxcr3*$^{-/-}$ mice were treated with an FDA-approved CCR5 inhibitor (maraviroc) with assessment of airway resistance, inflammatory cell recruitment by flow cytometry, whole lung gene expression and histology.

Results: A cohort of subjects with increased IFN-γ expression showed higher asthma severity. IFN-γ expression correlated with CXCR3 and CCR5 expression but in *Cxcr3*$^{-/-}$ and *Ccr5*$^{-/-}$ mice Type 1 inflammation was preserved in a murine SA model, most likely due to compensation by the other pathway. Incorporation of maraviroc in the experimental model blunted airway hyperreactivity, despite only mild effects on lung inflammation.

Conclusions: *IFNG* expression in asthmatic airways was strongly correlated with expression of both the chemokine receptors *CXCR3* and *CCR5.* While these pathways provide redundancy for establishing Type 1 lung inflammation, inhibition of the CCL5/CCR5 pathway with maraviroc

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provided unique benefits in reducing airway hyperreactivity. Targeting this pathway may be a novel approach for improving lung function in Type 1^{high} asthmatic individuals.

Clinical Implications: In Type 1 high asthma, CCR5 and CXCR3 provide redundant pathways for lung inflammation, but CCR5 inhibition provides a potential novel target for reducing airway hyperreactivity.

Capsule Summary:

Type 1 inflammation in asthma is associated with more severe disease, but inhibition of the CCL5/CCR5 pathway in these patients may provide a novel approach to reduce airway hyperreactivity

Keywords

CXCR3; CXCL9; CXCL10; CCL5; CCR5; IFN-γ; severe asthma; maraviroc

Introduction

Asthma is a common disease affecting 5–10% of the population in developed countries[1,2], and nearly 5% of patients have severe asthma (SA) with poor response to corticosteroid therapy. While great strides have been made in the management of Type 2 high asthma through the use of targeted biologic therapy[3], nearly 50% of asthma patients lack evidence of this phenotype[4]. For these patients as well as for those with elevated Type 2 inflammation who fail to respond to targeted biologic therapy, improved understanding of the underlying pathophysiology is key to identifying novel pathways and therapeutic targets.

We have previously shown that approximately 30% of SA patients have evidence of Type 1 inflammation marked by elevated airway levels of interferon-γ (IFN-γ) [5] C-X-C Ligand 10 (CXCL10, IP-10) is also elevated in these patients and associated with worse disease as evidenced by higher need for steroids and more frequent exacerbations [6] CXCL10 (along with sister ligands CXCL9 and CXCL11) binds to C-X-C Receptor 3 (CXCR3) to promote chemotaxis of Th1 cells[7, 8] as well as activation of eosinophils[9] and mast cells[10]. As Th1 cells produce IFN-γ that promotes CXCL10 expression, this can lead to a positive feed forward loop to augment local Type 1 inflammation, an important component of the immune viral response[11], but a driver of local pathology in autoimmunity[7]. Importantly, IFN-γ induction of CXCL10 is refractory to corticosteroids, making this a potential component of steroid resistance in severe asthma patients[6, 12, 13].

The CXCL10-CXCR3 pathway is not the only pathway for Th1 chemotaxis. C-C motif ligand 5 (CCL5, RANTES) acts through the C-C Receptor 5 (CCR5) receptor to promote homing of cells to sites of inflammation. While co-expression of both receptors is uncommon in peripheral blood, it is highly prevalent at sites of inflammation in autoimmune disease[14], and has been described in the lung [15].

In this study, we investigated the effect of chemotactic blockade in a previously described murine SA model characterized by elevated type 1 inflammation and minimal steroid response[5]. We demonstrate the important role that both chemotactic pathways play in establishing Type 1 inflammation in the lung. Finally, we show that inhibition of the CCL5/

CCR5 pathway provides unique benefits in improving airway hyperreactivity despite only limited improvement in lung inflammation.

Methods

Human Subjects

Data previously obtained from subjects enrolled in the Severe Asthma Research Program (SARP) I/II cohorts was used. In brief, non-smoking subjects between ages 18–60 with asthma from racially/ethnically diverse backgrounds were recruited. All subjects had FEV1 >60% predicted. Subjects meeting American Thoracic Society (ATS) 2000 definition of severe asthma were classified as having severe asthma (SA) with the remaining subjects classified as having mild to moderate asthma (MMA). Additional non-smoking subjects without asthma were recruited as healthy controls (HC). Subjects underwent regularly scheduled visits that included spirometry, exhaled nitric oxide (FeNO), sputum sample, clinical questionnaire and medication use questionnaire. Subjects underwent bronchoscopy with bronchial epithelial brushings and bronchoalveolar lavage. Samples were processed by microarray as previously published[6]. Immunohistochemistry data was obtained from endobronchial biopsies as previously published.[16] All data was previously obtained prior to the start of the current project.

Murine Models

C57BL/6 wild type (WT) mice (Cat#000664), mice deficient in CXCR3 *(Cxcr3*$^{-/-}$, Cat # 005427) and mice deficient in CCR5 *(Ccr5*$^{-/-}$ Cat# 005796) were purchased from the Jackson Laboratory. All animals were cared for according to the NIH Policy on Humane Care and Use of Laboratory Animals with adherence to the Guide for the Care and Use of Laboratory Animals. Mice were housed under pathogen-free conditions and underwent treatments between 8–10 weeks of age. Age-matched mixed sex mice were used in this study. As described previously[5], mice were exposed to a Type-1 dominant SA model.[17]Mice were sensitized to 25 μg house dust mite antigen (HDM) (low-endotoxin, Greer Laboratories cat # XPB70D3A2.5) and combined with 5μg cyclic-di-GMP (Axxora cat # BLG-C057–01) on days 1, 3 and 5. Mice were rested for 5 days and then subjected to 3 challenge sets involving 3 consecutive daily challenges with HDM and cyclic-di-GMP with 4 days of rest between each set. Each challenge set included 0.5μg cyclic-di-GMP with 25 μg HDM on day 1, followed by 25 μg HDM only on the following 2 days. Mice were then sacrificed on day 28 and the lungs were used for histology and preparation of whole lung homogenate. For flow cytometry studies, an abbreviated model was used with the mice sacrificed after the first challenge was completed and lungs processed for single cell suspension. For maraviroc studies, maraviroc was dosed in drinking water (300mg/L) beginning 24 hours prior to the initial model event (sensitization or challenge) and continued until harvest (Fig E5, A, in this article's Online Repository at www.jacionline.org).

Intracellular Staining and Flow Cytometry

Lungs of anesthetized mice were perfused with sterile PBS, removed and processed for single cell preparation as described previously.[5] Briefly, the lung tissues were digested in a collagenase A/DNase suspension and then dissociated on a gentleMACS dissociator.

A single cell suspension was prepared by passing the dissociated tissue through a 70 μm cell strainer, and then treated with RBC lysis buffer (BD Pharm Lyse™). Cells were then stimulated for 2.5 hours with PMA (50 ng/ml)/ionomycin (1 ug/ml) in the presence of brefeldin A and monensin. Cells were washed, resuspended in Hanks' balanced salt solution and stained with fixable viability dye eFIuor 780 (eBioscience). Cells were fixed overnight using a Foxp3/Transcription Factor Staining Buffer Set (eBioscience) and stained for surface markers as well as intracellular cytokines for 45 minutes in permeabilization buffer. Data were acquired on a FACSAria flow cytometer (BD Immunocytometry Systems) and analyzed using FlowJo software (Tree Star, Inc).

Measurement of Airway Resistance

Airway hyperresponsiveness (AHR) in mice was measured as previously described with some modifications.[5] Mice were anesthetized using Xylazine (12 mg/kg)/Sodium Pentobarbital (90 mg/kg) and Pancuronium (0.8 mg/kg) and subjected to the forced oscillation technique for measuring AHR using a Flexivent system (SCIREQ). Measurements of lung function were made following perturbation with increasing doses of methacholine (0–100mg/ml).

Statistical Analysis

Statistical Analysis was completed in GraphPad Prism 7.03 for all in vitro and murine studies. Human Data was analyzed using STATA SE 15. For parameters with normal distribution including murine studies, one-way ANOVA with Dunn's post-hoc test or Student's T-test (with Welch's correction for unequal standard deviations as needed) were used. For all human data, non-parametric analyses were used with Wilcoxon rank-sum testing for two group comparisons, Kruskal-Wallis test with Sidak's post-hoc testing for multigroup analysis of variance, Spearman's Non-parametric Correlation for comparison of association between continuous variables and Fisher's Exact testing for contingency groups. Exact p-values are reported throughout, analysis of variance for multigroup comparisons were considered significant at a $p<0.05$ (in these settings exact value for post-hoc testing is reported).

Results

IFN-γ is elevated in a sub-cohort of more severe asthma subjects

We examined previously obtained clinical and microarray data (obtained from bronchoalveolar lavage [BAL] cell pellets) from subjects enrolled in the Severe Asthma Research Program I/II (SARP I/II); 148 subjects had microarray data available for analysis. This included HC (n=38), subjects with MMA (n=69) and subjects with SA (n=42). As expected, significant differences were noted between asthma severity groups in age, BMI, Asthma Quality of Life Questionnaire (Juniper AQLQ, higher score indicates better quality), spirometry and markers of Type 2 inflammation (Table EI, online repository). Notably, there was no difference in race or sex between groups. While *IFNG* levels were not significantly different across groups (p=0.34), there was notably greater variance with increasing asthma severity (Fig 1, A, Fig E1, A, online repository). This suggested that a cohort of asthma subjects expressed notably elevated levels of IFN-γ compared to the healthy control group.

To characterize this IFN-γ high subset of subjects, we assigned a cutoff gene expression value of 7.467 based on two standard deviations above the healthy control (healthy control median 6.591, SD 0.438; Fig 1, A, dotted line). This divided the asthma cohort into IFN-γ high subjects (n = 21) and IFN-γ low (n = 90). Notably, a significantly higher proportion of subjects were IFN-γ high among those with SA than among those with MMA (28.6% vs 13.0%, p=0.05, Fig 1, B), a trend that held when subjects were divided into mild, moderate and severe categories (p=0.06, Fig E1, B, online repository). IFN-γ high subjects were significantly older, with a trend towards later age of onset of asthma (Table I). IFN-γ high subjects also showed a trend towards a lower forced expiratory volume at 1 second (FEV1) with a significant reduction in forced vital capacity (FVC) (Table I).

Comparing BAL cell differentials, IFN-γ high subjects had notably elevated lymphocyte and neutrophil content in their BAL samples (Fig 1, C) despite no difference in BAL total cell counts (Fig E1, C, online repository). There was no difference in eosinophil counts between groups (p=0.74, data not shown), although notably all subjects with an eosinophil percentage greater than 5% were in the IFN-γ low group. This supports a greater contribution of lymphocytes and neutrophils to the overall airway inflammation in IFN-γ high subjects. Some subjects also had endobronchial biopsy immunohistochemistry data that was also available for review (IFN-γ high n = 8, IFN-γ low n = 23). Reviewing these data showed a similar trend to elevation of $CD4^+$ cells in the endobronchial biopsies of IFN-γ high subjects along with a significant increase in $CD68^+$ cells consistent with increased number of macrophage/monocyte cells in this group. No differences were seen in these large airway samples in the presence of eosinophils, neutrophils or mast cells (Fig E2, online repository), suggesting that these differences are manifested primarily in the small airways.

To clinically characterize these subjects, we examined medication utilization and disease exacerbations. There was no difference noted in inhaled corticosteroid use between groups (Table I). There was an increased use of systemic oral corticosteroids in the IFN-γ high group (42.9% vs. 20.2%, p=0.05; Fig 1, D). There was also a trend towards more subjects having required multiple oral steroid bursts (45.0% vs. 25.3%, p=0.10; Fig 1, *E)* or having required an ED/Urgent Care evaluation for asthma in the prior 3 months (60% vs 41.9%, p=0.21; Fig E1, D, online repository) although neither reached statistical significance.

Limiting our analysis to only SA subjects, we observed significant differences in BAL cell differentials (increased lymphocyte and neutrophil percentages), between IFN-γ high and IFN-γ low groups, although clinical differences were no longer apparent (Table EII, online repository). However, as the significant majority of IFN-γ high subjects were moderate or severe (Fig E1, B, online repository), it follows that IFN-γ high subjects tended to be sicker with higher steroid requirements.

IFN-γ is correlated with the CXCL10/CXCR3 and CCL5/CCR5 chemotactic pathways.

To understand the pathways involved in Type 1 signaling in these subjects, we examined expression and correlation of IFN-γ with known chemotactic signals. While comparison of *CXCL9, CXCL10* and *CCL5* expression across all asthma severity groups showed no significant difference, expression of each of these genes was elevated in IFN-γ high subjects compared to IFN-γ low subjects (Fig 2, A, Fig E3, A, online repository). We also observed

strong correlation for each ligand with their cognate receptor (r = 0.75 and r = 0.69 for *CXCL9* and *CXCL10* with *CXCR3* respectively, r = 0.65 for *CCL5* with *CCR5,* p<0.001 for all measures; Fig E3, B, online repository). Examining co-expression with Type 2 and Type 3 cytokine pathways, IFN-γ high subjects showed no difference in *IL5* or *IL17A* expression, but a significant elevation in *IL4* was seen in the IFN-γ high subgroup, consistent with a prior description of elevated exhaled nitric oxide expression in SA subjects with high IFN-γ[18] (Fig 2, B).

When comparing chemotactic pathways with *IFNG* expression, we noted significant correlation with both receptors (r=0.78 and r = 0.57 for *CXCR3* and *CCR5* with *IFNG* respectively, p<0.001 for all measures; Fig 2, C). This suggested that both pathways might play a role in the recruitment of IFN-γ-expressing cells, as has been noted in other conditions [14, 15]. To confirm this possibility, we examined association between the two receptors, and noted strong correlation (r=0.69, p<0.001; Fig 2, D). These correlations further strengthened when restricted to SA subjects (Fig E4, A–C, online repository). This supported the hypothesis that both the CXCL10/CXCR3 and CCL5/CCR5 pathways contribute to IFN-γ upregulation in a severe asthma phenotype.

Cxcr3$^{-/-}$ mice and *Ccr5*$^{-/-}$ mice have a preserved severe asthma phenotype

To better understand the importance of each chemotactic pathway in Type 1 inflammation, we exposed mice to a murine SA model (Fig E5, A, online repository) that induces IFN-γ dependent AHR which is resistant to corticosteroid treatment, parallel with the same phenomenon observed in human severe asthma[5, 6, 12]. This model utilizes a bacterial second messenger molecule (cyclic-di-GMP) to achieve a Type-1 dominant immune profile, consistent with reports in severe asthmatic children of a Type 1 inflammatory signature paired with recovery in BAL of pathogenic bacteria and viruses despite an absence of overt infectious disease in these subjects.[17] WT and *Cxcr3*$^{-/-}$ mice were subjected to the SA model. Following sacrifice, whole lung RNA was isolated and assessed by qRT-PCR. *Cxcr3*$^{-/-}$ mice had higher lung expression of *Cxcl10* (p = 0.03) and a trend towards higher expression of *Cxcl9* (p = 0.08) and *Ifng* (p = 0.07) (Fig 3, A). No significant differences in *Il4, Il13,* or *Il17a* gene expression were detected between the two groups of mice (Fig 3, A). Histologic analysis of lung tissue sections showed similarity between *Cxcr3*$^{-/-}$ and WT mice at baseline (naïve) and also after being subjected to the SA model (Fig 3, B). To confirm our suspicion that the *Ccl5/Ccr5* pathway was potentially responsible for the persistent Type 1 inflammation in *Cxcr3*$^{-/-}$ mice we analyzed expression of *Cxcr3* and *Ccr5* mRNA in whole lung tissue RNA from mice subjected to the SA model. *Cxcr3*$^{-/-}$ mice displayed increased *Ccr5* expression compared to that detected in the WT mice (p = 0.02) (Fig 3, C). This suggested that increased chemotaxis via the CCL5/CCR5 pathway may functionally compensate for the loss of CXCL10/CXCR3 signaling.

To assess the importance of the CCL5/CCR5 pathway, we subjected WT and *Ccr5*$^{-/-}$ mice to the SA model. Whole-lung RNA analysis by qRT-PCR again showed persistent elevations in *Cxcl10, Ccl5, Ifng, Il4,* Il17, and *Il10* without any significant difference between WT and *Ccr5*$^{-/-}$ mice (Fig 4, A). Histological analysis revealed a similar profile of increased inflammation in the two groups of mice (Fig 4, B). Finally, examination of receptor

expression in *Ccr5*$^{-/-}$ mice revealed higher *Cxcr3* expression compared to that in WT mice (p=0.05, Fig 4, C). These data combined with the results obtained using *Cxcr3*$^{-/-}$ mice strongly suggested redundancy in the ability of these pathways to mediate chemotaxis leading to Type 1 inflammation, and is consistent with previously described co-expression of CXCR3 and CCR5 protein on T-cells in human lungs.[15]

CCR5 and CXCR3 blockade effectively decreases but does not eliminate Th1 lymphocyte recruitment

In order to address the apparent overlapping functions of the Cxcr3 and Ccr5 pathways in the SA model, we next examined the effect of chemokine receptor blockade on specific cell recruitment to the lung. We utilized an abbreviated mouse model for these studies, examining cell recruitment after the first challenge was completed (Fig E5, A, online repository). In both WT and *Cxcr3*$^{-/-}$ mice, we observed a similar degree of cellular recruitment with IFN-γ^+ cells forming the predominant component of the pulmonary T-lymphocyte population (gating strategy in Fig E5, B, online repository). We did not observe any significant difference in IFN-γ^+, IL-17A$^+$, IL-13$^+$ or IL-10$^+$ T-lymphocytes between WT and *Cxcr3*$^{-/-}$ mice, (Fig 5, A).

Next, to assess the ability of CCR5 blockade to attenuate Th1 chemotaxis to the lung, we utilized maraviroc, a small molecule inhibitor of Ccr5[19]. Mice received maraviroc via drinking water beginning prior to sensitization, and pulmonary cell recruitment was assessed after the first challenge. In WT mice, maraviroc had no significant effect on either total cell numbers in the lung or the percentage of IFN-γ^+ or IL-13$^+$ cells, although a trend towards reduction in both IFN-γ^+ and IL-17A$^+$ cell numbers was observed (Fig 5, B). These data suggested a limited efficacy for the drug in blocking T-lymphocyte lung chemotaxis when the CXCL10/CXCR3 pathway was intact. However, utilizing maraviroc in *Cxcr3*$^{-/-}$ mice to simulate a double knockout of the genes, we observed a substantial decrease in total cell numbers and in IFN-γ^+ (p=0.009) and IL-17A$^+$ (p=0.005) cells (Fig 5, C). These data support an essential component for both pathways in T cell recruitment to the lung and further supports the limited efficacy of single pathway blockade for reducing cellular inflammation.

Efficacy of CCR5 inhibition in blocking airway hyperreactivity (AHR)

Although both *Ccr5*$^{-/-}$ and *Cxcr3*$^{-/-}$ mice failed to exhibit a reduction in lung inflammation individually, we investigated the effect of pathway blockade on AHR. Since dissociation between lung inflammation and AHR has been observed in asthma[20], we had reason to suspect that single pathway blockade might still prove effective in this regard. AHR was assessed in our murine SA model utilizing a methacholine challenge protocol as previously described.[5] We observed no effect of single pathway deletion on AHR in the *Ccr5*$^{-/-}$and *Cxcr3*$^{-/-}$ mice (Fig 6, A). Given our data that single pathway deletion resulted in compensatory alternative pathway overexpression in genetic knockout mice (Fig 3, C, and Fig 4, C), we hypothesized that single pathway inhibition might be effective in WT mice without the problem of compensatory increase in the alternative pathway. To assess this, we again utilized the CCR5 inhibitor maraviroc in WT mice. Use of maraviroc in mice prior to sensitization with HDM (Maraviroc Base, Fig E5, online repository) resulted in a

significant reduction in AHR (Fig 6, B) compared to that in the untreated control mice, suggesting that in WT mice single pathway blockade could attenuate symptoms. Given these findings, we also assessed the treatment potential of CCR5 in allergen-sensitized mice. In this treatment mode (Maraviroc Treat), we again observed significant reduction in AHR compared to that in the untreated mice (Fig 6, B). To confirm our hypothesis that this benefit in AHR was dissociated from lung inflammation, we assessed cytokine expression and inflammation in the lungs. While we found no difference in cytokine expression in whole lung with pre-challenge maraviroc (Fig 6, C), we did observe a modest improvement in the degree of airway inflammation by histology, consistent with the changes we noted in T-lymphocyte cell populations observed by flow cytometry (Fig 5, B), although some degree of inflammation did remain (Fig 6, D). Overall, these data suggest a role for CCR5 in promoting AHR in T1 dominant severe asthma, but that the CXCL10/CXCR3 pathway may provide an alternate mechanism for AHR when overexpressed, which is in agreement with prior data in T2 driven models.[21]

Discussion

While Type 2 inflammation is now well described in asthma with multiple therapeutic options available, the understanding of mechanisms underlying airway inflammation and hyperreactivity in asthma patients with a more complex IFN-γ high inflammatory phenotype remains poor. We previously demonstrated a role for IFN-γ high/Type 1 high inflammation in a subset of asthma patients that is generally steroid unresponsive[5, 6 12] In this study, analysis of the SARP I/II microarray BAL data has revealed a Type-1 high group marked by increased expression of IFN-γ. These patients are marked by a more lymphocytic and neutrophilic sputum content and appear to have worse disease overall given the higher proportion of Type-1 high patients that are severe. Although markers of disease control (exacerbations, ED visits) did not reach statistical significance in our study, this is likely due to our study being underpowered for this outcome (only 21 subjects with Type-1 high asthma were identified); the overall trend, however, points towards greater exacerbation frequency and is consistent with our prior clinical data in CXCL10 high patients [6] as well as a recent publication implicating Th1 cells as the dominant source of worsening lung function in rhinovirus mediated asthma exacerbations.[22] While patients with relatively low Type-2 markers are often less responsive to steroids [4,23], even some patients with elevated Type 2 markers can remain poorly controlled despite steroids or Type 2 targeted biologics due to a mixed inflammatory profile, indicating a need for novel asthma therapies.

Improved understanding of the pathways involved in establishing Type 1 inflammation in asthma will be important to identify targets for novel therapeutics. To that end, we have previously shown CXCL10 elevation in Type-1 high asthma and posited that the IFN-γ/CXCL10/CXCR3 axis represented an important chemotactic factor to drive Th1 recruitment to the lung in a possible forward feeding signaling loop[11]. Prior studies in Type 2 predominant murine asthma models have shown mixed effects of CXCR3 deficiency, with improvement in an ovalbumin (OVA)-driven model[24] but worsening asthma in an HDM model [25] which may reflect variations in Type-1/Type-2 balance between these models. Here, we observed that *Cxcr3*$^{-/-}$ mice showed a trend towards greater overall and Type 1 inflammation in a SA model. While the endpoint responses in these studies are variable,

all of them including our own show a consistent increase in IFN-γ in the *Cxcr3*$^{-/-}$ mice regardless of the model used. As CXCR3 also plays a significant role in eosinophil[9, 26] and mast cell recruitment[10, 27], it is possible that the effect in the overall model is determined by the altered immune balance resulting from Type-2 reduction and Type-1 increase that appears to be consistently caused by loss of CXCR3.

Given the role of CXCR3 in Th1 recruitment[7], the consistent increase in IFN-γ levels in the lung suggested an alternative mechanism of establishing type 1 inflammation.CCR5, one of the principal chemotactic receptors for CCL5 (RANTES), is often co-expressed with CXCR3 on T-cells at sites of inflammation in autoimmune disorders [14] Although co-expression has been previously identified in the lung, this prior study had failed to show increased expression in asthma, which was likely due to the selection of only a small number of mild asthma subjects (n=4) [15] In our study we see strong correlation between *CXCR3, CCR5* and *IFNG* at the RNA level, correlations which increased with asthma severity. Turning to murine models, *Ccr5*$^{-/-}$ mice have been shown to have diminished airway inflammation in a Type 2 dominant model induced with OVA [28], but when we examined *Ccr5*$^{-/-}$ in our SA model we observed minimal effect on Type 1 or Type 2 inflammation. As CCR5 is also an important recruiter of eosinophils [29, 30], it is again possible that this discrepancy in results rests in the variable Type 1 and Type 2 pathway induction between these models.

Prior studies have shown a dissociation between AHR and lung inflammation, suggesting that the molecular pathways for these components may be linked but separable.[20] Here, we show that in mice deficient in one of these receptors, significant overexpression of the other receptor is able to maintain increased AHR. However, blockade of the CCR5 pathway alone in WT mice led to a significant reduction in AHR with both pre-sensitization and pre-challenge (treatment) dosing strategies, suggesting that inhibition of the CCL5/CCR5 pathway was effective in blunting AHR even after airway inflammation was already established. In a similar fashion, it is possible that blockade of CXCR3 alone in a WT mouse might also reduce AHR. Notably, our findings regarding CCR5 and AHR are in keeping with our recent study evaluating severe asthma immune phenotypes by deep immune profiling of BAL cells through cytometry time of flight (CyTOF). In this study, which identified a severe asthma subgroup with lymphocyte-predominant/Th1^{High} asthma immune profile, CD161^{+}CCR5^{+} were the most strongly associated cell type with airway obstruction assessed via FEV1.[31] Since maraviroc is an FDA-approved drug for the treatment of patients with HIV, our findings raise the exciting possibility that it could be repurposed for the treatment of a subset of patients with SA.

The mechanism for this effect on AHR could be related to mast cell recruitment and activation. AHR has previously been linked to mast cell presence in an OVA model, with IFN-γ playing a significant role in mast cell function,[27] and Th1, rather than Th2, cells are known to play a significant role in rhinovirus related worsening lung function in asthma, arguing for a link between Type 1 signaling and AHR.[22, 32] We previously described airway mast cell presence in our murine SA model.[6] In our recently published study, the subset of SA patients characterized by a Type 1^{High} T-cell dominated immune response also displayed a signature of increased mast cell gene expression in their BAL cells.[31] Mast

cells can express both CCR5[33] and CXCR3[10] suggesting a role for epithelial and airway smooth muscle cells in the production of the cognate chemokines[34] in recruitment of mast cells to the airways. In addition to inflammatory signaling via histamines, leukotrienes and prostaglandins, mast cells can exert direct influence on AHR via protease activity through the protease activated receptors (PAR-2),[5, 35] a function which may be dependent on local inflammatory milieu.[36] This suggests that the AHR mediating effects of CCR5 inhibition could also be related to alterations in this milieu rather than solely impaired chemotaxis given the post-sensitization efficacy that we observed. Further examination of the roles of CXCR3, CCR5, and their ligands in mast cell recruitment and function in asthma are needed to fully understand the therapeutic potential of these pathways.

One caveat with regard to our study is that the cross-sectional nature of the SARP I/II cohort makes conclusions regarding the natural history and persistence of the Type 1 high phenotype that we have proposed impossible to ascertain. Longitudinal cohort studies with repeated airway sampling will be critical to understanding the natural history and persistence of this phenotype over time. Furthermore, the cross-sectional assessment prevents any prospective assessment of asthma severity or exacerbations as they pertain to the Type 1 high phenotype. As shown in Fig 1, the variance in *IFNG* expression is wide in the SA group without a clear point of demarcation for *IFNG* high status. While our study relies on a cutoff point defined by expression levels in healthy controls, future work will investigate the continuum of *IFNG* expression as it relates to disease control and expression levels of other inflammatory pathways.

Additionally, the mechanisms underlying Type 1 inflammation mediated airway inflammation and hyperreactivity specifically also remain unclear. How CCR5 directly contributes to AHR and how CCR5 blockade affects other pathways involved in Type 1 high asthma remain unelucidated and an important focus of future work. Pairing BAL cell content/RNA signatures with airway level changes at both the molecular and cellular level will also provide new insights into potential mechanisms and serves as an exciting future research direction to address these issues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AHR	Airway Hyperreactivity
CCL5	C-C Ligand 5 (RANTES)
CCR5	C-C chemokine receptor 5

CXCL9	C-X-C Ligand 9 (MIG)
CXCL10	C-X-C Ligand 10 (IP-10)
CXCR3	C-X-C chemokine receptor 3
HDM	House Dust Mite
ICS	Intracellular Staining
IFN-γ	Interferon-gamma
MMA	Mild to Moderate Asthma
SA	Severe Asthma
WT	Wild Type

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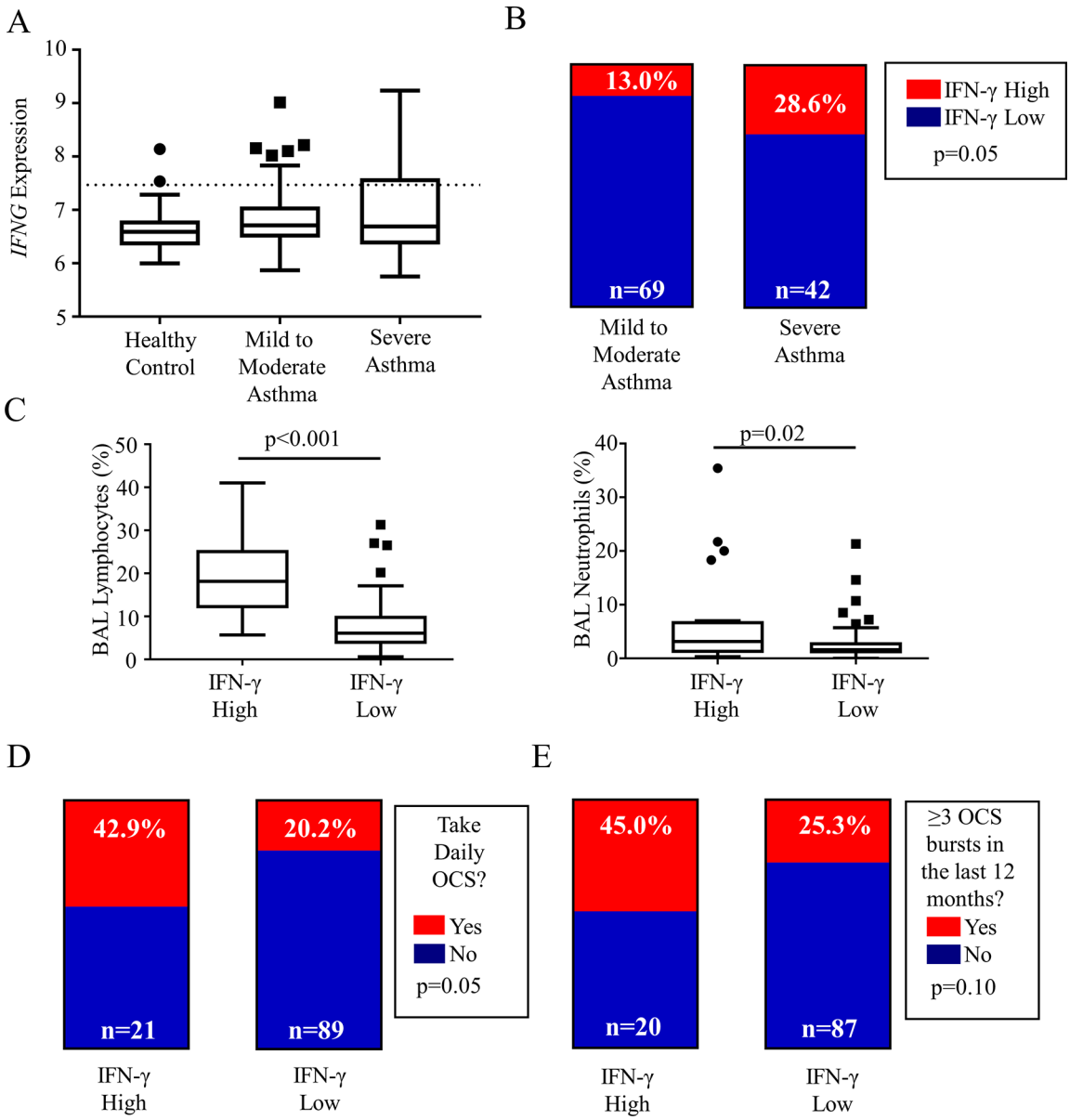

FIG 1:

IFN-γ is elevated in a sub-group of asthma patients with more severe disease. **A** Microarray data from SARP I/II bronchoalveolar lavage cell pellet RNA (BAL) was analyzed. *IFNG* expression in BAL is shown across asthma severity groups; while there was no statistical difference in median, a population of asthma patients with significantly elevated *IFNG* expression compared to healthy controls is noted; these patients were identified by a cutoff of two standard deviations above the healthy control median (y=7.467, dotted line). **B** After dividing the cohort into IFN-γ high and low, the prevalence of these groups was compared by asthma severity (Fisher's Exact test). **C** BAL Cell differentials (% cell content) were compared for lymphocytes and neutrophils between IFN-γ High and Low groups; Mann-Whitney U test. **D** and **E** Daily use of oral corticosteroid (OCS) and the presence of

frequent exacerbations (≥3 in the prior 12 months) were compared between IFN-γ High and Low Groups (Fisher's Exact Test)

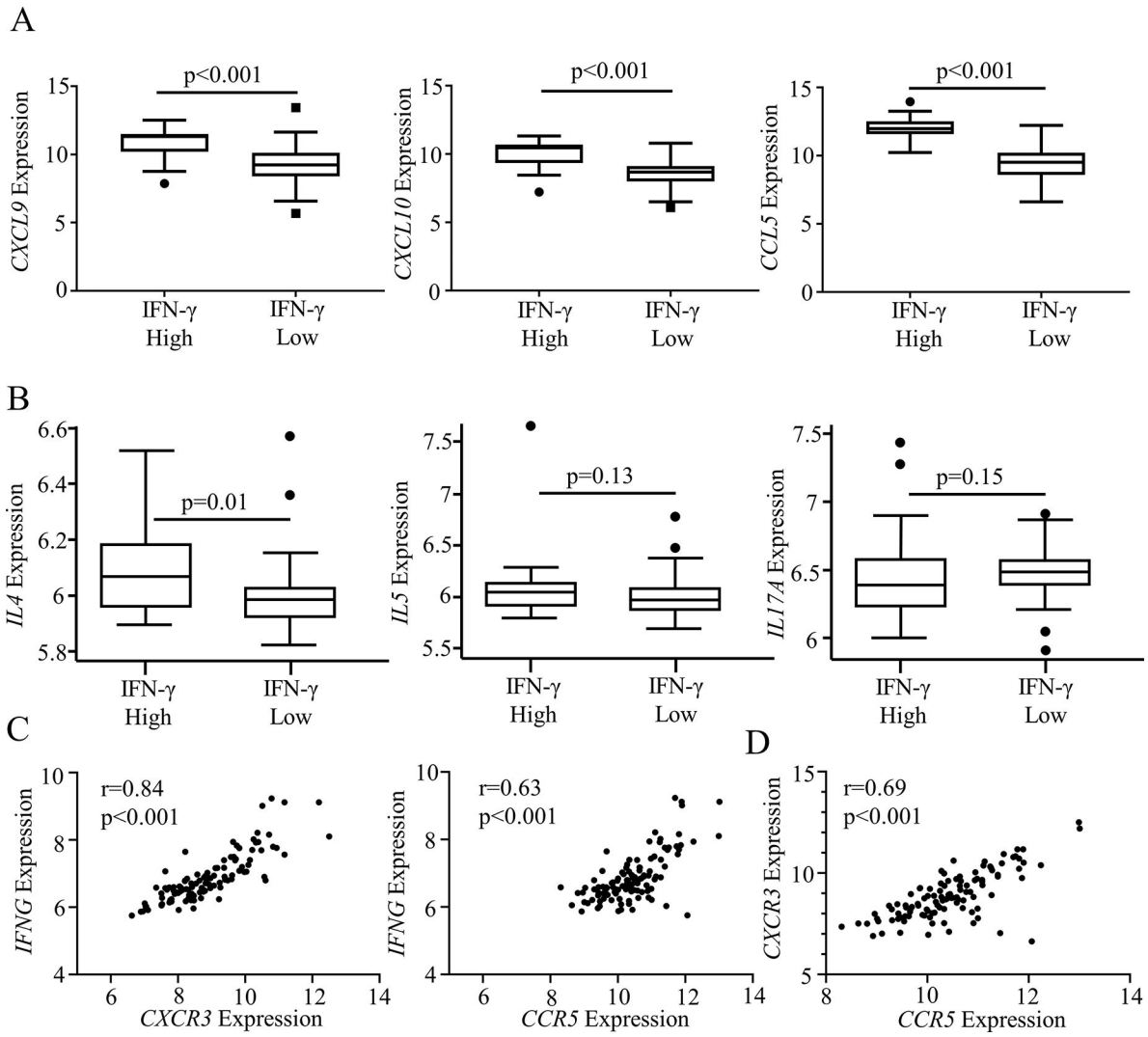

FIG 2:

There is a strong correlation between CXCR3 and CCR5 in asthma that suggests a dual chemotactic role. **A** Microarray data from SARP I/II bronchoalveolar lavage cell pellet RNA (BAL) was analyzed. *CXCL10* and *CCL5* expression in BAL was compared between IFN-γ High and Low groups (Mann-Whitney U test). **B** Expression of the Type 2 chemokine *IL4* was elevated in *IFNG* high subjects, while no difference was observed in the eosinophilic cytokine *IL5* or the Type 3 cytokine *IL17A* (Mann-Whitney U test). **C** Both *CXCR3* and *CCR5* correlate with *IFNG* suggesting a role in promoting Type 1 inflammation (Spearman Nonparametric Correlation). **D** There is strong correlation between both *CCR5* and *CXCR3* suggesting both pathways are upregulated in certain patients (Spearman Nonparametric Correlation).

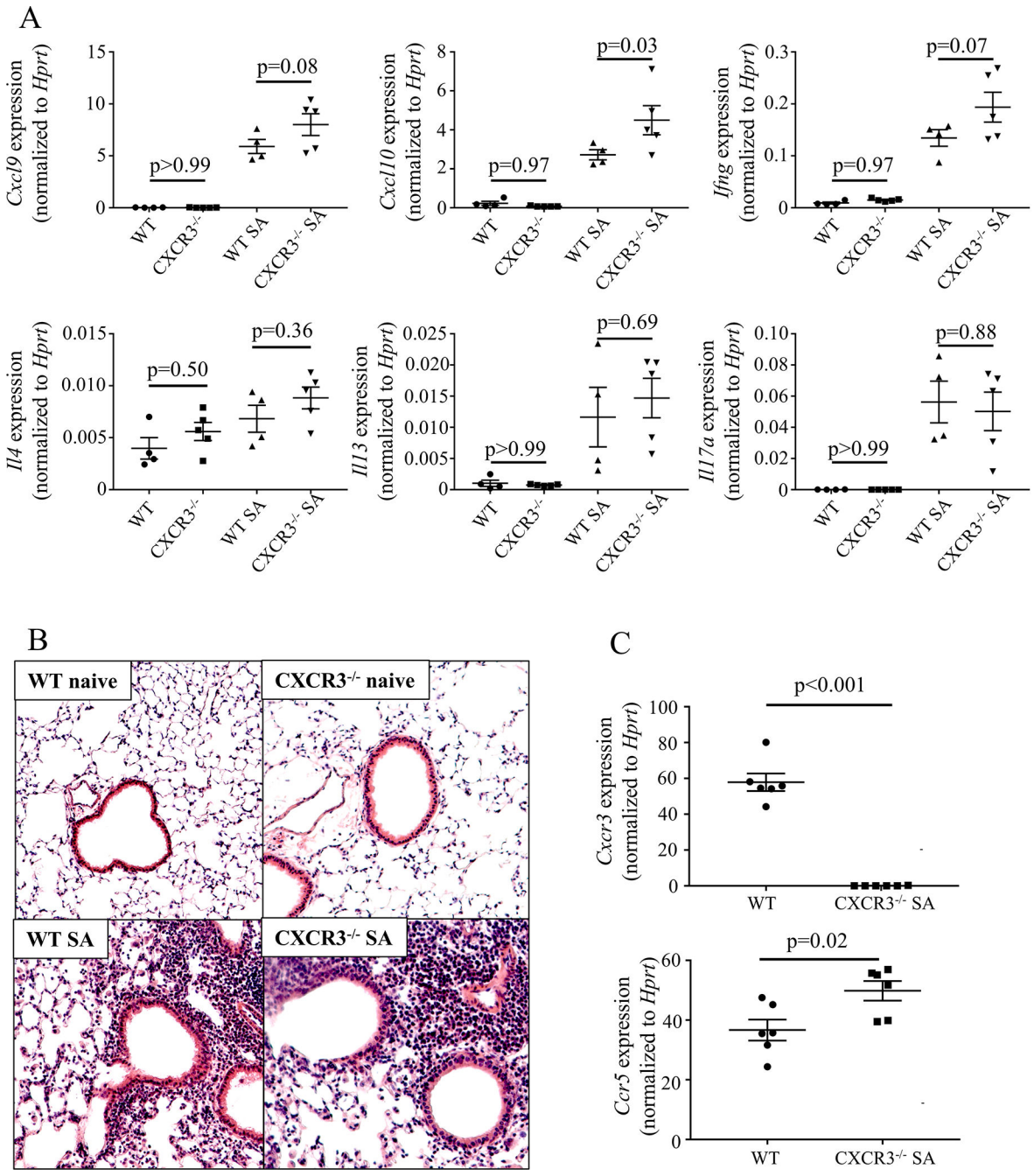

FIG 3:

Cxcr3$^{-/-}$ mice have a similar inflammatory profile and phenotype to C57Bl/6 mice in a murine severe asthma model. **A** Whole lung PCR compared cytokine expression between naïve and severe asthma model (SA) exposed WT and *Cxcr3*$^{-/-}$ mice (one-way ANOVA with $\alpha<0.05$ for each panel, Sidak's post-hoc test for within treatment comparison shown; data representative of two independent experiments). **B** H&E staining of lung sections shows similar inflammation between treatment groups in both WT mice and *Cxcr3*$^{-/-}$ mice (20x magnification, representative sections) **C** Whole lung PCR compared expression of the

Cxcr3 and *Ccr5* genes in WT and *Cxcr3*$^{-/-}$ mice (Student's T-test, data representative of 2 independent experiments).

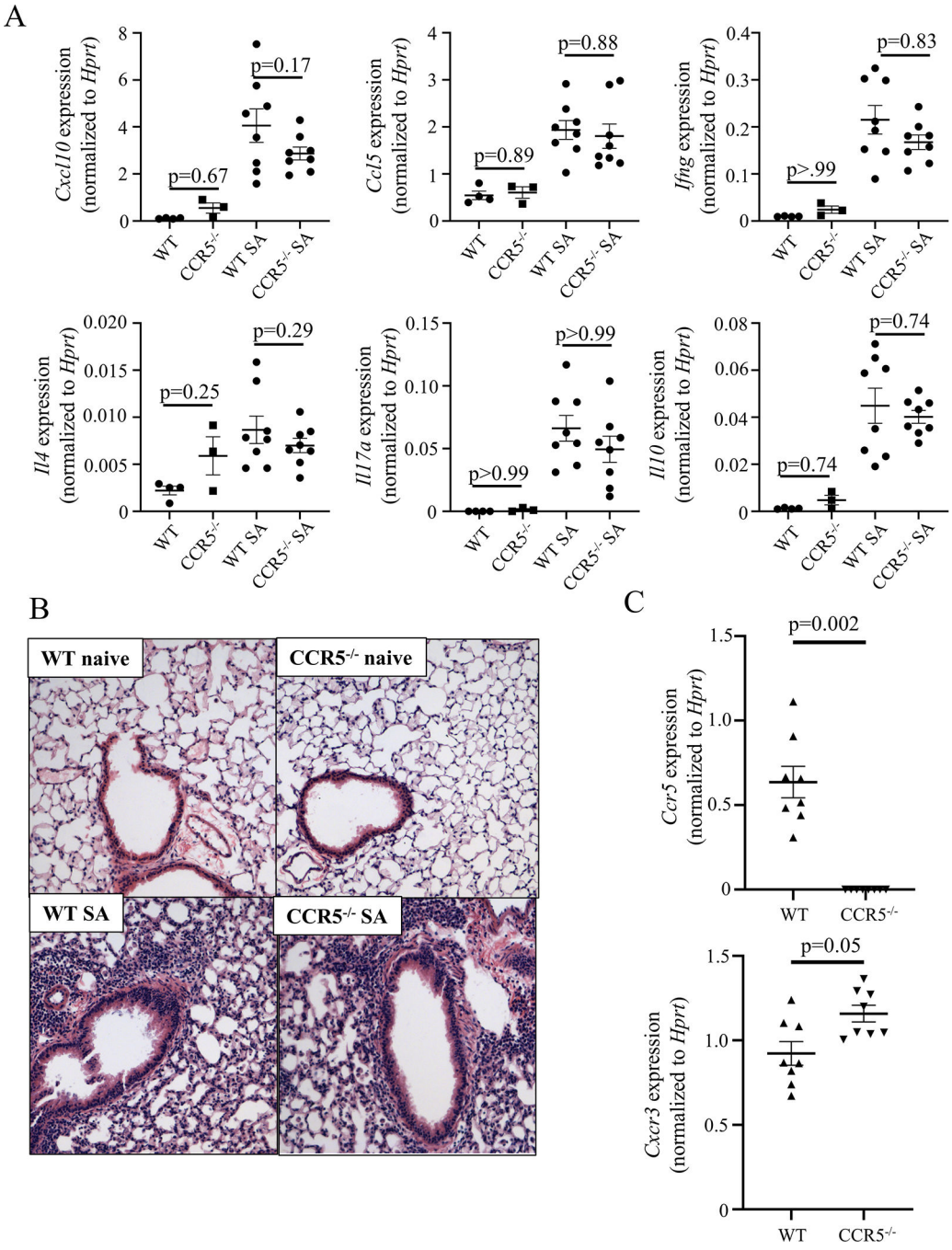

FIG 4:

Ccr5$^{-/-}$ mice also have a similar inflammatory profile and phenotype to WT mice in a murine severe asthma model. **A** Whole lung PCR compares cytokine expression between naïve and severe asthma model (SA) exposed WT mice and *Ccr5*$^{-/-}$ mice (One-way ANOVA with $\alpha<0.05$ is significant for each panel, Sidak's post-hoc test for within treatment comparison; data are pooled from 2 independent experiments). **B** H&E staining of lung sections shows similar inflammation between treatment groups in both WT mice and *Ccr5*$^{-/-}$ mice (20x magnification, representative sections). **C** Whole lung PCR compared

expression of the *Ccr5* and *Cxcr3* genes in WT and *Ccr5*$^{-/-}$ mice (Student's T-test, data pooled from 2 independent experiments).

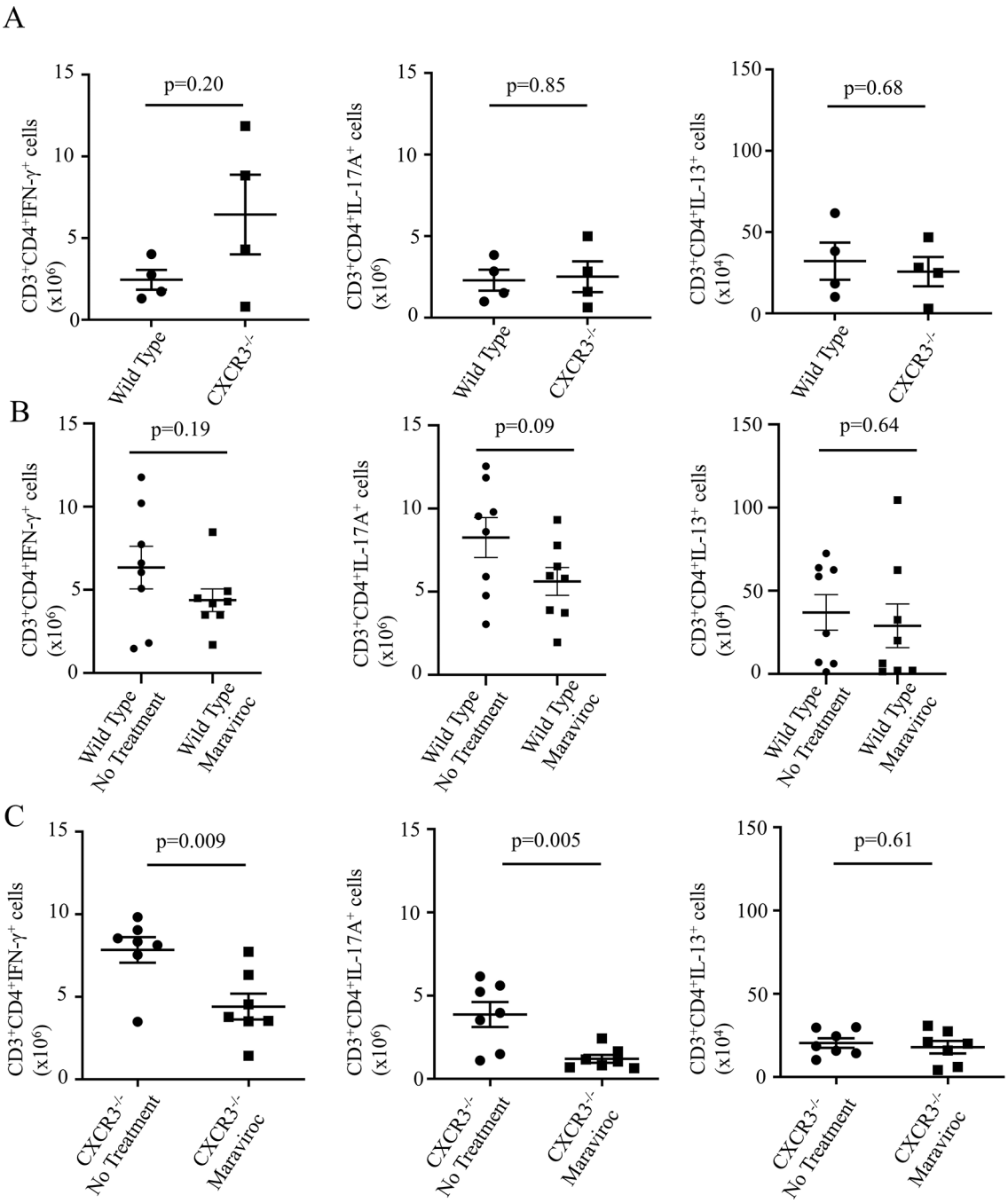

FIG 5:

Dual Receptor inhibition significantly reduces IFN-γ^+ cell recruitment to the lungs in a severe asthma model. **A** Assessment of lung T-lymphocyte ($CD3^+CD4^+$) cell populations by ICS in *Cxcr3*$^{-/-}$ mice shows no significant difference in T-cell subtype recruitment (Student's T-test, data representative of 2 independent experiments). **B** WT mice were treated with maraviroc or no treatment with a non-significant trend to decrease in IFN-γ^+ and IL-$17a^+$ cells noted in maraviroc treated mice with no change in IL-13^+ cells (Student's T-test, data pooled from 2 independent experiments). **C** *Cxcr3*$^{-/-}$ mice were treated with

maraviroc or no treatment with significant reductions in IFN-γ^+ and IL-17a$^+$ cells with maraviroc treatment and no change in IL-13$^+$ cells (Student's T-test, data pooled from 2 independent experiments).

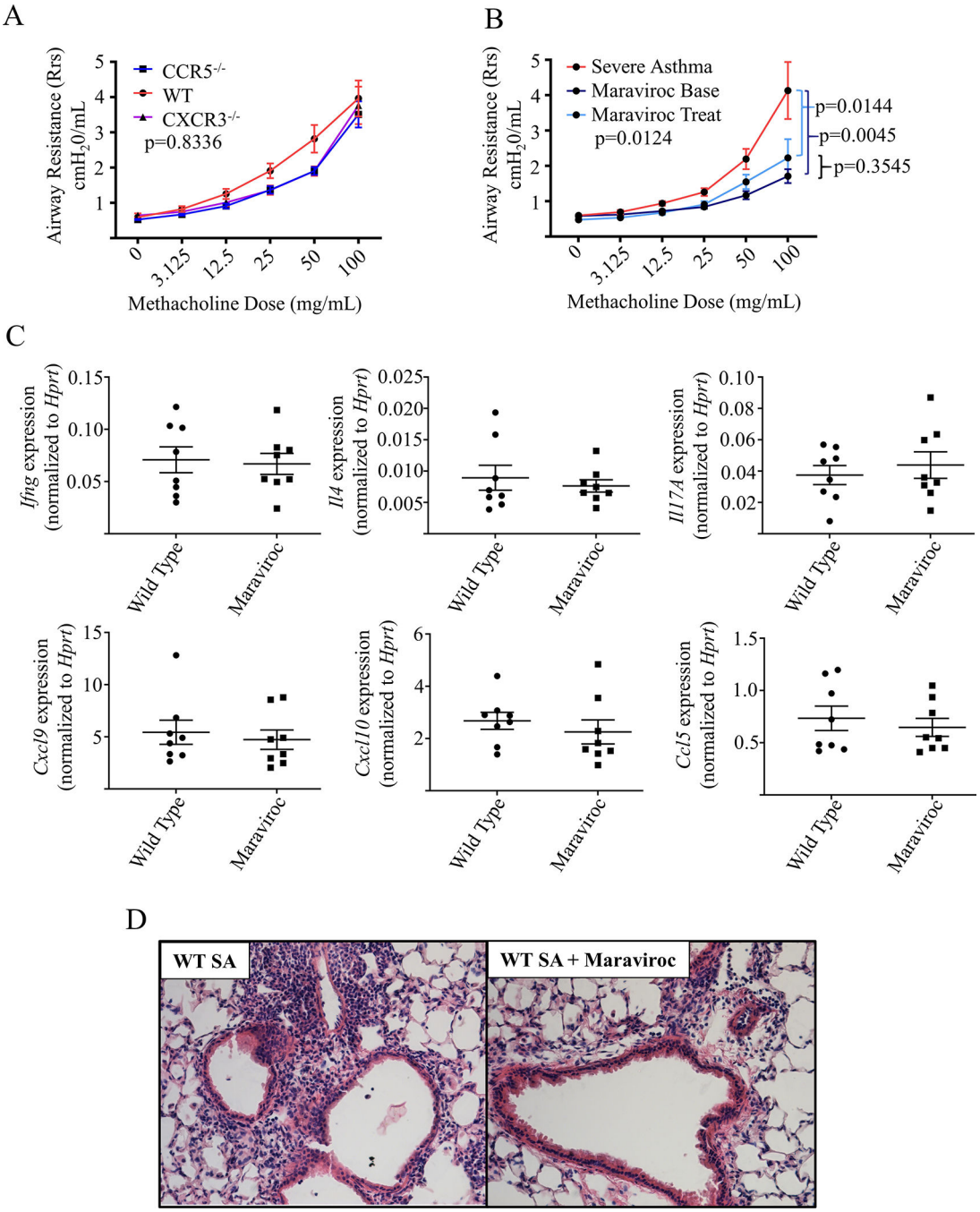

FIG 6:

Maraviroc effectively inhibits Airway hyperreactivity without modifying lung inflammation. **A** WT mice (WT, n = 7), *Cxcr3*$^{-/-}$ mice (n=5) and *Ccr5*$^{-/-}$ mice(n=6) were subjected to the SA model and underwent airway resistance testing with increasing concentrations of methacholine (data pooled from 2 separate experiments, Kruskall-Wallis testing). **B** WT mice were exposed to the SA model (Severe Asthma, n = 16) and a subset were treated with either maraviroc throughout the model beginning pre-sensitization (Maraviroc Base, n = 8) or post-sensitization but prior to challenges (Maraviroc Treat, n = 8) (data pooled

from 4 experiments, Kruskall-Wallis testing with Dunn's post hoc testing for intergroup comparisons). **C** WT and Maraviroc treated mice (Maraviroc) completed the full SA model and underwent assessment of whole lung cytokine expression by PCR with no significant differences observed. **D** Histology from WT and pre-challenge Maraviroc treated mice showed a modest improvement but residual inflammation in the maraviroc group (H&E staining, 20x magnification).

Table I:

Demographics and Clinical Parameters of IFN-γ high and low asthma patients.

		IFNG Low n=90	IFNG High n=21	p-value
Age at Enrollment (years)		**30.80(23.58–44.58)**	**43.53(34.38–51.00)**	**0.005**
Age at diagnosis (years)		7(3–12)	10.5(4–33)	0.16
Sex = 1		31 (34%)	7 (21%)	0.92
Race	1	26(20.8%)	6(27.3%)	0.82
	2	96(76.8%)	16(72.7%)	
	3	1(0.8%)	0(0%)	
	4	2(1.6%)	0(0%)	
BMI (kg/m^2)		29.04(25.14–34.35)	31.41(26.47–36)	0.23
Ever smoked		18(20.4%)	2(9.5%)	0.24
Atopy		85(75.2%)	18(81.82%)	0.51
Juniper AQLQ		4.87(3.57–5.53)	4.81(3.75–5.10)	0.37
FeNO (ppb)		32.5(20.0–62.3)	38(18.6–48.7)	0.34
IgE (IU/ml)		95.5(44.5–351.5)	166(43–280)	0.48
Sputum Eosinophils (%)		1.2(0.2–4.0)	1.7(0.7–3.6)	0.54
Blood Eosinophils (x10^3 cells/μL)		0.300(0.100–0.400)	0.150(0.100–0.300)	0.13
FEV1 (% predicted)		**80.0(64.0–97.0)**	**70.0(56.0–81.0)**	**0.05**
FVC (% predicted)		**92.0(77.5–103.0)**	**84.0(67.5–93.0)**	**0.02**
FEV1/FVC (x100)		72.7(64.2–80.4)	70.3(59.0–78.3)	0.19
ICS use		54(61.4%)	16(76.2%)	0.31
High Dose ICS		29(48.3%)	10(62.5%)	0.40
Anti-IgE Therapy		7(8.0%)	2(10.0%)	0.67

Continuous Variables are shown as median(25%–75%). Contingency Variables are shown as category(%). Continuous Variables analyzed by Mann-Whitney U-test, Contingency Variables with Fisher's Exact Test. Exact p-values shown for all tests. BMI = Body Mass Index, AQLQ = Asthma Quality of Life Questionairre, FeNO = Fraction of Exhaled Nitric Oxide, FEV1 = Forced Expiratory Volume at 1 Second, FVC = Forced Vital Capacity, ICS = Inhaled Corticosteroids.