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Chemokine Receptor 4 Plays a Key Role in T Cell Recruitment into the Airways of Asthmatic Patients

Pandurangan Vijayanand,* Kesta Durkin,* Guido Hartmann,† Jaymin Morjaria,* Gregory Seumois,* Karl J. Staples,* David Hall,‡ Christina Bessant,‡ Michelle Bartholomew,‡ Peter H. Howarth,* Peter S. Friedmann,* and Ratko Djukanović*†

T lymphocytes of the Th2 type are central orchestrators of airway inflammation in asthma. The mechanisms that regulate their accumulation in the asthmatic airways remains poorly understood. We tested the hypothesis that CCR4, preferentially expressed on T lymphocytes of the Th2 type, plays a critical role in this process. We enumerated by flow cytometry the CCR4-expressing T cells from blood, induced sputum, and biopsy samples of patients with asthma and control subjects. We showed a positive correlation between the numbers of peripheral blood CCR4+ T cells and asthma severity, provided evidence of preferential accumulation of CCR4+ T cells in asthmatic airways, and demonstrated that CCR4+ but not CCR4− cells from patients with asthma produce Th2 cytokines. Explanted airway mucosal biopsy specimens, acquired by bronchoscopy from subjects with asthma, were challenged with allergen and the explant supernatants assayed for T cell chemotactic activity. Allergen-induced ex vivo production of the CCR4 ligand, CCL17 was raised in explants from patients with asthma when compared with healthy controls. Using chemotaxis assays, we showed that the T cell chemotactic activity generated by bronchial explants can be blocked with a selective CCR4 antagonist or by depleting CCR4+ cells from responder cells. These results provide evidence that CCR4 might play a role in allergen-driven Th2 cell accumulation in asthmatic airways. Targeting this chemokine receptor in patients with asthma might reduce Th2 cell-driven airway inflammation; therefore, CCR4 antagonists could be an effective new therapy for asthma. This study also provides wider proof of concept for using tissue explants to study immunomodulatory drugs for asthma. The Journal of Immunology, 2010, 184: 4568–4574.

Asthma is one of the most common chronic diseases in the Western world, affecting >5 million people in the U.K. (1). The symptoms are thought to be the result of allergic inflammation of the airway, which is characterized by infiltrating eosinophils, mast cells, and basophils as prominent effector cells (2). T lymphocytes of the Th2 type (Th2 T cells), driven by chronic exposure to environmental airborne allergens, are increased in asthmatic airways and play a central role in orchestrating this inflammatory response by regulating IgE production, accumulation, and activation of eosinophils, as well as contributing to airway remodeling involving the epithelium and fibroblasts (3, 4). The accumulation of Th2 T cells in the lungs is essential for both the initiation and persistence of airway inflammation (3), and studies in asthmatic volunteers have shown marked increases in Th2 T cells in the lungs after in vivo allergen challenge (5). However, the mechanisms by which these cells accumulate within the asthmatic lungs are poorly understood, although a number of candidates, including the chemokine receptors CCR3, CCR4, and CCR8 and the PG D2 receptor CRTH2 (chemoattractant receptor-homologous molecule expressed on Th2 lymphocytes), have been implicated because of their preferential expression on T cells producing Th2 cytokines (6–12).

Targeted cellular movement in allergic inflammation likely involves an array of cytokines and other mediators, including histamine, tryptase and leukotrienes. Chemokines, a subfamily of cytokines that signal through 7-transmembrane G-protein-coupled receptors, have attracted particular interest in the hope that their selective inhibition might attenuate accumulation of those Th2 cells that are involved in disease, thereby providing more focused therapy (13). One of the main problems with this approach has been the redundancy of both chemokines and their receptors. More than 20 chemokine receptors and 40 chemokines have been characterized to date, with potential functional relevance to a variety of diseases, such as atherosclerosis, rheumatoid arthritis, lymphoma, cancer, and allergy (14). The levels of many of these chemokines have been found to be raised in asthmatic airways, be it in chronic disease or following acute exposure to an allergen (15, 16). However, without the benefit of functional studies to link the presence of these chemokines and their receptors to T cell accumulation, the findings have provided only circumstantial evidence for their role, and it has been unclear which chemoattractant pathway is integral to T cell accumulation.

Our previous work and that of others has identified increased T cell chemotactic activity in the asthmatic airways either in vivo, by sampling of the airway lining fluid by bronchoalveolar lavage (BAL) and sputum induction, or by using bronchial explants as an ex vivo model (15–17). Although these studies have provided useful insight into some of the mechanisms regulating T cell chemotactic activity,
they have not yet resolved the critical question of specificity and redundancy of the chemokine–chemokine receptor network. We hypothesized that CCR4, preferentially although not exclusively, expressed on human Th2 T cells (9, 11), and its ligands CCL17 and CCL22 (previously known as macrophage-derived chemokine and thymus and activation-regulated chemokine) (12), play key roles in T cell chemotaxis into the bronchial tissues in asthma.

Studies into the role of CCR4 in animal models of allergic lung inflammation, using gene deletion or neutralizing Abs, have provided conflicting results (18–21). Evidence in human asthma has remained circumstantial, being based on the demonstration that CCR4+ cell counts and the expression of the CCR4 ligands CCL17 and CCL22 increases in the airways after allergen challenge of mild, steroid-naive patients with asthma (5, 15, 22).

We have sought to place CCR4 within the context of chronic asthma and to relate its role to disease severity by quantifying the expression of CCR4 on circulating and airway T cells in patients with asthma ranging from mild steroid-naive to severe, requiring corticosteroids for symptom control. Using a human experimental asthma model, where bronchial mucosal tissue, obtained by fiberoptic bronchoscopy, can be studied ex vivo, we then sought to identify a functional role for CCR4 and its ligands. We re-created the in vivo process of allergen-driven migration of T cells from blood to the airways by challenging bronchial biopsies with allergen and assessing the released chemotactic activity using the same patient’s blood-derived CD45RO+ T cells (memory/activated T cells) as responder cells. In this ex vivo model, as a proof-of-principle experiment to study loss of function, we then used a highly selective CCR4 antagonist to determine whether the CCR4-chemokine axis plays a dominant role in the T cell chemotactic activity generated by allergen-challenged asthmatic airways.

Materials and Methods
Study subjects
The study was approved by the Ethics Committees of the Southampton University Hospitals Trust, and written informed consent was obtained from all subjects. Twenty-one healthy subjects and 56 with asthma (19 mild and steroid-naive, 25 moderate and treated with low-dose inhaled corticosteroids, and 12 severe taking oral or high-dose inhaled corticosteroids) meeting established diagnostic criteria were studied (23, 24). Allergic status of all study subjects was determined by skin-prick testing for common aeroallergens. All asthmatic subjects who underwent bronchoscopy had a positive result from a skin-prick test for house dust mites (Dermatophagoides pteronyssinus), which was used in the explant model as the allergen stimulant.

Acquisition of samples and processing
Airway lumen cells were acquired by sputum induction, a reproducible and widely used method that was performed in this study according to the recommendations of the European Respiratory Society (25). Sputum samples were solubilized using dithioerythritol to allow separation of the cellular and fluid phases. Cell aliquots were then processed for flow cytometric analysis as previously reported (4).

Bronchial mucosal samples were obtained by endobronchial biopsy from 47 subjects (14 healthy, 18 with mild asthma, and 15 with moderate asthma) using fiberoptic bronchoscopy conducted under local anesthesia according to the recommendations of the American Thoracic Society (26). Biopsies were initially weighed (to normalize cytokine–chemokine production for amounts of tissue) and then cultured for 24 h in AIM-V serum free media supplemented with 2 mM l-glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin, 2 mM mercaptoethanol, 0.5 g/ml fungizone, and 1 mM sodium pyruvate (Life Technologies, Paisley, U.K.). As many as eight biopsy specimens were taken from each subject, with two specimens being placed in wells (24-well plate) containing 550 ml of medium alone (unchallenged control explants) or medium supplemented with 5000 standardized quality units/ml of house dust mite (D. pteronyssinus) allergen (ALK, Horsholm, Denmark). After 24 h of culture, the conditioned medium (CM) was collected from both conditions and stored at −80°C until further analysis. Biopsy specimens from 10 patients with mild asthma and 10 with moderately severe asthma were also treated with collagenase to obtain dispersed T cells, as previously reported (4). In pilot experiments, this was shown not to affect CCR4 expression. Blood samples were collected and PBMCs were isolated by density centrifugation.

Flow cytometric analysis of CCR4 expression on peripheral blood and airway T cells
Cell surface expression of CCR4 on PBMCs, sputum cells, and dispersed bronchial biopsies was quantified by flow cytometry using either the FACScalibur for blood and sputum cells or the FACSAnia for tissue cells (BD Biosience, Oxford, U.K.) as described (4). The following combination of mAbs was used for analysis of peripheral blood CCR4+ cells: anti-CD45 PE-Cy5-conjugated and FITC-conjugated (BD Biosience), anti-CD3 APC-conjugated (BD Bioscience), anti-CCR4 PE-conjugated (R&D Systems, Abingdon, U.K.) and mouse IgG2a PE-conjugated isotype control Ab (R&D Systems). For the analysis of airway lumen (sputum) cells anti-CD3 (APC-conjugated; BD Bioscience), anti-CCR4 (PE-conjugated), and mouse IgG2a (PE-conjugated) isotype control Ab were used. Dispersed bronchial biopsy cells were analyzed with the following Abs: anti-CD3 (APC-conjugated), anti-CCR4 (PE-conjugated), and mouse IgG2a isotype control (PE-conjugated). Analysis was performed using Cellquest or Diva software (BD Bioscience). CCR4 expression on peripheral blood cells was shown as percentages of CD4+ T cells that were also CCR4+. In view of the effects of collagenase treatment on CD4, CCR4 expression was shown as a percentage of CD3+ T cells in the biopsy samples. For consistency, the same approach was taken in respect of sputum cells.

Measurement of cytokines and chemokines
The concentrations of chemokines (CCL1.11, CCL20, CCL19, CCL4, CCL2, and CCL5) in biopsy culture supernatants were determined using Search Light custom-made human chemokine array following the manufacturer’s instructions (Pierce, Northumberland, U.K.). The concentrations of multiple cytokines (IL-2, IL-4, IL-5, IL-10, IL-13, TNF-α, IFN-γ) and the CCR4 ligand chemokines (CCL17 and CCL22) in biopsy culture supernatants were determined using the Luminex assay (Bio-Rad, Hemel Hempstead, U.K.). Capture and detection Abs were from R&D Systems (CCL17, CCL22, TNF-α), Pharmingen, Oxford, U.K. (IL-2, IL-4 detection Ab, IL-5 detection Ab, IL-13), Endogen, Northumberland, U.K. (IFN-γ, IL-10, IL-4 detection Ab), and GlaxoSmithKline (Stevenage, U.K.; IL-5 capture Ab).

T cell chemotaxis assay
We re-created ex vivo the process of inhaled allergen-induced T cell migration from blood into the airways of patients with asthma. We used explant CM as the source of airway chemokines, which was produced in view of the effects of collagenase treatment on CD4, CCR4 expression was shown as a percentage of CD3+ T cells in the biopsy samples. For CCR4, the same approach was taken in respect of sputum cells.

Migration of memory T cells (CD4+CD45RO+) cells was tested for the following conditions: CM with allergen only (i.e., no tissue), CM from allergen-stimulated explants, and CM from allergen–stimulated explants, with the addition of the selective CCR4 antagonist (compound 1, which was synthesized by GSK). CCL22 was used as a positive control at 100 nM concentration. The CCR4 antagonist was used at 10 nM, a concentration that had been shown in prior experiments to be highly effective at inhibiting CCL22-induced chemotaxis. The specificity of the antagonist at this concentration was tested using a panel of chemokines that acted on different chemokine receptors and showed no effect on CCR5, CCR3, CCR2, and CXCR3 (Supplemental Fig. 5).

CCR4 depletion studies
CCR4+ T cells were depleted from PBMCs by magnetic automated cell sorting of cells prelabeled with anti-CCR4 PE-conjugated Ab (R&D Systems), which was used in the explant model as the allergen stimulant. We re-created ex vivo the process of inhaled allergen-induced T cell migration from blood into the airways of patients with asthma. We used explant CM as the source of airway chemokines, which was produced in view of the effects of collagenase treatment on CD4, CCR4 expression was shown as a percentage of CD3+ T cells in the biopsy samples. For CCR4, the same approach was taken in respect of sputum cells.

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Systems) and anti-PE microbeads (Miltenyi Biotec). PBMCs or PBMCs depleted of CCR4+ T cells from the same donors were stimulated for 7 d with D. pteronyssinus (house dust mite) allergen (ALK) at 5000 standardized quality units/ml or tetanus toxoid (1:11,000; Diftavax, Aventis Pasteur, Berkshire, U.K.). The doses and time-courses were previously shown to be optimal time for the assessment of cytokine production by PBMC (data not shown). Th2 and Th1 cytokines levels were measured in the supernatants using the Luminex assay as described above.

Statistical analysis

The levels of CCR4 expression were normally distributed across the subject populations studied, and parametric tests were applied for statistical comparisons. The data from each subject group were analyzed by ANOVA to study the statistical difference among the four groups, and each difference within two groups was further confirmed by the Scheffe post hoc test. For other comparisons (normally distributed values) between two groups, the Student two-tailed t test was applied. Cytokine and chemokine values from bronchial explant cultures were not normally distributed, so nonparametric tests were applied, with the Wilcoxon signed-ranks test and the Mann-Whitney U test being applied for comparison between paired and unpaired samples, respectively. Data are displayed in box and whisker plots to illustrate medians and the distribution.

Results

CCR4-expressing T cells are increased in the blood and airways of subjects with asthma

We first investigated the extent of CCR4 expression on peripheral blood CD4+ T cells by flow cytometry. To relate this to asthma severity, we studied patients with mild asthma treated with bronchodilators alone (n = 12), moderate asthma requiring inhaled corticosteroids (n = 12), and severe asthma treated with oral corticosteroids (n = 12). In healthy, nonatopic individuals, a mean ± SEM of 22.8% ± 1.4 of peripheral blood CD4+ T cells expressed CCR4. Although the numbers of CCR4+ T cells in patients with mild asthma (27.4% ± 2.6) were slightly but not significantly raised, significant increases were seen in moderate and severe asthma (mean 1.6-fold and 2.4-fold, respectively; Fig. 1A), thus showing a convincing relationship between CCR4 expression in blood and disease severity.

Analysis of airway lumen cells, sampled by sputum induction, showed that a mean of 40% of CD3+ T cells in healthy individuals was CCR4+. However, similar to blood, CCR4+ cell numbers were significantly higher in patients with asthma (>60%), albeit without any differences between mild, moderate, and severe disease (Fig. 1B). Using fiberoptic bronchoscopy, endobronchial biopsy specimens were taken from healthy volunteers and patients with mild or moderately severe asthma to assess whether enrichment of CCR4+ cells in asthma was also a feature in the mucosa. T cells were extracted by enzymatic dispersion, and flow cytometric analysis of CD3+ T cells was undertaken; this showed that CCR4+ T cells were also markedly enriched in the mucosa (Fig. 1C). When compared with healthy individuals, who had a mean ± SEM of 29% ± 3.1 of CCR4+ T cells, CCR4+ T cells were increased in both mild (65% ± 4.6 of CD3+ T cells) and moderately severe asthma (59% ± 3.6), despite treatment of the latter with inhaled corticosteroids.

CCR4+ T cells are the principal source of Th2 cytokines

Whereas Th2 cells have been shown to preferentially express surface CCR4, these observations have been made in vitro under non-physiologic polarizing conditions, using IL-4 and anti–IFN-γ Ab (10), which makes it difficult to extrapolate the findings to the in vivo setting of natural disease. Therefore, we selectively removed CCR4+ T cells by magnetic cell sorting from the PBMCs of donors with asthma and assessed the remaining CCR4− cells for cytokine production in response to stimulation with house dust mite allergen extract (D. pteronyssinus). This resulted in a significant (p < 0.05) reduction of the allergen-induced in vitro release of the Th2 cytokines, IL-4, IL-5 and IL-13, without affecting the Th1

![FIGURE 1. Increased CCR4 expression on T cells in patients with asthma, as assessed by flow cytometry. A. Flow cytometric analysis of blood CCR4+ T cells was performed on PBMCs and results expressed as percentages of CD4+ T cells (n = 12 in each group). B. Sputum was induced, and acquired samples were solubilized with dithioerythritol to obtain T cells residing in the bronchial lumen. The cellular fraction was analyzed as previously described (4). The results are also expressed as percentages of CD3+ T cells that are CCR4+ (n = 7 in each group). To analyze mucosal T cells, bronchial biopsies from healthy subjects (n = 6) and those with asthma (mild and moderate, n = 10 in each group) were treated with collagenase. Because this treatment affects surface CD4 expression, the expression of CCR4 was shown as a percentage of CD3+ T cells (C). Representative flow cytometric plots are shown for T cells expressing CCR4 in blood of a healthy subject (D), sputum of a patient with moderate asthma (E), and dispersed bronchial biopsy specimen from subject with moderate asthma (F). All data are shown as mean ± SEM; p value was obtained by ANOVA with Scheffe test. *p < 0.05; **p < 0.001.](http://www.jimmunol.org/ Downloaded from)
response to tetanus toxoid (Fig. 2, Supplemental Fig. 1). In addition, we performed intracellular flow cytometry to confirm that Th2 cytokine-producing cells are mainly confined to the CCR4+ peripheral blood CD4+ T cells (Supplemental Fig. 2). These results indicated that CCR4+ T cells are the major source of Th2 cytokines, and selective inhibition of their migration from blood to the airways would be predicted to inhibit Th2-dependent airway inflammation in patients with asthma without affecting the potential for Th1 responses if CCR4+ are selectively reduced in the lungs.

Bronchial mucosa of subjects with asthma releases Th2 cytokines after allergen challenge

Having observed abundant CCR4 expression in both the airways lumen and mucosa of patients with asthma, we used the bronchial explant model, which we have developed for ex vivo study of mechanisms of mucosal inflammation, to investigate the relevance of this finding on the production of Th2 cytokines in the airways (17, 27). Consistent with past observations during in vivo allergen challenge of individuals with asthma, the addition of house dust mite allergen extract resulted in significant release of Th2 cytokines IL-5 \( (p = 0.002) \) and IL-13 \( (p = 0.01) \) in the CM of bronchial explants from patients with mild asthma, but not healthy subjects, thus confirming the Th2 profile of explant tissue responses that mirror those seen in vivo (Fig. 3A, 3B). In contrast, patients with moderate asthma, who were well controlled with regular inhaled corticosteroids, displayed a cytokine profile similar to healthy subjects, suggesting effective Th2 cytokine suppression by corticosteroids in vivo (Fig. 3A, 3B).

**FIGURE 2.** Depletion of CCR4+ T cells from PBMCs from patients with atopic asthma inhibits the production of Th2 cytokines in response to stimulation with allergen, without affecting Th1 cytokine responses to tetanus toxoid. A, Whole PBMCs and PBMCs depleted of CCR4+ T cells from atopic asthmatics \( (n = 4) \) were stimulated by allergen \( (D. pteronyssinus [Der p]) \). IL-5, IL-13, and IL4 were measured in culture supernatants. B, The production of Th1/Th0 cytokines (IFN-\( \gamma \), TNF-\( \alpha \), and IL-2) responses was studied using tetanus toxoid to stimulate whole PBMCs and PBMCs depleted of CCR4+ T cells. \( *p < 0.05 \), Student \( t \) test in two-tailed analysis.

**CCR4 ligand (CCL17) is preferentially released by bronchial explants from subjects with asthma after allergen challenge**

We hypothesized that the accumulation of CCR4+ cells can result from enhanced recruitment by the CCR4 ligands CCL17 and CCL22 released by mucosal cells stimulated by allergen. Analysis of CM showed that spontaneous release (seen in unchallenged explants) of CCL17, but not CCL22, was higher in both mild and moderate asthma when compared with nonatopic control subjects (Fig. 3C, 3D), suggesting upregulation of CCL17 that had occurred in vivo as part of an ongoing (i.e., a chronic, inflammatory response that was partly steroid refractory). We speculate that the predominant cell types (epithelial and dendritic cells) responsible for the production of CCL17 and CCL22, respectively, might be differentially sensitive to the effects of steroid treatment. In addition, allergen stimulation of the explants induced a significant increase in CCL17 in mild asthma but had no effect in moderate asthma. Allergen stimulation had no effect on the release of other chemokines, CCL11, CCL20, CCL19, CCL4, CCL2, or CCL5, although comparison of spontaneous production showed a significant increase in CCL5, CCL2, CCL11, and CCL19 in mild but not in moderate asthma (Supplemental Fig. 3). The selective increase in the release of CCL17 after allergen challenge strongly suggested that asthmatic airways have the potential to preferentially chemoattract CCR4+ cells when stimulated with allergen, providing further support for the notion that the CCR4-chemokine axis plays a key role in allergen-induced asthma.

**Bronchial explants from subjects with asthma generate increased T cell chemotactic activity after allergen challenge**

We have previously shown that allergen stimulation of bronchial explants from both mild steroid-naive and moderate asthma patients treated with corticosteroids (but not healthy, nonatopic individuals) releases T cell chemotactic activities, the activity of which can be demonstrated in chemotaxis assays in which unselected CD3+ T cells are used as responder cells (17, 27). In this study, we have modified our method by selecting CD4+/CD45RO+ cells as responders based on pilot experiments showing that the majority of airway CD4+ T cells are of the CD45RO+ phenotype and that this population contains the CCR4+ cells (4). The CM from allergen-challenged explants derived from both mild and moderately severe asthma treated with inhaled corticosteroids contained significant T cell chemotactic activity when compared with medium alone; in contrast, no significant activity could be detected in CM from healthy subjects (Fig. 4A). Consistent with the finding of similar numbers of CCR4+ cells in the enzymatically treated biopsy specimens in mild and moderate asthma, the chemotactic activity in allergen-challenged explants was similar in these two patient categories (mean \( \pm \) SEM chemotactic indices of 1.90 \( \pm \) 0.17 and 1.85 \( \pm \) 0.15, respectively, with the chemotactic indices calculated as the numbers of T cells migrating in response to CM divided by the numbers migrating spontaneously.

**CCR4 antagonism blocks the enhanced T cell chemotactic activity generated by bronchial explants from subjects with asthma**

Although the above experiments demonstrated the potential for allergen-stimulated mucosal tissue from patients with asthma to recruit CCR4+ T cells, they did not provide evidence of specificity. Therefore, the dependency of the chemotactic activity on the CCR4-chemokine axis was further investigated by using a potent CCR4 antagonist.
FIGURE 3. Allergen induces the production of Th2 cytokines and the CCR4 ligand CCL17 in bronchial explants from asthmatic patients. Bronchial biopsy specimens were obtained from healthy subjects ($n = 14$), subjects with mild steroid-naive asthma ($n = 18$), and subjects with moderately severe asthma treated with inhaled corticosteroids ($n = 15$); these were stimulated ex vivo with *D. pteronyssinus* allergen for 24 h. Culture supernatants were assayed using a combination of Multiplex ELISA (by Luminex) and SearchLight for the Th2 cytokines, IL-5 (A) and IL-13 (B), the CCR4 ligands, CCL17 (C) and CCL22 (D), and several other chemokines of relevance to asthma (see Supplemental Material). Concentrations are shown relative to tissue weight. *p* < 0.05, by the Wilcoxon signed-ranks test and Mann-Whitney $U$ test for comparison between paired and unpaired samples.

Discussion

Chemokines and their receptors have been implicated in the pathogenesis of various human inflammatory diseases and are viewed as promising targets for novel immunomodulatory treatments (13). To date, the evidence for their role and their selection as targets for new drugs has been based mostly on in vitro studies and strengthened by functional studies in animal models of human diseases. Further evidence has been sought in descriptive studies in human volunteers in which only associations between chemokine–chemokine receptors could be established without firm cause–effect relationships. Although studies in animals provide extremely valuable novel insight into disease processes and early evidence for drug efficacy, their relevance to human disease remains uncertain until the pathologic findings have been reproduced in human disease. Furthermore, proof of concept for the efficacy of novel drugs cannot be obtained until these are tested in clinical trials, which are expensive and time-consuming.

In this translational study, we have tested the clinical relevance of CCR4 as a target in asthma. We first showed that CCR4 expression in T cells correlates with progression of asthma disease severity, thus providing circumstantial evidence for a role of CCR4 in asthma and suggesting that targeting CCR4 might have the greatest effect in patients with severe disease. This finding was consistent with previous observations in patients with an allergic inflammatory skin disease (28). Treatment of asthma with oral steroids has been shown previously not to increase the numbers of CCR4+ T cells in the blood (29); thus, the observed increased expression is linked to disease severity rather than corticosteroid treatment. This finding raised the possibility that the increased expression of CCR4 can be attributed to the expansion of Th2 cells, which could contribute to both chronic disease and allergen-induced exacerbations.

Contrary to our observation in blood T cells, our results of numbers of CCR4+ T cells in the airways do not show a severity-related gradient. It is possible that if the corticosteroid treatment of these subjects were reduced, the number of CCR4+ T cells would increase further in the airways in a pattern that is seen in the circulation, but such an intervention is not possible for ethical and safety reasons. However, there are two pertinent observations: 1) patients with mild asthma (corticosteroid naive) have increased numbers of CCR4+ T cells in the airways in a pattern that is seen in the circulation, but such an intervention is not possible for ethical and safety reasons. However, there are two pertinent observations: 1) patients with mild asthma (corticosteroid naive) have increased numbers of CCR4+ T cells in the airways when compared with healthy subjects; and 2) despite corticosteroid treatment, which is the current standard, both moderate and severe asthmatic patients have increased numbers of CCR4+ T cells when compared with healthy subjects. This raises the question as to whether the mechanisms responsible for the increased numbers of CCR4+ T cells in the airways of patients with asthma are...
The chemotactic activity for T cells produced by bronchial explant stimulated with the *Dermatophagoides pteronyssinus* (*Der p*) allergen in bronchial explants from steroid-naive (mild asthmatics) and steroid-treated asthmatics (moderate asthmatics) is dependent upon CCR4. (A) Explants from healthy subjects (*n* = 6), mild steroid-naive atopic asthmatics (*n* = 8) and moderately severe asthmatics treated with inhaled corticosteroids (*n* = 9) were treated with allergen. The CM was used in chemotaxis assays and CD45RO+ enriched cells from the same volunteer who donated the biopsies were used as responder cells. The dependency of the chemotactic response to CM was studied in all three subject groups using three approaches: (A) applying a specific CCR4 blocker (full bars), and (B) using CD45RO+ cells depleted of or enriched for CCR4+ cells. For comparison of effectiveness in mild asthma, separate explants (*n* = 4) were treated with 300 nM fluticasone, a standard corticosteroid used in asthma treatment (C). Chemotaxis index is calculated as the number of T cells migrating in response to CM divided by the number migrating spontaneously. The spontaneous migration in culture medium is given a chemotaxis index of 1.

Previous studies have provided a convincing case for surface CCR4 expression being a marker of Th2 cells (6, 7, 9–11). One limitation of these studies has been the fact that CCR4 expression was induced in nonphysiologic conditions, using cytokines that polarize T cells into the Th2 phenotype. Complementing these studies, we have now shown that the selective removal of these CCR4+ T cells from blood significantly reduced allergic inflammation, as shown by a marked reduction in the production of the Th2 cytokines IL-4, IL-5, and IL-13, but with no consequences for Th1 responses. In further experiments, using intracellular cytokine staining, we have shown that Th2 cytokines are predominantly expressed in CCR4+ T cells (Supplemental Fig. 2). This study suggests that inhibiting the migration of T cells to the asthmatic airway by targeting CCR4 might abrogate the allergic inflammation in the airways without affecting immune responses that serve to protect against infection. However, other cell types, including mast cells, basophils, eosinophils, and alternatively activated macrophages, could be an additional source of Th2-type cytokines, and it is possible that effective inhibition of CCR4+ Th2-cytokine producing T cells might leave sufficient Th2 cytokines from non-T cell sources to enable persistence of Th2-mediated inflammation. Furthermore, there is evidence that Th1 and Th17 cytokines play a role in asthma, the latter being possibly more relevant to neutrophilic forms of asthma (30). Asthma is now widely viewed as a syndrome composed of several phenotypes, some of which may be driven predominantly by Th2 cytokines; others might involve other cytokines to a greater extent. Thus, a clinical trial would be required to validate the concept that targeting CCR4+ T cells would have a significant effect on airway inflammation in asthma.

The presence of CCR4 on a high percentage of T cells, especially within the airway, also raises questions of possible additional function of this chemokine receptor. Indeed, studies by other authors and our own unpublished observations showing that naturally occurring FOXP3+ regulatory T cells also express CCR4 point to a potential role for CCR4 in the trafficking of these important regulators of immune function (31). These findings raise the question as to whether targeting CCR4 could have adverse consequences because of the reduction of FOXP3+ (forkhead box P3) regulatory T cells in the airways. It is possible that the optimal effect will require a balance between CCR4+ effector T cells and CCR4+ regulatory T cells. Furthermore, understanding the biological role of these naturally occurring regulatory T cells in the airway of subjects with asthma would help to clarify this issue. Reduced function and/or numbers of regulatory T cells have been implicated in the pathogenesis of a number of conditions, including allergy and autoimmune disorders (32).

This study in human asthma goes beyond description and correlation, and it provides new functional insight into the physiologic relevance of a fundamental immunologic process that maintains allergic inflammation in asthma. In addition, this study emphasizes the value of exploratory ex vivo approaches in humans as an intermediary step before contemplating clinical trials. This study provides a potential template design for proof-of-concept studies.
that also help select the patient subpopulation in which a CCR4 antagonist is likely to work best. We also propose that CCR4 expression on T cells could be an important diagnostic and prognostic biomarker of allergic diseases and could be readily used as an indicator of response to treatment with asthma drugs that target Th2 T cells. In summary, this study provides translational evidence that the CCR4-chemokine axis plays a key role in the trafficking of T cells into the human asthmatic airways.

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Disclosures
R.D. has received funding from GlaxoSmithKline for past conference participation and lecturing.

References
SUPPLEMENTAL DATA

Figure S1

a) IL-5

b) IFN-γ

IL-13

TNF

IL-4

IL-2

Tetanus Toxoid: - - + +
Der p: - - - -
CCR4+ T cells: + + - -
Figure S1: Whole PBMC and PBMC depleted of CCR4+ T cells from atopic asthmatics (n=4) were stimulated using allergen (*Dermatophagoides pteronyssinus* [Der p]) or tetanus toxoid for 7 days. IL-5, IL-13 and IL-4 measured from culture supernatants in response to stimulation to tetanus toxoid are shown in panel (a). The Th1/Th0 cytokines (interferon-γ, TNF-α and IL-2) measured from culture supernatants in response to allergen are shown in panel (b). The differences observed between the conditions illustrated do not reach statistical significance.
Figure S2: CCR4+ T cells are the major source of Th2 cytokines. CCR4+ and CCR4-memory CD4+ T cells were isolated from PBMC of healthy subjects (n=4) by FACS sorting. Cells were stimulated with Phorbol Myristate Acetate (PMA) (20ng/ml) and Ionomycin (1µM) for 6 hours with brefeldin A (5µg/ml) added for the last 2 hours. Cells were then fixed in 4% paraformaldehyde, permeabilised with 0.5% saponin and stained with fluorescently labeled antibodies to IL-4 (APC), IFN-gamma (PerCP-Cy5.5), and IL-13 (FITC) (BioLegend, CA). Stained cells were analyzed using the LSRII (BD Biosciences) flowcytometer. FACS data was analyzed on FlowJo software and contour plots with outliers from all the subjects are illustrated.
Figure S 3

CCR4 in T cell recruitment to human asthmatic airways

CCL11

CCL20

CCL19

CCL5

CCL4

CCL2
**Figure S3:** Bronchial explants from mild, steroid-naïve asthmatic release more chemokines (CCL11, CCL5, CCL20, CCL19, CCL2) either constitutively or following allergen stimulation when compared to healthy controls. Bronchial biopsies were obtained from healthy (n=14), mild steroid-naïve asthmatics (n=18) and moderately severe asthmatics treated with inhaled corticosteroids (n=15); these were stimulated *ex vivo* with *Der p* allergen for 24 hr. Culture supernatants were assayed using custom made SearchLight ELISA kit for the detection of chemokines CCL11, CCL5, CCL20, CCL19, CCL2. *, P<0.05 by the Wilcoxon-Signed-Ranks test and Mann-Whitney U test for comparison between paired and unpaired samples.
Structure of CCR4 antagonist

Compound 1

Selectivity of Compound 1

Compound 1 was tested for activity at 274 macromolecular targets including the chemokine receptors CCR1, 2, 4 and 8 and CXCR1, 2 and 3, 82 other G-protein coupled receptors and a variety of ligand-gated ion channels, neurotransmitter transporters, adhesion molecules, kinases and other enzymes. The pKi was <6 for the majority of these proteins. The table below indicates the affinity of compound 1 for those proteins where its pKi was >6. Compound 1 was 23-fold selective over CCR1 and at least 280-fold selective for CCR4 over the other targets tested.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>pKi*</th>
<th>Replicates</th>
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<tbody>
<tr>
<td>CCR4</td>
<td>9.02 ± 0.05</td>
<td>99</td>
</tr>
<tr>
<td>CCR1</td>
<td>7.65 ± 0.05</td>
<td>3</td>
</tr>
<tr>
<td>EP3</td>
<td>6.56 ± 0.16</td>
<td>4</td>
</tr>
</tbody>
</table>

CCR4 in T cell recruitment to human asthmatic airways
* The pKi was determined from the IC$_{50}$ of inhibition curves using the Cheng-Prusoff correction (Cheng & Prusoff, 1973*) assuming a competitive interaction.

*Cheng, Y-C & Prusoff, W (1973) Relationship between the inhibition constant (KI) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. Biochem. Pharmacol. 22, 3099-3108.
Figure S5: CCR4 antagonist (compound 1) is highly potent and selective. The potency and selectivity of the CCR4 antagonist was tested in chemotaxis assays performed using CD45RO+ CD4+ (memory) T cells as responder cells. Recombinant chemokines CCL22 (acting on CCR4), CXCL10 (acting on CXCR3), CCL5 (acting on CCR5, CCR3) and CCL2 (acting on CCR2) were used in varying concentrations and their ability to chemoattract CD45RO+ memory CD4+ T cells was tested in the presence (filled bars) and absence of the CCR4 blocker (empty bars). Chemotaxis index = numbers of T cells migrating in response to chemokines divided by numbers migrating spontaneously. The spontaneous migration in chemotaxis buffer is given a chemotaxis index of 1. Data are from one of three representative experiments.