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Compartmentalized chemokine-dependent regulatory T cell inhibition of allergic pulmonary inflammation

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Abstract

Background—Induction of endogenous regulatory T cells (Tregs) represents an exciting new potential modality for treating allergic diseases such as asthma. Tregs have been implicated in the regulation of asthma but the anatomic location where they exert their regulatory function, and the mechanisms controlling their migration necessary for their suppressive function in asthma are not known. Understanding these aspects of Treg biology will be important for harnessing their power in the clinic.

Objective—To determine the anatomic location where Tregs exert their regulatory function in the sensitization and effector phases of allergic asthma, and to determine the chemokine receptors that control the migration of Tregs to these sites *in vivo* in mice and in humans.

Methods—The clinical efficacy and the anatomic location of adoptively transferred chemokine receptor-deficient CD4⁺CD25⁺ Foxp3⁺ Tregs was determined in the sensitization and effector phases of allergic airway inflammation in mice. The chemokine receptor expression profile was determined on Tregs recruited into the human airway following bronchoscopic segmental allergen challenge of subjects with asthma.

Results—We show that CCR7, but not CCR4, is required on Tregs to suppress allergic airway inflammation during the sensitization phase. In contrast, CCR4, but not CCR7, is required on Tregs to suppress allergic airway inflammation during the effector phase. Consistent with our murine studies, humans with allergic asthma had an increase in CCR4 expressing functional Tregs in the lung following segmental allergen challenge.

Conclusion—The location of Treg function differs during allergic sensitization and during allergen-induced recall responses in the lung, and that this differential localization is critically dependent on differential chemokine function.

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Keywords

Asthma; regulatory T cells; chemokines; CCR4; CCR7; segmental allergen challenge

INTRODUCTION

Allergic asthma is a common immune-mediated disorder whose frequency in developed countries continues to escalate.¹ In most cases, airway inflammation characteristic of asthma is believed to result from an allergic-type reaction to an inhaled substance from the environment, resulting in an inappropriate airway Th2-type immune response². Despite an increased understanding of the pathological features of this disease, the mechanisms underlying the regulation of allergic asthma remain largely unknown, and as a result the development of new therapies for asthma has stalled.

Foxp3⁺ regulatory T cells (Tregs) have been shown to be major regulators of antigen-specific inflammation³, and have been implicated as key regulators of allergic disease. Murine models have provided strong evidence for an important role for Tregs in suppressing allergic airway inflammation. The adoptive transfer of CD4⁺CD25⁺ Tregs into sensitized mice before aerosolized allergen challenge has been shown to abrogate airway hyperresponsiveness⁴ and prevent allergic airway inflammation⁵. Furthermore, depletion of CD4⁺CD25⁺ Tregs in mice normally resistant to allergic airway inflammation resulted in increased airway eosinophilia, hyperresponsiveness, and IgE levels as well as Th2 cytokines in the lung after allergen sensitization and challenge.⁶ Finally, Foxp3⁺ Tregs are important for regulating chronic allergic inflammation in mice and are essential for establishing tolerance in the respiratory mucosa.⁷ Taken together, these data provide compelling evidence that Tregs play an important role in the regulation of allergic airway inflammation in mice.

Tregs have also been implicated in the regulation of human allergic asthma. A higher frequency of allergen-specific IL4-producing T cells and lower frequency of IL10-producing T cells was found in the peripheral blood of atopic asthmatics⁸. Children with allergic disease had fewer CD4⁺CD25^{hi} T cells in their blood compared to healthy controls, while children with moderate-severe asthma had increased numbers of CD4⁺CD25^{hi} T cells compared to those with milder disease, which correlated with increased Foxp3 and IL-10 mRNA expression.⁹ Consistent with this study, pediatric asthmatic patients were found to have a lower percentage of CD4⁺CD25^{hi} T cells in the BAL as compared with healthy children.¹⁰ Furthermore, this study demonstrated that inhaled corticosteroid treatment was associated with an increase in CD4⁺CD25^{hi} T cells in the peripheral blood and BAL. In addition, systemic or inhaled or glucocorticoid treatment of moderate-severe asthmatics increased the frequency of CD4⁺CD25⁺ Foxp3 IL-10 expressing T cells in the peripheral blood,¹¹ suggesting a link between the treatment of asthma and the induction of Tregs. These studies collectively highlight a potentially important role for Tregs in suppressing Th2 responses and allergic asthma, and suggest that individuals with allergic disease may have insufficient or non-functional regulatory T cell responses.

While Tregs have been implicated in the regulation of asthma, important questions relating to the anatomic location where Tregs exert their regulatory function in asthma, and the mechanisms that control the migration of Tregs necessary for them to perform this suppressive function *in vivo* remain unknown. Understanding this aspect of Treg biology will be important for harnessing their power in the clinic. In order to inhibit effector cell function, Tregs likely need to home to sites where effector T cells are generated and function. To do so, Tregs likely use the same homing molecules that naïve and effector T cells utilize.¹² In fact, CCR7 has been shown to be important for Treg homing and function to

the lymphoid compartment during the initiation of immune responses.¹³⁻¹⁶ Likewise, CCR4, CCR5 and CXCR3 have been shown to be important for Treg recruitment and function to peripheral sites of inflammation.^{12, 17-20} Given CCR7's role in lymph node (LN) homing, and CCR4's role in the recruitment of Th2 cells into the allergic lung,²¹ we hypothesized that if Tregs suppressed allergic pulmonary inflammation in the lymphoid compartment they would require CCR7 and if they suppressed allergic pulmonary inflammation in the lung they would require CCR4. Here we test this hypothesis by using "sensitization" and "effector" adoptive transfer models of allergic airway inflammation and CCR7- and CCR4-deficient Tregs.

METHODS

Mice

WT C57BL/6 mice (Thy-1.2, CD90.2), WT congenic C57BL/6 Thy-1.1 mice (CD90.1) and CCR4^{-/-} mice on C57BL/6 background were obtained from The Jackson Laboratory. CCR7^{-/-} mice²³ were backcrossed nine generations to C57BL/6 mice. OT-II mice were bred with CCR4^{-/-} and CCR7^{-/-} mice to generate CCR4^{-/-} OT-II and CCR7^{-/-} OT-II lines in the C57BL/6 background. Donor Thy1.2 C57BL/6 mice (OT-II, CCR4^{-/-} OT-II, and CCR7^{-/-} OT-II) were used at 6-8 wk of age, and recipient Thy1.1 C57BL/6 mice were used at 8-10 wk of age. Mice used for experiments were age and gender matched. The experimental protocol was approved by the Subcommittee of Research Animal Care of Massachusetts General Hospital according to institutional Animal Care and Use Committee guidelines before the start of the study.

mAbs

Anti-mouse CD4-PerCP (clone RM4-5), anti-mouse CD25-APC (clone PC61), anti-human CD4-PerCP, anti-human CD3-FITC, anti-human CD25-FITC, anti-human CCR4-PE, anti-human CXCR3-PE, anti-human CCR6-PE, and anti-human CCR7-PE were purchased from BD PharMingen. Anti-mouse Foxp3 PE (clone FJK-16S) and anti-human Foxp3-APC were purchased from eBiosciences.

Mouse Treg Cell isolation

CD4⁺ T cells were isolated from the LNs and spleen of WT, CCR4^{-/-} or CCR7^{-/-} OT-II donor mice by the EasySep mouse CD4⁺ T cell enrichment kit. CD4⁺CD25^{hi} cells were then isolated by FACS cell sorting (FACS Aria).

Human Treg suppression assay

CD4⁺CD25⁺ Tregs were isolated from the post-allergen challenge BAL of allergic asthmatic subjects by a CD4⁺CD25⁺ Treg kit (Miltenyi). CD4⁺ T cells were isolated from the blood of the same allergic asthmatic subject using RosetteSep CD4⁺ T cell enrichment cocktail (StemCell Technologies). CD4⁺ T cells isolated from the blood were incubated for 10 min at 37°C in 10 μ M CFSE (Invitrogen) in PBS, washed 3X with RPMI, and resuspended in complete RPMI. 10⁵ CFSE-labeled CD4⁺ cells were incubated with varying concentrations of BAL CD4⁺CD25⁺ Tregs. All cultures (except unstimulated control) were stimulated with CD3/CD28/IL2 bead kit (Miltenyi) for 5 days. Proliferation of CD4⁺ T cells was measured by assessing CFSE dilution by flow cytometry.

Mouse models of allergic airway inflammation

"Sensitization" model: Immediately after cell sorting, 1×10^5 FACS-purified CD4⁺CD25^{hi} cells in cold PBS (Mediatech) were injected into recipient WT C57BL/6 mice via the tail vein intravenously (i.v.). Mice were sensitized by two intraperitoneal (i.p.) injections of 10

μg of OVA (Sigma) adsorbed onto 1 mg aluminum hydroxide (Sigma) in 430 μl of saline (OVA-alum) on days 0 and 14. Three days after, mice were challenged by inhalation of OVA aerosol (1% w/v in PBS) for 20 min on four consecutive days (days 17-20), and tissue and BAL were harvested at day 21. *“Effector” model:* Briefly, WT host mice were sensitized on day 0 and day 14 with 100 μg OVA/alum i.p. Two days later on day 16, mice received 1×10^5 Tregs i.v. On days 17-20 mice were challenged with 1% OVA aerosol and tissue and BAL were harvested at day 21. Allergic airway inflammation was assessed by performing BAL cell counts and cytospin differentials and by analyzing hematoxylin and eosin (H&E) and Periodic acid-Schiff (PAS) stained lung sections using an inflammation score as detailed in the Online Repository. IL10, IL13, IL4 and IL5 protein levels in the BAL were determined with a Milliplex MAP cytokine immunoassay (Millipore Corp, St Charles, MI, USA).

Mouse Treg trafficking models

“Sensitization” model: 1×10^6 Thy1.2 Tregs were adoptively transferred i.v. into naïve WT Thy1.1 recipient mice. 24 h following adoptive transfer, recipient mice were immunized i.p. with OVA in alum. 5 days post-immunization, LN and lungs were harvested and the location of transferred Tregs was assessed using the congenic marker Thy1.2. *“Effector” model:* 1×10^6 Thy1.2 Tregs were adoptively transferred i.v. into WT Thy1.1 recipient mice two days following the second OVA and alum i.p. immunization and prior to aerosolized OVA challenges. 24 h after Treg transfer, recipient mice were challenged with aerosolized OVA on 2 consecutive days and LNs and lungs harvested 24 h after the last aerosolized OVA challenge. Treg localization was then assessed by flow cytometry using the congenic marker Thy1.2.

Quantitative real-time PCR

RNA was purified (RNeasy; Qiagen, Valencia, CA), DNase treated, converted to cDNA (Applied Biosystems, Carlsbad, CA), amplified in the presence of SYBR Green and analyzed using quantitative real time PCR (QPCR) (Mastercycler EP *Realplex*; Eppendorf, Hauppauge, NY).

Segmental allergen challenge of human allergic asthmatics

The study was approved by the Partners Healthcare Institutional Review Board and each subject provided written consent. All subjects met the American Thoracic Society's definition of asthma and had symptoms to cat or dust mite exposure with a corresponding positive skin prick test. Selection of subjects and bronchoscopy previously described²⁴. In brief, a prechallenge BAL was obtained from the lingula. Diluent (2 ml) was then administered to the anterior segment of the right upper lobe followed by administration of allergen (2 ml) to the lateral segment of the right middle lobe. Twenty-four hours later, BAL samples were obtained from the diluent and antigen challenged lung segments. (see OR Methods for details).

Statistical analysis

Student's *t* test (unpaired, two-tailed, $\pm$ SEM) was used to calculate significant levels for all murine experiments. For analysis of human samples, a nonparametric paired test was performed. $P < 0.05$ was considered to be statistically significant in all data.

RESULTS

CCR4^{-/-} and CCR7^{-/-} Tregs are functional *in vitro*

We first sought to determine if the lack of CCR4 and CCR7 would affect the functionality of Tregs. Tregs were isolated from the spleen and LN of naïve OT-II mice, which are transgenic for the T cell receptor recognizing ovalbumin peptide 323-339 (pOVA₃₂₃₋₃₉), as well as CCR4-deficient (CCR4^{-/-}) OT-II and CCR7-deficient (CCR7^{-/-}) OT-II mice by flow cytometry based cell sorting gating on CD4⁺CD25^{hi} cells (Fig. E1). Foxp3 immunostaining on this purified population revealed that they were approximately 90% Foxp3 positive. The number of CD4⁺CD25^{hi} cells recovered from the spleens and LNs of WT OT-II, CCR4^{-/-} OT-II and CCR7^{-/-} OT-II mice were similar as was the percent of Foxp3 cells in the purified cell populations from these strains of mice (Fig. E1). Varying concentrations of CD4⁺CD25^{hi} cells purified from WT OT-II, CCR4^{-/-} OT-II and CCR7^{-/-} OT-II mice were co-cultured with CFSE labeled splenic WT CD4⁺CD25⁻ T cells simulated with anti-CD3/CD28 antibodies in the presence of IL-2 in a Treg suppression assay. After 5 days, cell division was assessed by measurement of CFSE dilution. Tregs purified from WT OT-II, CCR4^{-/-} OT-II and CCR7^{-/-} OT-II mice were equally capable of inhibiting the proliferation of effector T cells, thereby demonstrating no intrinsic defect in suppressor function in Tregs isolated from CCR4^{-/-} and CCR7^{-/-} mice.

CCR7-expressing Tregs are needed to suppress allergic airway inflammation in naive mice

In the sensitization model, Tregs were freshly isolated from spleen and LNs of WT, CCR4^{-/-}, or CCR7^{-/-} OT-II mice and adoptively transferred into recipient WT mice one day prior to immunization. Host mice were then immunized twice with OVA in adjuvant (alum) two weeks apart. Three days after the second immunization, mice were challenged daily with four aerosolized doses of OVA (Fig. 1a). 24 h after the 4th aerosolized OVA challenge, BAL and lungs were harvested for analysis. As expected, OVA-challenged mice that did not receive Tregs revealed prominent eosinophilic airway inflammation. However, mice that received either WT or CCR4^{-/-} OT-II Tregs prior to immunization showed markedly suppressed eosinophilic inflammation. In marked contrast, mice that received CCR7^{-/-} OT-II Treg prior to sensitization showed minimal suppression of eosinophilic airway inflammation (Fig. 1). The BAL of mice that received WT and CCR4^{-/-} OT-II Tregs had significantly less total cells, lymphocytes, and eosinophils than control mice that did not receive Tregs or mice that received CCR7^{-/-} OT-II Tregs (Fig. 1b and Fig. 1c). Blinded scoring of lung histology was consistent with the BAL data and demonstrated a marked decrease in lung inflammation in mice injected with WT or CCR4^{-/-} OT-II Tregs compared to mice that received PBS or CCR7^{-/-} OT-II Tregs (Fig. 1d). Likewise, PAS staining revealed a marked decrease in mucous production in mice that received WT or CCR4^{-/-} Tregs compared to mice that received PBS or CCR7^{-/-} Tregs (Fig 1c). Finally, there was a significant reduction in the levels of the Th2 cytokines IL4, IL5 and IL13 and an increase in the anti-inflammatory cytokine IL10 in the BAL of mice transferred with WT OT-II Tregs compared to mice that received PBS or CCR7^{-/-} OT-II Tregs (Fig. 1e). Of note, antigen-nonspecific polyclonal Tregs were less effective at suppressing allergic airway inflammation than antigen-specific OT-II Tregs (Fig E2a), suggesting that Treg suppression of allergic airway inflammation during the sensitization phase is more effectively mediated in an antigen-specific manner. These data demonstrate that Tregs require CCR7, but not CCR4 expression, to suppress allergic pulmonary inflammation in the sensitization phase. These findings are also consistent with previous studies showing CCR7 deficiency results in an inability of Tregs to regulate the priming phase of an immune response.^{3, 15, 25-27}

CCR4-expressing Tregs are needed to suppress allergic airway inflammation in sensitized mice

We next studied the ability of Tregs to suppress allergic airway inflammation in sensitized mice ('effector model'). Freshly isolated Tregs were adoptively transferred i.v. into OVA immunized recipient WT mice two days after the second i.p. immunization (Fig. 2a). One day after Treg transfer, mice received 4 daily aerosolized OVA challenges. 24 h after the 4th aerosolized challenge, BAL and lungs were harvested for analysis. As before, mice that did not receive Tregs had prominent eosinophilic airway inflammation. However, in this model, eosinophilic inflammation was suppressed in mice that received either WT or CCR7^{-/-} OT-II Tregs after sensitization but was not suppressed in mice that received CCR4^{-/-} OT-II Treg after sensitization (Fig. 2). Analysis of cells isolated from BAL fluid from OVA-immunized and challenged mice demonstrated a marked decrease in the number of total cells and eosinophils in the BAL fluid of mice receiving either WT or CCR7^{-/-} OT-II Tregs compared with mice receiving either PBS or CCR4^{-/-} OT-II Tregs (Fig 2b). Additionally, blinded scoring of lung histology showed a marked reduction in eosinophilic lung inflammation and mucus production in mice injected with WT and CCR7^{-/-} OT-II Tregs compared to control PBS injected mice or mice that received CCR4^{-/-} OT-II Tregs (Fig 2c and 2d). Likewise, PAS staining revealed a marked decrease in mucous production in mice that received WT or CCR7^{-/-} Tregs compared to mice that received PBS or CCR4^{-/-} Tregs (Fig 2c). Finally, we observed a significant reduction in levels of the Th2 cytokines IL4, IL5 and IL13 and a marked increase in the anti-inflammatory cytokine IL10 in the BAL of mice that received WT OT-II Tregs, but not in the BAL of mice that received PBS or CCR4^{-/-} OT-II Tregs (Fig 2e). Airway inflammation in recipient mice that received CCR4^{-/-} OT-II Tregs in this model was no different than in mice that received no Tregs. As seen in the sensitization model, antigen-nonspecific polyclonal Tregs were less effective at suppressing allergic airway inflammation than antigen-specific OT-II Tregs (Fig E2b), suggesting that Treg suppression of allergic airway inflammation during the effector phase is also more effectively mediated in an antigen-specific manner. These data demonstrate that Tregs require CCR4 expression, but not CCR7 expression, to suppress allergic pulmonary inflammation in sensitized mice. These findings are consistent with a previous study demonstrating that CCR4 was required on CD4⁺CD25⁺ Tregs for suppression of spontaneous pulmonary inflammation in Foxp3-deficient *scurfy* mice.²⁸

Chemokine-dependent Treg location dictates function

To determine if the differential chemokine receptor dependence of Treg suppressor function in the sensitization and effector models was the result of differential location where Tregs need to function in the two models, we analyzed the location of WT, CCR4^{-/-} and CCR7^{-/-} Thy1.2 OT-II Tregs following adoptive transfer into WT Thy1.1 mice in the two models (Fig. 3a and 3d). In the sensitization model, WT and CCR4^{-/-} OT-II Tregs were found in equal numbers in the lymphoid compartment while CCR7^{-/-} OT-II Tregs were not found in LNs (Fig. 3b). WT and CCR4^{-/-} OT-II Tregs were not recovered from the lung while CCR7^{-/-} OT-II Tregs could perhaps migrate to the lung because they could not home to LNs. We also analyzed the pattern of CCR7 and CCR4 ligand expression in the LNs and lungs of mice in the sensitization model. Using RT-QPCR we found that the CCR7 ligands CCL19 and CCL21 were detected preferentially in LN and not in the lung. In contrast, the CCR4 ligands CCL17 and CCL22 were detected at very low levels in lung and not in the LN (Fig 3c). These data suggest that in the sensitization phase, CCR7 expression is required for Treg homing into the lymphoid compartment.

In the effector model, WT and CCR7^{-/-} OT-II Tregs were found in the lung in roughly equal numbers while the number of CCR4^{-/-} OT-II Tregs recovered from the lung were markedly reduced (Fig. 3e). Tregs of any genotype were not recovered from the LNs in

appreciable numbers. Furthermore, in this model, we found that the CCR4 ligands CCL17 and CCL21 were highly expressed in the lung while the CCR7 ligands were moderately expressed in the thoracic LN (Fig 3f). These data strongly suggest that CCR4 expression is required for Treg migration into the lung. Examination of CCR7 and CCR4 expression on WT Tregs isolated from the lung in this model after 2 aerosolized OVA challenges revealed that 75% of the Foxp3⁺ Tregs expressed CCR4 while only 17% expressed CCR7 (Fig 3g). Collectively, these results suggest that chemokine receptor expression on Tregs affects their homing ability, which dictates their functional capacities at different stages of the allergic airway response.

Tregs are enriched in BAL of human asthmatics after airway allergen challenge

To extend our murine studies on the role of chemokine receptors in Treg function to human asthma, 10 asthma patients with a history of mild to moderate asthma and allergy to dust mite or cat allergen were studied using a segmental allergen challenge protocol.²⁴ Characteristics of the subjects can be found in Table E1 in Online Repository.

Analysis of the BAL revealed a characteristic allergic airway response in the allergen challenged segment. In response to the allergen challenge, the total number of cells increased >9-fold, lymphocytes increased >4-fold and eosinophils increased >690-fold in the BAL (Fig. 4a). Although there was no difference in the percentage of CD4⁺ T cells following allergen challenge (Fig 4b), given the increase in total lymphocytes, the total number of CD4⁺ cells increased 5.5-fold in the BAL following allergen challenge (Fig. 4c), demonstrating a recruitment of CD4⁺ cells into the airways.

Within the CD4⁺ population in the BAL, we found that the percent of CD4⁺CD25⁺Foxp3⁺ Tregs following allergen challenge was no different than the percent of Tregs in either the pre-challenge or diluent (Fig 4d). However, the number of CD4⁺CD25⁺Foxp3⁺ Tregs in the post-allergen challenge BAL was approximately 10-fold greater than the number of Tregs in either the pre-challenge or diluent BAL (Fig. 4e). Together, these data suggest that even though there was marked recruitment of Tregs into the asthmatic lung following allergen challenge, the ratio of Tregs to other CD4⁺ T cells was not significantly altered.

CCR4 is expressed on Tregs recruited into the BAL following allergen challenge

To extend our observation on the role of CCR4 in Treg recruitment into the airway in allergic pulmonary inflammation in the mouse to human asthma, we examined the chemokine receptor expression profile on Tregs found in the BAL before and 24 hours after segmental allergen challenge (Fig. 4f). CD4⁺CD25⁺Foxp3⁺ Tregs in the pre-challenge, diluent and post-allergen challenge BAL were stained for CCR7, CCR6, CCR4 and CXCR3 surface expression. There was a >2-fold increase in the percent of Tregs expressing CCR4 in the BAL post-allergen challenge compared to the percent of Tregs expressing CCR4 in the pre-challenge BAL, suggesting that CCR4 contributes to the recruitment of Tregs into the asthmatic airway. In contrast, we found no enrichment in Tregs expressing CCR7, CCR6 or CXCR3 post-allergen challenge.

BAL Tregs from asthmatic subjects are functional

We next determined if the Tregs recovered from the BAL of asthmatic subjects following allergen challenge were functional using a Treg suppression assay (Fig. 4g). Autologous peripheral blood CD4⁺ T cell proliferation was inhibited up to 50% when cultured with an equivalent number of CD4⁺CD25⁺ Tregs. As expected, as the ratio of Tregs to T effectors decreased, T cell proliferation was inhibited to a lesser extent. These data confirm that Tregs recovered from the lung of allergic asthmatics are functional.

DISCUSSION

Using chemokine-receptor deficient Tregs in adoptive transfer experiments, we found that CCR7-dependent Treg homing to the LN was required to suppress the sensitization phase of allergic pulmonary inflammation. In contrast, in allergen sensitized mice, CCR7-dependent Treg homing to the LN was dispensable for suppression of the allergen recall response, and instead, CCR4-dependent Treg homing to the lung was required to suppress the recall response to an inhaled allergen and resultant allergic pulmonary inflammation. These data suggest that even though the lymphoid compartment is thought to be the primary site for the generation of effector T cells in recall responses, such as in aeroallergen challenge of sensitized mice⁴, peripheral tissue, such as the lung, is the site where Tregs are required to home to suppress recall responses. It will be of interest to determine if this is a general principle for where Tregs need to function in recall versus primary immune responses.

We extended our murine studies to humans and found that the number of CD4⁺CD25⁺Foxp3⁺ Tregs in the BAL of allergic asthmatic subjects increased significantly post-bronchoscopic allergen challenge, and that of all of the chemokine receptors tested, CCR4 was the one chemokine receptor increased on these recruited Tregs. These data suggest that in humans, like in the mouse, CCR4 may be critical for Treg recruitment into the asthmatic airway.

We also demonstrated that recruited Tregs into the asthmatic lung are functional in their ability to suppress CD4⁺ T effector cells. This raises the question that if functional Tregs are recruited into the airways of allergic asthma subjects following allergen challenge, then why do they still develop the signs and symptoms of asthma. One possible explanation is that there are too many effector CD4⁺ T cells recruited into the airways upon allergen challenge relative to the number of Tregs that are recruited into the airways, thereby overwhelming the function of the recruited Tregs. We found that the number of Tregs recruited into the inflamed airways post-challenge is 100-fold less than the number of total recruited CD4⁺ T cells recruited. Therefore, in allergic asthmatic individuals, the inability of Tregs to suppress the inflammatory response may in part be a result of too many recruited effector CD4⁺ T cells and too few recruited Tregs. This is consistent with studies demonstrating that manipulating the numbers of Tregs relative to the numbers of effector cells recruited into the lung using selective chemokine blockade can greatly influence the ability of Tregs to control pulmonary inflammation.²⁹

Our data offer insight into the conflicting data in the literature regarding the therapeutic utility of inhibiting CCR4 in allergic pulmonary inflammation. Using Th2 cell adoptive transfer models of allergic pulmonary inflammation, we previously demonstrated that CCR4 plays an important role in effector Th2 cell recruitment to the allergic lung.²¹ In contrast, when CCR4^{-/-} mice were studied in immunization and challenge models of allergic pulmonary inflammation, the results were conflicting but for the most part unimpressive.^{22, 30, 31} Our data suggest that in CCR4^{-/-} mice, Treg recruitment to the lung would also be inhibited along with Th2 cell recruitment thus perhaps negating the beneficial effects of CCR4 blockade on Th2 cell recruitment. We believe the fact that Tregs use the same chemokine receptor pathways as effector T cells to gain access to inflamed peripheral tissue may limit the usefulness of global chemokine inhibition and may partly explain some of the recent failures of chemokine based therapeutics. Our study suggests that the selective inhibition of chemokine receptors on effector T cells and/or the sparing of this inhibition on Tregs may be required for effective chemokine therapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Tregs	regulatory T cells
BAL	bronchoalveolar lavage
LN	lymph nodes
i.p.	intraperitoneal
i.v.	intravenous
WT	wild-type
H&E	hematoxylin
PAS	Periodic acid-Schiff

REFERENCES

1. Locksley RM. Asthma and allergic inflammation. *Cell*. 2010; 140:777–83. [PubMed: 20303868]
2. Larche M, Robinson DS, Kay AB. The role of T lymphocytes in the pathogenesis of asthma. *J Allergy Clin Immunol*. 2003; 111:450–63. quiz 64. [PubMed: 12642820]
3. Lahl K, Loddenkemper C, Drouin C, Freyer J, Arnason J, Eberl G, et al. Selective depletion of Foxp3+ regulatory T cells induces a scurfy-like disease. *J Exp Med*. 2007; 204:57–63. [PubMed: 17200412]
4. Strickland DH, Stumbles PA, Zosky GR, Subrata LS, Thomas JA, Turner DJ, et al. Reversal of airway hyperresponsiveness by induction of airway mucosal CD4+CD25+ regulatory T cells. *J Exp Med*. 2006; 203:2649–60. [PubMed: 17088431]
5. Kearley J, Barker JE, Robinson DS, Lloyd CM. Resolution of airway inflammation and hyperactivity after in vivo transfer of CD4+CD25+ regulatory T cells is interleukin 10 dependent. *J Exp Med*. 2005; 202:1539–47. [PubMed: 16314435]
6. Lewkowich IP, Herman NS, Schleifer KW, Dance MP, Chen BL, Dienger KM, et al. CD4+CD25+ T cells protect against experimentally induced asthma and alter pulmonary dendritic cell phenotype and function. *J Exp Med*. 2005; 202:1549–61. [PubMed: 16314437]
7. Curotto, de Lafaille MA.; Kutchukhidze, N.; Shen, S.; Ding, Y.; Yee, H.; Lafaille, JJ. Adaptive Foxp3+ regulatory T cell-dependent and -independent control of allergic inflammation. *Immunity*. 2008; 29:114–26. [PubMed: 18617425]
8. Akdis M, Verhagen J, Taylor A, Karamloo F, Karagiannidis C, Crameri R, et al. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. *J Exp Med*. 2004; 199:1567–75. [PubMed: 15173208]
9. Lee JH, Yu HH, Wang LC, Yang YH, Lin YT, Chiang BL. The levels of CD4+CD25+ regulatory T cells in paediatric patients with allergic rhinitis and bronchial asthma. *Clin Exp Immunol*. 2007; 148:53–63. [PubMed: 17349011]
10. Hartl D, Koller B, Mehlhorn AT, Reinhardt D, Nicolai T, Schendel DJ, et al. Quantitative and functional impairment of pulmonary CD4+CD25hi regulatory T cells in pediatric asthma. *J Allergy Clin Immunol*. 2007; 119:1258–66. [PubMed: 17412402]

11. Karagiannidis C, Akdis M, Holopainen P, Woolley NJ, Hense G, Ruckert B, et al. Glucocorticoids upregulate FOXP3 expression and regulatory T cells in asthma. *J Allergy Clin Immunol.* 2004; 114:1425–33. [PubMed: 15577848]
12. Bromley SK, Mempel TR, Luster AD. Orchestrating the orchestrators: chemokines in control of T cell traffic. *Nat Immunol.* 2008; 9:970–80. [PubMed: 18711434]
13. Eller K, Weber T, Pruenster M, Wolf AM, Mayer G, Rosenkranz AR, et al. CCR7 deficiency exacerbates injury in acute nephritis due to aberrant localization of regulatory T cells. *J Am Soc Nephrol.* 2010; 21:42–52. [PubMed: 19917782]
14. Forster R, Davalos-Misslitz AC, Rot A. CCR7 and its ligands: balancing immunity and tolerance. *Nat Rev Immunol.* 2008; 8:362–71. [PubMed: 18379575]
15. Schneider MA, Meingassner JG, Lipp M, Moore HD, Rot A. CCR7 is required for the in vivo function of CD4+ CD25+ regulatory T cells. *J Exp Med.* 2007; 204:735–45. [PubMed: 17371928]
16. Ueha S, Yoneyama H, Hontsu S, Kurachi M, Kitabatake M, Abe J, et al. CCR7 mediates the migration of Foxp3+ regulatory T cells to the paracortical areas of peripheral lymph nodes through high endothelial venules. *J Leukoc Biol.* 2007; 82:1230–8. [PubMed: 17698914]
17. Iellem A, Colantonio L, D'Ambrosio D. Skin-versus gut-skewed homing receptor expression and intrinsic CCR4 expression on human peripheral blood CD4+CD25+ suppressor T cells. *Eur J Immunol.* 2003; 33:1488–96. [PubMed: 12778466]
18. Yuan Q, Bromley SK, Means TK, Jones KJ, Hayashi F, Bhan AK, et al. CCR4-dependent regulatory T cell function in inflammatory bowel disease. *J Exp Med.* 2007; 204:1327–34. [PubMed: 17548518]
19. Hoerning A, Koss K, Datta D, Boneschansker L, Jones CN, Wong IY, et al. Subsets of human CD4(+) regulatory T cells express the peripheral homing receptor CXCR3. *Eur J Immunol.* 2011; 41:2291–302. [PubMed: 21538345]
20. Bystry RS, Aluvihare V, Welch KA, Kallikourdis M, Betz AG. B cells and professional APCs recruit regulatory T cells via CCL4. *Nat Immunol.* 2001; 2:1126–32. [PubMed: 11702067]
21. Mikhak Z, Fukui M, Farsidjani A, Medoff BD, Tager AM, Luster AD. Contribution of CCR4 and CCR8 to antigen-specific T(H)2 cell trafficking in allergic pulmonary inflammation. *J Allergy Clin Immunol.* 2009; 123:67–73. [PubMed: 19062085]
22. Chvatchko Y, Hoogewerf AJ, Meyer A, Alouani S, Juillard P, Buser R, et al. A key role for CC chemokine receptor 4 in lipopolysaccharide-induced endotoxic shock. *J Exp Med.* 2000; 191:1755–64. [PubMed: 10811868]
23. Forster R, Schubel A, Breitfeld D, Kremmer E, Renner-Muller I, Wolf E, et al. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell.* 1999; 99:23–33. [PubMed: 10520991]
24. Thomas SY, Banerji A, Medoff BD, Lilly CM, Luster AD. Multiple chemokine receptors, including CCR6 and CXCR3, regulate antigen-induced T cell homing to the human asthmatic airway. *J Immunol.* 2007; 179:1901–12. [PubMed: 17641057]
25. Menning A, Hopken UE, Siegmund K, Lipp M, Hamann A, Huehn J. Distinctive role of CCR7 in migration and functional activity of naive- and effector/memory-like Treg subsets. *Eur J Immunol.* 2007; 37:1575–83. [PubMed: 17474155]
26. Valmori D, Merlo A, Souleimanian NE, Hesdorffer CS, Ayyoub M. A peripheral circulating compartment of natural naive CD4 Tregs. *J Clin Invest.* 2005; 115:1953–62. [PubMed: 16007258]
27. Lim HW, Broxmeyer HE, Kim CH. Regulation of trafficking receptor expression in human forkhead box P3+ regulatory T cells. *J Immunol.* 2006; 177:840–51. [PubMed: 16818738]
28. Sather BD, Treuting P, Perdue N, Miazgowiec M, Fontenot JD, Rudensky AY, et al. Altering the distribution of Foxp3(+) regulatory T cells results in tissue-specific inflammatory disease. *J Exp Med.* 2007; 204:1335–47. [PubMed: 17548521]
29. Seung E, Cho JL, Sparwasser T, Medoff BD, Luster AD. Inhibiting CXCR3-dependent CD8+ T cell trafficking enhances tolerance induction in a mouse model of lung rejection. *J Immunol.* 2011; 186:6830–8. [PubMed: 21555535]
30. Kawasaki S, Takizawa H, Yoneyama H, Nakayama T, Fujisawa R, Izumizaki M, et al. Intervention of thymus and activation-regulated chemokine attenuates the development of allergic airway

- inflammation and hyperresponsiveness in mice. *J Immunol.* 2001; 166:2055–62. [PubMed: 11160256]
31. Conroy DM, Jopling LA, Lloyd CM, Hodge MR, Andrew DP, Williams TJ, et al. CCR4 blockade does not inhibit allergic airways inflammation. *J Leukoc Biol.* 2003; 74:558–63. [PubMed: 12960268]

Key Messages

- Tregs require CCR7 but not CCR4 to suppress the sensitization phase of allergic pulmonary inflammation.
- In contrast, Tregs require CCR4 but not CCR7 to suppress the recall response to an inhaled allergen.
- CCR4 is enriched on human CD4⁺CD25⁺Foxp3⁺ Tregs recruited into the asthmatic airway following segmental allergen challenge.

Capsule Summary

CCR7 and lymph node homing is required for Treg suppression of the sensitization phase of allergic pulmonary inflammation while CCR4 and lung homing is required for Treg suppression of the recall response to inhaled allergens.

Figure 1. CCR7, but not CCR4, is required on Tregs to suppress allergic airway inflammation in the sensitization model

(a) Schematic of sensitization model of Treg suppression (see Methods for details). (b) Total cells, eosinophils and lymphocytes in the BAL were enumerated at day 21 in WT mice that received PBS (Solid bars), WT OT-II Tregs (grey bars), CCR4^{-/-} OT-II Tregs (checked bars), or CCR7^{-/-} OT-II Tregs (white bars) i.v. on day -1 followed by OVA i.p. on days 0 and 14. Data are representative of three experiments. (c) Histopathological analysis of lung sections on day 21 stained with H&E (top) and PAS (bottom). Scale bars, 50 μ m. Data are representative of 3 independent experiments. (d) Blinded histopathological scoring of lung inflammation on day 21 from mice treated as above (* p <0.05 compared to PBS injected mice; n = 10 mice per group from three experiments). (e) Cytokines measured in the BAL. Data are mean $\pm$ SEM of 8 mice per group from two experiments (* p <0.05).

Figure 2. CCR4, but not CCR7, is required on Tregs to suppress allergic airway inflammation in the effector model

(a) Schematic of the effector model of Treg suppression (see Methods for details). (b) Total cells, eosinophils and lymphocytes in the BAL were enumerated on day 21 in OVA immunized mice that received PBS (Solid bars), WT OT-II Tregs (grey bars), CCR4^{-/-} OT-II Tregs (checked bars), or CCR7^{-/-} OT-II Tregs (white bars) on day 16. Data are representative of 3 experiments. (c) Histopathological analysis of lung sections stained with H&E (top) and PAS (bottom). Scale of bar 50 μ m. Data are representative of 3 independent experiments. (d) Blinded histopathological scoring of lung inflammation on day 21 from mice treated as above (* p <0.05 compared to PBS injected mice; n = 10 mice per group from 3 experiments). (e) Cytokines measured in the BAL. Data are a mean $\pm$ SEM of 8 mice per group from 2 experiments (* p <0.05).

Figure 3. Chemokine receptor-dependent trafficking of Tregs to LN and lung

(a) Schematic of experimental protocol. (b) Cells from thoracic LN and lung were analyzed for Foxp3⁺Thy1.2⁺ expression (gated on CD4⁺CD25⁺ cells) on day 5. Data are pooled from 4 independent experiments. (c) Chemokine expression in LNs (pooled thoracic and inguinal) and lungs from WT mice measured on day 5 by RT-QPCR (n=8 mice from 2 independent experiments). (d) Schematic of experimental protocol. (e) Percent of Foxp3⁺Thy1.2⁺ cells of CD4⁺CD25⁺Foxp3⁺ cells in lymphocyte gate isolated from the LN and lung on day 19. These data are pooled from four independent experiments. (f) Chemokine expression in the thoracic LN and lung from WT mice measured by RT-QPCR on day 19 (n=8 mice from 2 independent experiments). (g) Percent CCR4 and CCR7 cell surface expression on Tregs (gated on CD4⁺CD25⁺Foxp3⁺ cells in lymphocyte gate) recovered from the lung on day 21 in the effector model. Data are mean ± SEM.

Figure 4. Tregs recruited into the human allergic lung express CCR4 and are functional
Data from 10 allergic asthmatic subjects (a-f). (a) Number of lymphocytes, monocytes, neutrophils, and eosinophils from baseline and allergen challenge BAL (mean $\pm$ SEM). (b) The percent and (c) number of CD4⁺ cells recovered from the BAL pre-allergen challenge (pre) and 24 hours post-bronchoscopic segmental diluent (dil) or allergen (Ag) challenge (*p<0.05; n=10 subjects). (d) The percent of CD25⁺Foxp3⁺ Tregs of total CD4⁺ cells and (e) the number of CD4⁺CD25⁺Foxp3⁺ Tregs recovered from BAL pre- and 24 hours post bronchoscopic diluent or allergen challenge (*p<0.05; n=10 subjects). (f) Percent of Tregs (CD4⁺CD25⁺Foxp3⁺) expressing CCR4, CCR6, CCR7 and CXCR3 in the BAL. (*p<0.05; n=10 subjects). (g, h) Treg suppression assay. (g) Representative FACS plots and (h) quantitation of percent inhibition of T effector (Teff) proliferation in the presence or varying ratios of Teff to Tregs (n=3).