

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

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

Objective: We sought to determine the anatomic location at which Treg cells exert their regulatory function in the sensitization and effector phases of allergic asthma and to determine the chemokine receptors that control the migration of Treg cells to these sites *in vivo* in both mice and human subjects.

Methods: The clinical efficacy and anatomic location of adoptively transferred chemokine receptor-deficient CD4⁺CD25⁺ forkhead box protein 3–positive Treg cells was determined in the sensitization and effector phases of allergic airway inflammation in mice. The chemokine receptor expression profile was determined on Treg cells recruited into the human airway after bronchoscopic segmental allergen challenge of asthmatic patients.

Results: We show that CCR7, but not CCR4, is required on Treg cells to suppress allergic airway inflammation during the sensitization phase. In contrast, CCR4, but not CCR7, is required on Treg cells to suppress allergic airway inflammation during the effector phase. Consistent with our murine studies, human subjects with allergic asthma had an increase in CCR4-expressing functional Treg cells in the lungs after segmental allergen challenge.

Conclusion: The location of Treg cell function differs during allergic sensitization and allergen-induced recall responses in the lung, and this differential localization is critically dependent on differential chemokine function. (*J Allergy Clin Immunol* 2013;■■■■:■■■-■■■.)

Key words: Asthma, regulatory T cells, chemokines, CCR4, CCR7, segmental allergen challenge

Allergic asthma is a common immune-mediated disorder the frequency of which continues to escalate in developed countries.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 T_H2-type immune response.² Despite an increased understanding of the pathologic 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.

Forkhead box protein 3 (Foxp3)–positive regulatory T (Treg) cells have been shown to be major regulators of antigen-specific inflammation³ and implicated as key regulators of allergic disease. Murine models have provided strong evidence for an important role for Treg cells in suppressing allergic airway inflammation. The adoptive transfer of CD4⁺CD25⁺ Treg cells into sensitized mice before aerosolized allergen challenge has been shown to abrogate airway hyperresponsiveness⁴ and prevent allergic airway inflammation.⁵ Furthermore, depletion of CD4⁺CD25⁺ Treg cells in mice normally resistant to allergic airway inflammation resulted in increased airway eosinophilia, hyperresponsiveness, and IgE levels, as well as increased levels of T_H2 cytokines, in the lungs after allergen sensitization and challenge.⁶ Finally, Foxp3⁺ Treg cells 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 Treg cells play an important role in the regulation of allergic airway inflammation in mice.

Treg cells have also been implicated in the regulation of human allergic asthma. A higher frequency of allergen-specific IL-4–producing T cells and a lower frequency of IL-10–producing T cells was found in the peripheral blood of atopic asthmatic patients.⁸ Children with allergic disease had fewer CD4⁺CD25^{hi} T cells in their blood compared with healthy control subjects, whereas children with moderate-to-severe asthma had increased numbers of CD4⁺CD25^{hi} T cells compared with numbers seen in 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 bronchoalveolar lavage (BAL) fluid 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 fluid. In addition, systemic or inhaled glucocorticoid treatment of patients with moderate-to-severe asthma increased the frequency of CD4⁺CD25⁺ Foxp3 IL-10–expressing T cells in the

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Abbreviations used

BAL: Bronchoalveolar lavage
 CFSE: Carboxyfluorescein succinimidyl ester
 FACS: Fluorescence-activated cell sorting
 Foxp3: Forkhead box protein 3
 LN: Lymph node
 OVA: Ovalbumin
 PAS: Periodic acid–Schiff
 PE: Phycoerythrin
 qPCR: Quantitative real-time polymerase chain reaction
 Treg: Regulatory T
 WT: Wild-type

peripheral blood,¹¹ suggesting a link between the treatment of asthma and the induction of Treg cells. These studies collectively highlight a potentially important role for Treg cells in suppressing T_H2 responses and allergic asthma and suggest that patients with allergic disease might have insufficient or nonfunctional Treg cell responses.

Although Treg cells have been implicated in the regulation of asthma, important questions relating to the anatomic location at which Treg cells exert their regulatory function in asthmatic patients and the mechanisms that control the migration of Treg cells necessary for them to perform this suppressive function *in vivo* remain unknown. Understanding this aspect of Treg cell biology will be important for harnessing their power in the clinic. Treg cells likely need to home to sites where effector T cells are generated and function to inhibit effector cell function. To do so, Treg cells likely use the same homing molecules used by naive and effector T cells.¹² In fact, CCR7 has been shown to be important for Treg cell 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 cell 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 T_H2 cells in the allergic lung,²¹ we hypothesized that if Treg cells 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 Treg cells.

METHODS**Mice**

Wild-type (WT) C57BL/6 mice (Thy-1.2, CD90.2), WT congenic C57BL/6 Thy-1.1 mice (CD90.1), and CCR4^{−/−} mice on a C57BL/6 background were obtained from the Jackson Laboratory (Bar Harbor, Me). CCR7^{−/−} mice²³ were backcrossed 9 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 to 8 weeks of age, and recipient Thy1.1 C57BL/6 mice were used at 8 to 10 weeks of age. Mice used for experiments were age and sex 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–peridinin-chlorophyll-protein complex (clone RM4-5), anti-mouse CD25–allophycyanin (clone PC61), anti-human CD4–peridinin-chlorophyll-protein complex, anti-human CD3–fluorescein isothiocyanate, anti-human CD25–fluorescein isothiocyanate, anti-human CCR4–phycoerythrin (PE), anti-human CXCR3-PE, anti-human CCR6-PE, and anti-human CCR7-PE were purchased from BD Pharmingen (San Jose, Calif). Anti-mouse Foxp3-PE (clone FJK-16S) and anti-human Foxp3-allophycyanin were purchased from eBioscience (San Diego, Calif).

Mouse Treg cell isolation

CD4⁺ T cells were isolated from the LNs and spleens of WT, CCR4^{−/−}, or CCR7^{−/−} OT-II donor mice by using the EasySep mouse CD4⁺ T-cell enrichment kit (STEMCELL Technology, Vancouver, British Columbia, Canada). CD4⁺CD25^{hi} cells were then isolated by means of fluorescence-activated cell sorting (FACS; FACSria, BD Biosciences).

Human Treg cell suppression assay

CD4⁺CD25⁺ Treg cells were isolated from the postallergen challenge BAL fluid of allergic asthmatic subjects by using a CD4⁺CD25⁺ Treg kit (Miltenyi Biotec, Bergisch Gladbach, Germany). CD4⁺ T cells were isolated from the blood of the same allergic asthmatic subject by using the RosetteSep CD4⁺ T-cell enrichment cocktail (STEMCELL Technologies). CD4⁺ T cells isolated from the blood were incubated for 10 minutes at 37°C in 10 μmol/L carboxyfluorescein succinimidyl ester (CFSE; Invitrogen, Carlsbad, Calif) in PBS, washed 3 times with RPMI, and resuspended in complete RPMI. CFSE-labeled CD4⁺ cells (10⁵) were incubated with varying concentrations of BAL fluid CD4⁺CD25⁺ Treg cells. All cultures (except unstimulated controls) were stimulated with the CD3/CD28/IL2 bead kit (Miltenyi Biotec) for 5 days. Proliferation of CD4⁺ T cells was measured by assessing CFSE dilution with flow cytometry.

Mouse models of allergic airway inflammation

“Sensitization” model. Immediately after cell sorting, 1 × 10⁵ FACS-purified CD4⁺CD25^{hi} cells in cold PBS (Mediatech, Manassas, Va) were injected into recipient WT C57BL/6 mice through the tail vein intravenously. Mice were sensitized by 2 intraperitoneal injections of 10 μg of ovalbumin (OVA; Sigma, St Louis, Mo) adsorbed onto 1 mg of aluminum hydroxide (Sigma) in 430 μL of saline (OVA-alum) on days 0 and 14. Three days later, mice were challenged by means of inhalation of OVA aerosol (1% wt/vol in PBS) for 20 minutes on 4 consecutive days (days 17–20), and tissue and BAL fluid were harvested at day 21.

“Effector” model. Briefly, WT host mice were sensitized on days 0 and 14 with 100 μg of OVA/alum administered intraperitoneally. Two days later, on day 16, mice received 1 × 10⁵ Treg cells intravenously. On days 17 to 20, mice were challenged with 1% OVA aerosol, and tissue and BAL fluid were harvested at day 21. Allergic airway inflammation was assessed by performing BAL cell counts and cytospin differentials and by analyzing hematoxylin and eosin and periodic acid–Schiff (PAS)-stained lung sections using an inflammation score, as detailed in the **Methods** section in this article's Online Repository at www.jacionline.org. IL-10, IL-13, IL-4, and IL-5 protein levels in the BAL fluid were determined with a Milliplex MAP cytokine immunoassay (Millipore, St Charles, Mo).

Mouse Treg cell trafficking models

“Sensitization” model. Thy1.2 Treg cells (1 × 10⁶) were adoptively transferred intravenously into naive WT Thy1.1 recipient mice. Twenty-four hours after adoptive transfer, recipient mice were immunized intraperitoneally with OVA in alum. Five days after immunization, LNs and lungs were harvested, and the location of transferred Treg cells was assessed by using the congenic marker Thy1.2.

“Effector” model. Thy1.2 Treg cells (1×10^6) were adoptively transferred intravenously into WT Thy1.1 recipient mice 2 days after the second OVA and alum intraperitoneal immunization and before aerosolized OVA challenges. Twenty-four hours after Treg cell transfer, recipient mice were challenged with aerosolized OVA on 2 consecutive days, and LNs and lungs were harvested 24 hours after the last aerosolized OVA challenge. Treg cell localization was then assessed by means of flow cytometry with the congenic marker Thy1.2.

Quantitative real-time PCR

RNA was purified (RNeasy; Qiagen, Valencia, Calif), DNase treated, converted to cDNA (Applied Biosystems, Carlsbad, Calif), amplified in the presence of SYBR Green, and analyzed by using quantitative real-time polymerase chain reaction (qPCR; Mastercycler EP *Realplex*; Eppendorf, Hauppauge, NY).

Segmental allergen challenge of human allergic asthmatic patients

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 response. Selection of subjects and bronchoscopy were previously described.²⁴ In brief, a prechallenge BAL sample 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 the [Methods](#) section in this article’s Online Repository for details.

Statistical analysis

The Student *t* test (unpaired, 2-tailed, $\pm$ SEM) was used to calculate significant levels for all murine experiments. For analysis of human samples, a non-parametric paired test was performed. A *P* value of less than .05 was considered statistically significant for all data.

RESULTS

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

We first sought to determine whether the lack of CCR4 and CCR7 would affect the functionality of Treg cells. Treg cells were isolated from the spleens and LNs of naive OT-II mice, which are transgenic for the T-cell receptor recognizing OVA peptide 323-339 (pOVA₃₂₃₋₃₃₉), as well as CCR4^{-/-} OT-II and CCR7^{-/-} OT-II mice, by using flow cytometry-based cell-sorting gating on CD4⁺CD25^{hi} cells (see [Fig E1](#) in this article’s Online Repository at www.jacionline.org). Foxp3 immunostaining on this purified population revealed that they were approximately 90% Foxp3⁺. 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 percentage of Foxp3 cells in the purified cell populations from these strains of mice (see [Fig E1](#)). Varying concentrations of CD4⁺CD25^{hi} cells purified from WT OT-II, CCR4^{-/-} OT-II, and CCR7^{-/-} OT-II mice were cocultured with CFSE-labeled splenic WT CD4⁺CD25⁻ T cells simulated with anti-CD3/CD28 antibodies in the presence of IL-2 in a Treg cell suppression assay. After 3 days, cell division was assessed by means of measurement of CFSE dilution. Treg cells 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 Treg cells isolated from CCR4^{-/-} and CCR7^{-/-} mice.

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

In the sensitization model Treg cells were freshly isolated from spleens and LNs of WT, CCR4^{-/-} OT-II, or CCR7^{-/-} OT-II mice and adoptively transferred into recipient WT mice 1 day before immunization. Host mice were then immunized twice with OVA in adjuvant (alum) 2 weeks apart. Three days after the second immunization, mice were challenged daily with 4 aerosolized doses of OVA ([Fig 1, A](#)). Twenty-four hours after the fourth aerosolized OVA challenge, BAL fluid and lungs were harvested for analysis. As expected, OVA-challenged mice that did not receive Treg cells revealed prominent eosinophilic airway inflammation. However, mice that received either WT or CCR4^{-/-} OT-II Treg cells before immunization showed markedly suppressed eosinophilic inflammation. In marked contrast, mice that received CCR7^{-/-} OT-II Treg cells before sensitization showed minimal suppression of eosinophilic airway inflammation ([Fig 1](#)). The BAL fluid of mice that received WT and CCR4^{-/-} OT-II Treg cells had significantly fewer total cells, lymphocytes, and eosinophils than control mice that did not receive Treg cells or mice that received CCR7^{-/-} OT-II Treg cells ([Fig 1, B and C](#)). 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 Treg cells compared with mice that received PBS or CCR7^{-/-} OT-II Treg cells ([Fig 1, D](#)). Likewise, PAS staining revealed a marked decrease in mucus production in mice that received WT or CCR4^{-/-} Treg cells compared with that seen in mice that received PBS or CCR7^{-/-} Treg cells ([Fig 1, C](#)). Finally, there was a significant reduction in levels of the Th2 cytokines IL-4, IL-5, and IL-13 and an increase in levels of the anti-inflammatory cytokine IL-10 in the BAL fluid of mice transferred with WT OT-II Treg cells compared with those in mice that received PBS or CCR7^{-/-} OT-II Treg cells ([Fig 1, E](#)). Of note, antigen-nonspecific polyclonal Treg cells were less effective at suppressing allergic airway inflammation than antigen-specific OT-II Treg cells (see [Fig E2, A](#), in this article’s Online Repository at www.jacionline.org), suggesting that Treg cell suppression of allergic airway inflammation during the sensitization phase is more effectively mediated in an antigen-specific manner. These data demonstrate that Treg cells 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 Treg cells to regulate the priming phase of an immune response.^{3,15,25-27}

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

We next studied the ability of Treg cells to suppress allergic airway inflammation in sensitized mice (“effector” model). Freshly isolated Treg cells were adoptively transferred intravenously into OVA-immunized recipient WT mice 2 days after the second intraperitoneal immunization ([Fig 2, A](#)). One day after Treg cell transfer, mice received 4 daily aerosolized OVA challenges. Twenty-four hours after the fourth aerosolized challenge, BAL fluid and lungs were harvested for analysis. As

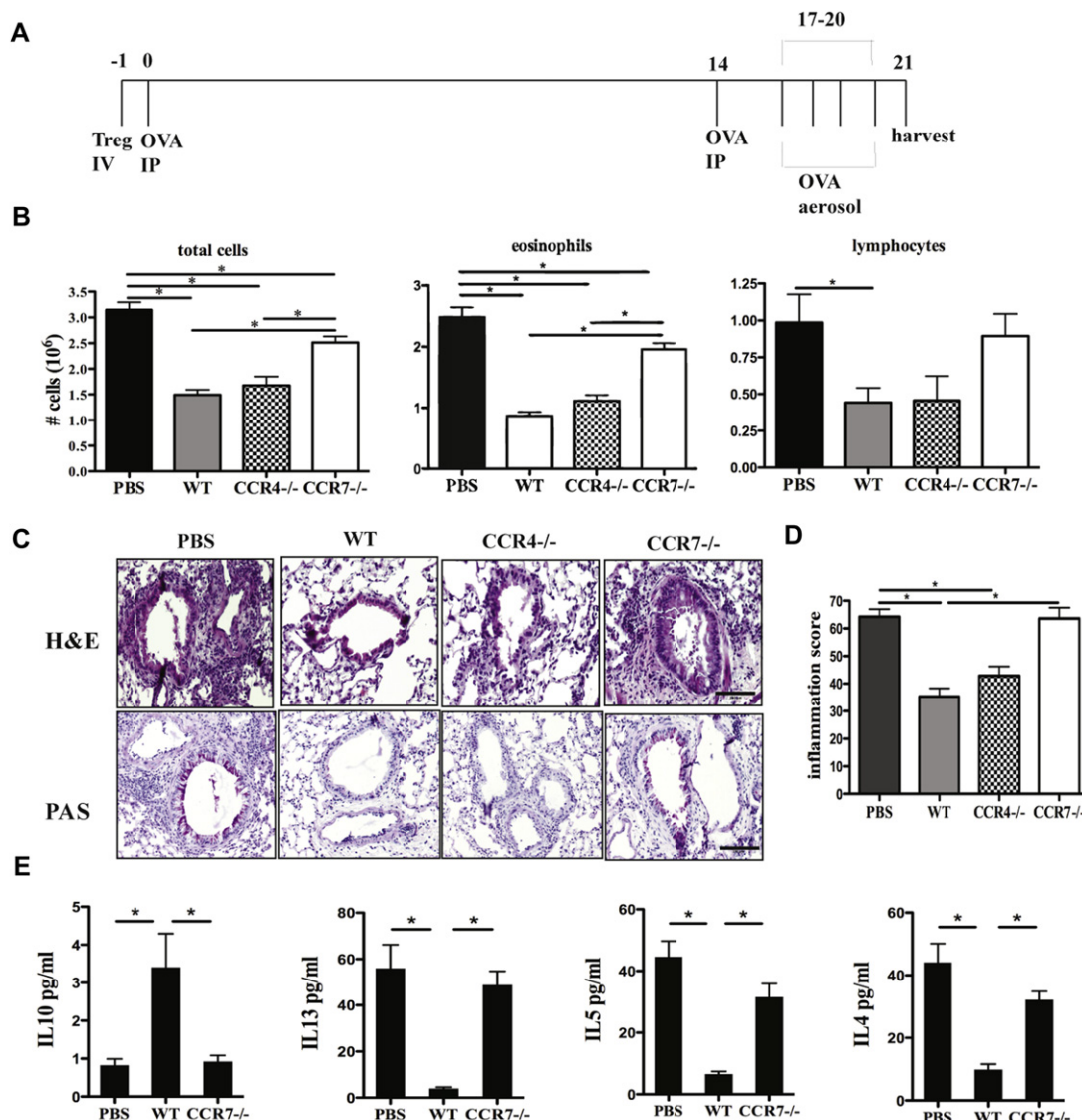


FIG 1. CCR7, but not CCR4, is required on Treg cells to suppress allergic airway inflammation in the sensitization model. **A**, Schematic of the sensitization model of Treg cell suppression (see the [Methods](#) section for details). *IP*, Intraperitoneal; *IV*, intravenous. **B**, Total cells, eosinophils, and lymphocytes in the BAL fluid were enumerated at day 21 in WT mice that received PBS (solid bars), WT OT-II Treg cells (gray bars), CCR4^{-/-} OT-II Treg cells (checked bars), or CCR7^{-/-} OT-II Treg cells (open bars) intravenously on day -1 followed by OVA administered intraperitoneally on days 0 and 14. Data are representative of 3 experiments. **C**, Histopathologic analysis of lung sections on day 21 stained with hematoxylin and eosin (H&E; top) and PAS (bottom). Scale bar = 50 μm. Data are representative of 3 independent experiments. **D**, Blinded histopathologic scoring of lung inflammation on day 21 from mice treated as above (n ≥ 10 mice per group from 3 experiments). *P < .05. **E**, Cytokines measured in the BAL fluid. Data are means ± SEMs of 8 mice per group from 2 experiments. *P < .05.

before, mice that did not receive Treg cells had prominent eosinophilic airway inflammation. However, in this model eosinophilic inflammation was suppressed in mice that received either WT or CCR7^{-/-} OT-II Treg cells after sensitization but was not suppressed in mice that received CCR4^{-/-} OT-II Treg cells after sensitization (Fig 2). Analysis of cells isolated from BAL fluid from OVA-immunized and OVA-challenged mice demonstrated a marked decrease in the numbers of total cells and eosinophils in the BAL fluid of mice receiving either WT or CCR7^{-/-} OT-II Treg cells compared with numbers found in mice receiving either PBS or CCR4^{-/-} OT-II Treg

cells (Fig 2, B). 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 Treg cells compared with control PBS-injected mice or mice that received CCR4^{-/-} OT-II Treg cells (Fig 2, C and D). Likewise, PAS staining revealed a marked decrease in mucus production in mice that received WT or CCR7^{-/-} Treg cells compared with mice that received PBS or CCR4^{-/-} Treg cells (Fig 2, C). Finally, we observed a significant reduction in levels of the Th2 cytokines IL-4, IL-5, and IL-13 and a marked increase in levels of the anti-inflammatory cytokine IL-10 in the

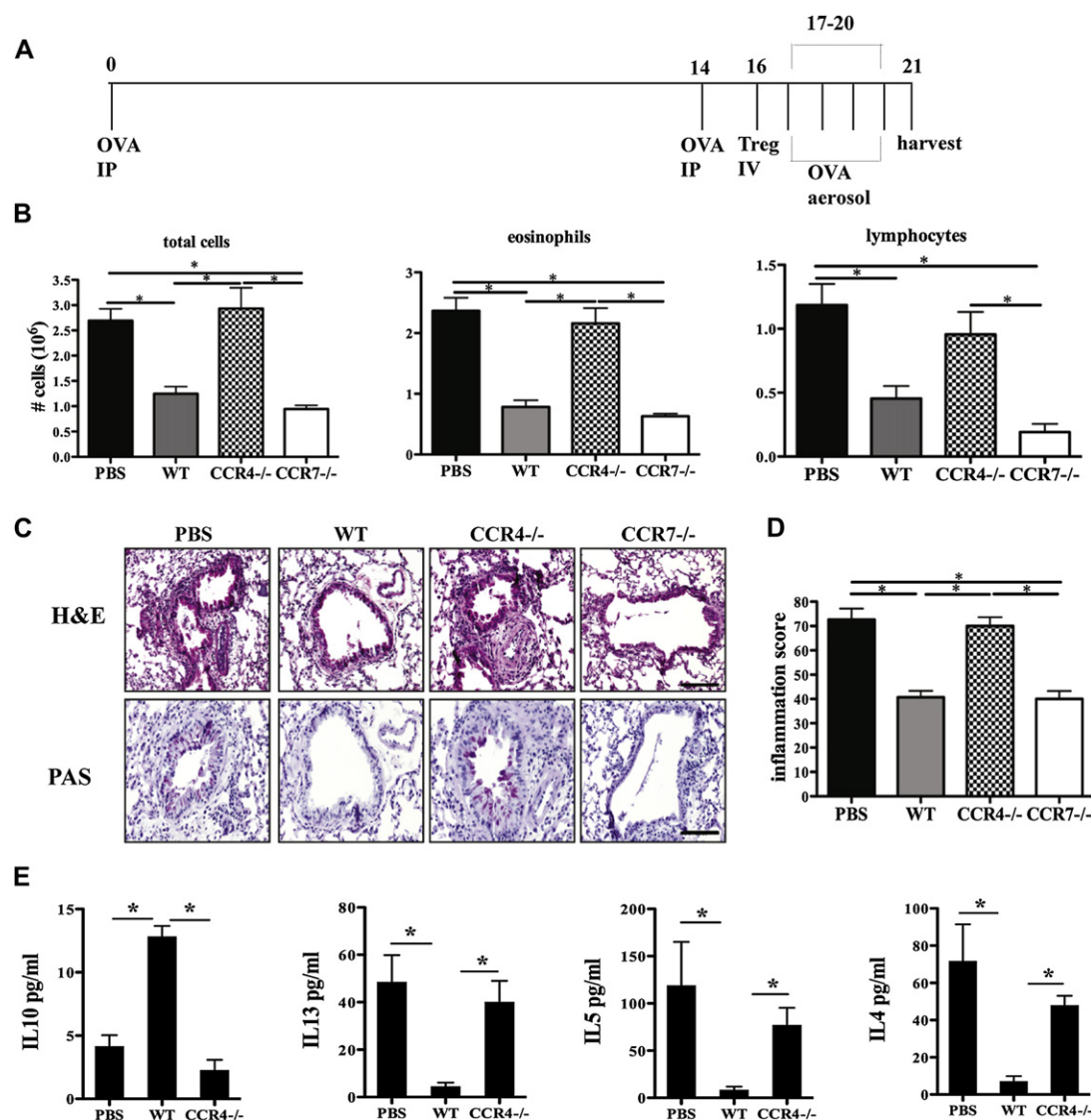


FIG 2. CCR4, but not CCR7, is required on Treg cells to suppress allergic airway inflammation in the effector model. **A**, Schematic of the effector model of Treg cell suppression (see the [Methods](#) section for details). IP, Intraperitoneal; IV, intravenous. **B**, Total cells, eosinophils, and lymphocytes in the BAL fluid were enumerated on day 21 in OVA-immunized mice that received PBS (solid bars), WT OT-II Treg cells (gray bars), CCR4^{-/-} OT-II Treg cells (checked bars), or CCR7^{-/-} OT-II Treg cells (open bars) on day 16. Data are representative of 3 experiments. **C**, Histopathologic analysis of lung sections stained with hematoxylin and eosin (H&E; top) and PAS (bottom). Scale bar = 50 μ m. Data are representative of 3 independent experiments. **D**, Blinded histopathologic scoring of lung inflammation on day 21 from mice treated as above ($n \geq 10$ mice per group from 3 experiments). * $P < .05$. **E**, Cytokines measured in the BAL fluid. Data are means $\pm$ SEMs of 8 mice per group from 2 experiments. * $P < .05$.

BAL fluid of mice that received WT OT-II Treg cells but not in the BAL fluid of mice that received PBS or CCR4^{-/-} OT-II Treg cells (Fig 2, E). Airway inflammation in recipient mice that received CCR4^{-/-} OT-II Treg cells in this model was no different than that in mice that received no Treg cells. As seen in the sensitization model, antigen-nonspecific polyclonal Treg cells were less effective at suppressing allergic airway inflammation than antigen-specific OT-II Treg cells (see Fig E2, B), suggesting that Treg cell suppression of allergic airway inflammation during the effector phase is also more effectively mediated in an antigen-specific manner. These data demonstrate that Treg cells 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⁺ Treg cells for suppression of spontaneous pulmonary inflammation in Foxp3-deficient *scurfy* mice.²⁸

Chemokine-dependent Treg cell location dictates function

To determine whether the differential chemokine receptor dependence of Treg cell suppressor function in the sensitization and effector models was the result of the differential location

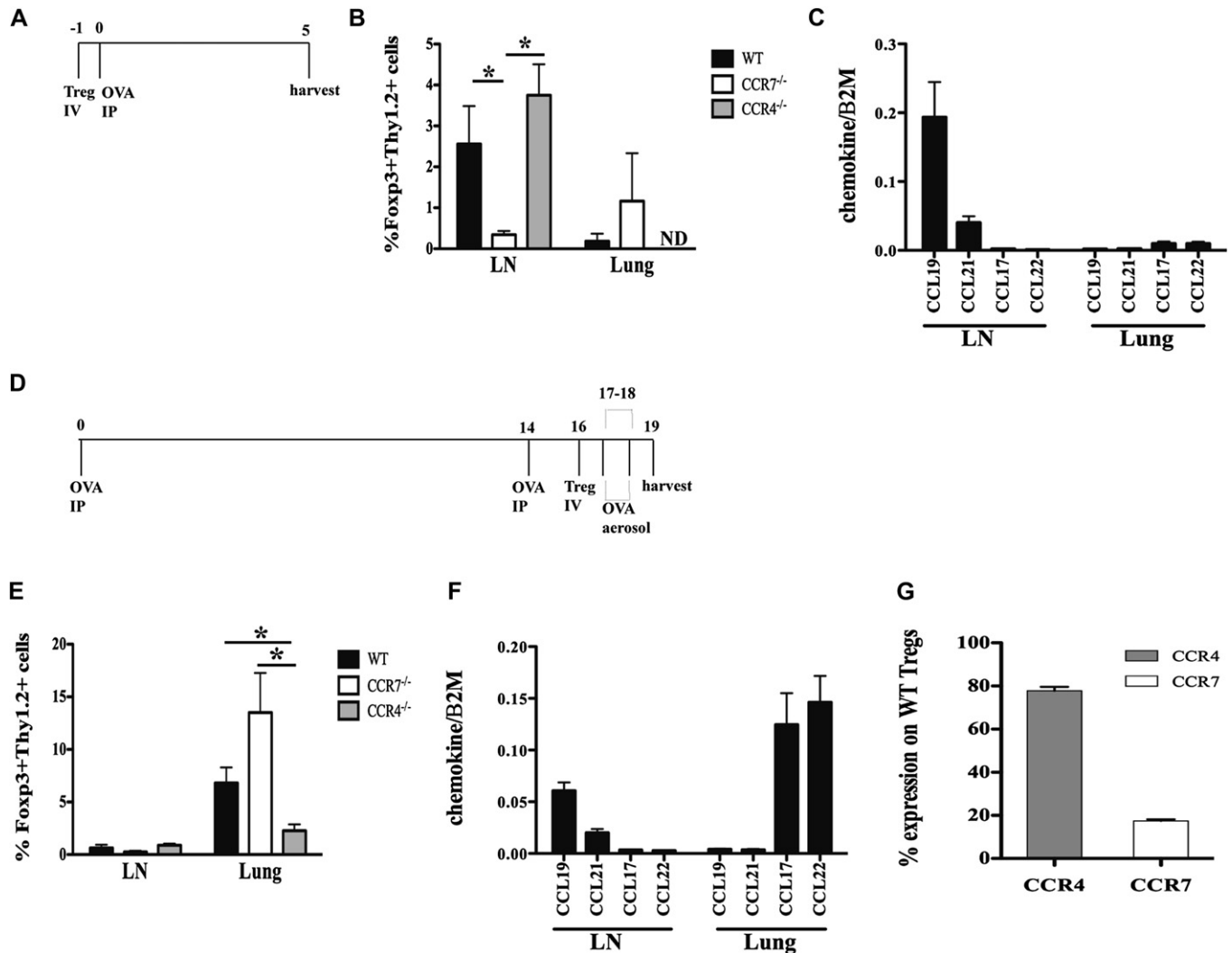


FIG 3. Chemokine receptor-dependent trafficking of Treg cells to LNs and lungs. **A**, Schematic of the experimental protocol. **B**, Cells from thoracic LNs and lungs were analyzed for Foxp3⁺Thy1.2⁺ expression (gated on CD4⁺CD25⁺ cells) on day 5. Data are pooled from 4 independent experiments. **C**, Chemokine expression in LNs (pooled thoracic and inguinal) and lungs from WT mice measured on day 5 by using qPCR (n = 8 mice from 2 independent experiments). **D**, Schematic of the experimental protocol. **E**, Percentage of Foxp3⁺Thy1.2⁺ cells of CD4⁺CD25⁺Foxp3⁺ cells in lymphocyte gate isolated from the LNs and lungs on day 19. Data are pooled from 4 independent experiments. **F**, Chemokine expression in the thoracic LNs and lungs from WT mice measured by using qPCR on day 19 (n = 8 mice from 2 independent experiments). **G**, Percentage of CCR4 and CCR7 cell-surface expression on Treg cells (gated on CD4⁺CD25⁺Foxp3⁺ cells in lymphocyte gate) recovered from the lungs on day 21 in the effector model. Data are means $\pm$ SEMs. **P* < .05. B2M, β_2 -Microglobulin; IP, intraperitoneal; IV, intravenous; ND, not determined.

where Treg cells need to function in the 2 models, we analyzed the location of WT, CCR4^{-/-}, and CCR7^{-/-} Thy1.2 OT-II Treg cells after adoptive transfer into WT Thy1.1 mice in the 2 models (Fig 3, A and D). In the sensitization model WT and CCR4^{-/-} OT-II Treg cells were found in equal numbers in the lymphoid compartment, whereas CCR7^{-/-} OT-II Treg cells were not found in LNs (Fig 3, B). WT and CCR4^{-/-} OT-II Treg cells were not recovered from the lung, whereas CCR7^{-/-} OT-II Treg cells were, perhaps because CCR7^{-/-} OT-II Treg cells could not home to the LNs. We also analyzed the pattern of CCR7 and CCR4 ligand expression in the LNs and lungs of mice in the sensitization model. Using qPCR, we found that the CCR7 ligands CCL19 and CCL21 were detected preferentially in LNs and not in the

lung. In contrast, the CCR4 ligands CCL17 and CCL22 were detected at very low levels in lungs and not in the LNs (Fig 3, C). These data suggest that in the sensitization phase CCR7 expression is required for Treg cell homing into the lymphoid compartment.

In the effector model WT and CCR7^{-/-} OT-II Treg cells were found in the lung in roughly equal numbers, whereas the numbers of CCR4^{-/-} OT-II Treg cells recovered from the lung were markedly reduced (Fig 3, E). Treg cells 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, whereas the CCR7 ligands were moderately expressed in the thoracic LNs (Fig 3, F). These

data strongly suggest that CCR4 expression is required for Treg cell migration into the lung. Examination of CCR7 and CCR4 expression on WT Treg cells isolated from the lung in this model after 2 aerosolized OVA challenges revealed that 75% of the Foxp3⁺ Treg cells expressed CCR4, whereas only 17% expressed CCR7 (Fig 3, G). Collectively, these results suggest that chemokine receptor expression on Treg cells affects their homing ability, which dictates their functional capacities at different stages of the allergic airway response.

Treg cells are enriched in BAL fluid of human asthmatic patients after airway allergen challenge

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

Analysis of the BAL fluid revealed a characteristic allergic airway response in the allergen-challenged segment. In response to allergen challenge, the total number of cells increased greater than 9-fold, lymphocyte numbers increased greater than 4-fold, and eosinophil numbers increased greater than 690-fold in the BAL fluid (Fig 4, A). Although there was no difference in the percentage of CD4⁺ T cells after allergen challenge (Fig 4, B), given the increase in total lymphocyte numbers, the total number of CD4⁺ cells increased 5.5-fold in the BAL fluid after allergen challenge (Fig 4, C), demonstrating a recruitment of CD4⁺ cells into the airways.

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

CCR4 is expressed on Treg cells recruited into the BAL fluid after allergen challenge

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

BAL fluid Treg cells from asthmatic subjects are functional

We next determined whether the Treg cells recovered from the BAL fluid of asthmatic subjects after allergen challenge were functional by using a Treg cell suppression assay (Fig 4, G). Autologous peripheral blood CD4⁺ T-cell proliferation was inhibited up to 50% when cultured with an equivalent number of CD4⁺CD25⁺ Treg cells isolated from the BAL fluid. As expected, as the ratio of Treg cells to effector T cells decreased, T-cell proliferation was inhibited to a lesser extent. These data confirm that Treg cells recovered from the lungs of allergic asthmatic patients are functional.

DISCUSSION

Using chemokine receptor-deficient Treg cells in adoptive transfer experiments, we found that CCR7-dependent Treg cell homing to the LNs was required to suppress the sensitization phase of allergic pulmonary inflammation. In contrast, in allergen-sensitized mice CCR7-dependent Treg cell homing to the LNs was dispensable for suppression of the allergen recall response, and instead, CCR4-dependent Treg cell 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 mice,⁴ peripheral tissue, such as the lung, is the site at which Treg cells are required to home to suppress recall responses. It will be of interest to determine whether this is a general principle for where Treg cells need to function in recall versus primary immune responses.

We extended our murine studies to human subjects and found that the number of CD4⁺CD25⁺Foxp3⁺ Treg cells in the BAL fluid of allergic asthmatic subjects increased significantly after bronchoscopic allergen challenge and that CCR4, of all of the chemokine receptors tested, was the one chemokine receptor increased on these recruited Treg cells. These data suggest that in human subjects, as in the mouse, CCR4 might be critical for Treg cell recruitment into the asthmatic airway.

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

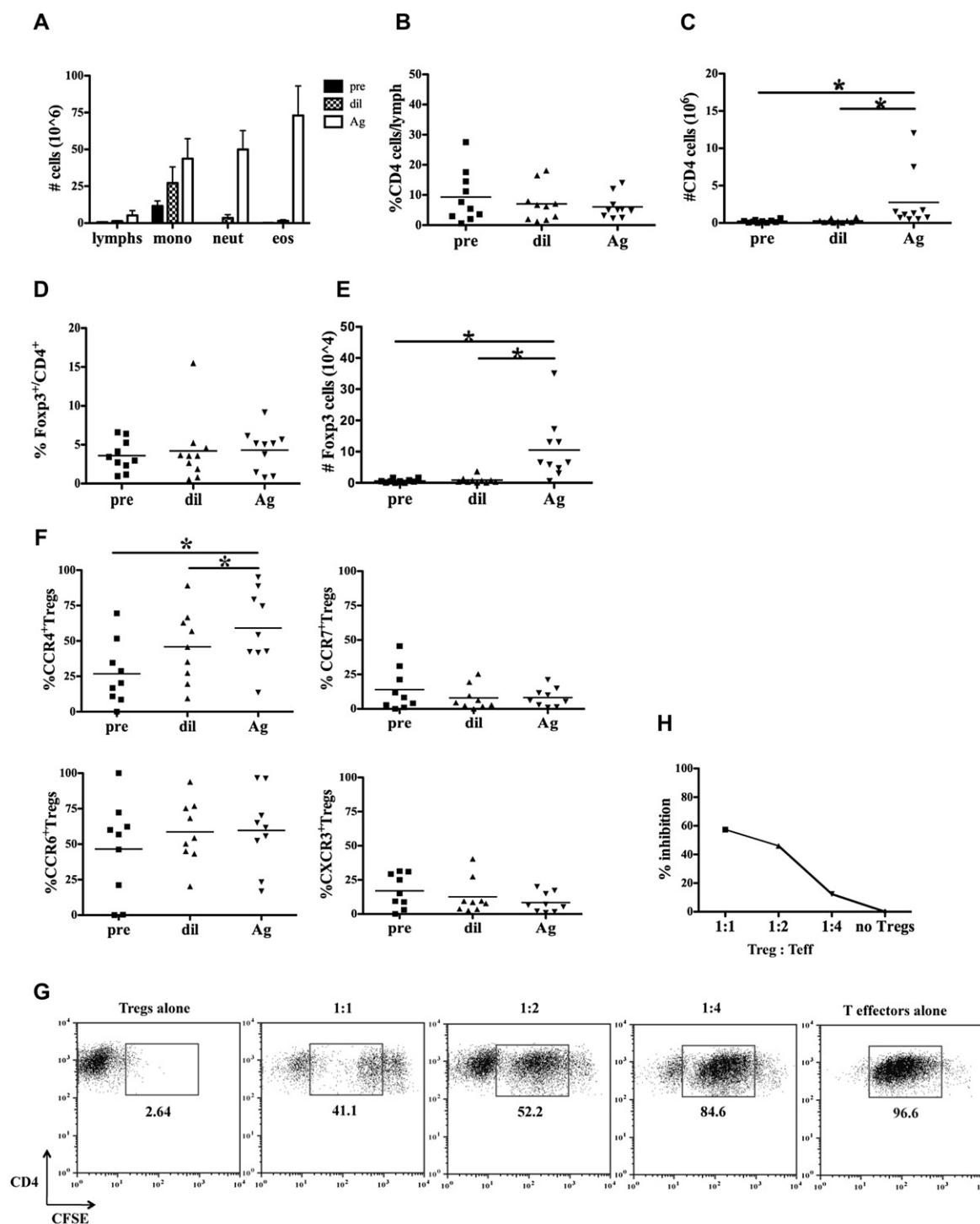


FIG 4. Treg cells recruited into the human allergic lung express CCR4 and are functional. Data throughout the figure are from 10 allergic asthmatic subjects in Fig 4, A-F. **A**, Numbers of lymphocytes, monocytes, neutrophils, and eosinophils from baseline and allergen-challenged BAL fluid (mean $\pm$ SEM). **B** and **C**, Percentages (Fig 4, B) and numbers (Fig 4, C) of CD4⁺ cells recovered from the BAL fluid before allergen challenge (*pre*) and 24 hours after bronchoscopic segmental diluent (*dil*) or allergen (*Ag*) challenge (*n* = 10 subjects). **P* < .05. **D** and **E**, Percentages of CD25⁺Foxp3⁺ Treg cells of total CD4⁺ cells (Fig 4, D) and numbers of CD4⁺CD25⁺Foxp3⁺ Treg cells (Fig 4, E) recovered from BAL fluid before and 24 hours after bronchoscopic diluent or allergen challenge (*n* = 10 subjects). **P* < .05. **F**, Percentages of Treg cells (CD4⁺CD25⁺Foxp3⁺) expressing CCR4, CCR6, CCR7, and CXCR3 in the BAL fluid (*n* = 10 subjects). **P* < .05. **G** and **H**, Treg cell suppression assay. Representative FACS plots (Fig 4, G) and quantitation of percentage inhibition of effector T (*Teff*) cell proliferation (Fig 4, H) in the presence or varying ratios of effector T cells to Treg cells (*n* = 3).

Our data offer insight into the conflicting literature regarding the therapeutic utility of inhibiting CCR4 in patients with allergic pulmonary inflammation. Using T_H2 cell adoptive transfer models of allergic pulmonary inflammation, we previously demonstrated that CCR4 plays an important role in effector T_H2 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 cell recruitment to the lung would also be inhibited along with T_H2 cell recruitment, thus perhaps negating the beneficial effects of CCR4 blockade on T_H2 cell recruitment. We believe the fact that Treg cells use the same chemokine receptor pathways as effector T cells to gain access to inflamed peripheral tissue might limit the usefulness of global chemokine inhibition and might 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, the sparing of this inhibition on Treg cells, or both might be required for effective chemokine therapeutics.

Key messages

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

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METHODS

Lung inflammation score

The amount of inflammation was assessed by a researcher blinded to the genotype of the adoptively transferred Treg cells. The evaluator assessed the inflammatory infiltrate around the airways. Each set of sections was given a score of 0 to 5 for inflammation (0, no inflammation; 1, 20% of airways with inflammation; 2, 20% to 40% of airways with inflammation; 3, 40% to 60% of airways with inflammation; 4, 60% to 80% of airways with inflammation; and 5, greater than 80% of airways with inflammation).

Cytospin

Cell differential counts for BAL fluid were determined by enumerating alveolar macrophages, neutrophils, eosinophils, and lymphocytes on cytocentrifuge preparations stained with a combination of Wright stain (EM Sciences, Hatfield, Pa) and Diff-Quick (Dade Behring, Deerfield, Ill).

Isolation of mouse tissue lymphocytes

BAL was performed by means of lavage of the whole lung through the trachea with 3×1 mL of PBS containing 0.6 mmol/L EDTA. Thoracic LNs were collected and dispersed into single-cell suspensions by means of passage through cell strainers. Lungs were either inflated with PBS/formalin for histology or perfused with PBS before digestion to obtain a single-cell suspension, as described previously.^{E1}

Flow cytometric staining for human Treg cells

For cell-surface expression, BAL fluid was blocked with 10% human serum and stained in 2% FCS. Cells were then stained for Foxp3 expression, according to the manufacturer's instructions (eBioscience). Cells were then fixed with 2% paraformaldehyde. Samples were run on a FACSCalibur (BD Biosciences) cytometer and analyzed with FlowJo software (TreeStar, Ashland, Ore). BAL fluid lymphocytes were analyzed with forward- and side-scatter properties, as previously described.^{E2}

Murine Treg cell suppression assay

CD4⁺ T cells were separated into CD25⁺ and CD25[−] fractions by staining with CD25-PE and sorting the CD25^{hi} cells by using flow cytometry (FACSARIA). Final CD4⁺CD25^{hi} cells were approximately 90% pure. WT CD4⁺CD25[−] cells were incubated for 10 minutes at 37°C in 10 μ mol/L CFSE (Invitrogen) in PBS, washed 3 times with RPMI, and resuspended in complete RPMI. In each culture well 10⁵ CFSE-labeled WT CD4⁺CD25[−] cells were incubated with varying concentrations of WT, CCR4^{−/−}, or

CCR7^{−/−} CD4⁺CD25^{hi} Treg cells. All cultures (except unstimulated control cultures) were stimulated with CD3/CD28 beads (Dynabeads, Invitrogen) and 20 ng/mL IL-2 (PeproTech, Rocky Hill, NJ) for 3 days. Proliferation of CD4⁺CD25[−] T cells was measured by assessing relative CFSE dilution with flow cytometry.

PCR primers used for qPCR

Specific primers for sequence detection of message for CCL19 were forward primer 5'-ATGCGGAAGACTGCTGCC-3' and reverse primer 5'-CGGAAGGCTTTCACGATGTT-3', those for CCL21 were forward primer 5'-TCCCGGCAATCCTGTTCTT and reverse primer 5'-CCTTCCTCAGGGTTTGACA-3', those for CCL17 were forward primer 5'-CAGGGATGCCATCGTGTTTC-3' and reverse primer 5'-CACCAATCTGATGGCCTTCTT-3', and those for CCL22 were forward primer 5'-TACATCCGT CACCTCTGCC-3' and reverse primer 5'-CGGTTATCAAAACAACGCAG-3'. Samples underwent amplification in the presence of SYBR Green (Applied Biosystems) and were analyzed by using real-time qPCR (Mastercycler EP *Realplex*; Eppendorf, Hauppauge, NY).

Allergen extracts for skin testing and segmental allergen challenge of human subjects

Standardized allergen extracts for cat hair and *Dermatophagoides pteronyssinus* were purchased from Greer Laboratories (Lenoir, NC). Standardized cat hair allergen extract contained 0.1 ng/mL endotoxin; standardized *D pteronyssinus* allergen extract contained 9 ng/mL endotoxin. All subjects underwent allergen skin prick testing and were found to have a positive skin prick test response to either cat or dust mite extract. The threshold level of allergen sensitivity was then determined by using skin prick test titration with serial 3-fold dilutions of either cat or dust mite extract. The lowest concentration of extract eliciting a positive prick skin test response was used as the allergen concentration for segmental allergen challenge. Subjects had not taken inhaled corticosteroids for at least 2 weeks, bronchodilators for 12 hours, and antihistamines for 1 week before undergoing procedures.

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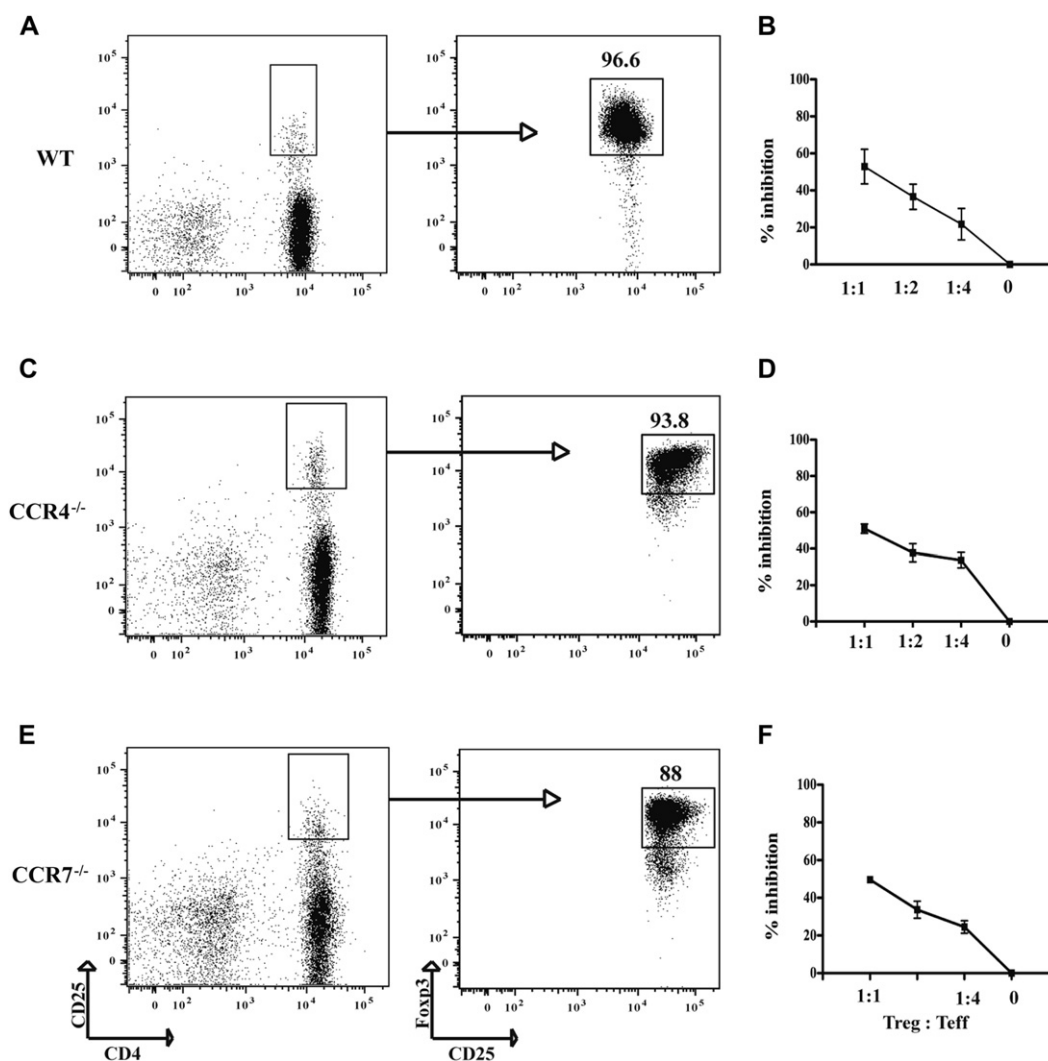


FIG E1. WT OT-II, CCR4^{-/-} OT-II, and CCR7^{-/-} OT-II Treg cells suppress effector T cells. **A, C, and E,** Representative FACS plots demonstrating the percentage of Foxp3 staining on CD4⁺CD25^{hi} sorted Treg cells isolated from the spleens and LNs of WT OT-II, CCR4^{-/-} OT-II, or CCR7^{-/-} OT-II mice before adoptive transfer. **B, D, and F,** Percentage inhibition of effector T (*Teff*) cell proliferation in the presence or absence of different ratios of sorted Treg cells isolated from WT OT-II, CCR4^{-/-} OT-II, or CCR7^{-/-} OT-II mice (n = 3 mice per group).

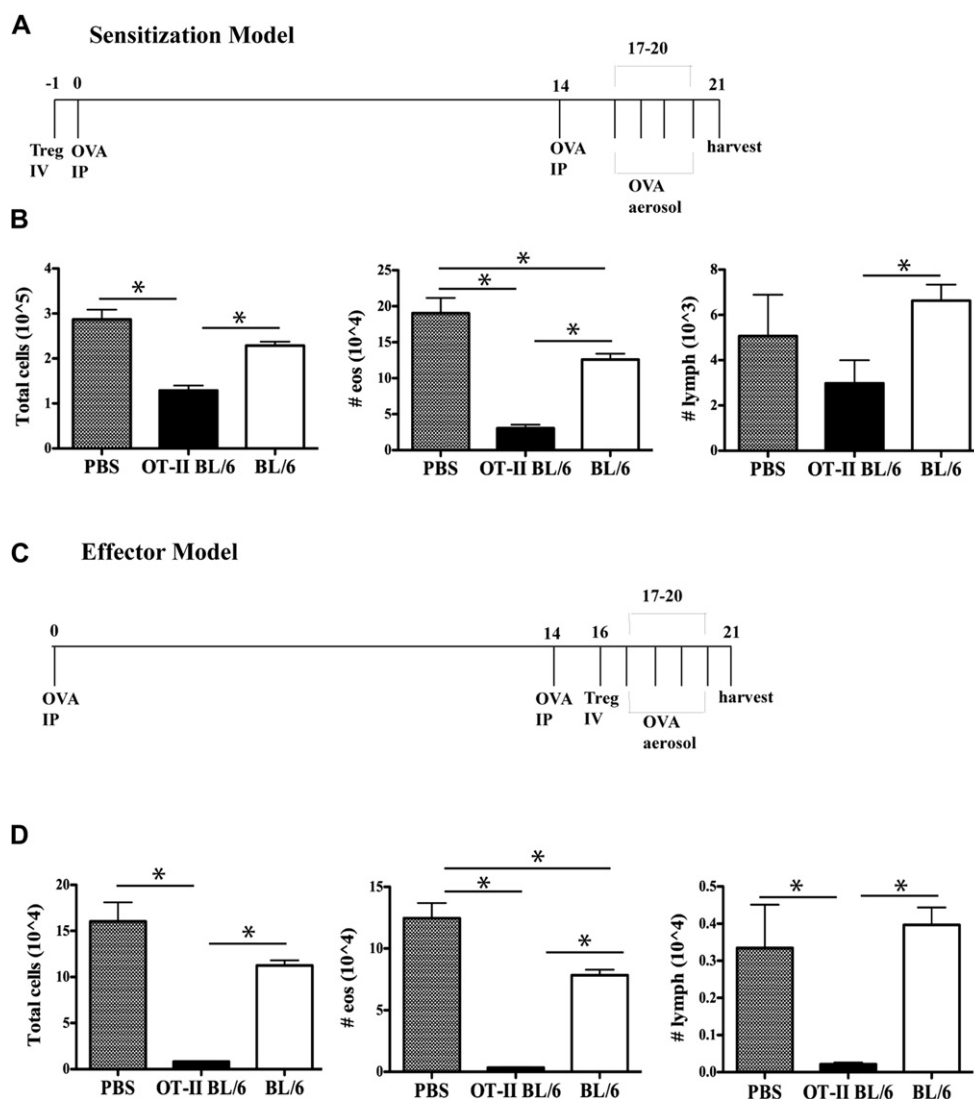


FIG E2. Antigen-specific Treg cells are more effective at suppressing allergic airway inflammation than polyclonal Treg cells. **A**, Schematic of the sensitization model of Treg cell suppression (see the [Methods](#) section for details). **B**, Total cells, eosinophils, and lymphocytes in the BAL fluid were enumerated at day 21 in C57BL/6 WT mice that received PBS (gray bars), WT OT-II Treg cells (solid bars), or WT polyclonal Treg cells for C57BL/6 mice (open bars) intravenously on day -1 followed by OVA administered intraperitoneally on days 0 and 14 and daily aerosolized OVA challenges on days 17 to 20. Data are representative of 2 experiments ($n = 6-7$ mice per group from 2 experiments). **C**, Schematic of the effector model of Treg cell suppression (see the [Methods](#) section for details). **D**, Total cells, eosinophils, and lymphocytes in the BAL fluid were enumerated on day 21 in OVA-immunized mice (intraperitoneally) that received PBS (gray bars), WT OT-II Treg cells (solid bars), or WT polyclonal Treg cells from C57BL/6 mice (open bars) on day 16 followed by daily aerosolized OVA challenges on days 17 to 20. Data are representative of 2 experiments ($n = 6-7$ mice per group from 2 experiments). * $P < .05$. IP, Intraperitoneal; IV, intravenous.

TABLE E1. Asthmatic subjects' characteristics, lung function test results, and allergen doses used in segmental allergen challenge

Subject no.	Allergen used	Allergen full dose	FEV ₁	FEV ₁ (% predicted)	FVC	FVC (% predicted)	Methacholine PC ₂₀
1	DP	123.3	3.27	ND	3.82	ND	ND
2	DP	370	2.86	106	3.55	113	ND
3	DF	370	2.77	97	3.57	106	ND
4	Cat	123.3	3.29	85	4.25	92	ND
5	DP	41	2.02	75	2.38	77	ND
6	DF	13.7	3.47	103	4.39	112	0.22
7	Cat	41	3.96	103	4.67	104	>25
8	DP	3333	3.59	106	3.83	101	1.69
9	DF	1111	3.38	86	4.34	91	2.52
10	DF	123.3	4.42	118	5.03	112	>25

Allergen doses are measured either in bioequivalent allergy units per milliliter for cat hair allergen or in arbitrary units per milliliter for *D pteronyssinus* and *D farinae* allergens. Allergen doses in segmental allergen challenge were based on responsiveness to skin testing. Subjects did not take corticosteroids for at least 2 weeks before the study. DF, *Dermatophagoides farinae*; DP, *Dermatophagoides pteronyssinus*; ND, not determined.