



Regulatory T Cells Migrate to Airways via CCR4 and Attenuate the Severity of Airway Allergic Inflammation

This information is current as of February 9, 2013.

Lucas Faustino, Denise Morais da Fonseca, Maisa Carla Takenaka, Luciana Mirotti, Esther Borges Florsheim, Marcia Grandó Guerreschi, João Santana Silva, Alexandre Salgado Basso and Momtchilo Russo

J Immunol published online 6 February 2013
<http://www.jimmunol.org/content/early/2013/02/06/jimmunol.1202354>

-
- Supplementary Material** <http://www.jimmunol.org/content/suppl/2013/02/06/jimmunol.1202354.DC1.html>
- Subscriptions** Information about subscribing to *The Journal of Immunology* is online at: <http://jimmunol.org/subscriptions>
- Permissions** Submit copyright permission requests at: <http://www.aai.org/ji/copyright.html>
- Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at: <http://jimmunol.org/cgi/alerts/etoc>



Regulatory T Cells Migrate to Airways via CCR4 and Attenuate the Severity of Airway Allergic Inflammation

Lucas Faustino,* Denise Morais da Fonseca,[†] Maisa Carla Takenaka,[‡] Luciana Mirotti,* Esther Borges Florsheim,* Marcia Grando Guerreschi,[‡] João Santana Silva,[†] Alexandre Salgado Basso,[‡] and Momtchilo Russo*

We have previously shown that regulatory T (Treg) cells that accumulate in the airways of allergic mice upregulate CC-chemokine receptor 4 (CCR4) expression. These Treg cells suppressed in vitro Th2 cell proliferation but not type 2 cytokine production. In the current study, using a well-established murine model of allergic lung disease or oral tolerance, we evaluated the in vivo activity of Treg cells in allergic airway inflammation with special focus on CCR4 function. We found that allergic, but not tolerant, mice treated with anti-CD25 Ab showed increased airway eosinophilia and IL-5- or IL-4-producing Th2 cells when compared with untreated mice. Notably, mice with CCR4 deficiency displayed an augmented airway allergic inflammation compared with wild-type or CCR2 knockout (KO) mice. The allergic phenotype of CCR4KO mice was similar to that observed in anti-CD25-treated mice. The exacerbated allergic inflammation of CCR4KO mice was directly associated with an impaired migration of Treg cells to airways and augmented frequency of pulmonary Th2 cells. Adoptive transfer of CD25⁺CD4⁺ T cells expressing high levels of CCR4, but not CCR4KO CD25⁺CD4⁺ T cells, attenuated the severe airway Th2 response of CCR4KO mice. Our results show that CCR4 is critically involved in the migration of Treg cells to allergic lungs that, in turn, attenuate airway Th2 activation and allergic eosinophilic inflammation. *The Journal of Immunology*, 2013, 190: 000–000.

Allergic asthma is characterized by airway eosinophilic inflammation associated with airway hyperresponsiveness, mucus hypersecretion, and elevated production of IgE (1, 2). The development of allergic responses is mediated by Th2 effector cells that secrete type 2 cytokines IL-4, IL-5, IL-9, and IL-13 responsible to the asthma phenotype (2).

Foxp3⁺ regulatory T (Treg) cells are essential for maintenance of immunological homeostasis and self-tolerance, and deficiency of Foxp3 results in spontaneous development of autoimmunity and other inflammatory disorders, including allergic reactions both in humans and mice (3–7). Usually, mucosal exposure to nonpathogenic Ags results in a state of hyporesponsiveness, known as mucosal tolerance, which is revealed upon immu-

nogenic contact with the same Ag. Indeed, it has been shown that oral or nasal Ag administration can efficiently inhibit pulmonary Th2-mediated responses (8–10). The involvement of Treg cells in preventing the development of asthma-like response was clearly shown in transgenic T/B monoclonal mice, which lack naturally occurring Treg cells (11). In these mice, previous oral exposure to OVA resulted in the generation of adaptive Foxp3-expressing Treg cells in the spleen and draining lymph nodes that efficiently inhibited the development of allergic airway disease in a TGF- β -dependent manner (11). It has been postulated that insufficient suppressive activity of Treg cells in allergic patients during birch pollen season or in atopic individuals underlines allergic airway inflammation (12, 13).

We have recently shown that Foxp3⁺ Treg cells accumulate in the lungs of allergic mice and that the majority of these cells upregulated CCR4, CD69, and CD54 and drastically downregulated CD62L, a phenotype that resemble effector/memory T cells, distinguishing from naive CCR4^{low}CD69[–]CD54^{low}CD62L^{high} Treg cells (14). Our in vitro studies showed that highly purified infiltrating lung Treg cells efficiently suppress pulmonary Th cell proliferation but fail to inhibit type 2 cytokine production (14). However, in vivo studies are still necessary to pinpoint and dissect the role of Treg cells in allergic processes or in tolerance.

Naive and activated Treg cells as other T cells can be distinguished by the expression of particular chemokine receptors and adhesion molecules responsible for their localization in lymph nodes or inflamed tissues (15, 16). Some studies indicate a crucial role of CCR4 in the pathogenesis of asthma by recruiting Th2 cells to the site of allergen exposure (17–19). However, other studies using knockout (KO) mice for CCR4 or Abs against these chemokine receptor gathered conflicting results (19–22).

In this study, we approached the function of Treg cells in allergic or tolerant mice by inactivating Treg cells in vivo with anti-CD25 mAb treatment or by the use of CCR4-deficient mice.

*Department of Immunology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, SP 05508-900, Brazil; [†]Department of Biochemistry and Immunology, School of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP 14049-900, Brazil; and [‡]Department of Microbiology, Immunology, and Parasitology, São Paulo School of Medicine, Federal University of São Paulo, São Paulo, SP 04023-062, Brazil

Received for publication August 22, 2012. Accepted for publication January 2, 2013.

This work was supported by Fundação de Amparo a Pesquisa do Estado de São Paulo and Conselho Nacional de Desenvolvimento Científico e Tecnológico.

L.F. designed and executed experiments and interpreted data; D.M.F., M.C.T., L.M., E.B.F., and M.G.G. participated in the execution of some experiments and interpretation of the data; M.R. designed the experiments and together with L.F., J.S.S., and A.S.B. interpreted data and wrote the manuscript. All authors contributed to the editing of the final draft.

Address correspondence and reprint requests to Prof. Momtchilo Russo, Department of Immunology, Institute of Biomedical Sciences, University of São Paulo, Avenida Professor Lineu Prestes 1730, São Paulo, SP 05508-900, Brazil. E-mail address: momrusso@icb.usp.br

The online version of this article contains supplemental material.

Abbreviations used in this article: BAL, bronchoalveolar lavage; KO, knockout; MHC II, MHC class II; MLN, mesenteric lymph node; PAS, periodic acid-Schiff; Teff, effector T; Treg, regulatory T; WT, wild-type.

Copyright © 2013 by The American Association of Immunologists, Inc. 0022-1767/13/\$16.00

We found that in allergic but not in tolerant mice, treatment with anti-CD25 mAb during allergen challenges resulted in more activated Th2 cells producing IL-5 when compared with wild-type (WT) animals. Likewise, CCR4 KO mice also showed more activated Th2 cells and IL-5 production. In addition, Treg cell migration into airways of CCR4-deficient mice was decreased compared with CCR4-sufficient or CCR2-deficient mice. In conclusion, airway allergic inflammation was more severe in anti-CD25-treated or CCR4-deficient mice than in their counterparts. Our results highlight the role of Treg cell migration via CCR4 pathway in the attenuation of allergic lung inflammation *in situ*.

Materials and Methods

Mice

Female C57BL/6 mice at 8–12 wk old, housed under specific pathogen-free conditions at the Department of Immunology, Biomedical Science Institute, University of São Paulo, São Paulo, Brazil, were used throughout the experiments. CCR4 KO mice were provided by the Department of Biochemistry and Immunology, School of Medicine of Ribeirão Preto, University of São Paulo, and were housed at the Department of Immunology, Biomedical Science Institute, University of São Paulo. Mice were treated according to Animal Welfare guidelines of the Biomedical Science Institute, University of São Paulo.

Induction of allergic airway disease and oral tolerance

Mice were sensitized and boosted by s.c. route with 4 µg chicken OVA/1.6 mg of aluminum hydroxide gel in 0.2 ml PBS at days 0 and 7. Airway inflammation was induced by two intranasal challenges with 10 µg OVA at days 14 and 21. Experiments were performed 24 h after the last challenge with OVA intranasally (day 22). Oral tolerance to OVA was induced by spontaneous intake of 1% OVA (grade V; Sigma-Aldrich) solution dissolved in sterile drinking water for 5 consecutive d before sensitization as previously described (11).

Anti-CD25 treatment

In selected experiments, mice were treated with 500 µg purified anti-CD25 mAb (PC61), with isotype control rat IgG1 mAb (GL113), or sterile PBS administered i.v. on days 13, 14, 15, and 20 to deplete and/or to inactivate CD25⁺ putative Treg cells (23–25). In our experimental protocol, the anti-CD25 treatment of allergic mice decreased >50% of infiltrating lung Foxp3⁺CD4⁺ T cells (Supplemental Fig. 4).

Bronchoalveolar lavage fluid and lung cell isolation

Mice were deeply anesthetized, the trachea was cannulated, and lungs were lavaged with 1 ml cold PBS. Total and differential cell counts of bronchoalveolar lavage (BAL) were determined by hemocytometer and cytopsin preparation stained with Instant-Prov (Newprov). After collection of BAL, lungs were perfused with 5 ml ice-cold PBS and removed. Lungs were minced and placed in 6 ml RPMI 1640 containing 2 mg/ml collagenase IV and 1 mg/ml DNase I (Sigma-Aldrich) and incubated at 37°C for 30 min. To make a single-cell suspension, the remaining tissue was forced through a 70-µm cell strainer. Cells were pelleted, and RBCs were lysed using 1 ml ACK lysing buffer (Life Technologies). The remaining cells were washed with RPMI 1640 containing 10% FBS, and viable cells were counted via trypan blue exclusion.

Isolation and adoptive transfer of preactivated CD25⁺CD4⁺ T cells

CD25⁺CD4⁺ T cells from C57BL/6 WT or CCR4KO mice obtained from spleens and lymph nodes were purified using mouse CD4⁺ T cell enrichment kit (EasySep) followed by sorting of stained CD25⁺ cells using an FACS Aria cell sorter (BD Biosciences). Before cell transfers, sorted CD25⁺CD4⁺ T cells (>98%) that expressed >80% of Foxp3 (data not shown) were preactivated by culturing the cells in wells coated with anti-CD3 (145-2C11; 2 µg/ml) and anti-CD28 (37.51; 2 µg/ml) mAb in the presence of rIL-2 (2.5 ng/ml) for 36 h. This procedure was performed to increase the CCR4 expression of WT CD25⁺CD4⁺ T cells as described by Saito et al. (26) and shown in Supplemental Fig. 3. Preactivated CD25⁺CD4⁺ T cells (5×10^5 cells) were then i.v. transferred into OVA/aluminum hydroxide gel-sensitized CCR4KO mice 24 h after the first OVA challenge, and the experiments were performed at day 22, 1 d after the second OVA challenge.

Flow cytometry analysis

Single-cell suspensions were preincubated with FcBlock (2.4G2) (BD Biosciences). Cells were stained with fluorochrome-conjugated Abs against surface markers CD3 (145-2C11), CD4 (RM4-5), CD8a (53-6.7), CD45RB (C363.16A), CD69 (H1.2F3), CCR4 (2G12), Siglec-F (E50-2440), Gr1 (RB6-8C5), and MHC class II (MHC II; M5/114.15.2) in staining buffer (PBS containing 2% FCS and 0.1% NaN₃) for 30 min on ice and then washed. For Foxp3 (FJK-16s), IL-5 (TRFK5), and IL-4 (11B11) staining, cells were permeabilized and subsequently stained using the Foxp3 Staining Buffer Set (eBioscience) according to the manufacturer's protocol. Cell acquisition was performed on an FACSCanto II instrument using FACSDiva software (BD Biosciences). For each sample, at least 300,000 events were collected. Data were analyzed using FlowJo software (Tree Star).

Determination of OVA-specific IgE and IgG1

OVA-specific Abs were assayed by sandwich ELISA as previously described (8). For OVA-specific IgE determinations, plates were coated with 2 µg/ml goat anti-mouse IgE Ab (Southern Biotechnology Associates). Serum samples were added followed by addition of biotin-labeled OVA. Bound OVA–biotin was revealed by streptavidin peroxidase conjugate (Sigma-Aldrich) as previously described (8). Hyperimmune serum was used as IgE standard and arbitrarily assigned as 10,000 U/ml. For OVA-specific IgG1, serum samples were plated on 96 wells previously coated with OVA (2 µg/well). The bound Abs were revealed with goat anti-mouse IgG1 followed by peroxidase-labeled rabbit anti-goat Abs (all from Southern Biotechnology Associates). The concentration of OVA-specific Ab was estimated by comparison with IgG1 standards run in parallel as previously described (8).

Cytokine determination

The levels of IL-4 and IL-5 in the BAL fluid were assessed by a sandwich ELISA kit according to the manufacturer's recommendations as previously described (8). Values are expressed as picograms per milliliter deduced from standards run in parallel with recombinant cytokines. Purified and biotinylated Abs to IL-4 and IL-5 kits were from BD OptEIA (BD Biosciences).

Lung histology

Lungs were perfused via the right ventricle with 10 ml cold PBS, removed, and immersed in 10% phosphate-buffered formalin for 24 h and then in 70% ethanol until embedding in paraffin. Tissues were sliced, and 5-µm sections were stained with hematoxylin/periodic acid-Schiff (PAS) for analysis of cellular inflammation and mucus production.

Statistical analysis

Statistical significance was assessed by either the two-tailed Student *t* test (two groups) or ANOVA for multiple groups with a post hoc Tukey test to determine the significance, **p* < 0.05, ***p* < 0.01, and ****p* < 0.001, all performed using Prism 5.0 software (GraphPad). Data are presented as means ± SEM.

Results

Anti-CD25 treatment exacerbates airway allergic inflammation

We have previously shown that Treg cells accumulate in the lungs after OVA challenge in allergic mice but not in mice made tolerant by previous OVA feeding (14). To assess the function of Treg cells *in vivo*, we treated the allergic or tolerant animals with four i.v. injections of anti-CD25 mAb (PC61) after allergic sensitization to deplete and/or to inactivate putative CD25⁺ Treg cells (23–25) as depicted in Fig. 1A. As expected, allergic but not tolerant mice developed allergic lung inflammation (Fig. 1). Notably, allergic but not tolerant mice treated with anti-CD25 mAb showed a higher number of eosinophils in the BAL fluid (Fig. 1B) and more intense parenchymal lung infiltrates than allergic mice not treated with anti-CD25 (PBS group) (Fig. 1C). More specifically, the percentage and number of eosinophils characterized as Siglec-F⁺Gr1^{int}MHC II⁺ cells were >2-fold higher in the lungs of allergic mice treated with anti-CD25 than those not treated (Fig. 1D, 1E) or treated with isotype control rat IgG1 mAb (GL113) (data not shown). As mentioned, the treatment of tolerant mice with anti-

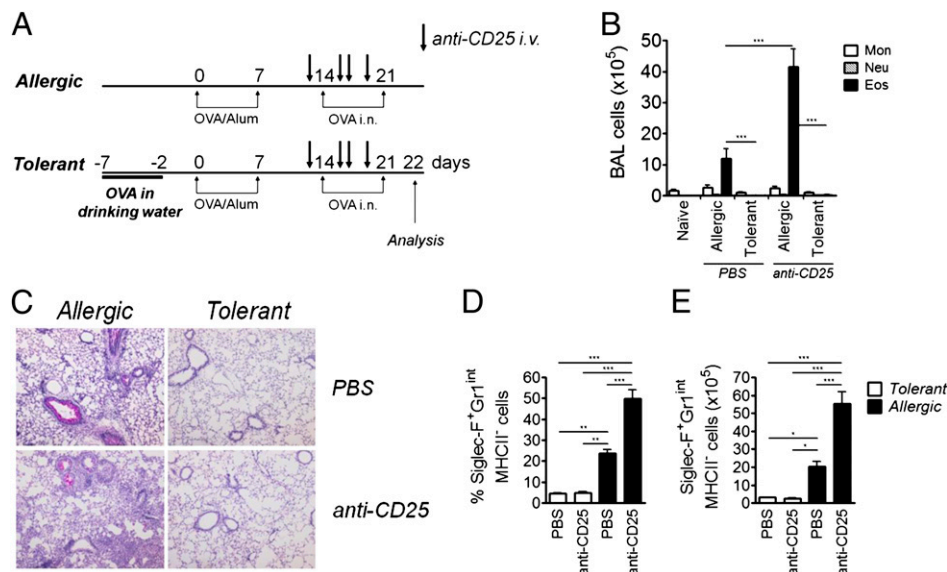


FIGURE 1. Anti-CD25 treatment aggravates airway eosinophilic inflammation. **(A)** Anti-CD25 treatment protocol of C57BL/6 mice induced to oral tolerance or airway allergic inflammation. Lung and BAL cells were harvested after 24 h of the last OVA challenge from mice treated with PBS or anti-CD25 mAb i.v. **(B)** Differential counts of cytospin preparations from BAL cells. **(C)** Representative lung sections stained with hematoxylin/PAS for analysis of cellular inflammation and mucus production (original magnification $\times 100$). Percentage **(D)** and number **(E)** of lung Siglec-F⁺Gr1^{int}MHC II⁺ cells representing eosinophils. Cells recovered from lung were stained for MHC II, Gr1, and Siglec-F and gated in MHC II⁺ cells. Values represent the means $\pm$ SEM for groups of three mice and are representative of two independent experiments. Significant differences, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, are shown.

CD25 did not modify the tolerance status as evidenced by reduced number of eosinophils in the BAL (Fig. 1B) and absence of lung allergic inflammation (Fig. 1C–E).

Anti-CD25 treatment increases effector Th cells in lung tissue

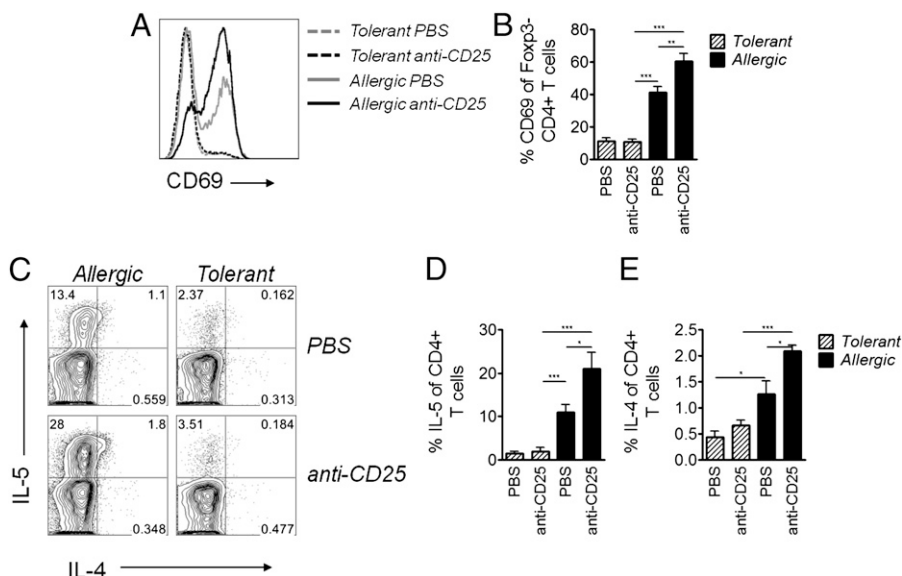
The above results suggested that anti-CD25 treatment probably inhibited Treg cell function (23). However, as activated T cells also express CD25, we were interested in determining the activation status of airway Th cells. For this, we evaluated the expression of the activation markers CD69 or CD25 as well as the production of IL-5 and IL-4 by infiltrating CD4⁺ T cells.

We found that anti-CD25 treatment of allergic mice increased the frequency of CD4⁺ T cells expressing CD69, whereas the frequency of CD4⁺CD25⁺Foxp3⁺ T cells was decreased when

compared with allergic PBS group (Fig. 2A, 2B, Supplemental Fig. 4A, 4B). Again, no differences were observed between tolerant mice treated or not with anti-CD25, which showed very low expression of activated T cells in the lungs when compared with allergic groups (Fig. 2A, 2B). The frequency of cells expressing CD69 and type 2 cytokines IL-5 and IL-4 in tolerant mice treated or not with anti-CD25 was almost absent (Fig. 2A–E). In contrast, the frequency of CD4⁺ T cells producing IL-5 or IL-4 increased in the lungs of anti-CD25-treated mice when compared with PBS group (Fig. 2C–E). Notably, the frequency of IL-5-producing CD4⁺ T cells was 10-fold higher when compared with T cells producing IL-4 (Fig. 2C–E).

We conclude that inactivation of putative CD25⁺ Treg cells with anti-CD25 treatment augments allergic lung inflammation by in-

FIGURE 2. Anti-CD25 treatment increases activated CD4⁺ T cells in allergic lungs. Representative FACS histogram **(A)** and percentage of lung Foxp3⁺CD4⁺ T cells expressing CD69 **(B)**. Cells recovered from lungs of tolerant or allergic mice treated with PBS or anti-CD25 mAb i.v. were stained for CD3, CD4, Foxp3, and CD69 and gated in Foxp3⁺CD4⁺CD3⁺ cells. **(C–E)** Percentage of lung CD4⁺ T cells expressing IL-5 and IL-4. Cells recovered from lung were stained for CD3, CD4, IL-5, and IL-4 and gated in CD4⁺CD3⁺ cells. Values represent the means $\pm$ SEM for groups of three mice and are representative of two independent experiments. Significant differences, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, are shown.



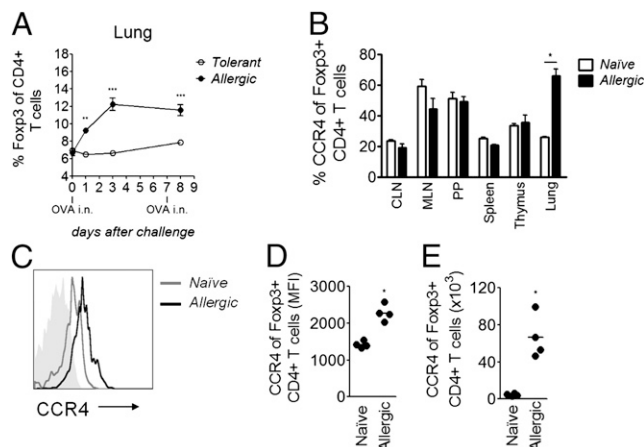


FIGURE 3. CCR4-expressing Treg cells accumulate in lung allergic inflammation. **(A)** Time course of Foxp3⁺CD4⁺ T (Treg) cells frequency in the lungs of tolerant and allergic mice. Cells recovered from lung were stained for CD4 and Foxp3 and gated in CD4⁺ cells. **(B)** Percentage of Foxp3⁺CD4⁺ T cells expressing CCR4. Cells recovered from cervical lymph nodes (CLN), MLN, Peyer's patches (PP), spleen, thymus, and lungs were stained for CD3, CD4, Foxp3, and CCR4 and gated in Foxp3⁺CD4⁺ cells. **(C)** Representative FACS histogram of lung Foxp3⁺CD4⁺ T cells expressing CCR4. **(D)** Mean fluorescence intensity (MFI) of CCR4 expressed by lung Foxp3⁺CD4⁺ T cells. **(E)** Number of Foxp3⁺CD4⁺ T cells expressing CCR4 in the lungs. Values represent the means $\pm$ SEM for groups of four mice and are representative of two independent experiments. Significant differences, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, are shown.

creasing the number of activated Th2 cells producing IL-5. In addition, we found that the vast majority of lung infiltrating CD4⁺ T cells were CD25 negative (Supplemental Fig. 4).

Foxp3⁺ Treg cells expressing CCR4 accumulate in the airways of allergic mice

We have previously shown that Treg cells accumulated in the lungs of allergic mice were phenotypically different from those present in lymphoid organs and could be characterized among other activation markers as CCR4⁺ (14). Therefore, we first monitored the appearance of Treg cells in the lungs of allergic or tolerant mice. Fig. 3 shows that the frequency of Foxp3⁺CD4⁺ T cells started to increase 24 h after the first OVA challenge, reaching maximum values with 3 d and plateauing thereafter in allergic but not tolerant mice (Fig. 3A). Next, we determined the frequency of Foxp3⁺ Treg cells expressing CCR4 in different organs of allergic or naive mice. We could not detect any difference between naive and allergic mice in

CCR4 expression of Treg cells present at draining cervical lymph nodes, mesenteric lymph nodes (MLN), Peyer's patches, thymus, and spleen (Fig. 3B). However, a significant difference in the distribution of Foxp3⁺ T cells expressing CCR4 between allergic and naive mice was detected in the lungs (Fig. 3B, 3E). In addition, the expression of CCR4 in Treg cells was more pronounced in allergic mice when compared with naive mice (Fig. 3C, 3D).

These results clearly indicate that Treg cells from allergic mice migrate to lung tissue after allergen challenge.

CCR4 deficiency impairs Treg cell migration to airways

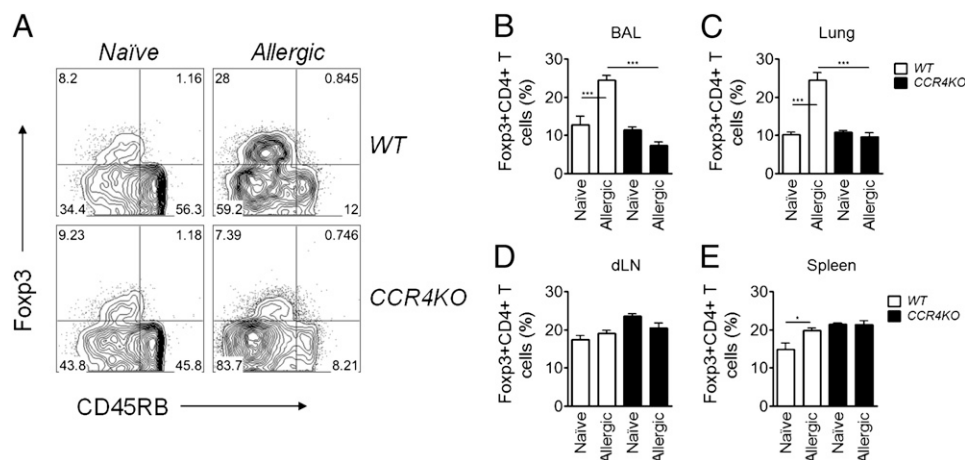
To assess the role of CCR4 in the migration of Treg or effector T (Teff)/memory T cells during airway allergic inflammation, we performed experiments in CCR4KO mice. We found that the percentage of Foxp3⁺CD4⁺ T cells was markedly decreased in the BAL and lungs of allergic CCR4KO mice, reaching values $\sim 8\%$ when compared with allergic WT mice that showed $>20\%$ values (Fig. 4A–C). No significant differences in Treg cells were observed between WT and CCR4KO mice in draining lymph nodes or spleen (Fig. 4D, 4E).

To further characterize the phenotype of CD4⁺ T cells present in the airways of allergic CCR4KO mice, we determined the frequency of Treg cells and activated CD4⁺ T cells. For comparison we used WT and CCR2KO mice because this chemokine receptor has also been shown to be expressed by Treg cells (27). We found that the frequency of Foxp3⁺CD4⁺ T cells expressing CD69 or with low expression of CD45RB (CD45RB^{low}), a marker of Teff/memory T cells, was increased in the airways of CCR4KO mice when compared with WT or CCR2KO mice (Fig. 5A, Supplemental Fig. 1). In contrast, the frequency of Treg cells in BAL and lungs was decreased in CCR4KO mice when compared with WT or CCR2KO mice (Fig. 5B, 5C). No differences were observed in activated T or Treg cells present in the spleen (Fig. 5A, 5D). Therefore, CCR4 deficiency affected profoundly the balance between Treg and activated CD4⁺ T cells in the airways as revealed by the ratio of Teff/Treg cells (Fig. 5E).

CCR4-deficient mice display severe eosinophilic lung inflammation

Because in allergic mice CCR4 deficiency resulted in an increased ratio of Teff versus Treg cells, we next determined the magnitude of allergic inflammation in these mice compared with WT mice. Notably, we found that CCR4KO mice showed an intense influx of eosinophils to airways as revealed by differential cell BAL counts (Fig. 6A). Indeed, the number of eosinophils was >3 -fold higher in the BAL of allergic CCR4KO mice ($24.2 \pm 3.9 \times 10^5$ cells) than in WT mice ($6.7 \pm 0.7 \times 10^5$ cells). In addition,

FIGURE 4. CCR4 deficiency impairs Treg cell migration to airways. **(A)** Percentage of lung CD4⁺ T cells expressing Foxp3 and CD45RB. Cells recovered from lungs of naive or allergic WT and CCR4-deficient mice were stained for CD3, CD4, Foxp3, and CD45RB and gated in CD4⁺CD3⁺ cells. Percentage of Foxp3⁺CD4⁺ T cells in BAL **(B)**, lung **(C)**, draining lymph nodes (dLN) **(D)**, and spleen **(E)**. Values represent the means $\pm$ SEM for groups of four mice and are representative of three independent experiments. Significant differences, $*p < 0.05$ and $***p < 0.001$, are shown.



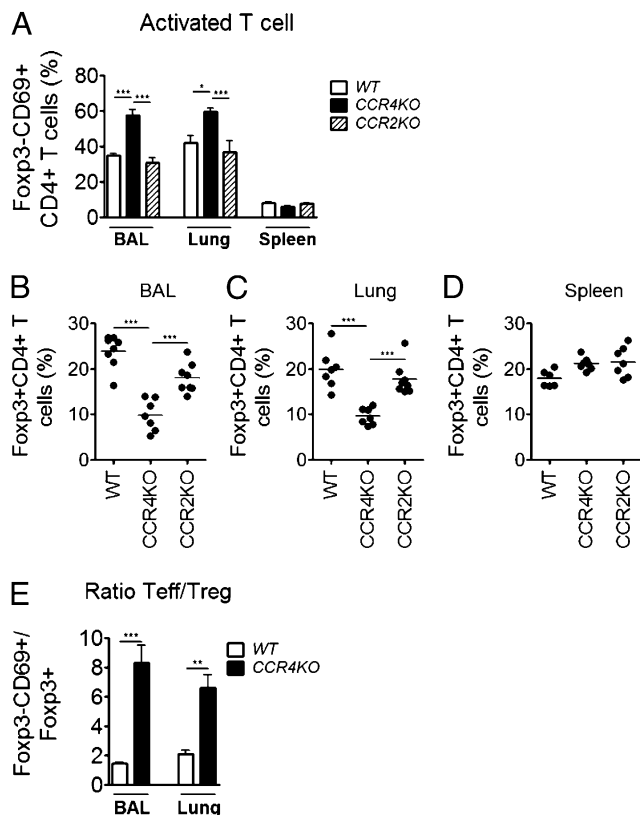


FIGURE 5. CCR4 deficiency alters the balance between Treg and activated CD4⁺ T cells in allergic airways. **(A)** Percentage of Foxp3⁺CD69⁺ CD4⁺ T cells in BAL, lung, and spleen of allergic WT, CCR4⁻, and CCR2KO mice. Percentage of Foxp3⁺CD4⁺ T cells in BAL **(B)**, lung **(C)**, and spleen **(D)**. **(E)** Ratio between Foxp3⁺CD69⁺CD4⁺ T (Teff) cells and Foxp3⁺CD4⁺ T (Treg) cells present in the BAL and lungs. Cells recovered from BAL and lung were stained for CD3, CD4, Foxp3, and CD69 and gated in CD4⁺CD3⁺ cells for analysis of Treg (Foxp3⁺) cells or gated in Foxp3⁺CD4⁺CD3⁺ cells for analysis of Teff (CD69⁺) cells. Values represent the means $\pm$ SEM for groups of four mice or more and are representative of three independent experiments. Significant differences, * p < 0.05, ** p < 0.01, and *** p < 0.001, are shown.

CCR4KO mice showed a more intense lung parenchymal inflammation characterized by diffuse cell infiltrates than WT mice (Fig. 6B). FACS analysis confirmed that the frequency and number of eosinophils (Siglec-F⁺Gr1^{int}MHC II⁺ cells) in lung tissue were more pronounced in CCR4KO than in WT mice (Fig. 6C–E). Consistent with the enhanced number of eosinophils in the airways, the BAL levels of IL-5 and the percentage of lung CD4⁺ T cells producing IL-5 were ~2-fold higher in allergic CCR4KO mice than in allergic WT mice (Fig. 6F, 6G, Supplemental Fig. 2A). In addition, the production of IL-4 by lung infiltrating CD4⁺ T cells was higher in CCR4KO mice than in WT mice (Fig. 6F, 6H, Supplemental Fig. 2B). However, the levels of OVA-specific IgE and IgG1 did not differ between both allergic groups (Supplemental Fig. 2C, 2D). Our results show that CCR4KO mice develop a more intense allergic lung inflammation than WT mice.

Adoptive transfer of WT CD25⁺CD4⁺ T cells to sensitized CCR4KO mice attenuates eosinophilic airway inflammation and lung T cells secreting type 2 cytokines

We reasoned that the aggravation of allergic lung inflammation by anti-CD25 or by CCR4 deficiency was due to diminished function of Treg cells in the lung. Therefore, to demonstrate the biological role of CCR4 molecule on Treg cell function in vivo, we per-

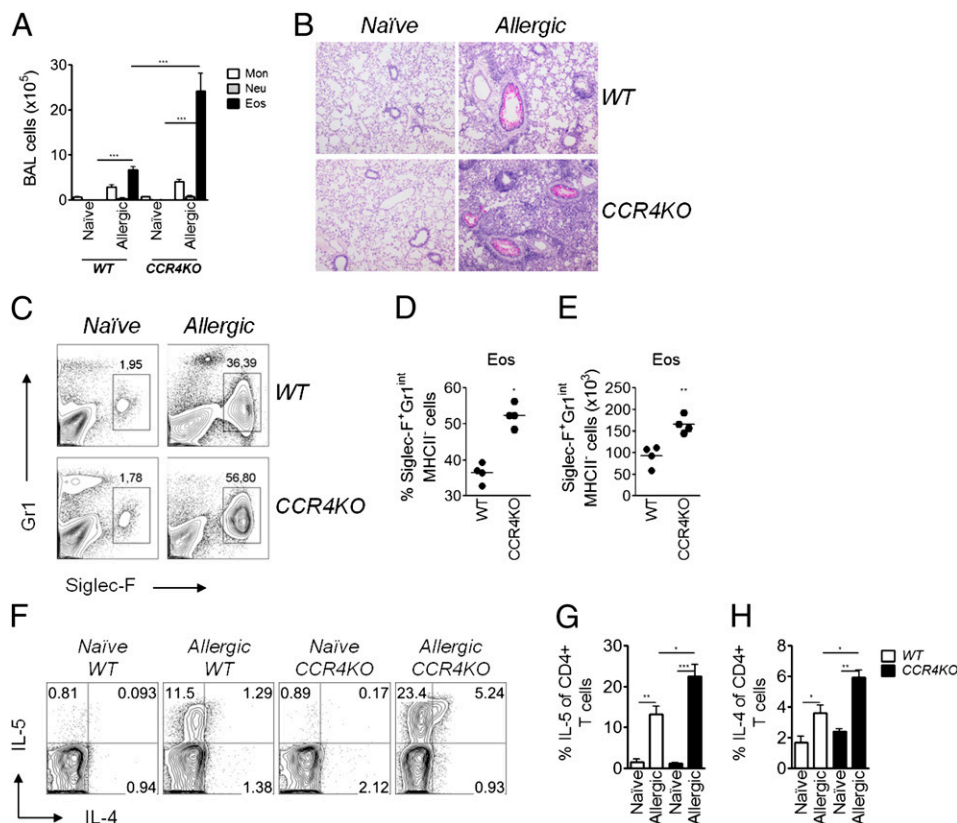
formed adoptive-transfer experiments of CD25⁺CD4⁺ T cells isolated from WT or CCR4-deficient mice into allergic CCR4KO mice. For this, we first preactivated isolated CD25⁺CD4⁺ T cells with anti-CD3/anti-CD28 mAb to upregulate the expression of CCR4, (Supplemental Fig. 3). Then we transferred these cells to sensitized CCR4KO mice 1 d after the first OVA challenge and performed the experiments 24 h after the second OVA challenge. We found that transfer of WT, but not CCR4-deficient, CD25⁺CD4⁺ T cells decreased significantly the total number of inflammatory cells and eosinophils in the BAL of allergic CCR4KO mice (Fig. 7A, 7B). Also, adoptive transfer of WT, but not CCR4-deficient, CD25⁺CD4⁺ T cells decreased the frequency and number of eosinophils (Siglec-F⁺Gr1^{int}MHC II⁺ cells) in the lungs (Fig. 7C–E). Notably, the percentage of CD4⁺ T cells producing IL-5 and IL-4 was markedly reduced in the lungs of mice that received CD25⁺CD4⁺ T cells from WT when compared with animals that received CCR4-deficient CD25⁺CD4⁺ T cells or none (Fig. 7F–H). Consistent with diminished allergic airway inflammation, the ratio between lung Th2 cytokine-producing cells and Foxp3⁺ Treg cells was lower in mice transferred with WT CD25⁺CD4⁺ T cells than with CCR4-deficient CD25⁺CD4⁺ T cells (Fig. 7I). Altogether, these results clearly highlight the suppressive role of CCR4⁺ Treg cells on Th2-mediated allergic airway inflammation.

Discussion

In the present work, we investigated the in vivo role of Treg cells in allergic mice or in mice made tolerant by previous Ag feeding. For this, we treated the animals with anti-CD25 mAb, a procedure that are known to deplete and/or inactivate Treg cell function (23–25). We found that anti-CD25 treatment did not alter the state of unresponsiveness induced by oral tolerance. In contrast, in allergic mice, the anti-CD25 treatment aggravated airway inflammation as evidenced by an increased number of lung activated T cells, IL-4-, IL-5-producing T cells, and eosinophils. It was previously reported that anti-CD25 treatment could directly abrogate effector CD25⁺ T cell responses during an inflammatory episode (28). We have shown that both lung CD25⁺ and CD25⁺ T cells are effector cells because they produce substantial amounts type 2 cytokines (14). In this study, we showed that the vast majority of lung-infiltrating CD4⁺ T cells were CD25⁺, which are not affected by anti-CD25 treatment. In addition, effector CD25⁺ T cells decreased after treatment. Therefore, the augmented effector Th2 activity observed after anti-CD25 treatment is exerted mainly by the lack of Treg function and effector CD25⁺ Th2 cells. Our results with anti-CD25 treatment during allergen challenges are in line with previous findings showing that anti-CD25 treatment before OVA sensitization exacerbates allergic airway inflammation and type 2 cytokine production (26). In our study, the anti-CD25 treatment was performed during the period of allergen challenges, and the fact that it exacerbated allergic inflammation indicates that this treatment is preferentially affecting Treg rather than Teff cell function.

The fact that Treg cells did not revert the tolerance state appear conflicting in light of our previous work showing that Treg cells via TGF- β are crucial for the establishment of oral tolerance (11). Indeed, it is well documented that oral tolerance is strictly related to the emergence of Treg cells (29–31). However, it should be noted that we treated the animals after the establishment of oral tolerance, and in this scenario, Treg cells may not play an active role as they play during the induction phase of tolerance when they inhibit the emergence of Teff cells (11, 32). In sharp contrast, our results with anti-CD25 treatment highlighted the active role that Treg cells exert on lung allergic inflammation, a condition in which Teff cells have been already

FIGURE 6. CCR4 deficiency exacerbates the allergic airway inflammation. **(A)** Differential counts of cytospin preparations from BAL cells of WT and CCR4-deficient mice. **(B)** Representative lung sections stained with hematoxylin/PAS for analysis of cellular inflammation and mucus production (original magnification $\times 100$). Percentage **(C, D)** and number **(E)** of lung Siglec-F⁺Gr1^{int}MHC II⁺ cells representing eosinophils. Cells recovered from lung were stained for MHC II, Gr1, and Siglec-F and gated in MHC II⁺ cells. **(F-H)** Percentage of lung CD4⁺ T cells expressing IL-5 or IL-4. Cells recovered from lung were stained for CD3, CD4, IL-5, and IL-4 and gated in CD4⁺CD3⁺ cells. Values represent the means $\pm$ SEM for groups of four mice and are representative of three independent experiments. Significant differences, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, are shown.



generated during the sensitization process. In this situation, we noticed the aggravation of airway allergic inflammation after anti-CD25 treatment, reinforcing the view of functional inactivation of Treg cells by anti-CD25 treatment. Because anti-CD25 mAb was given systemically (i.v.) and not in the airways, we could not determine whether Treg cells were exerting their suppressive activity in lymphoid organs such as draining lymph nodes or spleen, inhibiting the development of Teff cells, or in the lung tissue acting on emigrated Teff cells. However, we favor the notion that Treg cells attenuate allergic inflammation, exerting their activity in the lung tissue. First, we have previously shown that Foxp3⁺ Treg cells accumulate in the lungs of allergic mice and efficiently suppressed pulmonary T cell proliferation but not type 2 cytokine production (14). Second, our experiments with CCR4KO mice clearly showed that Treg cell migration to the lungs was impaired. In turn, these animals exhibited an asthma phenotype similar to the one observed with anti-CD25 treatment (i.e., they showed severe allergic airway inflammation with increased number of activated CD4⁺ T cells producing IL-5). Indeed, the more noticeable effects of anti-CD25 treatment or CCR4 deficiency were the increased infiltration of eosinophils and IL-5-producing T cells in the airways. In addition, micrographs of lung sections of anti-CD25-treated or CCR4KO mice showed a more dispersed cellular infiltrate occupying the lung parenchyma than that observed in their allergic counterparts. Other allergic parameters such as IgE production were not affected by anti-CD25 treatment or CCR4 deficiency, indicating that Treg cells may exert their suppressive activity in the lung instead of systemically.

Our results with CCR4KO mice are in line with other reports showing the role of CCR4 in Treg cell migration and function. For instance, CCR4KO mice failed to develop tolerance to allograft after administration of anti-CD154 and donor spleen cells, and this was associated with impaired recruitment of Foxp3-expressing T cells to the graft (33). In a model of inflammatory bowel dis-

ease, CCR4-deficient Treg cells failed to migrate to the MLN and could not prevent colitis (34). Notably, in noninflammatory conditions, mice with a complete loss of CCR4 on Treg cells develop severe inflammatory disease in the skin and lungs, suggesting a role of CCR4-positive Treg cells in the control of tissue homeostasis (35). In our model, we found that CCR4-expressing Foxp3⁺ Treg cells are naturally distributed in different tissues of naive mice, but were increased in the lungs of mice undergoing allergic airway inflammation, reinforcing the notion that allergic milieu triggers the migration of Treg cells into the airways that, in turn, exert a suppressive activity. It is likely, based on our findings and previous work, that lung Treg cells are attenuating lung inflammation by inhibiting the in situ proliferation of Teff lung cells.

Regarding allergic inflammation, CCR4 appears to be dispensable for Th2-mediated allergic lung disease because CCR4KO mice exhibited more T cell-producing IL-5 and IL-4 and eosinophils when compared with WT. Our results are in line with other reports showing that allergic airway disease was not impaired in mice deficient for CCR4 or after treatment with anti-CCR4 (21, 22). However, these findings conflict with other studies showing that CCR4 and its interaction with respective chemokines CCL17 (thymus and activation-regulated chemokine) and CCL22 (macrophage-derived chemokine) play a role in allergic inflammation (20, 36–43). These conflicting results on the role of CCR4 in allergy remain to be elucidated, but may be due to different experimental protocols and animals employed. Other chemokine receptors, besides CCR4, such as CCR8 and CCR3, are also expressed by Th2 cells and might have overlapping functions (44–48). Thus, it is possible that in our experiments with CCR4KO mice, CCR8 and/or CCR3 mediated the migration of inflammatory Th2 cells to the lungs that, in turn, promoted Th2-mediated allergic airway inflammation.

We postulated that the aggravation of allergic lung inflammation by anti-CD25 or CCR4 deficiency was due to diminished function

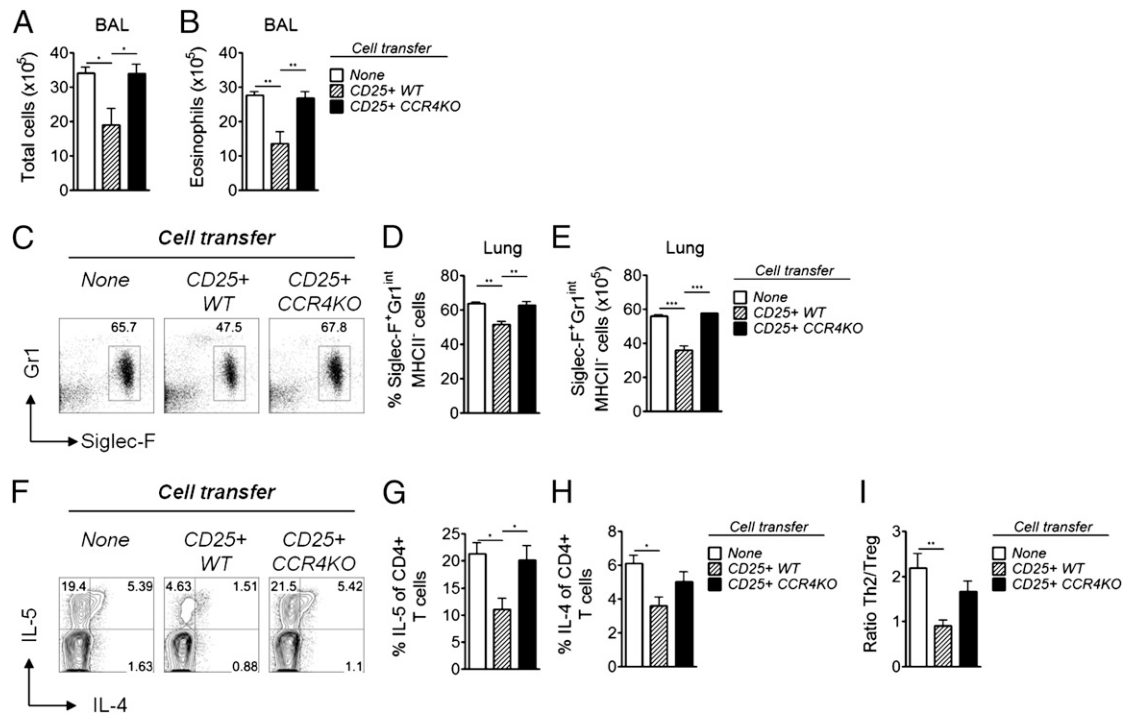


FIGURE 7. Adoptive transfer of WT CD25⁺CD4⁺ T cells attenuates allergic airway inflammation of CCR4KO mice. On day 15, after OVA sensitization and first OVA challenge, CCR4KO mice received i.v. 5×10^5 preactivated CD25⁺CD4⁺ T cells from WT or CCR4KO mice. At day 21, mice were challenged again with OVA, and 24 h later, BAL fluid and lung samples were collected for analysis. Total number of cells (**A**) and eosinophils (**B**) in the BAL. Percentage (**C**, **D**) and number (**E**) of lung Siglec-F⁺Gr1^{int}MHCII⁺ cells representing eosinophils. Cells recovered from lung were stained for MHCII, Gr1, and Siglec-F and gated in MHCII⁺ cells. (**F–H**) Percentage of lung CD4⁺ T cells expressing IL-5 and/or IL-4. Cells recovered from lung were stained for CD3, CD4, IL-5, and IL-4 and gated in CD4⁺CD3⁺ cells. (**I**) Ratio between IL-5⁺IL-4⁺Foxp3[−]CD4⁺ T (Th2) and Foxp3⁺CD4⁺ T (Treg) cells present in the lungs. Cells recovered from lung were stained for CD3, CD4, Foxp3, IL-5, and IL-4 and gated in CD4⁺CD3⁺ cells for analysis of Treg (Foxp3⁺) cells or gated in IL-5⁺IL-4⁺Foxp3[−]CD4⁺CD3⁺ cells for analysis of Th2 cells. Values represent the means $\pm$ SEM for groups of four mice. Significant differences, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, are shown.

of Treg cells in the lungs. To evaluate the role of putative Treg cells in vivo, we transferred preactivated WT CD25⁺CD4⁺ T cells that expressed high levels of CCR4 to allergic CCR4KO mice. We found that the transferred cells efficiently suppressed allergic lung inflammation as revealed by the decreased number of eosinophils and Th2 cells secreting type 2 cytokines. In contrast, preactivated CD25⁺CD4⁺ T cells from CCR4KO animals were without effect. These results clearly highlight the regulatory role exerted by CD25⁺CD4⁺ T cells expressing CCR4 in lung allergic inflammation.

Eosinophils play a central role in asthma (49–51), and clinically, asthma varies from mild to severe forms (52). Interestingly, it was shown in severe asthma with a follow-up of 5 y that high numbers of eosinophils could persist despite treatment with inhaled and oral corticosteroids (53). It is possible that inappropriate Treg cell function might underline an asthma phenotype (12). Our findings extend this concept by showing that impaired Treg cell migration to sites of allergic inflammation aggravates the severity of airway eosinophilic inflammation.

Finally, our work provides a warning against the validity of CCR4 as a therapeutic target in the treatment of asthma and opens up the possibility of exploiting CCR4-positive Treg cells for the control of lung eosinophilic inflammation.

Acknowledgments

We thank Paulo Albe for histological preparations, Daniela Teixeira for expert technical assistance in FACS sorting of CD25⁺ Treg cells, and Eliane Mello for anti-CD25 (PC61) mAb production and technical assistance.

Disclosures

The authors have no financial conflicts of interest.

References

- Temann, U. A., P. Ray, and R. A. Flavell. 2002. Pulmonary overexpression of IL-9 induces Th2 cytokine expression, leading to immune pathology. *J. Clin. Invest.* 109: 29–39.
- Foster, P. S., A. W. Mould, M. Yang, J. Mackenzie, J. Mattes, S. P. Hogan, S. Mahalingam, A. N. McKenzie, M. E. Rothenberg, I. G. Young, et al. 2001. Elemental signals regulating eosinophil accumulation in the lung. *Immunol. Rev.* 179: 173–181.
- Fontenot, J. D., M. A. Gavin, and A. Y. Rudensky. 2003. Foxp3 programs the development and function of CD4⁺CD25⁺ regulatory T cells. *Nat. Immunol.* 4: 330–336.
- Wildin, R. S., S. Smyk-Pearson, and A. H. Filipovich. 2002. Clinical and molecular features of the immunodysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome. *J. Med. Genet.* 39: 537–545.
- Read, S., V. Malmström, and F. Powrie. 2000. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25⁺CD4⁺ regulatory cells that control intestinal inflammation. *J. Exp. Med.* 192: 295–302.
- Curotto de Lafaille, M. A., S. Muriglan, M. J. Sunshine, Y. Lei, N. Kutchukhidze, G. C. Furtado, A. K. Wensky, D. Olivares-Villagómez, and J. J. Lafaille. 2001. Hyper immunoglobulin E response in mice with monoclonal populations of B and T lymphocytes. *J. Exp. Med.* 194: 1349–1359.
- Chatila, T. A., F. Blaeser, N. Ho, H. M. Lederman, C. Voulgaropoulos, C. Helms, and A. M. Bowcock. 2000. JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic dysregulation syndrome. *J. Clin. Invest.* 106: R75–R81.
- Russo, M., M. A. Nahori, J. Lefort, E. Gomes, A. de Castro Keller, D. Rodriguez, O. G. Ribeiro, S. Adriouch, V. Gallois, A. M. de Faria, and B. B. Vargaftig. 2001. Suppression of asthma-like responses in different mouse strains by oral tolerance. *Am. J. Respir. Cell Mol. Biol.* 24: 518–526.
- Akbari, O., R. H. DeKruyff, and D. T. Umetsu. 2001. Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nat. Immunol.* 2: 725–731.
- Mucida, D. S., D. Rodríguez, A. C. Keller, E. Gomes, J. S. Menezes, A. M. de Faria, and M. Russo. 2004. Decreased nasal tolerance to allergic asthma in mice fed an amino acid-based protein-free diet. *Ann. N. Y. Acad. Sci.* 1029: 361–365.

1 **Supplemental Figure legends**

2 **Supplemental Figure 1.** *CCR4-deficiency increases effector/memory CD4⁺ T cells in*
3 *allergic airways.* Percentage of Foxp3⁻CD69⁺CD4⁺ T cells in **(A)** BAL, **(B)** lung, **(C)**
4 draining lymph node (dLN) and **(D)** spleen of naïve or allergic wild-type (WT) and
5 CCR4-deficient mice. Percentage of Foxp3⁻CD45RB^{low}CD4⁺ T cells in **(E)** BAL, **(F)**
6 lung, **(G)** dLN and **(H)** spleen. Cells recovered from BAL, lung and spleen were stained
7 for CD3, CD4, Foxp3, CD69 and CD45RB and gated in Foxp3⁻CD4⁺CD3⁺ cells for
8 analysis of activated T (CD69⁺) cells or for analysis of effector/memory T (CD45RB^{low})
9 cells. Values represent the means ± SEM for groups of four and are representative of
10 three independent experiments. Significant differences **P*<0.05, ***P*<0.01, ****P*<0.001
11 are shown.

12
13 **Supplemental Figure 2.** *Type 2 cytokine production and OVA-specific antibodies in WT*
14 *and CCR4KO mice.* **(A)** IL-5 and **(B)** IL-4 in the BAL, **(C)** anti-OVA IgG1 and **(D)** anti-
15 OVA IgE in the serum were determined by ELISA. Values represent the means ± SEM
16 for groups of four mice and are representative of three independent experiments.
17 Significant differences **P*<0.05, ****P*<0.001 are shown.

18
19 **Supplemental Figure 3.** *Sorted CD25⁺CD4⁺ T cells up-regulate CCR4 expression upon*
20 *in vitro activation with anti-CD3 and anti-CD28.* Expression of CCR4 in isolated
21 CD25⁺CD4⁺ T cells preactivated or not (*None*) with immobilized anti-CD3 (2µg/ml) and
22 anti-CD28 (2µg/ml) mAb in the presence of recombinant IL-2 (2.5ng/ml) for 36h. Values
23 are representative of groups of four mice.

24

25 **Supplemental Figure 4. Anti-CD25 treatment decreases *Foxp3*⁺Treg cells. (A, B)**26 Percentage of *Foxp3* and CD25 in lung CD4⁺ T cells of tolerant or allergic C57BL/6

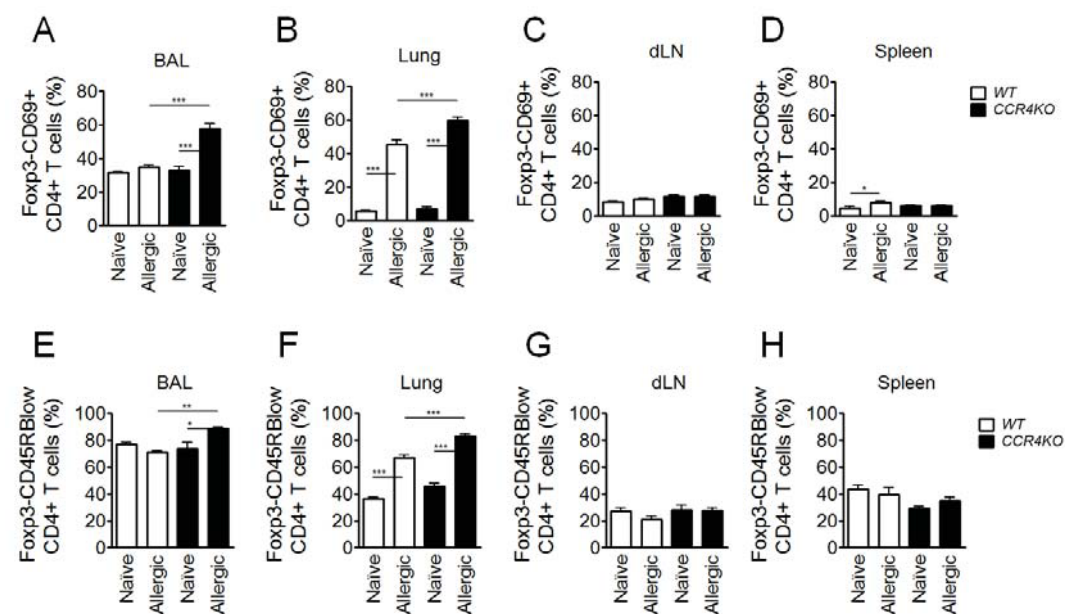
27 mice treated with PBS or anti-CD25 mAb i.v. Twenty -four hours of the last OVA

28 challenge the cells recovered from the lungs were stained for CD3, CD4, CD25 and

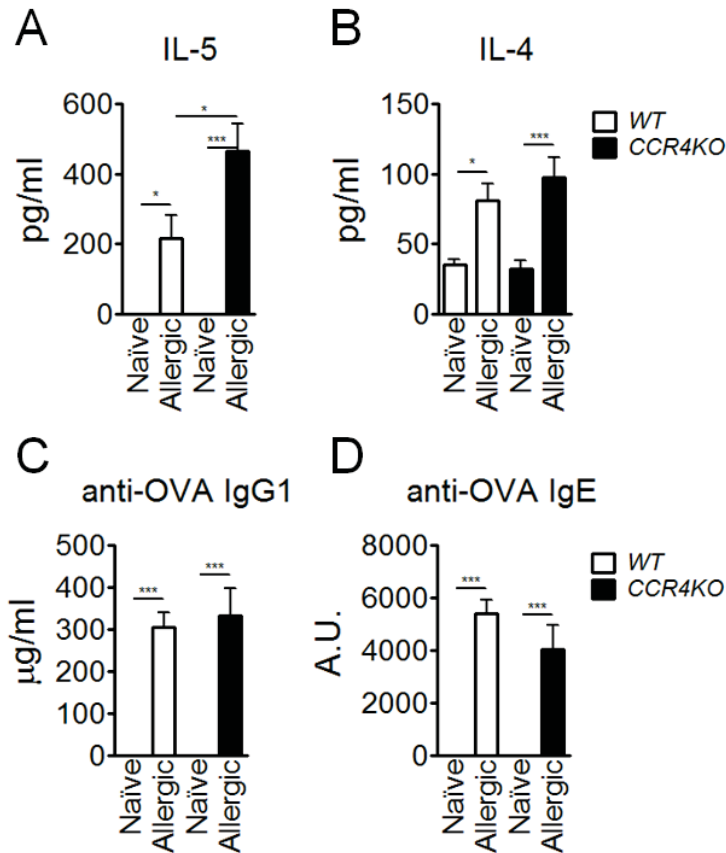
29 *Foxp3* and gated in CD4⁺CD3⁺ cells for flow cytometry analysis of *Foxp3* and CD25.30 Values represent the means $\pm$ SEM for groups of three mice and are representative of two31 independent experiments. Significant differences * $P < 0.05$, *** $P < 0.001$, are shown.

32

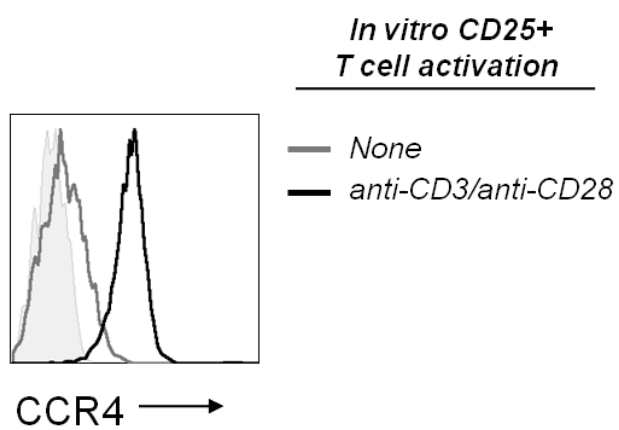
Supplemental Figure 1.



Supplemental Figure 2.



Supplemental Figure 3.



Supplemental Figure 4.

