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Contribution of CCR4 and CCR8 to antigen-specific Th2 cell trafficking in allergic pulmonary inflammation

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Abstract

Background—Recruitment of antigen-specific Th2 cells into the lung is critical for the development of allergic airway inflammation. Although CCR4 and CCR8 are preferentially expressed on Th2 cells and CCR4, CCR8 and CXCR3 ligands are increased in asthma, the specific relative contribution of these receptors to antigen-specific Th2 cell trafficking into the allergic lung is not known.

Objective—To determine the relative contribution of CCR4, CCR8 and CXCR3 to antigen-specific Th2 cell trafficking in a murine model of allergic pulmonary inflammation.

Methods—We used adoptive transfer experiments to compare the trafficking of wild type antigen-specific Th2 cells with antigen-specific Th2 cells deficient in CCR4, CCR8 or CXCR3.

Results—CCR4-deficient antigen-specific Th2 cells failed to traffic efficiently into the lung and the airways. In contrast, CCR8-deficient antigen-specific Th2 cells accumulated in these sites. Trafficking of CXCR3-deficient antigen-specific Th2 cells and CCR4-deficient and CCR8-deficient antigen-specific Th1 cells were comparable to their wild type counterparts. Approximately 60% of IL-4 producing antigen-specific T cells expressed CCR4. Disruption of CCR4-mediated antigen-specific Th2 cell trafficking decreased the levels of Th2-type cytokines in the airways and reduced airway eosinophilia and mucus production.

Conclusions—Our study demonstrates that CCR4 is required for the efficient entry of antigen-specific Th2 cells into the lung and the airways in murine model of allergic pulmonary inflammation.

Clinical implication—Inhibition of CCR4-mediated Th2 cell trafficking may contribute to asthma therapy.

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Capsule summary—CCR4 is required for efficient entry of Th2 cells into the allergic lung. Disruption of CCR4-mediated antigen-specific Th2 cell trafficking decreases airway Th2-type cytokines, eosinophilia and mucus production,

Keywords

CCR4; CCR8; CXCR3; asthma; chemokine; T cell trafficking

INTRODUCTION

The accumulation of Th2 cells in the airways following antigen challenge is critical for the development of allergic airway inflammation and has been shown to be due to their recruitment from draining lymph nodes and not from proliferation in the lung or airways¹. This recruitment process depends on chemoattractant receptor signaling on antigen-specific Th2 cells² in response to Stat6-inducible chemokines in the lung³.

CCR4 and CCR8 are two chemoattractant receptors preferentially expressed on Th2 cells^{4–7}, and therefore, are likely to participate in the recruitment of antigen-specific Th2 cells to sites of allergen exposure. In addition, CD4⁺ T cells that infiltrate the lung or the airways of asthmatics have been shown to express not only CCR4 and CCR8 but also CXCR3^{8–12}. Furthermore, CCR4, CCR8 and CXCR3 ligands are increased in the lung or the bronchoalveolar lavage (BAL) of asthmatics and in mouse models following allergen challenge^{11–16}. These studies implicate CCR4, CCR8 and CXCR3 in T cell trafficking in asthma but do not establish the specific functional roles of these receptors or their relative contribution to the trafficking of antigen-specific Th2 cells.

Despite the preponderance of indirect evidence in the literature for a critical role for CCR4 and CCR8 in the pathogenesis of asthma, studies utilizing CCR4-deficient or CCR8-deficient mice have led to conflicting results. While some studies show a decrease in markers of allergic airway inflammation^{12, 17–19}, other studies show no difference between wild type and CCR4-deficient or CCR8-deficient mice^{20–23}. This raises the possibility that CCR4 and CCR8 may play different roles on different cell types with the final outcome depending on the balance of the effect of CCR4 or CCR8 deficiency on different cells. For example, CCR4 and CCR8 are not only expressed on Th2 cells, which are sufficient to induce the asthma phenotype^{3, 24}, but are also expressed on Tregs, which attenuate the asthma phenotype^{25–27}. Therefore, to understand the role of CCR4 and CCR8 in the pathogenesis of asthma, one must examine the role of these receptors in each cell type. Furthermore, understanding the relative contribution of each chemokine receptor to the trafficking of antigen-specific Th2 cells and its degree of overlap with other receptors is a pre-requisite to assessing the efficacy of blocking any one receptor as a therapeutic strategy.

Here we compare the impact of CCR4, CCR8 or CXCR3 deficiency on the trafficking of antigen-specific Th2 cells using an adoptive transfer model of allergic pulmonary inflammation and define the relative contribution of CCR4, CCR8 and CXCR3 to antigen-specific Th2 cell trafficking in asthma pathogenesis.

Methods

Mice

Thy1.1⁺ OTII, Thy1.2⁺ OTII, and CXCR3^{-/-} mice were obtained as previously described²⁸. CCR8^{-/-} mice were a gift from Sergio A. Lira (Mount Sinai School of Medicine)¹⁷. CCR4^{-/-} and C57BL/6 mice were purchased from the Jackson Laboratory. All mice were in the C57BL/6 background. CCR4^{-/-}OTII, CXCR3^{-/-}OTII, CCR8^{-/-}OTII and

Thy1.1⁺Thy1.2⁺ mice were bred in our laboratory. Chemokine receptor-deficient OTII mice were generated by breeding male OTII mice with female chemokine receptor-deficient mice. The male mice from the first generation were bred again with female chemokine receptor-deficient mice. The chemokine receptor-deficient male mice in the second generation were identified by genotyping using specific primers ^{17, 22, 29}. All mice were age and gender matched and used at 6 to 8 weeks of age. Mice were housed under specific pathogen-free conditions. All experiments were done according to protocols approved by the Massachusetts General Hospital Subcommittee on Research Animal Care.

Generation of Th2 and Th1 cells

CD4⁺ T cells were isolated from spleens and pooled lymph nodes of OTII and chemokine receptor-deficient OTII mice using CD4 Dynabeads (Dyna). Purified CD4⁺ T cells (5 X 10⁵ cells/ml) were activated in the presence of irradiated (3,000 rads) splenocytes (2 X 10⁶ cells/ml), 1 ug/ml of pOVA_{323–339}, and 1.5 ug/ml of anti-CD28 (BD Pharmingen) in a 24 well plate. Th2 cells were generated by activating the cells in the presence of IL-4 (PeproTech) and anti-IFN- γ (R46A2). Th1 cells were generated by activating the cells in the presence of IL-12 (PeproTech) and anti-IL-4 (11B11) ²⁸. Cells were fed with IL-2 (PeproTech, 5–10 U/ml) initially on day 2 and then daily until used between days 5–7. Intracellular staining was performed as previously described ²⁸.

Adoptive transfer models

For competitive adoptive transfers, OTII (Thy1.1⁺) and chemokine receptor-deficient OTII (Thy1.2⁺) cells were mixed at a 1:1 ratio at 20 million cells/ml and injected into recipient Thy1.1⁺Thy1.2⁺ mice. For separate adoptive transfers, 5 million OTII (Thy1.2⁺) or chemokine receptor-deficient OTII (also Thy1.2⁺) Th2 cells were injected into separate Thy1.1⁺ recipient mice. All injections were performed at 0.5ml via the tail vein. One day after adoptive transfer, mice were given 3 daily 5% OVA (Sigma) challenges, for 20 minutes each day, using a nebulizer (Pulmo Aide). In some experiments, 2mg of bromodeoxyuridine (BrdU) at 10mg/ml was injected intraperitoneally before the third OVA challenge. Either 2 or 24 hours after the last challenge, BAL, lungs, thoracic lymph nodes (TLN) and spleens were isolated.

BAL and lymphocyte isolation

BAL was performed with six 0.5-ml aliquots of PBS with 0.6 mM EDTA. Lungs were minced and digested in RPMI medium containing 0.28 Wunsch U/ml Liberase Blenzyme (Roche) and 30 U/ml DNase (Sigma) for 45 min at 37 °C. Single cell suspensions from tissues were passed through 70-micron cell strainers (Fisher), lysed with RBC lysis buffer (Sigma) and washed. BAL differential cell counts were determined using Diff-Quik stained cytocentrifuged cell preparations.

Flow cytometry reagents

Fluorescent-conjugated anti-mouse monoclonal antibodies and 7-AAD were from BD Pharmingen. Staining for CCR4 was done with a chimeric CCL22-IgG Fusion protein from Daniel J. Campbell ³⁰ after blocking with rat and mouse serum (Sigma). Fluorescent-conjugated F(ab')₂ fragment, goat anti-human IgG (Fc specific, Jackson ImmunoResearch Laboratory) was used as the secondary reagent.

Airway Hyperreactivity

Mice were anesthetized with ketamine (80 mg/kg), intubated, placed in a whole-body plethysmograph (Buxco) and ventilated with a rodent ventilator (Harvard Apparatus). The average resistance and compliance were determined by software over 3 minutes after exposing

the mice to aerosolized methacholine (Sigma) at doubling concentrations from 0 to 16 mg/ml delivered in-line with the ventilator breaths.

Quantitative PCR

RNA was isolated from lung (Qiagen) and converted to cDNA and analyzed by quantitative PCR (QPCR) as previously described²⁸ with the Mx4000™ Multiplex Quantitative PCR System (Stratagene).

Cytokine and chemokine analysis

Cytometric bead array (BD Biosciences) and ELISA kits (R&D) were used per manufacturer's instructions.

Histology

Paraffin embedded lung tissue was stained with hematoxylin and eosin (H&E) or diastase periodic Schiff (PAS). A blinded investigator scored tissue sections based on extent of cellular infiltration and loss of normal lung architecture from 0= normal, 1= <25%, 2= 25–50%, 3= 50–75%, and 4= >75% of lung tissue with inflammation. PAS scores were assigned by a blinded investigator examining 10 consecutive fields per slide with 0<5% PAS positive goblet cells, 1=5–25%, 2=25–50%, 3=50–75%, 4>75% staining³¹.

Statistical analysis

All experiments were performed at least twice with a minimum of three mice per group per experiment. Each mouse was used as a data point. Pooled data are presented as mean $\pm$ s.e.m. Statistical significance was determined using the unpaired two-tailed Student's t-test in experiments comparing two groups and by ANOVA in experiments comparing three groups. $P < 0.05$ was considered significant.

Results

CCR4 deficiency diminishes antigen-specific Th2 cell trafficking into the lung and the airways while CXCR3 and CCR8 deficiency are not associated with a trafficking defect

In order to study the relative contribution of CCR4, CCR8 and CXCR3 to the trafficking of antigen-specific Th2 cells, we compared the homing of wild type OTII Th2 cells, which are transgenic for the T cell receptor recognizing OVA peptide_{323–339}, with the homing of OTII Th2 cells deficient in CCR4, CCR8 or CXCR3 into the lung and the airways *in vivo*, using a competitive adoptive transfer model³². We chose an adoptive transfer approach in order to isolate the effect of deficiency in CCR4, CCR8 or CXCR3 on antigen-specific Th2 cells from that in all other cell types³³. The competitive transfer provides a sensitive tool since the homing of different cell populations are directly compared in the same recipient mouse.

We generated wild type OTII (Thy1.1⁺), CCR4^{-/-}OTII (Thy1.2⁺), CCR8^{-/-}OTII (Thy1.2⁺), and CXCR3^{-/-}OTII (Thy1.2⁺) Th2 cells *in vitro* under Th2 polarizing conditions in the presence of IL-4 and anti-IFN- γ . We established that there was no difference between OTII Th2 cells and chemokine receptor-deficient OTII Th2 cells in differentiation, chemotaxis to CXCL12, activation and proliferation (See Figure E1 in the Online Repository). A mix containing equal numbers of wild type OTII (OTII) Th2 cells and either CCR4^{-/-}OTII, CCR8^{-/-}OTII or CXCR3^{-/-}OTII Th2 cells was prepared. Each pre-injection mix was stained for CD4, Thy1.1 and Thy1.2 to determine the ratio of OTII cells to chemokine receptor-deficient OTII cells. We then adoptively transferred the OTII and CCR4^{-/-}OTII cell mix, the OTII and CCR8^{-/-}OTII cell mix, and the OTII and CXCR3^{-/-}OTII cell mix intravenously into separate sets of recipient Thy1.1⁺Thy1.2⁺ mice. All recipient mice were given three daily

aerosolized OVA challenges. 24 hours after the last challenge, BAL, lungs, TLN and spleens were harvested and single cell suspensions were stained for CD4, Thy1.1 and Thy1.2. OTII cells were identified as CD4⁺Thy1.1⁺. Chemokine receptor-deficient cells were determined as CD4⁺Thy1.2⁺ and recipient cells were recognized as CD4⁺Thy1.1⁺Thy1.2⁺. Homing index was calculated as:

$$[\%KO - OTII/\%OTII]_{\text{tissue}}/[\%KO - OTII/\%OTII]_{\text{input}}.$$

We found that CCR4^{-/-}OTII Th2 cells had a decreased homing index into the BAL and the lung but not the TLN or spleen. The homing index for the OTII and CCR4^{-/-}OTII Th2 cell co-transfer was 0.56 for the BAL, 0.72 for the lung, 0.96 for the TLN, and 1.03 for the spleen (Fig 1, A and B). The percentage of CCR4^{-/-}OTII Th2 cells was 50% and 35% lower than the percentage of OTII Th2 cells in the BAL and the lung, respectively (p<0.05).

Surprisingly, CCR8^{-/-}OTII Th2 cells had an increased homing index into the BAL, lung and TLN but not the spleen. The homing index for the OTII and CCR8^{-/-}OTII Th2 cell co-transfer was 1.5 for the BAL, 1.7 for the lung, 1.6 for the TLN, and 0.96 for the spleen (Fig 1, A and B). The percentage of CCR8^{-/-}OTII Th2 cells was 43%, 74% and 53% higher than the percentage of OTII Th2 cells in the BAL, lung and TLN, respectively (p< 0.05).

In contrast to CCR4 and CCR8, CXCR3 deficiency did not alter antigen-specific Th2 cell trafficking. The homing index for the OTII and CXCR3^{-/-}OTII Th2 cell co-transfer was 1.09 for the BAL, 1.05 for the lung, 0.94 for the TLN, and 1.1 for the spleen (Fig 1, A). There was no difference in the percentage of OTII and CXCR3^{-/-}OTII Th2 cells in the BAL, lung, TLN and spleen.

We next determined that CCR4 and CCR8 deficiency did not impact antigen-specific Th1 cell trafficking. We generated OTII (Thy1.1⁺), CCR4^{-/-}OTII (Thy1.2⁺) and CCR8^{-/-}OTII (Thy1.2⁺) Th1 cells *in vitro* under Th1 polarizing conditions in the presence of IL-12 and anti-IL-4. We established that there was no difference between OTII Th1 cells and chemokine receptor-deficient OTII Th1 cells in differentiation, chemotaxis to CXCL12, activation and proliferation (data not shown). Competitive adoptive transfers were performed as described above. We observed that homing indices in the BAL and lung ranged from 0.99 to 1.1 (Fig 1, C) and there was no difference in the percentages of OTII Th1 cells and CCR4^{-/-}OTII or CCR8^{-/-}OTII Th1 cells in the BAL and lung.

CCR8 deficiency does not affect the *in vivo* proliferation of antigen-specific Th2 cells

Unlike CCR4^{-/-}OTII and CXCR3^{-/-}OTII Th2 cells, CCR8^{-/-}OTII Th2 cells accumulated in the TLN more than OTII Th2 cells. To determine whether differences in proliferation explain this observation, we compared the incorporation of BrdU ³⁴, a thymidine analogue, in OTII and CCR8^{-/-}OTII Th2 cells *in vivo*.

OTII (Thy1.2⁺) and CCR8^{-/-}OTII (Thy1.2⁺) Th2 cells were generated *in vitro* and adoptively transferred via the tail vein into separate Thy1.1⁺ C57BL/6 recipient mice. Mice were given two daily OVA challenges. Before the third OVA challenge, mice were injected with 2mg of BrdU intraperitoneally. The lung and the TLN were harvested two hours after BrdU injection and single cell suspensions were stained for CD4, Thy1.1 and Thy1.2. The extent of BrdU staining was determined in antigen-specific Th2 cells identified as CD4⁺Thy1.2⁺ cells in the lymphocyte gate. We analyzed BrdU incorporation after the third OVA challenge because the number of antigen-specific cells increases dramatically at this time point (data not shown). We gave a short pulse of BrdU in order to minimize the effect of T cell trafficking from one site to another.

There was no difference in the percentage of antigen-specific cells that were BrdU⁺ in the TLN or the lung between OTII Th2 cell recipients and CCR8^{-/-}OTII Th2 cell recipients (Fig 2, A and B). The rate of BrdU incorporation, and thus proliferation, was higher in the TLN compared with the rate of BrdU incorporation and proliferation in the lung for both OTII and CCR8^{-/-}OTII Th2 cell recipients over 2 hours (Fig 2, B) ($p < 0.005$), providing support that most antigen-specific Th2 cell proliferation occurs in the draining lymph node ¹.

The number of antigen-specific cells (CD4⁺Thy1.2⁺) was 3.2-fold higher in the TLN of CCR8^{-/-}OTII Th2 cell recipients compared with OTII Th2 cell recipients 2 hours after the third OVA challenge (Fig 2, C). As a result, despite equal rates of proliferation of OTII and CCR8^{-/-}OTII Th2 cells, the number of antigen-specific cells that were BrdU⁺ (CD4⁺Thy1.2⁺BrdU⁺) was 3.4-fold higher in the TLN of CCR8^{-/-}OTII Th2 cell recipients compared with OTII Th2 cell recipients (Fig 2, D). This indicates that more CCR8^{-/-}OTII Th2 cells enter the TLN and proliferate.

Between 2 to 24 hours after the third OVA challenge, the gap in the number of antigen-specific Th2 cells between CCR8^{-/-}OTII and OTII Th2 cell recipients decreased in the TLN but increased in the lung (Fig 2, E), suggesting that antigen-specific Th2 cells leave the TLN and accumulate in the lung over time.

Since CCR8 has been shown to protect T cells against apoptosis ³⁵, we compared staining with 7-AAD, an apoptosis marker, *in vivo* in OTII and CCR8^{-/-}OTII Th2 cells and observed no differences between the two groups (See Figure E2 in the Online Repository).

Disruption of CCR4-mediated and CCR8-mediated antigen-specific Th2 cell trafficking alters the expression of cytokines and chemokines in the BAL

We next evaluated the impact of interrupting CCR4-mediated and CCR8-mediated antigen-specific Th2 cell trafficking on the asthma phenotype. OTII, CCR4^{-/-}OTII and CCR8^{-/-}OTII Th2 cells were generated *in vitro* and adoptively transferred via the tail vein into separate recipient mice. Recipient mice were given daily aerosolized OVA challenges for three days. 24 hours after the last OVA challenge, lungs were harvested for histologic assessment, BAL total cell count and differential were determined, BAL cytokine and chemokine protein levels were measured and airway hyperresponsiveness was assessed.

We found that IL-4, IL-5, IL-13, CCL17 and CCL1 levels were markedly lower in CCR4^{-/-}OTII Th2 cell recipients compared with OTII Th2 cell recipients (Fig 3, A and B). In contrast, IL-4, IL-5, CCL22 and CCL1 levels were markedly higher in CCR8^{-/-}OTII Th2 cell recipients compared with OTII Th2 cell recipients. There was a trend towards increased levels of IL-13 in CCR8^{-/-}OTII Th2 cell recipients ($p = 0.07$) (Fig 3, A and B).

Majority of IL-4 producing antigen-specific Th2 cells express CCR4

To better understand the contribution of CCR4 on antigen-specific Th2 cells to the delivery of Th2 cytokines into the airway, we performed surface staining for CCR4 using a CCL22-IgG fusion protein ³⁰ along with intracytoplasmic staining for IL-4. We determined that 66% ($\pm 5\%$) of *in vitro* generated OTII Th2 cells express CCR4 (Fig 4, A). CCR4 staining with the CCL22-IgG fusion protein was specific as CCR4^{-/-}OTII Th2 cells had only 3% staining (Fig 4, A). Intracytoplasmic staining for IL-4 revealed that ~39% of *in vitro* generated OTII Th2 cells express IL-4 and ~30% express both IL-4 and CCR4 (Fig 4, B). On average, 62% ($\pm 8\%$) of IL-4 producing OTII Th2 cells express CCR4. To determine CCR4 expression of antigen-specific Th2 cells *in vivo*, BAL cells were isolated from Thy1.1⁺ recipient mice after adoptive transfer of OTII (Thy1.2⁺) Th2 cells and three OVA challenges. BAL cells were stained for CD4, Thy1.2 and CCR4 and antigen-specific cells were identified in the lymphocyte gate as

CD4⁺Thy1.2⁺. CCR4 staining was detected on 53% ($\pm$ 5) of CD4⁺Thy1.2⁺ in the BAL (Fig 4, C). Similar staining studies for CCR8 was not possible due to high background staining with commercially available polyclonal mouse anti-CCR8 antibodies (data not shown).

Absence of CCR4-mediated or CCR8-mediated antigen-specific Th2 cell trafficking modifies markers of allergic airway and pulmonary inflammation

As expected^{3, 24}, OTII Th2 cell recipient mice developed lung and airway eosinophilic inflammation, airway mucus hypersecretion and airway hyperresponsiveness. We found that BAL eosinophil counts were decreased by 53% in CCR4^{-/-}OTII and increased by 64% in CCR8^{-/-}OTII Th2 cell recipients compared with OTII Th2 cell recipient mice. Total cell count was also increased by 42% in CCR8^{-/-}OTII Th2 cell recipients (Fig 5, A). The histology and PAS scores were 33% and 30%, respectively, lower in CCR4^{-/-}OTII and 40% and 32%, respectively, higher in CCR8^{-/-}OTII Th2 cell recipients compared with OTII Th2 cell recipients (Fig 5, B and C). There was a trend towards a decrease in the percentage change in resistance by invasive plethysmography between CCR4^{-/-}OTII Th2 cell recipients and the other two groups at 1mg/ml and 2mg/ml of methacholine. However, this difference did not reach statistical significance. There was also no statistically significant difference between the three groups in percentage change in compliance as measured by invasive plethysmography (Fig 5, D and E).

Discussion

In this study, we have delineated the role of CCR4, CCR8 and CXCR3 in antigen-specific Th2 cell trafficking. We have shown that while CCR4 deficiency decreases antigen-specific Th2 cell trafficking into the allergic lung, CXCR3 deficiency has no impact and CCR8 deficiency leads to an accumulation of these cells. The roles of CCR4 and CCR8 were specific for antigen-specific Th2 cells as antigen-specific Th1 cell trafficking was not affected by CCR4 or CCR8 deficiency.

There are conflicting reports in the literature on the effect of CCR4 deficiency on allergic airway inflammation. CCR4^{-/-} mice sensitized with OVA and challenged 14 days later with five daily intranasal OVA doses developed airway inflammation, airway hyperresponsiveness and serum IgE levels comparable to wild type mice²². Moreover, the use of CCR4 blocking antibody did not inhibit allergic airway inflammation in a guinea pig model²³. However, airway hyperresponsiveness and eosinophilia were diminished in CCR4^{-/-} mice in a chronic *Aspergillus* model¹⁸, and in another report using CCL17 blocking antibody in OVA sensitized mice prior to OVA challenges¹⁹. There is also discrepancy on the effect of CCR8 deficiency on allergic airway inflammation. For example, three studies used an active immunization model with OVA and alum but only one found a difference between wild type and CCR8^{-/-} mice^{17, 20, 21}. In addition, there was no difference in BAL eosinophilia after adoptive transfer of CD4⁺ cells isolated from OVA-immunized wild type and CCR8^{-/-} mice²⁰, while CCR8 deficiency led to a decrease in allergic airway inflammation in a mast cell dependent model¹². In all of these reports, the CCR4 or CCR8 axis was interrupted in all cell types, including Tregs. The outcome of each study most likely differed based on the animal model used and the balance of the effect of CCR4 or CCR8 deficiency on multiple cell types. Our study is the first to isolate the role of CCR4 and CCR8 in the trafficking of antigen-specific Th2 cells from all other cell types by using competitive and separate adoptive transfer models.

Among the chemokine receptors studied, CCR4 was the only receptor required for the efficient recruitment of antigen-specific Th2 cells into the lung and the airways. We show that approximately 60% of antigen-specific Th2 cells express CCR4 prior to their adoptive transfer and after trafficking into the airways. This level of CCR4 expression is consistent with the 50% trafficking defect in CCR4^{-/-} antigen-specific Th2 cells. It is also similar to what has been

described in asthmatic subjects ^{9, 10}. We also demonstrate that approximately 60% of IL-4 producing antigen-specific Th2 cells express CCR4. This explains the dramatic decline in BAL Th2-type cytokines in CCR4^{-/-}OTII Th2 cell recipient mice compared with OTII Th2 cell recipients. In addition, CCR4^{-/-}OTII Th2 cell recipients had a 50% reduction in airway eosinophilia and a modest decrease in overall lung parenchymal inflammation and airway mucus production but no change in airway hyperresponsiveness. This indicates that the residual Th2 cells and Th2-type cytokines were sufficient to preserve some markers of allergic lung and airway inflammation. In this study, we focused on CCR4, CCR8 and CXCR3, and the relative contribution of other chemoattractant receptors to the residual Th2 cell trafficking remains to be determined.

We show that while CXCR3 had no impact on the trafficking of antigen-specific Th2 cells, CCR8^{-/-}OTII Th2 cells accumulated in the lung, BAL and TLN. This increased accumulation was not due to decreased apoptosis or increased rate of proliferation. However, while the rate of proliferation was the same between OTII and CCR8^{-/-}OTII Th2 cells, there were more CCR8^{-/-}OTII Th2 cells proliferating in the TLN. This resulted in more CCR8^{-/-}OTII Th2 cells being available to leave the TLN and accumulate in the lung over time. The reason for the increased trafficking of CCR8^{-/-}OTII Th2 cells into the TLN is not clear. However, we speculate that in the absence of CCR8 signaling, CCR8^{-/-} OTII Th2 cells are not responsive to constitutive CCR8 ligands expressed in organs, such as the skin ³⁶, and are thus more available for recruitment and subsequent proliferation in the TLN. These data demonstrate that the functional role of any given chemokine receptor and its ligands cannot be predicted from *in vitro* studies or studies of ligand and receptor expression and highlight the complexity of the chemokine system *in vivo*.

In summary, we have isolated the role of CCR4, CCR8 and CXCR3 on antigen-specific Th2 cells from the role of these receptors on all other cell types using an adoptive transfer model of allergic pulmonary inflammation. In so doing we have determined that CCR4 is required for the efficient entry of antigen-specific Th2 cells into the allergic lung and the airways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BAL	Bronchoalveolar lavage
CFSE	Carboxyfluorescein succinimidyl ester
BrdU	5-bromo-2-deoxyuridine
7-AAD	7-amino-actinomycin D
KO	Knock out

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Fig 1. Contribution of CCR4, CCR8 and CXCR3 to antigen-specific Th2 cell trafficking (A) Homing indices for the OTII and CCR4^{-/-} OTII (n=14 in 4 experiments), OTII and CXCR3^{-/-} OTII (n=6 in two experiments) and OTII and CCR8^{-/-} OTII Th2 cell co-transfers (n=17 in five experiments). (B) Sample flow cytometry plots. (C) Homing indices for OTII and CCR4^{-/-} OTII and OTII and CCR8^{-/-} OTII Th1 cell co-transfers. (n=6–9 in 2 to 3 experiments.)

Fig 2. Impact of CCR8 deficiency on in vivo proliferation of antigen-specific Th2 cells
 (A) Sample flow cytometry plots of BrdU incorporation by CD4⁺Thy1.2⁺ cells in OTII and CCR8^{-/-} OTII Th2 cell recipients. n=6–7 per group in two experiments. (B) Percentage of BrdU⁺CD4⁺Thy1.2⁺ cells. (C) Number of CD4⁺Thy1.2⁺ cells. (D) Number of BrdU⁺CD4⁺Thy1.2⁺ cells. (E) Fold difference, calculated as # of CD4⁺Thy1.2⁺ cells in CCR8^{-/-} OTII Th2 cell recipients: # of CD4⁺Thy1.2⁺ cells in OTII Th2 cell recipients at 2 and 24 hours after the third OVA challenge. n=6–7 per group per time in two experiments.

Fig 3. BAL cytokine and chemokine levels

(A) Th2 cytokine levels. (B) Chemokine levels. n=9–19 mice per group in two to four separate experiments. *p<0.05, **<0.005.

Fig 4. CCR4 expression by IL-4 producing antigen-specific T cells

(A) CCR4 expression on *in vitro* generated OTII and CCR4^{-/-} OTII Th2 cells. n=4 experiments.

(B) Intracytoplasmic staining for IL-4 and surface staining for CCR4. n=3 experiments.

(C) CCR4 expression on CD4⁺Thy1.2⁺ isolated from the BAL. n=6 in two experiments. Control on the x-axis is the secondary reagent used in CCR4 staining.

Fig 5. Markers of allergic airway and pulmonary inflammation

(A) BAL total cell count and differential, n=10–16. (B) Histology score, n=7–17. (C) PAS score, n=6–14. (D and E) airway hyperresponsiveness after methacholine (MC) challenges, n=7. *p<0.05, **p<0.005.