CC Chemokine Receptor (CCR)3/Eotaxin Is Followed by CCR4/Monocyte-derived Chemokine in Mediating Pulmonary T Helper Lymphocyte Type 2 Recruitment after Serial Antigen Challenge In Vivo

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Abstract

Isolated peripheral blood CD4 cells from allergic individuals express CC chemokine receptor (CCR)3 and CCR4 after expansion in vitro. In addition, human T helper type 2 (Th2) cells polarized in vitro selectively express CCR3 and CCR4 at certain stages of activation/differentiation and respond preferentially to the ligands eotaxin and monocyte-derived chemokine (MDC). However, controversy arises when the in vivo significance of this distinct expression is discussed. To address the functional role of CCR3/eotaxin and CCR4/MDC during the in vivo recruitment of Th2 cells, we have transferred effector Th cells into naive mice to induce allergic airway disease. Tracking of these cells after repeated antigen challenge has established that both CCR3/eotaxin and CCR4/MDC axes contribute to the recruitment of Th2 cells to the lung, demonstrating the in vivo relevance of the expression of these receptors on Th2 cells. We have shown that involvement of the CCR3/eotaxin pathway is confined to early stages of the response in vivo, whereas repeated antigen stimulation results in the predominant use of the CCR4/MDC pathway. We propose that effector Th2 cells respond to both CCR3/eotaxin and CCR4/MDC pathways initially, but that a progressive increase in CCR4-positive cells results in the predominance of the CCR4/MDC axis in the long-term recruitment of Th2 cells in vivo.

Key words: chemokines • effector T helper type 2 cells • migration • allergic airway disease • chemokine receptors

Introduction

T cells are critical mediators of inflammation, and as such their migration to inflammatory sites is a tightly controlled process involving a complex series of molecules expressed by a variety of cell types. This results in the delivery of functional subsets of cells to particular tissues or microenvironments. This is especially important for T cells, since effector T cells can be divided into distinct subsets based on their cytokine profiles and functional properties. Th1 cells characteristically produce IFN-γ and contribute to host defense against pathogens, whereas Th2 cells produce IL-4 and IL-5 and are associated with allergic reactions involving IgE, eosinophils, and basophils (1). Th2 cells and the cytokines they secrete are thought to be critically important for the development of injury during allergic reactions such as asthma. However, it is unclear how or why the Th2 subset migrates to the lung. Th2 cells have previously been distinguished from Th1 cells by virtue of their cytokine profile, although more recently a range of surface markers specific for Th2 cells has been defined (2, 3). Of particular importance to the question of selective Th subset migration to inflammation sites is the growing evidence that chemokine receptor expression is tightly regulated on Th cells, and that Th cell subsets express restricted receptors for chemokines (4). In accordance with this selective expression, Th1 and Th2 cells differentially migrate in response to the chemokines that bind to these receptors.

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Eotaxin and monocyte-derived chemokine (MDC)[1] are among the chemokines that seem to attract selectively Th2 but not Th1 cells (5, 6). Eotaxin is produced by epithelial cells and binds CC chemokine receptor (CCR)3 with high affinity and fidelity (7–9), whereas MDC is produced by macrophages and interacts specifically with CCR4 (10). CCR3 was originally described on eosinophils and basophils (8, 9), but has been documented as being present on human Th2 but not Th1 cells (5, 6). The fact that this particular Th cell subset expresses the receptor for a proeosinophilic chemokine is interesting in the context of the pathophysiology of allergic lung disease. The attraction of these Th2 cells by eotaxin may represent a mechanism by which an allergen-driven reaction escalates with the production of IL-4 and IL-5, both of which are necessary for the differentiation and activation of eosinophils. CCR4 has been similarly identified as a Th2-specific marker (11, 6), and its ligands, MDC and thymus and activation-regulated chemokine (TARC), have been shown to attract Th2 cells in preference to Th1 cells (6, 11–13). CCR8 is also selectively expressed on Th2 cells (14). Several groups have used T cells, either directly isolated from patients or generated in vitro, to show elegantly that CCR3 and CCR4 identify a subset of human T cells that exhibit a cytokine profile consistent with that of Th2 cells. However, the role of the CCR3/eotaxin or CCR4/MDC axes in attracting effector Th2 cells has not been established during in vivo inflammatory processes.

The aim of this study was to determine the functional importance of the eotaxin/CCR3 and MDC/CCR4 axes on the migration of antigen-specific Th2 cells in vivo using a T cell transfer model of allergen-induced lung injury in mice. We have used this in vivo model to show that eotaxin/CCR3 and MDC/CCR4 interactions play a critical, cooperative role in the homing of antigen-specific Th2 cells to the challenged lung, giving rise to eosinophilia and bronchial hyperresponsiveness (BHR). Moreover, we have determined that eotaxin/CCR3-mediated recruitment of Th2 cells in vivo is transient and progresses to an MDC/CCR4-dominated response that is maintained over time.

Materials and Methods

Mice. Mice expressing the transgene for the DO11.10 TCR-α/β, which recognizes residues 323–339 of chicken OVA in association with I-Ad, were provided by Dr. D. Loh (Washington University, St. Louis, MO [15]). Transfer recipients were 6–8-wk-old female BALB/cJ mice (The Jackson Laboratory).

In Vitro Polarization of T Cells. OVA-specific TCR-transgenic $CD4^+$ T cells were isolated from the spleen and cultured in complete RPMI 1640 medium with OVA323–339 (1 μg/ml) and mitomycin C–treated splenocytes. For Th1 phenotype development, recombinant murine IL-12 (40 ng/ml; Endogen) and neutralizing anti–IL-4 Ab (11B11, 20 μg/ml; R&D Systems) were added; for Th2 phenotype development, recombinant murine IL-4 (50 ng/ml) and anti–IL-12 (TOSH-2, 10 μg/ml; Endogen) were used. Cells were cultured for three rounds of antigenic stimulation under polarizing conditions. At this point, the cells were divided into two portions, with the majority being used to induce pulmonary inflammation as described below. A small sample (2×10^5 cells) from each culture was activated on immobilized anti-CD3 mAb (2C11, 10 μg/ml; PharMingen) in the presence of human (h)IL-2 (10 U/ml; Endogen) for 48 h to determine the integrity of the polarization. Culture supernatants were collected for measurement of IL-4, IL-5, and IFN-γ levels by ELISA (Endogen), and cell pellets were collected for RNA extraction and PCR analysis. Th2 cells produced high IL-4 and IL-5 levels but little IFN-γ, whereas Th1 cells produced high IFN-γ levels but little IL-4 and IL-5 (Th2 cells: 100–300 ng/ml IL-4, 50–150 ng/ml IL-5, and <20 pg/ml IFN-γ; Th1 cells: 7,000–15,000 ng/ml IFN-γ). Similarly, RNA expression analysis revealed that Th2 cells expressed predominantly IL-4 and IL-5 but little if any IFN-γ, whereas the reverse was true of Th1 cells.

Induction of Pulmonary Inflammation. In preparation for induction of allergic inflammation, Th1 or Th2 cells produced as described above were rested in hIL-2 (10 U/ml; Endogen) for 48 h before being washed in tissue culture medium. Recipient BALB/c mice were given 2×10^6 cells intravenously. 24 h later, mice were exposed to an aerosol of OVA (50 mg/ml, Grade V; Sigma Chemical Co.) for 20 min. Thereafter, mice were challenged daily and were killed 24 h after the last aeroallergen challenge on day 4, 7, or 14. Control mice received cells but were challenged with aerosolized PBS. After the mice were killed, bronchoalveolar lavage (BAL) was collected by cannulation of the trachea and lavage with 1 ml of PBS. Lungs were then inflated with optimum cutting temperature (OCT) compound and removed, and the right lobes of the lung were snap-frozen in liquid nitrogen while the left were fixed in 10% buffered formalin.

For blocking studies, mice were injected daily with 100 μg per mouse polyclonal rabbit antieotaxin Abs (7) or polyclonal rabbit anti–murine MDC (13) 30 min before OVA challenge. Mice were then killed at day 4 after T cell transfer (after three antigen challenges) or at day 7 (after six antigen challenges), as shown in Fig. 1.

Determination of BHR. Airway responsiveness was measured in Th2 recipient mice 24 h after the last aerosol challenge by recording respiratory pressure curves using whole body plethysmography (Buxco; EMKA Technologies) in response to inhaled methacholine (Sigma Chemical Co.) at concentrations ranging from 2.5 to 25 mg/ml for 1 min. Airway responsiveness was expressed in enhanced pause (P_{enh}), a calculated value that correlates with measurement of airway resistance, impedance, and intrapleural pressure in the same mouse: $P_{enh} = (t_e/t_{r1}) \times Pef/Pif$ (t_e, expiration time; t_r, relaxation time; Pef, peak expiratory flow; Pif, peak inspiratory flow).

BAL. Total BAL cells were counted, and aliquots (5×10^5 cells per slide) were pelleted onto glass slides by cytocentrifugation. A differential cell count was then performed after Wright-Giemsa staining (Fisher Diagnostics). Percentages of eosinophils, lymphocytes, neutrophils, and macrophages were determined by counting in eight randomly selected high-power fields (hpf; magnification: ×40; total area: 0.5 mm^2) and dividing this number by the total number of cells per hpf. To obtain the absolute number of each leukocyte subtype in the lavage, these percentages were multiplied by the total number of cells recovered from the BAL fluid.

In Vivo Measurement of Cytokine Production. Levels of the cytokines IL-4, IL-5, IFN-γ, IL-6, and IL-10 were determined in the lavage fluid of mice using ELISA kits (Endogen).

[1]*Abbreviations used in this paper:* AAD, allergic airway disease; BAL, bronchoalveolar lavage; BHR, bronchial hyperresponsiveness; CCR, CC chemokine receptor; hpf, high-power field(s); MDC, monocyte-derived chemokine; P_{enh}, enhanced pause; TARC, thymus and activation-regulated chemokine.

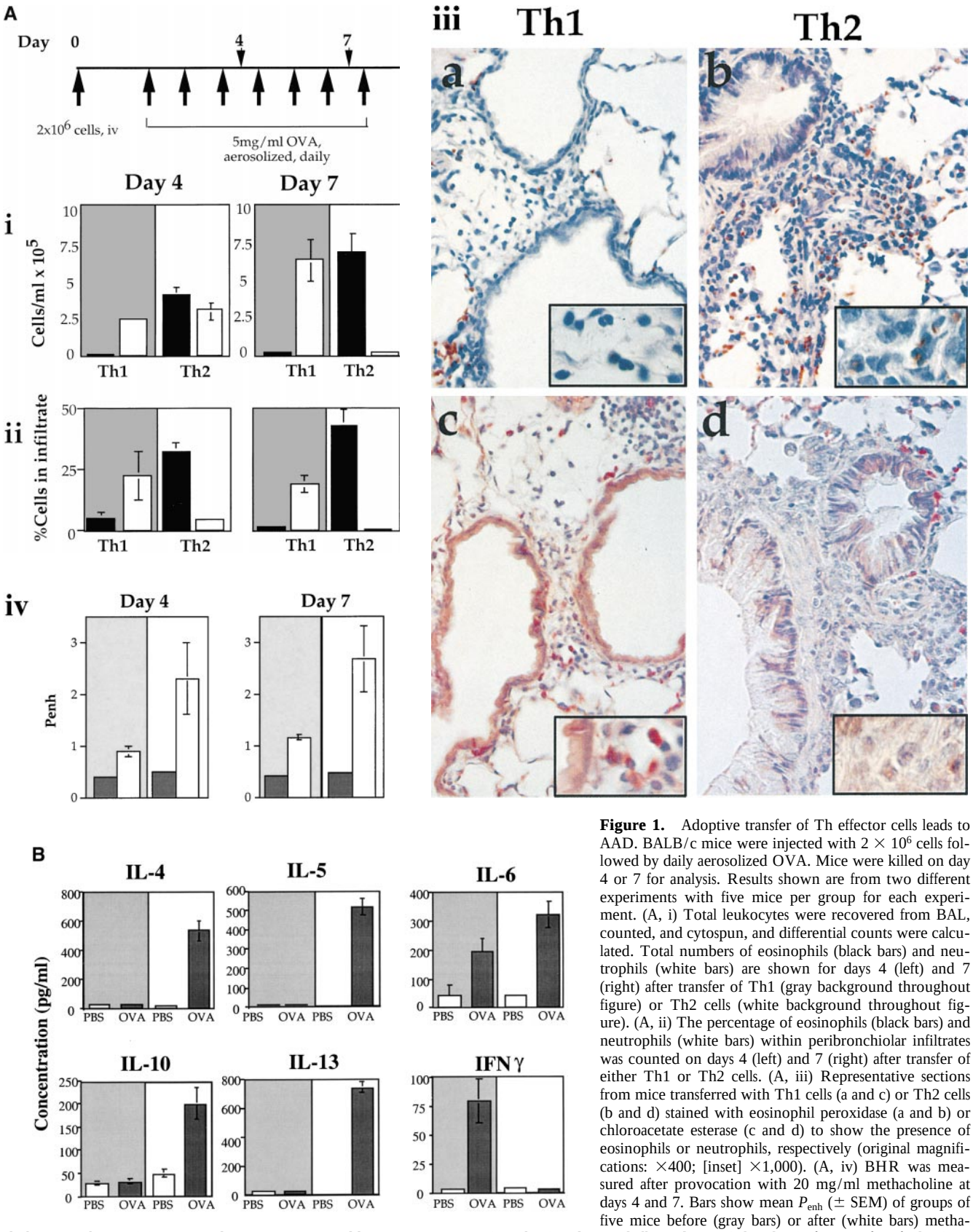

Figure 1. Adoptive transfer of Th effector cells leads to AAD. BALB/c mice were injected with 2×10^6 cells followed by daily aerosolized OVA. Mice were killed on day 4 or 7 for analysis. Results shown are from two different experiments with five mice per group for each experiment. (A, i) Total leukocytes were recovered from BAL, counted, and cytospun, and differential counts were calculated. Total numbers of eosinophils (black bars) and neutrophils (white bars) are shown for days 4 (left) and 7 (right) after transfer of Th1 (gray background throughout figure) or Th2 cells (white background throughout figure). (A, ii) The percentage of eosinophils (black bars) and neutrophils (white bars) within peribronchiolar infiltrates was counted on days 4 (left) and 7 (right) after transfer of either Th1 or Th2 cells. (A, iii) Representative sections from mice transferred with Th1 cells (a and c) or Th2 cells (b and d) stained with eosinophil peroxidase (a and b) or chloroacetate esterase (c and d) to show the presence of eosinophils or neutrophils, respectively (original magnifications: ×400; [inset] ×1,000). (A, iv) BHR was measured after provocation with 20 mg/ml methacholine at days 4 and 7. Bars show mean P_{enh} (± SEM) of groups of five mice before (gray bars) or after (white bars) methacholine stimulation. (B) BAL cytokines were measured by ELISA in OVA- (gray bars) and PBS-challenged mice (white bars) after transfer of Th1 (gray panel) or Th2 cells (white panel). Each bar represents the mean (± SEM) concentration in groups of five mice on day 4.

Measurement of Chemokine Ligand and Receptor Expression by PCR Analysis. PCR was performed using the Advantage® KlenTaq polymerase (Clontech Laboratories) according to the manufacturer's instructions. cDNA derived from 25 ng of total RNA was used for each 30-μl reaction containing 0.5 μM primers, 0.2 mM dNTP mix, 1× PCR reaction buffer, and 0.5 μl polymerase. Samples were amplified at 94°C for 30 s, 52–60°C for 1 min, and 68°C for 1 min for 20, 25, or 32 cycles. 10 μl of each reaction was loaded per well on 1.5% agarose gels. Primer sequences were as follows: for CCR3, 5′-TCTGTGGAATGAGTGGGGTTTTG and 5′-GTAATACGACTCACTATAGGGACTTCTGGATAGCGAGGACTG; for CCR4, 5′-ATCGTGCACGCGGTATTCTCC and 5′-GACGGGGTTAAGGCAGCAGTGA; for MDC, 5′-GGTGAAGAAGCTACTCCATAAACT and 5′-GTAATACGACTCACTATAGGGAGAAGGGATAGAGGGGAGGTA; and for eotaxin, 5′-TCTCCCTCCACCATGCAGAG and 5′-CAGATCTCTTTGCCCAACCT. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were purchased from Clontech Laboratories.

Lung Histology and Immunohistochemistry. The left lobe of the lungs was fixed in 10% neutral buffered formalin (NBF; J.T. Baker) and paraffin embedded. Sections (4 μm) were stained for cyanide-resistant peroxidase according to standard protocols (16), then counterstained with hematoxylin to depict eosinophils or with chloroacetate esterase to show neutrophils. The composition of infiltrates was then determined by counting the total number of infiltrating cells in five peribronchiolar fields and determining the percentage of eosinophils, neutrophils, and mononuclear cells. General morphology was assessed on hematoxylin and eosin–stained sections.

For determination of antigen-specific T cells within lung tissue, serial frozen sections (4 μm) were stained with either anti-CD4 (PharMingen) or an Ab specific for the transgenic TCR, KJ126 (17). Both of these Abs were biotinylated, and positive staining was detected using streptavidin-peroxidase (DAKO Corp.) followed by diaminobenzidine (DAB; Vector) before counterstaining in hematoxylin. Eosinophils were stained for cyanide-resistant peroxidase as described above.

The number of cells per hpf was obtained by counting positively stained cells (CD4 cells or eosinophils) in five fields per section at a magnification of 400. To calculate the percentage of $KJ126^+$ CD4 cells, CD4 cells were counted; the same field was located on the KJ126-stained serial section, and positive cells were enumerated. At least 250 CD4 cells were counted for each section, and the percentage of $KJ126^+$ CD4 cells was then calculated.

$CCR3^+$ or $CCR4^+$ Th2 cells were detected in lung sections by immunohistochemical staining using polyclonal Abs specific for the COOH terminus of CCR3 or CCR4 (Santa Cruz Biotechnology). Staining of primary Abs was visualized with a biotinylated donkey anti–goat Ig Ab (Jackson Immunochemicals) followed by streptavidin-peroxidase as described above. Positively stained Th2 cells were enumerated by locating an infiltrate in the serial KJ126-stained section and counting the percentage of KJ-stained cells that were either $CCR3^+$ or $CCR4^+$. At least 50 KJ126-stained cells were counted in each section from lungs obtained at days 4 ($n = 6$), 7 ($n = 4$), and 14 ($n = 4$), with between two and three lobes stained per mouse.

Results and Discussion

Adoptive Transfer of Th Effector Cells Leads to Allergic Airway Disease. The evaluation of the role of chemokines in the migration of Th2 cells in vivo during active immunization models of allergic airway disease (AAD) is complicated by the difficulty of tracking a small subset of effector Th2 cells that are specific for antigen during the course of the inflammatory response. To evaluate the role of the eotaxin and MDC chemotactic pathways in vivo, we sought a mouse model in which basic pathophysiological features of AAD (eosinophilia, interstitial inflammation, bronchial hyperresponsiveness, and cytokine production) could be induced in vivo upon transfer of well-characterized, easy-to-track, antigen-specific Th2 cells. Such an approach would enable us technically to address the hypothesis mentioned above, but interpretation of the results obtained would necessarily have to consider the clear differences and limitations of the experimental system chosen. Specifically, a mouse model system in which some of the important processes during the development of an AAD-type chronic inflammatory reaction are totally or partially "bypassed," whereas others (i.e., the migration, accumulation, and activation of Th2 cells to the airways and the pathophysiologies they elicit) are presumably maintained. Therefore, it is important to recognize when interpreting the data presented in this report that antigen presentation, antigen-presenting cell activation and migration, and activation, migration, and differentiation of naive T cells and that of their immature effector Th descendants, among others, are essential processes that most likely occur and progress differently in adoptive transfer models and active immunization models of AAD.

Several elegant models of AAD have been described whereby transfer of in vitro–polarized Th2 cells induces pulmonary eosinophilia (18–21). However, the Th cells used for these studies were, in general, polarized for short times in culture. Therefore, we set out to develop a system whereby Th cells were maximally polarized to ensure differential chemokine receptor expression, and thus induced multiple pathophysiological endpoints after transfer in vivo. Th2 or Th1 cells were generated in vitro after several rounds of polarizing cytokines before transfer in vivo, when mice received multiple serial in vivo antigen challenges. Accordingly, Th1 or Th2 cells were transferred intravenously to unsensitized BALB/c recipient mice, and changes in lung function were measured at various time intervals after antigen challenge. Mice were then killed at day 4 or 7, and the extent of inflammation was determined in the BAL and tissue (Fig. 1). Control mice that received cells but no antigen challenge showed no increase in cells either in the BAL or the tissue. Transfer of either Th1 or Th2 cells in conjunction with serial OVA challenge resulted in an increase in the total number of lavage leukocytes, as has been reported previously using similar protocols (18, 21). Staining of cytospins revealed a differential migration of leukocytes to BAL after Th2 transfer compared with Th1 transfer, in that transfer of Th2 cells initiated an eosinophilic infiltrate, whereas Th1 transfer initiated a neutrophilic infiltrate. Infiltration increased with challenge, peaking at day 7 (Fig. 1 A, i). Similar results were observed in lung tissue, in which Th1 transfer resulted in a perivascular and peribronchiolar infiltrate, composed largely of neutrophils, in conjunction with macrophages and lymphocytes. Conversely, Th2 transfer initiated

an eosinophilic infiltration to perivascular and peribronchiolar areas (Fig. 1 A, ii). The proportion of either eosinophils or neutrophils was 35% after Th2 cell transfer and 25% after Th1 cell transfer, respectively (Fig. 1 A, iii). Interestingly, this induction of pulmonary inflammation after transfer of antigen-specific Th2 cells was accompanied by a corresponding increase in BHR (Fig. 1 A, iv), although no such change was detected after Th1 cell administration. The polarized pathological response to transfer of Th1 or Th2 cells was reflected in the repertoire of cytokines in BAL fluid. Th1 cell transfer induced secretion of IFN-γ but low levels of IL-4, -5, -10, and -13, whereas Th2 transfer was associated with an increase in IL-4, -5, -6, -10, and -13, with no discernible increase in IFN-γ (Fig. 1 B, i).

Th2 Cells Preferentially Express CCR3 and CCR4 In Vivo As Well As In Vitro. Recent evidence has shown that effector Th cells are polarized with respect to their chemokine receptor expression as well as their cytokine production, and that Th2 cells preferentially express CCR3 and CCR4 (5, 6, 11). These findings were first confirmed in the murine in vitro–polarized Th cells used for this study. Th2 cells showed increased expression of CCR3 and CCR4 RNA after activation with anti-CD3 and IL-2 (Fig. 2 A). The increased expression of CCR3 after activation correlates well with an enhanced calcium-mobilization response to eotaxin stimulation on mouse activated Th2 cells (not shown) compared with nonactivated control cells. This is somewhat unexpected, as anti-CD3 stimulation, in contrast to CCR4, downregulates CCR3 expression in human Th2 cells (14). This difference could reflect a disparity between the mouse and the human system, or simply represent a different degree of differentiation/activation between the cells used in the experiments described here and those used by other investigators. Immunohistochemical staining for CCR3 and CCR4 showed that the majority of the cells were double positive after incubation with Th2-polarizing cytokines (Fig. 2 B), but not with Th1 cytokines (data not shown). With this protocol, all cells expressed both receptors simultaneously, although there was a variation in the degree of expression, with definite high and low expressing populations.

The decision concerning the activation state of the Th2 cells to be transferred in vivo is one that deserves comment. Different groups have used different conditions to polarize and activate Th2 cells before transfer in vivo (18–21). In general, these protocols are based on the transfer of Th cells that are polarized for short times in culture. In the system used here, Th cells were maximally polarized and activated to ensure differential chemokine receptor expression. A second reason for the degree of polarization and activation used here was to ensure the induction of multiple pathophysiological endpoints after transfer in vivo. When interpreting the results obtained in this report and comparing them with results obtained in others, it is critical to factor in and to compare such possible differences in activation and polarization of the transferred T cells, which could well represent different stages of disease initiation and progression and/or the cellular basis of different etiologies resulting in the same final chain of pathophysiological events.

To determine the expression pattern of CCR3 and CCR4 and their ligands in lung tissue after adoptive transfer, we performed PCR in pools of mRNA extracted from lungs at days 4 and 7 after Th cell transfer. We found that CCR3 and its ligand, eotaxin, are upregulated in lung RNA isolated after serial OVA challenge of mice after Th2 cell transfer, but not after Th1 transfer (Fig. 2 C). Higher CCR4 mRNA levels were also seen in mice after Th2 cell transfer compared with Th1 transfer. MDC mRNA was expressed after challenge in both Th1 and Th2 recipient mice, presumably since the main cell type producing this chemokine is the macrophage (10). We did not find any significant TARC expression after induction of pulmonary inflammation (data not shown); thus, MDC was used as the ligand for functional studies. The principal site of CCR3 expression is likely to be on eosinophils, and of CCR4 on macrophages, but these receptors have also been found on Th2 cells in vitro (5, 11).

To localize the expression of CCR3 and CCR4 with Th2 cells in vivo, we used immunohistochemical staining to determine that both $CCR3^+$ and $CCR4^+$ Th2 cells were indeed present in the lung after intravenous transfer of antigen-specific Th2 cells and subsequent allergen challenge (Fig. 2 D). Moreover, there was a greater proportion of Th2 cells that expressed CCR3 rather than CCR4 on day 4, whereas the converse was true for day 7, with a greater proportion of Th2 cells expressing CCR4 rather than CCR3 (Fig. 2 D). There were no CCR3- or CCR4-expressing effector Th cells after transfer of Th1 cells (data not shown). When this analysis was performed at day 14 (after 13 in vivo antigen stimulations), the vast majority (>95%) of Th2 cells found in the lung expressed CCR4, whereas there were only a small number (<5%) of CCR3-expressing Th2 cells (Fig. 2 D).

CCR3/Eotaxin and CCR4/MDC Function in a Coordinated Manner to Promote Th2 Cell Recruitment In Vivo. Based on this differential expression of CCR3 and CCR4, we formulated the hypothesis that CCR3/eotaxin and CCR4/MDC pathways play differential, coordinating roles in the development of pathology during allergic reactions. To test this hypothesis, we used neutralizing Abs to block the ligands for CCR3 and CCR4, eotaxin and murine MDC, respectively, after acute or repeated antigen challenge in mice injected with antigen-specific Th cells. It should be kept in mind that, although eotaxin is the main described ligand for CCR3 in both mice and humans, other chemokines such as monocyte chemoattractant protein 3 (MCP-3) have been shown to exert their effects in vitro at least partially through CCR3 binding. Similarly, TARC is yet another known ligand for CCR4. Therefore, blockage of eotaxin and MDC might not necessarily be equivalent to blockage of CCR3 or CCR4.

We have found previously that the antieotaxin and anti-MDC Abs used in this study block the specific migration of CCR3- or CCR4-expressing cells, both in vitro and in vivo (7, 13). Mice were killed on day 4 or 7, and the migration of antigen-specific Th cells to the lung was determined histologically. The donor Th1 and Th2 cells expressed a clonotypic TCR recognized by an mAb, making it possible to

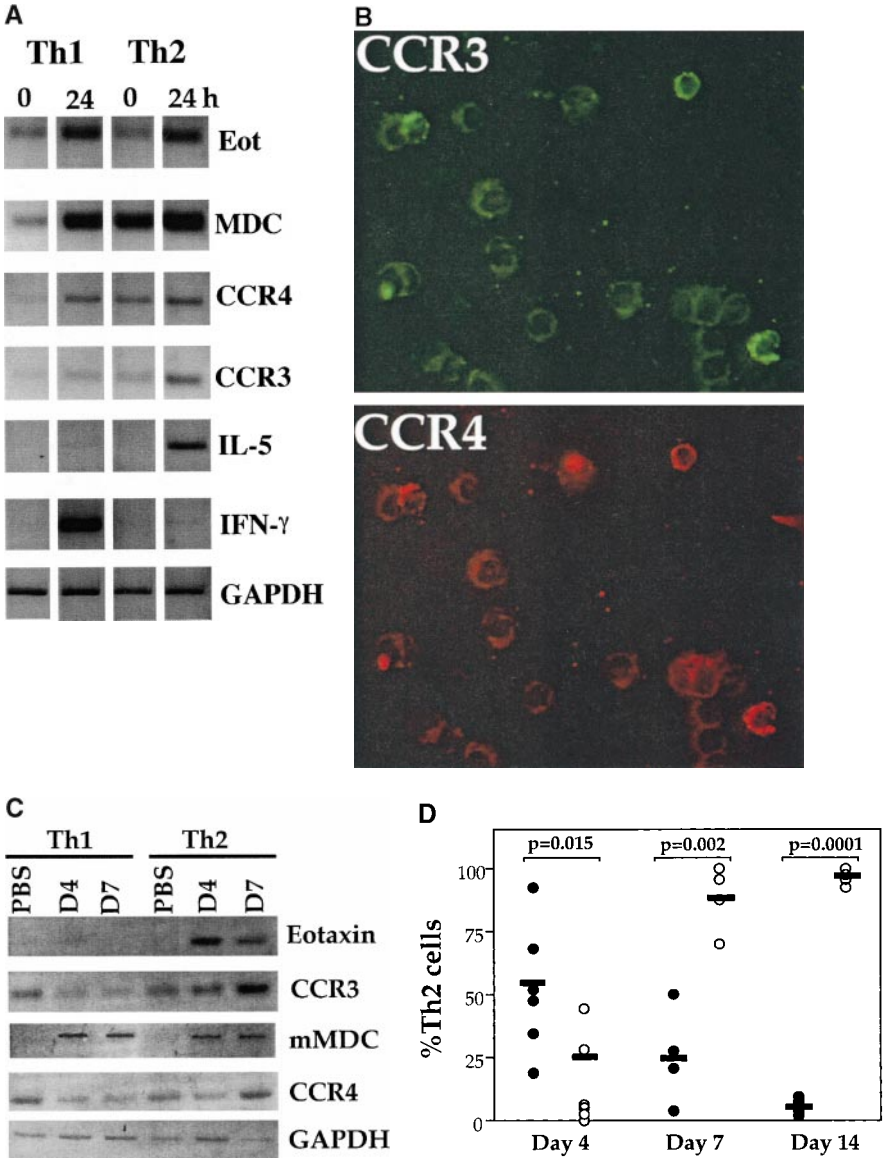

Figure 2. Chemokine receptor expression in donor Th cell populations, and in lungs after Th cell transfer. (A) Expression of chemokine receptors CCR3 and CCR4 and their respective ligands, eotaxin and MDC, were determined by PCR in effector Th cells after three rounds of polarization and activation with anti-CD3 Ab. Polarity of Th cells was checked by expression of IL-5 and IFN-γ in each population. (B) Protein expression of CCR3 and CCR4 was confirmed by immunohistochemical staining of cytospins prepared from third-round polarized cells. (C) Expression of chemokine receptors CCR3 and CCR4 and their respective ligands, eotaxin and MDC, was determined by PCR in lung RNA pooled from three mice transferred with either Th1 or Th2 cells and challenged with PBS or OVA. Levels were compared with those of the housekeeping gene, GAPDH. (D) Relative proportions of $CCR3^+$ and $CCR4^+$ Th2 cells in allergic lung tissue. CCR3- and CCR4-expressing Th2 cells were enumerated by locating an infiltrate in each KJ126-stained section and counting the percentage of KJ126-stained cells that were either $CCR3^+$ or $CCR4^+$ in serial sections. Sections were counted in lungs obtained at day 4 ($n = 6$), 7 ($n = 4$), or 14 ($n = 5$).

distinguish donor (antigen-specific) cells from host Th cells (17). We found that although the total number of CD4 cells was unaffected by blockage of either CCR3 or CCR4 ligands, the percentage of antigen-specific Th2 cells decreased by at least 50% (Fig. 3). In contrast, blockage of eotaxin or MDC had no effect on the migration of antigen-specific Th1 cells (Fig. 3 B). However, the specific effect of blocking one pathway versus the other differed at each time point (as discussed in detail below). These data show that eotaxin and MDC interactions with their specific receptors in vivo are critical for antigen-specific Th2 cell recruitment to the lung in this model.

To determine whether this selective blockage of antigen-specific Th cells by distinct chemokines affected eosinophil infiltration, BHR, and BAL cytokine production, we measured these parameters on day 4 after blockage with antieotaxin, or on day 7 after neutralization of MDC. Eosinophilia was decreased by blockage of either eotaxin or MDC (Fig. 4 A). This may stem from interference in the interaction of eotaxin with CCR3 on eosinophils, but also with CCR3 on Th2 cells, since the initiating event in eosinophil accumulation in this model occurs as a direct result of antigen-specific Th2 cell migration. BHR was also reduced after blockage of either eotaxin or MDC (although the latter did not reach statistical significance; Fig. 4 B). The decreased migration of antigen-specific Th cells also impaired the production of Th2-type cytokines, with significant decreases in IL-4 after blockage of eotaxin or MDC (Fig. 4 C). Neither treatment had any effect on IL-5 production. These results demonstrate unequivocally that Th2 cells alone can initiate pulmonary inflammation and that the recruitment of these essential cells via the CCR3/eotaxin (day 4) and CCR4/MDC (day 7) pathway is a critical event in the development of AAD. In this regard, it is important to note that eosinophilia and BHR were only moderately reduced (~35% inhibition of eosinophilia; data

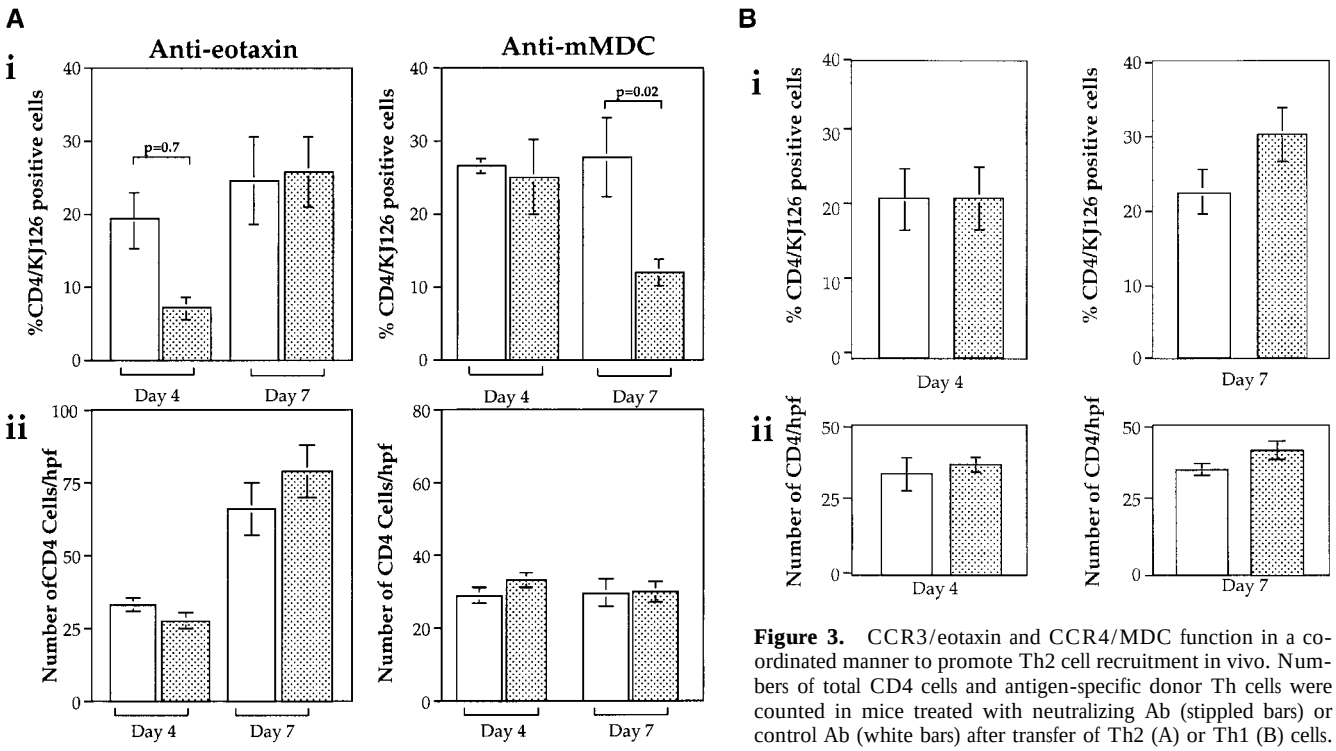

Figure 3. CCR3/eotaxin and CCR4/MDC function in a coordinated manner to promote Th2 cell recruitment in vivo. Numbers of total CD4 cells and antigen-specific donor Th cells were counted in mice treated with neutralizing Ab (stippled bars) or control Ab (white bars) after transfer of Th2 (A) or Th1 (B) cells. The percentage of antigen-specific cells was quantified by counting at least 250 $CD4^+$ cells in each section, and then counting the number of $KJ126^+$ cells in serial sections at a magnification of 400 for 10 mice in each group from two separate experiments (i). Total numbers of $CD4^+$ cells per hpf are shown (ii).

not shown) on day 7 after blockage of eotaxin (compared with ~75% inhibition of eosinophilia at day 4; Fig 4), indicating that other chemokines in addition to eotaxin participate in the final recruitment of eosinophils in vivo (data not shown).

The CCR4/MDC Axis Is Dominant in Mediating Recruitment of Th2 Cells after Repeated Allergen Exposure. We have shown that CCR3/eotaxin and CCR4/MDC mediate selective recruitment of antigen-specific Th cells during the allergic process. However, our results also show that these axes are important at different stages of the disease process. In our model, the CCR3/eotaxin pathway was critical in mediating the recruitment of Th2 cells after initial antigen stimulation in vivo, as determined by effective blockage at day 4. However, after repeated antigen stimulation (on day 7), the CCR3/eotaxin axis was superceded by the CCR4/MDC pathway, which was critical for Th2 migration by day 7. Further stimulation with antigen results in >95% of Th2 cells expressing CCR4 by day 14, but <5% expressing CCR3. This reinforces the view that chemokines and their receptors function in a tightly controlled, coordinated manner, as has been suggested in active immunization models of pulmonary inflammation (22). The interpretation of the results reported here in the context of the pattern of eotaxin and MDC expression in the lung during the development of AAD after active immunization (13) illustrates the complexity of hypothesizing expression and function correlations. For example, we have reported previously that the peak of MDC expression precedes that of eotaxin expression during the course of an active immunization model of AAD (13). Based on the results reported here, we propose that this maximal accumulation of MDC mRNA and protein probably correlates more with an early accumulation of monocytes/macrophages (which produce significant amounts of MDC [13]) in the lung in that particular model than with an early recruitment of Th2 cells. We hypothesize that lower levels of MDC present at later time points (13) are in turn critical for the recruitment of Th2 cells to the airways. Conversely, we hypothesize that maximal levels of eotaxin occurring at later time points during the course of the same active immunization models (7, 13) correlate better with final eosinophil recruitment, whereas lower levels of eotaxin present in the lung at early time points could be key to initiate Th2 accumulation in the lung.

The data presented here also strengthen the hypothesis that Th cells modulate their chemokine receptor expression according to the degree and extent of antigen stimulation and the cytokine milieu. Our experiments indicate that this indeed occurs during the in vivo allergic response. Moreover, our results show that this regulation of expression gives rise to functional consequences. These results are aligned with the data from in vitro experiments that show that Th2 cells lose CCR3 expression and preferentially gain CCR4 expression in response to repeated antigen stimulation (14).

Concluding Remarks. We have taken advantage of a mouse model of AAD based on the adoptive transfer of polarized effector Th cells to determine the functional importance of the chemokine receptor/ligand axes CCR3/eo-

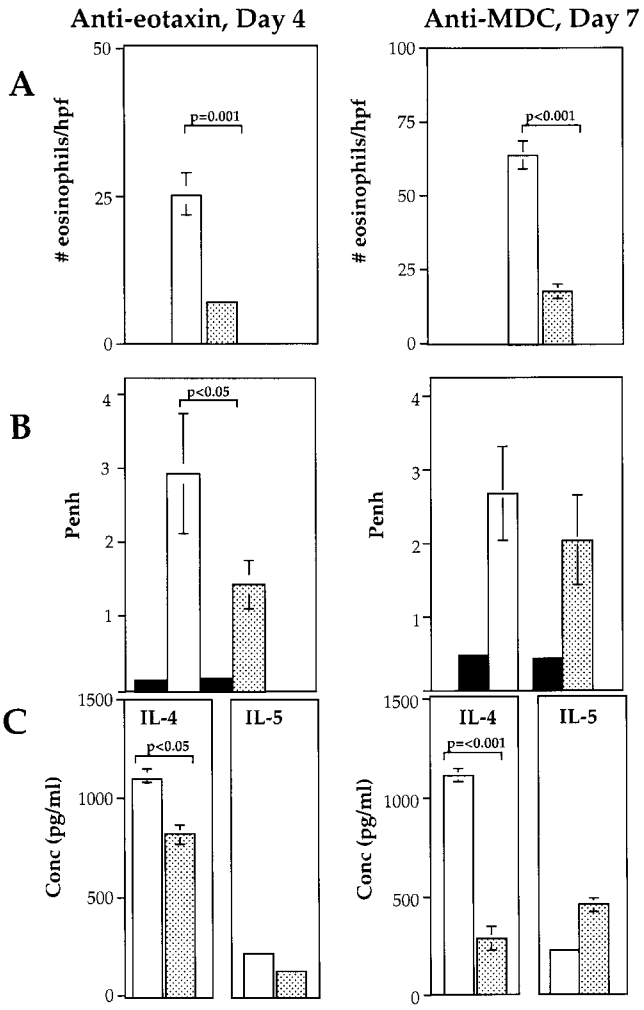

Figure 4. Decreased Th2 cell migration affects development of eosinophilia and BHR. The extent of eosinophilia (A), development of BHR (B), and levels of lavage cytokines (C) were determined in mice transferred with Th2 cells, challenged with OVA, and treated with neutralizing Abs to eotaxin (left) or MDC (right). Mice were given control Ab (white bars) and compared with Ab-treated mice (stippled bars). Numbers of eosinophils were counted in five hpf in cyanide-resistant, peroxidase-stained sections. BHR was measured as described in Materials and Methods and is shown as mean ± P_{enh} for each group of mice after stimulation with methacholine (white and stippled bars) or at baseline (black bars). Levels of IL-4 and IL-5 in BAL were determined by ELISA, and are calculated as mean ± SEM for each group.

taxin and CCR4/MDC in mediating the recruitment of antigen-specific Th2 cells during in vivo allergic reactions. We have shown that both eotaxin/CCR3 and MDC/CCR4 play a critical role in the homing of Th2 cells to the lung after antigen challenge. This finding emphasizes the relevance of previous in vitro results and demonstrates for the first time in vivo that CCR3 and CCR4 not only are markers of Th2 cells, but also have a critical pathophysiological significance in the development of AAD (as determined by their impact in BHR and eosinophilia). Moreover, we have determined that these pathways act in a coordinated cooperative manner, with the CCR3/eotaxin pathway being critical in the acute stages of a response after initial challenge. However, repeated antigen challenge results in an increased frequency of CCR4-expressing Th2 cells. Consequently, the CCR4/MDC pathway ultimately dominates in the recruitment of antigen-specific Th2 cells. Based on these findings, we would like to propose that it is the CCR4/MDC axis which is primarily responsible for the long-term recruitment of antigen-specific Th2 cells to target organs, such as airways, during chronic inflammatory responses in which there is repeated exposure to allergen.

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