Involvement of CCR5 in the passage of Th1-type cells across the blood-retina barrier in experimental autoimmune uveitis

Isabel J. Crane,*† Heping Xu,* Carol Wallace,* Ayyakkannu Manivannan,† Matthias Mack,‡ Janet Liversidge,* Gabriel Marquez,§ Peter F. Sharp,† and John V. Forrester*

Departments of *Ophthalmology and †Bio-Medical Physics and Bio-Engineering, University of Aberdeen Medical School, Foresterhill, United Kingdom; ‡Department of Internal Medicine II, University of Regensburg, Germany; and §Centro Nacional de Biotecnologia, UAM-Campus de Cantoblanco, Madrid, Spain

Abstract: Although the recruitment of T helper cell type 1 (Th1)/Th2 cells into peripheral tissues is essential for inflammation and the host response to infection, the traffic signals that enable the distinct positioning of Th1/Th2 cells are unclear. We have determined the role of CC chemokine receptor 5 (CCR5) in this using experimental autoimmune uveitis (EAU) as a model system. In EAU, Th1-like cells are preferentially recruited into the retina across the blood-retina barrier, partly as a result of expression of the adhesion molecules P-selectin glycoprotein ligand 1 (PSGL-1) on CD3+ T cells, infiltrating the retina, also expressed the chemokine receptor CCR5, and CCR5 ligands, macrophage-inflammatory protein-1α (MIP-1α), MIP-1β, and regulated on activation, normal T expressed and secreted (RANTES), were strongly expressed in the retina at peak EAU. Th1-like cells, polarized in vitro, expressed high levels of CCR5. The trafficking of these CCR5+ cells was examined by tracking them after adoptive transfer in real time in vivo at an early disease stage using scanning laser ophthalmoscopy. Treatment of the cells with antibody against CCR5 prior to transfer resulted in a reduction in their infiltration into the retina. However, rolling velocity, rolling efficiency, and adherence of the cells to retinal endothelium were not reduced. CCR5 is clearly important for Th1 cell recruitment, and this study demonstrates for the first time in vivo that CCR5 may act at the level of transendothelial migration rather than at the earlier stage of rolling on the endothelium. J. Leukoc. Biol. 79: 435–443; 2006.

Key Words: chemokines · chemokine receptor · cell trafficking · inflammation

INTRODUCTION

CD4 T helper (Th) cells are categorized into functionally distinct subsets, characterized by the patterns of cytokines they produce, thus providing a basis for polarized immune responses. Th1 cells, producing interferon-γ (IFN-γ), interleukin (IL)-2, and tumor necrosis factor α, are involved in monocyte/macrophage-mediated inflammatory responses, whereas Th2 cells, producing cytokines such as IL-4, IL-5, IL-13, and IL-10 [1, 2], are associated with increased and altered antibody production and mast cell and eosinophil proliferation and function. Th2 cells also negatively regulate Th1 cell-mediated responses, thus acting in an anti-inflammatory capacity. The recruitment of Th1/Th2 cells into peripheral tissues is essential for inflammation and the host response to infection. However, the traffic signals that enable the distinct positioning of Th1/Th2 cells are unclear.

In experimental autoimmune uveitis (EAU), an animal model for the human inflammatory eye disease endogenous posterior uveoretinitis, CD4+ T lymphocytes cross the blood-retina barrier (BRB) and initiate retinal destruction [3]. EAU is a Th1-type, organ-specific, autoimmune disease in which disease is induced by immunization of animals at distant sites with retinal antigens and appropriate adjuvants [4]. We have shown that Th1-like cells are preferentially recruited into the retina in EAU [5] during initiation and resolution of the disease, suggesting that reduction in Th1 cell numbers rather than an influx of Th2 cells was important for resolution. This selective recruitment of Th1 cells into the retina during inflammation was partly a result of up-regulation of the adhesion molecules P-selectin glycoprotein ligand 1 (PSGL-1) and lymphocyte function-associated antigen-1 (LFA-1) on Th1 cells [5] and intercellular adhesion molecule-1 (ICAM-1) and P/E-selectin on the endothelium of retinal venules, the site of leukocyte extravasation [5, 6].

Chemokines, molecules that direct the migration of T cells [7], are known to add specificity to the interactions initiated by adhesion molecules during the recruitment of leukocytes from the circulation during an inflammatory reaction. In an inflammatory situation, leukocytes respond to an activated endothelium by initial tethering, rolling, and then firm adhesion before diapedesis into the tissue [8, 9]. Chemokines on the endothelium [10] stimulate firm adhesion via integrin activation [11].
Th1 and Th2 cells have been shown to express chemokine receptors differentially. Th1 cells express CC chemokine receptor 5 (CCR5) and CXC chemokine receptor 3 (CXCR3) predominantly [12, 13], and Th2 cells express CCR3, CCR4, and CCR8 [12–14]. Which receptors are important for the trafficking of these cells to inflamed sites will depend on the chemokines present at the specific sites.

As Th1 cells are preferentially recruited during EAU, we predicted that CCR5 or CXCR3 would play a dominant role in this process, and in this study, we focus on the role of CCR5. We have previously shown that blocking macrophage-inflammatory protein-1α (MIP-1α; CC chemokine ligand 3 [CCL3]), a ligand for CCR5, inhibited the trafficking of labeled leukocytes to inflamed retina, leading to a reduction in disease severity [15]. However, these experiments did not examine the effects on Th cell subsets specifically and did not determine which of the chemokine receptors for MIP-1α, CCR1, or CCR5 was involved.

The importance of CCR5 in Th1 cell recruitment has been highlighted in recent studies. Preferential migration of Th1 clones to rheumatoid arthritis synovial tissue cell-derived supernatants was strongly inhibited by blocking regulated on activation, normal T expressed and secreted (RANTES; CCL5), a CCR5 ligand, but not CXC chemokine ligand 10 [CXCL10; IFN-inducible protein 10 (IP-10)], a CXCR3 ligand [16]. CCR5 has also been shown in mice to be important for the development of insulinitis and diabetes [17]. An examination of T cell recruitment to cutaneous sites has shown that although in vitro CCR5 appears to be less critical for chemotaxis of antigen-activated T lymphoblasts than CXCR3, in vivo, CCR5 and CXCR3 were equally important [18]. Another recent indication of the significance of CCR5 has been provided by the finding that preferential Th1 cell migration, induced by the binding of IL-16 to CD4, is enhanced in the presence of CCR5 [19].

As it is clearly difficult to accurately model in vitro all aspects of the complex interactions occurring as a leukocyte interacts with the endothelium in vivo, in this study, we have examined the role of CCR5 in Th1 cell recruitment at the BRB in vivo using a recently developed, noninvasive method, scanning laser ophthalmoscopy (SLO) [20]. This method enables real-time tracking of leukocytes in vivo under true physiological conditions in the retinal circulation. Subsequent confocal microscopy of retinal wholemounts from the same eye allows correlation of in vivo findings with extravascular retinal infiltration of cells. Our data show that CCR5 is required for Th1 cell recruitment in EAU and that it acts at the stage of transendothelial migration rather than the initial stages of rolling or adhesion.

MATERIALS AND METHODS

Induction of EAU

Female B10R.III mice, 8–12 weeks old (Biological Services Unit, University of Aberdeen, UK), were treated humanely according to the Animal License Act (UK). EAU was induced with a subcutaneous 50 μl injection into each thigh of 25 μg peptide 161–180 human interphotoreceptor retinoid-binding protein (IRBP; SIGPYISYLHPGNTILHVD; purity >85%, Sigma-Aldrich, Poole, UK), emulsified 1:1 with complete Freund’s adjuvant (CFA; H37Ra, Difco Laboratories, Detroit, MI) [21, 22].

Preparation of antigen-specific, Th1-polarized cells

CD4-positive cells were isolated from spleens 12–16 days after peptide immunization at peak EAU using magnetic cell sorter (MACS) positive selection (Miltenyi Biotec, Bergisch Gladbach, Germany). Antigen-presenting cells (APCs) were isolated from normal mouse spleen by depletion of CD4 and CD8 cells (MACS negative selection). APCs (2 × 10^5) were treated with mitomycin C (50 μg/ml, Sigma-Aldrich) at 37°C in the dark for 30 min. They were washed well before resuspending with CD4^+ cells in the ratio 1:2 in RPMI 1640 supplemented with 10% (v/v) fetal calf serum, 1 mM L-glutamine, 100 μg/ml streptomycin, and 100 IU/ml penicillin. The cell mix was cultured for 3 days with 50 μg/ml IRBP peptide and IL-12 (5 ng/ml), IFN-γ (20 ng/ml), and anti-IL-4 (1 μg/ml, clone 11B1), all from BD Biosciences PharMingen (Oxford, UK). Control cells were not cultured with IFN-γ and anti-IL-4 but with IL-10 (10 ng/ml) and anti-IFN-γ (2 μg/ml, clone XMG1.2, BD Biosciences PharMingen). Dead cells were removed by density centrifugation over Ficoll (Sigma-Aldrich), and culture was continued for 2 days further before analysis or use of the T cells.

The additional 2 days in culture allowed the cells to return to a resting state [23]. The resulting effector cells express levels of L-selectin and CD45RB comparable with naïve CD4 cells and not memory cells, low CD25 expression (data not shown) [23], indicating a resting state, and increased levels of CD44, showing that they are activated cells. The return to a resting state is important, as resting cells have been shown to actively traffic to a much greater extent than fully activated cells [23, 24]. These cells are thought to resemble mature effector T cells in vivo, which re-enter the circulation after a sessile, proliferative phase [24].

For cell tracking by SLO or confocal microscopy, the polarized population was labeled by incubation at 2 × 10^6 cells/ml with 40 μg/ml calcein-acetoxyethyl (AM) at 37°C for 30 min. Cells were washed three times and adjusted to 1 × 10^5 in 150 μl. For chemokine receptor-blocking studies, labeled cells were incubated for 1 h at 37°C with neutralizing antibody to CCR5 [25] or CCR9 [26] at 2.5 μg/ml prior to tail vein injection.

Adaptive transfer of cells

Resting antigen-specific, Th1-polarized cells (1 × 10^5) were injected via the tail vein into normal B10R111 mice to check that these cells were able to cause EAU. Control cells, cultured with IL-4 and anti-IFN-γ but with IL-4 (10 ng/ml) and anti-IL-4 were cultured for 3 days with 50 μg/ml IRBP peptide and IL-12 (5 ng/ml), IFN-γ (20 ng/ml), and anti-IL-4 (1 μg/ml, clone 11B1), all from BD Biosciences PharMingen (Oxford, UK). Control cells were not cultured with IFN-γ and anti-IL-4 but with IL-10 (10 ng/ml) and anti-IFN-γ (2 μg/ml, clone XMG1.2, BD Biosciences PharMingen). Dead cells were removed by density centrifugation over Ficoll (Sigma-Aldrich), and culture was continued for 2 days further before analysis or use of the T cells.

Flow cytometry

Intracellular staining of T cell-polarized subsets for IFN-γ and IL-4 was performed after restimulation of resting cells on a plate precoated with 2 μg/ml anti-CD3 overnight. Stained cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences PharMingen).

Cell surface expression of chemokine receptors on resting cells was detected by flow cytometric analysis. T cells were stained with specific antibodies to mouse CCR2 [25], CCR3 (BD Biosciences PharMingen), CCR4 (Alexis Corporation, Nottingham, UK), CCR5 [25], CCR9 [26], and CXCR3 [kind gift of Prof. Joshua Farber, National Institutes of Health (NIH), Bethesda, MD] and their isotype controls (BD Biosciences PharMingen) in 1% (w/v) bovine serum albumin/phosphate-buffered saline (PBS) at 4°C for 20 min. For comparison, CCR5 and CXCR3 were also examined on activated cells after initial polarization for 3 days, prior to allowing the cells to rest for 2 days. Flow cytometry was performed on a FACSCalibur (BD Biosciences PharMingen) and analyzed using CellQuest software (BD Biosciences PharMingen).

Analysis of chemokine mRNA expression in the retina

Retinas were dissected from the eyes of naïve animals and those immunized with IRBP peptide following perfusion with 30 ml PBS containing 10 U/ml...
heparin under terminal anesthesia. Retinas from the same animal were pooled, and RNA was isolated using RNA-Be (Biogenesis Ltd., Poole, Dorset, UK) according to the manufacturer’s protocol.

Poly A+ RNA from 3 μg total RNA was reverse-transcribed with 200 U Moloney murine leukemia virus reverse transcriptase (RT; Promega UK, Southampton). This cDNA (1 μL) was used in the polymerase chain reaction (PCR), and each PCR was carried out in a total volume of 25 μL containing 12.5 μL master mix (Promega UK) and 2.5 μL primer (10 μM) mix. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [28], MIP-1α, MIP-1β, and RANTES primers [29] were obtained from ThermoHybaid (Ulm, Germany). Primers were intron-spanning to allow discrimination of any genomic DNA.

Thirty-three cycles of amplification were performed, each cycle consisting of a denaturation step at 94°C for 50 s, annealing at 55°C for 1 min, and polymerization at 72°C for 1 min, 30 s. In the first cycle denaturation was carried out for 2 min, and in the final cycle, polymerization was for 5 min. After polymerization, samples were run on a 1.8% agarose gel (molecular biology grade, Promega UK) in Tris-borate-EDTA buffer (0.045M Tris-borate, 0.05% [v/v] ethidium bromide. Relative abundance of product was assessed by calculating the ratios of the cytokine band to the GAPDH band for each sample using the GeneGenius software (Syngene, Cambridge, UK).

**SLO and image analysis**

Mice with EAU, 9 days after immunization with peptide, were anaesthetized, pupils dilated, and contact lenses fitted as described [20]. Sodium fluorescein [100 μL 0.05% (v/v), Sigma-Aldrich Co., Ltd. (Poole, UK)] was injected via the tail vein, followed by 1 × 107 calcein-AM (Molecular Probes Europe BV, Leiden, The Netherlands)-labeled resting cells in 150 μL complete medium. SLO images were recorded simultaneously on videotape (S-VHS) and digitally at 25 frames/s, as described previously [20]. For each eye, three regions of interest containing one to three veins/venules were recorded for at least 30 min after cell injection. Video analysis was carried out offline as described elsewhere [30]. Rolling leukocytes and those not interacting with the endothelium were counted in each venule. Rolling cells were defined as those cells with a velocity below the critical velocity [5, 31, 32]. The rolling efficiency was calculated at 2 min-intervals as the percentage of labeled rolling cells among the total number of labeled cells that entered a venule. The sticking efficiency was determined as the percentage of labeled cells becoming firmly adherent for at least 20 s compared with the total number of labeled cells that rolled in a venule during the same time interval. Rolling velocities of 40 randomly chosen, rolling, labeled cells in retinal venules were measured in digital images [20].

**Retinal wholemounts for confocal microscopy**

Fifty minutes after injection of labeled resting cells and SLO, the anaesthetized mice were injected via the tail vein with 100 μL Evans Blue solution [2% (w/v) in PBS, Sigma-Aldrich Co., Ltd.] After 10 min, animals were killed by CO2 inhalation, and eyes were immersed in 2% (w/v) paraformaldehyde for 1 h. Retinas were removed, washed twice in PBS for 15 min, spread on clean glass slides, and mounted vitreous-side up [33]. Mounts were examined using a confocal laser-scanning microscope, LSM 510 (Carl Zeiss, Gotingen, Germany).

**Immunohistochemistry**

Eyes were frozen, and cryostat sections were cut, fixed, and stained as described previously [15, 34]. Briefly, sections were incubated with primary antibody, rat monoclonal antibody immunoglobulin G (IgG2b) to mouse CCR5 [25] at 30 μg/ml at 4°C for 1 h at room temperature. Isotype control antibody was rat IgG2b (Serotec Ltd., Oxford, UK). Sections were then incubated for 30 min each, first with biotinylated rabbit anti-rat antibody and second, with streptavidin, biotinylated alkaline phosphatase complex (both Dako Ltd., Cambridge, UK) before addition of Fast Red substrate solution.

Sections were prepared in the same way for confocal microscopy and stained dually for CCR5 and CD3 with antibody to mouse CCR5 as above, detected using fluorescein isothiocyanate (FITC)-conjugated antibody to rat Ig (1:100, Serotec Ltd., Oxford, UK). Sections were then incubated for 30 min each, first with biotinylated rabbit anti-rat antibody and second, with streptavidin, biotinylated alkaline phosphatase complex (both Dako Ltd., Cambridge, UK) before addition of Fast Red substrate solution.

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**Data analysis**

Probability values were calculated using Dunnett’s multiple comparison test or Student’s unpaired two-tailed t-test. Probability values of P < 0.05 were considered significant.

**RESULTS**

**Analysis of Th1-polarized antigen-specific lymphocytes**

Intracellular staining for IFN-γ and IL-4 in antigen-specific T cells cultured in Th1-polarizing conditions was performed after overnight restimulation of resting cells with anti-CD3 as described previously [5]. Flow cytometric analysis showed that the majority of the cells, when stimulated, produced IFN-γ, and few produced IL-4 (Fig. 1). Adoptive transfer of resting Th1-like cells into normal mice confirmed that they induced uveitis. Anterior and posterior uveitis was evident with detection of clinical signs of anterior uveitis as early as 3 days post-transfer (data not shown). Examination of H&E-stained sections 7 days post-transfer revealed that when 1 × 107 cells were transferred, all animals exhibited signs of disease with inflammatory cells infiltrating the rod outer segments, the nuclear layers, and the vitreous, vasculitis, and areas of retinal detachment (Fig. 2a). The mean infiltrative grade was 3 using a histological cellular infiltration grading system (maximal grade 6) [35]. Adoptive transfer of control cells, which had been cultured with Th2-polarizing cytokines [5], did not show any clinical or histological signs of disease, even when the number of transferred cells was increased to 2 × 107 (Fig. 2b).

Flow cytometric analysis of chemokine receptor expression by the resting Th1-polarized population showed that CCR5, CXCR3, and CCR3 were strongly expressed with little CCR2, CCR4, or CCR8 expression (Fig. 3). A comparison of the expression of CCR5 and CXCR3 on CD4+ cells before and after rest showed that CCR5 on 6.2% (SD 0.9) and CXCR3 on 37.3% (SD 7.4) of cells before resting and CCR5 on 84.2% (SD 0.5) and CXCR3 on 57.9% (SD 6.4) cells after resting.

**CCR5 expression in EAU**

We have shown previously that clinical signs of disease appear from Day 9 post-immunization (p.i.), with peak disease at approximately Day 12, and that active disease lasts for 2–3 weeks [36]. Histological examination has shown that at Day 9 p.i., inflammatory cell infiltration and perivasculitis are present, and infiltrating cells are distributed in the ganglion layer and vitreous. Most of the photoreceptor layer remains normal at this time, but there is some focal photoreceptor cell damage [36]. Cryostat sections from animals at Day 10 p.i. were stained using antibodies to CCR5 and showed CCR5 expression on infiltrating cells throughout the retina and within the vitreous (Fig. 4, a and b). The percentage of infiltrating...
cells within the vitreous expressing CCR5 was $44.01 \pm 9.21$ (SD). CCR5 expression was not detected where there was no inflammatory cell infiltrate (data not shown). Dual fluorescent staining of sections showed that the majority of infiltrating CD3-positive cells was also CCR5-positive (Fig. 4, c and d). Although the majority of CCR5-positive cells was also positive for CXCR3, some cells expressed CCR5 only (Fig. 4, e and f).

**MIP-1α,β and RANTES expression in perfused retina**

To determine the relevance of CCR5 expression on retinal-antigen-specific, Th1-polarized T cells, the expression of mRNA for the CCR5 ligands, MIP-1α, MIP-1β (CCL4), and RANTES, in perfused retina was determined by routine PCR. In normal retina from animals that had been immunized with PBS and CFA without IRBP-peptide, low levels of RANTES and MIP-1α mRNA could be detected and no MIP-1β. However, mRNA for each of these chemokines was strongly expressed in retinas from animals with EAU at Day 10 or Day 13 p.i. (Fig. 5).

**Effect of treatment with anti-CCR5 on in vivo cell trafficking of Th1-polarized cells**

The rolling velocity and rolling efficiency of injected, labeled, Th1-polarized cells in retinal venules were examined by SLO in recipient animals with EAU 9 days after immunization with peptide. Neutralizing antibody to CCR5 had no significant effect ($P>0.05$) on either of these parameters (Fig. 6). Sticking efficiency, the percentage of rolling cells that became firmly adherent, also remained unchanged ($P>0.05$), $11.1\% \pm 2.2$ (SE) without antibody and $12.5\% \pm 6.2$ (SE) with anti-CCR5 treatment.

**Effect of treatment with anti-CCR5 on infiltration of cells into the retina**

The infiltration of labeled, Th1-polarized cells into the retina of animals with EAU (9 days after immunization), 1 h after introduction of the cells via the tail vein, was examined by confocal microscopy of retinal wholemounts. When the labeled cells were incubated prior to injection with neutralizing antibody to CCR5, there was a significant decrease ($P=0.006$) in the number of cells that infiltrated compared with untreated cells (Fig. 7). However, when these cells were treated in the same way with anti-CCR8, there was no decrease ($P=0.8$) in the number of cells that infiltrated the retina (data not shown). Both of these antibodies have been shown in previous studies to have neutralizing activity [25, 26]. Flow cytometric analysis of cell surface-bound antibody at 1, 2, 4, and 24 h after antibody incubation showed that antibody remained bound for
4 h but had been lost at 24 h (data not shown), indicating that neutralization was effective over the course of the experiment.

DISCUSSION

We show that there is marked expression of CCR5 on mononuclear cell infiltrates present in the inflamed retina in EAU. This suggests that CCR5 has a role in the infiltration of these cells into the retina and may be involved in their recruitment from the circulation and possibly their interaction with the endothelium. Although CCR5 has previously been shown to be expressed in the inflamed eye in EAU, these studies examined mRNA from enucleated eyes and did not define the location of this expression, the cells with which it is associated, or whether it is reflected in protein expression at the cell surface [37, 38].

CCR5 has recently been shown to be as important for T cell recruitment to cutaneous sites as CXCR3, but this could only be demonstrated in vivo [18]. In this study, we also use an in vivo system to demonstrate that CCR5 plays a role in the recruitment of antigen-specific Th1-polarized cells across the specialized BRB and into the retina during an inflammatory reaction. However, the function of CCR5 appears to be confined to the transendothelial migration of T cells into the inflamed retina and has no part to play in tethering, rolling, or sticking to the endothelium. This contrasts with previous studies of neutrophil recruitment in vivo, where CC chemokines have been shown to be involved in tethering and rolling of neutrophils via selectins and integrins [39, 40] as well as their firm adherence [41].

These findings that CCR5 may be more important for infiltration of the Th1-polarized cells rather than their rolling behavior at the BRB is supported by an in vitro study, which has shown that CCR5 is more important for spreading and polarization under flow conditions than initial arrest but that blocking CCR5 action inhibits transendothelial chemotaxis [42]. CCR1 was important for arrest of cells whether they had high levels of CCR1 and low CCR5 (as for the monocytes) or whether (as for the T cells) they had low CCR1 and high CCR5 [42]. This led to the suggestion that chemokine receptors, rather than being redundant, have specialized roles in distinct steps of leukocyte trafficking. However, these data were generated using in vitro assays of cell adhesion or transmigration across human microvascular endothelial cells in response to RANTES or MIP-1β. Our results provide the first evidence that this selective role for chemokines in the process of transendothelial migration also occurs in vivo. In addition, it has been shown that CCR5 is recycled after internalization following chemokine interaction, whereas CCR1 is not, suggesting a more prolonged role for CCR5 on the Th1 cell as the cell migrates into the tissue rather than simply at the stage of recruitment from the circulation [42].

Our finding that CCR5 has no role in the rolling and adherence of Th1-like cells on the retinal endothelium suggests that the CCR5 ligands MIP-1α, MIP-1β, and RANTES are not involved in the rolling of these cells at this site through this

Fig. 3. Flow cytometry analysis of chemokine receptor expression on resting Th1-polarized cells, which were stained with specific antibodies to mouse chemokine receptors and their isotype controls at 4°C for 20 min. Histograms show expression of CCR2, CCR5, CCR3, CCR8, CCR4, and CXCR3. The x-axis is mean fluorescent intensity, and the y-axis is cell number. The shaded histograms are the isotype control readings. Data shown are representative of three independent experiments.
receptor. However, we have previously shown by tracking labeled splenocytes in mice with EAU that MIP-1α/H9251 is directly involved in the interaction of leukocytes with the endothelium at the BRB. Reduction in leukocyte velocity, seen at sites of infiltration in EAU, was inhibited by anti-MIP-1α/H9251, and the number of rolling leukocytes was much less [15]. It is likely, therefore, that MIP-1α may affect Th1 cell rolling through its other receptor CCR1. MIP-1α may thus have dual functions in this model: to enhance P-selectin/PSGL-1 and LFA-1/ICAM-1 interactions in early adhesion via CCR1 and to mediate migration through the endothelium via CCR5.

In addition to MIP-1α, the other ligands for CCR5, RANTES, and MIP-1β are present in the retina as EAU reaches its peak of severity. Anti-CCR5 treatment will abrogate the response of the Th1-polarized cells via CCR5 to each of these chemokines. Whether one of these ligands in particular is important for the CCR5 response of these cells or whether the combination of these chemokines [43] is fundamental to their recruitment is not known. A recent study showed that neutralization of RANTES, as leukocytes started to accumulate in the eye during EAU, led to a reduction in the Th1-type response, although disease was exacerbated, possibly as a result of a reduction in the number of CD8⁺ T cells [44]. CCR5 may also enhance Th1 cell recruitment by binding CD4 and facilitating binding and signaling of the T cell chemoattractant, IL-16 [19]. Up-regulation of IL-16 mRNA has been reported in the retina in EAU [38].

We and others have shown that CXCR3 is strongly expressed on Th1 cells and is also likely to be important for Th1 cell recruitment into the retina [38, 45]. The CXCR3 ligands, IP-10 and monokine induced by IFN-γ (CXCL9), are present at high levels in the eye during EAU [37, 38]. In vivo, IP-10 and MIP-1α had comparable T cell recruiting activities to cutaneous sites [18]. A study of T cells from the peripheral blood of multiple sclerosis patients showed that CCR5 but not CXCR3 was up-regulated compared with normal controls [46].
This accounted for the increased migration of Th1 cells to RANTES and MIP-1α in vitro, indicating that CCR5 may be more important than CXCR3 for Th1 cell recruitment in some disease situations.

In our study, the blocking of CCR5 only resulted in a 31% inhibition of Th1 cell infiltration into the retina, suggesting that CXCR3 ligands may also contribute to transmigration. We have shown that the majority of infiltrating cells expressing CCR5 also expresses CXCR3, suggesting that these two receptors, like CCR5 and CCR1, may also affect different aspects of Th1 cell migration. Cells are known to respond to a combination of chemotactic signals to reach the appropriate site [43]. Some infiltrating cells were identified, which expressed CCR5 only, but it is possible that these cells have lost CXCR3 once in the retina [45].

Comparison of resting and activated cells showed an increase in CCR5 and CXCR3 expression once cells had been allowed to return to a resting state. This is consistent with the increased ability of resting cells to migrate [23, 24], which we have also observed in the retina (data not shown). In a study about the ability of adoptively transferred T cell receptor transgenic Th1 cells to cause intraocular inflammation, CCR5 mRNA expression was shown to be increased in activated cells and remain high through resting and reactivation, although cell surface expression was not examined [45]. CXCR3 was increased at the resting stage, as in our study, but this did not correlate with the ability of these cells to cause disease [45]. Differences in the findings of the two studies indicate that variation in the mode of induction of the Th1 cells and in disease development may alter chemokine receptor expression, possibly via cytokines. Studies are planned to determine the role of CXCR3 in the preferential recruitment of Th1 cells using this model system and are awaiting the availability of a neutralizing anti-mouse CXCR3 antibody.

In conclusion, this study shows the importance of the chemokine receptor CCR5 in the recruitment of Th1-type cells
across the BRB and demonstrates for the first time in vivo that CCR5 may be more important for infiltration of cells across the BRB rather than for rolling on the retinal vasculature in the initial stages of recruitment.

ACKNOWLEDGMENTS

These studies were funded by The Wellcome Trust, Grant 057311. I. J. C. and H. X. contributed equally to this study.

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