Abnormal immune response of CCR5-deficient mice to ocular infection with herpes simplex virus type 1

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Ocular herpes simplex virus type 1 (HSV-1) infection elicits a strong inflammatory response that is associated with production of the \( \beta \) chemokines CCL3 and CCL5, which share a common receptor, CCR5. To gain insight into the role of these molecules in ocular immune responses, the corneas of wild-type (WT) and CCR5-deficient (CCR5\( \cdot \cdot \cdot \) mice were infected with HSV-1 and inflammatory parameters were measured. In the absence of CCR5, the early infiltration of neutrophils into the cornea was diminished. Associated with this aberrant leukocyte recruitment, neutrophils in CCR5\( \cdot \cdot \cdot \) mice were restricted to the stroma, whereas in WT mice, these cells trafficked to the stroma and epithelial layers of the infected cornea. Virus titres and cytokine/chemokine levels in the infected tissue of these mice were similar for the first 5 days after infection. However, by day 7 post-infection, the CCR5\( \cdot \cdot \cdot \) mice showed a significant elevation in the chemokines CCL2, CCL5, CXCL9 and CXCL10 in the trigeminal ganglion and brainstem, as well as a significant increase in virus burden. The increase in chemokine expression was associated with an increase in the infiltration of CD4 and/or CD8 T cells into the trigeminal ganglion and brainstem of CCR5\( \cdot \cdot \cdot \) mice. Surprisingly, even though infected CCR5\( \cdot \cdot \cdot \) mice were less efficient at controlling the progression of virus replication, there was no difference in mortality. These results suggest that, although CCR5 plays a role in regulating leukocyte trafficking and control of virus burden, compensatory mechanisms are involved in preventing mortality following HSV-1 infection.

INTRODUCTION

Both innate and adaptive immunity are involved in controlling the replication and spread of herpes simplex virus type 1 (HSV-1) following corneal infection in mice. Initially, neutrophils are recruited to the infected site by the local production of chemotactic cytokines and the expression of adhesion molecules, including intercellular adhesion molecule 1 and platelet endothelial cell adhesion molecule 1 (Su \textit{et al}., 1996; Tang & Hendricks, 1996; Thomas \textit{et al}., 1997). Although the recruitment of neutrophils is protective, as shown by an increase in virus titre in the absence of these cells (Tumpey \textit{et al}., 1996), soluble mediators, including matrix metalloproteinase 9, nitric oxide, vascular endothelial growth factor, tumour necrosis factor alpha (TNF-\( \alpha \)) and interleukin 1 (IL-1), secreted by neutrophils or resident cells contribute to the pathology in the cornea (Zheng \textit{et al}., 2001; Lee \textit{et al}., 2002; Biswas \textit{et al}., 2004). In addition, HSV-1 DNA contains CpG motifs that are immunostimulatory and have been found to induce angiogenesis, thus promoting neovascularization in the normally avascular cornea (Zheng \textit{et al}., 2002; Lundberg \textit{et al}., 2003). Therefore, not only is virus clearance required for a successful outcome favouring the host, but controlling the pro-inflammatory response is essential in preserving the visual axis.

Pro-inflammatory cytokines (e.g. TNF-\( \alpha \), IL-1 and IL-6) produced in response to HSV-1 infection (He \textit{et al}., 1999) activate integrin expression (Laudanna \textit{et al}., 2002) and the production of chemokines (Rollins, 1997; Luster, 1998). Within the cornea, the chemokines macrophage inflammatory protein (MIP)-1\( \alpha \) (CCL3) and MIP-2\( \alpha \) (CXCL1) have been implicated in the pathogenic outcome of HSV-1 infection, herpetic stromal keratitis (Tumpey \textit{et al}., 1998a; Banerjee \textit{et al}., 2004). However, the expression of these chemokines is delayed relative to the production of gamma interferon (IFN-\( \gamma \))-inducible protein 10 (IP-10, CXCL10), a chemokine expressed constitutively in the cornea and upregulated in response to HSV-1 infection (Su...
et al., 1996; Carr et al., 2003). CXCL10 may function as an ocular sentinel chemokine that regulates the initial inflammatory response following HSV-1 infection (Carr et al., 2003; Wickham et al., 2004). Neutralization of CXCL10 dramatically reduces cellular infiltration into the cornea and suppresses the expression of CCL3, as well as RANTES (CCL5) (Carr et al., 2003; Wickham et al., 2004). HSV-1 infection of peritoneal cells and primary splenic and trigeminal ganglion (TG) cell cultures leads to the production of CCL5 (Melchjorsen et al., 2002; D. J. J. Carr, unpublished observation). However, the role of CCL5 in the inflammatory cascade following HSV-1 infection remains unclear.

CCL5 induces the migration of a variety of leukocyte types, including T cells, monocytes, dendritic cells and natural killer cells, by binding to the chemokine receptors CCR1, CCR3 and CCR5 on these cells (Zlotnik & Yoshie, 2000; Appay & Rowland-Jones, 2001). CCL5 shares the CCR5 receptor with CCL3 and, because CCR5 is expressed on T cells (Mack et al., 2001), a cell type reported to be involved in protection against HSV-1 replication and spread after corneal infection (Ghiassi et al., 2000), the effects that CCR5 deficiency in mice might have on the course and outcome of corneal HSV-1 infection were investigated.

METHODS

Virus and cells. African green monkey kidney fibroblasts (Vero cells, ATCC CCL–81, from the ATCC) were propagated in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), gentamicin (Invitrogen) and antibiotic/antimycotic solution (Invitrogen) at 37 °C in 5% CO2 and 95% humidity. HSV-1 stocks (McKrae strain) were propagated in Vero cells as described by Harland & Brown (1998). Stocks were stored at 80 °C for 1 min and were washed 3 times at room temperature, corneas were removed with PBS containing 0.5 ml of 4% paraformaldehyde (Sigma) in PBS. Following 15 min incubation at room temperature, corneas were removed from the eyes, placed in 0.5 ml 4% paraformaldehyde and incubated overnight at room temperature. The following day, corneas were washed 3 times with 1:0 ml PBS containing 1% Triton X-100 (Sigma) for 10 min. After the last wash, corneas were incubated with PBS containing 10% horse serum for 60 min at room temperature. Next, the corneas were incubated with 1–2 μg fluorescein isothiocyanate (FITC)-conjugated anti-HSV-1 antibody (Dako), FITC-conjugated anti-Ly-6G/6C (Gr-1) (BD Pharmingen), phycoerythrin (PE)-conjugated anti-Mac-3 (BD Pharmingen), FITC-conjugated anti-CD3 (BD Pharmingen) and/or Alexa fluor 546-conjugated anti-CCL5 (Lane et al., 2000) antibodies in 100 μl PBS for 180 min at 37 °C in the dark. After the incubation period, the antibody-containing solution was removed and the corneas were washed with 3 × PBS containing 1% Triton X-100. Next, 50 μl mounting medium containing DAPI (4,6-diamidino-2-phenylindole; Vector Laboratories) was added to each sample and samples were incubated at 4 °C overnight in the dark. The following day, corneas were placed onto coverslips and an incision was made in each cornea, encompassing 50% of the tissue (to facilitate the flattening of the cornea onto the slide). Slides were placed on top of the coverslips, removing all air pockets, and they were kept at 4 °C in the dark until analysis by confocal microscopy. Alexa fluor 546-conjugated control IgG from normal serum and FITC-conjugated mouse IgG2a antibody were used as isotypic controls. Control and anti-CCL5 antibody was labelled with Alexa fluor 546 according to the manufacturer’s suggestions (Molecular Probes). One experiment, 500 μg recombinant CCL5 or CCL2 was pre-incubated with the Alexa fluor 546-conjugated anti-CCL5 antibody prior to addition to the HSV-1-infected cornea samples to show specificity of the anti-CCL5 antibody. For CD3 T-cell detection, corneas from mice 7 days p.i. were dissected and fixed for 30 min in

Spleen and lymph node cell cultures. Cells from the spleen, CLN, mesenteric lymph nodes (MLN) and iliac/inguinal lymph nodes (ILN) were removed from WT and CCR5–/– mice 7 days p.i. Cells from the spleen and lymph nodes were crushed through a 70 μm cell strainer (BD Biosciences) and the strainer was flushed with 5-0 ml RPMI 1640 medium containing 10% FBS. Red blood cells were lysed with 0.84% NH4Cl. Cells were then counted by using trypan blue and plated onto 24-well microtitre plates (Greiner Bio-One) at a concentration of 1-5 × 10⁶ cells per well. Cells were stimulated with heat-inactivated HSV-1 (m.o.i. of 0-5). Following a 5 day incubation period, the supernatant was collected and assayed for IL-6, IL-10, IL-12p70, IFN-γ and CCL5 using a Bio-Plex suspension array system and murine cytokine 5-plex assay (Bio-Rad). The sensitivity of the array system was 2 pg per tissue for each of the targeted analytes.

ELISA for measuring cytokine/chemokine levels in infected tissue. Detection of IFN/monocyte chemotactic protein 1 (MCP-1, CCL2), CCL3, CCL5, monokine-induced by IFN-γ (MIG, CXCL9), CXCL10 and IFN-γ in HSV-1-infected and uninfected tissue was performed by using commercially available kits (R&D Systems) according to the manufacturer’s instructions. The sensitivity for the detection of the chemokines/ cytokines ranged from 4-9 to 15-0 pg per tissue. Each sample was assayed in duplicate along with a standard provided in the kit to generate a standard curve used to determine the amount of targeted cytokine/chemokine. Standard curves did not fall below a correlation coefficient of 0-9950.

Whole-mount preparation. Eyes were removed from infected and uninfected mice and placed into 1-5 ml microcentrifuge tubes containing 0-5 ml 4% paraformaldehyde (Sigma) in PBS. Following 15 min incubation at room temperature, corneas were removed from the eyes, placed in 0-5 ml 4% paraformaldehyde and incubated overnight at room temperature. The following day, corneas were washed 3 times with 1:0 ml PBS containing 1% Triton X-100 (Sigma) for 10 min. After the last wash, corneas were incubated with PBS containing 10% horse serum for 60 min at room temperature. Next, the corneas were incubated with 1–2 μg fluorescein isothiocyanate (FITC)-conjugated anti-HSV-1 antibody (Dako), FITC-conjugated anti-Ly-6G/6C (Gr-1) (BD Pharmingen), phycoerythrin (PE)-conjugated anti-Mac-3 (BD Pharmingen), FITC-conjugated anti-CD3 (BD Pharmingen) and/or Alexa fluor 546-conjugated anti-CCL5 (Lane et al., 2000) antibodies in 100 μl PBS for 180 min at 37 °C in the dark. After the incubation period, the antibody-containing solution was removed and the corneas were washed with 3 × PBS containing 1% Triton X-100. Next, 50 μl mounting medium containing DAPI (4,6-diamidino-2-phenylindole; Vector Laboratories) was added to each sample and samples were incubated at 4 °C overnight in the dark. The following day, corneas were placed onto coverslips and an incision was made in each cornea, encompassing 50% of the tissue (to facilitate the flattening of the cornea onto the slide). Slides were placed on top of the coverslips, removing all air pockets, and they were kept at 4 °C in the dark until analysis by confocal microscopy. Alexa fluor 546-conjugated control IgG from normal serum and FITC-conjugated mouse IgG2a antibody were used as isotypic controls. Control and anti-CCL5 antibody was labelled with Alexa fluor 546 according to the manufacturer’s suggestions (Molecular Probes). One experiment, 500 μg recombinant CCL5 or CCL2 was pre-incubated with the Alexa fluor 546-conjugated anti-CCL5 antibody prior to addition to the HSV-1-infected cornea samples to show specificity of the anti-CCL5 antibody. For CD3 T-cell detection, corneas from mice 7 days p.i. were dissected and fixed for 30 min in
4% paraformaldehyde, then washed five times with PBS. Corneas were then blocked for 2 h with 1:100 FcBlock (CD16; BD Pharmingen) in PBS-BGEN (3% BSA, 0.25% gelatin, 0.025% Nonidet-P40 and 5 mM EDTA) with 5% rat serum and subsequently incubated overnight at 4°C with 100 μl 1:40 FITC-conjugated anti-CD3 (BD Pharmingen) in PBS-BGEN. Corneas were then washed five times with PBS, fixed with 1% paraformaldehyde for 30 min at 4°C and rinsed five times with PBS. The corneas were then soaked overnight in Vectashield mounting medium containing DAPI, then mounted on slides for subsequent analysis by confocal microscopy.

Confocal microscopy. Corneas (n=2 per group per time point) were imaged by using an Olympus IX81-FV500 epifluorescence/confocal laser-scanning microscope with a UApo 40× water immersion lens. Samples were excited with 405, 488 and 546 nm wavelength lasers. Scanning images were taken with a step size of 2 μm in the z axis and image analysis was performed by using FLOWVIEW software (Olympus). CD3 + -stained cells were enumerated from 10 corneas per group of mice, consisting of central and peripheral sections.

Cornea- and TG-cell suspensions. For CD11b + cell staining at 3 and 7 days p.i., anaesthetized mice were perfused with PBS and corneas and TG were removed and incubated with collagenase type I solution (3 mg ml -1; Sigma) in PBS at 37°C. Every 20 min for 60 min, the tissue was triturated using a p1000 Pipetman (Gilson). Following the incubation period, the digested tissue was washed through a 70 μm cell strainer (BD Biosciences) and the cell strainer was flushed with 5 ml RPMI 1640 medium supplemented with 10% FBS. The resultant cells were washed twice with PBS containing 1% BSA and counted by using trypan blue. For CD4 and CD8 T-cell infiltration into the TG, the TG were removed from perfused mice on day 6 p.i. and subjected to homogenization using a Dounce homogenizer. Following homogenization, samples were passed through the 70 μm cell strainer, which was then flushed with 5 ml RPMI 1640 medium supplemented with 10% FBS.

Flow cytometry. Single-cell suspensions of CLN (inferior and superior), MLN, ILN, spleen, cornea and TG were prepared and placed in 5 ml polystyrene round-bottom tubes (Becton Dickinson). CLN cells (5×10 6 cells per tube) or cell suspensions of cornea or TG were incubated with anti-mouse CD16/32 (Fcγ II/III receptor; 2.4G2) (BD Pharmingen) for 20 min at 4°C. Following the incubation period, cells were washed twice in 1 ml PBS containing 1% BSA (4°C) and labelled with 1–2 μg of the following antibodies obtained from BD Pharmingen: FITC- or PE-conjugated anti-CD3 (clone 17A2), FITC-conjugated anti-CD4 (clone RM4-5), FITC-conjugated anti-CD8α (clone 53-6.7), FITC-conjugated anti-CD69 (clone H1.2F3), PE-conjugated anti-CD25 (clone 3C7) and/or PE-conjugated anti-CD11b (clone M1/70). To determine anti-HSV-1-specific CD8 + T cells, CLN cells were incubated with FITC-conjugated HSV-1 glycoprotein B F98-503 (H-2K b peptide; SSIEFARL; Core Facility, Baylor College of Medicine, Houston, TX, USA) and PE-conjugated anti-CD8. After labelling with the antibody or antibody combinations, cells were incubated for 30 min at 4°C in the dark. After the incubation period, cells were washed twice in 1 ml PBS containing 1% BSA (4°C) and resuspended in 1% paraformaldehyde. In the case of analysis of TG or brainstem samples for T-cell infiltration, cells were triple-labelled with PE-conjugated anti-CD3, FITC-conjugated anti-CD4 or -CD8, and PE-Cy5-conjugated anti-CD45 (clone 30-F11; BD Pharmingen). Cells were assayed on a FACSCalibur instrument (Becton Dickinson) using WinMDI data-analysis software (J. Trotter, The Scripps Research Institute, La Jolla, CA, USA). For TG and brainstem T-cell infiltration, cells were gated on the CD45 + population and percentages of CD4 and CD8 T cells were determined by using this gate setting. Isotypic-control antibodies were included in the analysis to establish background fluorescence levels.

Adoptive transfer. One million CLN cells obtained from C57BL/6 or CCR5 −/− mice infected with HSV-1 (1000 p.f.u. per eye) 6 days previously were introduced intravenously into CCR5 −/− mice on day 3 p.i. in a volume of 50 μl. At day 7 p.i., recipient CCR5 −/− mice were anaesthetized and perfused with PBS. Following perfusion, corneas, TG and brainstems were removed, homogenized and assessed for virus yields by plaque assay.

Statistics. One-way ANOVA and Tukey’s post hoc t-test were used to determine the significance (P<0.05) of differences between WT and CCR5 −/− groups for each parameter measured. However, survival studies were analysed by the non-parametric Mann–Whitney rank-order test. All statistical analyses were performed with the program GBSTAT (Dynamic Microsystems).

RESULTS

HSV-1 infection in CCR5 −/− mice

To determine whether CCR5 expression is involved in the host response against ocular HSV-1 infection, WT and CCR5 −/− mice were infected with 500 p.f.u. HSV-1 and assayed for virus titres in the cornea, TG and brainstem at various times p.i. At days 3 and 5 p.i., there was no significant difference in the quantity of virus recovered from infected tissues with the exception of the TG. Specifically, at day 5 p.i., the TG of CCR5 −/− mice contained 3722±10000 p.f.u., whereas WT mice yielded only 4275±1135 p.f.u. (P<0.05; n=9 per group). By day 7 p.i., virus titres were significantly elevated in the cornea, TG and brainstems of CCR5 −/− mice relative to WT controls (Fig. 1a). Surprisingly, even though CCR5 −/− mice possessed significantly more virus in the cornea and nervous system at day 7 p.i., mortality in these animals was not higher than in WT mice and even showed a delay (Fig. 1b).

CD11b + cell migration to the cornea and TG following HSV-1 infection

An acute inflammatory reaction characterized by infiltrating leukocytes predominantly composed of neutrophils has been reported in the cornea within the first 24 h following HSV-1 infection (Thomas et al., 1997). CD11b + macrophages and neutrophils have also been reported to infiltrate the TG within 72 h following ocular HSV-1 infection (Shimeld et al., 1995; Liu et al., 1996). As CCR5 deficiency has previously been reported to affect macrophage trafficking (Kuziel et al., 2003), we sought to determine whether the absence of CCR5 would alter leukocyte infiltration in the cornea during acute HSV-1 infection. At 72 h p.i., there was a significant reduction in the percentage of CD11b + cells in the cornea and TG of CCR5 −/− mice relative to WT cornea and TG (Fig. 1c). This difference was lost, however, at day 7 p.i., although both lines showed a higher percentage of CD11b + cells in the cornea and TG relative to the 72 h time point (Fig. 1d).
Levels of cytokines and chemokines in infected tissue

To further define differences between WT and CCR5−/− mice, levels of CCL2, CCL3, CCL5, CXCL9, CXCL10, IL-12 and IFN-γ were assessed by ELISA. Associated with the delayed infiltration of CD11b+ cells into the cornea of CCR5−/− mice at day 3 p.i., CXCL9 levels were reduced (4±4 pg per cornea) compared with WT control mice (21±7 pg per cornea). Within the TG of CCR5−/− mice, both CXCL9 and CXCL10 were reduced compared with WT controls at day 3 p.i. (Fig. 2a). However, no other differences in chemokine/cytokine levels were found between WT and CCR5−/− mouse cornea or TG samples at day 3 p.i. By day 7 p.i., changes in chemokine expression became more evident in the nervous system. Specifically, even though there were no changes in the level of cytokines or chemokines expressed in the cornea of CCR5−/− compared with WT mice, CCL2, CCL5, CXCL9 and CXCL10 levels were significantly higher in the TG of CCR5−/− mice compared with the WT controls at day 7 p.i. (Fig. 2b). Likewise, CCL2 and CCL5 levels were elevated in the brainstems of CCR5−/− mice compared with WT animals (Fig. 2c). In contrast to the above-mentioned chemokines, CCL3, IFN-γ and IL-12 levels were similar in CCR5−/− and WT mice in all tissues at day 7 p.i. (data not shown). Collectively, changes in the expression of selected chemokines in the nervous system were found to mirror virus loads recovered at day 7 p.i.

Fig. 1. Sensitivity of CCR5−/− mice to HSV-1 infection. C57BL/6 (WT) and CCR5−/− mice were infected with 500 p.f.u. HSV-1 per eye and evaluated for virus titre and the host response to infection. (a) At day 7 p.i., mice (n=11–15 per group) were euthanized and the cornea, TG and brainstem (BS) were removed by using a dissecting microscope, homogenized and assayed for infectious virus by plaque assay. Data are expressed as the mean p.f.u. (log10) ± SEM and are pooled from three to five experiments with n=3 or 4 mice per group per experiment. Filled bars, WT; empty bars, CCR5−/−. (b) Mice (n=23–25 per group) were monitored and recorded for survival over 30 days p.i. Mean values ±SEM are shown; data were pooled from three experiments with n=6–10 mice per group per experiment. The difference between survival of infected WT and CCR5−/− mice was significant on day 8 p.i. (P<0.05). Dashed line, C57BL6; solid line, CCR5−/−. (c, d) Mice (n=8–16 per group) were euthanized at day 3 (c) or day 7 (d) p.i. and the corneas and TG were removed and processed for composition of CD11b+ cells by flow cytometry. Percentages of the gated population ±SEM are shown. Empty bars, WT; filled bars, CCR5−/−. For all parts, *P<0.05 between WT and CCR5−/−.
CCL5 expression in the cornea

Although no differences were detected in the amount of CCL5 in the cornea of HSV-1-infected WT and CCR5\(^{-/-}\) mice, the results did not determine the location of expression relative to the influx of infiltrating leukocytes. Therefore, a whole-mount approach was undertaken to consider the three-dimensional aspect of CCL5 expression in the cornea over time between WT and CCR5\(^{-/-}\) mice.

Within 24 h p.i., CCL5 expression was detected within the epithelial layer and stroma of the cornea in both CCR5\(^{-/-}\) (data not shown) and WT (Fig. 3a) mice. CCL5 expression was co-localized with HSV-1 antigen within the epithelium, whereas other sites within the epithelial layer and stroma were found to express only CCL5, with no apparent viral antigen present.

Fig. 2. (a) CCR5\(^{-/-}\) mice express less CXCL9 and CXCL10 in the TG at day 3 p.i. C57BL/6 (WT) and CCR5\(^{-/-}\) mice (\(n=8\) mice per group for CXCL9 and \(n=15\) per group for CXCL10) were infected with 500 p.f.u. HSV-1 per eye. At day 3 p.i., mice were euthanized and TG were removed and assayed for the indicated chemokine or cytokine by ELISA. Mean values ± SEM are shown; data were pooled from two to four experiments with \(n=3\) or 4 mice per group per experiment. Chemokine/cytokine expression in the TG (b) and brainstem (c) of HSV-1-infected mice is shown. Mice (\(n=8–15\)/group) were infected with 500 HSV-1 p.f.u. per eye and euthanized 7 days p.i. TG and brainstems were removed, homogenized and assayed for cytokine/chemokine levels by ELISA. Mean values ± SEM are shown in pg per TG (b) and pg per brainstem (c) for each analyte taken from four or five mice per group per experiment. Filled bars, WT; empty bars, CCR5\(^{-/-}\). For all parts, **\(P<0.01\) and *\(P<0.05\) between WT and CCR5\(^{-/-}\).
but not CCL2 (Fig. 3b). Likewise, isotypic controls for anti-HSV-1 and anti-CCL5 antibodies showed no reactivity with infected (Fig. 3a) or uninfected (not shown) corneas.

Although a reduction in the infiltration of CD11b+ cells within the cornea was found previously during the early time frame of acute HSV-1 infection comparing CCR5−/− with WT mice as determined by flow cytometry (Fig. 1c, d), we also sought to determine whether there were differences in the distribution of cells infiltrating the cornea relative to CCL5 expression in the WT and CCR5−/− mice. During the course of the first 5 days p.i., two distinct differences were found in the presentation of infiltrating cells. Specifically, consistent with the flow-cytometry data measuring CD11b+ cells, there was a reduction in Gr-1+ /Mac-3− neutrophil recruitment in the stroma of CCR5−/− mice compared with WT mice, which was most noticeable at day 3 p.i. (Fig. 4). In addition, neutrophils from the WT mice were found to infiltrate the epithelial layer of the cornea by day 5 p.i., whereas the Gr-1+/Mac-3− cells from CCR5−/− mice were rarely found to infiltrate into this layer (Fig. 4). Both WT and CCR5−/− mice appeared to show equal distribution of CCL5 expression in the cornea, with co-localization of neutrophils and CCL5 expression most evident by day 5 p.i. (Fig. 4). However, it is worth noting the absence of co-localization of CCL5 with Gr-1+ cells in the epithelial layers of the cornea in CCR5−/− mice compared with WT mice at day 5 p.i.

As T cells express CCR5 (Mack et al., 2001) and are central in controlling HSV-1 spread (Ghiasi et al., 2000), CD3+ T-cell infiltration was evaluated in the cornea and nervous system, comparing HSV-1-infected WT and CCR5−/− groups. Within the cornea, there was a modest but insignificant increase in T-cell infiltration in the centre and a reduction in infiltrating T cells in the peripheral areas in WT mice compared with CCR5−/− animals (Fig. 5). In contrast to the cornea, there was a significant increase in the percentage of CD8 T cells residing in the TG and CD4 and CD8 T cells residing in the brainstems of HSV-1-infected CCR5−/− mice compared with the infected WT controls (Fig. 6).

**CLN lymphocytes from CCL5−/− mice have a muted response to virus antigen, but dampen virus replication in the brainstem following adoptive transfer**

As the initial adaptive immune response would develop in the draining lymph nodes, the CLN was evaluated for cell number and cell reactivity to virus antigen. Although the percentage of CD4+ and CD8+ T lymphocytes was similar in WT and CCR5−/− CLN, remarkably, the total number of cells recovered from the CCR5−/− lymph nodes was threefold higher than that found in WT mice (Fig. 7a). In response to heat-inactivated HSV-1, CLN cells from virus-infected CCR5−/− mice produced profoundly less IL-6, IL-10 and especially IFN-γ than CLN cells from WT mice.

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**Fig. 4. Reduction of Gr-1+ cell recruitment in CCR5−/− corneas following HSV-1 infection.** C57BL/6 WT and CCR5−/− mice were infected with 500 p.f.u. HSV-1 per eye. Mice were euthanized at the indicated time p.i. and inspected for Gr-1+ cell infiltration and CCL5 expression by confocal microscopy using FITC-conjugated anti-Gr-1+ antibody (green) and Alexa fluor 546-conjugated anti-CCL5 antibody (red). Note that the corneas from uninfected mice showed no detectable CCL5 expression or Gr-1+ cells. In separate experiments, Gr-1+ cells were found not to be Mac-3+. This figure is representative of two experiments for each time point for each group of mice evaluated. Bar, 20 µm.
CCL5 levels were not significantly different between CCR5<sup>−/−</sup> mice and WT controls (Fig. 7b). Likewise, similar levels of activation markers (CD25 and CD69) were expressed on the CLN T cells from WT and CCR5<sup>−/−</sup> mice (data not shown). These changes were specific for the CLN cells, as splenic-cell responses to HSV-1 antigen were similar between WT and CCR5<sup>−/−</sup> cells (Fig. 7b). Neither MLN nor ILN cells stimulated with HSV-1 antigen showed appreciable levels of cytokines generated (<50 pg ml<sup>−1</sup>). To further assess the functionality of the CLN cells from WT and CCR5<sup>−/−</sup> mice, an adoptive-transfer experiment was performed. Specifically, CLN cells from HSV-1-infected WT or CCR5<sup>−/−</sup> mice were inoculated into HSV-1-infected CCR5<sup>−/−</sup> mice and the recipient animals were then assessed for virus titre 4 days later. CCR5<sup>−/−</sup> recipients of CCR5<sup>−/−</sup> CLN cells showed a reduction in HSV-1 in the brainstem compared with recipients of WT CLN cells (Fig. 8). Noticeable changes in virus yields were not found in the

![Fig. 5. Aberrant T-cell recruitment into the cornea of CCR5<sup>−/−</sup> mice following ocular HSV-1 infection. C57BL/6 (WT) and CCR5<sup>−/−</sup> mice (n=5 mice per group) were infected with 500 p.f.u. HSV-1 per eye. Mice were euthanized 7 days p.i. and inspected for CD3<sup>+</sup> cell infiltration into the cornea by using FITC-conjugated anti-CD3<sup>+</sup> antibody (green) by confocal microscopy. Nuclear staining is represented by DAPI (blue). Arrows point to cells. Image represents a composite image of the cornea. Mean values ± SEM are given; n=10 per group. Bar, 16 μm.](http://vir.sgmjournals.org)

![Fig. 6. Increase in T-cell infiltration into the nervous system of CCR5<sup>−/−</sup> mice following HSV-1 infection. C57BL/6 (WT) and CCR5<sup>−/−</sup> mice (n=6 or 7 mice per group) were infected with 500 p.f.u. HSV-1 per eye. Mice were euthanized 7 days p.i. and inspected for CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> T-cell infiltration into the TG and brainstem (BS) by flow cytometry. (a) Representative result comparing CD8 T-cell infiltration in the TG and BS of WT and CCR5<sup>−/−</sup> mice. (b) Summary of the analysis. Mean values ± SEM are shown. Empty bars, WT; filled bars, CCR5<sup>−/−</sup>. *P<0.05 between WT and CCR5<sup>−/−</sup>.](http://vir.sgmjournals.org)
cornea or TG of recipient CCR5−/− animals (Fig. 8). Similar percentages of HSV-1-specific CD8+ T cells were found within the CLN population of WT (36 ± 5%) and CCR5−/− (34 ± 3 ± 4·2%) mice, indicating that the efficiency of virus clearance in the brainstem was not reflected by input HSV-1-specific CD8 effector T cells.

**DISCUSSION**

Based on the expression of chemokines previously reported to be present in HSV-1-infected corneas of mice, including CCL3 (Su et al., 1996) and CCL5 (Carr et al., 2003), it was hypothesized that eliminating CCR5 expression would have a significant impact on the capacity of the host to mount an inflammatory response to the virus. CCR5−/− mice exhibited reduced neutrophil infiltration in the cornea early after infection (i.e. day 3 p.i.). This reduction correlated with reduced CXCL9 levels, but not with levels of other chemokines. In fact, one candidate chemokine associated with Gr-1+ neutrophil chemotaxis, CCL3 (Wolpe et al., 1988), was not detectable in the cornea of HSV-1-infected WT or CCR5−/− mice until day 5 p.i. and the levels were not significantly different between the knockout and WT animals. Although in the present study it is difficult to

**Fig. 7.** Lack of response to recall antigen in CCR5−/− mice. C57BL/6 (WT) and CCR5−/− mice were infected with 500 p.f.u. HSV-1 per eye and euthanized 7 days p.i. CLN were removed and cells were counted by using trypan blue. (a) Total cell count for lymph nodes obtained from WT and CCR5−/− mice (n=15 per group) and the percentage of CD4+ and CD8+ T cells within the lymph-node population (n=10 per group). For left-hand side plot: filled bars, WT; empty bars, CCR5−/−. Mean values ±SEM are shown. **P<0·01 between WT and CCR5−/−. Dotted line, mean number of total leukocytes from uninfected CLN. (b) Cytokine/chemokine production by CLN or spleen cells (1·5 × 10^6 cells per well) obtained from mice 7 days p.i. and incubated with heat-inactivated HSV-1 (7·5 × 10^5 p.f.u.) for 5 days. Supernatants were collected from the stimulated cells and assayed for cytokine/chemokine production by using the BioPlex suspension array system or sandwich ELISA. Bars, mean ±SEM for each cytokine/chemokine (n=5–10 per group per analyte). *P<0·05 between WT and CCR5−/−.

**Fig. 8.** CLN cells from CCR5−/− mice suppress HSV-1 infection in the brainstem in CCR5−/− recipient animals. One million CLN cells from day 6 HSV-1-infected C57BL/6 (WT) or CCR5−/− mice (n=6 per group) were inoculated intravenously into CCR5−/− mice infected 3 days previously with HSV-1 (1000 p.f.u. per eye). At day 7 p.i., recipient animals were euthanized and the cornea, TG and brainstem (BS) were removed, homogenized and assayed for HSV-1 titres by plaque assay. Mean HSV-1 titres ±SEM are shown. Filled bars, WT; empty bars, CCR5−/−.
interpret the relationship between CXCL9 expression and neutrophil infiltration, neutrophils have been reported to generate CXCL9 (Gasperini et al., 1999). Therefore, it is tempting to speculate that the reduction in CXCL9 levels observed in the cornea of CCR5−/− mice during the early course of infection may simply reflect the reduction in neutrophil trafficking into the tissue.

In contrast to differences in cell infiltration between infected WT and CCR5−/− mice at day 3 p.i., neutrophil recruitment into the cornea was similar between these strains at day 7 p.i. However, the virus titre in the infected tissue was elevated in the CCR5−/− mice. As neutrophils contribute to the restriction of virus replication in the cornea (Tumpey et al., 1996; Thomas et al., 1997), the higher virus load in CCR5−/− mice at day 7 p.i. might reflect the earlier decrease in neutrophil recruitment into the cornea of CCR5−/− mice found at day 3 p.i. In addition, a unique feature found with the CCR5−/− mice was the lack of neutrophil penetration into the epithelial layers of the cornea, in contrast to the corresponding cells in WT mice at day 5 p.i. If neutrophils that normally suppress local virus replication are prevented from entering the corneal epithelium where virus initially replicates and subsequently spreads (Tumpey et al., 1996; Thomas et al., 1997), it would be expected that more virus would eventually be found at this site, which is consistent with the present data. Thus, it is likely that the increase in virus titre in the cornea of CCR5−/− mice at day 7 p.i. is due to defective neutrophil trafficking to the cornea and penetration of the epithelium during the earlier stages of infection. Even though redundancy in the chemokine network ultimately overcomes deficiency in CCR5 expression in the knockout mice, the results do point to a central role for CCR5 in the early stages of neutrophil recruitment into the cornea and potential trafficking of T lymphocytes into the cornea by day 7 p.i.

Blocking CCL5 or CCR5 expression has previously been reported to significantly dampen the inflammatory process in the central nervous system during mouse hepatitis virus infection (Lane et al., 2000; Glass & Lane, 2003; Glass et al., 2004), disseminated Cryptococcus neoformans infection (Huffnagle et al., 1999) and Plasmodium berghei infection (Belnoue et al., 2003). In contrast, in pulmonary infection models with influenza A virus (Dawson et al., 2000) or Mycobacterium tuberculosis (Algood & Flynn, 2004), CCR5−/− mice exhibit an exaggerated inflammatory response. Although in our model of ocular HSV-1 infection of CCR5−/− mice there was an early delay in accumulation of CD11b+ cells in the cornea and TG, by day 7 p.i. the cell numbers rebounded in the TG and levels of chemokines surpassed those observed in WT mice.

Implied by the increased virus yields in CCR5−/− mice is the prediction that the disease process might result in significantly higher mortality. In sharp contrast to this notion, survival studies showed that CCR5−/− mice were equally or less susceptible to HSV-1-induced death than WT mice. Selective expression of chemokines/cytokines, including IL-4, IL-6 and CCL2, that have been associated with neuroinflammation or end-stage encephalitides have been investigated (Huang et al., 2002; Buch et al., 2004; Kalehna et al., 2004; Kurt-Jones et al., 2004; Roberts et al., 2004). However, no differences were found in the levels of these soluble factors in the brains of WT and CCR5−/− mice that might explain the delay in mortality observed in CCR5−/− animals (data not shown). However, an increase was found in the percentage of CD4 and/or CD8 T cells recruited to the TG and brainstems of CCR5−/− mice. Although the CD8 T-cell effector activity of the infiltrating cells was not measured, a previous study has reported that CCR5 serves as a negative regulator of antiviral CD8 T-cell activity (De Lemos et al., 2005). In the present study, it was found that the transfer of CCR5−/− CLN cells into HSV-1-infected CCR5−/− mice dampened virus yield in the brainstems of recipients. Further studies are warranted to better understand the level of participation of the CD8 T lymphocytes from CCR5−/− mice in monitoring HSV-1 infection.

Confocal microscopic analysis showed that, whilst CCL5 was not detectable in HSV-1-infected mice in uninfected corneas, which is consistent with the ELISA data, CCL5 was detected in both the epithelial layers and stroma of the cornea. The expression co-localized with HSV-1 antigen. However, there were numerous sites within the cornea that expressed CCL5, but not HSV-1 antigen, suggesting that resident cells do express CCL5 during virus infection. To the best of our knowledge, this is the first reported mapping of CCL5 expression in the cornea following a virus infection. Understanding the relevance of chemokine and chemokine-receptor expression within the cornea during an infectious process will significantly enhance our capacity to formulate strategies and therapeutics that take advantage of the host response to clear the infection with nominal collateral damage to the visual axis.

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