Title

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Reduced Macrophage Infiltration and Demyelination in Mice Lacking the Chemokine Receptor CCR5 Following Infection with a Neurotropic Coronavirus

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Studies were performed to investigate the contributions of the CC chemokine receptor CCR5 in host defense and disease development following intracranial infection with mouse hepatitis virus (MHV). T cell recruitment was impaired in MHV-infected CCR5$^{-/-}$ mice at day 7 postinfection (pi), which correlated with increased ($P \leq 0.03$) titers within the brain. However, by day 12 pi, T cell infiltration into the CNS of infected CCR5$^{-/-}$ and CCR5$^{+/+}$ mice was similar and both strains exhibited comparable viral titers, indicating that CCR5 expression is not essential for host defense. Following MHV infection of CCR5$^{+/+}$ mice, greater than 50% of cells expressing CCR5 antigen were activated macrophage/microglia (determined by F4/80 antigen expression). In addition, infected CCR5$^{-/-}$ mice exhibited reduced ($P \leq 0.02$) macrophage (CD45^{high}F4/80^{+}) infiltration, which correlated with a significant reduction ($P \leq 0.001$) in the severity of demyelination compared to CCR5$^{+/+}$ mice. These data indicate that CCR5 contributes to MHV-induced demyelination by allowing macrophages to traffic into the CNS.   © 2001 Academic Press

Key Words: chemokine; chemokine receptor; demyelination; multiple sclerosis; macrophage; neuroimmunology.

INTRODUCTION

CCR5 is a member of the CC chemokine receptor family that is expressed on various hematopoietic cells, including lymphocytes and macrophages (Raport *et al.,* 1996). Chemokines that are capable of binding to CCR5 include CCL3 (MIP-1α), CCL4 (MIP-1β), and CCL5 (RANTES) (Boring *et al.,* 1996; Meyer *et al.,* 1996; Raport *et al.,* 1996; Zlotnik and Yoshie, 2000). Recent studies have implicated CCR5 as an important molecule in regulating the immune response as well as leukocyte trafficking following microbial infection. Mice deficient in CCR5 (CCR5$^{-/-}$) exhibit altered T cell activity and impaired macrophage function (Sato *et al.,* 1999; Zhou *et al.,* 1998). Furthermore, macrophage trafficking in response to antigen is impaired in CCR5$^{-/-}$ mice, indicating that CCR5 is required for migration of this population of cells (Huffnagle *et al.,* 1999).

Mouse hepatitis virus (MHV) is a positive-strand RNA virus that is a member of the *Coronaviridae* family. MHV infection of the CNS results in an acute encephalomyelitis followed by a chronic demyelinating disease that is similar to the pathology observed in the human demyelinating disease multiple sclerosis (MS) (Houtman and Flemming, 1996; Lane and Buchmeier, 1997). A robust expression of chemokine genes occurs within the brains and spinal cords of MHV-infected mice which precedes then accompanies leukocyte infiltration (Lane *et al.,* 1998). Among the chemokines expressed within the CNS of MHV-infected mice are the CCR5 ligands CCL3, CCL4, and CCL5, suggesting an important role for these chemokines in attracting leukocytes into the CNS. In support of this are studies demonstrating that treatment of MHV-infected mice with neutralizing antisera specific for CCL5 resulted in reduced lymphocyte infiltration into the brain and enhanced viral recovery (Lane *et al.,* 2000). Moreover, anti-CCL5 treatment resulted in decreased macrophage infiltration, which correlated with a significant decrease in the severity of demyelination. Therefore, these data suggested that CCL5 contributes to demyelination in MHV-infected mice by attracting macrophages into the CNS following binding and signaling of chemokine receptors expressed on the surface of these cells.

The present study was undertaken in order to better understand how CCR5 contributes to disease following MHV infection of the CNS. To this end, we have evaluated the severity of neurologic disease following MHV infection of CCR5$^{-/-}$ mice (Andres *et al.,* 2000). The results presented indicate that MHV infection of the CNS of CCR5$^{-/-}$ mice results in the development of an effective immune response, as characterized by cytokine and chemokine gene expression and clearance of virus from the CNS. However, both macrophage infiltration and demyelination was significantly reduced in MHV-infected CCR5$^{-/-}$ mice compared to CCR5$^{+/+}$ mice, indicating that CCR5 contributes to the pathogenesis of MHV-induced demyelination by attracting macrophages into the CNS.

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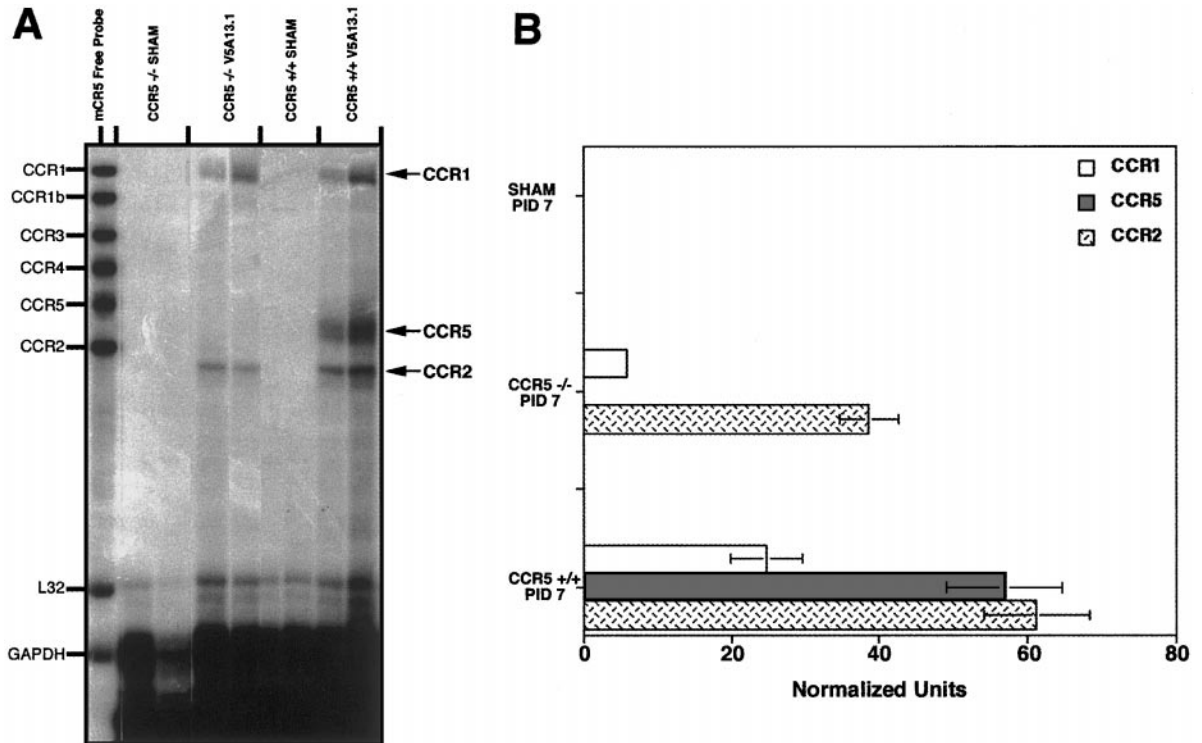
FIG. 1. (A) Chemokine receptor mRNA expression in the brain following MHV infection. CCR5$^{+/+}$ and CCR5$^{-/-}$ mice were infected with MHV or sterile saline (sham), brains isolated at 7 days pi, and total RNA was subjected to RPA. The free probe set is indicated in the lefthand margin. These fragments run higher than protected fragments due to additional cloning sequences. Each lane indicates a single mouse and infected (V5A13.1) or sham mice are indicated at the top of the autoradiograph. Note the decreased levels of mRNA transcripts for CCR1 and CCR2 within infected CCR5$^{-/-}$ mice compared to CCR5$^{+/+}$ mice. CCR5 transcripts are absent in CCR5$^{-/-}$ mice. The RPA shown is representative of three separate experiments. Sample sizes are as follows: CCR5$^{+/+}$, $n = 2$; CCR5$^{-/-}$, $n = 2$; sham, $n = 2$ for both groups of mice. (B) Quantitative analysis of chemokine receptor mRNA transcripts. Densitometric analysis of each lane representing a brain sample from an individual mouse was performed on the scanned autoradiograph (A). Data are presented as the average ±SEM.

RESULTS

### MHV-induced CNS disease and CCR5 expression

MHV infection of C57BL/6 mice results in an acute encephalomyelitis characterized by viral replication in neurons and glial cells. Approximately 40% of infected mice die during the acute stage of disease and surviving mice develop a severe demyelinating disease characterized by persistence of viral RNA and antigen within white matter tracts accompanied by mononuclear cell infiltration and white matter destruction. Chemokines are expressed within the CNS of MHV-infected mice and these molecules contribute to both host defense and disease development through attraction of T cells and macrophages (Lane et al., 1998, 2000; Liu et al., 2000, 2001). In order to characterize the chemokine receptor profile in the brains of MHV-infected mice, total RNA was isolated at day 7 postinfection (pi) and subjected to RPA using a multitemplate probe set containing mouse chemokine receptor riboprobes. The results shown in Fig. 1 clearly demonstrate increased expression of mRNA transcripts for CCR1, CCR2, and CCR5 in CCR5$^{+/+}$ mice following MHV infection of the CNS. As expected, CCR5 transcripts

were not expressed in CCR5$^{-/-}$ mice. In addition, there was an approximate twofold reduction in mRNA transcript levels for both CCR1 and CCR2 in CCR5$^{-/-}$ mice. Neither sham-infected CCR5$^{+/+}$ nor CCR5$^{-/-}$ mice exhibited increased expression for any of the chemokine receptors tested. Confocal microscopy revealed that greater than 55% of cells positive for CCR5 also expressed F4/80 (an antigen expressed by activated microglia/macrophages) (Fig. 2). CCR5 antigen was not detected on F4/80-positive cells within the brains of CCR5$^{-/-}$ mice. Although not determined, it is likely that T cells as well as activated astrocytes constitute the remaining population of CCR5-positive cells, as these cells have been shown to express this receptor (Mack et al., 2001; Dorf et al., 2000).
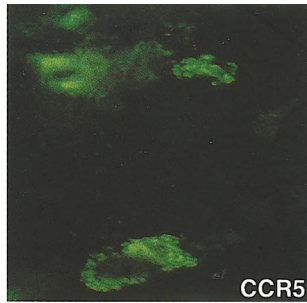
MHV infection of CCR5$^{-/-}$ mice

To determine the contribution of CCR5 to host defense and neurologic disease following MHV infection of the CNS, CCR5$^{-/-}$ and CCR5$^{+/+}$ mice were infected with MHV and the severity of disease was evaluated. There were no marked differences in either clinical disease severity or mortality, with approximately 60% of CCR5$^{-/-}$
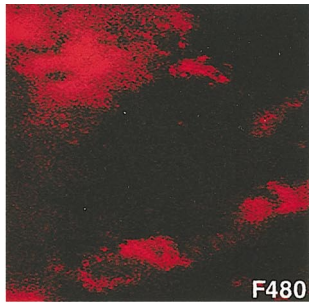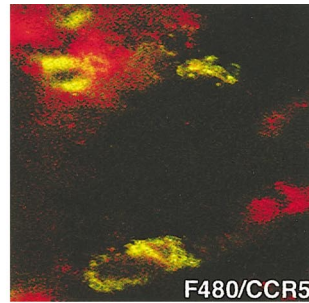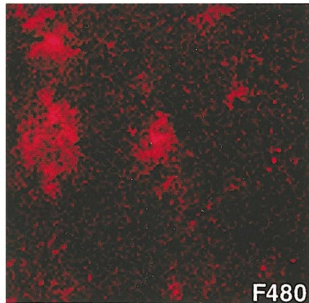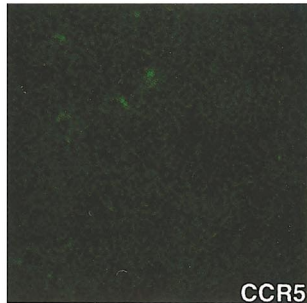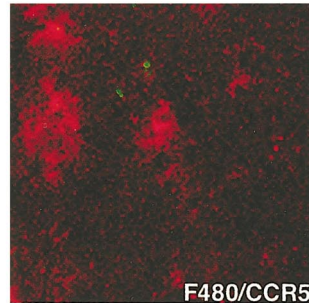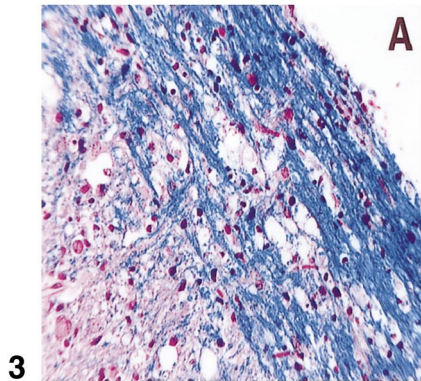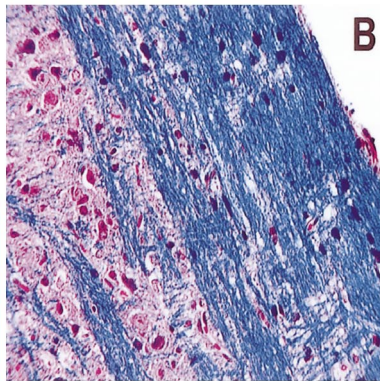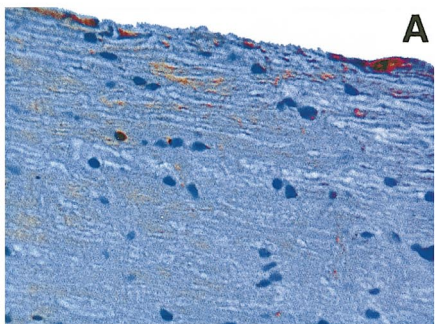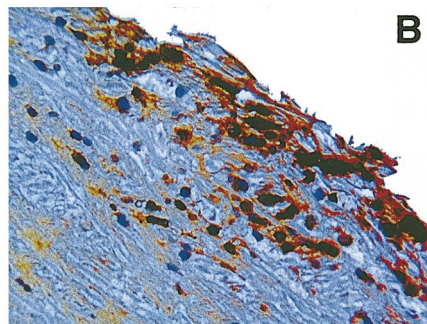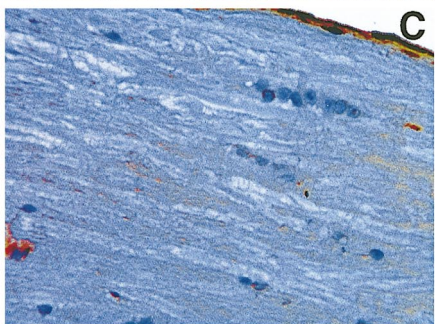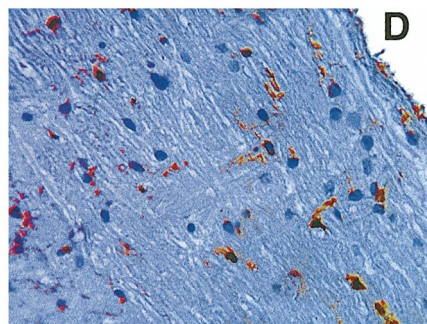
TABLE 1

Reduced Leukocyte Infiltration in CCR5$^{-/-}$ Mice

Mouse type	Days postinfection	Brain titer (PFU/gram tissue-Log$_{10}$) (mean ± SEM)	Average cell recovery	CD4$^+$ (mean ± SEM)	CD8$^+$ (mean ± SEM)	F4/80$^+$ (mean ± SEM)
CCR5$^{+/+}$	7	5.1 ± 0.1 (9)[a]	9.9 × 10^5	19.5 ± 1.3 (13)[c]	22.1 ± 1.6 (13)	27.2 ± 2.4 (13)
	12	2.2 ± 0.2 (12)	7.8 × 10^5	21.6 ± 1.7 (3)	16.5 ± 0.9 (3)	29.1 ± 1.5 (3)
CCR5$^{-/-}$	7	5.9 ± 0.1 (12)[b]	7.7 × 10^5	12.2 ± 1.9 (8)[b]	14.1 ± 1.6 (8)[b]	18.8 ± 2.3 (8)[b]
	12	2.5 ± 0.1 (9)	1.1 × 10^6	21.0 ± 2.3 (3)	21.5 ± 3.7 (3)	11.8 ± 0.7 (3)[d]

[a] Parentheses indicate numbers of mice used. Data presented represent five independent experiments.
[b] $P \leq 0.03$ when compared to CCR5$^{+/+}$ mice.
[c] Data are presented as the percentage of positive cells within the gated population.
[d] $P \leq 0.001$ when compared to CCR5$^{+/+}$ mice.

and CCR5$^{+/+}$ mice surviving until day 12 pi. Analysis of viral titers within the brains revealed a fivefold increase in virus present ($P \leq 0.03$) in CCR5$^{-/-}$ mice (5.9 ± 0.1 PFU/g, Log$_{10}$, $n = 12$) compared to CCR5$^{+/+}$ mice (5.1 ± 0.1 PFU/g, $n = 9$) at day 7 pi (Table 1). However, by day 12 pi, there was not a significant difference in viral burden between CCR5$^{-/-}$ mice (2.5 ± 0.1 PFU/g, $n = 9$) and CCR5$^{+/+}$ mice (2.2 ± 0.2 PFU/g, $n = 12$) (Table 1). In addition, no differences were detected in the distribution of viral antigen within the brains of infected CCR5$^{+/+}$ or CCR5$^{-/-}$ mice at either day 7 or day 12 pi, indicating that lack of CCR5 did not alter the cellular tropism of the virus (data not shown). Collectively, these data indicate that CCR5 is not essential in host defense or clearance of virus from the brains of MHV-infected mice.

Mononuclear cell infiltration

The increased viral titers within the brains of CCR5$^{-/-}$ mice at day 7 pi suggested that there may be reduced levels of infiltrating T cells present during acute disease, as both CD4$^+$ and CD8$^+$ T cells are required for optimal clearance of virus from the CNS (Williamson and Stohlman, 1990; Lane et al., 2000). FACS analysis of infiltrating mononuclear cells at day 7 pi revealed decreased infiltration ($P \leq 0.03$) of CD4$^+$ T cells (12.2 ± 1.9%, $n = 8$) and CD8$^+$ T cells (14.1 ± 1.6%, $n = 8$) into the brains of CCR5$^{-/-}$ mice compared to CCR5$^{+/+}$ mice (CD4$^+$, 19.5 ± 1.3%, $n = 13$; CD8$^+$, 22.1 ± 1.6%, $n = 13$) (Table 1). By

day 12 pi, however, there were no significant differences in the infiltration of CD4$^+$ or CD8$^+$ T cells into the CNS of infected CCR5$^{-/-}$ or CCR5$^{+/+}$ mice (Table 1). Macrophage/microglial staining as assessed by F4/80 antigen expression was decreased by 30 and 60% in CCR5$^{-/-}$ mice at day 7 (CCR5$^{+/+}$, 27.2 ± 2.4%, $n = 13$; CCR5$^{-/-}$, 18.8 ± 2.3%, $n = 8$, $P \leq 0.03$) and day 12 pi (CCR5$^{+/+}$, 29.1 ± 1.5%, $n = 3$; CCR5$^{-/-}$, 11.8 ± 0.7%, $n = 3$; $P \leq 0.001$), respectively (Table 1). Collectively, these data indicate that T cell infiltration into the CNS is affected during acute disease in CCR5$^{-/-}$ mice, yet these cells are ultimately able to enter the CNS and participate in elimination of infectious virus. Further, these data indicate that macrophage/microglial activation and/or infiltration is compromised in the absence of CCR5.

Reduced macrophage infiltration and myelin destruction in CCR5$^{-/-}$ mice

Previous studies have demonstrated that macrophages are important contributors to demyelination in MHV-infected mice (Lane et al., 2000; Wu and Perlman, 1999; Wu et al., 2000). In an effort to distinguish between infiltrating macrophages and resident microglia, flow analysis was performed using FITC-conjugated F4/80 antibodies and PE-conjugated CD45 antibodies. Infiltrating macrophages have been found to exhibit a CD45^{high}F4/80$^+$ phenotype, whereas microglia are CD45^{low}F4/80$^+$ (Ford et al., 1995; Fife et al., 2000). Such

FIG. 2. F4/80-positive cells express CCR5. CCR5 expression on F4/80-positive cells was demonstrated using confocal microscopy. Representative staining from brains of MHV-infected CCR5$^{+/+}$ and CCR5$^{-/-}$ mice at day 7 pi for F4/80 alone (red cells), CCR5 alone (green cells), or dual-labeled F480/CCR5-positive cells (yellow cells) is shown. Cells shown were present within the brain parenchyma of infected mice. Original magnification, ×400.

FIG. 3. Demyelination is reduced in CCR5$^{-/-}$ mice. Representative LFB staining of spinal cords of MHV-infected CCR5$^{+/+}$ and CCR5$^{-/-}$ mice at 12 days postinfection. CCR5$^{+/+}$ mice (A) display mononuclear infiltration and myelin destruction, in contrast to CCR5$^{-/-}$ mice (B), which have limited cellular infiltration and compact myelin. Original magnification, ×200.

FIG. 4. F4/80 staining is decreased in white matter tracts of MHV-infected CCR5$^{-/-}$ mice. Representative staining for F4/80 antigen within white matter tracts of MHV-infected CCR5$^{+/+}$ mice (B) or CCR5$^{-/-}$ mice (D). F4/80-positive cells are stained brown. Note heavy infiltration within white matter tracts of CCR5$^{+/+}$ mice compared to CCR5$^{-/-}$ mice. F4/80 staining of sham-infected CCR5$^{+/+}$ mice (A) and CCR5$^{-/-}$ mice (C) are included for controls. Original magnification, ×200.

TABLE 2

Diminished Macrophage Infiltration Correlates with Decreased Demyelination in CCR5$^{-/-}$ Mice

Mouse type	Day postinfection	Average cell recovery	CD45^{high}/F480+[b] (mean $\pm$ SEM)	DM[c] (mean $\pm$ SEM)
CCR5$^{+/+}$	12	9.33×10^5 (4)[a]	$1.3 \times 10^5 \pm 1.2 \times 10^4$	2.2 $\pm$ 0.6 (9)
CCR5$^{-/-}$	12	8.21×10^5 (5)	$4.5 \times 10^4 \pm 8.2 \times 10^{3d}$	0.9 $\pm$ 0.4 (11)[e]

[a] Parentheses indicate number of mice used.
[b] Data are presented as the percentage of positive cells within the gated population.
[c] Data presented are from three independent experiments.
[d] $P \le 0.02$ when compared to CCR5$^{+/+}$ mice.
[e] $P \le 0.001$ when compared to CCR5$^{+/+}$ mice.

analysis revealed an approximate threefold reduction ($P \le 0.02$) in numbers of infiltrating macrophages present within the CNS of infected CCR5$^{-/-}$ mice at day 12 ($4.5 \times 10^4 \pm 8.2 \times 10^3$, $n = 5$) compared CCR5$^{+/+}$ mice ($1.3 \times 10^5 \pm 1.2 \times 10^4$, $n = 4$) (Table 2). In order to determine if limited macrophage infiltration into the CNS of infected CCR5$^{-/-}$ mice correlated with a reduction in demyelination, spinal cords from infected CCR5$^{+/+}$ and CCR5$^{-/-}$ mice at day 12 pi were stained with luxol fast blue (LFB) and the severity of demyelination was evaluated. As shown in Fig. 3B, infected CCR5$^{-/-}$ mice displayed reduced mononuclear cell infiltration into white matter tracts which was accompanied by a marked reduction in the severity of demyelination compared to CCR5$^{+/+}$ mice (Fig. 3A). Quantitative analysis revealed a significant reduction ($P \le 0.001$) in demyelination in CCR5$^{-/-}$ mice (0.9 ± 0.4, $n = 11$) when compared to CCR5$^{+/+}$ mice (2.2 ± 0.6, $n = 9$) at day 12 pi (Table 2). Immunohistochemical staining was performed on spinal cord sections from both strains of mice to determine if macrophage/microglial infiltration into white matter tracts was reduced. The data presented in Fig. 4 reveal intense F4/80 staining within white matter tracts of CCR5$^{+/+}$ mice, while staining in CCR5$^{-/-}$ mice is notably reduced.

Cytokine and chemokine gene expression in CCR5$^{+/+}$ and CCR5$^{-/-}$ mice

We next evaluated the cytokine and chemokine gene expression patterns in the CNS of MHV-infected CCR5$^{+/+}$ and CCR5$^{-/-}$ mice in an attempt to correlate the reduction in demyelination in CCR5$^{-/-}$ mice with the presence and/or the absence of specific mRNA signals. There was no obvious difference in the cytokine transcript profile between the two strains of mice at either 7 or 12 days pi (Fig. 5A). Moreover, quantification of signal intensities revealed similar levels of transcripts for the cytokines LT-β, TNF-α, IFN-γ, and IFN-β at days 7 and 12 pi between the strains of mice (Fig. 5B). MHV-infected CCR5$^{+/+}$ and CCR5$^{-/-}$ mice expressed a similar chemokine profile, with CCL2 (MCP-1), CCL3, CCL5, and CXCL10 (IP-10) being expressed at days 7 and 12 pi (Fig.

6A). Analysis of transcript levels revealed comparable mRNA abundance for each chemokine at day 7 in both strains of mice (Fig. 6B). However, by day 12 pi, CCR5$^{-/-}$ mice exhibited increased levels (approximately twofold) of mRNA transcripts for CCL5 compared to CCR5$^{+/+}$ mice.

DISCUSSION

Animal models of demyelination, including experimental autoimmune encephalomyelitis (EAE), Theiler's virus-induced demyelination, and MHV-induced demyelination, have provided valuable information with regard to understanding the immunopathological mechanisms contributing to human demyelinating diseases, such as MS. We have used the MHV model of viral-induced demyelination to better understand the complex mechanisms by which chemokines and chemokine receptors contribute to neuroinflammation, host defense, and myelin destruction. Through use of specific neutralizing antibodies we have demonstrated important roles for CXCL9 (Mig) and CXCL10 in promoting a protective Th1 response following MHV infection characterized by CD4$^+$ and CD8$^+$ T cell infiltration and IFN-γ production (Liu et al., 2000, 2001). Further, we have shown that CCL5 serves to attract macrophages into the CNS which then contribute to myelin destruction (Lane et al., 2000). Therefore, chemokines clearly influence leukocyte migration and infiltration into the CNS following MHV infection and have a profound effect on the outcome of disease with regard to host defense and demyelination.

The present study was undertaken to provide information regarding the role of chemokine receptors to MHV-induced neurologic disease. Instillation of MHV into the brains of C57BL/6 mice resulted in increased mRNA expression of the chemokine receptors CCR1, CCR2, and CCR5 (Fig. 1). Ligands for these receptors that are expressed within the CNS of MHV-infected mice include CCL2, CCL3, CCL4, CCL5, and CCL7 (MCP-3) (Lane et al., 1998). Therefore, these data suggest that expression of chemokine receptors by either resident cells of the CNS and/or inflammatory cells in response to chemokine expression may influence the immune response. We
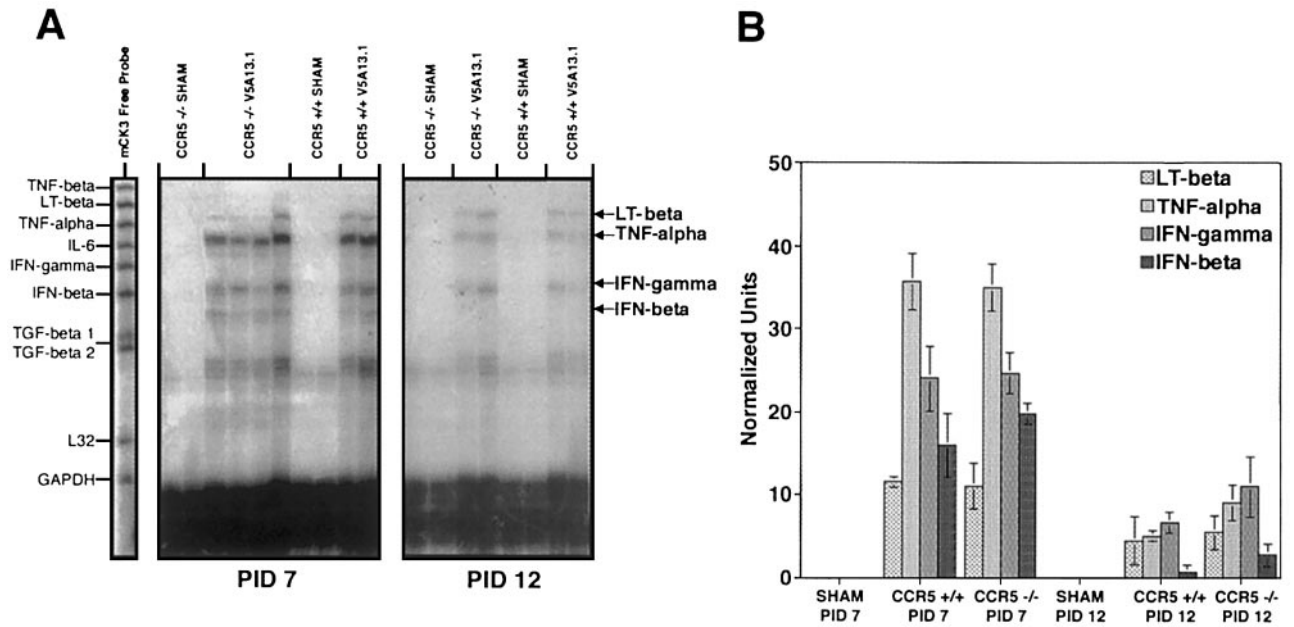
FIG. 5. (A) Cytokine gene expression in CCR5$^{+/+}$ and CCR5$^{-/-}$ mice. Mice were infected with either MHV or sterile saline (sham) by ic injection, brains isolated at days 7 or 12 pi, and total RNA was subjected to RPA analysis. Shown is a representative RPA from two separate experiments. Sample sizes are as follows: day 7 pi, CCR5$^{+/+}$, $n = 2$; CCR5$^{-/-}$, $n = 4$; day 12 pi, CCR5$^{+/+}$, $n = 2$; CCR5$^{-/-}$, $n = 2$. Results from sham mice are shown from both groups at days 7 and 12 pi, $n = 2$. (B) Quantitative analysis of cytokine mRNA transcripts expressed. Densitometric analysis of each lane representing a brain sample from an individual mouse was performed on the scanned autoradiograph (A). Data are presented as the average ±SEM.

elected to examine the role of CCR5 in MHV-induced disease in more detail as we have recently determined that CCL5 is important in both host defense and disease

development (Lane *et al.,* 2000). In addition, the CCR5 ligand CCL3 is expressed, albeit at lower levels, during acute disease, suggesting that this chemokine may also
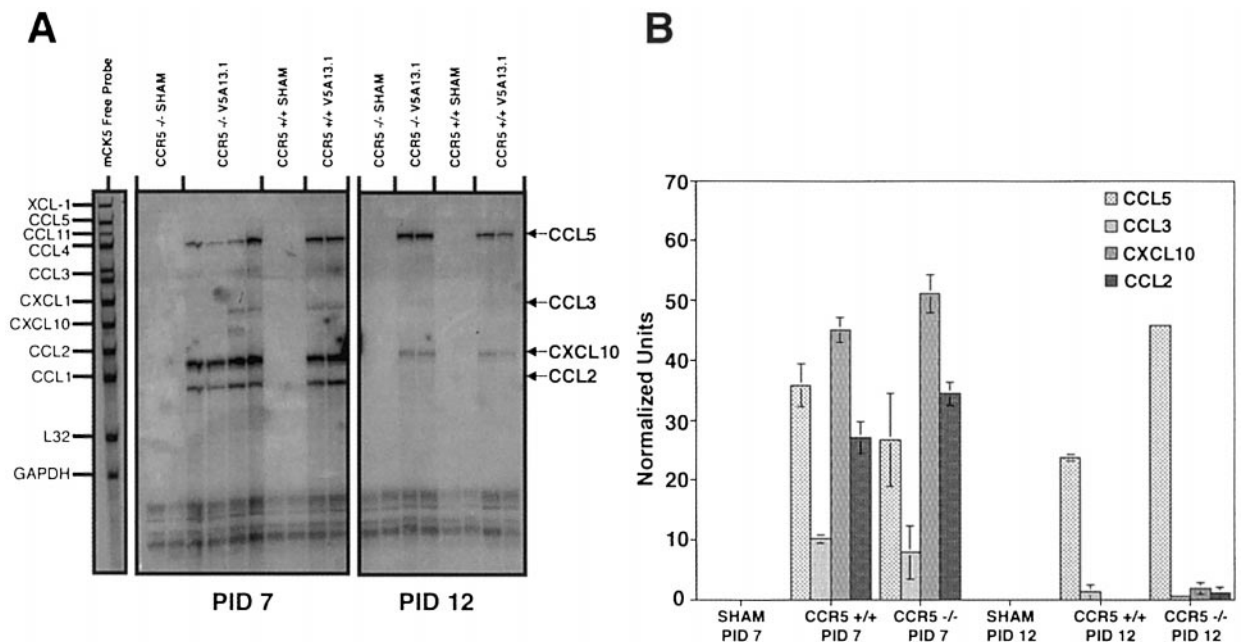
FIG. 6. (A) Chemokine gene expression in CCR5$^{+/+}$ and CCR5$^{-/-}$ mice. Representative RPA showing chemokine gene expression in either CCR5$^{+/+}$ or CCR5$^{-/-}$ mice at days 7 and 12 pi with MHV. RNA samples from Fig. 5 were used to examine chemokine mRNA transcripts in the autoradiograph presented. (B) Quantitative analysis of chemokine mRNA transcripts expressed is shown. Densitometric analysis of each lane representing a brain sample from an individual mouse was performed on the scanned autoradiograph (A). Note the approximate twofold increase in the level of CCL5 mRNA transcripts in CCR5$^{-/-}$ mice compared to CCR5$^{+/+}$ at day 12 pi. Data are presented as the average ±SEM.

contribute to neuroinflammation following viral infection of the CNS. In support of a role for CCR5 in host defense following viral infection are studies by Dawson *et al.* (2000) that have shown increased mortality in CCR5$^{-/-}$ mice following influenza A viral infection. Further, CCR5 expression is considered important in host defense following infection with *Cryptococcus neoformans* and *Listeria monocytogenes,* as CCR5$^{-/-}$ mice exhibited increased mortality and enhanced microbial recovery from infected tissues (Huffnagle *et al.,* 1999; Zhou *et al.,* 1998). Moreover, recent studies have revealed important roles for the chemokine receptors CCR1 and CCR2 in mediating host defense through leukocyte recruitment following viral infection (Dawson *et al.,* 2000; Domachowske *et al.,* 2000). Collectively, these data suggest that chemokine receptor expression represents an important component of host defense systems following microbial infection by regulating leukocyte migration to sites of infection and replication.

MHV infection of CCR5$^{-/-}$ mice revealed reduced CD4$^+$ and CD8$^+$ T cell infiltration into the CNS at day 7 pi, which correlated with enhanced viral recovery from the brain (Table 1). The delay in response to infection during acute disease is similar to findings by Sato *et al.* (1999) that demonstrated a decreased host response following *Leishmania donovani* infection of CCR5$^{-/-}$ mice compared to CCR5$^{+/+}$ mice. However, by day 12 pi no differences in CD4$^+$ or CD8$^+$ T cell levels were observed within the brains of infected CCR5$^{+/+}$ or CCR5$^{-/-}$ mice and both strains of mice exhibited comparable viral titers. These data suggest that CCR5 is important in directing T cell migration into the CNS during acute disease but is not required for T cell infiltration at later times. This may be the result of differential expression of CCR5 by T cells, which is dictated by the stage of disease, e.g., acute versus chronic. Alternatively, it is possible that CCR5 signaling is important in attracting T cells during acute disease but that T cells respond to different chemotactic signals at later stages of disease. More importantly, these data clearly indicate that CCR5 is not essential in host defense following MHV infection of the CNS. In addition, these studies illustrate the importance of the challenging antigen/microbe with regard to the role of CCR5 in contributing to host defense and the outcome of infection.

Analysis of cytokine and chemokine gene expression indicated that no differences in profiles for any of these factors existed at either day 7 or day 12 pi (Figs. 5 and 6). The fact that CCR5$^{-/-}$ mice displayed levels of IFN-γ mRNA similar to those of CCR5$^{+/+}$ mice despite close to a 40% reduction in the number of T cells present in the brains suggests that T cell production of IFN-γ is increased in the absence of CCR5. These results are consistent with those of Zhou *et al.* (1998) that demonstrated enhanced IFN-γ production by T cells obtained from CCR5$^{-/-}$ mice following *in vitro* stimulation. Despite

equivalent levels of IFN-γ mRNA between the two strains of mice at day 7, CCR5$^{-/-}$ mice exhibited increased viral titers compared to CCR5$^{+/+}$ mice. IFN-γ has been determined to exert a potent antiviral effect in MHV-infected mice, leaving these data to be somewhat surprising. However, it is possible that reduced levels of IFN-γ protein are present in infected CCR5$^{-/-}$ mice and/or that expression of other antiviral effector molecules such as perforin are reduced compared to CCR5$^{+/+}$ mice (Lin *et al.,* 1997). CCR5$^{-/-}$ mice expressed increased levels of mRNA transcripts for CCL5 at day 12 when compared to CCR5$^{+/+}$ mice. These data suggest that CCR5-mediated and/or other chemokine receptor signaling pathways regulate CCL5 expression, as has been suggested to occur in other chemokine/receptor interactions (Sallusto *et al.,* 2000).

It is important to note that MHV-infected CCR5$^{-/-}$ mice displayed a marked decrease in mRNA transcript levels for CCR1 and CCR2 compared to infected CCR5$^{+/+}$ mice (Fig. 1). The mechanism(s) responsible for this decrease in chemokine receptor expression is not clear. These receptors are expressed on activated T cells as well as macrophages (Murphy *et al.,* 2000). Therefore, reduced transcript levels for these receptors may simply reflect the lowered levels of infiltrating T cells and macrophages within the brains of infected CCR5$^{-/-}$ mice at day 7 pi. Alternatively, it is possible that in the MHV model system, CCR5 signaling may modulate expression of other chemokine receptors, e.g., CCR1 and/or CCR2, through the release of soluble factor(s) which regulate chemokine receptor expression on inflammatory cells as well as resident cells of the CNS.

Macrophage/microglia migration into spinal cord white matter tracts was markedly lowered within CCR5$^{-/-}$ mice when compared to CCR5$^{+/+}$ mice (Fig. 4). The overall reduction in activated macrophage (CD45^{high}F4/80$^+$) infiltration into the CNS of CCR5$^{-/-}$ mice correlated with significant reduction in the severity of demyelination compared to CCR5$^{+/+}$ mice at day 12 pi (Table 2). These data support and extend earlier studies that have shown an important role for macrophages in participating in myelin destruction within MHV-infected mice (Wu and Perlman, 1999; Wu *et al.,* 2000; Lane *et al.,* 2000). More importantly, the data presented in this paper clearly indicate that CCR5 is important in contributing to demyelination, in part by allowing macrophage migration into the CNS following MHV infection. This is further supported by the demonstration that the majority of F4/80-positive cells present within the brains of MHV-infected mice express CCR5 (Fig. 2). Collectively, these data suggest that ligand binding, e.g., CCL5 and/or CCL3, and signaling to CCR5 results in macrophage migration and infiltration into the CNS. However, the fact that we have previously demonstrated that CCL3 is expressed only at low levels during acute disease and is not detectable during chronic demyelination, whereas

robust expression of CCL5 is detected during both phases of disease, suggests that CCL5 may be the primary CCR5 signaling chemokine in this model. This is supported by earlier studies that showed an important role for CCL5 in attracting macrophages into the CNS following MHV infection (Lane et al., 2000). Therefore, the data presented in this study suggest that one mechanism by which CCL5 contributes to demyelination is via attracting macrophages into the CNS through CCR5-mediated signaling pathways. Additional evidence supporting this is provided by the observation that even in the presence of increased CCL5 expression at day 12 pi, demyelination is reduced in CCR5$^{-/-}$ mice. This suggests that CCL5 does not directly exert a toxic effect on oligodendrocytes (the myelin-producing cell of the CNS) but contributes to disease by attracting CCR5-positive macrophages into the brains and spinal cords.

Our data are in contrast with those of a recent study by Tran et al. (2000), which reports that CCR5$^{-/-}$ mice were able to develop EAE following immunization with myelin oligodendrocyte glycoprotein. No differences were detected between CCR5$^{-/-}$ mice and CCR5$^{+/+}$ mice with regard to production of Th1 cytokines, kinetics and severity of disease, or leukocyte infiltration into the CNS. This is in contrast to the results presented in this paper, which clearly demonstrate reduced macrophage infiltration and demyelination in MHV-infected CCR5$^{-/-}$ mice. Further, Siebert et al. (2000) show that CCR5$^{-/-}$ mice are indistinguishable from their wild-type counterparts with respect to myelin density and spinal cord macrophage infiltration following transection injury. Collectively, these results illustrate the differences in the roles of chemokines and chemokine receptors with regards to different models of neurodegenerative disease. Although CCL5 is expressed within the brains of mice with EAE, Kennedy et al. (1998) demonstrated that antibody-mediated neutralization of CCL5 did not affect disease outcome. In contrast, anti-CCL5 treatment of MHV-infected mice resulted in diminished macrophage migration to the CNS and reduced demyelination (Lane et al., 2000). Therefore, these differences emphasize the importance of using different models of demyelination to study how chemokine/chemokine receptor expression and usage influence disease outcome.

The combination of previous studies with the current data allows for a model presentation with regard to chemokine expression as it relates to host defense and disease development following MHV infection of the CNS (Lane et al., 1998; Liu et al., 2000, 2001). The T cell chemoattractant CXCL10 is expressed very early (day 1 pi) following MHV infection and serves to attract antiviral T cells into the CNS. Infiltration of activated T cells results in increased expression of IFN-γ that promotes expression of another T cell chemoattractant, CXCL9. Both CXCL9 and CXCL10 are important sentinel molecules and serve to protect the host by attracting antiviral

T cells into the CNS. Expression of CCL5 increases as T cells accumulate and this chemokine serves to attract additional T cells and macrophages into the CNS by binding and signaling to CCR5 that is expressed on the surface of these cells. Following the acute stage of disease, activated T cells are retained within the CNS and express CCL5, which results in macrophage accumulation and myelin destruction. Finally, these data support and extend earlier studies from our laboratory and others which indicate that strategies designed to target chemokines and/or their receptors may offer unique opportunities for the treatment of neuroinflammatory diseases such as MS.

MATERIALS AND METHODS

Virus and mice

The MHV strain V5A13.1 was kindly provided by M. Buchmeier (Scripps Research Institute, La Jolla, CA) (Dalziel et al., 1986). Age-matched (5–7 weeks) CCR5$^{+/+}$ and CCR5$^{-/-}$ mice (8th generation backcrossed to C57BL/6, H-2^{b} background) were used for all experiments. Following anesthetization by inhalation of methoxyflurane (Pitman-Moor Inc., Washington Crossing, NJ), mice were injected intracranially (ic) with 10 PFU of MHV suspended in 30 μl of sterile saline (Lane et al., 1998, 2000). Control (sham) animals were injected with 30 μl of sterile saline alone. Animals were sacrificed at days 7 and 12 pi, at which point brains and spinal cords were removed for analysis in studies described. One half of each brain was used for plaque assay on the DBT astrocytoma cell line to determine viral burden (Hirano et al., 1978; Lane et al., 1998). The remaining halves were used for histologic analysis, stored at −80°C for RNA isolation, or used for FACS analysis (Lane et al., 2000).

Immunohistochemistry

Rat anti-mouse F4/80 (Serotec, Oxford, England) [1:50 dilution in phosphate-buffered saline containing 10% normal goat serum (NGS)] was used for the detection of activated macrophage/microglia (Lane et al., 2000). A biotinylated secondary antibody (1:300 dilution, Vector Laboratories, Burlingame, CA) was used for visualization. Staining was performed on 6-μm-thick frozen sections fixed in 95% ethanol for 10 min at −20°C. The ABC Elite (Vector Laboratories) staining system was used according to the manufacturer's instructions, and diaminobenzidine (DAB) was used as a chromogen. All slides were counterstained with hematoxylin, dehydrated, and mounted. Staining controls were (i) omission of primary antibodies from the staining sequence and (ii) treatment of sham-infected mice with primary and secondary antibodies.

T cell isolation and flow cytometry

Cells were obtained from brains of CCR5$^{+/+}$ and CCR5$^{-/-}$ mice at 7 and 12 days pi and a single cell suspension was obtained using a previously described method (Lane *et al.*, 2000). Antibodies used for flow cytometry in these studies included FITC rat anti-mouse CD4, CD8 (Pharmingen, San Diego, CA), F4/80 (Serotec), and PE-conjugated rat anti-mouse CD45 (Pharmingen). In all cases, an isotype-matched FITC-conjugated antibody was used. Cells were incubated with antibodies for 45 min at 4°C, washed, and fixed in 1% paraformaldehyde. Following fixation, cells were analyzed on a FACStar (Becton–Dickinson, Mountain View, CA) (Lane *et al.*, 2000; Liu *et al.*, 2000). Data are presented as the percentage of positive cells within the gated population.

Confocal microscopy

Primary Abs (diluted in PBS containing 10% normal horse serum) used for dual fluorescent detection of cellular Ags were as follows: rat anti-mouse F4/80 (Serotec) at 1:50 and goat anti-mouse CCR5 (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:50. For F4/80 primary Ab, a TRITC-conjugated secondary Ab was used (1:50; Sigma, St. Louis, MO). For CCR5 primary Ab, a FITC-conjugated secondary Ab was used (1:50; Zymed, South San Francisco, CA). Staining was performed on 6-μm frozen sections fixed in acetone for 10 min at −20°C. Dual stained slides were then subjected to confocal microscopy using a Bio-Rad MRC UV laser-scanning confocal microscope (Bio-Rad, Richmond, CA.).

Ribonuclease protection assay

Total RNA was extracted from brains of MHV-infected CCR5$^{+/+}$ and CCR5$^{-/-}$ mice at days 7 and 12 pi. Cytokine, chemokine, and chemokine receptor transcripts were analyzed using the mCK-3, mCK-5, and mCR-5 multitemplate probe sets, respectively (Pharmingen). RPA analysis was performed with 15 μg of total RNA using a previously described protocol (Lane *et al.*, 1998, 2000). Probes for L32 and GAPDH are included to verify consistency in RNA loading and assay performance. For quantification of signal intensity, autoradiographs were scanned and individual chemokine, cytokine, or chemokine receptor transcript bands were normalized as the ratio of band intensity to the L32 control (Lane *et al.*, 1998, 2000; Liu *et al.*, 2000). Analysis was performed using NIH Image 1.61 software.

Histology

Spinal cords were removed at 7 and 12 days pi and either fixed by immersion overnight in 10% normal buffered Formalin (NBF) for paraffin embedding or directly embedded in OCT compound (Sakura Finetek, Torrence, CA) for cryosectioning. The severity of demyelination was determined by LFB staining of spinal cords and analyzed with a light microscope. LFB-stained spinal cord sections were coded and read blind by two investigators. Demyelination was scored as follows: 0, no demyelination: 1, mild inflammation accompanied by loss of myelin integrity; 2, moderate inflammation with increasing myelin damage; 3, numerous inflammatory lesions accompanied by significant increase in myelin stripping; and 4, intense areas of inflammation accompanied by numerous phagocytic cells engulfing myelin debris (Lane *et al.*, 2000). Scores were averaged and presented as means ±SEM.

Statistical analysis

Statistically significant differences between groups of mice were determined by *t* test using Sigma-Stat 2.0 software and *P* values of ≦0.05 were considered significant.

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