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Ligand up-regulation does not correlate with a role for CCR1 in pathogenesis in a mouse model of non–lymphocyte-mediated neurological disease

Meryll E Corbin, Susan Pourciau, Timothy W Morgan, Marc Boudreaux, and Karin E Peterson

Department of Pathobiological Sciences, School of Veterinary Medicine, Louisiana State University, Baton Rouge, Louisiana, USA

CCR1 ligands, including CCL3, CCL5, and CCL7, are up-regulated in a number of neurological disorders in humans and animal models. CCR1 is expressed by multiple cell types in the central nervous system (CNS), suggesting that receptor signaling by neuronal cell types may influence pathogenesis. In the current study, the authors used a mouse model of retrovirus infection to study the contribution of CCR1 to neuropathogenesis in the absence of lymphocyte recruitment to the CNS. In this model, infection of neonatal mice with the neurovirulent retrovirus Fr98 results in increased expression of proinflammatory chemokines in the CNS, activation of glial cells, and development of severe neurological disease. Surprisingly, no difference in neuropathogenesis was observed between CCR1-sufficient and CCR1-deficient mice following infection with the neuropathogenic virus Fr98. CCR1 was also not necessary for control of virus replication in the brain or virus-induced activation of astroglia. Additionally, CCR1 deficiency did not affect the up-regulation of its ligands, CCL3, CCL5, or CCL7. Thus, CCR1 did not appear to have a notable role in Fr98-induced pathogenesis, despite the correlation between ligand expression and disease development. This suggests that in the absence of inflammation, CCR1 may have a very limited role in neuropathogenesis. *Journal of NeuroVirology* (2006) **12,** 241–250.

Keywords: brain; CCL3; CCL5; CCR1; mouse; retrovirus

Introduction

Increased expression of many proinflammatory cytokines and chemokines has been detected in the brain and/or cerebrospinal fluid of patients with several neurological disorders. For example, CCL2 (monocyte chemoattractant protein-1; MCP-1), CCL3 (macrophage inflammatory protein-1_; MIP-1α), and CCL5 (RANTES) have been associated with pathogenesis in Alzheimer's disease (AD), multiple sclerosis (MS), and human immunodeficiency virus (HIV)-associated dementia (HAD) (Kelder *et al*, 1998; McManus *et al*, 1998; Schmidtmayerova *et al*, 1996; Van Der Voorn *et al*, 1999; Xia *et al*, 1998, 2000). One function of chemokine expression is the recruitment of lymphocytes and other inflammatory cells to sites of infection and/or damage, including the central nervous system (CNS) (Dufour *et al*, 2002; Glass *et al*, 2005; Huang *et al*, 2002; Youssef *et al*, 1999). In addition, several studies have demonstrated that multiple cell types in the brain express chemokine receptors, indicating that chemokines may have a role in activating and regulating functions of intrinsic brain cells (Bajetto *et al*, 1999, Bonavia *et al*, 2003; Cowell and Silverstein, 2003; Flynn *et al*, 2003; Klein *et al*, 1999; Rezaie *et al*, 2002; Tran *et al*,

Address correspondence to Karin E. Peterson, Department of Pathobiological Sciences, School of Veterinary Medicine, Skip Bertman Drive, Louisiana State University, Baton Rouge, LA 70803, USA. E-mail: kpeterson@vetmed.lsu.edu

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2004). Chemokines added to *in vitro* cultures induced activation of astroglia and neurons and altered cytokine/chemokine production by these cells (Andjelkovic *et al*, 2002; Bonavia *et al*, 2003; Klein *et al*, 1999; Luo *et al*, 2002). Chemokines also induced calcium flux in neurons and were shown to protect neurons from *N*-methyl-d-asparate (NMDA) and HIV tat–induced cell death (Eugenin *et al*, 2003; Klein et al., 1999). Thus, the induction of chemokines in response to infection or damage to the CNS may alter the basal activation state and/or normal functions of these cell types and thereby contribute to neuropathogenesis.

We have studied the contribution of chemokines to neuropathogenesis in the absence of substantial lymphocytic inflammation by utilizing a mouse model of polytropic retrovirus infection. In this model, infection of neonatal mice with the neurovirulent retrovirus Fr98 results in the development of severe neurological disease characterized by ataxia, seizures, and/or death (Portis *et al*, 1995). Increased mRNA and protein expression of multiple proinflammatory cytokines and chemokines is detected in the brain of Fr98-infected mice (Peterson *et al*, 2001, 2004; Peterson and Chesebro, 2006). This cytokine and chemokine response is not simply associated with virus infection because infection with a highly-related but avirulent retrovirus, Fr54, does not induce chemokine expression (Peterson *et al*, 2001; Peterson and Chesebro, 2006). Despite the significant increase in chemokine expression in the CNS following Fr98 infection, no substantial infiltrate of lymphocytic cells is observed in the brain by histological examination and no increase in gene expression of T-cell or B-cell markers is detected (Peterson *et al*, 2001; Portis *et al*, 1995; Robertson *et al*, 1997). Instead, the primary neuroinflammatory pathology associated with Fr98-induced disease is microglial nodules and the activation of microglia and astrocytes (Portis *et al*, 1995; Robertson *et al*, 1997). As Fr98 primarily infects microglia and endothelia, but not neurons, the mechanism of neuropathogenesis appears to be indirect (Portis *et al*, 1995; Robertson *et al*, 1997).

Of the 10 cytokines and chemokines up-regulated prior to the onset of Fr98-induced disease, 3 interact with the chemokine receptor CCR1. *Ccl3* and *Ccl5* mRNA and protein levels are up-regulated in Fr98-infected mice compared to mock or Fr54-infected controls (Peterson *et al*, 2001; Peterson and Chesebro, 2006). Kinetic analysis revealed that mRNA expression of *Ccl3* and *Ccl5* occurs prior to the onset of clinical disease in Fr98-infected mice, suggesting a possible role for these factors in the induction of pathogenesis (Peterson *et al*, 2001). mRNA expression of a third ligand, *Ccl7* (MCP-3), is also up-regulated prior to disease but returns to basal levels by the time of clinical signs appear (Peterson *et al*, 2004). In the current study, we used knockout mice to examine the role of CCR1 in the neuroinflammatory response to Fr98 infection as well as in the development of neurological disease.

Results

Fr98-induced up-regulation of CCR1 and CCR1 ligands

Both CCL3 and CCL5 bind to multiple chemokine receptors, including CCR1 and CCR5 (Boring *et al*, 1996; Gao *et al*, 1993; Neote *et al*, 1993). Previous studies demonstrated that CCR5 was not required for Fr98-induced neurological disease (Peterson *et al*, 2004). To analyze the potential role of CCR1 in Fr98 infection, we backcrossed CCR1-deficient C57BL/6 mice with susceptible 129SvEv mice for a minimum of seven generations. To confirm that Fr98 infection induced up-regulation of chemokines in CCR1+/− mice, brain tissue from clinically ill Fr98-infected CCR1+/− mice was analyzed by multiplex bead array. Increased protein levels of CCL2, CCL3, CCL4 (MIP-1β), and CCL5 were detected in Fr98-infected CCR1+/− mice as compared to age-matched uninfected controls (Figure 1A). *Ccr1* mRNA expression in CCR1+/− mice was analyzed by quantitative real-time polymerase chain reaction (PCR) analysis. A significant increase in *Ccr1* mRNA expression was observed in Fr98-infected mice as compared to uninfected controls (Figure 1B). This increase was not consistently detected at the protein level by Western blot detection (data not shown), possibly due to the sensitivity of the assay. However, the increase in *Ccr1* mRNA and the increase in protein expression of CCR1 ligands suggests that CCR1 stimulation may contribute to Fr98 mediated pathogenesis.

Cellular expression of Ccl3 *and* Ccl5

To determine if the CCR1 ligands, *Ccl3* and *Ccl5*, were produced by the same cell types in the CNS, we analyzed sagittal brain tissue sections from Fr98 and mock-infected mice by *in situ* hybridization and immunohistochemistry. Cells expressing *Ccl3* or *Ccl5* mRNA were found in multiple regions of the brain and were generally located in the proximity of, but not in, blood vessels (Figure 2). *Ccl3*-positive cells were found to dual stain with the microglia/macrophage marker Iba1 and had processes indicating that these cells were microglia (Figure 2A). *Ccl5*-positive cells were found in clusters and did not appear to be microglia cells as they lacked Iba1 positive processes (Figure 2B). A few of the *Ccl5*-positive cells appeared to be macrophages as they were also Iba1 positive, but did not have Iba1 processes (Figure 2B). Thus, although *Ccl3* and *Ccl5* are both up-regulated by Fr98 infection, they appear to be produced by different cell types.

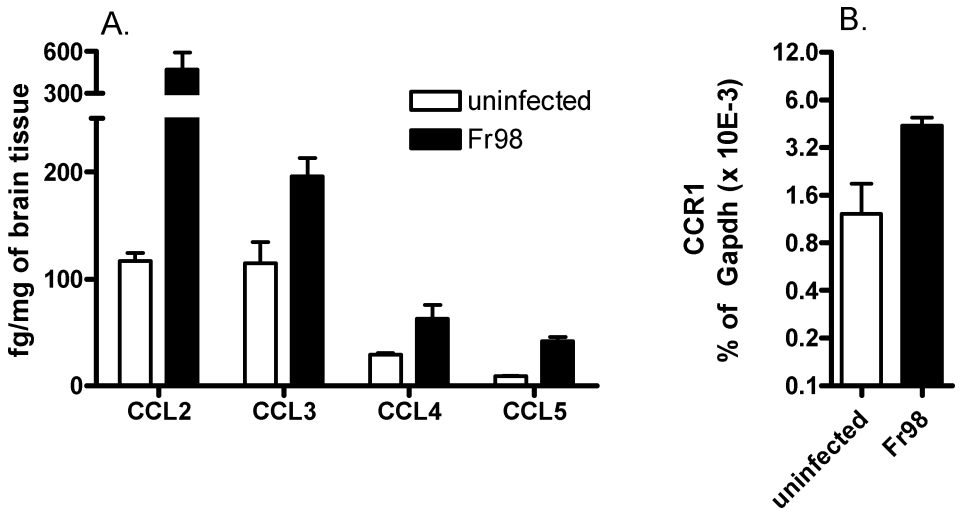

Figure 1 **(A)** Chemokine protein levels in brain tissue from uninfected and Fr98-infected CCR1+/− mice. Neonatal CCR1+/− mice were infected by i.p. inoculation of 10^4 FFU of Fr98 within 24 h of birth. Mice were then followed for the development of clinical signs of severe ataxia, seizures, or death. Brain tissue from Fr98-infected mice at the time of disease and age-matched uninfected controls were processed for multiplex protein analysis as described in Materials and Methods. Protein levels were determined using an in-plate standard curve and subsequently converted to fg/mg of brain tissue. Results are the mean ± standard error of four mice per group. Statistical analysis was done by Mann-Whitney analysis. Statistical differences between uninfected and Fr98-infected groups were $P < .05$ for all four chemokines. **(B)** Ccr1 mRNA expression in uninfected and Fr98-infected CCR1+/− mice. Mice were infected as described in **(A)**. Brain tissue was processed for mRNA as described in Materials and Methods. Ccr1 mRNA expression was detected as described in Materials and Methods. Data are the mean ± standard error for eight mice per group. Statistical analysis was done by Mann-Whitney analysis. Statistical difference between uninfected and Fr98-infected mice was $P = .002$.

Effect of CCR1 deficiency on Fr98-induced neurological disease

To analyze the role of CCR1 in neuropathogenesis, heterozygous CCR1+/− and CCR1−/− littermates were infected with Fr98 within 24 h of birth and followed for the development of clinical neurological disease. No significant difference in the development of neurological disease was observed in CCR1−/− mice compared to CCR1+/− mice (Figure 3). Additionally, no significant difference in disease development was observed between Fr98-infected CCR1+/− mice and the parental 129SvEv strain (Figure 3). Similar to previous findings with Fr98 virus infection, saggital sections of cerebrum, cerebellum, and brainstem from virus-infected CCR1+/− and CCR1−/− mice did not contain substantive histologic evidence of neuronal degeneration or inflammation as compared to age matched uninfected controls (data not shown). Thus, despite the up-regulation of two CCR1 ligands and *Ccr1* mRNA following Fr98-infection, CCR1 does not appear to play a significant role in Fr98-induced neurological disease.

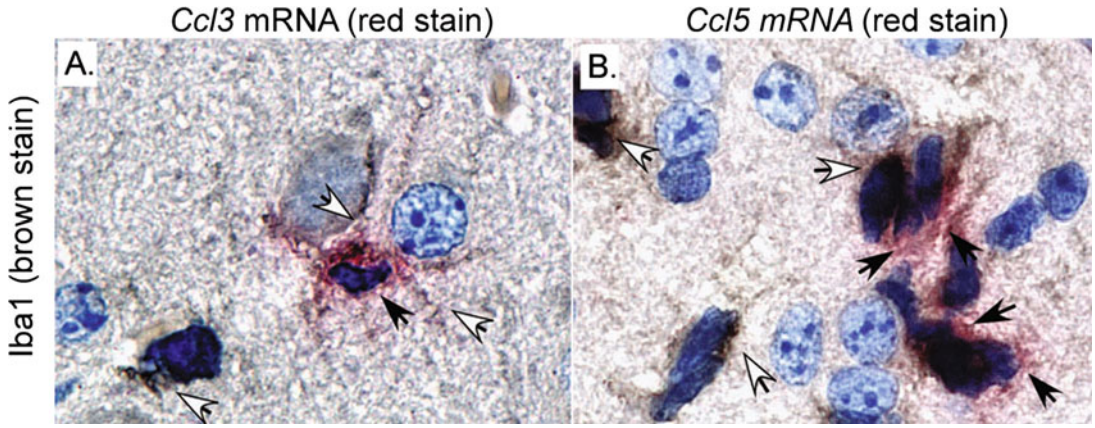

Figure 2 Cellular localization of Ccl3 and Ccl5 mRNA expression in the brain. Midsagittal sections from Fr98-infected 129SvEv mice were analyzed for Ccl3 **(A)** and Ccl5 **(B)** mRNA expression by *in situ* hybridization (red cytoplasmic color, black arrows). No Ccl3 or Ccl5 positive cells were observed in mock-infected mice (data not shown). Sections were then incubated with anti-Iba1 antibodies for the detection of microglia and macrophages (brown color, white arrows). Sections were counterstained with hematoxylin. Photos were taken with a digital camera. 1000× magnification.

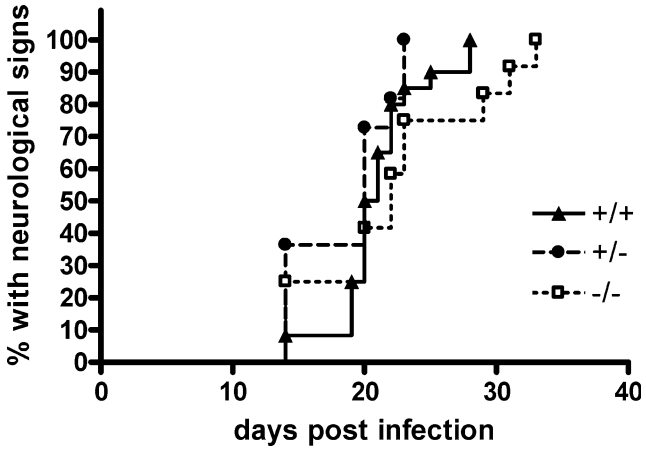

Figure 3 Development of neurological disease in CCR+/− and CCR1−/− 129SvEv mice infected with Fr98. Neonatal CCR1+/− and CCR1−/− littermates were infected by i.p. inoculation of 10^4 FFU of Fr98 within 24 h of birth. Mice were then followed for the development of clinical signs of severe ataxia, seizures, or death. At the time of clinical disease or at 21 days of age, mice were genotyped for CCR1 wild-type and knockout alleles. Data are presented as the percentage of mice with severe ataxia or dead of 11 CCR1+/− mice and 12 CCR1−/− mice. Statistical analysis was done with the Kaplan-Meir survival curve analysis. The *P* value for the two-tailed logrank (Mantel-Haenszel) test for trend was .093.

Increased expression of the CCR1 ligands CCL3, CCL5, and CCL7 in wild-type and knockout mice

To determine if CCR1 had an autocrine affect in the upregulation of the CCR1 ligands *Ccl3, Ccl5*, and *Ccl7*, we analyzed mRNA expression of these genes in heterozygous and homozygous CCR1-deficient mice. Interestingly, mRNA levels of *Ccl3, Ccl5*, and *Ccl7* were upregulated to a similar extent in both Fr98-infected CCR1+/− and CCR1−/− mice compared to CCR1+/− and CCR1−/− uninfected controls (Figure 4). Thus, CCR1 was not necessary for the upregulation of CCR1 ligands following Fr98 infection.

Effect of CCR1 deficiency on Fr98-induced astrocyte activation

In vitro cultured astrocytes express CCR1 and are activated in response to stimulation with CCL3 and CCL5 (Luo *et al*, 2002). Activation of astrocytes

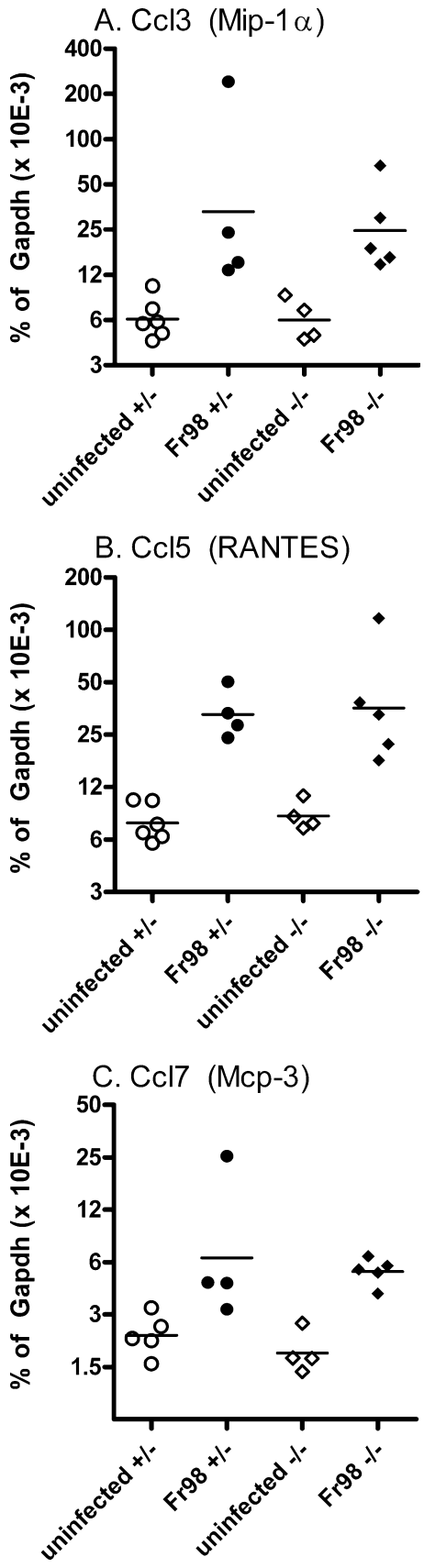

Figure 4

Figure 4 Expression of Ccl3 **(A)** or Ccl5 **(B)** and Ccl7 **(C)** mRNA following polytropic retrovirus infection. Brain tissue was removed from uninfected and Fr98-infected CCR1+/− and CCR1 −/− mice at 17 to 30 days post infection and processed for mRNA as described in Materials and Methods. mRNA expression was determined using quantitative PCR with triplicate wells for each sample. Data are shown as the gene expression as % of Gapdh mRNA expression. Each symbol represents an individual animal. Statistical analysis was done using a one-way ANOVA with the Newman-Keuls post-test. The *P* values for uninfected versus Fr98-infected CCR1+/− or CCR1−/− mice was <.05 for all three chemokines. No significant difference ($P > .05$) was observed between Fr98-infected CCR1+/− mice and Fr98-infected CCR1−/− mice. Data shown represent one of two similar experiments.

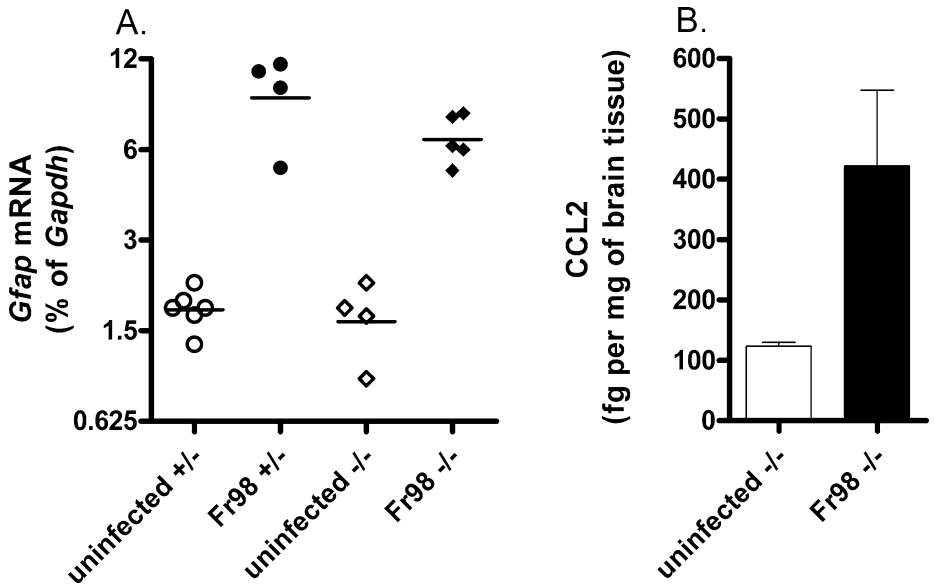

Figure 5 **(A)** Expression of Gfap mRNA following polytropic retrovirus infection. mRNA expression was analyzed as described for Figure 4. Data are shown as the gene expression as % of Gapdh mRNA expression. Each symbol represents an individual animal. Data shown represents one of two similar experiments. Statistical analysis was done using a one-way ANOVA with the Newman-Keuls post-test. The *P* values for uninfected versus Fr98-infected CCR1+/− or CCR1−/− mice was <.001. No statistical difference was found between Fr98-infected CCR1+/− mice and Fr98-infected CCR1−/− mice. **(B)** CCL2 protein levels in uninfected and Fr98-infected CCR1 deficient mice. Data are shown as the fg of CCL2 per mg of brain tissue. Analysis was completed as described for Figure 1A. Results are the mean ± standard error of four mice per group. Statistical analysis was done by Mann-Whitney analysis, with a *P* value of .028 for uninfected versus Fr98-infected CCR1−/− mice.

is one of the primary pathologies associated with Fr98 infection (Portis *et al*, 1995; Robertson *et al*, 1997). To examine if CCR1 was involved in Fr98-induced activation of astrocytes, we analyzed heterozygous and homozygous CCR1-deficient mice for the expression of glial fibrillary acidic protein (GFAP), which is up-regulated on astrocytes following activation. No difference was observed in Fr98-induced activation of astrocytes between heterozygous and homozygous CCR1-deficient mice either by real-time PCR analysis (Figure 5A) or immunohistochemistry (data not shown). Additionally, CCL2, which is primarily produced by astrocytes (Peterson *et al*, 2004), was significantly up-regulated in Fr98-infected CCR1-deficient mice compared to uninfected controls (Figure 5B). The level of CCL2 expression in Fr98-infected CCR1−/− mice was similar to levels in Fr98-infected wild-type mice (Figure 1A). Thus, CCR1 does not appear to play a role in Fr98-induced astrocyte activation, as measured by either GFAP or CCL2 expression.

Effect of CCR1 on virus replication in the CNS

CCR1, as well as other chemokine receptors, are often involved in controlling virus infections, by regulating the trafficking of immune cells to the sites of infection. To determine if CCR1 altered virus replication in the CNS, we analyzed virus levels in the brain by real-time PCR and immunohistochemistry. No difference in virus gag RNA levels was observed between heterozygous and homozygous CCR1-deficient mice (Figure 6). Additionally, no notable difference in virus spread or virus infection of endothelia or microglia was observed between heterozygous and homozygous CCR1-deficient mice by immunohistochemistry analysis (data not shown). Thus, despite the increased expression of the CCR1 ligands CCL3, CCL5, and CCL7 during the course of Fr98 infection of the CNS, it appears that CCR1 does not influence the pathogenesis of Fr98 infection, as deficiency in CCR1 does not impact the infection or pathogenesis of the virus and does not appear to be necessary for the induction of the innate immune response.

Discussion

In the current study, we analyzed the effect of the chemokine receptor CCR1 on the nonlymphocyte mediated neuroinflammation induced by the murine retrovirus Fr98. Previous studies had shown a correlation between the expression of CCR1 ligands, CCL3 and CCL5, and the development of neurological disease (Peterson *et al*, 2001; Peterson and Chesebro, 2006). Surprisingly, a significant difference in disease development was not observed between CCR1 deficient mice and CCR1 heterozygous mice (Figure 3). Additionally, CCR1 did not appear to have a significant role in the induction of the neuroinflammatory response to Fr98 infection, as CCR1 deficiency did not alter the up-regulation of proinflammatory chemokines (Figure 4) or astrocyte activation (Figure 5) following Fr98 infection.

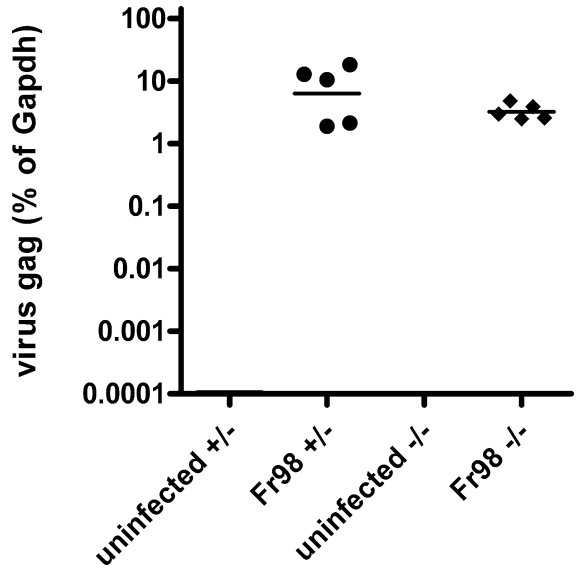

Figure 6 Expression of virus gag mRNA in CCR1 + /− and CCR1−/− mice infected with Fr98. mRNA expression was analyzed as described for Figure 4. Data are shown as the gene expression as a ratio of Gapdh mRNA expression. Each symbol represents an individual animal. Data shown represents one of two similar experiments. Statistical analysis was done using a one-way ANOVA with the Newman-Keuls post-test. The P values for uninfected versus Fr98-infected CCR1+/− or CCR1 − /− mice was <.001. The difference between virus gag expression in Fr98-infected CCR1+/− mice and Fr98-infected CCR1−/− mice was not significant ($P > .05$).

Furthermore, CCR1 did not appear to be important for the regulation of virus infection, as no difference in virus levels were observed in CCR1+/− or CCR1−/− mice (Figure 6).

CCR1 protein is detected in the rodent brain as early as 3 days post birth, with peak expression between 7 to 21 days of age (Cowell and Silverstein, 2003). CCR1 has been detected on neuronal cells, including Purkinje cells and molecular interneurons, as well as astrocytes and endothelia in neonates (Cowell and Silverstein, 2003). As murine astrocytes have been shown to express CCR1, and to migrate in response to CCL3 stimulation (Tanabe *et al*, 1997), it was surprising that CCR1 deficiency did not affect Fr98-induced activation of astrocytes. A possible explanation for this is the presence of CCR5 on astrocytes (Dorf *et al*, 2000). CCR5 binds to CCL3 and may induce a similar activation signal to astrocytes following Fr98 infection (Boring *et al*, 1996). Alternatively, other mechanisms such as direct virus stimulation or the production of other cytokines such as tumor necrosis factor α (TNFα) may play a more prominent role in the activation of astrocytes.

The promiscuous nature of chemokines and chemokine receptors may also account for the lack of an effect of CCR1 deficiency on Fr98 neuropathogenesis. As stated above, CCR5 is an additional receptor for CCL3 (Boring *et al*, 1996). Moreover, CCR3, CCR4, and CCR5 are additional receptors for CCL5 and CCR2 is an additional receptor for CCL7 (Blanpain *et al*, 2001; Kurihara and Bravo, 1996). Previous studies with CCR5-deficient mice demonstrated that CCR5 is also not required for Fr98 mediated pathogenesis (Peterson *et al*, 2004). In contrast, CCR2, which has a less promiscuous set of ligands, did contribute to Fr98 pathogenesis (Peterson *et al*, 2004). Possibly, the deficiency in CCR1 is overcome by the ability of CCR1 ligands to signal through CCR5, and vice versa.

CCR1 has been shown to play an nonredundant role in the neuropathogenesis in other models of neuropathogenesis, including experimental autoimmune encephalitis (EAE). In EAE, CCR1-deficient mice had significantly reduced incidence and severity of clinical signs compared to wildtype controls (Rottman *et al*, 2000). This was associated with decreased cellular infiltrates in the CNS of CCR1-deficient mice (Rottman *et al*, 2000). Unlike EAE, Fr98-induced neuropathogenesis is not associated with substantial influx of inflammatory cells in the brain, possibly due to the neonatal age of virus infection (Portis *et al*, 1995). Thus, CCR1 may play a critical role in the recruitment of inflammatory cells to the CNS, but may not influence pathogenesis in the absence of inflammation.

This study also indicates that CCR1 does not affect Fr98 infection and replication in the brain. CCR1 was important for recruiting immune cells to the site of virus infection and eliciting antiviral host defense for both paramyxovirus and herpes simplex virus (Domachowske *et al*, 2000b; Sorensen and Paludan 2004), but was not responsible for regulating host defense or inflammation following respiratory syncytial virus or West Nile virus infections (Domachowske *et al*, 2000a; Glass *et al*, 2005). In the instance of West Nile virus, the host response was mediated by CCR5, rather than CCR1 (Glass *et al*, 2005). In the case of Fr98 infection, a strong adaptive immune response does not appear to be generated. Additionally, lymphocytes do not appear to play a role in regulating Fr98 virus levels in the brain, as no significant numbers of CD3 or B cells are detected in the brain following Fr98 infection. Thus, the lack of a strong adaptive immune response to Fr98 may limit the role of specific chemokine or chemokine receptors in controlling virus infection. Instead, virus replication and spread may be limited by the requirement of cell division for infection and replication by murine retroviruses. Possibly, the limited number of dividing microglial cells in the brain along with the lack of infiltrating cell types may suppress the replication of Fr98 in the CNS and limit the spread of the virus in lieu of protective immune responses.

Materials and methods

Mice

CCR1-deficient C57BL/6 mice were a gift from Phil Murphy (National Institute of Allergy and Infectious Diseases, National Institutes of Health) and were

backcrossed for seven generations or more with 129S6 (129SvEv) mice. Mice were subsequently intercrossed to generate CCR1+/− and CCR1−/− breeder pairs. These mice were then mated to produce litters of 50% CCR1+/− and 50% CCR1−/− mice. All mice were maintained at Louisiana State University School of Medicine or at Rocky Mountain Laboratories. All animal experiments were carried out in accordance with the regulations of the Louisiana State University Animal Care and Use Committee and the guidelines of the National Institutes of Health.

Detection of CCR1 wild-type and knockout alleles
CCR1 wild-type alleles were detected using the forward primers m3 × s300 (GCT GTC TCT GAT CTG GTC TTC CTT) and m3 × s600 (GAG TTC ACT CAC CGT ACC TGT AGC) and reverse primer m3×RT3 (TTT GAC CTT CTT CTC ACT GGG TCT TC) to generate products of 480 and 180 bp . CCR1-neomycin alleles were detected using the forward primer m3 × s300 and reverse primers m3 × RT3 and AneoPCR (TGG GTG GAG AGG CTT TTT GCT TCC TCT TGC) to generate products of 1.4 kb and 150 bp. Tail DNA was purified using Qiagen Tail Isolation columns (Qiagen, Valencia, CA) or Genomic DNA Isolation columns (Sigma Aldrich, St. Louis, MO) following manufacturers' instructions. Products were amplified using the following conditions; 94°C for 3 min, followed by 25 cycles of 94°C for 30 s, 60°C for 30 s and 70°C for 30 s, followed by 72°C for 10 min.

Virus infection of mice
The construction of virus clone Fr98 has been previously described (Portis *et al*, 1995). Virus stocks were prepared from the supernatants of confluent cultures of Fr98-infected *Mus dunni* fibroblast cells. Virus titers were determined by focus forming assays using the envelope specific monoclonal antibodies 514 and 720 (Robertson *et al*, 1991). Mice were infected within 24 h of birth with 100 μl of cell culture supernatant containing 10^4 focus-forming units (FFU) of Fr98. Mice were observed daily for clinical signs of CNS disease, which was characterized by obvious signs of ataxia and/or seizures (Portis *et al*, 1995).

Histological analysis
Histologic samples were immersion fixed in 3.7% neutral-buffered formalin for 24 h and then embedded in paraffin. Paraffin-embedded blocks of hemisected brain were cut in 4-μm para-central sagittal sections that included cerebrum, cerebellum, and brainstem. These sections were adhered to a glass slide and stained with hematoxylin and eosin (H&E) using an automated histologic stainer. Slides were examined for neuronal degeneration and histopathologic evidence of inflammation.

Multiplex assay for chemokine expression
Infected mice and uninfected controls were exsanguinated by axillary incision under deep isoflurane anesthesia, followed by cervical dislocation. Brains were removed from infected mice at the time of neurological disease and at similar time points for age-matched controls. Brain tissue was then divided into two sections by midsagittal dissection. Tissue sections were immediately frozen in liquid nitrogen and stored at −80°C or placed in 3.7% neutral-buffered formalin and processed for histology. To generate tissue homogenates for analysis by multiplexing, samples were weighed and then homogenized in 400 μl of Bio-Plex cell lysis solution (Bio-Rad Laboratories, Hercules, CA) containing Complete Mini protease inhibitors (Roche Applied Science, Indianapolis, IN) and 2 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich). Samples were homogenized using Kontes Disposable Pellet Pestles and a cordless motor (Fisher Scientific, Hampton, NH) and diluted to 400 μg/ml in lysis buffer. Cellular debris was removed by centrifugation at 4500 × g for 15 min at 4°C. Samples were analyzed for cytokine protein expression using a Bio-Rad x-plex assay or BioSource 20 plex assay (BioSource, Camarillo, CA) on a Bio-Plex instrument (Bio-Rad) following manufacturers' instructions. Samples were calculated as pg/ml using standard curve from in-plate standards and subsequently converted to fg/mg of brain tissue.

Preparation of RNA for real-time PCR analysis
Total RNA from frozen sagittal sections of brain tissue was prepared using TriZol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Total RNA for each sample was subsequently treated with DNase (Ambion, Austin, TX) for 30 min to remove any contaminating DNA, and purified over Zymo RNA cleanup columns (Zymo Research, Orange, CA). RNA was reverse-transcribed using iScript reverse transcription kit (Bio-Rad) following manufactures' instructions. The reverse transcription reaction was diluted four fold in RNase free water prior to use in real-time PCR reactions.

Real-time PCR analysis of gene expression
Primers for real-time PCR analysis are shown in Table 1 and were designed using Primer3 software (Rozen and Skaletsky, 2000). Primer pairs were blasted against the GenBank, European Molecular Biology Laboratory (EMBL), DNA Data Bank of Japan (DDBJ), and Protein Data Bank databases using the National Center for Bioinformatics (NCBI) website to confirm specificity. Reactions were run in triplicate using SYBR green mix with Rox (Bio-Rad) in a 10 μl volume with approximately 10 ng of DNase-treated total RNA and 500 nM each of forward and reverse primers. Samples were run on an ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). Melting curve analysis

Table 1 List of real-time PCR primers

Gene	*Forward primer*	*Reverse primer*
Ccl3	ACCATGACACTCTGCAACCA	GATGAATTGGCGTGGAATCT
Ccl5	GGTTTCTTGATTCTGACCCTGTA	AGGACCGGAGTGGGAGTAG
Ccl7	TTCTGTGCCTGCTGCTCATA	TTGGGGATCTTTTGTTTCTTG
Gapdh	AACGACCCCTTCATTGAC	TCCACGACATACTCAGCAC
Gfap	CGTTTCTCCTTGTCTCGAATGAC	TCGCCCGTGTCTCCTTGA
Fb29gag	CCCGTGGCGGATTCTACT	TCGGAGAAAGAGGGGTTGTTA

demonstrated a single product for each primer pair. Lack of DNA contamination was confirmed by running reactions without reverse transcriptase. Untranscribed controls were either negative for all genes after 40 cycles or had at least a 1000-fold lower expression level than the analyzed samples. The cycle number at which each sample reached a fixed fluorescence threshold (C_T) was used to quantify gene expression. Data were calculated as the difference in C_T value ($\log_2$) for *Gapdh* minus the C_T value of the gene of interest for each sample ($\Delta C_T = C_T$ *Gapdh* − C_T gene of interest) to control for variations in RNA amounts in each sample. The data are presented as a percentage of *Gapdh* expression for each gene of interest.

Generation of probes for in situ *hybridization*
Digoxigenin (DIG)-labeled sense and antisense probes were generated using the DIG RNA labeling kit (Roche Molecular Biochemicals), T7 or T3 polymerase, and 1 μg of linearized template DNA for *Ccl3* or *Ccl5* (BD Biosciences) following manufacturers' instructions. DIG-labeled probes were purified over RNAeasy columns (Qiagen, Valencia, CA). Probe concentration was calculated by limiting serial dilutions of the probes on nylon membrane with comparison to a known standard (Roche Molecular Biochemicals).

In situ *hybridization*
In situ hybridization was performed as previously described (Peterson *et al*, 2004). DIG-labeled probes were detected with alkaline phosphatase (AP)-labeled anti-DIG antibodies (Roche Molecular Biochemicals) using the Fast-Red substrate (Roche Molecular Biochemicals). Sections were then either counterstained with hematoxylin or used for immunohistochemistry. For subsequent immunohistochemical analysis, sections were blocked in 1% normal goat serum (NGS) in phosphate buffered saline (PBS). Sections were then incubated with a 1/200 dilution of rabbit anti-Iba1 (Wako Chemicals, Richmond, VA), in 1% NGS. Iba1-positive cells were detected using horse radish peroxidase (HRP)-conjugated anti-rabbit antibodies (Zymed Laboratories, San Francisco, CA). Sections were developed with 3′3′ diaminobenzidine (DAB) substrate (Sigma-Aldrich), counterstained with hematoxylin and coverslipped.

Statistical Analysis
All statistical analysis was completed using GraphPad Prism software (San Diego, CA).

References

Andjelkovic AV, Song L, Dzenko KA, Cong H, Pachter JS (2002). Functional expression of CCR2 by human fetal astrocytes. *J Neurosci Res* **70:** 219–231.

Bajetto A, Bonavia R, Barbero S, Florio T, Costa A, Schettini G (1999). Expression of chemokine receptors in the rat brain. *Ann N Y Acad Sci* **876:** 201–209.

Blanpain C, Buser R, Power CA, Edgerton M, Buchanan C, Mack M, Simmons G, Clapham PR, Parmentier M, Proudfoot AE (2001). A chimeric MIP-1alpha/RANTES protein demonstrates the use of different regions of the RANTES protein to bind and activate its receptors. *J Leukoc Biol* **69:** 977–985.

Bonavia R, Bajetto A, Barbero S, Pirani P, Florio T, Schettini G (2003). Chemokines and their receptors in the CNS: expression of CXCL12/SDF-1 and CXCR4 and their role in astrocyte proliferation. *Toxicol Lett* **139:** 181–189.

Boring L, Gosling J, Monteclaro FS, Lusis AJ, Tsou CL, Charo IF (1996). Molecular cloning and functional expression of murine JE (monocyte chemoattractant protein 1) and murine macrophage inflammatory protein 1alpha receptors: evidence for two closely linked C—C chemokine receptors on chromosome 9. *J Biol Chem* **271:** 7551–7558.

Cowell RM, Silverstein FS (2003). Developmental changes in the expression of chemokine receptor CCR1 in the rat cerebellum. *J Comp Neurol* **457:** 7–23.

Domachowske JB, Bonville CA, Gao JL, Murphy PM, Easton AJ, Rosenberg HF (2000a). MIP-1 alpha is produced but it does not control pulmonary inflammation in response to respiratory syncytial virus infection in mice. *Cell Immunol* **206:** 1–6.

Domachowske JB, Bonville CA, Gao JL, Murphy PM, Easton AJ, Rosenberg HF (2000b). The chemokine macrophage-inflammatory protein-1 alpha and its receptor CCR1

control pulmonary inflammation and antiviral host defense in paramyxovirus infection. *J Immunol* **165:** 2677–2682.

Dorf ME, Berman MA, Tanabe S, Heesen M, Luo Y (2000). Astrocytes express functional chemokine receptors. *J Neuroimmunol* **111:** 109–121.

Dufour JH, Dziejman M, Liu MT, Leung JH, Lane TE, Luster AD (2002). IFN-gamma-inducible protein 10 (IP-10; CXCL10)-deficient mice reveal a role for IP-10 in effector T cell generation and trafficking. *J Immunol* **168:** 3195–3204.

Eugenin EA, D'Aversa TG, Lopez L, Calderon TM, Berman JW (2003). MCP-1 (CCL2) protects human neurons and astrocytes from NMDA or HIV-tat-induced apoptosis. *J Neurochem* **85:** 1299–1311.

Flynn G, Maru S, Loughlin J, Romero IA, Male D (2003). Regulation of chemokine receptor expression in human microglia and astrocytes. *J Neuroimmunol* **136:** 84–93.

Gao JL, Kuhns DB, Tiffany HL, McDermott D, Li X, Francke U, Murphy PM (1993). Structure and functional expression of the human macrophage inflammatory protein 1 alpha/RANTES receptor. *J Exp Med* **177:** 1421–1427.

Glass WG, Lim JK, Cholera R, Pletnev AG, Gao JL, Murphy PM (2005). Chemokine receptor CCR5 promotes leukocyte trafficking to the brain and survival in West Nile virus infection. *J Exp Med* **202:** 1087–1098.

Huang D, Tani M, Wang J, Han Y, He TT, Weaver J, Charo IF, Tuohy VK, Rollins BJ, Ransohoff RM (2002). Pertussis toxin-induced reversible encephalopathy dependent on monocyte chemoattractant protein-1 overexpression in mice. *J Neurosci* **22:** 10633–10642.

Kelder W, McArthur JC, Nance-Sproson T, McClernon D, Griffin DE (1998). Beta-chemokines MCP-1 and RANTES are selectively increased in cerebrospinal fluid of patients with human immunodeficiency virus-associated dementia. *Ann Neurol* **44:** 831–835.

Klein RS, Williams KC, Varez-Hernandez X, Westmoreland S, Force T, Lackner AA, Luster AD (1999). Chemokine receptor expression and signaling in macaque and human fetal neurons and astrocytes: implications for the neuropathogenesis of AIDS. *J Immunol* **163:** 1636–1646.

Kurihara T, Bravo R (1996). Cloning and functional expression of mCCR2, a murine receptor for the C-C chemokines JE and FIC. *J Biol Chem* **271:** 11603–11607.

Luo Y, Berman MA, Zhai Q, Fischer FR, Abromson-Leeman SR, Zhang Y, Kuziel WA, Gerard C, Dorf ME (2002). RANTES stimulates inflammatory cascades and receptor modulation in murine astrocytes. *Glia* **39:** 19–30.

McManus C, Berman JW, Brett FM, Staunton H, Farrell M, Brosnan CF (1998). MCP-1, MCP-2 and MCP-3 expression in multiple sclerosis lesions: an immunohistochemical and in situ hybridization study. *J Neuroimmunol* **86:** 20–29.

Neote K, DiGregorio D, Mak JY, Horuk R, Schall TJ (1993). Molecular cloning, functional expression, and signaling characteristics of a C—C chemokine receptor. *Cell* **72:** 415–425.

Peterson KE, Chesebro B (2006). Influence of proinflammatory cytokines and chemokines on the neuropathogenesis of oncornavirus and immunosuppressive lentivirus infections. *Curr Top Microbiol Immunol* **303:** 67–95.

Peterson KE, Errett JS, Wei T, Dimcheff DE, Ransohoff R, Kuziel WA, Evans L, Chesebro B (2004). MCP-1 and CCR2 contribute to non-lymphocyte-mediated brain disease induced by Fr98 polytropic retrovirus infection in mice: role for astrocytes in retroviral neuropathogenesis. *J Virol* **78:** 6449–6458.

Peterson KE, Robertson SJ, Portis JL, Chesebro B (2001). Differences in cytokine and chemokine responses during neurological disease induced by polytropic murine retroviruses map to separate regions of the viral envelope gene. *J Virol* **75:** 2848–2856.

Portis JL, Czub S, Robertson S, McAtee F, Chesebro B (1995). Characterization of a neurologic disease induced by a polytropic murine retrovirus: evidence for differential targeting of ecotropic and polytropic viruses in the brain. *J Virol* **69:** 8070–8075.

Rezaie P, Trillo-Pazos G, Everall IP, Male DK (2002). Expression of beta-chemokines and chemokine receptors in human fetal astrocyte and microglial co-cultures: potential role of chemokines in the developing CNS. *Glia* **37:** 64–75.

Robertson MN, Miyazawa M, Mori S, Caughey B, Evans LH, Hayes SF, Chesebro B (1991). Production of monoclonal antibodies reactive with a denatured form of the Friend murine leukemia virus gp70 envelope protein: use in a focal infectivity assay, immunohistochemical studies, electron microscopy and western blotting. *J Virol Methods* **34:** 255–271.

Robertson SJ, Hasenkrug KJ, Chesebro B, Portis JL (1997). Neurologic disease induced by polytropic murine retroviruses: neurovirulence determined by efficiency of spread to microglial cells. *J Virol* **71:** 5287–5294.

Rottman JB, Slavin AJ, Silva R, Weiner HL, Gerard CG, Hancock WW (2000). Leukocyte recruitment during onset of experimental allergic encephalomyelitis is CCR1 dependent. *Eur J Immunol* **30:** 2372–2377.

Rozen S, Skaletsky HJ (2000). Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* **132:** 365–386.

Schmidtmayerova H, Nottet HS, Nuovo G, Raabe T, Flanagan CR, Dubrovsky L, Gendelman HE, Cerami A, Bukrinsky M, Sherry B (1996). Human immunodeficiency virus type 1 infection alters chemokine beta peptide expression in human monocytes: implications for recruitment of leukocytes into brain and lymph nodes. *Proc Natl Acad Sci U S A* **93:** 700–704.

Sorensen LN, Paludan SR (2004). Blocking CC chemokine receptor (CCR) 1 and CCR5 during herpes simplex virus type 2 infection in vivo impairs host defence and perturbs the cytokine response. *Scand J Immunol* **59:** 321–333.

Tanabe S, Heesen M, Berman MA, Fischer MB, Yoshizawa I, Luo Y, Dorf ME (1997). Murine astrocytes express a functional chemokine receptor. *J Neurosci* **17:** 6522–6528.

Tran PB, Ren D, Veldhouse TJ, Miller RJ (2004). Chemokine receptors are expressed widely by embryonic and adult neural progenitor cells. *J Neurosci Res* **76:** 20–34.

Van Der Voorn P, Tekstra J, Beelen RH, Tensen CP, Van Der Valk P, De Groot CJ (1999). Expression of MCP-1 by reactive astrocytes in demyelinating multiple sclerosis lesions. *Am J Pathol* **154:** 45–51.

Xia MQ, Bacskai BJ, Knowles RB, Qin SX, Hyman BT (2000). Expression of the chemokine receptor CXCR3 on neurons and the elevated expression of its ligand IP-10

in reactive astrocytes: in vitro ERK1/2 activation and role in Alzheimer's disease. *J Neuroimmunol* **108:** 227–235.

Xia MQ, Qin SX, Wu LJ, Mackay CR, Hyman BT (1998). Immunohistochemical study of the beta-chemokine receptors CCR3 and CCR5 and their ligands in normal and Alzheimer's disease brains. *Am J Pathol* **153:** 31–37.

Youssef S, Wildbaum G, Karin N (1999). Prevention of experimental autoimmune encephalomyelitis by MIP-1alpha and MCP-1 naked DNA vaccines. *J Autoimmun* **13:** 21–29.