Increased proinflammatory cytokine and chemokine responses and microglial infection following inoculation with neural stem cells infected with polytropic murine retroviruses

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Abstract

Proinflammatory cytokines and chemokines are often detected in brain tissue of patients with neurological diseases such as multiple sclerosis (MS), HIV-associated dementia (HAD) and Alzheimer’s disease (AD). We have utilized a mouse model of retrovirus-induced neurological disease to examine how these proinflammatory responses contribute to neuropathogenesis. In previous studies with this model, a correlation was found between neurovirulence and cytokine and chemokine expression. However, it was unclear whether the induction of these cytokines and chemokines was in response to specific virus envelope determinants or was regulated by the level of virus infection in the brain. In the current study, we demonstrated that multiple polytropic retroviruses induced cytokine and chemokine mRNA expression following increased virus levels in the brain. Increased virus levels of polytropic viruses also correlated with increased neuropathogenesis. In contrast, the ecotropic retrovirus, FB29, did not induce cytokine or chemokine mRNA expression or neurological disease, despite virus levels either similar to or higher than the polytropic retroviruses. As polytropic and ecotropic viruses utilize different receptors for entry, these receptors may play a critical role in the induction of these innate immune responses in the brain.

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Introduction

Insult or injury to the central nervous system (CNS) is often associated with the activation of the innate immune response and the production of proinflammatory cytokines and chemokines (Bajetto et al., 2001; Mennicken et al., 1999). Additionally, increased cytokine/chemokine expression correlates with pathogenesis in several neurological disorders including multiple sclerosis (MS), Alzheimer’s disease (AD), and HIV-associated dementia (HAD) (Conant et al., 1998; Eikelenboom et al., 2002; Glass et al., 1993; Gonzalez et al., 2002; Mahad and Ransohoff, 2003; McManus et al., 1998; Sun et al., 2003; VanDer et al., 1999; Wesselingh et al., 1993; Xia and Hyman, 1999). Therefore, it is important to determine the factors that trigger the induction of proinflammatory responses and to identify which cytokines contribute to the pathogenic process in the brain.

We have used the mouse model of polytropic retrovirus infection to examine the contribution of proinflammatory cytokines in the development of neurological disease. In this model, intraperitoneal (i.p.) infection of neonatal mice with the retrovirus, Fr98, results in the development of a neurological disease with clinical signs of severe ataxia and seizures (Portis et al., 1995). The main cell types infected by virus are endothelial cells, microglia and macrophages (Poulsen et al., 1999; Poulsen et al., 1998; Robertson et al., 1997). The primary pathology associated with disease development is gliosis with activated astrocytes and microglia and no detectable increase in lymphocytic infiltration as measured by immunohistochemistry or mRNA analysis (Peterson et al., 2001; Portis et al., 1995; Robertson et al., 1997). Despite the lack of lymphocytic
infiltration, the development of disease correlates with increased production of proinflammatory cytokines in the brain. Increased expression of $\text{Tnf}$($\alpha$), $\text{Ccl2}$($\text{MCP-1}$), $\text{Ccl3}$($\text{MIP-}\alpha$), $\text{Ccl4}$($\text{MIP-}\beta$), $\text{Ccl5}$($\text{RANTES}$) and $\text{Ccx10}$($\text{IP-10}$) mRNA was detected 2 to 3 days prior to the onset of Fr98-induced neurological disease (Peterson et al., 2001). In addition, $\text{IL-1x}$ and $\text{Ccx11}$($\text{MIP-2}$) mRNA expression was upregulated at the time of clinical signs of disease (Peterson et al., 2001). Knockout mice and antibody blocking experiments have demonstrated a role for at least two of these cytokines, $\text{Tnf}$($\alpha$) and $\text{Ccl2}$($\text{MCP-1}$), in Fr98-mediated neurological disease (Peterson et al., 2004a, 2004b).

Intraperitoneal injection of neonatal mice with another murine polytropic retrovirus, Fr54, does not induce either neurovirulence or cytokine upregulation, despite the presence of virus in the brain (Portis et al., 1995). Fr54 differs from Fr98 primarily in the envelope protein and lacks both neurovirulent determinants encoded by the Fr98 envelope gene (Portis et al., 1995). Additionally, Fr54 infects endothelium and microglia/macrophages in the brain at a two to three-fold lower level than Fr98 (Hasenkugl et al., 1996; Robertson et al., 1997). Thus, either virus-specific envelope sequences or virus-specific differences in infection of target cells could be determining factors in the induction of proinflammatory cytokines and chemokines and neurological disease.

In a previous study, intraventricular inoculation of Fr54-infected neural stem cells (NSC) resulted in less endothelial cell infection, but an increase in spread of the virus to resident microglia/macrophages, resulting in a two to three-fold increase of virus in the CNS, and was associated with a severe clinical brain disease (Poulsen et al., 1999). However, the neuroinflammatory response induced by Fr54-NSC was not examined, and therefore, it was not clear whether the pathogenesis of this disease was similar to the disease induced by Fr98. In the current study, we studied the levels and sources of proinflammatory cytokines and chemokines induced in vivo by Fr54-NSC as well as by NSC infected with other polytropic or ecotropic murine retroviruses. The results indicated that viral envelope sequences associated with polytropic envelope receptor specificity played an important role in induction of cytokines and chemokines associated with this brain disease. Cytokine and chemokine mRNA analysis of microglia cells from Fr54-NSC-infected mice demonstrated that these cells produced $\text{Ccl3}$($\text{MIP-}\alpha$) and $\text{Ccl4}$($\text{MIP-}\beta$) mRNA in response to Fr54 infection, while astrocytes were the source of $\text{Ccl2}$($\text{MCP-1}$) mRNA.

Results

**Increased infection of microglia by Fr54 results in chemokine expression**

In previous studies, inoculation of Fr54-infected NSC (Fr54-NSC) directly into the ventricles of neonatal mice resulted in increased virus spread to microglia/macrophages and the development of neurological disease (Poulsen et al., 1999). To determine if Fr54-NSC inoculation also induced upregulation of proinflammatory cytokines and chemokines associated with Fr98-induced disease, we analyzed mRNA expression in Fr54-NSC-inoculated mice. Similar to previous results (Poulsen et al., 1999), mice inoculated with Fr54-NSC developed clinical signs of severe ataxia and seizures by 3 to 4 weeks post-inoculation (Fig. 1A). The amount of virus present in the brain of Fr54-NSC-inoculated mice was three-fold higher than that observed in Fr54 i.p.-infected mice and slightly higher than the levels observed in Fr98 i.p.-infected mice, as measured by virus gag protein (Fig. 1B) or virus gag mRNA (data not shown). In addition, a high level of microglial infection was observed in Fr54-NSC-inoculated mice (Fig. 2A) as reported previously (Poulsen et al., 1999). To confirm that microglia were actually infected with Fr54, tissue sections from Fr54-NSC-inoculated mice were dual-stained for viral RNA by in situ hybridization (Fig. 2B, black arrows) and for microglia/macrophages using the marker, Iba1 (Fig. 2B, white arrows). Many of the virus-infected cells were positive for Iba1 indicating that brain microglia/macrophages were infected with Fr54. However, some virus-infected cells were not Iba1-positive, and these were possibly either the inoculated NSC, infected endothelia or oligodendrocytes.

Analysis of mRNA levels revealed that Fr54-NSC inoculation induced the same cytokines at similar levels to those observed in Fr98-infected mice (Fig. 1C). In contrast, mock-infected NSC (mock-NSC) inoculation did not induce neurological disease or upregulation of the proinflammatory cytokines. Thus, increasing the level of microglial infection by Fr54 correlated with the upregulation of proinflammatory cytokines in the brain and the development of clinical disease.

**NSC inoculation of the ecotropic retrovirus, FB29, does not induce disease**

The ability of Fr54 to induce cytokines and cause neurological disease suggested that any retrovirus at a high viral burden in the brain may be able to upregulate cytokine production. Therefore, NSC were infected with another avirulent murine retrovirus, FB29. FB29 contains the same LTR, gag and pol genes as Fr54 and Fr98, but uses a different receptor to infect its target cells such as microglia and endothelia (Portis et al., 1995). Interestingly, the amount of virus gag protein in the brains of mice inoculated with FB29-infected NSC (FB29-NSC) was 10-fold higher than that observed in either Fr54-NSC or Fr98 i.p. inoculated mice (Fig. 1B), with most of the infected cells appearing to be microglia by immunohistochemistry (data not shown). However, these mice did not display any signs of neurological disease (Fig. 1A). Despite the high virus levels in the brain, mRNA for only one chemokine, $\text{Ccl2}$($\text{MCP-1}$), was elevated by FB29-NSC inoculation (Fig. 1C). This level was significantly lower than that found in Fr54-NSC-inoculated mice (Fig. 1C) and was not consistently upregulated (Fig. 3D). Thus, a high retrovirus load was not sufficient for the induction of most proinflammatory cytokines and chemokines in the brain.
NSC-inoculation of other polytropic viruses induces cytokine/chemokine expression

The primary difference between Fr54 and FB29 is the envelope protein with Fr54 encoding a polytropic envelope and FB29 encoding an ecotropic envelope. To determine if high virus titers of other polytropic retroviruses induced clinical disease as well as cytokine and chemokine responses in the brain, we inoculated mice with NSC infected with two additional polytropic viruses, EC and MN2PTb, that normally replicate at low levels in the CNS. EC is a virus which induces a slow form of neurological disease with limited induction of cytokines and chemokines (Hasenkrug et al., 1996; Poulsen et al., 1998). MN2PTb is an avirulent virus isolated after a Moloney virus infection. MN2PTb contains a polytropic env similar, but not identical, to Fr54 (data not shown). Fr98-infected NSC were also used as an additional positive control. High levels of virus gag protein p30 were found following inoculation of NSC infected by each of the viruses tested (Fig. 3A). NSC inoculation increased the pathogenicity of all four polytropic viruses, but not the ecotropic virus, FB29 (Table 1). In addition, significant upregulation of cytokine and chemokine mRNA expression was noted in all of the mice inoculated with polytropic virus infected NSC (Fr98-NSC, Fr54-NSC, EC-NSC and MN2PTb-NSC) compared to mock-NSC or FB29-NSC (Figs. 3B–I). Thus the ability to induce CNS disease appeared to be common to all polytropic viruses tested so far, although there remain important virulence differences among these polytropic viruses.

Coinfection of Fr54 and FMuLV57 also induces CNS disease with increased cytokine and chemokine expression

Coinfection of polytropic retroviruses and ecotropic retroviruses has been reported to enhance the spread of polytropic retrovirus through pseudotyping of the polytropic genome (Lavignon and Evans, 1996). We used coinfection of Fr54 with an avirulent ecotropic virus, FMuLV57, as an alternative method to increase Fr54 infection of microglia cells to rule out the possibility that the Fr54-NSC were the source of the increased cytokines and chemokines in the brain. Mice...
infected with both Fr54 and FMuLV57 had a 20-fold increase in the amount of infectious Fr54 virus in the brain compared to mice infected with Fr54 alone (Fig. 4B), and this coinfection resulted in the development of neurological disease similar to that observed with Fr54-NSC inoculation (Fig. 4A). Coinfection also induced a significant upregulation of proinflammatory cytokine and chemokine genes compared to infection with Fr54 or FMuLV57 alone (Fig. 3C). Thus, infected NSC were not required for cytokine and chemokine expression induced by Fr54, indicating that these cells were not the source of the cytokines and chemokines in the CNS.

Production of cytokines and chemokines by microglia and astrocytes

The influence of increased virus infection of microglia on Fr54-induced cytokine expression suggests that the infected microglia/macrophages may be the primary cells responsible for the production of cytokines and chemokines. To test this possibility, we enriched microglia/macrophages (MG) from brain tissue of mock-NSC and Fr54-NSC-inoculated mice by percoll gradient. The resulting cell population was greater than 90% CD11b-positive cells by immunohistochemical staining (Fig. 2C) and flow cytometry (data not shown), contained both infected and uninfected cells (Fig. 2D) and was highly enriched for the MG gene F4/80 (Fig. 5A). Surprisingly, the only cytokine or chemokine mRNAs consistently detected in the MG population from Fr54-NSC-inoculated mice were Ccl3 (MIP-1α) and Ccl4 (MIP-1β) (Fig. 5B, data not shown). There was inconsistent expression of Ccl5 (RANTES) and Tnf (TNFα) (Fig. 5B, data not shown). The high levels of Ccl3 (MIP-1α) and Ccl4 (MIP-1β) mRNA in the microglia/macrophage population suggested that these cells may be the primary source of these...
chemokines. In contrast, mRNA for chemokines such as Ccl2(MCP-1) and Cxcl10(IP-10), found increased in the whole brain, was not detected in the purified MG population at all. Similar results were also observed in mice infected with Fr98 by the i.p. route (Fig. 5C).

In a previous study, astrocytes were found to be the primary source of Ccl2(MCP-1) mRNA in Fr98-infected mice (Peterson et al., 2004a, 2004b). Analysis of brain tissue sections from Fr54-NSC-inoculated mice also demonstrated Ccl2(MCP-1)-positive astrocytes by in situ hybridization and immunohistochemical analysis (Figs. 2E, F). These cells were located in multiple areas including the thalamus, hippocampus, cortex, cerebellum and brainstem. In contrast, no Ccl2(MCP-1)-positive cells were detected in the brain of mice infected with Fr54 using the i.p. route, although a few Ccl2(MCP-1)-positive cells were present in the brain stem. The production of Ccl3(MIP-1α) and Ccl4(MIP-1β) mRNA by microglia and Ccl2(MCP-1) mRNA by astrocytes in both Fr54-NSC and Fr98-inoculated mice suggested that there was a common pathway for cytokine and chemokine induction by both
viruses, which correlated with high levels of MG infection and clinical CNS disease.

Discussion

In the current study, a high level of infection of microglia by Fr54 and other polytropic retroviruses, either by coinfection or by inoculation of infected NSC, resulted in disease associated with the upregulation of proinflammatory chemokines and cytokines. However, microglial infection to similar levels by the ecotropic murine retrovirus, FB29, did not induce a similar upregulation of cytokine or chemokine expression (Fig. 1C). The difference between Fr54 and FB29 in cytokine and chemokine induction may result from virus stimulation of the cellular receptor for Fr54, but not the receptor for FB29. FB29, like other ecotropic retroviruses, uses the Solute Carrier Family 7 (cationic amino acid transporter) member 1 (SLC7a1, also known as CAT1) to infect cells (Kim et al., 1991; Wang et al., 1991). In contrast, polytropic viruses such as Fr54 and Fr98 utilize the cellular receptor, xenotropic/polytropic receptor 1 (XPR1, also known as RMC1) (Tailor et al., 1999; Yang et al., 1999). Although the actual function of XPR1 is unknown, it is predicted to be involved in G protein signal transduction based on similarities to the yeast protein, SYG1, and the presence of an SPX domain (Yang et al., 1999). Possibly, virus envelope binding of XPR1, but not SLC7a1, induces microglial activation, initiating a cytokine/chemokine cascade in the brain. Differences in receptor signaling could also explain the inability of NSC infected with the amphotropic retrovirus, 4070A, to induced neurological disease, despite high levels of virus infection (Traister and Lynch, 2002).

Table 1
Polytropic retrovirus-infected neural stem cells increase severity of neurological disease

<table>
<thead>
<tr>
<th>Virus</th>
<th>Tropism</th>
<th>Route of infection</th>
<th>Intraperitoneal</th>
<th>Intraventricular (NSC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fr54</td>
<td>Polytropic</td>
<td>No disease</td>
<td>25 dpi</td>
<td></td>
</tr>
<tr>
<td>Fr98</td>
<td>Polytropic</td>
<td>15 dpi</td>
<td>12 dpi</td>
<td></td>
</tr>
<tr>
<td>EC</td>
<td>Polytropic</td>
<td>37 dpi</td>
<td>18 dpi</td>
<td></td>
</tr>
<tr>
<td>MN2PTb</td>
<td>Polytropic</td>
<td>No disease</td>
<td>60 dpi</td>
<td></td>
</tr>
<tr>
<td>FB29</td>
<td>Ecotropic</td>
<td>No disease</td>
<td>No disease</td>
<td></td>
</tr>
</tbody>
</table>

* Cell-free virus was inoculated ip (10⁴ FFU).
* Virus-infected neural stem cells were inoculated into the lateral ventricles of the brain as previously described (Poulsen et al., 1999).
* Mean day post-infection for onset of neurological disease.

Fig. 4. Induction of neurological disease and cytokine expression following Fr54 coinfection with FMuLV57. Neonatal IRW mice were inoculated with 10⁴ FFU of FMuLV57 or Fr54 or coinfected with 10⁴ FFU of each virus. (A) Mice were followed for clinical signs of ataxia and seizures as described in Fig. 1A. Data are presented as the percentage of mice with ataxia for 6 to 12 mice per group. (B) FFU of virus obtained from brain homogenates of mice infected with either Fr54, FMuLV57 or both viruses. Data are presented as the mean±standard error of FFU per 100 μg of brain tissue for four mice per group. Statistics were performed using Mann-Whitney test. A significant (p<0.03) difference was observed between the number of polytropic FFU in FMuLV57/Fr54 infected mice compared to Fr54 infected mice. (C) Cytokine mRNA expression in the brains of mice at 21 days post-inoculation was analyzed as described for Fig. 1C. Data are the mean±standard error of four to eight mice per group. Statistical analysis was performed using a one-way ANOVA with the Newman–Keuls post-test. The P value for FMuLV57/Fr54 infected mice versus either Fr54 or FMuLV57 infected mice was <0.001 for CCL2, CCL4, CCL5, CXCL10 and IL-1α; <0.01 for CCL3 and was not significant (>0.05) for CXCL1 and TNFα.
The ability of Fr54, but not FB29, to induce cytokine and chemokine expression may also be due to factors other than the direct interaction of the envelope with the virus receptors. For example, independent of interactions with the viral receptors, the envelope may be toxic to certain cell types including neurons or glia, leading to the release of factors that stimulate cytokine and chemokine production. In contrast, the neuropathogenesis of the ecotropic murine retroviruses, FrCasE and Moloney ts1, has been linked to misfolding of the envelope protein and the induction of endoplasmic reticulum stress responses (Dimcheff et al., 2003; Dimcheff et al., 2004; Kim et al., 2004; Liu et al., 2004). However, so far we have found no evidence supporting a similar mechanism of pathogenesis with the polytropic murine retroviruses (data not shown). It is also possible that polytropic viruses induce a strong type I interferon response, as suggested by the production of Cxcl10(IP10) mRNA (Fig. 3I). The production of type I interferons may initiate an antiviral response that leads to the subsequent production of other proinflammatory cytokines and chemokines.

Although infected microglia appear to have a critical role in cytokine and chemokine induction in the brain, they do not appear to be the sole or even the primary source. For example, Ccl2(MCP-1) is produced by astrocytes after Fr54-NSC (Fig. 2D) and Fr98 infection as well as other retrovirus infections and neurological diseases (Conant et al., 1998; Peterson et al., 2004a, 2004b; VanDer et al., 1999). In the instance of Fr54-NSC and Fr98-induced disease, dual staining for Ccl2(MCP-1) mRNA and virus envelope protein demonstrated that Ccl2(MCP-1)-producing astrocytes were not infected (data not shown) (Peterson et al., 2004a, 2004b). Possibly, microglia activated by retrovirus infection produce soluble factors such as CCL3(MIP-1α) and CCL4(MIP-1β) that stimulate astrocytes to produce CCL2(MCP-1). Alternatively, retroviral envelope proteins may interact with cellular receptors on astrocytes and stimulate these cells to produce CCL2(MCP-1).

The production of Ccl3(MIP-1α) and Ccl4(MIP-1β) mRNA by the microglia/macrophage population in the brains of Fr98 and Fr54-NSC-inoculated mice suggest that these chemokines play an important role in neurovirulence. Additionally, increased expression of these two chemokines correlated with neurovirulence induced by Fr98, Fr54-NSC and Fr54/FMuLV57 inoculation. Both CCL3(MIP-1α) and CCL4(MIP-1β) bind to the chemokine receptor CCR5 (Boring et al., 1996). However, mice deficient in CCR5 infected with Fr98 developed neurological disease with the same severity and kinetics as wild-type mice (Peterson et al., 2004a, 2004b). Therefore, CCR5 is not required for this disease. Nevertheless, CCL3(MIP-1α), which binds to both CCR5 and CCR1, may contribute to pathogenesis by signaling through the CCR1 receptor (Boring et al., 1996; Post et al., 1995). In contrast, since CCR5 is the primary receptor for CCL4(MIP-1β), this chemokine may not play an essential role in this model.

The present study indicated that the proinflammatory cytokine/chemokine response to virus infection can be influenced both by the amount of virus in the brain as well as the identity of viral envelope proteins. Similarly, the ability of HIV variants to induce pathogenic cytokine/chemokine responses in the brain may be dependent upon the level of HIV infection as well as the ability of HIV envelope to directly
stimulate its coreceptors, such as CCR5 and CXCR4, on microglia and macrophages. In vitro studies have demonstrated that envelope variants of HIV can differ in their ability to induce CCL3(MIP-1α), CCL4(MIP-1β), CCL5(RANTES) and TNFα in macrophages (Choe et al., 2001). Thus, the lack of increased cytokine or chemokine production in some patients with detectable HIV in the brain may be due to the inability of a particular HIV variant to activate microglia and/or macrophages.

Materials and methods

Mice

Inbred Rocky Mountain White (IRW) mice were bred and housed at the Rocky Mountain Laboratories animal facility. All animal experiments were carried out in accordance with the regulations of the Rocky Mountain Laboratories Animal Care and Use Committee and the guidelines of the NIH.

Viruses and intraperitoneal (i.p.) infection and antibody treatment of mice

The construction of virus clones FB29, Fr54, EC and Fr98 has been previously described (Portis et al., 1995). Virus stocks were prepared from the supernatants of confluent 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). For intraperitoneal inoculation, IRW mice were injected with $10^4$ focus forming units (FFU) of virus within 24 h of birth. 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).

Virus inoculation using neural stem cells

The use of the neural stem cell line C17.2 as a vehicle for delivery of virus to the brain has previously been reported (Lynch et al., 1999; Poulsen et al., 1999; Traister and Lynch, 2002). C17.2 neural stem cells (NSC) were maintained in DMEM with 1 mM sodium pyruvate and 10% fetal calf serum in Primaria tissue culture flasks. NSCs were infected by culturing $1 \times 10^5$ cells with 4 μg/ml polybrene and $10^5$ FFU of Fr54 or FB29 for 48 h. Cells were passaged until confluent infected. Cells were analyzed for virus infection by immunofluorescence using the antibodies 514 or 720 (Robertson et al., 1991). Prior to inoculation, non-confluent flasks of Fr54, FB29, or mock-infected NSCs were harvested using 0.25% trypsin in 0.01% EDTA, washed twice in phosphate-buffered saline (PBS), and resuspended at 7.5 × 10^6 cells per ml in PBS with 0.2% trypan blue. Mice, within 24 h of birth, were anesthetized by hypothermia, and 4 μl of NSC suspension was injected into each ventricle using a Hamilton syringe with a 30-gauge needle for a final inoculum of $6 \times 10^5$ NSC per mouse. Correct inoculation of cells into the ventricles was monitored by observing trypan blue staining in the ventricles. C17.2 neural stem cells were previously modified to produce β-gal as a method of detection (Lynch et al., 1999). Immunohistochemistry analysis of β-gal expression from sagittal and coronal brain tissue sections indicated the presence NSC in multiple regions of the brain including the olfactory bulbs, cortex, hippocampus, thalamus, and cerebellum (Poulsen et al., 1999). Morphological analysis indicated these did not undergo apoptosis (data not shown). Immunohistochemistry analysis of virus protein expression was performed to confirm the spread of virus from NSC to microglia as previously described (Poulsen et al., 1999). NSC-inoculated mice were followed for clinical signs for 9 weeks post-inoculation and were recorded as having disease when signs of severe ataxia and/or seizures were apparent. Statistical analysis of disease development was done using Kaplan-Meir survival curve analysis using Graph-Pad Prism software.

Enrichment and characterization of microglia/macrophages

Infected mice were exsanguinated by axillary incision under deep isoflurane anaesthesia. Brain tissue was immediately removed from mice and placed in individual wells of a 6-well plate containing 6 ml of 2% FBS diluted in phosphate-buffered saline (PBS) on ice. Each brain was minced with scissors and transferred to an ice-cold Dounce homogenizer. Brain tissue was disrupted with a loose fitting pestle using 5 to 10 strokes per sample. The homogenate was then passed through a 100-μm nylon cell strainer (BD Biosciences, San Jose, CA) and resuspended in 4 ml of 70% percoll/PBS. The cell suspension was then layered underneath a 0 to 35% step percoll gradient using two 15-ml tube gradients for each brain sample. The gradients were then centrifuged at 2300 RPM for 45 min at room temperature. Following centrifugation, the layer of cells at the 0–35% interface and 35–70% interface were separately collected, rinsed in PBS and either seeded on BD Falcon 8-chamber slides (BD Biosciences), analyzed by flow cytometry, or processed for RNA. RNA was extracted using the Statagene (La Jolla, CA) miniprep RNA isolation kit following manufactures recommended protocol. For flow cytometric analysis, cells were labeled using anti-F4/80 monoclonal antibodies generated from the hybridoma HB-198(ATCC) with a FITC-labeled anti-rat secondary antibody and analyzed on a BD FACScan (BD Biosciences). Cells were greater than 90% positive for F4/80 by flow cytometric analysis. Cells added to 8-chamber slides were incubated in macrophage serum-free media (Invitrogen) for three to five days, fixed with 95% ethanol and stained using rat anti-mouse CD11b or goat anti-gp70 antibodies. Positive cells were detected using horse radish peroxidase (HRP)-conjugated anti-rat or anti-goat antibodies (Zymed Laboratories) and developed using the substrate, 3-amino-9-ethylcarbazole (AEC) (Sigma).

ELISA Assay for virus gag protein

Infected mice were exsanguinated by axillary incision under deep isoflurane anesthesia. Brains were removed from infected mice at the time of neurological disease or as indicated and

divided into two sections by mid-sagittal dissection. Tissue sections were immediately frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\). For analysis of virus gag protein levels, brain tissue was homogenized in 0.5\% NP-40 containing 0.01 M Tris base, 0.15 M NaCl, 0.001 M EDTA (pH 7.4) using Kontes Disposable Pellet Pestles (Fisher Scientific) and cordless motor to a final concentration of 10\% weight/volume. The homogenates were spun for 1 min at 14,000 RPM to remove debris and stored at \(-20^\circ\text{C}\) until use. Brain homogenates were analyzed for virus gag levels using the p30 virus gag ELISA assay previously described (Poulsen et al., 1999; Wehrly and Chesebro, 1997). In short, 96-well Immulon II plates were coated with 100 \(\mu\text{l}/\text{well}\) of the rat monoclonal antibody 48 and Fr54 foci were detected using the monoclonal antibody 514 (Evans and Morrey, 1987). In short, brain tissue was removed from infected mice, polytropic and ecotropic retroviruses were performed as previously described (Evans and Morrey, 1987; Sitbon et al., 1985). In short, brain tissue was removed from infected mice, homogenized and overlaid onto NIH 3T3 cells. FMuLV57 foci were detected using the monoclonal antibody 48 and Fr54 foci were detected using the monoclonal antibody 514 (Evans and Morrey, 1987; Sitbon et al., 1985).

**Focal immunoassay for ecotropic and polytropic retroviruses**

Focal immunoassays for the detection and differentiation of polytropic and ecotropic retroviruses were performed as previously described (Evans and Morrey, 1987; Sitbon et al., 1985). In short, brain tissue was removed from infected mice, homogenized and overlaid onto NIH 3T3 cells. FMuLV57 foci were detected using the monoclonal antibody 48 and Fr54 foci were detected using the monoclonal antibody 514 (Evans and Morrey, 1987; Sitbon et al., 1985).

**RNase protection assay**

Total RNA from brain tissue was prepared using Trizol reagent (Invitrogen, Carlsbad, CA) according to manufacturers’ instructions. For examination of mRNA from NSC, confluent flasks of cells were lysed using Trizol reagent and processed for RNA according to manufacturers’ instructions. RNA was quantified by spectrophotometry at 260 nm diluted to equal concentration in hybridization buffer (BD Biosciences) and stored at \(-80^\circ\text{C}\) until use. The RNA was then analyzed for specific cytokine mRNA using the RiboQuant System (BD Biosciences) as previously described (Peterson et al., 2001). \(^{32}\)P-labeled protected probe was quantified for each gene of interest using a STORM PhosphorImager (Amersham Biosciences, Piscataway, NJ) and Image Quant software. Data were normalized to \(L32\) mRNA expression for each sample and then calculated as a ratio of gene expression relative to gene expression in the positive control (RNA from Fr98-infected mice).

**Preparation of RNA for real-time reverse transcriptase PCR analysis**

Total RNA from the cerebrum or cerebellum/brainstem regions was isolated as described above for the RNase Protection assay. The RNA was then treated with DNase (Ambion, Austin, TX) for 30 min to remove any contaminating DNA, and purified over RNAeasy columns (Qiagen).

**Primers and probes for gene detection**

\(F4/80\) mRNA expression was detected using the forward primer F4/80-1958F (5\′-TTA CGA TGG AAT TCT CCT TGT ATA TCA-3′), the reverse primer F4/80-2051R (5′-CAC AGC AGG AAG GTG GTT ATG-3′), and probe F4/80-2001T (6FAM-AGT CAT CTC CCT GTG ATG TCT CCT GTG-3′). \(G\)ap mRNA expression was detected using the forward primer GFAP-16F (5′-CGT TTC TCC TTG TCG ATG-3′), the reverse primer GFAP-112R (5′-TGCC CCC GTG TCT CCT TGA-3′), and probe GFAP-42T (6FAM-TCC ACT CCC TGC CAG GGT GGA CTT-TAMRA). Primer and probes from Rodent GAPDH Control Reagent kit (Applied Biosystems) were used to detect \(Gapdh\) mRNA expression.

**Real-time PCR analysis of gene expression**

Reactions were run in triplicate using the one-step RT-PCR master mix (Applied Biosystems) in a 10 \(\mu\text{l}\) volume with approximately 10 ng of DNase-treated total RNA, 500 nM of forward and reverse primers and 250 nM of probe on an ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). Lack of DNA contamination was confirmed by running reactions without reverse transcriptase. 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 (\(\Delta C_T=C_T\) Gene of interest \(-C_T\) Gapdh gene of interest) to control for variations in RNA amounts in each sample. The data are presented as the fold expression of the gene of interest relative to \(Gapdh\). For example, a value of one indicates the same level of mRNA expression as \(Gapdh\) (\(\Delta C_T=0\)), whereas a value of two indicates a two fold higher level of mRNA expression than \(GAPDH\) (\(\Delta C_T=1\)).

**Generation of probes for in situ hybridization**

The generation of \(Ccl2\) (MCP-1) RNA in situ probes has been previously described (Glabinski et al., 1996; Peterson et al., 2004a, 2004b). Template DNA for the generation of \(Ccl2\)-specific probes was kindly provided by Richard Ransohoff. DIGoxigenin (DIG)-labeled sense and anti-sense probes were generated using the DIG RNA labeling kit (Roche Molecular Biochemicals), T7 or T3 polymerase and 0.5 to 1 \(\mu\)g of linearized template DNA following manufacturer’s 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). Sense-strand probes were used as negative controls.
In situ hybridization

In situ hybridization was performed as previously described (Peterson et al., 2004a, 2004b). In brief, brain tissues from Fr98, Fr54-NSC, Fr54 or mock-NSC-inoculated mice were removed at the time of clinical disease or the indicated times. Tissues were embedded in paraffin and processed for in situ hybridization analysis as described. DIG labeled sense and anti-sense probes were diluted 1/100 in hybridization buffer with 20 μl of probe used per section. DIG-labeled sense strands were used as a negative control. The sections were incubated at 95 °C for 4 min, and hybridized overnight at 56 °C. Non-hybridized probe was digested with 20 μg/ml of RNase A (Roche) in 500 mM NaCl, 10 mM Tris pH 8.0 with subsequent rinsing in 2XSSC and incubating sections in 1X SSC, 50% formamide at 56 °C to remove any remaining unbound probe. DIG-labeled probes were detected with alkaline phosphate (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 immunohistochemistry analysis, sections were blocked in 1% normal goat serum (NGS) in PBS. Sections were then incubated with a 1/1000 dilution of rabbit anti-anti-globulin acidic protein (GFAP) (Dako, Glostrup, Denmark) or a 1/100 dilution of rabbit anti-Iba1 (Wako, Richmond, VA), in 1% NGS. GFAP-positive cells were detected using AP-conjugated anti-rabbit antibodies (Jackson Immunoresearch, West Grove PA). Sections were developed with DAB substrate (Sigma-Aldrich, St. Louis, MO), counterstained with hematoxylin and coverslipped.

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References