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Intracerebral infection with murine cytomegalovirus induces CXCL10 and is restricted by adoptive transfer of splenocytes

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The brain's intrinsic immune system consists of glial cells that produce cytokines and chemokines in response to stimulation with cytomegalovirus (CMV). The present experiments were undertaken to determine whether this intrinsic glial cell response alone is sufficient to control CMV infection of the central nervous system (CNS) or whether effector cells from the somatic immune system are also required. Following stereotactic, intracerebroventricular (icv), injection of murine cytomegalovirus (MCMV) into immunocompetent (C.B-17) mice, viral spread in the brain was limited to the cells of the ventricular walls and the infection was resolved by 10 days post infection (p.i.). In contrast, icv infection of immunodeficient (C.B-17 SCID/bg) mice resulted in viral spread from the ventricles throughout the brain parenchyma and these mice succumbed to lethal disease. Adoptive transfer of total splenocytes from major histocompatibility complex (MHC)-matched, MCMV-primed animals restricted intracerebral viral infection to the periventricular cells and reduced levels of reporter gene expression from the viral genome. Peripheral immune cell transfer also protected immunodeficient animals from lethal disease. Depletion of Thy 1.2^+ cells from MCMV-primed splenocytes abolished the protective effect of adoptive transfer. Viral expression was found to be fourfold greater in the brains of animals given Thy 1.2–depleted splenocytes than from those receiving total undepleted cells. As MCMV infection proceeded in the brains of immunodeficient mice, levels of the T-cell chemoattractants CXCL10 and CCL2 remained elevated, whereas CXCL10 levels waned in the brains of animals receiving transferred splenocytes. Taken together, these results demonstrate the ability of T lymphocytes to restrict intracerebral viral spread and indicate that intrinsic glial cell responses alone are insufficient to control MCMV brain infection. *Journal of NeuroVirology* (2004) **10,** 152–162.

Keywords: chemokines; encephalitis; MCMV

Introduction

Previous *in vitro* studies from our laboratory have demonstrated that both astrocytes and microglial cells produce chemokines in response to infection with human cytomegalovirus (CMV) (Cheeran *et al*, 2001, 2003). In addition, CMV stimulates microglial cells to produce select cytokines, including some that have been shown to possess direct antiviral activity on fully permissive brain cells (Cheeran *et al*, 2001; Pulliam *et al*, 1995). Whether CMV infection of the central nervous system (CNS) is controlled by this intrinsic immune response of the brain or by immune cells recruited from the periphery in response to chemokines produced by glial cells remains unknown. The present study used a murine model of CMV brain infection to define the contribution of endogenous, CNS-specific, and peripheral immune responses to defense of the brain against CMV infection.

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Distinct organ-specific mechanisms have been shown to exist for the control of murine cytomegalovirus (MCMV) infections. These studies have clearly demonstrated that lymphocytes play a major role in protecting mice against MCMV infection, and a hierarchy of immune control of CD8+, natural killer (NK), and CD4+ cells has been proposed (Polic *et al*, 1998). The CD8+ T-cell subset is widely recognized as a primary effector cell capable of defending many tissues from productive viral infection (Jonjic *et al*, 1989; Koszinowski *et al*, 1990; Reddehase *et al*, 1985). Adoptive transfer experiments have verified that the host defense function of the CD8+ lymphocyte subset is not restricted to sites in which intravenously infused lymphocytes usually home (e.g., lungs and spleen), but also occurs in sites to which antiviral effector cells specifically migrate, such as the adrenal glands (Reddehase *et al*, 1988). Although CD8+ T cells have the ability to clear most tissues from productive viral infection, elimination of the virus from salivary glands requires CD4+ lymphocytes (Jonjic *et al*, 1989). This CD4+ cell–mediated mechanism of viral clearance from salivary glands has subsequently been shown to require interferon (IFN)-γ (Lucin *et al*, 1992) and tumor necrosis factor (TNF)-α (Pavic *et al*, 1993). In the absence of CD8+ lymphocytes, CD4+ cells have been shown to acquire compensatory protective antiviral activity that is not present in normal mice recovering from viral infection (Jonjic *et al*, 1990). Despite the extensive literature on the contribution of T cells to host defense, the role of lymphocytes in defense of brain tissue against CMV remains unknown.

The importance of innate immunity in control of MCMV, especially during the crucial early stage of infection, has been demonstrated in a number of mouse strains that differ greatly in their susceptibility. Unlike BALB/c mice, depletion of CD8+ lymphocytes in C57/B6 mice has little effect on viral clearance (Polic *et al*, 1996). However, *in vivo* depletion of NK cells using the anti-NK1.1 monoclonal antibody (mAb) PK136 increases the susceptibility of C57/B6 mice to infection with MCMV (Scalzo *et al*, 1992). This may be due to the fact that C57/B6 mice carry the *CMV-1*^{r} gene at the NK locus, which makes them fivefold more resistant to MCMV infection than strains with the *CMV-1*^{s} allele (Scalzo *et al*, 1990). In C.B-17 SCID mice, NK cells delay but fail to protect from lethal MCMV disease (Welsh *et al*, 1991). The mechanisms by which NK cells counteract viral infection are organdependent. For example, in the spleen, NK cells control MCMV via a perforin-dependent cytotoxic mechanism, whereas in the liver these same cells produce IFN-γ as a major mediator in the suppression of infection (Tay and Welsh, 1997). In the CNS of neonatal mice, innate immune responses mediated by NK cells, as well as nitric oxide (NO) derived from brain macrophages, have been shown to play a role in protecting the developing brain from MCMV infection (Kosugi *et al*, 2002).

Because neuroimmune responses to viral infections are poorly understood and are likely regulated by the local microenvironment of the CNS (Wenkel *et al*, 2000), we initiated this study to characterize the contributions of both the intrinsic (brain) and peripheral (spleen) immune systems to host defense against MCMV brain infection. During CNS viral infection, leukocyte trafficking from the blood into the brain is usually preceded by chemokine production from activated glial cells (Asensio and Campbell, 1997; Lane *et al*, 1998), and we previously have shown by *in vitro* studies that activated lymphocytes migrate towards supernatants from CMV-stimulated microglia (Cheeran *et al*, 2003). Peripheral immune effector cells responding to glial cell–produced chemotactic factors may play a major role in the blockade of intracerebral viral spread (Cheeran *et al*, 2001). The present experiments were undertaken to determine whether the glial cell immune response alone is sufficient to defend the brain against CMV infection or if effector cells from the somatic immune system are also required.

Results

Uncontrolled viral spread throughout the brain in immunodeficient mice

To examine spread of MCMV in the murine brain, animals were infected by injecting salivary gland-passaged RM461 (3×10^5 $TCID_{50}$), a recombinant virus expressing *Escherichia coli* β-galactosidase under the control of the human ie1/ie2 promoter/enhancer (Stoddart *et al*, 1994), into the right lateral ventricle. This model of ventricular CMV infection mimics viral dissemination from the cerebrospinal fluid into the brain and avoids confounding problems associated with systemic disease prior to CNS infection. At various times following infection, animals were sacrificed using isoflurane vapor, perfused with fixative (4% paraformaldehyde in 0.5 M phosphate buffer), and thick sections (50 μM) of brain tissue were cut for staining with X-gal. When immunocompetent mice were examined, Lac-Z gene expression (i.e., blue staining indicative of infection) was detected in the brains of animals sacrificed at 3, 5, and 7 days post infection (p.i.) and was absent from the CNS by days 9 p.i. (Figure 1A). In contrast, MCMV-infected immunodeficient SCID/bg mice exhibited marked spread of blue staining as the infection progressed throughout the brain by 7 to 9 days p.i. (Figure 1A) . Serial coronal sections of brains from immunodeficient mice representing areas both rostral and caudal to the injection site show the infection spreading extensively throughout the brain by 9 days p.i. (Figure 1B). In contrast, examination of brains of immunocompetent mice revealed cells that stained X-gal–positive largely restricted to

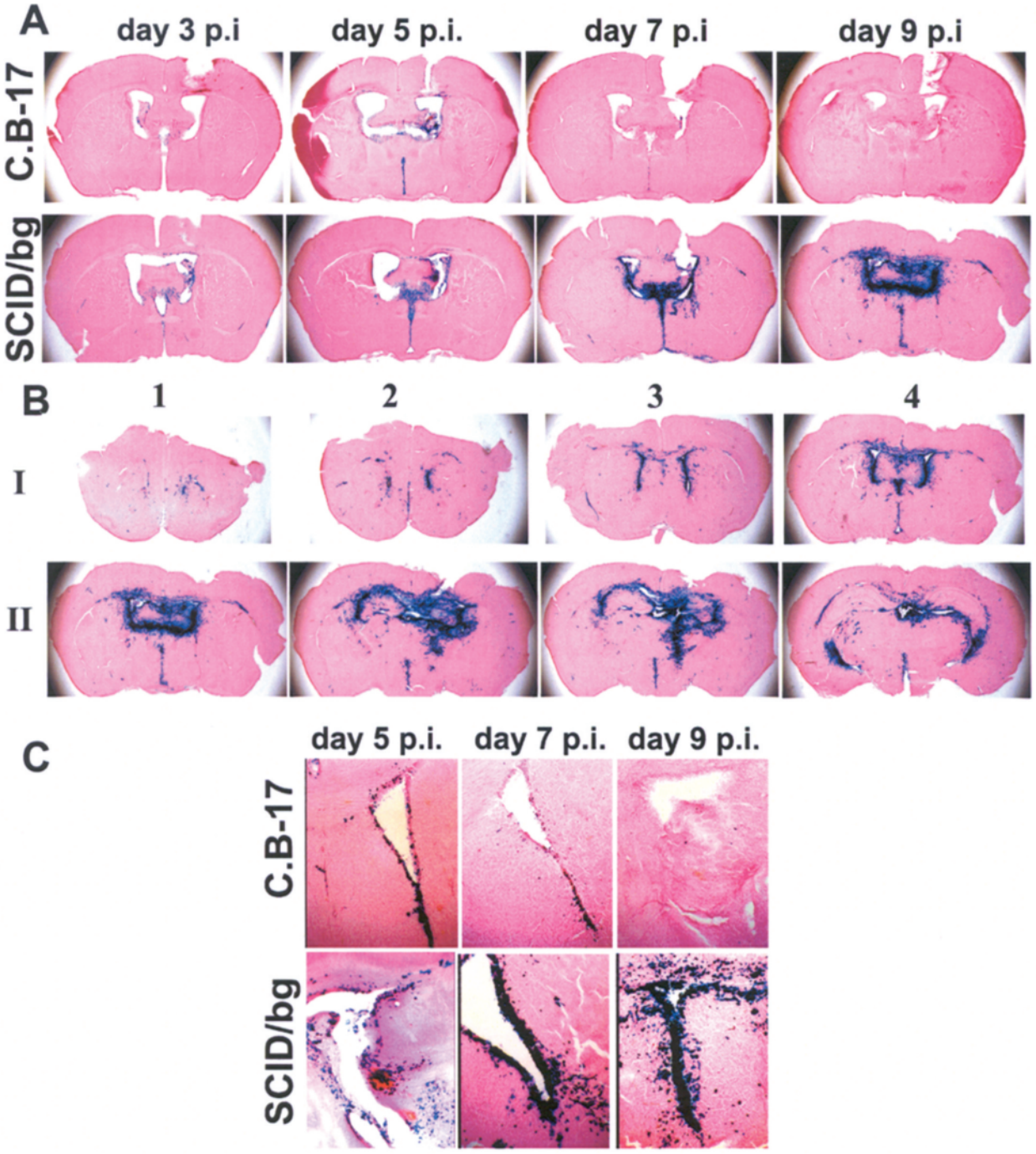

Figure 1 Viral spread into the brain parenchyma is controlled in immunocompetent but not immunodeficient animals. (**A**) Immunocompetent (C.B-17) and immunodeficient (C.B-17 SCID-bg) mice were inoculated with MCVM RM461 (3×10^5 $TCID_{50}$/10 μl). Brains were removed at 3, 5, 7, and 9 days p.i. and coronal sections (50 μM) were processed for *in situ* staining with X-gal (1 mg/ml). Infected cells appear blue in the photomicrographs. (**B**) Serial coronal sections through the infected brain of an immunodeficient animal representing areas (I) rostral and (II) caudal to the injection site showing viral spread into areas distal to the ventricles (I-1 and II-4) by 9 days p.i. (**C**) Histochemical detection of β-galactosidase expressing periventricular cells in both C.B-17 and C.B-17 SCID/bg mice at 5, 7, and 9 days p.i.

the periventricular area, and the virus cleared over time (Figure 1C). On the other hand, MCMV spread from the periventricular region into cells of the brain parenchyma of immunodeficient mice by 9 days p.i. (Figure 1C).

Splenocytes from MCMV-primed mice restrict brain infection

We next examined the contribution of splenocytes from immunized mice to the containment of MCMV brain infection. Aseptically collected

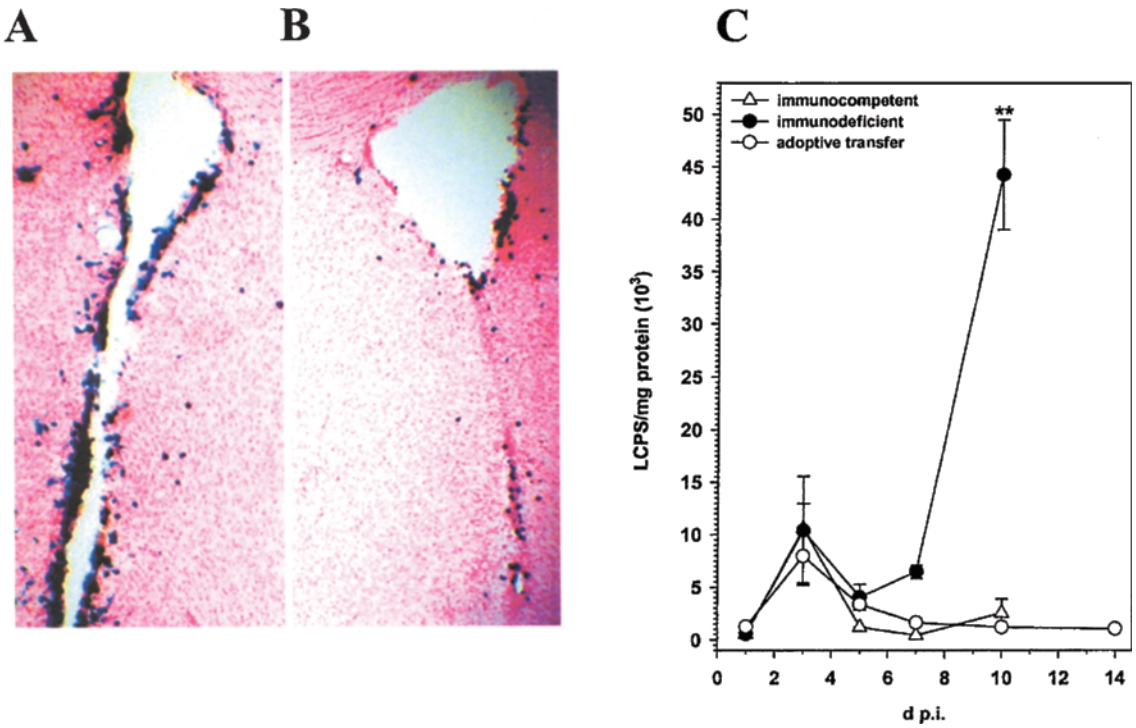

Figure 2 Adoptive transfer of splenocytes restricts intracerebral viral spread and reduces reporter gene expression in the CNS. Splenocytes (1×10^7 cells) obtained from MCMV (Smith strain)-primed, MHC-matched (H-2^d) mice were transferred into immunodeficient C.B-17 SCID/bg mice 24 h prior to icv infection with MCMV RM461 (3×10^5 $TCID_{50}$). Histochemical detection (X-gal) for β-galactosidase expressing periventricular cells in (**A**) C.B-17 SCID/bg mice and (**B**) identical mice receiving adoptive transfer of splenocytes 24 h prior to MCMV infection at 9 days p.i. (**C**) Kinetics of viral expression in brain tissue extracts obtained from immunocompetent mice (*open triangles*), immunodeficient mice (*closed circles*), and immunodeficient mice receiving adoptively transferred splenocytes (*open circles*). At the indicated times following icv infection with MCMV RM461, groups of animals were perfused with cold PBS and total brain tissue was homogenized in TPER (Pierce, Rockford, IL). Reporter gene expression from the viral genome was measured using a chemiluminescence assay (Tropix Gal-Screen; Applied Biosystems, Foster City, CA). Data are expressed as mean ± SEM luminescent counts per second (LCPM/mg protein) from two to five animals per time point.

splenocytes from major histocompatibility complex (MHC)-matched BALB/c (H-2^d) mice, collected 8 days following intraperitoneal administration of MCMV (Smith strain), were transferred into immunodeficient C.B-17 SCID/bg mice via tail vein injection. Recipient mice were given 1×10^7 spleen cells 24 h prior to intracerebroventricular (icv) infection with MCMV RM461. In the brains of immunodeficient mice without transferred splenocytes, blue staining cells indicative of viral infection were seen at 9 days p.i. (Figure 2A). When mice that had received adoptively transferred splenocytes were examined, noticeably fewer infected cells were observed at the same time point p.i., as the infection was in the process of being cleared (Figure 2B). Quantitative analysis of the kinetics of reporter gene expression from the viral genome confirmed these qualitative staining results. When extracts of infected brain tissue were examined using a chemiluminescence assay, reporter gene expression was controlled in the brains of both immunocompetent mice and in immunodeficient animals that had received adoptively transferred splenocytes. However, expression from the viral genome was found to increase as the infection proceeded in immunodeficient mice (Figure 2C). When examined at 8 days p.i., MCMV-infected SCID/bg mice displayed significantly higher levels (36-fold) of reporter gene expression in the CNS than did infected splenocyte-treated immunodeficient animals ($P < .01$, Student's t test).

MCMV brain infection is lethal in immunodeficient mice

We next analyzed the effect of immune reconstitution of immunodeficient mice on survival rate following MCMV brain infection. Stereotactic, icv inoculation of MCMV into the right lateral ventricle resulted in a lethal infection in immunodeficient mice (Figure 3). These animals became moribund by 6 to 10 days p.i. and succumbed to lethal disease by day 9. All animals do not succumb to infection in exactly the same time frame, but still very few make it past day 9 and none survive past day 11. There are no rare survivors. In contrast, immunocompetent C.B-17 mice completely recovered (6 to 8 days p.i) from an identically administered viral infection. Adoptive transfer of splenocytes from MCMV-immunized MHC-matched donors into C.B-17 SCID/bg mice 24 h prior to an identical icv infection with RM461 markedly increased survival (Figure 3). Thus, adoptive transfer

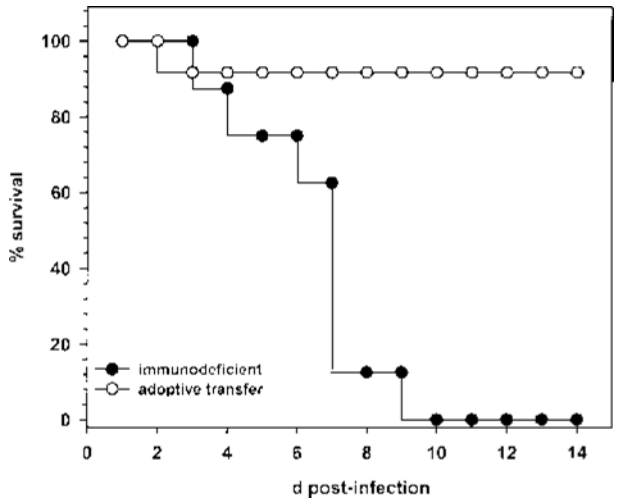

Figure 3 Transfer of splenocytes protects immunodeficient animals from lethal disease. MCMV RM461 (3×10^5 $TCID_{50}$ units in 10 μl) was stereotaxically injected slowly into the right lateral ventricle of immunodeficient (*closed circles*), and splenocyte-treated immunodeficient (*open circles*) mice (6 to 8 weeks old). Survival plot of infected mice followed over a 14-day time course. Survival is expressed as percent of mice examined; groups contained 10 animals each.

of splenocytes into SCID/bg mice was found to confer an immunocompetent phenotype to immunedeficient animals, capable of controlling lethal MCMV infection.

T lymphocytes infiltrate the brains of infected, adoptive-transfer recipients

Further experiments were designed to determine which type of splenocyte was infiltrating the CNS in response to MCMV brain infection. Ribonuclease protection assay (RPA) was performed using the RiboQuant multiprobe set for cell surface markers (Pharmingen, San Diego, CA) on RNA samples extracted from the brains of animals receiving transferred splenocytes at 5 days p.i. RPA analysis demonstrated the prominent presence of CD3+ mRNA in the brain (Figure 4A). In subsequent experiments, we performed immunofluorescence staining of MCMV-infected brain sections using anti-CD3 antibody at 1, 3, 5, and 7 days p.i. (Figure 4B). In addition, double-staining of brain sections from the infected animals following adoptive transfer with X-gal (for expression from the viral genome) and anti-CD3 surface antigen demonstrates that T lymphocytes are present near MCMV-infected cells, as well as throughout the infected brains (Figure 4C).

T lymphocytes control viral expression in the brain and protect against lethal disease

In the next set of experiments, we examined the functional significance of this T lymphocyte infiltration into the MCMV-infected brain. The approach we implemented was to remove all T lymphocyte subsets from total spleen cell preparations by immunomagnetic depletion, using Dynabeads Mouse pan T kit (Dynal, Lake Success, NY), prior to adoptive transfer. When extracts of infected brain tissue were examined using a chemiluminescence assay, reporter gene expression was found to be fourfold greater ($^*P < .05$ *versus* total spleen cells) in the animals receiving Thy 1.2^+-depleted splenocytes when compared to those receiving total splenocytes (Figure 5A). The efficiency of magnetic depletion of T lymphocytes from total splenocytes was determined to be 82% (33% CD3+ cells in total splenocytes *versus* 6% CD3+ cells following magnetic depletion) by flow cytometry (Figure 5B). Further depletion experiments went on to demonstrate that removal of Thy 1.2^+ cells prior to adoptive transfer abolished the ability of splenocytes to protect immunodeficient animals against lethal disease, as well as control MCMV-brain infection (Figure 5C).

Effect of adoptive transfer on chemokine levels in the CNS

Glial cells orchestrate immune cell recruitment to focal areas of viral infection within the brain and synchronize various immune cell functions through a regulated network of cytokines and chemokines. Consequently, we went on to examine temporal differences in CNS expression of chemokine mediators in response to MCMV brain infection in immunodeficient animals with or without adoptively transferred splenocytes. Because recruitment of lymphocytes plays a critical role in resolving MCMV infection in visceral organs (Jonjic *et al*, 1989; Koszinowski *et al*, 1990; Reddehase *et al*, 1985), we investigated the *in vivo* production of several lymphocyte chemoattractants, CXCL10 (interferon-γ–inducible protein-10, IP-10), CCL-2 (monocyte chemoattractant protein, MCP)-1, and CXCL9 (monokine induced by interferon-γ, MIG) in the brains of MCMV-infected mice.

In the brains of mice that had received adoptively transferred splenocytes, chemokine expression was found to coincide with viral expression reaching peak levels at 3 days p.i. followed by a decline (Figure 6). Surprisingly, as the infection progressed, higher levels of viral-induced chemokines were detected in the brains of immunodeficient mice than in the brains of splenocyte-treated immunodeficient animals (Figure 6A, B). Before immunodeficient animals succumbed to MCMV infection (i.e., 10 days p.i.), they expressed markedly greater amounts of CXCL10 and CCL2 than lymphocyte-treated SCID/bg mice that survived infection (Figure 6A, B). CXCL9 was not detected in the brains of infected, immunodeficient mice, but high levels were found in the brains of animals that had received adoptively transferred splenocytes (Figure 6C). These enzyme-linked immunosorbent assay (ELISA) results show that the actual level of chemokine per milligram total brain protein varies for the individual chemokine studied. However, it is presently unknown how the level of a particular chemokine correlates with its biological

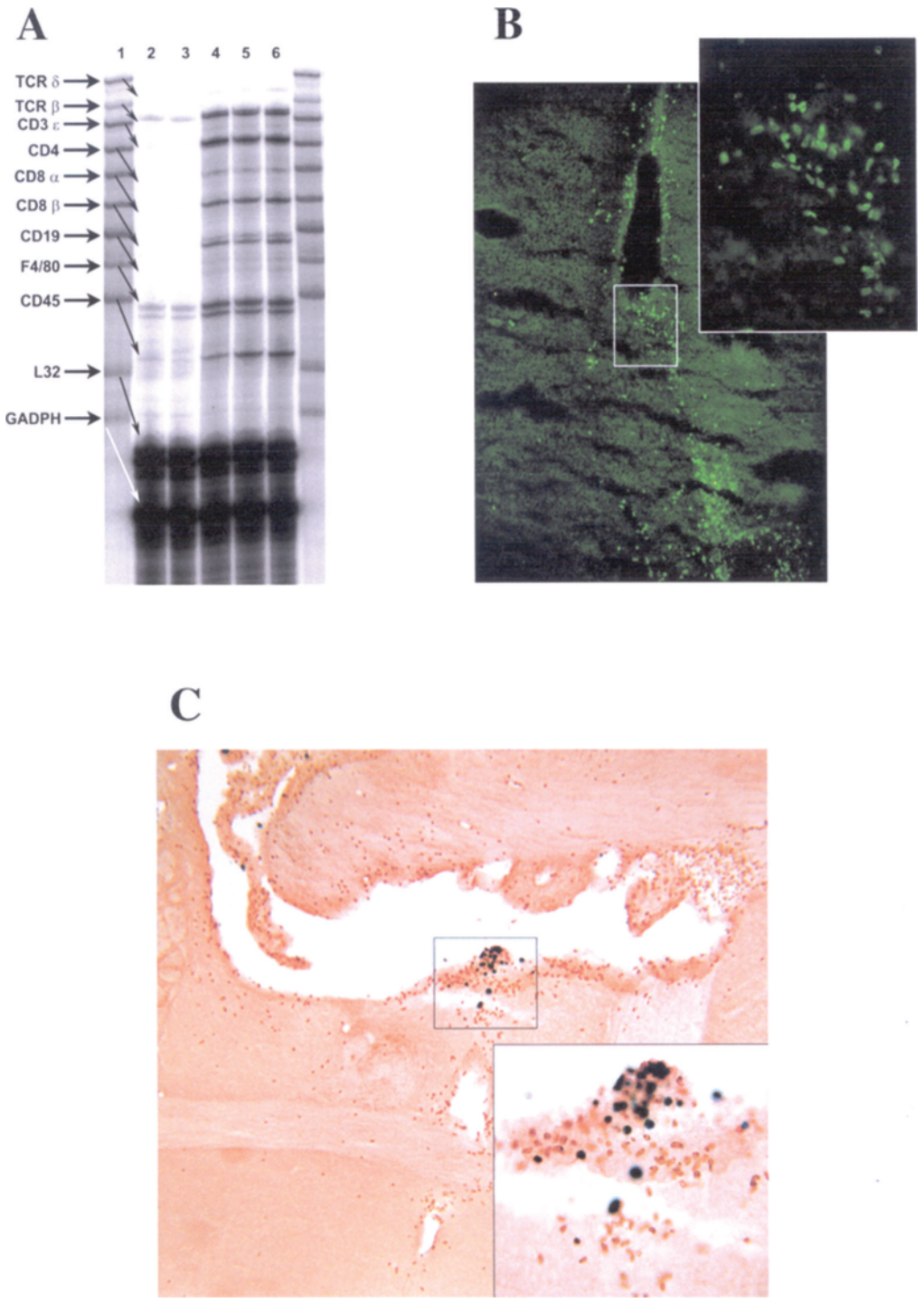

Figure 4 T lymphocytes infiltrate the brains of infected, adoptive transfer recipients. (**A**) Immunodeficient mice were infected with MCMV by the icv route 24 h after receiving adoptively transferred splenocytes. RNA was extracted from brain tissue 5 days following infection and analyzed by RPA using a multiprobe set for cell surface markers (Pharmingen). Lanes: 1, unprotected probe; 2 and 3, sham (saline)-inoculated; 4–6, MCMV-infected, 5 days p.i. (**B**) Immunofluorescence staining of MCMV-infected brain using rat anti-mouse-CD3 (R&D systems, Minneapolis, MN; 1:200) and FITC-labeled secondary antibody (Vector Laboratories, Burlingame, CA). (**C**) Double-staining for viral expression using X-gal (1 mg/ml), followed by immunohistochemical detection of CD3 cell surface antigen (rat anti-mouse-CD3; R&D systems; 1:200 and HRP-labeled secondary antibody with AEC substrate; Vector Laboratories) in the brains of MCMV-infected mice.

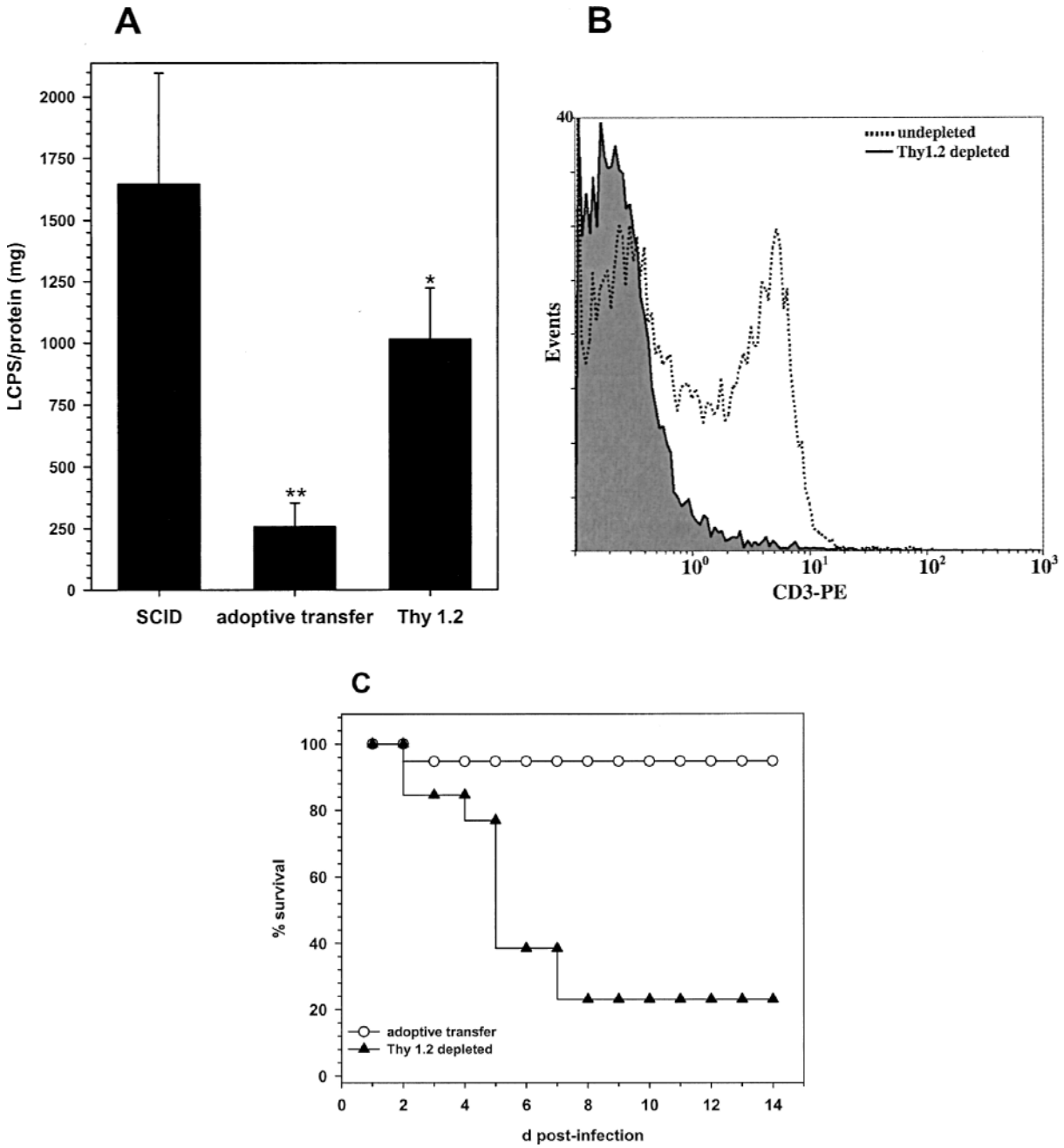

Figure 5 T lymphocytes control viral expression in the brain and protect against lethal disease. (**A**) Expression from the viral genome was measured in the CNS of animals receiving splenocytes that had been depleted of Thy 1.2^+ cells (Thy 1.2) prior to adoptive transfer. All T lymphocyte subsets were removed from total spleen cell preparations by immunomagnetic depletion. Viral expression data are presented as mean ± SEM LCPS from four to seven animals, 5 days p.i. ($^{**}P < .01$ *versus* SCID; $^{*}P < .05$ *versus* total spleen cells; Student's *t* test). (**B**) Efficiency of magnetic T-cell depletion from total splenocytes was assessed by flow cytometry using a PE-conjugated anti-mouse CD3 Mab (BD Biosciences, san Diego, CA). (**C**) Depletion of Thy 1.2^+cells prior to adoptive transfer abolishes the ability of splenocytes to protect immunodeficient animals against lethal disease. Survival is expressed as percent of mice examined; groups contained 10 to 15 animals each.

activity and its ability to induce splenocyte trafficking into the brain. In the CNS of infected, immunocompetent C.B.-17 mice, chemokine expression declined as viral infection was controlled, similar to the production profiles observed in the brains of adoptive transfer recipients (Figure 6D).

Thy 1.2^+ depletion limits CXCL9 production in the brains of recipient mice

We next examined the effect of depleting T cells from total splenocytes prior to adoptive transfer on CXCL9 production in the brains of recipient mice. CXCL9 production in the animals receiving Thy

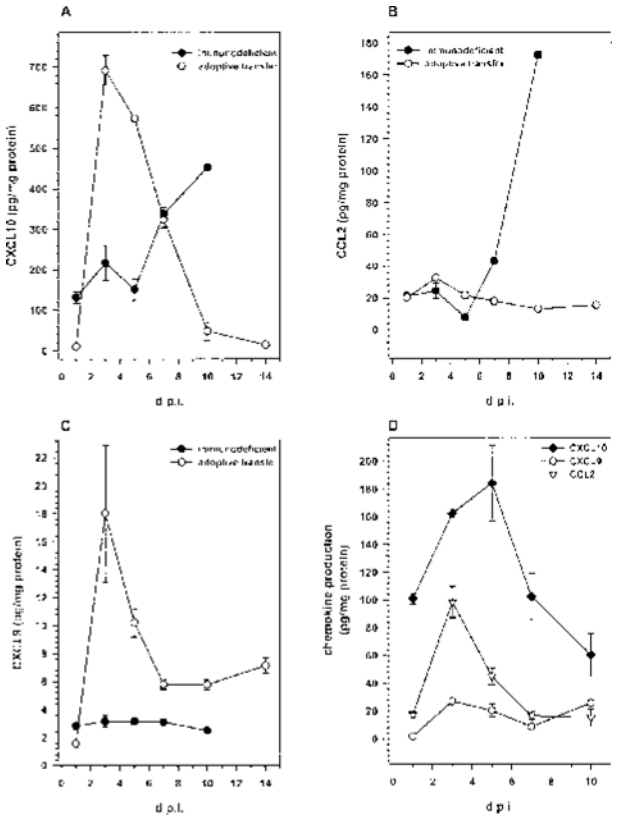

Figure 6 Adoptive transfer of splenocytes limits chemokine production in the brains of immunodeficient mice. (**A**) CXCL10 (IP-10), (**B**) CCL2 (MCP-1), and (**C**) CXCL9 (MIG) production in the CNS of MCMV-infected mice. Extracts of brain tissue were obtained from MCMV-infected immunodeficient mice treated with (*open circles*) or without (*closed circles*) adoptively transferred total spleen cells at 1, 3, 5, 7, and 10 days p.i. and chemokine levels in the brain were measured by ELISA. Data correspond to mean ± SEM chemokine levels from two to five animals per time point.

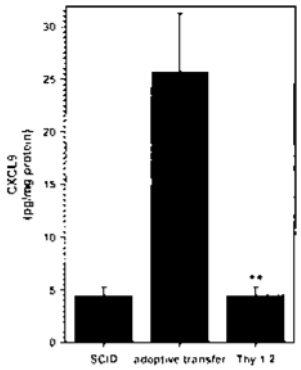

Figure 7 Depletion of Thy 1.2^{+} splenocytes prior to adoptive transfer reduces CXCL9 production in the brains of recipient mice. T lymphocyte subpopulations (Thy 1.2) were depleted from total splenocytes prior to adoptive transfer into immunodeficient C.B-17 SCID/Bg mice. Animals were infected by the icv route 24 h after the adoptive transfer. CXCL9 levels in the brain were measured at 5 days p.i. Data are presented as mean ± SEM chemokine level from each group, groups contained between 7 and 10 animals each. $^{**}P < .01$, Student's *t* test.

1.2–depleted splenocytes was significantly reduced when compared to those receiving undepleted cells (fivefold reduction; $^{**}P < .01$, Student's *t* test) and was similar to levels observed in animals without transferred splenocytes (Figure 7).

Discussion

Over the past several years, our laboratory has used cultures of primary human brain cells to investigate the interaction of glial cells with human CMV (Cheeran *et al*, 2000, 2003; Lokensgard *et al*, 1999, 2002). Although limited by their nature, *in vitro* experiments were used to study these glial cell responses because of the strict species tropism of human CMV for human cells. *In vivo* experimental infection of mice with MCMV has served as a useful model that imitates many facets of human CMV pathogenesis. These two CMVs share numerous similarities and MCMV pathogenesis in mice mimics many features of human CMV disease. Analogous to the situation in humans, immunocompetent mice are not susceptible to CMV encephalitis, whereas mice with a severe immune deficit (C.B-17 SCID/bg) develop uncontrolled viral brain infection analogous to that seen in patients with advanced acquired immuno-deficiency syndrome (AIDS) (Arribas *et al*, 1996). In the present study, we used icv-infected, adult SCID/bg mice as an animal model of CMV brain infection in the context of a severely immunocompromised host and demonstrated a role for cells from the somatic immune system in controlling this devastating brain infection.

In humans, the pathological manifestations of CMV encephalitis include two main forms: microglial nodule formation and ventriculoencephalitis (Arribas *et al*, 1996). By using a reporter gene–containing virus to track the pattern of viral spread within the murine CNS at various times p.i., we determined that in the brains of immunocompetent animals MCMV infection was largely restricted to cells of the ventricular walls, with minimal viral spread into the cerebral parenchyma. MCMV brain infection in these mice was cleared by 9 days p.i. In sharp contrast, in immunodeficient SCID/bg mice, extensive, uncontrolled viral spread from the ventricles throughout the brain parenchyma was observed, demonstrating that intrinsic glial cell responses alone are insufficient to control MCMV brain infection in these mice. Moreover, the immunodeficient mice succumbed to lethal MCMV disease. Taken together, these results demonstrate that following injection of MCMV into the cerebral ventricles viral infection begins in the periventricular cells, and in the absence of immune control, subsequently spreads into the brain parenchyma. A similar pattern of viral spread has recently been reported in the brains of newborn mice (Kosugi *et al*, 2002).

We also demonstrated that adoptive transfer of spleen cells from MCMV-primed, MHC-matched donor mice restricts viral spread within the brain. In newborn mice, innate immune responses, mediated both by NK cells as well as NO derived from macrophages, are thought to contribute to viral elimination from the brain (Kosugi *et al*, 2002). The present experiments used adult mice with the beige (bg) mutation. These bg mice possess normal numbers of NK cells, but exhibit defective NK activity and have been shown to be highly susceptible to infection with MCMV (Shellam *et al*, 1981). In this study, transfer of splenocytes protected immunodeficient animals from lethal disease, although we do not know what role NK cells versus other populations of lymphocytes played in conferring this protection. Our chemiluminescence data, quantifying reporter gene activity in the brain, show what appears to be biphasic control of expression from the MCMV genome. It is likely that the innate immune response plays an important role in the first phase of this control. It appears that the innate immune response initially (within the first week) clears the virus, even without adoptive transfer, and that the transferred splenocytes function to reduce viral expression at later time points. Further experiments are needed to characterize the different cell populations responsible for these innate and adaptive immune responses.

Through ribonuclease protection assay and immunocytochemistry, we have demonstrated that T lymphocytes are a predominant cell type that infiltrates the brain during MCMV infection. Furthermore, through depletion studies, T cells were shown to control viral expression in the brain and protect animals against lethal MCMV disease. Because trafficking of leukocytes from the blood into the brain is usually preceded by chemokine production from activated glial cells (Glabinski and Ransohoff, 1999), we also investigated the production of several key chemokines in response to infection of the brain with MCMV. CXCL10 has been shown to be involved in the recruitment of IFN-γ–producing lymphocytes into the brain (Farber, 1997; Liu *et al*, 2001), and this chemokine has also been demonstrated to be critical in providing host defense against viral infection of the CNS (Liu *et al*, 2000). Following icv infection of immunodeficient mice with MCMV, levels of CXCL10 as well as CCL2 in the CNS coincided with active viral expression and remained elevated throughout the time course of infection. In immunodeficient animals that received adoptively transferred splenocytes, however, the levels of these chemokines waned as viral infection was controlled and the mice recovered from disease. Our previous *in vitro* studies have shown that infection with human CMV elicits the production of CXCL10 from primary microglial cells, but not from astrocytes (Cheeran *et al*, 2003). Results from the present study suggest that glial cells also produce this important chemokine *in vivo*. Interestingly, CXCL9 was not detected in the brains of MCMV-infected immunodeficient mice that did not receive splenocytes. This finding indicates that viral infection alone does not stimulate CXCL9 production from brain cells, but that production of this chemokine requires either the physical presence of peripheral immune cells or soluble factors derived from these immune effectors (e.g., IFN-γ). A similar chemokine-to-cytokine-to-chemokine cascade has been reported for MCMV infection of the liver where CXCL9 expression depended upon the presence of MIP-1α, NK cells, and IFN-γ (Salazar-Mather *et al*, 2000). In any event, the production of chemokines, such as CXCL10 and CCL2, by endogenous brain cells indicates that they could be key mediators in recruitment of immune effector cells towards sites of MCMV infection within the brain. Taken together, the results of this study demonstrate the ability of effector cells from the somatic immune system to restrict intracerebral viral spread and indicate that intrinsic glial cell responses alone are insufficient to control MCMV brain infection.

Materials and methods

Viruses and animals

MCMV RM461, a recombinant virus expressing *E. coli* β-galactosidase under the control of the human ie1/ie2 promoter/enhancer (Stoddart *et al*, 1994), was kindly provided by Dr. Edward S. Mocarski (Stanford University). Salivary gland–passaged, sucrose gradient–purified virus was used for all icv infections. Stocks of MCMV Smith strain (ATCC, Rockville, MD) used to prime donor animals, were grown and titered by $TCID_{50}$ assay on NIH 3T3 fibroblasts. BALB/c, C.B-17, and C.B-17 SCID/bg mice were obtained from Charles River Laboratories (Wilmington, MA).

Intracerebroventricular infection

To develop an *in vivo* CMV brain infection model, female C.B17 or C.B17-SCID/bg mice (8 to 10 weeks old) were anesthetized using a combination of ketamine and xylazine (100 mg and 10 mg/kg body weight, respectively) and immobilized on a small animal stereotactic instrument equipped with a Cunningham mouse adapter (Stoelting, Wood Dale, IL). The skin and underlying connective tissue were reflected to expose reference sutures (sagittal and coronal) on the skull. The sagittal plane was adjusted such that the bregma and lambda were positioned at the same coordinates on the vertical plane. Salivary gland passaged MCMV RM461 (3×10^5 $TCID_{50}$ units in 10 μl) was injected into the right lateral ventricle at 0.9 mm lateral and 0.5 mm caudal to the bregma and 3.0 mm ventral to the skull surface using a Hamilton syringe (10 μl) fitted to a 25-G cannula. The injection was delivered over a period of 3 to 5 min. The opening in the skull was sealed with bone

wax and the skin was closed using 9 mm wound clips (Stoelting).

Adoptive transfer
Spleen cells were aseptically collected from MHC-matched BALB/c (H-2^d) mice 8 days following intraperitoneal injection of MCMV Smith strain. Splenocytes were depleted of red blood cells by treatment with 0.87% ammonium chloride for 2 min followed by the addition of an equal volume of phosphate-buffered saline (PBS) containing 2% fetal bovine serum. The cells were then washed and spun down twice. Total spleen cells (1×10^7) obtained from MHC-matched (H-2^d), MCMV-infected mice (8 days p.i.) were adoptively transferred via tail vein injection into immunodeficient C.B-17 SCID/bg mice 24 h prior to icv infection with RM461 ($3 \times 10^5 TCID_{50}$).

β-Galactosidase detection
In situ staining for Lac-Z expression was performed using X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside; Roche Applied Science, Indianapolis, IN) as a substrate at a concentration of 1 mg/ml. Reporter gene expression from the viral genome was quantified in whole brain tissue extracts (homogenized in tissue protein extraction reagent, TPER; Pierce, Rockford, IL) using a chemiluminescence assay for β-galactosidase according to the manufacturer's instructions (Tropix Gal-Screen; Applied Biosystems, Foster City, CA).

Ribonuclease protection assay
To determine which surface markers were present on cells infiltrating the CNS, total RNA was extracted (Qiagen, Valencia, CA) from MCMV-infected mice and assayed by using a RiboQuant multiprobe set for cell surface markers (Pharmingen, San Diego, CA) according to the manufacturer's instructions.

ELISA
A previously described sandwich ELISA–based system (Peterson *et al*, 1997) was used to measure levels of CXCL9, CXCL10, and CCL2 in murine brains at various times following MCMV infection. ELISA plates were coated with goat anti-mouse CXCL9 and CXCL10 (1 μg/ml; R&D Systems, Minneapolis, MN), or hamster anti-mouse CCL2 (Pharmingen) capture antibody at 2 μg/ml overnight at 4°C. The plates were washed and blocked with 1% bovine serum albumin (BSA) in PBS for 1 h at 37°C. Serial dilutions of recombinant chemokines were used to generate a standard concentration curve. Brain tissue extracts were incubated in capture antibody–coated wells for 2 h at 37°C. Biotin-labeled goat anti-mouse CXCL9 or CXCL10 antibody (1 μg/ml; R&D Systems) or hamster anti-mouse CCL2 (Pharmingen) was added for 90 min at 37°C followed by horseradish peroxidase–conjugated streptavidin (1:4,000; Jackson Immunoresearch, West Grove, PA) for 45 min. A chromogenic substrate (K-blue; Neogen Corporation, Lexington, KY) was then added for 10 to 20 min at room temperature. Color development was stopped with 1 M H_2SO_4. Levels of chemokines in murine brain tissue were estimated from the standard concentration curve using absorbance values at 450 nm. The sensitivity of the ELISA was 7.5 pg/ml, 3.7 pg/ml, and 7.5 pg/ml for CXCL10, CCL2, and CXCL9, respectively.

References

Arribas JR, Storch GA, Clifford DB, Tselis AC (1996). Cytomegalovirus encephalitis. *Ann Intern Med* **125:** 577–587.

Asensio VC, Campbell IL (1997). Chemokine gene expression in the brains of mice with lymphocytic choriomeningitis. *J Virol* **71:** 7832–7840.

Cheeran MC, Hu S, Gekker G, Lokensgard JR (2000). Decreased cytomegalovirus expression following proinflammatory cytokine treatment of primary human astrocytes. *J Immunol* **164:** 926–933.

Cheeran MC, Hu S, Sheng WS, Peterson PK, Lokensgard JR (2003). CXCL10 production from cytomegalovirus-stimulated microglia is regulated by both human and viral interleukin-10. *J Virol* **77:** 4502–4515.

Cheeran MC, Hu S, Yager SL, Gekker G, Peterson PK, Lokensgard JR (2001). Cytomegalovirus induces cytokine and chemokine production differentially in microglia and astrocytes: antiviral implications. *J NeuroVirol* **7:** 135–147.

Farber JM (1997). Mig and IP-10: CXC chemokines that target lymphocytes. *J Leukoc Biol* **61:** 246–257.

Glabinski AR, Ransohoff RM (1999). Sentries at the gate: chemokines and the blood-brain barrier. *J NeuroVirol* **5:** 623–634.

Jonjic S, Mutter W, Weiland F, Reddehase MJ, Koszinowski UH (1989). Site-restricted persistent cytomegalovirus infection after selective long-term depletion of CD4+ T lymphocytes. *J Exp Med* **169:** 1199–1212.

Jonjic S, Pavic I, Lucin P, Rukavina D, Koszinowski UH (1990). Efficacious control of cytomegalovirus infection after long-term depletion of CD8+ T lymphocytes. *J Virol* **64:** 5457–5464.

Kosugi I, Kawasaki H, Arai Y, Tsutsui Y (2002). Innate immune responses to cytomegalovirus infection in the developing mouse brain and their evasion by virus-infected neurons. *Am J Pathol* **161:** 919–928.

Koszinowski UH, Del Val M, Reddehase MJ (1990). Cellular and molecular basis of the protective immune response to cytomegalovirus infection. *Curr Top Microbiol Immunol* **154:** 189–220.

Lane TE, Asensio VC, Yu N, Paoletti AD, Campbell IL, Buchmeier MJ (1998). Dynamic regulation of alpha- and beta-chemokine expression in the central nervous system during mouse hepatitis virus-induced demyelinating disease. *J Immunol* **160:** 970–978.

Liu MT, Chen BP, Oertel P, Buchmeier MJ, Armstrong D, Hamilton TA, Lane TE (2000). The T cell chemoattractant IFN-inducible protein 10 is essential in host defense

against viral-induced neurologic disease. *J Immunol* **165:** 2327–2330.

Liu MT, Keirstead HS, Lane TE (2001). Neutralization of the chemokine CXCL10 reduces inflammatory cell invasion and demyelination and improves neurological function in a viral model of multiple sclerosis. *J Immunol* **167:** 4091–4097.

Lokensgard JR, Cheeran MC, Gekker G, Hu S, Chao CC, Peterson PK (1999). Human cytomegalovirus replication and modulation of apoptosis in astrocytes. *J Hum Virol* **2:** 91–101.

Lokensgard JR, Cheeran MC, Hu S, Gekker G, Peterson PK (2002). Glial cell responses to herpesvirus infections: role in defense and immunopathogenesis. *J Infect Dis* **186(Suppl 2):** S171–S179.

Lucin P, Pavic I, Polic B, Jonjic S, Koszinowski UH (1992). Gamma interferon-dependent clearance of cytomegalovirus infection in salivary glands. *J Virol* **66:** 1977–1984.

Pavic I, Polic B, Crnkovic I, Lucin P, Jonjic S, Koszinowski UH (1993). Participation of endogenous tumour necrosis factor alpha in host resistance to cytomegalovirus infection. *J Gen Virol* **74:** 2215–2223.

Peterson PK, Hu S, Salak-Johnson J, Molitor TW, Chao CC (1997). Differential production of and migratory response to beta chemokines by human microglia and astrocytes. *J Infect Dis* **175:** 478–481.

Polic B, Hengel H, Krmpotic A, Trgovcich J, Pavic I, Luccaronin P, Jonjic S, Koszinowski UH (1998). Hierarchical and redundant lymphocyte subset control precludes cytomegalovirus replication during latent infection. *J Exp Med* **188:** 1047–1054.

Polic B, Jonjic S, Pavic I, Crnkovic I, Zorica I, Hengel H, Lucin P, Koszinowski UH (1996). Lack of MHC class I complex expression has no effect on spread and control of cytomegalovirus infection in vivo. *J Gen Virol* **77:** 217–225.

Pulliam L, Moore D, West DC (1995). Human cytomegalovirus induces IL-6 and TNF alpha from macrophages and microglial cells: possible role in neurotoxicity. *J NeuroVirol* **1:** 219–227.

Reddehase MJ, Jonjic S, Weiland F, Mutter W, Koszinowski UH (1988). Adoptive immunotherapy of murine cytomegalovirus adrenalitis in the immunocompromised host: CD4-helper-independent antiviral function of CD8-positive memory T lymphocytes derived from latently infected donors. *J Virol* **62:** 1061–1065.

Reddehase MJ, Weiland F, Munch K, Jonjic S, Luske A, Koszinowski UH (1985). Interstitial murine cytomegalovirus pneumonia after irradiation: characterization of cells that limit viral replication during established infection of the lungs. *J Virol* **55:** 264–273.

Salazar-Mather TP, Hamilton TA, Biron CA (2000). A chemokine-to-cytokine-to-chemokine cascade critical in antiviral defense. *J Clin Invest* **105:** 985–993.

Scalzo AA, Fitzgerald NA, Simmons A, La Vista AB, Shellam GR (1990). Cmv-1, a genetic locus that controls murine cytomegalovirus replication in the spleen. *J Exp Med* **171:** 1469–1483.

Scalzo AA, Fitzgerald NA, Wallace CR, Gibbons AE, Smart YC, Burton RC, Shellam GR (1992). The effect of the Cmv-1 resistance gene, which is linked to the natural killer cell gene complex, is mediated by natural killer cells. *J Immunol* **149:** 581–589.

Shellam GR, Allan JE, Papadimitriou JM, Bancroft GJ (1981). Increased susceptibility to cytomegalovirus infection in beige mutant mice. *Proc Natl Acad Sci U S A* **78:** 5104–5108.

Stoddart CA, Cardin RD, Boname JM, Manning WC, Abenes GB, Mocarski ES (1994). Peripheral blood mononuclear phagocytes mediate dissemination of murine cytomegalovirus. *J Virol* **68:** 6243–6253.

Tay CH, Welsh RM (1997). Distinct organ-dependent mechanisms for the control of murine cytomegalovirus infection by natural killer cells. *J Virol* **71:** 267–275.

Welsh RM, Brubaker JO, Vargas-Cortes M, O'Donnell CL (1991). Natural killer (NK) cell response to virus infections in mice with severe combined immunodeficiency. The stimulation of NK cells and the NK cell-dependent control of virus infections occur independently of T and B cell function. *J Exp Med* **173:** 1053–1063.

Wenkel H, Streilein JW, Young MJ (2000). Systemic immune deviation in the brain that does not depend on the integrity of the blood-brain barrier. *J Immunol* **164:** 5125–5131.