T cell–mediated restriction of intracerebral murine cytomegalovirus infection displays dependence upon perforin but not interferon-γ

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The authors have previously reported that adoptive transfer of splenocytes suppresses murine cytomegalovirus (MCMV) brain infection following intracerebroventricular injection of immunodeficient mice and that depletion of Thy 1.2+ T lymphocytes abolishes this suppressive effect. Here the authors report that splenocytes depleted of CD4+ T lymphocytes prior to adoptive transfer retained their ability to control viral expression in the brain. In sharp contrast, depletion of the CD8+ T-cell population prior to transfer abolished the suppressive effect, with sixfold greater expression in the brain than when undepleted splenocytes were used. The authors went on to examine the contributions of cytokine- and perforin-mediated mechanisms in controlling MCMV brain infection using splenocytes from major histocompatibility (MHC)-matched IFN-γ-knockout (GKO), and perforin-knockout (PKO) mice. When used in adoptive transfer studies, splenocytes from GKO mice controlled viral expression; however, cells from PKO mice could not control reporter gene expression or viral DNA replication in brain tissues. The authors have previously reported that the levels of the T-cell chemoattractant CXCL10 are highly elevated in the brains of MCMV-infected mice. Here the authors found that the receptor for this ligand, CXCR3, was not essential in mediating the suppressive effects of adoptive transfer. These data indicate that peripheral CD8+ T cells control MCMV brain infection through a perforin-mediated mechanism and that neither IFN-γ nor CXCR3 play a critical role in this neuroprotective response.

Keywords: encephalitis; MCMV; perforin

Introduction

We have previously reported that adoptive transfer of splenocytes from primed immunocompetent mice into immunodeficient (C.B-17 SCID/bg) recipients restricted murine cytomegalovirus (MCMV) brain infection following subsequent stereotactic intracerebroventricular (icv) injection (Cheeran et al, 2004). Further experiments went on to demonstrate that peripheral CD3+ T lymphocytes infiltrated the MCMV-infected brain and that depletion of the Thy1.2+ T-cell population prior to adoptive transfer abolished the protective effect (Cheeran et al, 2004). Despite an extensive literature on the contribution of T cells to host defense, the mechanism by which these lymphocytes defend the brain against MCMV remains unknown.

Neuroimmune responses to viral infections of the brain must clear the invading pathogen while simultaneously preventing extensive damage to this
vital nonregenerating tissue (Patterson et al, 2002). T lymphocytes are known to mediate their antiviral functions by a variety of mechanisms, including the production of tumor necrosis factor-α and interferon (IFN)-γ, as well as through cytotoxic mechanisms involving Fas/FasL and perforin (Finke et al, 1995; Guidotti and Chisari, 2000; Medana et al, 2000; Ruby and Ramshaw, 1991; Tishon et al, 1995). T lymphocytes use different immune mechanisms tailored to control viral infections in particular organs, and it is becoming increasingly clear that target cell type–specific mechanisms within particular tissues also exist. For example, in the central nervous system (CNS), IFN-γ production by infiltrating T lymphocytes has been shown to play an important role in clearance of viral infections from neurons without concomitant neuronal loss (Binder and Griffin, 2001; Kundig et al, 1993; Patterson et al, 2002; Tishon et al, 1995). However, T cells control mouse hepatitis virus replication in microglia and astrocytes through perforin-mediated cytolyis, whereas they use IFN-γ–mediated mechanisms to regulate viral replication in oligodendrocytes (Bergmann et al, 2004; Stohlman et al, 2002). It is likely that other CNS pathogens also encounter similar diverse neuroimmune responses at the level of cellular tropism.

T lymphocytes are known to be the primary effector cells capable of defending many tissues from productive CMV infection (Jonjic et al, 1989; Koszinowski et al, 1990; Reddehase et al, 1985), but when operating within the CNS, cytotoxic destruction of infected target cells may induce irreparable brain damage. The present study was undertaken to determine which T-cell subset is responsible for mediating the suppressive effect of adoptive transfer in our brain infection model and to determine the effector mechanism(s) used by these lymphocytes to control MCMV infection of the CNS.

Results

**CD8-positive splenocytes control MCMV brain infection**

To determine which T lymphocyte subpopulation was responsible for mediating the suppressive effect of adoptive transfer on MCMV brain infection, either CD4+ (L3T4) or CD8+ (Lyt-2) cells were separately removed from total splenocytes obtained from primed mice by immunomagnetic depletion. Following depletion, the splenocytes were transferred into immunodeficient C.B-17 SCID/bg mice via tail-vein injection 24 h prior to icv injection of MCMV. When reporter gene expression in the infected brains was assessed using a chemiluminescence assay at 6 days post icv infection, we found that cells which had been depleted of the CD4+ T-lymphocyte population prior to adoptive transfer retained the ability to control viral expression in the brain, similar to undepleted splenocytes (Figure 1A). In sharp contrast, depletion of the CD8+ T cells prior to adoptive transfer abolished the subsequent suppressive effect on viral brain infection, with sixfold greater expression than adoptive transfer using undepleted splenocytes (Figure 1A). The results obtained by reporter gene expression assay were further corroborated by measuring viral load in brain tissue homogenates obtained from animals in each treatment group using real-time PCR (6 days post infection). The amount of MCMV DNA was standardized to β-actin and is presented as mean ± SEM normalized copy number from pooled data using three to five animals per group. (C) Efficiency of magnetic depletion of CD8+ or (D) CD4+ cells from total splenocytes was assessed by flow cytometry using a PE-conjugated anti-murine CD8 or anti-CD4 specific Mab (BD Biosciences, San Diego, CA).
the CD8\(^+\) T cells prior to adoptive transfer abolished the subsequent suppressive effect on viral brain infection were obtained using chemiluminescence assay and real-time PCR. Because the efficiency of magnetic depletion can be variable, we carried out flow cytometry studies using anti-mouse CD8 and CD4 monoclonal antibodies (MAbs) to measure the depletion of each T-cell subset from total splenocytes prior to performing adoptive transfer. These studies demonstrated that the efficiency of magnetic depletion was >99% for both CD8 and CD4 cells (Figure 1C and D, respectively).

**Control of MCMV brain infection is not mediated by IFN-\(\gamma\)**

We next examined the contribution of IFN-\(\gamma\) in controlling MCMV brain infection using splenocytes from IFN-\(\gamma\)-knockout (GKO) BALB/c mice. When splenocytes from GKO mice were transferred into immunodeficient mice 24 h prior to icv infection, these cells retained their ability to suppress viral expression in the brain and the levels of reporter gene expression observed at 6 days post infection (p.i.) were similar to those seen using splenocytes from wild-type mice (Figure 2).

**T cell–mediated restriction of MCMV brain infection displays dependence upon perforin**

When splenocytes from perforin-knockout (PKO) mice were used in adoptive transfer experiments, reporter gene expression from the viral genome was found to be 6.21-fold greater than in the brains of infected mice which received splenocytes from wild-type animals at 5 days p.i. (Figure 3A). The failure of CD8\(^+\) lymphocytes from PKO mice to control MCMV infection was confirmed by quantifying the level of viral DNA replication in the brain using real-time PCR. Immunodeficient mice receiving splenocytes from PKO mice prior to infection displayed 6.92-fold more MCMV DNA in their brains than mice receiving splenocytes from wild-type animals (Table 1). In addition to real-time RT-PCR experiments, we have found significantly elevated levels of granzyme B transcripts in the brains of adoptive transfer recipients given splenocytes from either wild-type or PKO

| Table 1 Effect of adoptive transfer on viral DNA replication in the brain |
|-------------------------------|-----------------|---------|-----------------|
| Treatment                      | \(-\Delta C_T\) | Average | Fold increase\(\ast\) |
| Scid/Bg                        | 4.31            | 4.30    | 13.37           |
| Adoptive transfer              | 0.1             | 0.56    | 1               |
| PKO                            | 3.38            | 3.35    | 6.92            |

\(\ast\)Over animals given wild-type splenocytes.
mice (Figure 3B). These results demonstrated that PKO mice are not deficient in granzyme B production and that the levels of granzyme B detected in the brain correlate well with lymphocyte infiltration (shown below), but not necessarily protection against MCMV.

*Depletion of the CD8\(^+\) lymphocyte subset or the knockout of perforin does not affect T-cell infiltration into the brains of adoptive transfer recipients*

CD8-depleted splenocytes as well as splenocytes from PKO mice were unable to control MCMV brain infection. To determine if these splenocytes were defective in their ability to infiltrate the brain parenchyma, we performed dual histochemical (i.e., X-gal) and immunohistochemical (i.e., anti-CD3) staining experiments to examine the presence of virus and CD3\(^+\) lymphocytes in brain sections obtained from animals receiving either total splenocytes (Figure 4A) or splenocytes following depletion of the CD8\(^+\) T-cell subset (Figure 4B). In analogous experiments, brain sections from mice receiving transferred splenocytes from PKO mice were stained in an identical manner (Figure 4C). The results obtained clearly demonstrated that neither the depletion of the CD8\(^+\) cell subset nor the knockout of perforin deleteriously affected the ability of these lymphocytes to infiltrate the brain.

*CXCR3 is not essential in mediating the suppressive effects of adoptive transfer*

We have previously reported that levels of the T-cell chemoattractant CXCL10 (IFN-\(\gamma\)-inducible protein of 10 kDa, IP-10) are highly elevated in the brains of MCMV-infected mice [Cheeran et al, 2004]. In this study, we examined whether the receptor for this ligand, CXCR3, was essential in mediating the suppressive effects of adoptive transfer. When splenocytes from CXCR3-knockout mice were used in adoptive transfer experiments, these cells retained their ability to suppress brain infection and levels of expression from the viral genome in the CNS were similar to those detected in mice receiving wild-type splenocytes at 5 days post icv injection (Figure 5).

The data expressed as luminescent counts per second (LCPS/mg) are generated by an enzymatic reaction in which \(\beta\)-galactosidase cleaves a chemiluminescent substrate. This is why level of the raw counts obtained depends upon the reaction conditions. The LCPS obtained fluctuate widely between experiments and even change within a single experiment over time. Therefore, the raw luminescence counts themselves are meaningless and comparisons can only be made by examining relative levels between groups in the same experiment.

**Discussion**

Using our previously described model of MCMV brain infection (Cheeran et al, 2004), we first asked which Thy 1.2\(^+\) T-cell subset was responsible for mediating the suppressive effect of adoptive transfer. By individually depleting either the CD4\(^+\) or CD8\(^+\) T-cell population prior to splenocyte transfer, it was
determined that the CD8+ subset was the peripheral immune cell type responsible for restricting MCMV brain infection. These results clearly demonstrated that CD8+ T cells, and not CD4+ lymphocytes, are the principal effectors of MCMV clearance from the brain. However, a dependence on CD4+ T-helper cells for the initial clonal expansion of CD8+ cells during the immune priming phase cannot be discounted.

We went on to investigate the mechanism(s) of MCMV suppression in the CNS using splenocytes from primed GKO mice. Following adoptive transfer via tail-vein injection, it was found that splenocytes from GKO mice were able to suppress MCMV brain infection as capably as cells obtained from wild-type animals. It is likely that T lymphocytes use distinct immune mechanisms to control viral replication in particular types of brain cells. For example, in the mouse hepatitis virus model, CD8+ T cells have been shown to possess the ability to inhibit virus replication in all brain cell types; however, viral inhibition by these lymphocytes is mediated through different, cell type–specific mechanisms (Bergmann et al., 2004). Cytolytic activity in the absence of IFN-γ suppresses the infection in astrocytes, but not oligodendrocytes; whereas lymphocytes that secreted IFN-γ, but lacked cytolytic activity, inhibited replication in oligodendrocytes, but not astrocytes (Bergmann et al., 2004). In our MCMV infection model, we have found that astrocytes support productive viral replication, although we cannot rule out the involvement of other brain cell types.

Perforin is a cytolytic protein present in the granules of T lymphocytes and natural killer cells. When splenocytes from PKO animals were used in adoptive transfer experiments, they were unable to control viral expression in the brain. This failure of splenocytes deficient in the perforin pathway to control MCMV brain infection was confirmed using real-time PCR to quantify the extent of viral DNA replication in the CNS. Interestingly, the levels of viral expression in the brain were actually higher in PKO mice than in mice not receiving any adoptive transfer of splenocytes. There are many possible explanations for why more expression from the viral genome is observed in the PKO recipient mice than in the SCID/bg controls and it is interesting that similar results are observed following staining with X-gal (i.e., more blue cells present in brain sections from perforin knockouts). It could be that the splenocytes from PKO mice respond to greater amounts of virus in the brain by producing more cytokines, which stimulate promoter activity, and, therefore, more reporter gene expression is observed. We do not know if the increased reporter gene expression observed in the brains of PKO splenocyte recipients worsens the outcome, relative to nonrecipients, because MCMV brain infection is lethal in both groups.

In an experimental model of MCMV retinitis, it has been reported that mice deficient in the perforin cytotoxic pathway, but not the Fas/FasL pathway, are highly susceptible, indicating the importance of the perforin pathway in providing protection against MCMV in the eye (Dix et al., 2003). Interestingly, in the same study higher MCMV titers were observed in the livers of PKO mice, indicating that the perforin pathway may also provide protection against virus–induced hepatitis (Dix et al., 2003). Results reported here using our brain infection model are consistent with the idea that CD8+ lymphocytes use perforin to control viral replication in astrocytes (Bergmann et al., 2004).

It is clear that glial cells and lymphocytes communicate through chemokine mediators. We have previously reported that levels of the chemokines CXCL10 and CXCL9 (monokine induced by IFN-γ, MIG) are highly elevated during MCMV brain infection. Both of these chemokines are recognized by the receptor CXCR3 (Loetscher et al., 1996), which has been found on infiltrating T cells (Balashov et al., 1999; Simpson et al., 2000; Sorensen et al., 2002). Interactions between CXCL10, produced by glial cells in response to viral infection, and CXCR3, present on T cells, may guide infiltrating lymphocyte effectors to sites of viral infection in the brain. We tested the hypothesis that T cells from CXCR3-deficient mice would not control MCMV brain infection, because of defective trafficking to this infection site. However, the results obtained clearly show that splenocytes from CXCR3-knockout mice retain the ability to protect against MCMV brain infection following transfer into immunodeficient recipients.

The involvement of the perforin pathway in protection of mice against MCMV brain infection described here strengthens the conclusion that, although the
brain was once considered immunoprivileged, infiltrating T lymphocytes can use classic perforin-mediated pathways to clear viral infections from this vital site.

**Materials and methods**

**Viruses**

RM461, a recombinant MCMV expressing *Escherichia coli* β-galactosidase under the control of the human CMV ie1/ie2 promoter/enhancer (Stoddart et al, 1994), was kindly provided by 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 50% tissue culture infective dose (TCID<sub>50</sub>) assay on NIH 3T3 fibroblasts.

**Mice**

BALB/c and C.B-17 SCID/bg mice were obtained from Charles River Laboratories (Wilmington, MA). GKO BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). PKO BALB/c mice were a generous gift from Stephen Stohlman (University of Southern California). CXCR3-knockout BALB/c mice were kindly provided by Bao Lu and Craig Gerard (Harvard University).

**ICV infection of SCID/bg mice**

Icv infection of mice was performed as previously described (Cheeran et al, 2004). Briefly, female 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 passed MCMV RM461 (3 × 10<sup>5</sup> TCID<sub>50</sub> units in 10 μl), was injected into the right lateral ventricle at 0.9 mm lateral, 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 major histocompatibility complex (MHC)-matched BALB/c, GKO, PKO, or CXCR3-deficient mice 8 days following intraperitoneal injection of tissue-culture passaged 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. L3T4 (CD4<sup>+</sup>) and Lyt-2 (CD8<sup>+</sup>) cells were separately removed from total splenocytes obtained from primed mice by immunomagnetic depletion using Dynabeads according to the manufacturer’s instructions (Dynal, Lake Success, NY). Spleen cells (1 × 10<sup>7</sup>) obtained from MHC-matched, MCMV-primed mice (8 days p.i.) were then transferred via tail vein injection into immunodeficient C.B-17 SCID/bg mice 24 h prior to icv infection with RM461 (3 × 10<sup>6</sup> TCID<sub>50</sub>).

**β-Galactosidase detection**

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). 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.

**Real-time PCR**

DNA was extracted from brain tissue homogenates using the TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The DNA was eluted in water and stored at −80°C until quantification using real-time PCR. Primers for MCMV were designed from the gene encoding glycoprotein B (GenBank accession no. M86302, 5′-CGCT GGTTCGTTATTCGTTCTGTCAG-T3′ and 5′-CTGTTCTGTTGTCG CAGTTCTC-3′, 112-bp product). Primers recognizing the housekeeping gene β-actin were designed from the mouse β-actin DNA sequence (GenBank accession no. NM_007393, 5′-GGGCGATCCGGTCTCGGAG-3′ and 5′-GATGTCACGCAGATTCCC-3′, 100-bp product).

Total RNA was extracted from brain tissue homogenate using TRizol according to the manufacturer’s separate instructions for RNA tissue extraction. Reverse transcription of 5 μg of RNA into cDNA was carried out using SuperScript II reverse transcriptase according to the manufacturer’s specifications. Granzyme B primer sequences were taken from the mouse mRNA sequence of granzyme B (GenBank accession no. NM_013542, 5′-TCGACGGCTATGGCGCTTTAC-3′ and 5′-GAGGAGCACTCAGCAGAAGG-3′, 98-bp product). Primers recognizing the hypoxanthine guanine phosphoribosyl transferase 1 (HPRT) cDNA were designed from the murine HPRT mRNA sequence (GenBank accession no. BC083145, 5′-TGCTCAAGATGTCATGGAAG-3′ and 5′-AATCCAG CAGGTACGAAAGG-3′, 95-bp product).
PCR was carried out with the LightCycler FastStart DNA Master SYBR Green I kit (Roche Applied Science, Indianapolis, IN) following the manufacturer’s specifications. The 20-μl final reaction volume consisted of premade reaction mix (SYBR Green I dye, reaction buffer Taq DNA polymerase, and dNTPs), 0.3 microM of each primer, and 0.5 ng of DNA in water. For the amplification of cDNA targets, 2μl of a 10-fold dilution of the reverse transcription reaction was used for the starting template. Amplification and detection of products was performed using the LightCycler 2.0 thermocycler (Roche Applied Science) with the following schedule: polymerase activation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 10 s, annealing at 55°C for 5 s, and extension at 72°C for 5 s. A melting curve was performed to assess primer specificity and product quality by denaturation at 95°C, annealing at 65°C, and melting at a rate of 0.1°C/s to 95°C. The relative levels of viral DNA were quantified using the 2(-Delta Delta Ct) method (Livak and Schmittgen, 2001).

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