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Excess neutrophil infiltration during cytomegalovirus brain infection of interleukin-10-deficient mice

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Abstract

Wild-type mice control murine cytomegalovirus (MCMV) brain infection, but identical infection is lethal to animals deficient in interleukin (IL)-10. Here, we report that MCMV-infected IL-10 knockout (KO) mice displayed a marked increase in neutrophil infiltration into the infected, IL-10-deficient brain when compared to wild-type animals. Enhanced microglial cell activation, determined by MHC class II up-regulation, overexpression of CXCL2, and elevated P-selectin mRNA levels were observed. In vivo blocking of CXCL2 attenuated neutrophil infiltration and significantly improved the outcome of infection. Collectively, these data indicate that the absence of IL-10 results in pathologic neutrophil infiltration into MCMV-infected brains.

Keywords

IL-10; microglia; CXCL2; neutrophil

1. Introduction

IL-10 is a prototypical anti-inflammatory cytokine that suppresses cellular immunity and inhibits the synthesis and release of proinflammatory mediators (Howard et al., 1992; Moore et al., 2001). In the central nervous system, IL-10 has been shown to improve outcomes in models of stroke (Dietrich et al., 1999) and experimental autoimmune encephalomyelitis (Zhang et al., 2004). We have recently shown that absence of this single cytokine turns a benign MCMV brain infection lethal, without increased viral load in the brain (Cheeran et al., 2007). Lethal MCMV brain infection is associated with elevated levels of cytokines and chemokines, especially IFN- γ , and the IFN- γ -inducible chemokines CXCL9 and CXCL10 (Cheeran et al., 2007). Paradoxically, despite elevated cytokine and chemokine levels in the brain following MCMV infection of IL-10 deficient mice there is reduced lymphocyte infiltration (Cheeran et al., 2009).

IL-10 exerts a wide spectrum of biological activities implicated in the regulation of inflammatory and immune responses. Among the different cell types affected by interleukin-10, monocyte/macrophages and lymphocytes appear to be particularly modified

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with regard to their function, morphology, and phenotype (Cassatella, 1998). One of the main functions of anti-inflammatory cytokines such as IL-10 is thought to be their role as a host mechanism to limit tissue damage and turn off proinflammatory responses (Mills, 2004). Because resident glial cells and infiltrating lymphocytes communicate through cytokine and chemokine mediators, the well-documented neuroprotective action of anti-inflammatory cytokines is likely related to their ability to inhibit chemokine-driven neuroinflammation.

The recruitment of specific leukocyte subsets from the periphery into distinct tissues is mediated by sequential interaction of different adhesion and signaling molecules on leukocytes and the endothelial cells lining the vessel walls (Luster et al., 2005). CXCL2 (a homologue of human IL-8 or MIP-2 β) is a member of the C-X-C chemokine family. Although the role of CXCL2 in neuroinflammation is poorly understood, studies have demonstrated that CXCL2 is a potent chemoattractant and activator of neutrophils (Yoshimura et al., 1987). This activation may result in increased respiratory burst activity, production of bioactive lipids, and release of lysosomal enzymes, potentially contributing to tissue injury (Baggiolini et al., 1994; Harada et al., 1996). CXCL2 also regulates neutrophil adhesion to endothelial cells (Huber et al., 1991), facilitating tissue infiltration of these cells.

Microglial cells and astrocytes are the key sources of CXCL2 within the brain (Babcock et al., 2003; Rubio et al., 2006). Mononuclear phagocytes are activated following viral infection and produce a wide variety of cytokines and chemokines, which may either play a protective role or initiate exaggerated immune responses (Cheeran et al., 2001). Previous studies from our laboratory have shown that, there is heightened neuroinflammation and altered lymphocyte recruitment during MCMV brain infection in the context of IL-10 deficiency (Cheeran et al., 2007; Cheeran et al., 2009). In addition to producing chemokines which attract lymphocytes, activation of microglial cells may also be a key component that induces influx of neutrophils. Furthermore, CXCL2 is found in the brain during viral infections (Rubio et al., 2006), and antibody neutralization of CXCL2 can alter the host response in models of both viral (Yan et al., 1998) and bacterial (Greenberger et al., 1996; Kernacki et al., 2000) infection. In this study, we hypothesized that hyper-activation of microglial cells due to IL-10 deficiency would lead to increased neutrophil egress in response to MCMV brain infection.

2. Materials and Methods

2.1. Virus and animals

RM461, a MCMV expressing *E. coli* β -galactosidase under the control of the human *ie1/ie2* promoter/enhancer (Stoddart et al., 1994) was kindly provided by Edward S. Mocarski. The virus was maintained by passage in weanling female BALB/c mice. Salivary gland-passed virus was then grown in NIH 3T3 cells for 2 passages, which minimized any carry over of salivary gland tissue. Infected 3T3 cultures were harvested at 80 to 100% cytopathic effect and subjected to three freeze-thaw cycles. Cellular debris was removed by centrifugation ($1,000 \times g$) at 4°C, and the virus was pelleted through a 35% sucrose cushion (in Tris-buffered saline [50 mM Tris-HCl, 150 mM NaCl, pH 7.4]) at $23,000 \times g$ for 2 h at 4°C. The pellet was resuspended in Tris buffered saline containing 10%FBS. Viral stock titers were determined on 3T3 cells as 50% tissue culture infective doses (TCID₅₀) per milliliter. This virulent, salivary gland-passaged, sucrose gradient-purified virus was used for all intracerebroventricular (icv) infections. Mock-infected 3T3 cultures were processed in exactly the same manner as viral stocks and were used to sham-infect mice. For in vitro infection of microglia, a cell culture-passed and purified MCMV (RM461) was used. In all experiments, microglial cultures treated with mock and heat inactivated virus were used as controls for nonspecific cell stimulation. Heat-inactivation of virus was done at 95°C for 30

m, and uv-inactivation was achieved by placing sucrose-purified viral stocks or virus-infected cell culture supernatants in a tissue culture dish at a distance of 8 cm from a 256-nm uv light source, on ice, for 45 min. There was no virus detected following heat inactivation or uv-treatment in viral stocks treated in this manner. BALB/c mice were obtained from Charles River Laboratories (Wilmington, MA), while IL-10 KO animals were purchased from The Jackson Laboratory (Bar Harbor, ME).

2.2. Intracerebroventricular infection of mice

Infection of mice with MCMV was performed as previously described (Cheeran et al., 2004). Briefly, female mice (8–10 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 Co., 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. Virulent, salivary gland-passaged MCMV RM461 (1.5×10^5 TCID₅₀ units in 10 μ l), 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 μ l) fitted to a 27 G needle. The injection was delivered over a period of 3–5 min. The opening in the skull was sealed with bone wax and the skin was closed using 9 mm wound clips (Stoelting Co., Wood Dale, IL).

2.3. Isolation of brain leukocytes and Flow Cytometry

Leukocytes were isolated from MCMV-infected murine brains using a previously described procedure with minor modifications (Ford et al., 1995; Marten et al., 2000). Briefly, brain tissues harvested from 4–6 animals were minced finely in RPMI (2 g/L D-glucose and 10mM HEPES) and mechanically disrupted (in Ca/Mg free HBSS) at room temperature for 20 min. Single cell preparations from infected brains were resuspended in 30% Percoll and banded on a 70% Percoll cushion at 900 $\times$ g at 15°C. Brain leukocytes obtained from the 30–70% Percoll interface were stained with anti-mouse immune cell surface markers for 45 min at 4°C (CD45-Allophycocyanin (APC), CD11b-APC-CY7, Ly-6G-FITC, MHC Class II-phycoerythrin (PE), Ly-6C-FITC, F4/80-APC, and CD3-PE-Cy7, BD Biosciences, San Jose, CA) and analyzed by flow cytometry using a BD FACSCanto. Live leukocytes were gated using forward scatter and side scatter parameters and analyzed using FlowJo software (TreeStar, Inc.).

2.4. Murine microglial cell cultures

Microglial cells were isolated from cerebral cortices of 1-d-old mice as previously described (Chao et al., 1996; Suzumura et al., 1987). Briefly, after a 30-min trypsinization (0.25%), dispersed cortical cells were plated in Falcon 75 cm² culture flasks in DMEM (Sigma, St. Louis, MO) containing 10% heat inactivated fetal bovine serum (FBS; Hyclone, Logan, UT), penicillin (100 U/ml) and streptomycin (100 μ g/ml) (Sigma, St. Louis, MO). Medium was changed at d 1 and 4 after plating. On d 8 of culture, flasks were shaken for 20 min in an orbital shaker at 180–200 rpm to remove oligodendrocytes. On d 12–14 of culture, floating microglia were harvested by gentle shaking and seeded into 24- or 48-well culture plates ($1\text{--}2 \times 10^4$ cells/well) and washed after 60 min. Adherent microglial cells were $\geq 95\%$ pure, as determined by MAC-1 (microglial cell marker; Roche, Indianapolis, IN) and glial fibrillary acidic protein (GFAP-marker for astrocytes; Sigma) antibody staining (Giulian et al., 1986; Hassan et al., 1991).

2.5. Chemokine ELISA

A sandwich enzyme-linked immunosorbent assay (ELISA)-based system previously described (Peterson et al., 1997) was used to quantify CXCL2 levels from microglial cell culture supernatants. ELISA plates were coated with a rat anti-mouse CXCL2 capture antibody (R&D Systems, Minneapolis, MN) at 1 µg/ml overnight at room temperature (RT). The plates were washed (0.05% Tween-20 in phosphate-buffered saline, PBS) and blocked with 1% BSA in PBS for 1 h at 37°C. All samples were incubated in antibody-coated ELISA plates for 2 h at RT. After washing, detection antibody (biotinylated goat anti-mouse CXCL2 antibodies, 75 ng/ml; R&D Systems) was added for 90 min at RT, followed by streptavidin horseradish peroxidase (HPO) conjugate (1:200; R&D systems, Minneapolis MN) for 20 min. A chromogenic substrate (K-blue; Neogen, Lexington, KY) was then added for 10–20 min at room temperature. Absorbance values at 450 nm were used to quantify levels of CXCL2 in microglial culture supernatants based on the standard concentration curve.

2.6. Anti-CXCL2 Ab treatment

Anti-CXCL2 Ab treatment of IL-10 KO mice was done as described previously with minor modifications (Diab et al., 1999; Tumpey et al., 2002). Mice were treated with polyclonal goat anti-mouse CXCL2 (R&D systems, Minneapolis, USA) and its effect on neutrophil infiltration was assessed using flow cytometry at 3 d p.i. We also investigated if *in vivo* blocking of CXCL2 improved the survival of IL-10 KO and wild-type animals. Mice were given 10µg of pAb CXCL2 daily for 3 d and the first dose was given 2 hours prior to the icv infection with MCMV. Control animals received similar doses of goat IgG.

2.7. In-vivo depletion of neutrophils

Depletion of neutrophils in mice was done according to a previously described method with minor modifications (Stirling et al., 2009). Briefly, functional grade purified anti-mouse Ly-6G, clone RB6–8C5 or rat IgG antibodies were purchased from BioXcell (West Lebanon, NH USA). Animals received either 100 µg intraperitoneal injections of anti-Ly-6G or rat IgG at 2 h prior to the MCMV infection and every other day thereafter. Antibody-treated mice were bled prior to the infection to determine the efficacy of depletion by flow cytometry.

2.8. Real-time PCR for CXCL2 and P-selectin

Total RNA were extracted from brain tissue homogenates with Trizol reagent (Invitrogen, Carlsbad, CA). One µg RNA was DNase (Ambion, Applied Biosystems, Austin, TX) treated and reverse transcribed to cDNA with SuperScript™ III (Invitrogen), dNTP (GE Healthcare, Piscataway, NJ) and oligo (dT)_{12–18} (Promega, Madison, WI). Real-time PCR was performed in Mx3000p (Stratagene, La Jolla, CA) with SYBR® Premix Ex Taq™ (Takara, Madison, WI), primers and cDNA according to manufacturer's protocol. Reaction conditions for qPCR were as follows: initial denaturation at 95°C for 15 sec, amplification for 40 cycles at 95°C for 10 sec, 60°C for 10 sec and 72°C for 10 sec followed by dissociation curve analysis (1 cycle at 95°C for 60 sec, 55°C for 30 sec and 95°C for 30 sec) to verify PCR product specificity. Primer sequences used and product sizes were: sense 5' GCCAAGGGTTGACTTCAAGAAC 3' and antisense 5' CAGTTAGCCTTGCCTTTGTTCA 3' for CXCL2, 177 bp; sense 5'-ATGCCTGGCTACTGGACACT-3' and antisense 5'-CTTCATCGCACATGAACTGG-3' (Atkinson et al., 2006) for P-selectin, 155 bp; sense 5'-TGCTCGAGATGTCATGAAGG-3' and antisense 5'-AATCCAGCAGGTCAGCAAAG-3', for hypoxanthine guanine phosphoribosyl transferase (HPRT)-1 as housekeeping gene, 294bp. After normalizing to HPRT-1 expression (ΔC_t = target gene C_t – HPRT C_t) and then to control group ($\Delta\Delta C_t$ =

treatment $\Delta Ct - C \Delta Ct$), relative quantification using $2^{-\Delta\Delta Ct}$ was calculated as fold change of target mRNA expression vs. control.

3. Results

3.1. Increased neutrophil infiltration with IL-10-deficiency

In our initial experiments, we analyzed brain infiltrating leukocytes for the presence of neutrophils using flow cytometry. It has been previously shown that the brain infiltrating leukocytes can be phenotyped based on their expression of CD45 and CD11b (Cheeran et al., 2009; Marques et al., 2008) and infiltrating neutrophils were identified within this population as a CD45^(hi)CD11b⁽⁺⁾Ly-6G⁽⁺⁾MHC II⁽⁻⁾ cell-type. At 3 d p.i., brain-infiltrating leukocytes were isolated from MCMV-infected wild-type and IL-10 KO animals using a percoll gradient and were analyzed by flow cytometry for the presence of neutrophils. The representative dot plots are shown from brain infiltrating cells that were gated on CD45^(hi)CD11b⁽⁺⁾ cells and were analyzed for Ly-6G⁽⁺⁾MHC II⁽⁻⁾ cells representing neutrophils (Fig. 1A). Data from these experiments showed significantly elevated numbers of CD45^(hi)Ly-6G⁽⁺⁾MHC II⁽⁻⁾ cells in the brains of IL-10 KO mice when compared to wild-type controls, $6.97 \pm 0.3\%$ versus $1.71 \pm 0.3\%$, respectively, $p < 0.01$ Student's *t* test (Fig. 1B). A significant increase in the absolute numbers of infiltrating neutrophils was also observed in the IL-10 KO mice ($2.98 \pm 0.6 \times 10^4$ versus $1.2 \pm 0.1 \times 10^4$, $p = 0.03$) (Fig. 1C). There was no significant difference between wild-type and IL-10 KO animals in any other cell type that was studied including CD45^(int)CD11b⁽⁺⁾Ly-6C⁽⁺⁾, CD45^(hi)CD11b⁽⁺⁾F4/80⁽⁺⁾ and CD11b^(int) cells. To determine if replication-competent virus was essential for neutrophil infiltration, mice were infected with mock and uv-inactivated MCMV (RM461). In these experiments, both IL-10 KO and wild-type animals showed minimal infiltration and significantly lower numbers of neutrophils when compared to virus-infected mice. The absolute numbers of neutrophils in sham and uv-inactivated virus infected mice were 1220 ± 257 and 4186 ± 1956 in IL-10 KO mice versus 998 ± 458 and 4277 ± 1448 in wild-type animals, respectively.

3.2. Enhanced activation of microglial cells in IL-10 KO animals

To compare microglial cell activation in the brains of MCMV-infected wild-type versus IL-10-deficient mice, MHC class II expression was used as an indicator of activation. In these experiments, MHC class II expression on the CD45^(int)CD11b⁽⁺⁾ microglial cell population was evaluated using flow cytometry (Fig 2). There was no difference in the absolute counts of CD45^(int)CD11b⁽⁺⁾ cells that were analyzed for MHC II expression. Although microglial cells from MCMV-infected wild-type mice expressed elevated levels of MHC class II ($44.94 \pm 2.0\%$), this molecule was expressed at significantly higher levels on cells obtained from IL-10 KO mice ($68.17 \pm 0.7\%$) at 3 d p.i., (Fig. 2A). Mean fluorescence intensity of MHC II expression was also measured between the groups and was found to be significantly higher among microglia isolated from IL-10 KO mice ($57.00 \pm 2.2\%$ versus $143.00 \pm 2.84\%$, respectively, $p < 0.01$ Student's *t* test) (Fig. 2B).

3.3. IL-10 treatment attenuates MCMV-induced CXCL2 production by microglia

The anti-inflammatory property of IL-10 was tested on murine microglia responding to MCMV infection. In these studies, we investigated the ability of microglial cells to produce CXCL2 following MCMV infection by measuring its levels in culture supernatants using ELISA. Purified microglia were exposed to mock (i.e., virus-free 3T3 cell extract), heat-inactivated, and as well as to tissue culture passed MCMV (RM461). We first established a dose response curve for CXCL2 production by microglia and observed that a multiplicity of infection (MOI) of 5 produced the highest level of CXCL2 (1147.00 ± 79.0 pg/ml) at 24 h p.i. (Fig. 3A). Microglia infected with mock or heat-inactivated virus, or those left

uninfected, demonstrated very minimal CXCL2 production in microglia (19.68 ± 6.67 , 87.66 ± 21.21 and 13.48 ± 7.73 pg/ml, respectively) (Fig 3B). We then opted to analyze a time course for production of CXCL2 by microglia in response to MCMV infection. Microglia were either mock- or MCMV-infected at an MOI of 5 and supernatants were collected at various time points to measure CXCL2 by ELSIA. At 8, 24, 48, and 72 h p.i., we observed sustained production of CXCL2 at 24, 48 and 72 h p.i. with concentrations of 641.67 ± 58.30 , 627.67 ± 29.14 and 610.6 ± 46.36 pg/ml, respectively (Fig. 3C). We then investigated the effect of IL-10 on MCMV-induced CXCL2 production. Supernatants from infected and uninfected microglial cultures pretreated with IL-10 were analyzed for CXCL2 production by ELISA at 24 h p.i. IL-10 pre-treatment was found to inhibit MCMV-induced CXCL2 production from murine microglia. Pre-treatment of microglia with 10 and 30 ng/ml of IL-10 markedly reduced the production of CXCL2 (Fig. 3D) at 24 h p.i. (274.83 ± 35.74 pg/ml). Taken together, these results demonstrate that IL-10 can significantly inhibit the production of virus-induced CXCL2 by microglia.

3.4. Increased mRNA levels of CXCL2 and P-selectin with IL-10 deficiency

We have previously shown that absence of IL-10 during viral brain infection results in exaggerated cytokine and chemokine responses, specifically production of IFN- γ and the interferon-inducible chemokines CXCL9 and CXCL10 (Cheeran et al., 2007). In the next set of experiments, we hypothesized that the observed increased neutrophil infiltration into the brains of IL-10 KO mice was driven by over expression of the chemoattractant ligand CXCL2 and the adhesion molecule P-selectin. To test this hypothesis, whole brain homogenates obtained from MCMV-infected wild-type and IL-10 KO mice were measured for CXCL2 and P-selectin mRNA using quantitative real-time RT-PCR. Brain homogenates from IL-10 KO mice infected displayed 48.79 ± 10.5 fold more CXCL2 expression than sham-infected, whereas wild-type mice had a 23.95 ± 6.2 fold increase over sham infected (Fig. 4A). P-selectin mRNA induction was 9.89 ± 1.9 fold higher in IL-10 KO mice, while Wt mice had 1.95 ± 0.9 fold increase over their respective sham control (Fig. 4B). mRNA expression levels were significantly higher for both neutrophil trafficking mediators, CXCL2 and P-selectin in virus-infected IL-10 KO mice compared to virus-infected Wt animals. mRNA expression for CXCL2 and P-selectin was normalized to HPRT (hypoxanthine guanine phospho-ribosyl transferase), a house-keeping gene which was detected in similar amounts in both Wt and IL-10 KO mice.

3.5. Reduced neutrophil infiltration following treatment with anti-CXCL2 Ab

To investigate the potential contribution of CXCL2 production to the recruitment of neutrophils, we went on to treat IL-10 KO mice with neutralizing polyclonal goat anti-mouse CXCL2 (Diab et al., 1999; Tumpsey et al., 2002) and assess the effect on neutrophil infiltration using flow cytometry. For these studies, 10 μ g of anti-CXCL2 was administered daily for 3 d, the first dose was administered 2 h prior to the infection. Control IL-10 KO animals received similar doses of goat IgG. As described above, brain mononuclear cells isolated from treatment groups were gated on CD45^(hi)CD11b⁽⁺⁾ and were analyzed for Ly-6G⁽⁺⁾MHC⁽⁻⁾, representing neutrophils (Fig. 5A). Data from these experiments showed that anti-CXCL2 Ab treatment of IL-10 mice significantly attenuated the influx of neutrophils into the brain when compared to IgG treated control animals ($4.28 \pm 0.1\%$ vs. $7.31 \pm 0.5\%$, respectively) (Fig. 5B). The absolute number of infiltrating neutrophils was also significantly reduced ($4.2 \pm 0.1 \times 10^4$ versus $2.5 \pm 0.1 \times 10^4$, $p = 0.01$) with anti-CXCL2 treatment at 3 d p.i (Fig. 5C).

3.6. Improved outcome following anti-CXCL2 treatment

Since in vivo blocking of CXCL2 resulted in significantly reduced neutrophil infiltration into infected brains, we then opted to discern the contribution of CXCL2 to the acute phase

of lethal infection with MCMV. We treated IL-10 KO and wild-type mice with polyclonal goat anti-mouse CXCL2 as described above. We observed these animals for survival against lethal MCMV infection. The data show that anti-CXCL2 treatment improved the outcome of infection in IL-10 KO animals. 60% of the IL-10 KO ($p=0.0004$, Logrank test) that were treated with anti-CXCL2 survived lethal infection indicating reduced infiltration of neutrophils had a beneficial effect on the outcome of viral infection (Fig. 6). Although wild-type mice survive MCMV brain infection, they were also included in this study as internal controls. As expected, all (100%) of the wild-type animals treated with anti-CXCL2 survived.

3.7. Complete depletion of neutrophil exacerbates MCMV brain infection

To determine the effect of complete peripheral neutrophil depletion on the survival of IL-10 KO and wild-type mice, cell depletion studies were performed by administration of rat anti-mouse Ly-6G MAb or rat IgG2b. As shown in Fig. 7A, we first observed that there was 100% depletion of neutrophils in the peripheral blood prior to MCMV infection. However, IL-10 KO mice depleted of neutrophils in this manner did not show any improvement in clinical outcome over IgG-treated mice. Viral infection was lethal in both IgG- and antibody-treated IL-10 KO animals; however, we observed that clinical manifestations, such as seizures, were more pronounced in neutrophil depleted IL-10 KO animals prior to death. Wild-type mice depleted of neutrophils also succumbed to MCMV infection with similar clinical manifestations, while IgG-treated wild-type mice survived the infection (Fig. 7B).

4. Discussion

Results from studies presented here have helped elucidate the role of the anti-inflammatory cytokine IL-10 in host response to viral brain infection. Previous reports from our laboratory have shown that MCMV brain infection is lethal to IL-10 KO mice and dysregulated cytokine and chemokine responses occur in the brain. We have also previously reported that there is no significant difference in viral titer in the brain between wild-type and IL-10 KO mice (Cheeran et al., 2007). We observed that, despite elevated chemokine levels, recruitment of lymphocytes into the brain was decreased in IL-10-deficient mice when compared to wild-type, possibly through differential expression of adhesion molecule CD62L on lymphocytes of IL-10 KO and wild-type mice (Cheeran et al., 2009; Lund et al., 2008). In further attempts to identify the role of additional peripheral immune cell populations, we investigated neutrophil infiltration into the brains of MCMV-infected IL-10 deficient mice. The goal of this study was to evaluate the role of IL-10 deficiency and associated factors in recruitment of neutrophils into the brain in response to viral encephalitis. We showed using flow cytometry of mononuclear cell infiltrates obtained from brain homogenates that there was greater accumulation of neutrophils in the brain of IL-10 KO mice compared to wild-type animals.

The innate immune response is the first line of host defense against pathogens and is mediated to a large extent by phagocytes including macrophages and dendritic cells. The innate immune system recognizes microorganisms via a limited number of germline-encoded pattern-recognition receptors (PRRs) which recognize microbial components, known as pathogen-associated molecular patterns (PAMPs). Different PRRs react with specific PAMPs, show distinct expression patterns, activate specific signaling pathways, and lead to distinct antipathogen responses. DNA viruses, including herpes simplex virus 1 (HSV-1), HSV-2, and murine cytomegalovirus (MCMV), contain genomes that are rich in CpG-DNA motifs, which activate inflammatory cytokines and type I IFN secretion by stimulation of TLR9 (Krug et al., 2004).

Neutrophils play a critical role in the innate immune response to pathogens and their various roles in defense and damage have been well-documented. Circulating neutrophils can be rapidly mobilized to respond to proinflammatory, chemotactic signals induced within injured or infected tissue. Although neutrophils are usually considered important participants to combat extracellular bacterial infections, they are also prominent components of inflammatory responses induced by numerous viral infections (Andrews et al., 1999; Sakai et al., 2000; Wang and Forsyth, 2000). However, in absence of the anti-inflammatory milieu provided by IL-10 to suppress immune responses, their increased recruitment could contribute to pathology (Jeong et al., 2009).

Early neuroimmune responses to MCMV have previously been shown to be dominated by a macrophage influx in both wild-type and IL-10 KO mice. These infiltrating CD45^(hi)CD11b⁽⁺⁾ cells were found to be activated, as assessed by up-regulation of MHC class II expression. Resident microglia (i.e., CD45^(int)CD11b⁽⁺⁾ cells), which normally do not express MHC class II, become activated either in response to the virus itself or in response to IFN- γ , which has been shown to induce MHC class II expression (Hamo et al., 2007). In this study, we show that MHC II expression on microglia at 3 d p.i. is significantly higher in the brains of IL-10 KO mice, which indicates exaggerated neuroinflammatory processes.

Murine neutrophils respond to chemotactic signals such as CXCL2 or macrophage inflammatory protein (MIP)-2 (Diab et al., 1999). Increased neutrophil recruitment into the brains of mice devoid of IL-10 raised the question of whether up-regulation of CXCL2 in response to MCMV infection was different in IL-10 KO mice when compared to wild-type animals. To address this, we examined the ability of murine microglia to produce CXCL2 in response to MCMV infection. The culture supernatants tested at various time points had elevated levels of CXCL2 protein and its synthesis was inhibited by IL-10 treatment. Our laboratory has previously reported similar studies in which human CMV-induced chemokines (Cheeran et al., 2003) and herpes simplex virus (HSV)-1 (Marques et al., 2004) induced pro-inflammatory mediators were down-regulated following IL-10 treatment. The in vitro studies reported here suggest that IL-10 can attenuate CXCL2 synthesis in resident microglial cells and down-regulation of CXCL2 production may reduce the number of neutrophils migrating into the brain.

We also report elevated mRNA levels for CXCL2 in IL-10 KO mice. It has previously been shown that CXCL2 expression in the brain is also up-regulated during other viral infections such as neurotropic JHM strain of mouse hepatitis virus (Zhou et al., 2002). Similar findings have been reported in Theiler's virus infection (Rubio et al., 2006). In the present study, significant blocking of neutrophil recruitment into the brain was observed following anti-CXCL2 treatment, demonstrating the role for enhanced CXCL2 expression. A second important observation was that anti-CXCL2 treatment not only reduced neutrophil infiltration, but also significantly increased mean survival time and prevented lethal infection in 60% of IL-10 KO mice. We measured neutrophil influx into the brain following administration of a neutralizing antibody to CXCL2. The antibody used in these studies was neutralizing for neutrophil chemotaxis, but expression levels of CXCL2 in the brain should be unaltered by antibody administration. Additionally, neutrophil entry into the brain was not completely inhibited, indicating that there may be additional signaling components that aid neutrophil egress such as CXCL1/KC and CXCL5-6/LIX6, which are also functional homologues of IL-8. Previous investigators have shown that antibody neutralization of endogenous CXCL2 can alter the host response in models of bacterial infection (Diab et al., 1999; Seebach et al., 1995) and lung injury initiated by immune complexes (Shanley et al., 2000). CXCL2 is also involved in the development of autoimmune disease (Schimmer et al., 1997).

As reported in the literature, complete depletion of neutrophils in certain viral infections (Lundberg et al., 2008; Milligan et al., 2001) has not proven to be beneficial. This could be possibly due to delayed pathogen clearance, which may result in increased viral titers in affected organs. Although wild-type mice survive MCMV infection, in this study we included these animals in the pan neutrophil depletion experiments. We made an attempt to deplete all neutrophils, however, these animals developed severe clinical signs such as seizures and complete depletion of neutrophils was not shown to improve clinical outcome of infection in either of the phenotypes studied. These neutrophil depletion and chemokine blocking studies showed that neutrophils were essential in optimum number, while excess neutrophil infiltration, as in case of IL-10 KO mice, was pathologic.

Rapid neutrophil recruitment to sites of infection is driven by chemokines, which subsequently increase adhesion molecule expression (Michel and Neal, 1999). Interactions between neutrophils and endothelial adhesion molecules promote disassembly of tight junctions, permitting these cells to extravasate and enter damaged or infected tissue. It has been shown that human cytomegalovirus infection of endothelial cells increases the expression of adhesion molecules and platelet aggregation (Rahbar and Soderberg-Naucler, 2005). Here, we report the over expression of P-selectin in the brains of IL-10 KO mice is associated with increased neutrophil infiltration into the brain. Several different reports demonstrated enhanced expression of adhesion molecules such as ICAM-1, VCAM-1, and MadCAM-1 during IL-10 deficiency in colitis models using IL-10 KO mice (Kawachi et al., 2000).

Our previous experiments using this model have demonstrated the importance of the anti-inflammatory effects offered by IL-10 to favorable outcome of MCMV brain infection. Deletion of this one gene results in lethal brain infection, which is associated with dysregulated neuroimmune responses. Our previous findings using this IL-10 KO infection model include, exaggerated cytokine/chemokine responses, altered immune cell recruitment into the brain, and differential expression of adhesion molecules such as CD62L on immune cells. In this study, we report elevated levels of CXCL2 in the brains of IL-10 KO mice, which result in excess neutrophil infiltration. We also demonstrate that blocking of CXCL2 reverses this lethal effect due to excess neutrophils in the brain. Reducing neutrophil recruitment through chemokine blockade lead to a more favorable outcome than complete depletion of these cells, which also had adverse effects. This study reiterates the crucial role of IL-10 during MCMV encephalitis which we have previously reported.

The CNS has numerous mechanisms to minimize destructive inflammatory responses, including the blood brain barrier (Wedmore and Williams, 1981), an absence of a lymphatic drainage, and low MHC expression (Hickey, 1999). Although these mechanisms limit the potential for immune mediated pathology, they can be overcome as in encephalitis induced by either pathogens or autoimmune reactions (Hickey, 1999; Irani and Griffin, 1996). Proper regulation of neuroimmune responses appears to be crucial in controlling immunopathological brain damage associated with clearing viral infection. The neuroprotective effects of anti-inflammatory cytokines are believed to be mediated through downregulation of brain inflammation. It is thought that the neuroprotective action of IL-10 is related to its ability to inhibit chemokine-driven neuroinflammation, turn off proinflammatory responses, and limit damage to brain tissue. Neutrophils are potent mediators of acute inflammation for several reasons including their ability to rapidly release preformed antimicrobial and proinflammatory molecules, as well as their sheer number (Wedmore and Williams, 1981). In this study, we demonstrate that IL-10 deficiency during lethal MCMV encephalitis results in excess neutrophil recruitment and that *in vivo* blocking of a neutrophil recruiting chemokine results in reduced infiltration of the brain and improved survival.

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References

- Andrews DM, Matthews VB, Sammels LM, Carrello AC, McMinn PC. The severity of murray valley encephalitis in mice is linked to neutrophil infiltration and inducible nitric oxide synthase activity in the central nervous system. *J Virol.* 1999; 73:8781–8790. [PubMed: 10482632]
- Atkinson C, Zhu H, Qiao F, Varela JC, Yu J, Song H, Kindy MS, Tomlinson S. Complement-dependent P-selectin expression and injury following ischemic stroke. *J Immunol.* 2006; 177:7266–7274. [PubMed: 17082645]
- Babcock AA, Kuziel WA, Rivest S, Owens T. Chemokine expression by glial cells directs leukocytes to sites of axonal injury in the CNS. *J Neurosci.* 2003; 23:7922–7930. [PubMed: 12944523]
- Baggiolini M, Dewald B, Moser B. Interleukin-8 and related chemotactic cytokines--CXC and CC chemokines. *Adv Immunol.* 1994; 55:97–179. [PubMed: 8304236]
- Cassatella MA. The neutrophil: one of the cellular targets of interleukin-10. *Int J Clin Lab Res.* 1998; 28:148–161. [PubMed: 9801925]
- Chao CC, Hu S, Sheng WS, Bu D, Bukrinsky MI, Peterson PK. Cytokine-stimulated astrocytes damage human neurons via a nitric oxide mechanism. *Glia.* 1996; 16:276–284. [PubMed: 8833198]
- Cheeran MC, Gekker G, Hu S, Min X, Cox D, Lokensgard JR. Intracerebral infection with murine cytomegalovirus induces CXCL10 and is restricted by adoptive transfer of splenocytes. *J Neurovirol.* 2004; 10:152–162. [PubMed: 15204920]
- Cheeran MC, Hu S, Palmquist JM, Bakken T, Gekker G, Lokensgard JR. Dysregulated interferon-gamma responses during lethal cytomegalovirus brain infection of IL-10-deficient mice. *Virus Res.* 2007; 130:96–102. [PubMed: 17624463]
- Cheeran MC, Hu S, Sheng WS, Peterson PK, Lokensgard JR. CXCL10 production from cytomegalovirus-stimulated microglia is regulated by both human and viral interleukin-10. *J Virol.* 2003; 77:4502–4515. [PubMed: 12663757]
- Cheeran MC, Hu S, Yager SL, Gekker G, Peterson PK, Lokensgard JR. Cytomegalovirus induces cytokine and chemokine production differentially in microglia and astrocytes: antiviral implications. *J Neurovirol.* 2001; 7:135–147. [PubMed: 11517386]
- Cheeran MC, Mutnal MB, Hu S, Armien A, Lokensgard JR. Reduced lymphocyte infiltration during cytomegalovirus brain infection of interleukin-10-deficient mice. *J Neurovirol.* 2009; 1–9. [PubMed: 19462266]
- Diab A, Abdalla H, Li HL, Shi FD, Zhu J, Hojberg B, Lindquist L, Wretling B, Bakhiet M, Link H. Neutralization of macrophage inflammatory protein 2 (MIP-2) and MIP-1 α attenuates neutrophil recruitment in the central nervous system during experimental bacterial meningitis. *Infect Immun.* 1999; 67:2590–2601. [PubMed: 10225925]
- Dietrich WD, Danton G, Hopkins AC, Prado R. Thromboembolic events predispose the brain to widespread cerebral infarction after delayed transient global ischemia in rats. *Stroke.* 1999; 30:855–861. discussion 862. [PubMed: 10187891]
- Ford AL, Goodsall AL, Hickey WF, Sedgwick JD. Normal adult ramified microglia separated from other central nervous system macrophages by flow cytometric sorting. Phenotypic differences defined and direct ex vivo antigen presentation to myelin basic protein-reactive CD4⁺ T cells compared. *J Immunol.* 1995; 154:4309–4321. [PubMed: 7722289]
- Giulian D, Baker TJ, Shih LC, Lachman LB. Interleukin 1 of the central nervous system is produced by ameboid microglia. *J Exp Med.* 1986; 164:594–604. [PubMed: 3487617]
- Greenberger MJ, Strieter RM, Kunkel SL, Danforth JM, Laichalk LL, McGillicuddy DC, Standiford TJ. Neutralization of macrophage inflammatory protein-2 attenuates neutrophil recruitment and bacterial clearance in murine *Klebsiella pneumoniae*. *J Infect Dis.* 1996; 173:159–165. [PubMed: 8537653]

- Hamo L, Stohlman SA, Otto-Duessel M, Bergmann CC. Distinct regulation of MHC molecule expression on astrocytes and microglia during viral encephalomyelitis. *Glia*. 2007; 55:1169–1177. [PubMed: 17600339]
- Harada A, Mukaida N, Matsushima K. Interleukin 8 as a novel target for intervention therapy in acute inflammatory diseases. *Mol Med Today*. 1996; 2:482–489. [PubMed: 8947914]
- Hassan NF, Rifat S, Campbell DE, McCawley LJ, Douglas SD. Isolation and flow cytometric characterization of newborn mouse brain-derived microglia maintained in vitro. *J Leukoc Biol*. 1991; 50:86–92. [PubMed: 2056249]
- Hickey WF. Leukocyte traffic in the central nervous system: the participants and their roles. *Semin Immunol*. 1999; 11:125–137. [PubMed: 10329499]
- Howard M, O'Garra A, Ishida H, de Waal Malefyt R, de Vries J. Biological properties of interleukin 10. *J Clin Immunol*. 1992; 12:239–247. [PubMed: 1512298]
- Huber AR, Kunkel SL, Todd RF 3rd, Weiss SJ. Regulation of transendothelial neutrophil migration by endogenous interleukin-8. *Science*. 1991; 254:99–102. [PubMed: 1718038]
- Irani DN, Griffin DE. Regulation of lymphocyte homing into the brain during viral encephalitis at various stages of infection. *J Immunol*. 1996; 156:3850–3857. [PubMed: 8621923]
- Jeong ES, Won YS, Kim HC, Cho MH, Choi YK. Role of IL-10 deficiency in pneumonia induced by *Corynebacterium kutscheri* in mice. *J Microbiol Biotechnol*. 2009; 19:424–430. [PubMed: 19421001]
- Kawachi S, Jennings S, Panes J, Cockrell A, Laroux FS, Gray L, Perry M, van der Heyde H, Balish E, Granger DN, Specian RA, Grisham MB. Cytokine and endothelial cell adhesion molecule expression in interleukin-10-deficient mice. *Am J Physiol Gastrointest Liver Physiol*. 2000; 278:G734–G743. [PubMed: 10801266]
- Kernacki KA, Barrett RP, Hobden JA, Hazlett LD. Macrophage inflammatory protein-2 is a mediator of polymorphonuclear neutrophil influx in ocular bacterial infection. *J Immunol*. 2000; 164:1037–1045. [PubMed: 10623854]
- Krug A, French AR, Barchet W, Fischer JA, Dzionek A, Pingel JT, Orihuela MM, Akira S, Yokoyama WM, Colonna M. TLR9-dependent recognition of MCMV by IPC and DC generates coordinated cytokine responses that activate antiviral NK cell function. *Immunity*. 2004; 21:107–119. [PubMed: 15345224]
- Lund JM, Hsing L, Pham TT, Rudensky AY. Coordination of early protective immunity to viral infection by regulatory T cells. *Science*. 2008; 320:1220–1224. [PubMed: 18436744]
- Lundberg P, Ramakrishna C, Brown J, Tyszka JM, Hamamura M, Hinton DR, Kovats S, Nalcioğlu O, Weinberg K, Openshaw H, Cantin EM. The immune response to herpes simplex virus type 1 infection in susceptible mice is a major cause of central nervous system pathology resulting in fatal encephalitis. *J Virol*. 2008; 82:7078–7088. [PubMed: 18480436]
- Luster AD, Alon R, von Andrian UH. Immune cell migration in inflammation: present and future therapeutic targets. *Nat Immunol*. 2005; 6:1182–1190. [PubMed: 16369557]
- Marques CP, Cheeran MC, Palmquist JM, Hu S, Urban SL, Lokensgard JR. Prolonged microglial cell activation and lymphocyte infiltration following experimental herpes encephalitis. *J Immunol*. 2008; 181:6417–6426. [PubMed: 18941232]
- Marques CP, Hu S, Sheng W, Cheeran MC, Cox D, Lokensgard JR. Interleukin-10 attenuates production of HSV-induced inflammatory mediators by human microglia. *Glia*. 2004; 47:358–366. [PubMed: 15293233]
- Marten NW, Stohlman SA, Bergmann CC. Role of viral persistence in retaining CD8(+) T cells within the central nervous system. *J Virol*. 2000; 74:7903–7910. [PubMed: 10933698]
- Michel CC, Neal CR. Openings through endothelial cells associated with increased microvascular permeability. *Microcirculation*. 1999; 6:45–54. [PubMed: 10100188]
- Milligan GN, Bourne N, Dudley KL. Role of polymorphonuclear leukocytes in resolution of HSV-2 infection of the mouse vagina. *J Reprod Immunol*. 2001; 49:49–65. [PubMed: 11137112]
- Mills KH. Regulatory T cells: friend or foe in immunity to infection? *Nat Rev Immunol*. 2004; 4:841–855. [PubMed: 15516964]
- Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol*. 2001; 19:683–765. [PubMed: 11244051]

- Peterson PK, Hu S, Salak-Johnson J, Molitor TW, Chao CC. Differential production of and migratory response to beta chemokines by human microglia and astrocytes. *J Infect Dis.* 1997; 175:478–481. [PubMed: 9203678]
- Rahbar A, Soderberg-Naucler C. Human cytomegalovirus infection of endothelial cells triggers platelet adhesion and aggregation. *J Virol.* 2005; 79:2211–2220. [PubMed: 15681423]
- Rubio N, Sanz-Rodriguez F, Lipton HL. Theiler's virus induces the MIP-2 chemokine (CXCL2) in astrocytes from genetically susceptible but not from resistant mouse strains. *Cell Immunol.* 2006; 239:31–40. [PubMed: 16684516]
- Sakai S, Kawamata H, Mantani N, Kogure T, Shimada Y, Terasawa K, Sakai T, Imanishi N, Ochiai H. Therapeutic effect of anti-macrophage inflammatory protein 2 antibody on influenza virus-induced pneumonia in mice. *J Virol.* 2000; 74:2472–2476. [PubMed: 10666283]
- Schimmer RC, Schrier DJ, Flory CM, Dykens J, Tung DK, Jacobson PB, Friedl HP, Conroy MC, Schimmer BB, Ward PA. Streptococcal cell wall-induced arthritis. Requirements for neutrophils, P-selectin, intercellular adhesion molecule-1, and macrophage-inflammatory protein-2. *J Immunol.* 1997; 159:4103–4108. [PubMed: 9379001]
- Seebach J, Bartholdi D, Frei K, Spanaus KS, Ferrero E, Widmer U, Isenmann S, Strieter RM, Schwab M, Pfister H, Fontana A. Experimental *Listeria* meningoencephalitis. Macrophage inflammatory protein-1 alpha and -2 are produced intrathecally and mediate chemotactic activity in cerebrospinal fluid of infected mice. *J Immunol.* 1995; 155:4367–4375. [PubMed: 7594596]
- Shanley TP, Vasi N, Denenberg A. Regulation of chemokine expression by IL-10 in lung inflammation. *Cytokine.* 2000; 12:1054–1064. [PubMed: 10880252]
- Stirling DP, Liu S, Kubes P, Yong VW. Depletion of Ly6G/Gr-1 leukocytes after spinal cord injury in mice alters wound healing and worsens neurological outcome. *J Neurosci.* 2009; 29:753–764. [PubMed: 19158301]
- Stoddart CA, Cardin RD, Boname JM, Manning WC, Abenes GB, Mocarski ES. Peripheral blood mononuclear phagocytes mediate dissemination of murine cytomegalovirus. *J Virol.* 1994; 68:6243–6253. [PubMed: 8083964]
- Suzumura A, Mezitis SG, Gonatas NK, Silberberg DH. MHC antigen expression on bulk isolated macrophage-microglia from newborn mouse brain: induction of Ia antigen expression by gamma-interferon. *J Neuroimmunol.* 1987; 15:263–278. [PubMed: 3110208]
- Tumpey TM, Fenton R, Molesworth-Kenyon S, Oakes JE, Lausch RN. Role for macrophage inflammatory protein 2 (MIP-2), MIP-1alpha, and interleukin-1alpha in the delayed-type hypersensitivity response to viral antigen. *J Virol.* 2002; 76:8050–8057. [PubMed: 12134010]
- Wang SZ, Forsyth KD. The interaction of neutrophils with respiratory epithelial cells in viral infection. *Respirology.* 2000; 5:1–10. [PubMed: 10728725]
- Wedmore CV, Williams TJ. Control of vascular permeability by polymorphonuclear leukocytes in inflammation. *Nature.* 1981; 289:646–650. [PubMed: 7464931]
- Yan XT, Tumpey TM, Kunkel SL, Oakes JE, Lausch RN. Role of MIP-2 in neutrophil migration and tissue injury in the herpes simplex virus-1-infected cornea. *Invest Ophthalmol Vis Sci.* 1998; 39:1854–1862. [PubMed: 9727408]
- Yoshimura T, Matsushima K, Oppenheim JJ, Leonard EJ. Neutrophil chemotactic factor produced by lipopolysaccharide (LPS)-stimulated human blood mononuclear leukocytes: partial characterization and separation from interleukin 1 (IL 1). *J Immunol.* 1987; 139:788–793. [PubMed: 3298433]
- Zhang X, Koldzic DN, Izikson L, Reddy J, Nazareno RF, Sakaguchi S, Kuchroo VK, Weiner HL. IL-10 is involved in the suppression of experimental autoimmune encephalomyelitis by CD25+CD4+ regulatory T cells. *Int Immunol.* 2004; 16:249–256. [PubMed: 14734610]
- Zhou J, Stohman SA, Atkinson R, Hinton DR, Marten NW. Matrix metalloproteinase expression correlates with virulence following neurotropic mouse hepatitis virus infection. *J Virol.* 2002; 76:7374–7384. [PubMed: 12097550]

FIG. 1. Increased neutrophil infiltration into the brains of MCMV-infected IL-10 KO mice
Brain-infiltrating leukocyte subpopulations obtained from both wild-type (Wt) and IL-10 deficient (IL-10 KO) mice were examined using anti-CD45, anti-CD11b, anti-Ly-6G and anti-MHC-II Abs and analyzed using flow cytometry. (A) Representative dot plots from Wt and IL-10 KO mice. Average ratios of the cell population expressing a high level of each marker are presented from 3 independent experiments ($n = 3-5$ animals each). (B) Positive staining cells that are gated on $CD45^{(hi)}CD11b^{(+)}$ expressed as percentage of the as well as the (C) absolute number of infiltrating $CD45^{(hi)}CD11b^{(+)}$ Ly-6G $^{(+)}$ MHC-II $^{(-)}$ cells in the brains of MCMV-infected wild-type (Wt) versus IL-10 KO (IL-10 KO) mice were determined at 3 d p.i. Data showing the mean ($\pm$ SEM) absolute number of cells infiltrating

the brain were pooled from three to five experiments. $*p < 0.05$ and $**p < 0.01$ versus wild-type.

FIG. 2. Enhanced microglial cell activation following MCMV infection of IL-10 KO animals
Brain mononuclear cells, isolated through percoll gradient centrifugation, were stained using anti-CD45, anti-CD11b, and anti-MHC-II surface marker Abs. (A) Up-regulation of MHC class II expression on CD45^(int)CD11b⁽⁺⁾ resident microglia in response to viral brain infection. CD45^(int)/CD11b⁽⁺⁾ resident microglia in single cell suspensions of brain tissue obtained from MCMV-infected wild-type (Wt) and IL-10 KO (IL-10 KO) mice at 3 d.p.i. were stained with anti-MHC class II Abs and analyzed for expression using flow cytometry. Relative percentages, expressed as mean $\pm$ SEM, of MHC class II⁺ cells within the CD45^(int)CD11b⁽⁺⁾ population are presented. An overlay of histograms from isotype (shaded), Wt (solid line) and from IL-10 KO (dotted line) is also shown. (B) Data presented show mean fluorescent intensity (MFI) of MHC-II binding on microglia from Wt versus IL-10 KO mice. ** $p < 0.01$ versus Wt

FIG. 3. Inhibition of MCMV-induced CXCL2 production by the anti-inflammatory cytokine IL-10

(A) Primary murine microglial cell cultures were infected with MCMV (MOI=1, 2, and 5). Cell culture supernatants were collected at 24 h p.i. and CXCL2 was measured using ELISA. (B) Microglia were either uninfected (media) or infected (mock, heat-inactivated virus and MCMV) at an amount equivalent to MOI = 5 and cell culture supernatants were collected at 24 h p.i. and tested for CXCL2 production. (C) Induction of CXCL2 protein in microglia was measured at various time points (8, 24, 48, and 72 h post-infection) following mock and virus treatments (MOI = 5). For Fig 3 (A–C), data are expressed as mean ($\pm$ SD) CXCL2 levels from pooled data obtained during two separate experiments. * $p < 0.05$

(versus media (Fig 3A and C), mock-infected and heat inactivated (Fig 3 B) control cells). (D) Murine microglia were treated with 10 and 30 ng/ml of IL-10 for 24 h prior to infection with MCMV (MOI = 5). Cell culture supernatants were collected (24 h p.i.) and CXCL2 production was quantified using an ELISA. Data are expressed as mean $\pm$ SD of triplicate samples from two independent experiments using microglia. $**p < 0.01$ versus untreated

FIG. 4. Over expression of CXCL2 and P-selectin mRNA in the brains of IL-10 deficient mice
MCMV-induced (A) CXCL2 and (B) P-selectin mRNA expression was assessed using quantitative real-time RT-PCR on total RNA extracted from whole brain homogenates of wild-type (Wt) and IL-10 KO mice and from respective sham controls at 3 d p.i. mRNA levels were normalized to HPRT and are presented as mean $\pm$ SD normalized expression from pooled data obtained using three animals per group from 3 independent experiments. * $p < 0.05$ versus Wt infected with MCMV.

FIG. 5. Blocking CXCL2 decreases neutrophil infiltration into MCMV-infected brains

A polyclonal Ab to murine CXCL2 (R&D Systems, Minneapolis, MN) was used for neutralization studies. IL-10 KO mice ($n = 3$) were injected i.p. with 10 μ g of CXCL2 Ab 2 h prior to *icv* infection with MCMV, and at 2 and 3 d p.i. Control IL-10 KO mice ($n = 2$) were similarly treated with goat IgG. Brain infiltrating neutrophils were detected by flow cytometry as described above (3 d p.i.). (A) Representative dot plots from IL-10 KO mice treated with goat IgG or anti-CXCL2 Abs are shown. (B) Ratios of the cell population expressing a high level of each marker are presented. (C) The neutrophils are presented as absolute number of infiltrating CD45^(hi)CD11b⁽⁺⁾Ly-6G⁽⁺⁾MHC-II⁽⁻⁾ cells in the brains of MCMV-infected IL-10 KO mice treated with goat IgG or anti-CXCL2. Data show the mean

($\pm$ SEM) absolute number of cells infiltrating the brain. **, $p < 0.01$ versus IL-10 KO mice treated with goat IgG from 3 identical experiments (n=3)

FIG. 6. In-vivo blocking of CXCL2 confers a survival advantage

MCMV was injected into the right lateral ventricle of animals which were treated as described above with either anti-CXCL2 pAb (IL-10 KO mice, black circles, $n = 10$; and wild-type mice, open triangles, $n=6$) or goat IgG (IL-10 KO mice, open circles, $n = 10$; and wild-type mice, black triangles, $n=6$). Data are expressed as percent of mice in each group surviving at the indicated time point, followed over the 15 d time-course of the experiment.

* $p < 0.0004$ versus IgG Logrank test

FIG. 7. Complete depletion of neutrophils has no beneficial effect on outcome

(A) Prior to the infection, blood samples collected from the retro-orbital plexus were analyzed for presence of neutrophils ($\text{Ly-6G}^{+}\text{MHC II}^{-}$) by flow cytometry, analysis of $\text{CD45}^{\text{hi}}\text{CD11b}^{+}$ gated cells are shown in this representative dot plot. (B) MCMV was injected into the right lateral ventricle of animals which were treated with either anti-Ly-6G MAb (IL-10 KO mice, black circles, $n = 6$; and wild-type mice, open triangles, $n=6$) or rat IgG (IL-10 KO mice, open circles, $n = 6$; and wild-type mice, black triangles, $n=6$) following the same dosing schedule as described in the materials and methods. (i.e., $100 \mu\text{g}/\text{i.p.}$ 2 h prior to infection and every other day thereafter). Data are expressed as percent survival in each group at the indicated time point, followed over the 6 d time-course of the experiment.