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Journal of Neuroimmunology 194 (2008) 18–26

Journal of Neuroimmunology

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Role of IFN-γ responsiveness in CD8 T cell-mediated viral clearance and demyelination in coronavirus-infected mice

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Received 19 June 2007; received in revised form 12 October 2007; accepted 31 October 2007

Abstract

Immunocompetent, but not RAG1$^{-/-}$ mice infected with MHV–JHM develop demyelination. Transferred CD8 T cell-enriched splenocytes reconstitute demyelination, and this ability is dependent on donor IFN-γ. We used IFN-γR1$^{-/-}$ mice to examine the target of IFN-γ in CD8 T cell-mediated demyelination. In IFN-γR1$^{-/-}$RAG1$^{-/-}$ recipients, demyelination is decreased, but not eliminated, while viral titers are significantly increased when compared to IFN-γR1$^{+/+}$RAG1$^{-/-}$ recipients. IFN-γR1$^{-/-}$ CD8 T cells retain virus-specific effector function regardless of IFN-γR1 expression. Although IFN-γR1 responsiveness is critical for maximal demyelination, increased levels of infectious virus coupled with adoptive transfer of CD8 T cells may result in myelin destruction independent of IFN-γR1 expression.

Keywords: Viral infection; Demyelination; CD8 T cells; IFN-gamma

1. Introduction

Infection of the central nervous system (CNS) with viruses and other pathogens changes the normally quiescent CNS microenvironment to a pro-inflammatory state (Bergmann et al., 2006; Griffin, 2003; Hickey, 2001). After the initial innate response to a viral infection, effector CD8 T cells traffic to the inflamed CNS, playing a critical role in viral clearance through lytic and non-lytic antiviral mechanisms. However, these mechanisms are tightly regulated in the CNS during infection as well as in the resting state to prevent unnecessary autoimmune destruction of this vital, non-regenerative tissue. Furthermore, mechanisms that protect the CNS from immune-mediated damage may ultimately lead to incomplete viral clearance, persistent CNS infection, and chronic disease. Therefore, the generation of appropriate immune responses to infection results in clearance of virus while protecting the function of host tissues.

The neurotropic mouse hepatitis virus, strain JHM (MHV–JHM) is a well-studied pathogen that upsets the balance between viral clearance and regulation of host immune responses in the CNS (Bergmann et al., 2006; Perlman and Dandekar, 2005; Stohlman et al., 1998; Templeton and Perlman, 2007). MHV–JHM is a member of the family coronaviridae, which includes pathogens that are responsible for an array of human diseases ranging in pathogenicity from the relatively innocuous common cold to the more damaging severe acute respiratory syndrome (SARS). Immunocompetent mice infected with an attenuated MHV–JHM variant, J2.2v-1, also develop demyelinating disease (Fleming et al., 1987; Stohlman et al., 1998). Demyelination, a hallmark of the human disease multiple sclerosis (MS), is characterized by dense infiltration of macrophages into areas of white matter of the CNS, in some instances appearing in direct contact with myelin-stripped axons in MS lesions (Trapp et al., 1998) and in the spinal cord of MHV–JHM-infected mice (Templeton et al., in press). It has been hypothesized that, in addition to genetic factors which increase susceptibility, environmental factors such as chronic viral infection may play

Abbreviations: CNS, central nervous system; MHV, mouse hepatitis virus.

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doi:10.1016/j.jneuroim.2007.10.030

an important role in the etiology of MS (Hemmer et al., 2002). Studies that examine the pathogenesis of an autoimmune model of MS, experimental autoimmune encephalomyelitis (EAE), have largely focused on CD4 T cells as mediators of demyelinating disease. However, increasing evidence suggests that CD8 T cells also play an important role in autoimmune myelin destruction (Brisebois et al., 2006; Huseby et al., 2001). Therefore, CD8 T cells are implicated in both viral clearance and autoimmune destruction of myelin in the CNS.

When immunodeficient RAG1$^{-/-}$, SCID, or irradiated mice (that lack adaptive immunity) are infected with J2.2v-1, no demyelination develops and mice succumb to severe encephalitis characterized by high levels of infectious virus in the CNS (Wang et al., 1990; Wu and Perlman, 1999). However, transfer of splenocytes from MHV–JHM-immunized B6 mice infected RAG1$^{-/-}$ mice reconstitutes demyelination (Wu et al., 2000). In this model, development of demyelination is T cell-mediated, as depletion of both populations from the donor splenocyte suspension abrogates disease. Removal of either CD4 or CD8 T cells from the donor population does not abrogate demyelination, although recipient RAG1$^{-/-}$ mice exhibit distinct clinical courses. Mice receiving CD4 T cells (CD8 depleted) develop more severe encephalitis with little demyelination around 6–7 days post-transfer. After receiving CD8 T cell-enriched (CD4 T cell-depleted) splenocytes, infected RAG1$^{-/-}$ mice develop extensive demyelination, and display only a mild encephalitis when compared to mice receiving CD4 T cell-enriched splenocytes. CD4 and CD8 T cells mediate demyelination by different mechanisms. When CD8 T cell-enriched splenocytes are isolated from an MHV–JHM-immunized IFN-γ$^{-/-}$ donor, demyelination is significantly reduced when compared to mice receiving wild type CD8 T cell-enriched splenocytes (Pewe et al., 2002; Pewe and Perlman, 2002). Transfer of IFN-γ$^{-/-}$ CD4 T cell-enriched splenocytes into infected RAG1$^{-/-}$ mice results in increased demyelination (Pewe et al., 2002).

IFN-γ is a pleiotropic antiviral cytokine that is critical for CD8 T cell-mediated clearance of MHV–JHM from oligodendrocytes (Parra et al., 1999). In addition to activation of antiviral genes in infected and adjacent uninfected CNS cells, IFN-γ induces MHC expression and the production of chemokines such as the macrophage chemoattractant CCL2 (MCP-1) (Tran et al., 2000). After recruitment of macrophages by CCL2 and other cytokines/chemokines, IFN-γ acts to increase both production of nitric oxide and phagocytic function, (Schroder et al., 2004) which increases the demyelinating potential of these cells. In further support of the connection between IFN-γ-production and demyelination, Huseby et al. (2001) demonstrated that antibody blockade of IFN-γ in a CD8 T cell-mediated EAE resulted in decreased disease. A recent study of a spontaneous model of CD8 T cell-mediated demyelination using mice that constitutively express the T cell costimulatory molecule CD86 in microglia also supports this connection, as IFN-γR$^{-/-}$ mice did not develop disease (Brisebois et al., 2006). Collectively, these results show that IFN-γ is a central component in both CD8 T cell-mediated viral clearance and demyelination. Previous studies have focused on the requirement for IFN-γ, but not on the target of IFN-γ. Herein, we use MHV–JHM-infected RAG1$^{-/-}$ mice sufficient or deficient in IFN-γ receptor expression, to determine the target cell for IFN-γ expressed by CD8 T cells. Unexpectedly, our results showed that enhanced virus replication with subsequent demyelination occurred in the absence of IFN-γR expression in recipient mice.

2. Materials and methods

2.1. Virus

The neuroattenuated variant of MHV–JHM, J2.2v-1, provided by Dr. J. Fleming (University of Wisconsin, Madison), was used in all studies. Infectious virus isolated from brains was titered by dilution of the homogenates on Hela cells which express the receptor for MHV–JHM (Hela-MHVR).

2.2. Animals

Pathogen-free 5–6 week old C57Bl/6 (B6) mice were purchased from the National Cancer Institute (Bethesda, MD). Mice deficient in the recombinase activating gene 1 (RAG1$^{-/-}$) or in the α-chain of the IFN-γ receptor complex (IFN-γR1$^{-/-}$) were obtained from Jackson Laboratories (Bar Harbor, ME) and bred at the University of Iowa, as were mice transgenic for production of enhanced green fluorescent protein (GFP) under control of the β-actin promoter. Single knockout mice were crossed to obtain mice deficient in both IFN-γR1 and RAG1. Mice were screened for IFN-γR1 and RAG1 deficiency by PCR screening as previously described (Huang et al., 1993; Mombaerts et al., 1992; Pewe and Perlman, 2002). All animal studies were approved by the University of Iowa Animal Care and Use Committee.

2.3. Adoptive transfer model

RAG1$^{-/-}$ or IFN-γR1$^{-/-}$ RAG1$^{-/-}$ mice were infected with 1×10^3 PFU 2.2v-1 by intracranial injection. Adoptive transfer of 5×10^6 splenocytes from B6, GFP+, or IFN-γR1$^{-/-}$ mice immunized i.p. with wild type MHV–JHM to infected RAG1$^{-/-}$ or IFN-γR1$^{-/-}$ RAG1$^{-/-}$ mice was performed as previously described (Wu and Perlman, 1999).

2.4. Antibodies

The following mAbs were used in flow cytometric analysis or immunofluorescence microscopy (all purchased from BD Biosciences, San Diego, CA, unless stated): unconjugated and biotinylated rat anti-F4/80 (cl BM-8 Caltag Laboratories, Burlingame, CA); biotinylated rat anti-CD11c (mAb HL3); PE-conjugated rat anti-IFN-γ (mAb XMG1.2); FITC-conjugated and biotinylated rat anti-CD8 (mAb 53-6.7); purified rat anti-FcγRIII/II Ab (mAb 2.4G2); unconjugated anti-MHV nucleocapsid protein (51B88.2. Provided by Dr. M. Buchmeier, The Scripps Research Institute, La Jolla, CA).

2.5. Immunofluorescence staining and fluorescence microscopy

Eight micrometer sections were prepared from 4% *p*-formaldehyde-fixed, snap frozen spinal cords. Sections were blocked

with 10% horse serum in PBS, prior to incubation with primary antibody that detected macrophages/microglia (biotinylated rat anti-F4/80 (cl BM-8), 1:200), dendritic cells (biotinylated hamster anti-mouse CD11c (HL3), 1:200), T cells (biotinylated anti-rat CD4 and biotinylated anti-rat CD8 (BD Biosciences) for 1 hour at room temperature. After washing, sections were incubated with streptavidin (SA)-HRP (1:1000, Jackson ImmunoResearch, West Grove, PA) for an additional hour. Sections were amplified using TSA-Cy3 (Tyramide Signal Amplification, 1:100; Perkin-Elmer, Boston, MA) for 5 min. Samples were photographed with a Leitz DMRB microscope with a fluorescence attachment.

2.6. Histology, immunohistochemistry and quantification of demyelination

Mouse spinal cords were prepared and stained for demyelination, macrophage infiltration, and viral infection as previously described (Xue et al., 1999). For examination of myelin integrity, 8 μm sections were stained with luxol fast blue and counterstained with hematoxylin and eosin. The amount of demyelination was quantified as previously described, using ImageJ software (NIH, Bethesda, MD). For immunohistochemistry, rehydrated spinal cord sections were incubated with anti-rat F4/80 (1:500) to identify macrophages or mouse anti-MHV nucleocapsid protein (1:10,000) overnight, followed by isotype specific biotinylated antibody, followed by streptavidin–HRP. HRP was visualized using the Sigma FAST 3,3-Diaminobenzidine visualization kit (Sigma-Aldrich, St. Louis, MO).

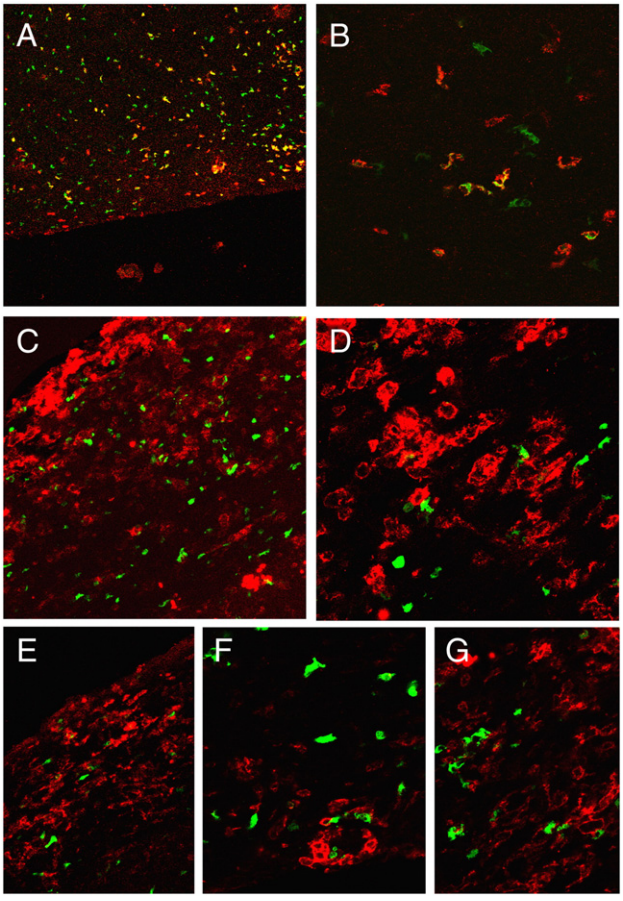

Fig. 1. Donor T cells, but not macrophages and DCs, traffic to the spinal cord in infected RAG1$^{-/-}$ recipient mice. J2.2.v-1-infected RAG1$^{-/-}$ mice received splenocytes from MHV–JHM-immune B6 mice expressing GFP in all nucleated cells. At day 8 post-transfer, spinal cords were harvested and sections were immunostained for CD4 and CD8 (red, A and B), F4/80 (red, C and D), or CD11c (red, E–G) using biotinylated rat antibodies, followed by SA-HRP and TSA-Cy3 amplification. Transferred GFP$^+$ cells are visible as green. Although F4/80$^+$and CD11c$^+$cells are proximal to GFP$^+$splenocytes (C–G), only cells expressing T cell markers are GFP$^+$(A and B). Samples depicted are representative of four mice. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.7. Preparation of leukocytes

Blood was collected in 1 ml of ACK lysis buffer and leukocytes counted after red blood cells lysis. CNS-derived leukocytes were isolated from B6 mice with acute encephalitis or subacute/chronic encephalomyelitis as previously described (Castro et al., 1994). Briefly, animals were sacrificed and perfused with sterile 1× PBS. Brain tissues were mechanically homogenized using frosted glass slides. Cells were suspended in 30% Percoll (Pharmacia, Piscataway, NJ) and centrifuged at 800 × g at 4 °C for 30 min. Percoll and lipid layers were aspirated and the cell pellet was washed twice and counted. Leukocytes in the CNS were identified by expression of CD45 using flow cytometry. Flow cytometry was performed on FACScan Flow Cytometer (BD Biosciences, Mountain View, CA).

2.8. Intracellular cytokine staining and FACS analysis

CNS-isolated cells were incubated in the presence of D^b-binding S510 peptide or control peptide for 4 hours at 37 °C in the presence of Golgiplug (brefeldin A, BD Biosciences). After incubation, cells were incubated with FITC-conjugated anti-CD8 mAb incubated in buffer containing 10% rat serum and anti-FcγRIII/II mAb. For detection of intracellular IFN-γ, cells were washed, fixed, permeabilized, and incubated with PE-conjugated anti-IFN-γ mAb as previously described (Wu et al., 2000). For phenotypic analysis, cells derived from the CNS were blocked and then incubated with specific mAbs, followed by flow cytometric analysis.

2.9. Statistics

A two-tailed unpaired Student *t* test was used to analyze differences in mean values between groups. All results are expressed as means ± SEM. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Donor T cells, but not macrophages and DCs, traffic to the spinal cord in infected RAG1$^{-/-}$ recipient mice

J2.2v-1-infected RAG1$^{-/-}$ mice that receive splenocytes from MHV–JHM immune B6 mice develop demyelination, while demyelination is absent in infected mice that receive splenocytes

depleted of CD4 and CD8 T cells (Wu et al., 2000). Thus, T cells are necessary for induction of demyelination in J2.2v-1-infected RAG1$^{-/-}$ mice. However, since demyelination is characterized by dense infiltration of F4/80^{+} and CD11c^{+} macrophages/microglia and DCs, it is possible that transferred phagocytic cells play a role in myelin destruction. Therefore, in the absence of an activating factor such as IFN-γ responsiveness in recipient mice, donor IFN-γR^{+} phagocytic cells may be able to compensate in the presence of T cells to mediate demyelinating disease. To rule out the presence of donor F4/80^{+} and CD11c^{+} macrophages as a contributing factor in the diseased spinal cord of chronically infected RAG1$^{-/-}$ mice, we transferred donor splenocytes from MHV–JHM immune mice expressing green fluorescent protein (GFP) (Fig. 1). At 8 days post-transfer, the spinal cords of infected RAG1$^{-/-}$ mice exhibited infiltration of GFP^{+} cells, many of which were positive for the T cell markers CD4 and CD8 (Fig. 1A, B). However, F4/80^{+} macrophages were uniformly GFP^{-} (Fig. 1C, D). Furthermore, CD11c^{+} cells were also GFP^{-}, and were clustered in the spinal cord near GFP^{+} cells (Fig. 1E, G) or appeared in a circular, perivascular arrangement (Fig. 1). Therefore, F4/80^{+} and CD11c^{+} cells in the MHV–J2.2v-1-infected spinal cord were not donor-derived.

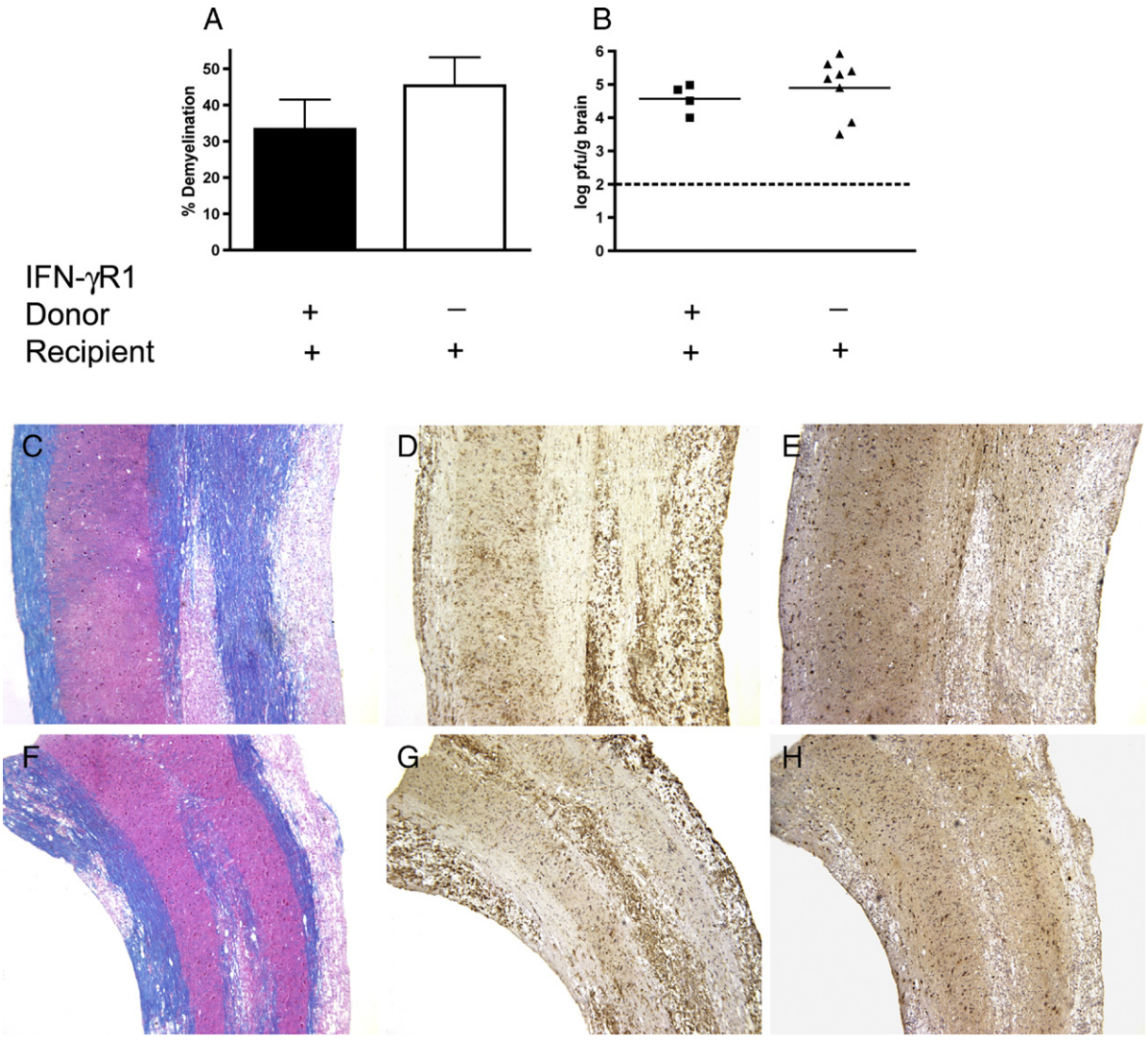

Fig. 2. IFN-γ donor responsiveness does not contribute to CD8 T cell-mediated demyelination. Infected RAG1$^{-/-}$ mice receiving CD8-enriched splenocytes i.v. from MHV–JHM-immune B6 IFN-γR1$^{-/-}$ or IFN-γR1$^{+/+}$ mice were sacrificed on days 12–15 post-transfer. Spinal cords were harvested and sections were stained for demyelination with luxol fast blue, (C and F). Immunohistochemistry was performed on adjacent sections to detect macrophage infiltration and localization of viral antigen using anti-F4/80 (D and G) and anti-MHV N ag (E and H), respectively, followed by biotinylated secondary antibody, and SA-HRP treatment with peroxidase substrate visualization. Demyelination was not significantly increased in mice that received cells from IFN-γR1$^{-/-}$ (white bar, A, and F) compared to IFN-γR1$^{+/+}$ donors (black bar, A, and C) ($p > 0.05$). Macrophage infiltration was associated with areas of demyelination (IFN-γR1$^{+/+}$, D, IFN-γR1$^{-/-}$ G), and the amount of virus detected in brain homogenates and distribution of viral antigen near areas of demyelination (IFN-γR1$^{+/+}$, squares, B, tissue section, E. IFN-γR1$^{-/-}$, triangles, B, tissue section H) were similar between groups ($p > 0.05$). Virus was detected in 4/6 and 8/8 mice receiving IFN-γR1$^{+/+}$and IFN-γR1$^{-/-}$ splenocytes, respectively. The dotted line (B) depicts the level of detection of infectious virus by plaque assay. Data are presented as a summary of two experiments.

3.2. IFN-γ donor responsiveness does not contribute to CD8 T cell-mediated demyelination

Previous results demonstrated that IFN-γ expression by donor CD8 T cells was critical for demyelination in J2.2v-1-infected RAG1$^{-/-}$ mice (Pewe and Perlman, 2002). Although macrophages in these spinal cords are recipient-derived (Fig. 1), macrophage and microglia activation may be a consequence of autocrine activation of CD8 T cells by IFN-γ. We examined this possibility by transferring IFN-γR1$^{-/-}$ CD8 T cell-enriched (CD4 depleted) splenocytes into J2.2v-1-infected RAG1$^{-/-}$ mice, and analyzed the amount of demyelination and virus levels in the CNS. Our results showed no significant increase in demyelination in infected recipients of IFN-γR1$^{-/-}$ compared to wild type CD8 T cell-enriched splenocytes (Fig. 2A, C, F). In addition, the recruitment of macrophages to areas of demyelination was equivalent in both groups (Fig. 2D, G), and the ability of recipient mice to clear virus from the CNS was not significantly altered (Fig. 2B, E, H). Thus, donor IFN-γ responsiveness does not significantly contribute to CD8 T cell-mediated demyelination or viral clearance.

3.3. IFN-γ recipient responsiveness contributes to CD8 T cell-mediated viral clearance and demyelination

In order to determine the requirement for IFN-γR1 in recipient cells, including macrophages and microglia, we adoptively transferred MHV–JHM-immune B6 CD8 T cell-enriched splenocytes into infected mice deficient in both IFN-γR1 and RAG1 (IFN-γR1$^{-/-}$ RAG1$^{-/-}$) or into control IFN-γR1$^{+/+}$RAG1$^{-/-}$ mice. As expected, demyelination was decreased in recipient mice that lacked IFN-γR1 when compared to IFN-γR1 sufficient mice

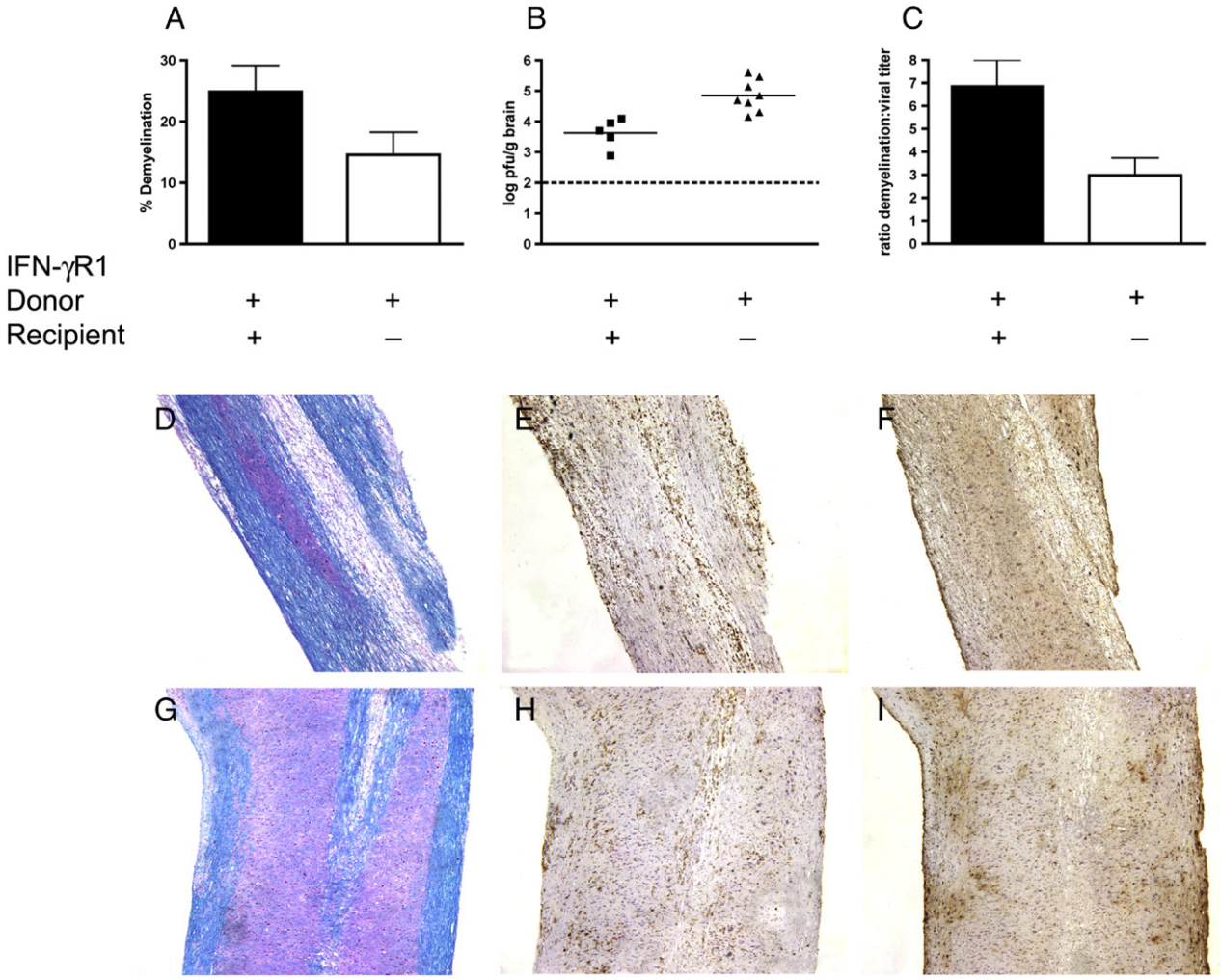

Fig. 3. IFN-γ recipient responsiveness contributes to CD8 T cell-mediated viral clearance and demyelination with wild type donor cells. Infected IFN-γR1$^{-/-}$ RAG1$^{-/-}$ mice receiving CD8-enriched splenocytes i.v. from MHV–JHM-immune B6 mice were sacrificed on days 8–10 post-transfer, and spinal cord sections were stained for demyelination with luxol fast blue (C and F). Immunohistochemical analysis of adjacent sections detected macrophage infiltration (E and H) and viral antigen (F and I), as described in Fig. 2. Demyelination was slightly decreased in IFN-γR1$^{-/-}$ RAG1$^{-/-}$ recipient mice (white bar, A, and G) compared to IFN-γR1$^{+/+}$RAG1$^{-/-}$ recipients (black bar, A, and D), although this difference was not statistically significant ($p>0.05$). The amount of infectious virus in brain homogenates ($p=0.0013$) and distribution of virus antigen near areas of demyelination was increased (IFN-γR1$^{+/+}$, squares, B, tissue section, F. IFN-γR1$^{-/-}$, triangles, B, tissue section I). Virus was detected in 5/5 IFN-γR1$^{+/+}$and 8/8 IFN-γR1$^{-/-}$ recipient mice. Macrophage infiltration was generally localized to areas of demyelination in both groups (IFN-γR1$^{+/+}$, E. IFN-γR1$^{-/-}$, H). When the ratio of demyelination to viral titer was compared for each sample, the differences between recipients were significant (IFN-γR1$^{+/+}$ black bar, IFN-γR1$^{-/-}$ white bar, C, $p=0.0126$). Data are presented as a summary of two experiments.

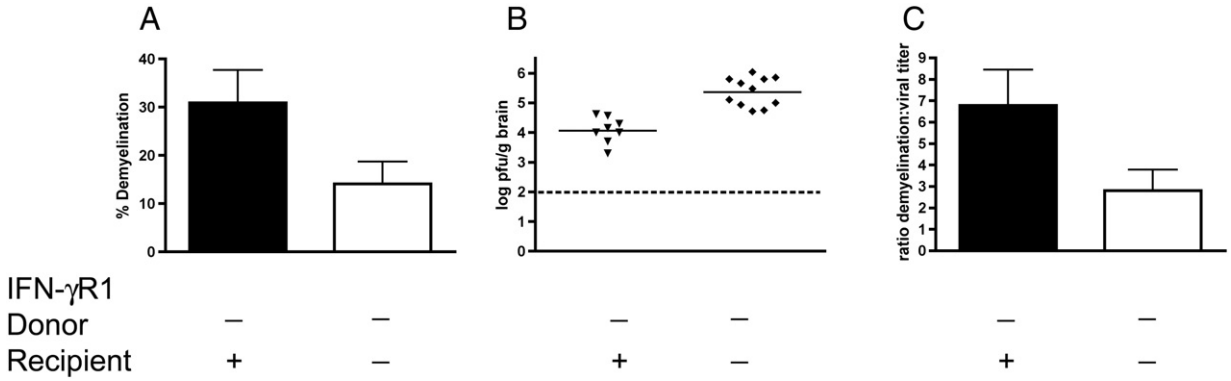

Fig. 4. IFN-γ recipient responsiveness contributes to CD8 T cell-mediated viral clearance and demyelination when donor cells lack IFN-γR1. Infected IFN-γR1$^{-/-}$ RAG1$^{-/-}$ mice receiving CD8 T cell-enriched splenocytes from MHV–JHM immune B6 IFN-γR1$^{-/-}$ mice were sacrificed on days 8–10 post-transfer (described in Fig. 2 and depicted in Figs. 2 and 3). Demyelination was decreased in IFN-γR1$^{-/-}$ RAG1$^{-/-}$ recipient mice (white bar, A) compared to IFN-γR1$^{+/+}$RAG1$^{-/-}$ recipients (black bar, A), although the difference between recipients was not statistically significant ($p>0.05$). The amount of infectious virus in brain homogenates ($p<0.0001$) and distribution of virus antigen near areas of demyelination was increased (IFN-γR1$^{+/+}$, squares, B. IFN-γR1$^{-/-}$, triangles, B). Virus was detected in 8/8 IFN-γR1$^{+/+}$ and 11/11 IFN-γR1$^{-/-}$ recipient mice. When the ratio of demyelination to viral titer was compared for each sample, the decrease in demyelination in IFN-γR1$^{-/-}$ recipients was magnified (IFN-γR1$^{+/+}$ black bar, IFN-γR1$^{-/-}$ white bar, C, $p=0.0425$). Data are presented as a summary of three experiments.

(Fig. 3A, D, G) while macrophage infiltration remained associated with areas of demyelination in both groups (Fig. 3E, H). Furthermore, viral titers were higher in recipient mice lacking IFN-γR1, up to one and a half log pfu/ml higher when compared to infected IFN-γ responsive RAG1$^{-/-}$ mice (Fig. 3B, F, I). Viral titers in the CNS of RAG1$^{-/-}$ mice that received IFN-γR1$^{-/-}$ CD8 T cells were comparable to previously published CNS titers of infected RAG1$^{-/-}$ mice that did not receive splenocytes (Wu and Perlman, 1999).

In previous reports of J2.2v-1 infection, viral clearance was determined to be IFN-γ or IFN-γR dependent (Gonzalez et al., 2006; Parra et al., 1999). Our observation of increased viral titers in infected IFN-γR1$^{-/-}$ RAG1$^{-/-}$ recipients support this conclusion (Fig. 3B, F, I). Therefore, an increase in virulence may also increase demyelination in the absence of IFN-γ responsiveness. When demyelination and viral titer are calculated as a ratio (demyelination:viral titer) for individual mice, the difference between IFN-γR1$^{-/-}$ recipients and IFN-γR1$^{+/+}$ recipients is increased (Fig. 3C). These differences reflect the combination of increased demyelination with lower virus loads in RAG1$^{-/-}$ recipients compared to decreased demyelination and higher virus loads in IFN-γR1$^{-/-}$ RAG1$^{-/-}$ recipients.

There is a five-fold decrease in demyelination when CD8 T8 T cell-enriched splenocytes from IFN-γ$^{-/-}$ as opposed to B6 donors were transferred into J2.2v-1-infected RAG1$^{-/-}$ recipients (Pewe and Perlman, 2002). In IFN-γR1 deficient recipients, however, the decrease in CD8 T cell-mediated demyelination was diminished. Although donor macrophages and DCs do not traffic to the spinal cord in MHV–J2.2v-1-infected mice (Fig. 1), IFN-γR1^{+} donor cells present in the transferred population may compensate for the lack of IFN-γ responsiveness of recipient mice, thus increasing demyelination. To eliminate this potentially complicating factor, we transferred IFN-γR1$^{-/-}$ donor CD8 T cell-enriched splenocytes into J2.2v-1-infected IFN-γR1$^{-/-}$ RAG1$^{-/-}$ mice or RAG1$^{-/-}$ mice. Similar to mice receiving wild type splenocytes, infected IFN-γR1$^{-/-}$ RAG1$^{-/-}$ mice receiving IFN-γR1$^{-/-}$ CD8 T cell-enriched splenocytes exhibited decreased demyelination and increased virus titers when compared to RAG1$^{-/-}$ recipients (Fig. 4A, B). Furthermore, when the ratio of demyelination to

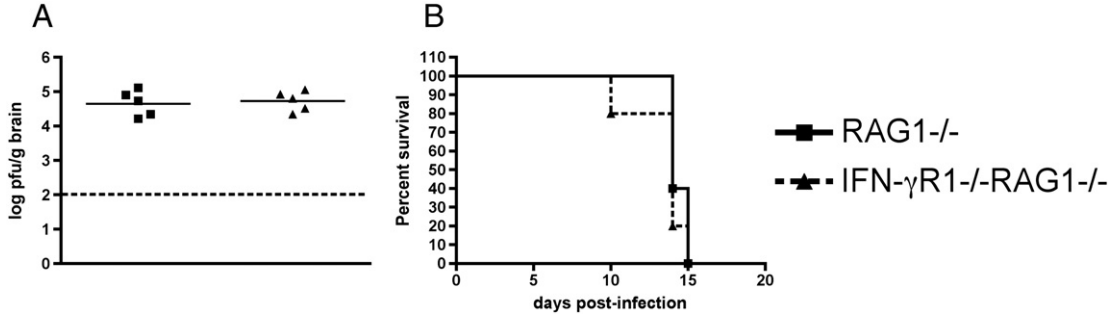

Fig. 5. IFN-γ responsiveness does not contribute to viral clearance in the absence of CD8 T cells. Brains fromJ2.2v-1-infected IFN-γR1$^{-/-}$ RAG1$^{-/-}$ or IFN-γR1$^{+/+}$RAG1$^{-/-}$ mice were harvested 4 days post-infection. There were no A differences in brain viral loads between IFN-γR1$^{+/+}$RAG1$^{-/-}$ (squares, A) and IFN-γR1$^{-/-}$ RAG1$^{-/-}$ (triangles, A) mice ($p>0.05$). Mice infected for a longer time period displayed equivalent rates of survival (IFN-γR1$^{+/+}$, solid line with squares, B. IFN-γR1$^{-/-}$, dotted line with triangles, B). Data are presented as a summary of two experiments.

viral titer is compared between IFN-γR1$^{-/-}$ and IFN-γR1$^{+/+}$ recipients of CD8 T cell-enriched IFN-γR1$^{-/-}$ splenocytes, the difference between groups is increased (Fig. 4C). Therefore, IFN-γ recipient responsiveness contributes to both CD8 T cell-mediated demyelination and viral clearance in RAG1-deficient mice.

3.4. IFN-γ responsiveness does not contribute to viral clearance in the absence of CD8 T cells

J2.2-v1-infected RAG1$^{-/-}$ mice do not develop demyelination despite the presence of high levels of infectious virus in the CNS (Wu and Perlman, 1999). It is possible that NK cells, which are capable of producing significant amounts of IFN-γ, may contribute to viral clearance in the absence of T cells. To test this possibility, we infected RAG1$^{-/-}$ mice or IFN-γR1$^{-/-}$ RAG1$^{-/-}$ mice with MHV–J2.2v-1 and compared virus titers in the brain at day 4 post-infection. As expected, viral titers were equivalent in both groups (Fig. 5A), suggesting that viral clearance is not mediated by an IFN-γ-dependent mechanism in the absence of CD8 T cells. Furthermore, no differences were detected in clinical disease or mortality between infected RAG1$^{-/-}$ and IFN-γR1$^{-/-}$ RAG1$^{-/-}$ mice (Fig. 5B).

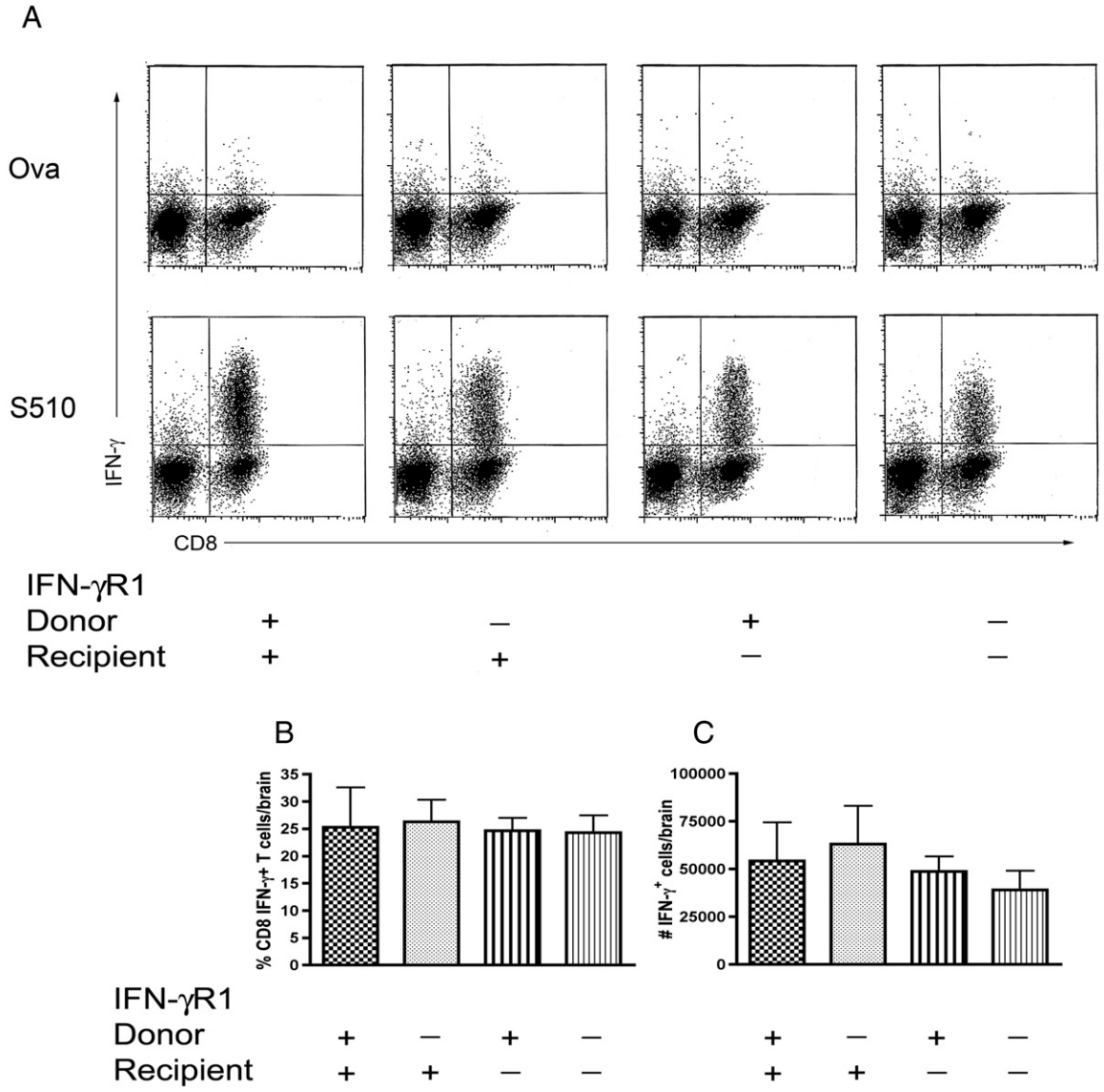

Fig. 6. Equivalent virus-specific CD8 T cell responses in MHV–JHM-infected mice. Lymphocytes were isolated from the brains of J2.2v-1-infected RAG1$^{-/-}$ mice 8–10 days after receiving CD8 T cell-enriched splenocytes from MHV–JHM immune mice. Lymphocytes were incubated with control peptide or a soluble peptide derived from the CD8 T cell immunodominant D^{b}-restricted epitope found within the spike glycoprotein of MHV–JHM, spanning amino acids 510–518 (S510). The analysis revealed no differences between donor or recipient groups based on IFN-γR1 expression, in either frequency (A,B) or total number (C) of virus-specific T cells from the CNS of infected mice (checkered bar, B6 donor — RAG1$^{-/-}$ recipient. Shaded bar, IFN-γR1$^{-/-}$ donor — RAG1$^{-/-}$ recipient. Vertical lined bar, B6 donor — IFN-γR1$^{-/-}$ RAG1$^{-/-}$ recipient. Statistical comparison of groups did not reveal any significant differences ($p > 0.05$). Dot plots are representative of each group, while graphical data are presented as a summary of three experiments, 4–6 mice per group.

3.5. Equivalent MHV–JHM-specific CD8 T cell responses in infected IFN-γR$^{-/-}$ RAG1$^{-/-}$ and IFN-γR1$^{+/+}$RAG1$^{-/-}$ mice

Although T cell IFN-γ responsiveness regulates antigen induced cell death, (Lohman and Welsh, 1998; Refaeli et al., 2002) a recent study demonstrated that IFN-γ also acts indirectly on other cells as a negative feedback regulator of T cell responses (Feuerer et al., 2006). Therefore, the ability of antigen-specific T cells to traffic to the CNS, exhibit their effector functions, and survive *in situ* may be altered in the absence of IFN-γ responsiveness in recipient cells, thus decreasing both CD8 T cell-mediated macrophage activation and viral clearance. To examine this possibility, we assessed virus-specific CD8 T cell number and function by measuring IFN-γ production by cells harvested from the infected RAG1$^{-/-}$ CNS after direct ex vivo stimulation with an MHV–JHM-specific CD8 T cell peptide. At days 8–10 post-transfer, equivalent frequencies (Fig. 6A, B) and total numbers (Fig. 6C) of CD8 T cells were isolated from RAG1$^{-/-}$ or IFN-γR1$^{-/-}$ RAG1$^{-/-}$ mice, regardless of donor responsiveness to IFN-γ. Furthermore, the number of virus-specific CD8 T cells in pre-transfer splenocyte suspensions were comparable in IFN-γR1$^{+/+}$and IFN-γR1$^{-/-}$ donors (data not shown), and were consistent with previously published results (Wu et al., 2000). Based on these results, we conclude that decreased demyelination and increased viral titers are not a result of decreased virus-specific CD8 T cell responses in J2.2v-1-infected IFN-γR1$^{-/-}$ RAG1$^{-/-}$ mice.

4. Discussion

T cells are required for the demyelination that is detected in the J2.2v-1-infected RAG1$^{-/-}$ CNS after adoptive transfer of splenocytes,, as depletion of T cells abrogated demyelination (Pewe and Perlman, 2002; Wu et al., 2000). In addition, our results indicate that macrophages and CD11c^{+}DCs of donor origin do not traffic to the spinal cord in infected mice (Fig. 1).

A requirement for IFN-γ recipient responsiveness in CD8 T cell-mediated demyelination is consistent with previous studies that examined the role of IFN-γ in MHV–JHM infection. IFN-γ expressed by virus-specific CD8 T cells is critical for demyelination in infected mice, while IFN-γ also plays a role in demyelination mediated by bystander CD8 T cells and γδ T cells (Dandekar et al., 2004, 2005; Pewe and Perlman, 2002). Brisebois et al. (2006) examined IFN-γ responsiveness in mice engineered to constitutively express the costimulatory molecule CD86 in microglia. These mice develop spontaneous demyelination, which is abrogated if mice are IFN-γR1-deficient; these mice also lacked the characteristic microglial activation observed in wild type mice. This result suggests that microglia are a target of IFN-γ, and thus, final effectors of demyelination.

Microglia/macrophages are present in demyelinating lesions in MS and in animal models of MS such as EAE, and in mice infected with Theiler's murine encephalomyelitis virus (TMEV) and MHV–JHM (Hemmer et al., 2002). Direct recruitment and activation of macrophages/microglia in the spinal cord of MHV–JHM-infected mice are sufficient to induce demyelination in the absence of T or B cells, since myelin destruction is detected in RAG1$^{-/-}$ mice infected with a recombinant MHV–JHM that expresses the macrophage chemokine CCL2 (MCP-1) (Kim and Perlman, 2005). In wild type mice, IFN-γ-mediated activation and recruitment of macrophages and microglia to sites of viral infection may also occur indirectly through other resident CNS cells. Astrocytes are the resident CNS cell type most likely to facilitate indirect cell recruitment and activation, as CCL2 is produced by astrocytes after IFN-γ treatment (Hayashi et al., 1995; Weiss et al., 1998).

In addition to a role in demyelination, IFN-γ responsiveness in recipient mice is important for CD8 T cell-mediated viral clearance (Figs. 3 and 4). This result is consistent with previous studies which demonstrated IFN-γ and IFN-γ responsiveness are critical for MHV–JHM clearance from myelin-producing oligodendrocytes of the CNS (Gonzalez et al., 2006; Parra et al., 1999). The observation that IFN-γR1 deficiency in infected RAG1$^{-/-}$ recipient mice does not decrease demyelination as extensively as IFN-γ deficiency in transferred CD8 T cells may be explained by enhanced virus replication in the first group (Pewe and Perlman, 2002). Further, IFN-γ deficiency is limited to the donor population in CD8 T cell-enriched splenocyte transfers. Other non-T cells present in RAG1-deficient mice are capable of producing IFN-γ, (Schroder et al., 2004) and thus may contribute to viral clearance in the absence of CD8 T cell IFN-γ production. In IFN-γR1$^{-/-}$ RAG1$^{-/-}$ mice, however, this alternative mechanism is not possible, as the effects of IFN-γ on any host cell type are eliminated, regardless of the source of IFN-γ. Thus, diminished virus clearance may result in substantial demyelination even in the absence of IFN-γR1. The primary mechanism for virus clearance from myelin-producing cells is IFN-γ-mediated (Bergmann et al., 2006) and our results suggest that IFN-γR1 expression in infected recipient mice is likely more critical for CD8 T cell-mediated viral clearance than for CD8 T cell-mediated demyelination.

Future studies are needed to determine the specific IFN-γ-responsive cell types that mediate CD8 T cell-mediated demyelination and/or viral clearance. Transgenic mice engineered to express a dominant-negative IFN-γR1 under the control of an oligodendrocyte-specific reporter demonstrated decreased viral clearance, with no decrease in demyelination (Gonzalez et al., 2006). Also, expression of a dominant-negative IFN-γR1 in astrocytes had no effect on viral clearance or demyelination (Hindinger et al., 2005). Our results demonstrating that recipient IFN-γR1 expression contributes to demyelination together with the observation of decreased microglial activation and spontaneous demyelination in IFN-γR1$^{-/-}$ mice described by Brisebois et al. (2006) suggest that IFN-γR1 expression by macrophages/microglia is important for maximal CD8 T cell-mediated demyelination. Although a transgenic mouse with macrophage-specific IFN-γR inhibition has been developed, microglia were not specifically examined in this study, (Dighe et al., 1995). Therefore, the importance of macrophage and microglial-specific IFN-γ responsiveness in CD8 T cell-mediated demyelination during MHV–JHM infection will require further study.

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