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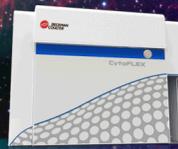
Cutting Edge: CD8 T Cell-Mediated Demyelination Is IFN- γ Dependent in Mice Infected with a Neurotropic Coronavirus

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CUTTING EDGE

Cutting Edge: CD8 T Cell-Mediated Demyelination Is IFN-γ Dependent in Mice Infected with a Neurotropic Coronavirus[1]

Lecia Pewe and Stanley Perlman[2]

Mice infected with the murine coronavirus, mouse hepatitis virus, strain JHM (MHV) develop an immune-mediated demyelinating encephalomyelitis. We showed previously that adoptive transfer of MHV-immune splenocytes depleted of either CD4 or CD8 T cells to infected $RAG1^{-/-}$ recipients (mice deficient in recombination activation gene 1) resulted in demyelination. Herein we show that transfer of CD8 T cell-enriched splenocytes from MHV-immune IFN-$\gamma^{-/-}$ donors resulted in a substantial decrease in demyelination (4.8% of the white matter of the spinal cord compared with 26.3% in those receiving cells from C57BL/6 donors). Similar numbers of lymphocytes were present in the CNS of recipients of either C57BL/6 or IFN-$\gamma^{-/-}$ CD8 T cells, suggesting that IFN-γ was not crucial for lymphocyte entry into the CNS. Rather, IFN-γ was critical for optimal activation or migration of macrophages or microglia into the white matter in the context of CD8 T cell-mediated demyelination. *The Journal of Immunology,* 2002, 168: 1547–1551.

Accumulating data from multiple studies show that either CD4 or CD8 T cells are able to mediate demyelination in experimental models of demyelination, including rodents infected with specific strains of mouse hepatitis virus or Theiler's encephalomyelitis virus or animals with experimental autoimmune encephalomyelitis (EAE).[3] In each of these models, demyelination is in large part immune-mediated, with CD4 and CD8 T cells making different contributions to the process of demyelination (1–5). Furthermore, although originally considered primarily a CD4 T cell-mediated disease, CD8 T cells may also have a significant role in the disease

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[3] Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; MHV, mouse hepatitis virus; MCP, macrophage chemoattractant protein; *RAG1*, recombination activation gene 1; p.t., post-transfer; S_{510}, residues 510–518 of the surface glycoprotein; CCL, C-C chemokine ligand.

process in patients with the human demyelinating disease, multiple sclerosis (MS) (6).

Mice infected with the JHM strain of mouse hepatitis virus (MHV) develop a fatal encephalitis, unless the infection is modified by treatment with antivirus Abs or T cells or by infection with attenuated strains (7, 8). One such strain, MHV 2.2-V-1, causes demyelination in immunocompetent C57BL/6 (B6) mice with less mortality than in the parental strain. When SCID or recombination activation gene 1-deficient $(RAG1)^{-/-}$ mice were infected with MHV 2.2-V-1, they succumbed to encephalitis 12–14 days postinoculation without evidence of significant demyelination in their spinal cords. Adoptive transfer of splenocytes from B6 mice into infected SCID or $RAG1^{-/-}$ mice resulted in the appearance of demyelination ~7 days post-transfer (p.t.) (9, 10). The development of demyelination was most consistent in $RAG1^{-/-}$ mice when splenocytes were transferred from donors previously immunized with MHV (10). Transfer of cells from naive donors only sporadically resulted in demyelination in this model.

In subsequent experiments, we showed that depletion of both CD4 and CD8 T cells from the donor populations abrogated the appearance of demyelination whereas depletion of only one T cell subset did not. Recipients of CD4 T cell-enriched populations developed severe encephalitis (hunching, lethargy, ruffled fur) 6–7 days p.t., whereas recipients of CD8 T cells remained largely asymptomatic until 7–10 days p.t., when they developed signs of hind limb weakness. More extensive demyelination developed in recipients of CD8 T cell-enriched populations (5). Little is known about the effector molecules important for CD4 or CD8 T cell-mediated demyelination in this model. Previous studies indicated that no single effector molecule was required for MHV-induced demyelination. Perforin, TNF-α, IFN-γ, inducible nitric oxide synthase, and IL-10 were studied in these reports (8).

A conclusion from one study was that IFN-γ, a key proinflammatory molecule, was critical for virus clearance from infected oligodendrocytes, although not for the development of demyelination (11). Overexpression of IFN-γ has also been shown to enhance recruitment of cells to the MHV-infected CNS, perhaps via activation of endothelial cells (12). Recent studies indicate that IFN-γ also has a suppressive role in CNS inflammatory disease and in chemokine regulation (13). Because CD4 or CD8 T cell-enriched populations were able to cause demyelination but with different patterns of clinical disease, we reasoned that each subset might cause demyelination using a different set of effector molecules. For these reasons, we decided to reinvestigate the roles of IFN-γ, perforin, and TNF-α in MHV-induced demyelination in the context of CD8 T cell enrichment. In this report, we show that

demyelination is largely abrogated when CD8 T cells from IFN-$\gamma^{-/-}$ mice are transferred.

Materials and Methods

Virus

The neuroattenuated variant of the JHM strain of MHV, MHV 2.2-V-1, generously provided by Dr. J. Fleming (University of Wisconsin, Madison, WI), was used in all studies.

Animals

Pathogen-free B6 mice were obtained from the National Cancer Institute (Bethesda, MD). $RAG1^{-/-}$ mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred at the University of Iowa (Ames, IA). IFN-$\gamma^{-/-}$ mice were obtained from The Jackson Laboratory.

Adoptive transfer model

$RAG1^{-/-}$ mice were infected with 1×10^3 PFU MHV 2.2-V-1 by intracranial injection. Adoptive transfer of 5×10^6 splenocytes from B6 or IFN-$\gamma^{-/-}$ mice immunized i.p. with wild-type MHV to infected $RAG1^{-/-}$ mice was performed as previously described (10). Wild-type MHV was used for immunization to maximize the anti-MHV immune response in donor animals. A total of 85 MHV 2.2-V-1-infected RAG1$^{-/-}$ mice were used in these experiments; 35 received B6, 25 received IFN-$\gamma^{-/-}$, 18 received perforin$^{-/-}$, 3 received TNF-$\alpha^{-/-}$, and 4 received TNF-α-perforin$^{-/-}$ CD8 T cell-enriched splenocytes. No infectious virus could be detected by plaque assay in the transferred cells (10).

Complement depletion

Donor splenocytes depleted of CD4 T cells were prepared for adoptive transfer by two rounds of complement lysis, as previously described (5). Under these conditions, depletion was >98% and no MHV-specific CD4 T cells were detected in the CNS of infected recipient mice at 10 days p.t. (5).

Immunohistochemistry

Sections were stained for macrophages/microglia with rat anti-F4/80 mAb (CI:A3-1; Serotec, Oxford, U.K.) as previously described (5).

Flow cytometry

Lymphocytes were prepared from the CNS as described previously (14). Briefly, single-cell suspensions were blocked with purified anti-mouse CD16/CD32 (mAb 2.4G2) (BD PharMingen, San Diego, CA) in 10% rat serum. For two- or three-color flow cytometric analysis, cells were stained with combinations of the following Abs: FITC-conjugated rat anti-mouse CD8, CD45 (BD PharMingen); PE-conjugated rat anti-mouse CD11b (Mac-1; BD PharMingen); and biotinylated anti-CD8 Ab. Biotinylated Ab was detected by avidin-APC (Molecular Probes, Eugene, OR). In all cases, an isotype-matched FITC- or PE-conjugated Ab was used. Flow cytometry was performed on a FACScan (BD Biosciences, San Jose, CA) or an EPICS 753 (Beckman Coulter, Fullerton, CA) at the University of Iowa FACS facility (Ames, IA).

Intracellular staining for IFN-γ or TNF-α

Lymphocytes were prepared from the CNS and stimulated with peptide-coated EL-4 cells for Ag presentation to CD8 T cells in the presence of brefeldin A or monensin (Golgiplug or Golgistop; BD PharMingen). Peptides corresponding to the immunodominant CD8 T cell epitope recognized in B6 mice (residues 510–518 of the surface glycoprotein (S_{510})) (15, 16)

or OVA$_{257–264}$ (OVA$_{257}$, irrelevant peptide) were used at a final concentration of 1 μM. Cells were processed for IFN-γ or TNF-α expression as previously described (5). Lymphocytes harvested from CNS tissue of single mice or pools of two to four mice were used in these analyses. The absolute number of Ag-specific cells was calculated by multiplying the fraction of Ag-specific CD8 T cells by the fraction of CD8 T lymphocytes by the total number of cells per brain.

Tetramers

Tetramers of MHC class I (H-2D^b) peptide S_{510} conjugated with avidin-PE (Molecular Probes) were obtained from the National Institute of Allergy and Infectious Diseases Tetramer Core Facility, Atlanta, GA.

Imaging and quantification of demyelination

Demyelination was quantified using Vtrace software (Image Analysis Facility, University of Iowa, Ames, IA), as previously described (5).

Results

Demyelination is markedly reduced in recipients of IFN-γ$^{-/-}$ CD8 T cell-enriched splenocytes

MHV-infected $RAG1^{-/-}$ mice develop clinical disease consisting of lethargy, ruffled fur, wobbly gait, and hind limb paresis 7–9 days after adoptive transfer of undepleted MHV-immune B6 or IFN-γ$^{-/-}$ splenocytes. Examination of spinal cords revealed 15–20% demyelination of the white matter of the spinal cord in recipients of either B6 or IFN-γ$^{-/-}$ cells (data not shown).

In contrast, greater morbidity was observed in recipients of IFN-γ$^{-/-}$ CD8 T cell-enriched splenocytes than in those receiving similar populations from B6 mice. We showed previously that MHV-infected recipients of B6 CD8 T cell-enriched splenocytes developed mild disease characterized by hind limb paresis/paralysis with few signs of encephalitis (5). Mice often survived to day 15 p.t. or longer. In contrast, recipients of IFN-γ$^{-/-}$ CD8 T cell-enriched splenocytes showed clinical signs of encephalitis (lethargy, ruffled fur, wasting, hunching) by 9–10 days p.t. and did not survive past 12 days p.t. Limb weakness, observed in mice with extensive demyelination, was not a prominent feature of disease in these animals. Virus titers were similar in recipients of CD8 T cells from B6 or IFN-γ$^{-/-}$ mice (Table I), making it unlikely that inefficient virus clearance in recipients of IFN-γ$^{-/-}$ cells accounted for the observed differences in clinical disease.

Consistent with these clinical observations, recipients of IFN-γ$^{-/-}$ CD8 T cells exhibited significantly less demyelination when compared with those receiving cells from B6 mice (Table I and Fig. 1, A and C). Ten days after transfer of B6 CD8 T cell-enriched splenocytes, $26.3 \pm 6.0\%$ of the white matter of the spinal cord showed evidence of demyelination with extensive macrophage infiltration (Fig. 1B). In contrast, demyelination was markedly reduced in recipients of IFN-γ$^{-/-}$ CD8 T cell-enriched splenocytes, encompassing, on the average, $4.8 \pm 1.3\%$ of the white matter. Most strikingly, we detected equal or greater numbers of activated macrophages/microglia in the gray matter, but greatly reduced

Table I. *Demyelination and virus titers in recipients of CD8 T cells at 9–12 days p.t.*

Enrichment Group	% Demyelination (no.)	No. of Samples with Detectable Virus	Titer (log$_{10}$ PFU/g tissue $\pm$ SE)[a]
B6	26.3 ± 6.0 (11)	9/9	4.89 ± 0.25
IFN-γ$^{-/-}$	4.8 ± 1.3 (11)[b]	4/4	4.85 ± 0.21
TNF-α$^{-/-}$	37.3 ± 8.3 (3)	3/3	5.41 ± 0.1
Perforin$^{-/-}$	15.5 ± 2.1 (14)	12/12	5.29 ± 0.16
Perforin-TNF-α$^{-/-}$	17.8 ± 2.3 (4)	4/4	5.21 ± 0.11

[a] No statistical difference was observed for viral titers among recipients of B6 and other populations.

[b] A significant difference was observed in the amount of demyelination between recipients of CD8 T cell-enriched B6 and IFN-γ$^{-/-}$ populations ($p < 0.05$) as measured by Student's t test, but not between recipients of B6 and other populations.
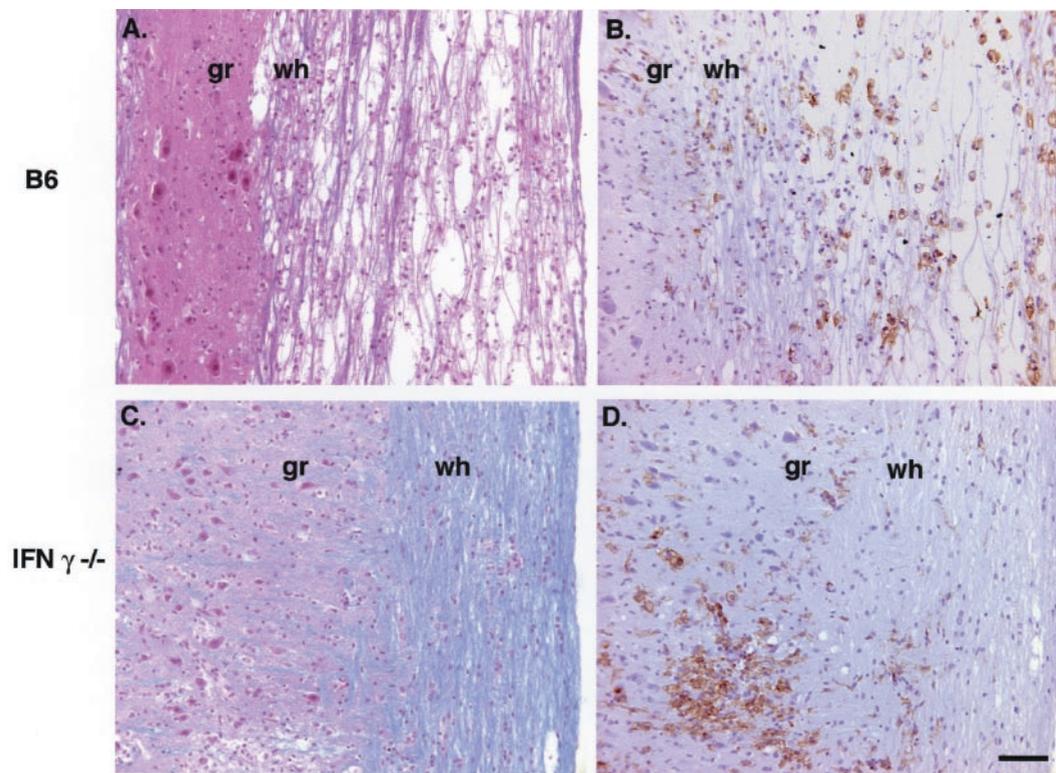
FIGURE 1. Decreased demyelination in recipients of IFN-$\gamma^{-/-}$ CD8 T cell-enriched splenocytes. MHV-infected *RAG1*$^{-/-}$ mice received CD8 T cell-enriched populations from immune B6 (*A* and *B*) or IFN-$\gamma^{-/-}$ (*C* and *D*) donors at 4 days postinoculation. Mice were sacrificed 10–11 days p.t. Spinal cords were stained with LFB for myelin (*A* and *C*) or with anti-F4/80 Ab for macrophages/microglia (*B* and *D*). Demyelination was greatly diminished in recipients of IFN-$\gamma^{-/-}$ cells, with a concomitant decrease in the number of macrophages/microglia infiltrating into the white matter. However, we still detected activated macrophages/microglia in the gray matter in these mice (*D*). gr, Gray matter; wh, white matter. Bar, 200 μm.

numbers in the white matter, in recipients of IFN-$\gamma^{-/-}$ cells when compared with mice receiving B6 splenocytes (Fig. 1, *B* and *D*; quantification is described below). The amount of demyelination was greater in recipients of IFN-$\gamma^{-/-}$ cells than in infected *RAG1*$^{-/-}$ mice in the absence of adoptive transfer in which only 1–2% of the spinal cord white matter showed evidence of myelin destruction (10). Thus, donor cell-derived IFN-γ was critical, but not absolutely required, for CD8 T cell-mediated demyelination.

To determine whether this effect was specific for IFN-γ or whether any defect in CD8 T cell effector function would affect demyelination, CD8 T cell-enriched populations from perforin$^{-/-}$, TNF-$\alpha^{-/-}$, or perforin-TNF-$\alpha^{-/-}$ donors were transferred to MHV-infected RAG1$^{-/-}$ mice (Table I). Less demyelination was observed in recipients of perforin$^{-/-}$ CD8 T cell-enriched splenocytes (15.5% $\pm$ 2.1) than in recipients of B6 cells (26.3% $\pm$ 6.0), but the difference did not reach statistical significance ($p = 0.07$). At least as much demyelination was detected in recipients of TNF-$\alpha^{-/-}$ CD8 T cells as in those receiving B6 cells. As in recipients of perforin$^{-/-}$ CD8 T cell-enriched populations, 17.8 $\pm$ 2.3% of the white matter of the spinal cord was demyelinated in recipients of cells from perforin-TNF-$\alpha^{-/-}$ donors. Thus, perforin may have

a role in demyelination, but its absence does not have as profound an effect as the absence of IFN-γ in the donor cell population.

Infiltration of CD8 T cells is the same in recipients of B6 or IFN-$\gamma^{-/-}$ CD8 T cells

One explanation for these results is that in the absence of IFN-γ in donor cells, recruitment of CD8 T cells or other inflammatory cells is inefficient. Our immunohistochemical analyses suggested that macrophage/microglia (Fig. 1, *B* and *D*), but not CD8 T cell (data not shown), infiltration into the white matter was diminished in recipients of IFN-$\gamma^{-/-}$ cells as compared with those receiving B6 cells. To quantify these results more precisely, we analyzed CNS-derived mononuclear populations for CD8 T cells and macrophages/microglia (Table II). The number of MHV-specific CD8 T cells in the CNS was assessed using S$_{510}$ tetramers. As shown in Table II, similar numbers of tetramer S$_{510}$-positive CD8 T cell lymphocytes were detected in the CNS in recipients of either IFN-$\gamma^{-/-}$ or B6 donor cells.

To assess the functional status of these cells, we measured TNF-α expression after peptide S$_{510}$ stimulation in recipients of B6 or IFN-$\gamma^{-/-}$ splenocytes and IFN-γ expression in recipients of

Table II. *Ag specificity of CD8 T cells in recipient mice after adoptive transfer*

Mouse	% S$_{510}$[a,b]	No. of S$_{510}$[c]	% S$_{510}$ Tetramers[a]	No. of S$_{510}$ Tetramers[c]	CD45^{high}CD11b$^+$
B6	8.1 $\pm$ 0.7 (7)	1.9 $\pm$ 0.5 $\times$ 10^4	68.0 $\pm$ 2.6 (4)	1.2 $\pm$ 0.2 $\times$ 10^5	3.6 $\pm$ 1.0 $\times$ 10^4 (7)
IFN-$\gamma^{-/-}$	6.4 $\pm$ 2.2 (3)	1.6 $\pm$ 0.6 $\times$ 10^4	47.5 $\pm$ 3.0 (3)	1.2 $\pm$ 0.3 $\times$ 10^5	2.0 $\pm$ 0.3 $\times$ 10^4 (8)

[a] Numbers of single mice or groups of two to four mice analyzed (numbers in parentheses). Values are shown after subtraction of background staining.
[b] Measured by intracellular staining for IFN-γ (B6) or TNF-α (B6 and IFN-$\gamma^{-/-}$). Percentage of total CD8 T cells is shown.
[c] Absolute number $\pm$ SE of virus-specific cells calculated as described in *Materials and Methods*.

B6 donor cells. In control experiments, we showed that assays for IFN-γ or TNF-α identified nearly identical numbers of epitope S_{510}-specific T cells in the CNS of recipients of undepleted B6 populations (IFN-γ 16.7 ± 2.4% vs TNF-α 15.4 ± 2.2%, $n = 7$). Consequently, data from experiments in which epitope S_{510}-specific T cells were detected with either cytokine are included in Table II. In other experimental systems, more pathogen-specific primary CD8 T cells expressed IFN-γ than TNF-α after peptide stimulation directly ex vivo, but memory cells expressed both cytokines. After secondary infection, most CD8 T cells expressed both cytokines (17, 18). These results suggest that after immunization and adoptive transfer, CD8 T lymphocytes harvested from the CNS of recipient mice function as secondary effector cells. Only a fraction of cells secreted IFN-γ or TNF-α in response to peptide S_{510}, suggesting that in both cases, only a minority of cells were capable of cytokine effector function. This number was, however, similar in recipients of B6 or IFN-γ$^{-/-}$ CD8 T cell-enriched splenocytes.

Decreased macrophage/microglia infiltration into the CNS of recipients of IFN-γ$^{-/-}$ CD8 T cells

In contrast, macrophage/microglia staining and FACS analysis revealed a decrease in the number of activated CD11b$^+$CD45^{high} cells in the CNS of recipients of IFN-γ$^{-/-}$ cells when compared with those receiving cells from B6 donors (B6, 3.6 ± 1.0 × 10^4; IFN-γ$^{-/-}$, 2.0 ± 0.3 × 10^4; Table II) although these differences were not statistically significant ($p = 0.10$). In another approach, we counted the number of macrophages/microglia in the gray and white matter of sagittal sections of spinal cords from three mice that received B6 CD8 T cell-enriched splenocytes and three that received IFN-γ$^{-/-}$ cells. For this purpose, all of the F4/80$^+$ cells in 1.25-mm-wide cross-sections at eight levels within spinal cords were counted. Consistent with the FACS analysis, the number of macrophages/microglia present in each cross-sectional area in recipients of IFN-γ$^{-/-}$ CD8 T cells was decreased by 45% (B6 vs IFN-γ$^{-/-}$, 179.4 ± 9.7 vs 80.3 ± 7.4, $p < 0.0001$). Furthermore, there was a 6- to 7-fold reduction in the number of F4/80$^+$ cells in the white matter (B6 vs IFN-γ$^{-/-}$, 145.2 ± 10.1 vs 21.9 ± 4.0, $p < 0.0001$) but more cells in the gray matter (B6 vs IFN-γ$^{-/-}$, 34.1 ± 3.9 vs 58.4 ± 5.5, $p < 0.001$) of recipients of IFN-γ$^{-/-}$ cells than in that of those receiving cells from B6 donors. These results suggest that CD8 T cell-derived IFN-γ is important in both macrophage/microglia activation and in migration of these cells into the white matter of the spinal cord.

Discussion

CD4 T cells were, until recently, considered the primary effector cells in rodents with the autoimmune demyelinating disease EAE and in patients with MS. These conclusions were based on many studies showing that myelin-specific CD4 T cells were able to mediate demyelination (3, 19). More recently, the generality of these conclusions has been called into question by studies showing that myelin-specific CD8 T cells were able to induce a severe and progressive form of EAE (1, 2). Furthermore, CD8 T cells are the predominant cells infiltrating demyelinating lesions in patients with MS (6). CD8 T cells also induce extensive demyelination in mice infected with Theiler's encephalomyelitis virus (4). In addition, normal (9) or reduced (20) amounts of demyelination were detected in mice genetically deficient in CD4 T cells after MHV infection, consistent with a role for CD8 T cells in this process. Our results extend these reports and support the conclusion that CD8 T cells are able to mediate demyelination independent of CD4 T cells. They also show that IFN-γ is critical for CD8 T cell-mediated demyelination, in agreement with the conclusions of

Huseby et al. (1). Furthermore, our data show that IFN-γ is important for macrophage/microglia activation or migration into the white matter of the CNS in the absence of CD4 T cells but is not required for the migration of CD8 T cells into the CNS. Demyelination was not reduced when undepleted IFN-γ$^{-/-}$ splenocytes were transferred, in agreement with previous results (11), nor is demyelination diminished in recipients of IFN-γ$^{-/-}$ CD4 T cells when compared with those receiving B6 cells (data not shown).

IFN-γ has multiple effects in CNS inflammation, both as a proinflammatory molecule and in regulating leukocyte trafficking and T cell population dynamics (21–24). In its proinflammatory role, it is involved in such functions as macrophage activation, up-regulation of MHC class I and class II expression, and induction of adhesion molecules important for entry into the CNS. As a proinflammatory mediator, IFN-γ is involved in the induction of expression of chemokines, such as C-C chemokine ligand (CCL) 2/macrophage chemoattractant protein (MCP)-1, CCL7/MCP-3, CCL4/macrophage-inflammatory protein-1β, CCL5/RANTES, CXC ligand 9/monokine induced by IFN-γ and CXC ligand 10/IFN-inducible protein 10/cytokine-responsive gene 2, which are chemoattractants for monocytes/macrophages and lymphocytes and are up-regulated in the CNS of MHV-infected mice (25). To determine whether reduced expression of any of these chemokines might be involved in the diminished macrophage/microglia activation or migration observed after transfer of IFN-γ$^{-/-}$ CD8 T cells, we assayed CNS samples for chemokine RNA expression. We found that each of these chemokines was expressed at equivalent or higher levels in recipients of IFN-γ$^{-/-}$ CD8 T cells than in those receiving B6 CD8 T cells (data not shown). This indicated that none was sufficient for macrophage/microglia infiltration or activation.

In summary, we showed that IFN-γ is critical for CD8 T cell-mediated demyelination in MHV-infected mice, most likely by facilitating macrophage activation or migration into the CNS. Our results may be relevant for understanding the human disease MS, because administration of recombinant IFN-γ worsened clinical disease in patients (26). This model system should be useful for delineating the precise mechanism of action of IFN-γ in the demyelinating process.

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