Immunopathology and Infectious Diseases

Inhibition of Interferon-γ Signaling in Oligodendroglia Delays Coronavirus Clearance without Altering Demyelination

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Infection of the central nervous system (CNS) by the neurotropic JHM strain of mouse hepatitis virus (JHMV) induces an acute encephalomyelitis associated with demyelination. To examine the anti-viral and/or regulatory role of interferon-γ (IFN-γ) signaling in the cell that synthesizes and maintains the myelin sheath, we analyzed JHMV pathogenesis in transgenic mice expressing a dominant-negative IFN-γ receptor on oligodendroglia. Defective IFN-γ signaling was associated with enhanced oligodendroglial tropism and delayed virus clearance. However, the CNS inflammatory cell composition and CD8+ T-cell effector functions were similar between transgenic and wild-type mice, supporting unimpaired peripheral and CNS immune responses in transgenic mice. Surprisingly, increased viral load in oligodendroglia did not affect the extent of myelin loss, the frequency of oligodendroglial apoptosis, or CNS recruitment of macrophages. These data demonstrate that IFN-γ receptor signaling is critical for the control of JHMV replication in oligodendroglia. In addition, the absence of a correlation between increased oligodendroglial infection and the extent of demyelination suggests a complex pathobiology of myelin loss in which infection of oligodendroglia is required but not sufficient. (Am J Pathol 2006, 168:796–804; DOI: 10.2353/ajpath.2006.050496)

The central nervous system (CNS), which is composed of highly specialized cells with limited renewal capacity, is the target of many viral infections.[1,2] Although the host immune response to viral CNS infections is often effective at limiting acute disease, it can also contribute to immunopathology.[3] The definition of immune effector mechanisms that control virus replication within specific subsets of CNS cells has been facilitated by analysis of models differing in cellular tropism. For example, viruses that preferentially infect and/or persist in neurons are generally controlled by noncytolytic effector strategies, including neutralizing antibodies (Ab) and interferon-γ (IFN-γ).[2] By contrast, acute lymphocytic choriomeningitis virus infection, which results in inflammation of the choroid plexus and meninges, is controlled by CD8+ T cells that use perforin-dependent cytolysis.[3,4] However, during chronic lymphocytic choriomeningitis virus infection in which virus persists predominantly in neurons, cytolytic CD8+ T-cell mechanisms are ineffective with recovery mediated by Ab and cytokines.[5,6] The neurotropic coronavirus, mouse hepatitis virus (MHV) strain JHM (JHMV), induces an acute encephalomyelitis associated with myelin loss; however, an insufficient immune response results in viral persistence.[7,8] JHMV initially infects ependymal cells, but as infection progresses it becomes polytropic and infects astrocytes, microglia, and macrophages.[8] As infection enters the white matter, oligodendroglia are primary targets of JHMV replication.[8] A concerted immune response, involving cellular and humoral effector mechanisms, controls acute and chronic JHMV infection.[7,9–11] JHMV infection induces CNS recruitment of inflammatory cells comprising components of both the innate and adaptive immune responses. Virus-specific CD4+ and CD8+ T cells are both present within the inflamed CNS.[9] However, CD8+ T cells are directly associated with the control of infectious virus.[9,10] By contrast CD4+ T cells appear to play an auxiliary role by promoting CD8+ T cell expansion and survival[12] and

have been implicated in the physiopathology of myelin loss.[13,14] JHMV replication in microglia, macrophages, and astrocytes is controlled via a perforin-dependent mechanism.[10,15] By contrast, IFN-γ, but not perforin, is critical for the control of virus replication in oligodendroglia, the cells that synthesize and maintain CNS myelin.[16] Whether a noncytolytic mechanism, ie, IFN-γ, directly controls JHMV replication in oligodendroglia and how signaling via this mediator impacts myelin loss are unknown. Virally induced demyelination has also been suggested to be the result of virus-induced oligodendroglial dysfunction or death.[17–20] However, virus alone is insufficient to mediate myelin destruction following JHMV infection, and both T cells and macrophages are necessary for demyelination.[10,13,21,22]

To determine the contribution of IFN-γ signaling in oligodendroglia to both immune-mediated control of JHMV replication and the subsequent myelin loss, pathogenesis of JHMV-infected transgenic (Tg) mice expressing a dominant-negative IFN-γ receptor (dnIFN-γR) specifically on oligodendroglia was compared to syngeneic wild-type (Wt) mice. The results demonstrate that IFN-γ signaling in oligodendroglia is required for virus control, eliminating potential secondary effects. Surprisingly, the high rate of infection did not increase oligodendroglial apoptosis or influence myelin loss, despite the presence of highly activated T cells capable of expressing anti-viral effector functions. These data thus indicate that, although CNS virus infection of glial cells associated with T-cell inflammation precipitates demyelination, extensive JHMV infection of oligodendroglia *per se* is not directly associated with cell death or myelin loss, even in the presence of activated T cells and macrophages.

Materials and Methods

Animal Model

Homozygous Tg mice on the H-2^b background expressing a dnIFN-γR1 under control of the proteolipid protein promoter, designated PLP-25/B6, were bred and maintained in the animal facilities of the University of Southern California. The transgene is expressed exclusively on oligodendroglia and severely impairs IFN-γ signaling.[23] Syngeneic Wt C57BL/6 (H-2^b) mice were purchased from the National Cancer Institute (Frederick, MD). Mice of both sexes were used at 6 to 7 weeks of age, and no gender-dependent differences were detected. All procedures were performed in compliance with protocols approved by the Keck School of Medicine Institutional Animal Care and Use Committee.

Virus Infection and Plaque Assay

Mice were infected with the neutralizing monoclonal antibody (mAb)-derived variant 2.2v-1 of the neurotropic JHMV strain of MHV.[24] Virus was propagated in the presence of mAb J.2.2, and titers were determined by plaque assay on monolayers of the delayed brain tumor murine astrocytoma as previously described.[9,24] Mice were in-

jected in the left brain hemisphere with 30 μl containing 250 plaque forming units of JHMV in endotoxin-free Dulbecco's modified phosphate-buffered saline (PBS). Infected mice were scored daily for clinical signs as follows: 0) no clinical signs or healthy, 1) hunched back, 2) partial hind limbs paralysis, 3) complete hind limbs paralysis, and 4) moribund or death.[24] To determine CNS virus replication, brains were dissected in the midsagittal plane, and one-half brain was homogenized in RPMI 1640 medium containing 25 mmol/L HEPES, pH 7.2, using Tenbroeck homogenizers. Cells were recovered by centrifugation at 500 × g for 7 minutes, and supernatants were stored at −70°C until use. Virus titers were determined by plaque assay on delayed brain tumor monolayers as previously described.[9,12]

Analysis of CNS Mononuclear Cells

CNS cells were obtained from homogenized brains and separated from myelin by differential centrifugation on Percoll gradients (Amersham Biosciences, Uppsala, Sweden) as previously described.[9] Briefly, homogenates were suspended in RPMI containing 25 mmol/L HEPES, pH 7.2, and adjusted to 30% Percoll. Cells were isolated by centrifugation at 800 × g for 25 minutes at 4°C onto a 1-ml 70% Percoll cushion. Cells recovered from the 30/70% interface were washed and counted using trypan blue exclusion. Cells were examined for surface molecule expression by flow cytometry. Nonspecific binding was inhibited by incubation with mouse Fc block CD16/CD32 (Clone 2.4G2) and a mixture of normal goat, human, mouse, and rat serum for 10 minutes on ice. All mAbs were obtained from BD Pharmingen (San Diego, CA) unless otherwise noted. Cells were stained for 30 minutes at 4°C with mAb specific for CD4 (L3T4, clone GK1.5), CD8a (Ly-2, clone 53-6.7), CD19 (1D3), CD45 (Ly-5, clone 30-F11), and NK 1.1 (PK136). Macrophages were identified as F4/80$^+$ (Serotec, Oxford, UK). Cells were washed with PBS containing 1% bovine serum albumin and fixed in 2% paraformaldehyde before analysis using a FACSCalibur and CellQuest Software (BD Biosciences, Mountain View, CA). Intact cells were included according to the side scatter versus forward scatter profiles. At least 1 × 10^4 cells were acquired in each sample.

Virus-specific CD8$^+$ T cells were detected with fluorescein isothiocyanate-labeled anti-CD8 mAb and phycoerythrin-labeled D^{b}S510 tetramers.[9] Antigen-specific intracellular IFN-γ production was determined by incubating 1 × 10^6 CNS-derived cells in 100 μl of RPMI 1640 supplemented with 10% fetal calf serum, 1 μmol/L S510 peptide, representing the H-2D^b immunodominant JHMV-specific CD8$^+$ T-cell epitope,[9,25] and 1 μg/ml monensin (BD Pharmingen) for 5 hours at 37°C. Peptide was omitted in control samples. Following surface staining, cells were fixed and permeabilized using Cytofix/Cytoperm kit (BD Pharmingen) and stained with a fluorescein isothiocyanate-labeled IFN-γ-specific mAb (clone XMG1.2) as recommended by the supplier (BD Pharmingen).

Virus-Specific Antibody Responses

Virus-specific Ab responses were determined by enzyme-linked immunosorbent assay as described previously.[16,26] Briefly, serial twofold serum dilutions were adsorbed onto JHMV-coated plates overnight at 4°C. Virus-specific Abs were detected with biotinylated anti-mouse IgG, anti-mouse IgM (Jackson ImmunoResearch Laboratories, West Grove, PA), anti-mouse IgG1 or anti-mouse IgG2a (Southern Biotech, Birmingham, AL), streptavidin/peroxidase (Sigma-Aldrich, St. Louis, MO), and 2,2-azino-di(3-ethylbenzthiazoline)sulfonic acid as substrate. Optical densities were determined at 405 nm, and the titers were calculated as reciprocal of the highest dilution that exceeded three standard deviations over mean negative control. Neutralization titers were determined as previously described.[26] Briefly, following heat inactivation (56°C for 30 minutes), serial twofold serum dilutions were incubated with 200 plaque forming units of JHMV in 96-well tissue culture plates for 90 minutes at 37°C. Delayed brain tumor cells (9×10^4 cell per well) were added before incubation at 37°C and 5% of CO_2 for 48 hours.[26]

Histopathology

Brains and spinal cords were removed and either fixed with Clark's solution (75% ethanol and 25% glacial acetic acid) and embedded in paraffin or snap frozen. Serial paraffin or frozen sections from tissues obtained at 5, 7, 10, 14, 16, 18, 21, and 30 days post infected (p.i.) were stained with either hematoxylin and eosin for inflammation or luxol fast blue for demyelination. The relative extent of myelin loss in serial longitudinal sections was determined by demarcating the demyelinated area in luxol fast blue-stained serial sections under a dissecting microscope. Sections were digitized with an Epson 2000 flatbed scanner at 720 dpi, and the percentage area of demyelination was calculated with Optimas image analysis software (Media Cybernectics, Silver Spring, MD) and an LCD drawing slate (Wacom Technology Corp., Vancouver, WA).

Viral antigen in oligodendroglia was co-localized in frozen sections of spinal cord obtained from mice perfused with 3% paraformaldehyde in PBS. Oligodendroglia were identified with anti-adenomatus ployposis coli mAb (Ab-7 Oncogene Research Products, Cambridge, MA) and visualized with peroxidase a NovaRED substrate kit (Vector Laboratories, Burlingame, CA). Viral antigen was detected with mAb J.3.3, specific for the viral nucleocapsid protein,[10,15,16] and visualized with the peroxidase SG substrate kit (Vector Laboratories). Axonal integrity was examined in paraffin spinal cord sections using anti-phosphoneurofilament mAb SMI-31 (Sternberger Monoclonals Inc., Lutherville, MD), followed by peroxidase Vectastain ABC kit (Vector Laboratories) and 3,3'-diaminobenzidine (Sigma-Aldrich). Infiltrating cells were identified by immunoperoxidase staining of acetone-fixed frozen sections with F4/80 (Serotec), anti-CD8 (Ly-2), and anti-CD4 (L3T4) mAbs (BD Pharmingen). Primary mAbs were detected by immunoperoxidase staining with a biotinylated rabbit anti-rat Ab (Vector Laboratories) and a Vectastain ABC kit and aminoethyl carbazol as chromogen (Vector Laboratories).

For identification of viral antigen, paraffin sections were stained with mAb J.3.3 followed by peroxidase Vectastain ABC kit (Vector Laboratories) and 3,3'-diaminobenzidine (Sigma-Aldrich). Viral antigens were quantified manually in serial spinal cord sections and are reported as positive cells/mm². Data were compared using Mann-Whitney U-test. Apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling using the ApopTag detection kit (Serological Corp., Norcross, GA). Briefly, serial frozen sections were fixed in 1% paraformaldehyde in PBS, pH 7.4, and post-fixed in ethanol-acetic acid (2:1). Endogenous peroxidase activity was quenched with 3% H_2O_2 in PBS. Slides were incubated 1 hour at 37°C with terminal deoxynucleotidyl transferase-digoxigenin-deoxynucleoside triphosphate mixture, and an anti-digoxigenin peroxidase conjugated-Ab and Vector NovaRed as substrate (Vector Laboratories) were used to detect end-labeled DNA fragments. Slides were counterstained with hematoxylin. Numbers of apoptotic cells were quantified manually and reported as positive cells/mm².

Results

IFN-γ Signaling Is Required for Effective Viral Clearance

Infected Tg and Wt mice were compared to determine how impaired oligodendroglia responses to INF-γ-influenced JHMV pathogenesis. No differences in clinical disease or mortality were detected comparing Wt and Tg mice up to day 30 p.i. (data not shown). Following infection clinical symptoms were initially apparent in both Tg and Wt mice by day 6 p.i. and peaked at days 10 to 11 p.i. Virus replication was examined to determine whether diminished IFN-γ signaling in oligodendroglia altered overall CNS virus replication or clearance. Consistent with initial JHMV replication in ependymal cells, astrocytes, and microglia,[8] similar levels of infectious virus were detected at days 5 and 7 p.i. (Figure 1) Although infectious virus within the CNS began to decline in both groups by day 10 p.i. (Figure 1), immune-mediated clearance was less efficient in Tg mice. Infectious virus was low, but detectable, in the CNS of ~40% of Wt mice at day 14 p.i. and dropped to undetectable levels in all Wt mice by day 16 p.i. By contrast, all infected Tg mice harbored infectious virus in the CNS at day 14 p.i., and infectious virus was detected in five of six Tg mice at day 16 p.i. Although infectious virus was still recovered from 23% of Tg mice at day 21 p.i., complete CNS clearance was achieved at day 30 p.i. (Figure 1). These data suggest that the decreased ability of oligodendroglia to respond to IFN-γ had a dramatic effect on infectious virus clearance from the CNS, without affecting clinical disease or mortality. The anti-viral Ab response plays no role in controlling acute JHMV infection in Wt mice but is
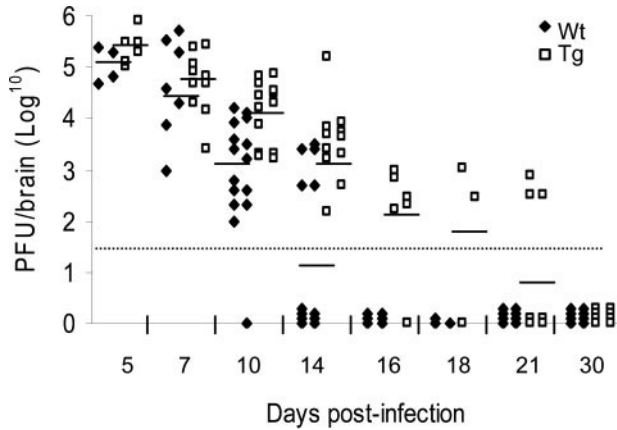
Figure 1. Virus replication within the CNS. Virus replication in the CNS at different time points p.i. determined in brain homogenates prepared from individual Wt and Tg mice by plaque assay. **Bars** indicate the average, and the **noncontinuous** line represents the limit of detection the assay.

Figure 3. Phenotypes within the CNS-infiltrating cells. The phenotype and frequencies of the cells contained within the CD45^{hi} inflammatory cells within the CNS were identified with mAbs specific for T and natural killer cells. Values are calculated as percentages of the specific population by setting CD45^{hi} cells equal to 100%. Bars depict the individual populations within the CNS. Data represent one of five similar experiments.

critical in maintaining CNS persistence.[11,26–28] Anti-viral Ab induction in Tg and Wt mice was compared to ensure that the delay in controlling infectious virus did not correlate with a defect in humoral immunity. No differences in kinetics, magnitude, or isotype of total anti-viral Ab or levels of serum virus neutralizing Ab were detected when comparing Tg and Wt mice (data not shown).

Impaired Oligodendroglia IFN-γ Signaling Does Not Affect CNS Inflammation

CNS recruitment of inflammatory cells was analyzed to determine whether differences in numbers or phenotype correlated with delayed virus clearance in Tg mice. Total numbers of cells recovered from the CNS were similar in both groups, with maximal numbers present at day 10 p.i. (Figure 2). Consistent with the increased cellularity, bone marrow-derived (CD45^{hi}) CNS-infiltrating cells were maximal at day 10 p.i. (Figure 2) and decreased by day
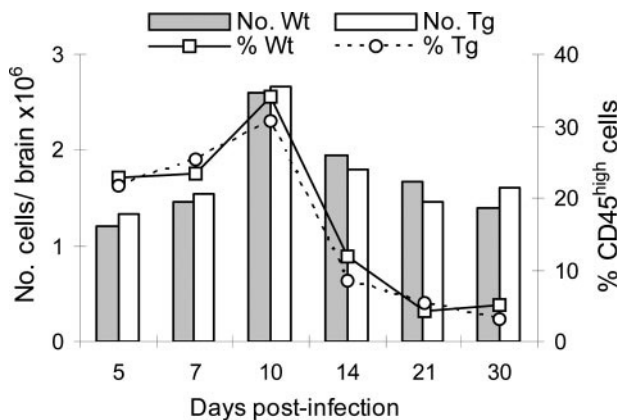

Figure 2. Composition of CNS inflammatory cells in JHMV-infected mice. Flow cytometric analysis shows inflammatory cells derived from dissociated brains isolated via Percoll step gradients. Bone marrow-derived inflammatory and resident CNS cells were distinguished by CD45 expression. Total CNS cells per brain; **bars** represent Wt and Tg mice. Percentage of CNS-infiltrating cells; **lines** represent CD45^{hi} cells for Wt and Tg mice. Data are derived from one representative of five experiments each containing a minimum of three mice per group.
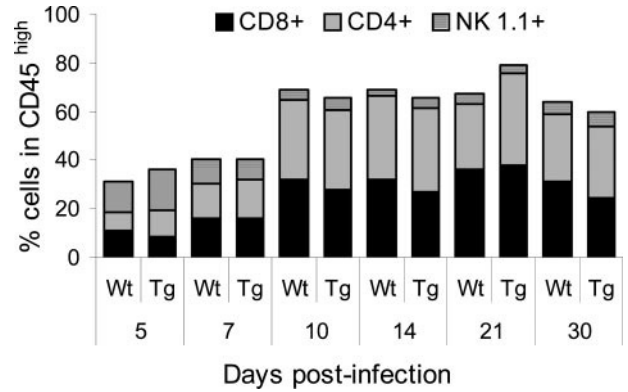
14 p.i. in both groups (Figure 2). Despite the continued presence of infectious virus in the CNS of Tg mice between days 14 and 21 p.i. (Figure 1), no differences in either yields or percentages of CD45^{hi} cells were detected (Figure 2). In addition, CD45^{hi} inflammatory cells remained within the CNS of both groups until day 30 p.i. (Figure 2), consistent with previous data.[9,11,29] Natural killer cells peaked at day 5 p.i. and then declined but remained detectable within the CNS-infiltrating population until day 30 p.i. in both groups (Figure 3). T cells initially represented a small percentage of CNS-infiltrating cells at days 5 and 7 p.i. CD4$^+$ and CD8$^+$ T cells rapidly increased, reaching maximum levels at day 10 p.i. (Figure 3), consistent with a dominant role of CD8$^+$ T cells in clearance of infectious virus.[9–11] Similar populations of CD4$^+$ and CD8$^+$ T cells were retained in the CNS of both Wt and Tg mice (Figure 3). Although an increased percentage of CD4$^+$ T cells in the CNS of infected Tg mice was detected at day 21 p.i., the difference did not reach statistical significance. These data indicate that neither overall inflammatory cell recruitment nor the composition was altered by the inability of oligodendroglia to signal via the IFN-γR.

Virus-Specific CD8$^+$ T Cell Response

Despite similar total CD8$^+$ T cell recruitment, a deficiency in virus-specific CD8$^+$ T cell induction or recruitment could account for ineffective control of viral CNS replication in Tg mice. No differences in the frequency of virus-specific CD8$^+$ T cells by tetramer staining were detected in cervical lymph nodes between days 5 and 21 p.i. in both groups (data not shown). Similar activation and recruitment of the CD8$^+$ T cells from the cervical lymph nodes[30] is consistent with previous data demonstrating equivalent peripheral immune responses comparing Tg and Wt mice.[23] Within the CNS virus-specific CD8$^+$ T cells comprised ~10% of the CD8$^+$ T-cell population at day 5 p.i. and increased to ~40% at day 7 p.i. in both the Tg and Wt mice (Figure 4). Virus-specific CD8$^+$ T cells in the CNS increased further to ~60% of CD8$^+$ T cells at
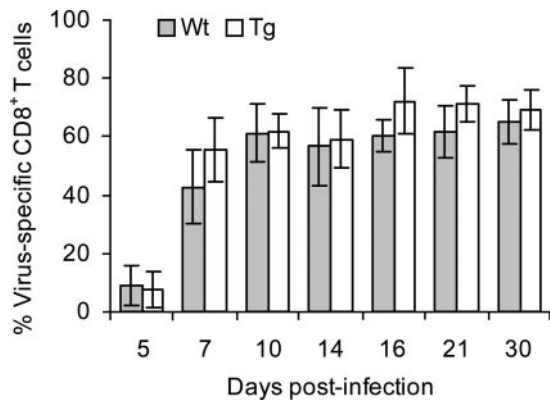
Figure 4. CNS-infiltrating virus-specific CD8^{+} T cells. Percentage of virus-specific S510 tetramer^{+}/CD8^{+} T cells within the CNS-infiltrating CD8^{+} T cell population in Wt and Tg mice ± SD. Values are calculated as a percentage of the specific population by setting CD45^{hi}/CD8^{+}-infiltrating cells equal to 100%. Data represent the mean of five separate experiments containing at least three mice per time point.

day 10 p.i. and were retained at relatively constant levels until day 30 p.i. (Figure 4) even as inflammation declined (Figure 2). Furthermore, no differences in either frequency or total numbers of virus-specific CD8^{+} T cells secreting IFN-γ were detected (Table 1). In addition, no difference in *ex vivo* virus-specific CD8^{+} T cell-mediated cytolysis was detected within the CNS-derived cell populations at 7 and 14 days p.i. (data not shown). Therefore, similar recruitment of virus-specific T cells into the CNS of both Tg and Wt mice suggests that the decreased ability to clear infectious virus was not due to a defect in CD8^{+} T cell priming, expansion, trafficking, or expression of effector function.

Preferential Virus Infection of Oligodendroglia

Infection of mice with a systemic defect in IFN-γ secretion results in delayed virus clearance, increased mortality, and preferential virus infection of oligodendroglia.[16] The less severe clinical disease phenotype of the infected dnIFN-γR1 Tg mice, compared to IFN-γ-deficient mice,[16] despite delayed virus clearance, suggested that restricted IFN-γ signaling by oligodendroglia may alter virus replication and/or tropism. Viral antigen was initially present in ependymal cells and astrocytes with few infected oligodendroglia in both groups (Figure 5, A and B). Viral antigen increased in these cell types and included a minimal number of infected microglia and macrophages at day 7 p.i., consistent with previous analysis of JHMV infection.[8] The number of virus-infected cells

Table 1. Virus-Specific CD8^{+} T Cells Recruited in the CNS

		IFN-γ positive cells	
		%*	Total × 10[4†]
Day 7	Wt	27.2	3.9
	Tg	31.6	4.7
Day 14	Wt	34.0	5.6
	Tg	44.3	6.7

*Percentages of IFN-γ positive cells are based on CD45^{hi}/CD8.
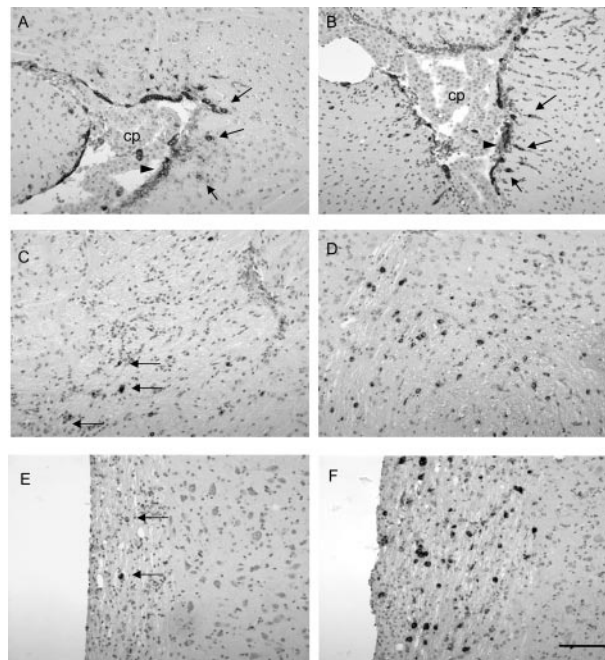†Total number is based on total cellular yield per brain.

Figure 5. Influence of IFN-γ signaling on viral antigen distribution. Virus-infected cells in brains from infected Wt (**A**) and Tg mice (**B**) at day 5 p.i. detected with a mAb specific for the viral nucleocapsid protein are shown. **Arrowheads** indicate antigen-positive ependymal cells, **arrows** indicate antigen-positive parenchymal cells, and **cp** indicates the choroids plexus. Note virus dissemination into the parenchyma. Scattered infected cells with a morphology consistent with oligodendroglia in brain from Wt mice at 10 days p.i. (**C**) and Tg mice (**D**) at day 10 p.i. Shown are virus-infected cells in spinal cord white matter tracks of Wt (**E**) and transgenic mice (**F**) at 18 days p.i. Scale bar = 100 μm.

decreased in Wt mice by day 10 p.i. (Figure 5C), as infectious virus was cleared (Figure 1). Although an increased number of virus-infected cells was detected in brains of Tg mice at day 10 p.i. (Figure 5D), only few infected cells remained in the brain of either the Tg or Wt mice at days 14 and 16 p.i. (data not shown). Consistent with the descending dissemination of JHMV,[8] viral antigen was detected in spinal cords of both groups of mice at day 7 p.i. However, even at this early time point differences in the number and type of virus-infected cells were evident. Virus-infected cells decreased in spinal cord of Wt mice, with only scattered infected oligodendroglia detected by 18 p.i. (Figure 5E). By contrast, the number of infected cells within the white matter of Tg mice remained constant or increased following day 10 p.i. By day 18 p.i. spinal cords of Tg mice contained large numbers of virus-infected cells (Figure 5F). Quantification of virus-infected cells in spinal cords at day 18 p.i. revealed an average of 2.5 ± 2.6 infected cells/mm^2 in Wt mice compared to an average of 28.3 ± 11 infected cells/mm^2 ($P < 0.001$) in Tg mice. The morphology and distribution of infected cells along white matter tracks was compatible with oligodendroglia; indeed, oligodendroglia identity was confirmed by co-expression of viral antigen and antigen-presenting cells (Figure 6G). Nevertheless, only scattered infected cells were detected in the spinal cords of either group at day 30 p.i. These data imply that the absence of IFN-γ signaling delays clearance of virus from oligodendroglia. However, eventual
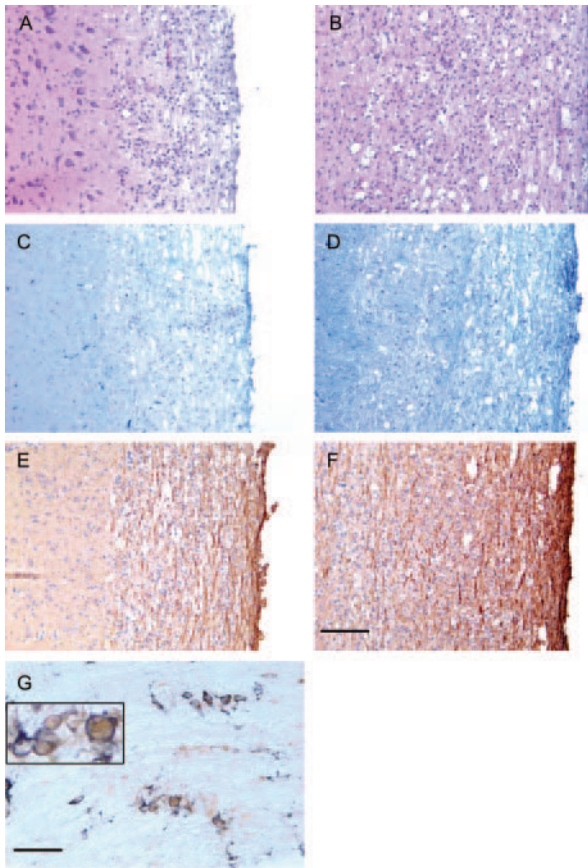
Figure 6. JHMV infection induced CNS inflammation and demyelination. Spinal cords from Wt (**A**, **C**, and **E**) and Tg mice (**B**, **D**, and **F**) 18 days p.i stained with H&E (**A** and **B**) or luxol fast blue (**C** and **D**). Extensive white matter inflammation associated with demyelination in both Wt (**A** and **C**) and Tg mice (**B** and **D**). Axons within the demyelinated lesions in Wt (**E**) and Tg (**F**) mice were visualized with anti-phosphoneurofilament antibody. Scale bar = 100 μm. Co-localization of viral antigen in oligodendrocytes (**G**) is shown, using virus-specific mAb J.3.3 (red) and anti-adenomatus ployposis coli (gray) at 18 days p.i. Scale bar = 50 μm. The **inset** shows higher magnification of double-positive cells in spinal cord of Tg mice.

control of infected oligodendroglia suggested that the loss of the infected cells may be due to cell death and/or limited spread via one or more Ab-dependent mechanisms.[11,27,28]

Increased Oligodendroglia Infection Does Not Alter Demyelination

Inflammatory cells localized to both the perivascular spaces and brain parenchyma in Wt and Tg mice, with no evident differences in the amount of cellular infiltration (data not shown). Inflammatory cells were predominantly located within the spinal cord white matter (Figure 6, A and B) associated with demyelinating lesions at day 18 p.i. (Figure 6, C and D). Demyelination in both groups was initially evident by day 10 p.i.; however, despite the increased number of virus-infected oligodendroglia (Figure 5), no differences in severity of myelin loss were detected (Figure 6, C and D). The similar extent of demyelination in infected Wt and Tg mice was confirmed by determining the relative percentage of myelin loss. In Wt

mice, the percentage of myelin loss determined in serial longitudinal spinal cord sections was 8.3% ± 3.7. Similarly, 10.9% ± 5.7 was detected in the spinal cords of the Tg mice. Consistent with the similar pattern of myelin loss, no evidence for axonal loss was apparent within the areas of demyelination in either group (Figure 6, C and D). Although primary demyelination with axonal sparring induced by JHMV infection is immune mediated,[10,22] it has recently been associated with early axonal degeneration.[31] However, no evidence of differential axonal loss was detected in the infected Tg mice (Figure 6, E and F), despite the increased number of infected oligodendroglia (Figure 5).

Apoptosis participates in the pathogenesis of both CNS infections and autoimmune diseases and partially regulates CNS inflammation via death of inflammatory cells.[18,32] Death of CNS-resident cells, especially oligodendroglia, has also been suggested to influence demyelination.[33–35] Antigen recognition by CD8+ T cells induces apoptosis of JMHV-infected cells,[36] and apoptotic oligodendroglia have been noted during CNS infection by a related MHV strain.[33] However, the majority of CNS cells undergoing apoptosis during JHMV infection are lymphocytes.[12,15] Apoptotic cells in infected Tg and Wt mice were compared to determine whether the increase in virus-infected oligodendroglia correlated with an increase in apoptotic cells. Surprisingly, the limited number of apoptotic cells, mainly scattered along white matter tracks, was similar in Wt (Figure 7A) and Tg mice (Figure 7B). Furthermore, the morphology and distribution of apoptotic cells was consistent with infiltrating mononuclear cells rather than glial cells, similar to previous data.[12,15] Lastly, the frequency of apoptotic cells confirmed that increased oligodendroglia infection in Tg mice did not result in a concomitant increase in cell death. An average of 11.0 ± 3.5 apoptotic cells/mm^2 was found in the spinal cords of Wt mice compared to an average of 14.0 ± 3.9/mm^2 in the infected Tg mice. Macrophages within the CNS-infiltrating CD45^{hi} cell populations were quantified to determine whether similar myelin loss was related to diminished recruitment of phagocytes.[22] The relative percentage of CNS-infiltrating macrophages was initially high at days 7 and 10 p.i., decreased following virus clearance, and remained relatively stable at low levels until day 30 p.i. in both groups (Figure 7C). In addition, no distinct distribution of F4/80$^+$ cells was detected by immunohistochemistry (data not shown). These data suggest that enhanced infection of oligodendroglia is not associated with either increased glial cell apoptosis or altered macrophage infiltration. JHMV infection of oligodendroglia impaired in IFN-γ responsiveness does not affect cellular survival and thus does not result in exacerbated demyelination.

Discussion

CNS infections require the host to balance immune responses capable of controlling the pathogen while minimizing potential damage to resident cells that have little or no capacity for regeneration.[1,2] Thus, it is to the host's
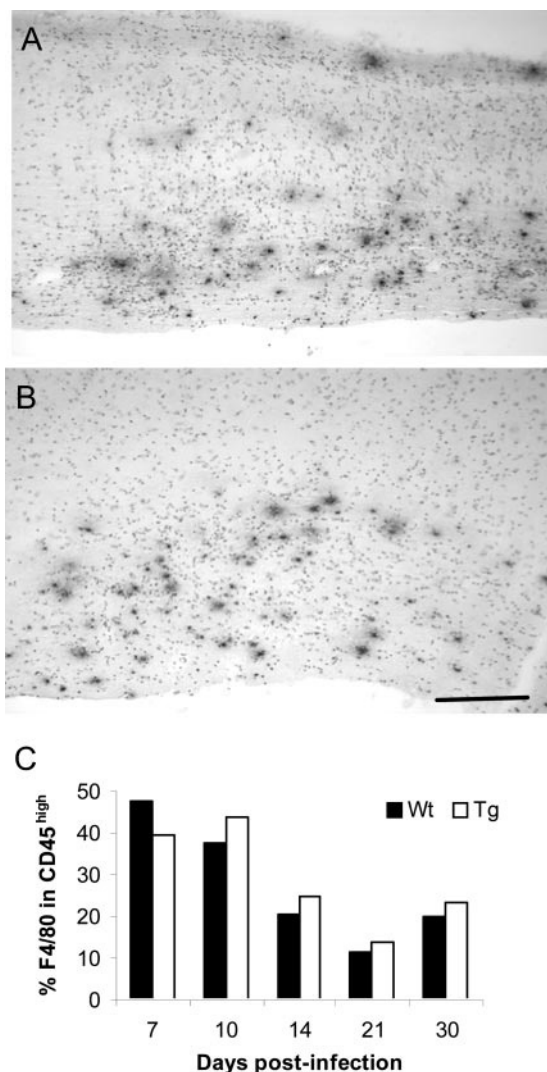
Figure 7. Contribution of apoptosis and infiltrating macrophages to CNS demyelination. Apoptotic cells in the spinal cords of Wt (**A**) and Tg (**B**) mice at 14 days p.i. detected by TUNEL. Scale bar = 200 μm. Percentage of CD45^{hi} F4/80$^+$ CNS-infiltrating macrophages (**C**) in JHMV-infected Wt (full bar) and Tg mice (empty bar). Values calculated as percentage F4/80$^+$ by setting CD45^{hi} bone marrow-derived inflammatory cells equal to 100%. Data represent one of five similar experiments.

advantage to control viral infection of neurons and glia via nonlytic mechanisms such as cytokines or neutralizing Abs.[2] The data in this report demonstrate that IFN-γ signaling controls JHMV replication in oligodendroglia during acute infection. Two additional novel findings are illustrated. First, increased infection of oligodendroglia defective in IFN-γ signaling did not correlate with increased cell death or myelin loss. Second, although IFN-γ is important to control virus replication in oligodendroglia during acute infection, additional mechanisms participate in the elimination of virus from the same cells in a temporally delayed fashion.

CD8$^+$ T cells are the primary effectors controlling JHMV replication within the CNS.[7,10,37] Emphasizing the unique biology of the CNS, distinct effector mechanisms control JHMV replication within specific cell types. Perforin-mediated cytolysis, but not Fas/Fas-L-dependent

signaling, is crucial for control of JHMV replication in astrocytes, macrophages, and microglia.[10,15,38] By contrast, perforin-mediated cytolysis does not appear to contribute to JHMV control in oligodendroglia *in vivo*,[10,15] although it has been demonstrated *in vitro*.[39,40] Similar to other CNS-resident cells, oligodendroglia in the naïve CNS do not express major histocompatibility complex molecules. However, major histocompatibility complex class I up-regulation on oligodendroglia during JHMV infection[41] supports their potential to act as targets. One hurdle for the inability to demonstrate perforin-mediated oligodendroglial death *in vivo* may reside in their unknown capacity to present antigen or resist T cell effector function. Analysis of JHMV infection in IFN-γ-deficient mice and immunocompromised recipients of virus-specific IFN-γ-deficient T cells suggested that IFN-γ controls JHMV replication in oligodendroglia, independent of cytolysis.[10,16] Indeed, similar to their participation in the control of other CNS viral infections,[2,42] IFN-γ-secreting T cells are recruited into the CNS during JHMV infection, and IFN-γ mRNA peaks coincident with viral clearance.[9,13,37,43] Therefore, a novel Tg mouse expressing a dnIFN-γR1 on oligodendroglia[23] was used to directly examine IFN-γ-dependent signaling mechanisms operating at the single-cell-type level within the CNS during JHMV infection.

The diminished ability of oligodendroglia to respond to IFN-γ signaling did not affect acute CNS virus replication due to the paucity of oligodendroglia infected during the early acute phase.[8] No defects in viral clearance from other cell types within the CNS supported the cell-specific effect of transgene expression.[23] However, impaired JHMV clearance from oligodendroglia became evident from increased antigen-positive cells restricted to oligodendroglia as infection progressed in Tg mice. JHMV-infected dnIFN-γR1 Tg mice showed no differences in the number, activation status, or phenotype of CNS-infiltrating cells compared to Wt mice, supporting fully functional peripheral activation of immune effectors in the dnIFN-γR1 Tg mice[23] and contrasting with the increased CD8$^+$ T cells infiltrating the CNS of JHMV-infected IFN-γ-deficient mice.[16] Similarly, CD8$^+$ T-cell IFN-γ secretion and cytolysis were similar in Tg mice compared to Wt mice during acute infection. Consistent with previous data,[9,27,44] cytolysis was down-regulated by CNS-retained CD8$^+$ T cells, and the capacity for viral antigen-induced IFN-γ secretion was retained in both groups of mice. Furthermore, no differences in CNS distribution of CD4$^+$ or CD8$^+$ T cells were detected when comparing infected Tg and Wt mice (data not shown). Therefore, the selective decrease in IFN-γ signaling demonstrates the critical impact of this cytokine on virus clearance from oligodendroglia during acute infection. Despite the delay, ultimate clearance of viral antigen from the oligodendroglia suggests that limited residual IFN-γ signaling[23] may slowly control virus replication. Alternatively, delayed virus clearance coincides with peak anti-viral Ab responses[11,26] and suggests that Ab may participate in the eventual control of JHMV replication in oligodendroglia. This concept is consistent with recent data suggesting an increased frequency of infected oligodendroglia in the

absence of humoral immunity[11,27] and expression of viral proteins on the surface of infected oligodendroglia.[45]

Myelin loss is a prominent pathological hallmark of both multiple sclerosis and JC virus infection in humans and is associated with experimental CNS viral infections, including JHMV, Theiler's murine encephalomyelitis virus, and Semliki Forest virus.[8,13,46–48] Cytolytic viral infection, T-cell-mediated cytotoxicity directed against oligodendroglia, a requirement for macrophages, and autoantibody specific for virus proteins expressed on the cell surface[18,45,46,49–52] have all been suggested to account for virus-induced myelin loss. It is intriguing that the absence of IFN-γ signaling leads to increased numbers of infected oligodendroglia without significantly affecting either the extent or distribution of demyelination. Although myelin loss during JHMV infection was initially attributed to direct cytolytic viral infection,[20] it has since been associated with CNS inflammatory responses.[10,13,21,53] The latter is exemplified by minimal or no demyelination in immunodeficient mice infected with JHMV despite extensive viral infection of oligodendroglia.[10,13] A contribution of oligodendroglial infection, dysfunction, and/or death to the process of demyelination in the presence of immune effector functions can nevertheless not be excluded. For example, oligodendroglial apoptosis is implicated in demyelination associated with JC virus infection[17] and multiple sclerosis.[54] Consistent with these data, Fas is expressed by oligodendroglia within multiple sclerosis lesions[34]; furthermore, the absence of Fas/FasL interactions and apoptosis are implicated in mediating resistance of *lpr*-, *gld*-, and caspase 11-deficient mice to experimental autoimmune encephalomyelitis-induced demyelination.[35,55] Although MHV infection induces apoptosis *in vitro*,[56] analysis of Fas/FasL interactions during JHMV infection failed to detect any role in pathogenesis,[38] and apoptosis within the CNS appeared to be restricted to the inflammatory cell population.[12,15] Similarly, although perforin-mediated cytolysis has been implicated in virus-induced immunopathology,[3] the absence of perforin does not affect JHMV-induced demyelination.[15] Likewise, in other models of virus-induced demyelination, infection of oligodendroglia does not correlate with the severity of demyelination or apoptosis.[19,57] Macrophages are also important components of CNS infiltration during coronavirus infection and are associated with myelin loss.[18,21,22] However, the number and distribution of CNS-infiltrating macrophages, as well as other components of the inflammatory response, were similar in JHMV-infected Tg and Wt mice, consistent with similar levels of demyelination. The absence of alterations in inflammatory components despite enhanced numbers of infected oligodendrocytes further suggests that oligodendrocytes tolerate infection by a cytoplasmically replicating RNA virus without overt evidence for release of chemokines or factors that signal destructive effector mechanisms. This notion is consistent with the prominent secretion of chemokines by astrocytes,[58] as well as limited matrix metalloproteinase production by resident cells[59] in this model. It remains to be investigated whether obvious discrepancies in linking oligodendroglia infection with demyelination during human JC virus infection and the murine JHMV model reside in distinct nature of viruses and their replication strategies or a necessity for IFN-γ signaling in oligodendrocytes to enhance the demyelinating process.

In conclusion, our data are consistent with a model in which IFN-γ directly participates in the control of JHMV replication in oligodendroglia during acute infection. Although demyelination following JHMV infection requires infection of oligodendroglia[24] and is immune-mediated,[10,21,22,53] the results demonstrate a clear absence of correlation between the number of infected cells and the extent of myelin loss. This suggests that infection of this critical CNS cell type, although an indispensable requirement,[24,44] does not necessarily directly contribute to myelin loss. Finally, the delayed but complete clearance of virus from oligodendroglia supports a role for humoral control of virus replication in this cell type independent of IFN-γ signaling mechanisms.

Acknowledgments

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