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Regular Article

# Transplantation of glial-committed progenitor cells into a viral model of multiple sclerosis induces remyelination in the absence of an attenuated inflammatory response

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## Abstract

Transplantation of remyelination-competent cells represents a promising strategy for the treatment of demyelinating diseases. As the environment dictates the success or failure of remyelination, it is critical to understand the role that the immune system plays in transplant-mediated remyelination. In this study, we evaluated the severity of neuroinflammation following transplantation of glial-committed progenitor cells into the spinal cords of mice chronically infected with mouse hepatitis virus (MHV), a model in which T cells and macrophages are critical in amplifying the severity of demyelination. Transplantation was performed following viral persistence in which inflammation and demyelination are established and clinical disease is evident. Mice were sacrificed 10 and 21 days following progenitor cell transplantation and the effect on neuroinflammation evaluated. Treatment did not alter accumulation of T cells or macrophages within the CNS as compared to control mice. Moreover, progenitor cell implantation did not affect local cytokine/chemokine gene expression in the CNS. Finally, remyelination associated with transplantation did not result in an imbalance of T<sub>H</sub>1-associated cytokine production by virus-specific T cells. These studies demonstrate that progenitor cell-mediated remyelination is not the result of modulating the composition of the cellular infiltrate nor cytokine expression by virus-specific T cells and suggest that remyelination may not depend on amelioration of the inflammatory response or alteration of cytokine secretion by virus-specific T cells.

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## Introduction

Multiple sclerosis (MS) is a chronic demyelinating disease characterized by inflammation in the central nervous system (CNS) leading to demyelination, axonal loss, and disability (Sospedra and Martin, 2005). Many treatments are geared

towards reducing neuroinflammation with the ultimate goal of reducing progression of disease (Rizvi and Agius, 2004). However, these treatments often do not address the neurodegenerative phase of disease. Remyelination can occur spontaneously in the adult human brain as evidenced by shadow plaques, in which large regions of white matter undergo remyelination with characteristically thin myelin sheaths (Halfpenny et al., 2002). However, remyelination does not occur uniformly within a lesion or across many lesions. Two major processes have been proposed to explain the failure of remyelination in MS, notably: (i) difficulties in recruiting oligodendrocyte precursor cells (OPCs) to areas of active demyelination and (ii) inhibition in differentiation of OPCs into oligodendrocytes capable of remyelination (Franklin, 2002). In

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each of these two processes signaling by growth factors, cytokines/chemokines, and extracellular matrix molecules are thought to contribute to an environment that is not conducive for promoting remyelination.

Studies in animal models have proven invaluable for identifying new methods for inducing remyelination in animals with established demyelination. For example, transplantation of stem cells into animal models of acute demyelination results in remyelination (Cao et al., 2002). Transplant of rodent embryonic stem cells into myelin-deficient shiverer mice results in cellular migration in the spinal cord, differentiation into oligodendrocytes and astrocytes, and myelination of axons (Brustle et al., 1999). Similarly, transplant of human embryonic stem cell-derived oligodendrocyte progenitor cells into myelin-deficient shiverer mice results in oligodendrocyte differentiation and remyelination (Nistor et al., 2005). Other models of demyelination have also reported reduced demyelination following transplantation of stem cells. Injection of adult neuronal precursors into mice with experimental autoimmune encephalomyelitis (EAE) resulted in recovery from disease and a significant decrease in the level of demyelination (Pluchino et al., 2003). Similarly, transplantation of stromal bone marrow cells into demyelinated rat spinal cord resulted in remyelination (Akiyama et al., 2002).

This study utilized mouse hepatitis virus (MHV, a murine coronavirus) as a model for MS to define the impact of transplantation on the inflammatory response associated with disease in the CNS. Infection of susceptible mice with MHV results in an acute encephalomyelitis followed by an immune-mediated demyelinating disease. Similar to MS, demyelination in MHV-infected mice is associated with impaired motor skills that often are associated with immune cell infiltration in white matter tracts (Lane and Buchmeier, 1997). Due to the similarities in both clinical and histologic disease between MS and MHV-induced demyelination, the MHV model is considered a relevant model for studying the underlying immunopathological mechanisms contributing to ongoing myelin destruction as well as clinical disease. Recently, a study by Totoiu et al. (2004) used the MHV model of chronic demyelination to determine if cellular transplantation modulated disease severity. Transplantation of glial-committed progenitor cells into MHV-infected mice with on-going demyelination and established clinical disease resulted in remyelination and improved locomotion. These studies highlight the efficacy of cellular transplantation therapy in promoting remyelination in a model in which there is extensive cellular infiltration and demyelination and provide us with the opportunity to investigate the role that inflammation plays in this regenerative process. Here, we report that cellular transplantation resulting in remyelination does not alter inflammation in the CNS nor modulate the ongoing immune response to MHV. These studies imply that modulation of the inflammatory environment is not required for remyelination, a finding that has profound implications for the development of cellular replacement strategies to treat demyelinating diseases.

## Materials and methods

### *Mice and infection*

Age-matched (5–7 weeks) C57BL/6 male mice (H-2<sup>b</sup> background) were used for all studies (National Cancer Institute, Bethesda, MD). Following anesthetization with ketamine (Phoenix, St. Joseph, MO), mice were injected intracranially (i.c.) with 500 PFU of MHV strain J2.2-V.1 suspended in 30  $\mu$ l of sterile Hank's balanced salt solution (HBSS). Control animals (sham) were injected with 30  $\mu$ l HBSS alone. Animals were euthanized at 22 and 33 days post-infection (p.i.) (corresponding to 10 or 21 days after transplantation) at which point brains, spinal cords, and spleens were removed for analysis.

### *Transplantation*

Cells for transplant were cultured from the striata of postnatal day 1 C57BL/6 mice as previously described and cultured for 6 days before transplantation (Totoiu et al., 2004). The striata region of the brain was chosen because it contains periventricular areas which are rich in neurogenic stem cells. Twelve days after i.c. infection with MHV,  $2.4 \times 10^5$  cells were injected in 2  $\mu$ l of DMEM (Gibco-Invitrogen) at T8 of the spinal cord (Totoiu et al., 2004). Control animals received i.c. infection with MHV and injection of DMEM alone at T8 of the spinal cord (Totoiu et al., 2004).

### *Histology*

Animals were euthanized by inhalation of halothane (Sigma, St. Louis, MO) 10 or 21 days following transplantation and fixed by cardiac perfusion with 4% paraformaldehyde (Fisher Scientific) or 4% glutaraldehyde (Fisher Scientific, Pittsburgh, PA) in 0.1 M PBS, pH 7.4. The length of spinal cord extending 6 mm cranial and 6 mm caudal to the site of implantation was extracted and processed for resin (glutaraldehyde fixed samples) or cryostat sectioning (paraformaldehyde fixed samples) as previously described (Totoiu et al., 2004). Briefly, 2-mm blocks were cut from 6 mm spinal cord sections both cranial and caudal to the site of transplantation, and even blocks were processed for immunohistochemistry, and odd numbered blocks were processed for resin embedding and toluidine blue staining for a total of 3 blocks cut on either side of the transplantation site. For resin sectioning, blocks were exposed to 1% OsO<sub>4</sub> (Electron Microscopy Sciences, Fort Washington, PA), dehydrated in ascending alcohols and embedded in Spurr resin (Electron Microscopy Sciences) according to standard protocols. Transverse 1-mm sections were cut from the cranial face of each block, stained with alkaline toluidine blue, cover slipped, and examined by light microscopy on an Olympus A $\times$ -80 microscope using OLYMPUS MicroSuite B3SV software. The state of myelination was determined by assessing the thickness of the myelin sheath in relation to the axon diameter (Guy et al., 1989; Hildebrand and Hahn, 1978). Demyelinated, remyelinated, and normally myelinated axons were counted

within  $4 \times 3750 \mu\text{m}^2$  areas, totaling  $15,000 \mu\text{m}^2$ , on each tissue section using the  $100\times$  objective with a  $2\times$  optical zoom representing approximately 10% of the total area of remyelination within transplanted animals, which was determined by measuring the total area of remyelination sections from each block in each animal using the  $40\times$  objective and averaging areas from all animals in each group. These quantitative assessments were conducted throughout the region extending 6 mm cranial and 6 mm caudal to the transplantation site. The number of demyelinated axons, remyelinated axons, the total number of axons, and the percentage of remyelinated axons were determined for each of the four regions on each tissue block, averaged, then averaged across animals within each group for each tissue block as previously described (Totoiu et al., 2004). Statistical analyses were conducted using SPSS software (Chicago, IL). For cryostat sectioning, blocks were cryoprotected in 30% sucrose solution in PBS, embedded in OCT (Fisher Scientific) and frozen sectioned in the transverse plane at  $20 \mu\text{m}$  on a JUNG CM3000 Leica cryostat for anti-BrdU staining as previously described (Totoiu et al., 2004).

#### *Mononuclear cell preparation and flow cytometry*

Cells were obtained from pooled brains and spinal cords of mice based on a described protocol (Pewe et al., 1998). A single cell suspension was obtained by grinding the tissue in sterile tissue culture plates on ice in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum. Cell suspensions were transferred to 15-ml conical tubes and Percoll (Amersham, Uppsala, Sweden) was added for a final concentration of 30%. One ml of 70% Percoll was underlaid, and the cells were spun at  $1300 \times g$  for 30 min at  $4^\circ\text{C}$ . Cells were removed from the interface, washed, and used for flow cytometry. FC receptors were blocked by incubating cells with a 1:200 dilution of rat anti-mouse CD16/32 (Fc $\gamma$  III/II receptor) (Pharmingen, San Diego, CA) for 15 min at  $4^\circ\text{C}$  then subjected to staining. Activated macrophages/microglia were identified by dual staining with FITC-conjugated rat anti-mouse F4/80 (C1:A3-1; Serotec, Oxford, England) and PE-conjugated CD45 (Pharmingen). The presence of CD8<sup>+</sup> T cells recognizing the immunodominant CD8 epitope located within the surface (S) glycoprotein within residues 510–518 (S510–518) was determined using PE-conjugated D<sup>b</sup>/S510–518 major histocompatibility class I tetramer (Trifilo et al., 2003). Cell surface expression of CD8 was determined using APC-conjugated rat anti-mouse CD8 (Pharmingen). Cells were incubated with antibodies for 30 min at  $4^\circ\text{C}$ , washed, and analyzed on a FACScaliber (Becton Dickinson, Mountain View, CA). The presence of virus-specific CD4<sup>+</sup> T cells was determined by intracellular cytokine staining for IFN- $\gamma$  (PE-conjugated anti-IFN- $\gamma$ , XMG1.2, Pharmingen) following 5-h stimulation with the immunodominant CD4 epitope present within the matrix (M) glycoprotein spanning residues 133–147 (M133–147) in medium containing GolgiStop using the Cytofix/Cytoperm kit (Pharmingen) according to the manufacturer's protocol (Glass et al., 2004). Cells were washed and blocked with PBS containing 10% FBS and a 1:200 dilution

of CD16/32. Cells were then stained with PE-conjugated CD4 for 30 min at  $4^\circ\text{C}$ , followed by fixing and staining for intracellular IFN- $\gamma$  using for 30 min (Glass et al., 2004). Cells were washed and analyzed on a FACScaliber. Data are presented as the percent positive within the gated population and then numerated based on the number of total live cells per tissue. Total numbers of cells were calculated by multiplying the percentage of positive live cells by the total number of isolated cells. The frequency of virus-specific cells was determined by dividing the total number of antigen-specific cells by the total number of gated cells.

#### *Ribonuclease protection assay (RPA)*

Total RNA was extracted from brains and spinal cords of MHV-infected mice 33 days p.i., 21 days post-transplant. Cytokine, chemokine, and chemokine receptor transcripts were analyzed using custom probesets (Pharmingen). RPA analysis was performed with  $12 \mu\text{g}$  of total RNA using a previously described protocol (Lane et al., 1998, 2000). Probes for L32 and GAPDH were included to verify consistency in RNA loading and assay performance. For quantification of signal intensity, autoradiographs were scanned, and individual transcript bands were normalized as the ratio of band intensity to L32 (Lane et al., 1998, 2000; Liu et al., 2000). Analysis was performed using NIH Image 1.61 software.

#### *ELISA*

Splenocytes were obtained from MHV-infected mice 21 days post-transplant and stimulated with the MHV epitopes specific for either CD4<sup>+</sup> T cells (M133–147) or CD8 epitopes (S510–518 and S598–605) (Held et al., 2004). As control, an ovalbumin (OVA) peptide spanning residues 323–339 (OVA323–339) was used. After 48 h, supernatants were collected, and production of IFN- $\gamma$  and IL-10 was determined using Quantikine M Mouse IFN- $\gamma$  Immunoassay kit (R&D Systems, Minneapolis, MN) and mouse IL-10 DuoSet (R&D Systems). Samples were measured in duplicate and cytokine concentrations presented as picograms per milliliters.

#### *Statistics*

Statistical differences between groups of mice were determined by Student's *t* test, and values of  $P \leq 0.05$  were considered significant.

## **Results**

### *Transplantation of glial-committed progenitor cells results in remyelination*

Glial-committed progenitor cells were dissected from the striatal region of the brains of day 1 postnatal C57BL/6 mice and cultured for 6 days before use in transplant, resulting in the growth of numerous neurospheres containing progenitor cells



(Figs. 1A, B). These cells were characterized by immunostaining for NG2 (data not shown), confirming that the majority of cells are oligodendrocyte progenitor cells as previously reported (Totoiu et al., 2004). Differentiation of these cells in vitro resulted in approximately 60–70% oligodendrocytes, 20–30%

astrocytes, and 5% other cell types in agreement with previous studies (Totoiu et al., 2004). Our results agree with previous findings that transplantation of glial-committed progenitor cells to mice chronically infected with MHV (day 12 p.i.) resulted in substantial remyelination 21 days post-transplant as evident by

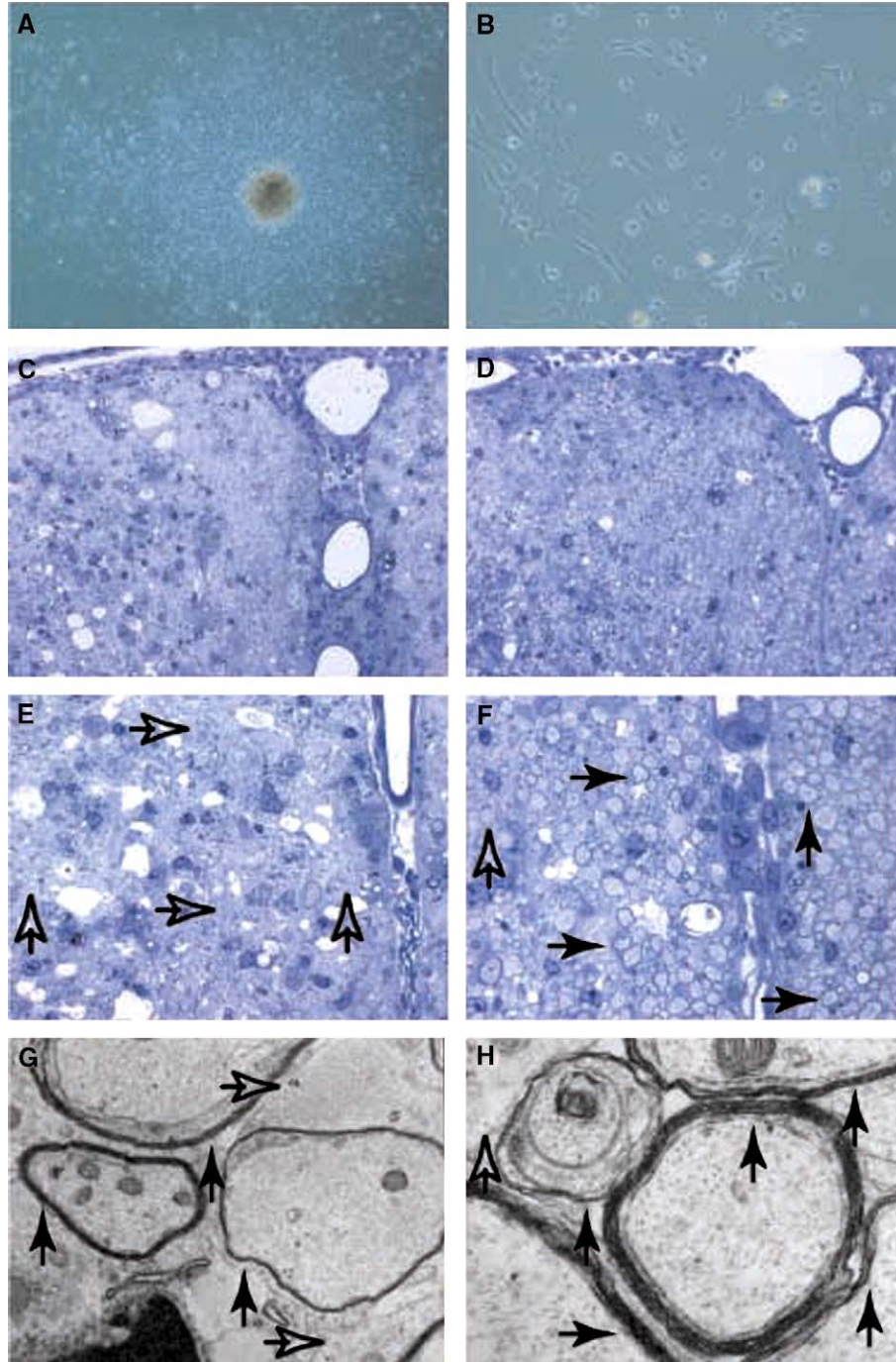


Fig. 1. Transplantation of glial-committed progenitor cells results in remyelination. Glial-committed progenitor cell cluster was cultured for 6 days in non-adherent growth factor containing media before transplant into animals. Shown are representative phase contrast photographs of cells growing in neurospheres (A) and cellular morphology (B) consistent with oligodendrocyte progenitor cells (Totoiu et al., 2004). Spinal cords were removed 21 days after transplant, and myelination was assessed by toluidine blue staining of transverse sections of mice receiving either DMEM (C and E), or cells (D and F). Shown are representative pictures of the ventral column and demyelinated axons are indicated by open arrows and remyelinated axons by closed arrows. Remyelination was further evaluated by electron microscopy in mice receiving glial-committed progenitor cells, and demyelinated axons are indicated by open arrows and remyelinated axons by closed arrows (G, H). 40 $\times$  magnification for panel A, 200 $\times$  magnification for panels B–D, 1000 $\times$  magnification for panels E, F, 39,000 $\times$  magnification for panels G, H.

toluidine blue staining (Figs. 1C–F) and electron microscopy (Figs. 1G, H).

### Infiltration of mononuclear cells into the CNS

Given the importance of the inflammatory response in initiating and maintaining disease in MHV-infected mice (Pewe and Perlman, 2002; Pewe et al., 2002; Wu et al., 2000), we sought to determine if one mechanism by which transplanted progenitor cells promoted remyelination was through modifying the severity of inflammation. To address this question, we first evaluated leukocyte infiltration into the CNS by flow cytometry at 10 days post-transplantation of glial-committed progenitor cells or DMEM as a control (22 days p.i.) (Fig. 2). The overall frequency of infiltrating of CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, and macrophages (F4/80<sup>+</sup> CD45<sup>high</sup>) was not altered in mice receiving progenitor cells as compared to control mice (Fig. 2J). Infiltration of virus-specific T cells was also investigated. The frequency of CD4<sup>+</sup>

T cells specific for the immunodominant epitope present within the matrix (M) protein at residues 133–147 (M133–147) was assessed by intracellular cytokine staining for IFN- $\gamma$  after stimulation with the M33–147 peptide (Xue et al., 1995). The frequency of M133–147-specific-CD4<sup>+</sup> T cells was not modulated following transplant of glial-committed progenitor cells as compared to control mice (Fig. 2K). Similarly, infiltration of virus-specific CD8<sup>+</sup> T cells was determined using MHC tetramers containing the immunodominant CD8 epitope present within the surface (S) glycoprotein at residues 510–518 (S510–518) (Castro and Perlman, 1995). The results of this analysis demonstrated that transplantation of cells did not alter the frequency of S510–518-specific CD8<sup>+</sup> T cells in the CNS when compared to control mice (Fig. 2L).

To confirm the lack of immunomodulation following stem cell transplant, we also looked at a later time point, 21 days following transplant (33 days p.i.). Analysis of inflammatory infiltrates into the CNS at 21 days post-transplant yielded similar results to that seen at 10 days post-transplant (Fig. 3).

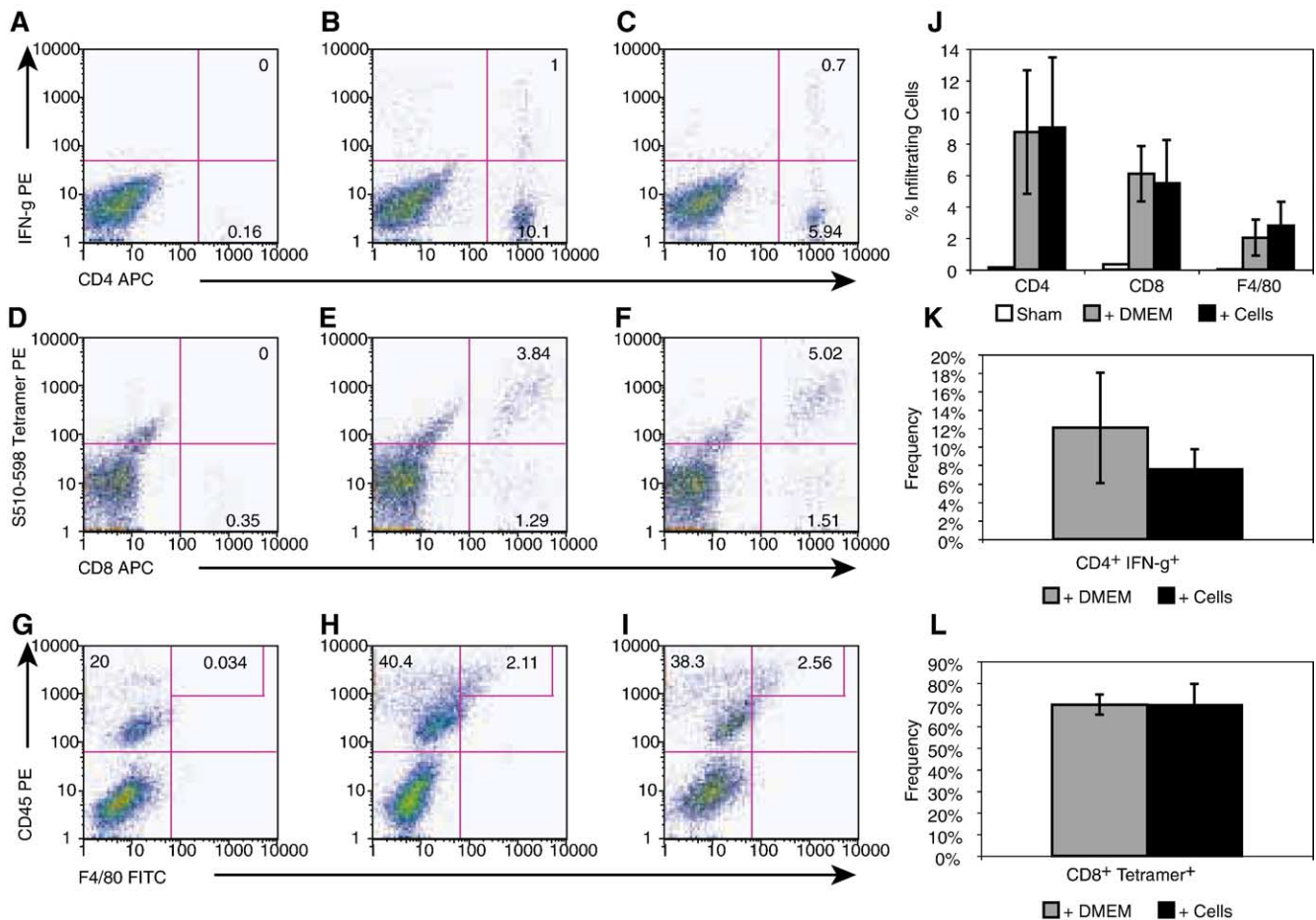


Fig. 2. Flow analysis of leukocyte infiltration into the CNS 10 days post-transplant shows that there is no modulation of inflammatory populations within the CNS following transplant. Leukocytes were isolated from CNS of sham and infected mice that received transplant; shown are representative dot plots of sham-infected mice (A, D, and G), mice receiving DMEM (B, E, and H), and mice receiving cells (C, F, and I). Intracellular cytokine staining for IFN- $\gamma$  was performed to determine the frequency of M133–147-specific CD4<sup>+</sup> T cells. Shown are representative FACS plots for sham mice (A), mice receiving DMEM (B), and mice receiving cells (C). The average frequency of virus-specific CD4<sup>+</sup> T cells is similar between mice receiving DMEM and mice receiving cells (K). Tetramer staining for S510–518 was used to identify virus-sp CD8<sup>+</sup> T cells in sham mice (D), mice receiving DMEM (E) and cells (F). The average frequency of virus-specific CD8<sup>+</sup> T cells is similar between mice as F4/80<sup>+</sup> CD45<sup>high</sup> cells. Shown are representative dot plots from sham mice (G), mice receiving DMEM (H) and cells (I). Analysis of percent of CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, and F4/80<sup>+</sup> CD45<sup>high</sup> cells in the CNS reveals that there are no differences in infiltration of these cell types following transplant of cells (J).

Indeed, the percentage of infiltrating CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, and CD45<sup>high</sup> F4/80<sup>+</sup> macrophages were equivalent in mice receiving cells or DMEM (Fig. 3A). Further, the frequency of virus-specific CD4<sup>+</sup> T cells, as assessed by intracellular cytokine staining for IFN- $\gamma$  following stimulation with peptide M133–147, was similar in mice receiving cells or control DMEM (Fig. 3B). In addition, the frequency of virus-specific CD8<sup>+</sup> T cells, assessed by staining with the S510–518 tetramer, was identical in mice receiving cells or DMEM (Fig. 3C).

#### Expression of cytokines, chemokines, and chemokine receptors

To further characterize the impact of transplantation of glial-committed progenitor cells on inflammation, we evaluated the expression of mRNA transcripts for chemokines, chemokine receptors, and cytokines within the CNS of experimental groups of mice. Total RNA was isolated from spinal cords and

brains of experimental groups of mice at 21 days post-transplant (33 days p.i.) and expression of inflammatory factors and receptors were analyzed by RNase protection assay (RPA) (Fig. 4). Numerous chemokines are expressed in the spinal cord at 33 days p.i., with CCL5, CCL2, CCL3, CCL4, and CXCL10 being readily detected in the spinal cord while only CCL5 and CXCL10 were detected in the brain. Transplantation of glial-committed progenitor cells did not alter chemokines expressed in either the spinal cord (Fig. 4B) or the brain (C). Similar levels of mRNA transcripts for chemokine receptors CXCR4, CCR5, and CCR2 were detected within both the spinal cord (E) and brain (F) of mice receiving cells or DMEM. Additionally, expression of cytokines within the CNS was not modulated following cell transplant (Figs. 4H, I) with low level expression of both TNF- $\alpha$  and IL-1 $\alpha$  observed in both groups of experimental animals.

#### *T<sub>H</sub>1/T<sub>H</sub>2 profile is unaffected in transplanted mice*

Lastly, we sought to investigate any effect transplantation of glial-committed progenitor cells to MHV-infected mice may have on T<sub>H</sub>1/T<sub>H</sub>2 response to viral antigens and cytokine production. Spleens were removed from mice 21 days post-transplant (33 days p.i.) and pulsed with MHV CD8 epitopes S510–518 and S598–605, MHV CD4 epitope M133–147, or control peptide for 48 h following which secretion of IFN- $\gamma$  (T<sub>H</sub>1 associated) and IL-10 (T<sub>H</sub>2 associated) were measured in cell supernatants by ELISA (Fig. 5). Responses to CD8 and CD4 epitopes were not altered in splenocytes from mice receiving cell transplant, nor was there any skewing of the T<sub>H</sub>1 response in favor of increased T<sub>H</sub>2 cytokine production. One out of five mice receiving cells did produce a low amount of IL-10 in response to MHV CD8 and CD4 epitopes, however, this difference was not significant.

#### Discussion

Therapeutic targets in MS include inflammation, demyelination, and axonal loss in chronic stages. Transplantation of gliogenic cell populations has the potential of addressing the latter two targets. Several reports have demonstrated the utility of transplantation therapies in experimental models of demyelination. Early studies by [Brustle et al. \(1999\)](#) showed that injection of embryonic stem cells into the spinal cord of myelin-deficient rats results in numerous myelinated axons 2 weeks post-transplant. Similarly, transplantation of clonal mouse neural stem cells (NSCs) into mice after spinal cord injury has been shown to support axonal regeneration ([Lu et al., 2003](#)). Successful transplants have been demonstrated using cells from a variety of sources. Bone marrow stromal cells were cultured in vitro and used for intrathecal transplantation into rats with gamma-irradiation or ethidium bromide lesions, resulting in remyelination and increased conduction velocity 3 weeks following transplant ([Akiyama et al., 2002](#)). Further, transplantation of NSCs into various myelin deficient animals, such as the myelin-deficient rat ([Hammang et al., 1997](#)), shiverer mouse ([Mitome et al., 2001](#); [Yandava et al., 1999](#)), and the MAG/Fyn-

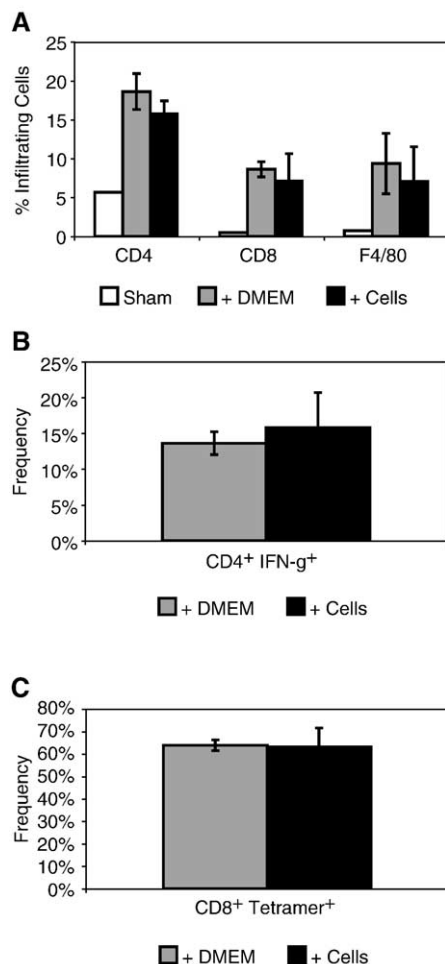


Fig. 3. Flow analysis of leukocyte infiltration into the CNS 21 days post-transplant shows that there is no modulation of inflammatory populations within the CNS following transplant when compared to control mice. Leukocytes were isolated from the CNS of sham and infected mice which received a transplant of DMEM or cells, and flow cytometry was performed to determine infiltration of CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, and F4/80<sup>+</sup> CD45<sup>high</sup> (macrophages) (A). Intracellular cytokine staining for IFN- $\gamma$  was performed to determine the frequency of M133–147-specific CD4<sup>+</sup> T cells (B); and tetramer staining for S510–518 was used to identify antigen-specific CD8<sup>+</sup> T cells (C).



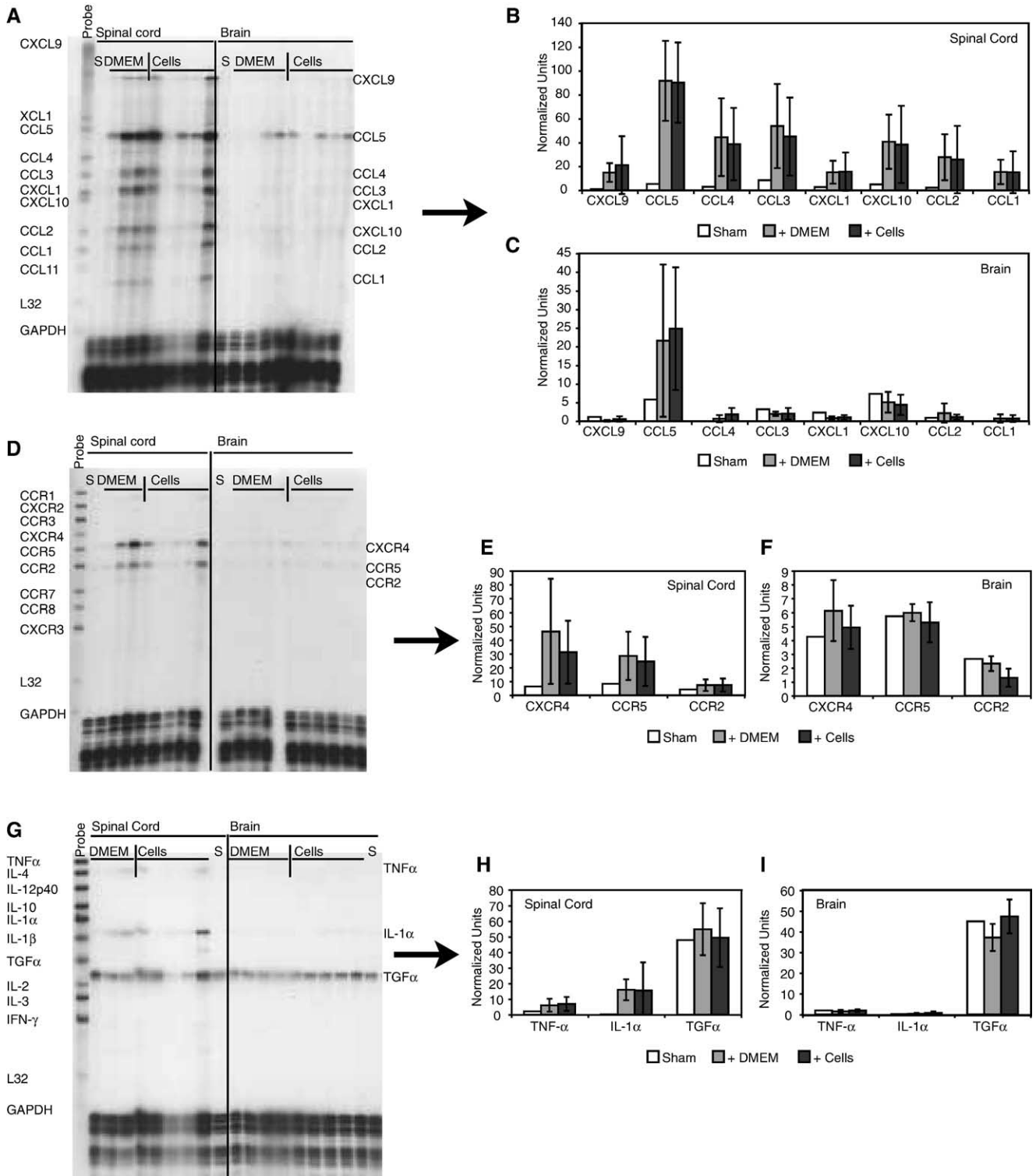


Fig. 4. Expression of inflammatory factors and receptors in the CNS is not changed following transplant of glial-committed progenitor cells. Total RNA was isolated from the spinal cords and brains of sham mice (S) and mice 33 days p.i., 21 days following transplant of DMEM or cells and analyzed by RNase protection assay for chemokines (A–C), chemokine receptors (D–F), and cytokines (G–I). Each lane contains RNA extracted from the spinal cord or brain, where indicated, of a different mouse. Data are presented as mean  $\pm$  standard deviation.

deficient mouse (Ader et al., 2001) results in remyelination. Additional studies using NSCs isolated from mouse spinal cord showed that these cells have long-term survival in vivo, surviving up to 15 weeks following transplantation into

hypomyelinated MAG/Fyn-deficient mice (Ader et al., 2004). Collectively, these studies clearly demonstrate the efficacy of transplant-mediated CNS remyelination in animal models of dysmyelination and hypomyelination. Nonetheless, it is



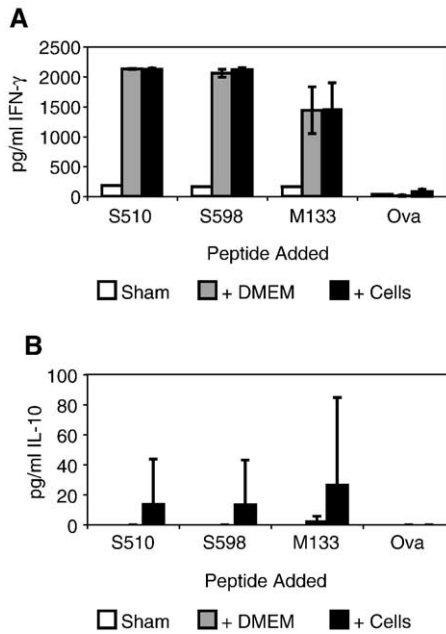


Fig. 5. Cytokine response to MHV-peptides is unaltered following transplant of glial-committed progenitor cells. Splenocytes were taken from mice 33 days p.i. (21 days post-transplant) and cultured with MHV CD8 epitopes (S510–518 and S598–605), MHV CD4 epitopes (M133–147), or OVA peptide for 48 h. Production of IFN- $\gamma$  (A) and IL-10 (B) was determined by ELISA. Data are expressed as means  $\pm$  standard deviation ( $n = 4-5$ ).

important to note that many animal models do not contain critical features of MS, including astrogliosis and the presence of inflammatory infiltrates.

Given the inflammatory nature of MS and the fact that current human therapies for MS are immunosuppressive in nature (Rizvi and Agius, 2004), it is critical to understand the influence, if any, that inflammation has on the ability of a transplanted cell population to affect remyelination. Studies using EAE-induced demyelination have evaluated the efficacy of transplant-mediated remyelination in an inflammatory environment. Transplantation of clonal oligodendrocyte progenitor cells (OPCs) into rats prior to induction of EAE results in cell migration within the CNS localizing to inflammatory lesions and surviving up to 50 days post transplant, weeks after recovery from disease (Tourbah et al., 1997). Likewise, transplantation of adult neural stem cells to mice after onset of EAE resulted in targeted migration of cells to areas of damage, improved clinical disease, as well as reduced demyelination and axonal loss (Pluchino et al., 2003). Following remyelination of the rat spinal cord by mouse oligodendroglial cells, and subsequent rejection of those cells by removal of immunosuppression, host oligodendrocyte remyelination was extensive and rapid, indicating that the inflammation associated with cell rejection did not impede repair (Blakemore et al., 1995). These studies demonstrate that remyelination is possible during inflammation. Indeed, several studies indicate that inflammation enhances survival of transplanted cells, migration of transplanted cells, and remyelination (Foote and Blakemore, 2005; O'Leary and Blakemore, 1997; Tourbah et al., 1997), and it has been suggested that oligodendrocyte progenitors within

chronic multiple sclerosis lesions may require induction of inflammation to enable their proliferation and differentiation into myelinogenic cells (Foote and Blakemore, 2005). Thus, it is important to understand the immune response during instances of successful remyelination in our effort to develop strategies to overcome remyelination failure.

Mechanisms by which transplanted cells facilitate remyelination include replenishing the OPC pool which ultimately enhances remyelination; suppressing local inflammatory gene expression thereby limiting access of inflammatory cells into the CNS; or through amplification of growth factors associated with stimulating endogenous cells to initiate repair. The timing of remyelination observed in previous studies suggests that transplanted cells may directly remyelinate damaged axons (Totoiu et al., 2004). Furthermore, transplantation of OPCs derived from human embryonic stem cells into the *shiverer* model of dysmyelination results in differentiation of these cells into oligodendrocytes and results in compact myelin formation providing evidence that transplanted progenitors can differentiate and myelinate naked axons (Nistor et al., 2005).

The present study examined the possibility that progenitor cell-induced remyelination was associated with attenuation of CNS inflammation which is strongly associated with ongoing myelin destruction in mice persistently infected with MHV. Transplant-mediated attenuation of inflammation would improve clinical score, might alter the capability of endogenous cells to affect remyelination, and is an important consideration in designing therapeutic approaches to treat human disease. A comprehensive overview of infiltration of leukocytes into the CNS was performed by FACS analysis, in which we found that intrathecal transplantation of progenitor cells into mice chronically infected with MHV did not modulate T cell or macrophage infiltration into the CNS at either 10 or 21 days following transplant (Figs. 2 and 3). Moreover, a systematic assessment of expression of chemokines, chemokine receptors, and cytokines expressed in the CNS by RPA support the finding that inflammation is not modulated following progenitor cell transplantation (Fig. 4). Further, MHV-specific immunity was not modulated following transplantation. Infiltration of antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> was similar in mice receiving transplant of progenitor cells or DMEM (Figs. 2 and 3). Additionally, T<sub>H</sub>1-cytokine expression by mouse splenocytes stimulated with MHV epitopes was not skewed in mice following transplantation (Fig. 5).

These studies provide a detailed analysis of the state of the immune response following transplant and provide strong evidence that transplantation of glial-committed progenitor cells does not result in immune suppression. This is in contrast to a study in which prophylactic transplant of neural precursor cells (NPCs) into rats with acute EAE had immunosuppressive effects (Einstein et al., 2003). This study reported reduced perivascular infiltrates in mice receiving NPCs as well as decreased proliferation of lymph node cultures when co-cultured with NPCs. It is noteworthy that our studies differ from this report in that we evaluated the therapeutic effects of transplantation for treating demyelinating disease induced by a

virus (MHV) in which the transplant was performed after disease onset. The timing of transplant is likely to be critical in influencing the development of an immune response or an ongoing immune response. Our finding that transplantation of glial-committed progenitor cells does not ameliorate inflammation are novel in that other therapies both clinical and experimental target inflammation to improve clinical disease. Specifically, experimental neutralization of chemokine CCL5 in mice chronically infected with MHV results in improved clinical and histological disease and reduces infiltration of leukocytes into the CNS as well as reduces infiltration of antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells into the CNS (Glass et al., 2004). It is possible that combining therapeutic neutralization of CCL5 with transplantation of glial-committed progenitor cells would synergize resulting in a greater degree of remyelination. Studies are underway to evaluate the benefit of such a combined therapy.

Our findings indicate that transplant-mediated remyelination is not mediated or accompanied by a reduced inflammatory response in the CNS. This is the first time in which repair and remyelination in mice chronically infected with MHV has been documented in the absence of reduced inflammation. These data suggest that remyelination can occur within an inflammatory environment and is not dependent upon modulation of inflammation. Other factors that may influence remyelination by transplanted glial-committed progenitor cells include expression of growth factors and extracellular matrix molecules. Further, these studies characterize the effect of transplantation on the immune response in the CNS, an important step in developing transplant-mediated therapies to treat demyelinating disorders. Importantly, transplantation does not reactivate the inflammatory process within the CNS. Additional studies are underway to understand mechanisms by which transplanted cells promote remyelination.

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## References

- Ader, M., Schachner, M., Bartsch, U., 2001. Transplantation of neural precursor cells into the dysmyelinated CNS of mutant mice deficient in the myelin-associated glycoprotein and Fyn tyrosine kinase. *Eur. J. Neurosci.* 14, 561–566.
- Ader, M., Schachner, M., Bartsch, U., 2004. Integration and differentiation of neural stem cells after transplantation into the dysmyelinated central nervous system of adult mice. *Eur. J. Neurosci.* 20, 1205–1210.
- Akiyama, Y., Radtke, C., Kocsis, J.D., 2002. Remyelination of the rat spinal cord by transplantation of identified bone marrow stromal cells. *J. Neurosci.* 22, 6623–6630.
- Blakemore, W.F., Crang, A.J., Franklin, R.J., Tang, K., Ryder, S., 1995. Glial cell transplants that are subsequently rejected can be used to influence regeneration of glial cell environments in the CNS. *Glia* 13, 79–91.
- Brustle, O., Jones, K.N., Learish, R.D., Karam, K., Choudhary, K., Wiestler, O. D., Duncan, I.D., McKay, R.D., 1999. Embryonic stem cell-derived glial precursors: a source of myelinating transplants. *Science* 285, 754–756.
- Cao, Q., Benton, R.L., Whittemore, S.R., 2002. Stem cell repair of central nervous system injury. *J. Neurosci. Res.* 68, 501–510.
- Castro, R.F., Perlman, S., 1995. CD8<sup>+</sup> T-cell epitopes within the surface glycoprotein of a neurotropic coronavirus and correlation with pathogenicity. *J. Virol.* 69, 8127–8131.
- Einstein, O., Karussis, D., Grigoriadis, N., Mizrahi-Kol, R., Reinhartz, E., Abramsky, O., Ben-Hur, T., 2003. Intraventricular transplantation of neural precursor cell spheres attenuates acute experimental allergic encephalomyelitis. *Mol. Cell. Neurosci.* 24, 1074–1082.
- Foote, A.K., Blakemore, W.F., 2005. Inflammation stimulates remyelination in areas of chronic demyelination. *Brain* 128, 528–539.
- Franklin, R.J., 2002. Why does remyelination fail in multiple sclerosis? *Nat. Rev., Neurosci.* 3, 705–714.
- Glass, W.G., Hickey, M.J., Hardison, J.L., Liu, M.T., Manning, J.E., Lane, T.E., 2004. Antibody targeting of the CC chemokine ligand 5 results in diminished leukocyte infiltration into the central nervous system and reduced neurologic disease in a viral model of multiple sclerosis. *J. Immunol.* 172, 4018–4025.
- Guy, J., Ellis, E.A., Kelley, K., Hope, G.M., 1989. Spectra of G ratio, myelin sheath thickness, and axon and fiber diameter in the guinea pig optic nerve. *J. Comp. Neurol.* 287, 446–454.
- Halfpenny, C., Benn, T., Scolding, N., 2002. Cell transplantation, myelin repair, and multiple sclerosis. *Lancet Neurol.* 1, 31–40.
- Hammang, J.P., Archer, D.R., Duncan, I.D., 1997. Myelination following transplantation of EGF-responsive neural stem cells into a myelin-deficient environment. *Exp. Neurol.* 147, 84–95.
- Held, K.S., Chen, B.P., Kuziel, W.A., Rollins, B.J., Lane, T.E., 2004. Differential roles of CCL2 and CCR2 in host defense to coronavirus infection. *Virology* 329, 251–260.
- Hildebrand, C., Hahn, R., 1978. Relation between myelin sheath thickness and axon size in spinal cord white matter of some vertebrate species. *J. Neurol. Sci.* 38, 421–434.
- Lane, T.E., Buchmeier, M.J., 1997. Murine coronavirus infection: a paradigm for virus-induced demyelinating disease. *Trends Microbiol.* 5, 9–14.
- Lane, T.E., Asensio, V.C., Yu, N., Paoletti, A.D., Campbell, I.L., Buchmeier, M.J., 1998. Dynamic regulation of alpha- and beta-chemokine expression in the central nervous system during mouse hepatitis virus-induced demyelinating disease. *J. Immunol.* 160, 970–978.
- Lane, T.E., Liu, M.T., Chen, B.P., Asensio, V.C., Samawi, R.M., Paoletti, A.D., Campbell, I.L., Kunkel, S.L., Fox, H.S., Buchmeier, M.J., 2000. A central role for CD4(+) T cells and RANTES in virus-induced central nervous system inflammation and demyelination. *J. Virol.* 74, 1415–1424.
- Liu, M.T., Chen, B.P., Oertel, P., Buchmeier, M.J., Armstrong, D., Hamilton, T.A., Lane, T.E., 2000. The T cell chemoattractant IFN-inducible protein 10 is essential in host defense against viral-induced neurologic disease. *J. Immunol.* 165, 2327–2330.
- Lu, P., Jones, L.L., Snyder, E.Y., Tuszynski, M.H., 2003. Neural stem cells constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury. *Exp. Neurol.* 181, 115–129.
- Mitome, M., Low, H.P., van den Pol, A., Nunnari, J.J., Wolf, M.K., Billings-Gagliardi, S., Schwartz, W.J., 2001. Towards the reconstruction of central nervous system white matter using neural precursor cells. *Brain* 124, 2147–2161.
- Nistor, G.I., Totoiu, M.O., Haque, N., Carpenter, M.K., Keirstead, H.S., 2005. Human embryonic stem cells differentiate into oligodendrocytes in high purity and myelinate after spinal cord transplantation. *Glia* 49, 385–396.
- O’Leary, M.T., Blakemore, W.F., 1997. Oligodendrocyte precursors survive poorly and do not migrate following transplantation into the normal adult central nervous system. *J. Neurosci. Res.* 48, 159–167.
- Pewe, L., Perlman, S., 2002. Cutting edge: CD8 T cell-mediated demyelination is IFN-gamma dependent in mice infected with a neurotropic coronavirus. *J. Immunol.* 168, 1547–1551.

- Pewe, L., Xue, S., Perlman, S., 1998. Infection with cytotoxic T-lymphocyte escape mutants results in increased mortality and growth retardation in mice infected with a neurotropic coronavirus. *J. Virol.* 72, 5912–5918.
- Pewe, L., Haring, J., Perlman, S., 2002. CD4 T-cell-mediated demyelination is increased in the absence of gamma interferon in mice infected with mouse hepatitis virus. *J. Virol.* 76, 7329–7333.
- Pluchino, S., Quattrini, A., Brambilla, E., Gritti, A., Salani, G., Dina, G., Galli, R., Del Carro, U., Amadio, S., Bergami, A., Furlan, R., Comi, G., Vescovi, A.L., Martino, G., 2003. Injection of adult neurospheres induces recovery in a chronic model of multiple sclerosis. *Nature* 422, 688–694.
- Rizvi, S.A., Agius, M.A., 2004. Current approved options for treating patients with multiple sclerosis. *Neurology* 63, S8–S14.
- Sospedra, M., Martin, R., 2005. Immunology of multiple sclerosis. *Annu. Rev. Immunol.* 23, 683–747.
- Totoiu, M.O., Nistor, G.I., Lane, T.E., Keirstead, H.S., 2004. Remyelination, axonal sparing, and locomotor recovery following transplantation of glial-committed progenitor cells into the MHV model of multiple sclerosis. *Exp. Neurol.* 187, 254–265.
- Tourbah, A., Linnington, C., Bachelin, C., Avellana-Adalid, V., Wekerle, H., Baron-Van Evercooren, A., 1997. Inflammation promotes survival and migration of the CG4 oligodendrocyte progenitors transplanted in the spinal cord of both inflammatory and demyelinated EAE rats. *J. Neurosci. Res.* 50, 853–861.
- Trifilo, M.J., Bergmann, C.C., Kuziel, W.A., Lane, T.E., 2003. CC chemokine ligand 3 (CCL3) regulates CD8(+)-T-cell effector function and migration following viral infection. *J. Virol.* 77, 4004–4014.
- Wu, G.F., Dandekar, A.A., Pewe, L., Perlman, S., 2000. CD4 and CD8 T cells have redundant but not identical roles in virus-induced demyelination. *J. Immunol.* 165, 2278–2286.
- Xue, S., Jaszewski, A., Perlman, S., 1995. Identification of a CD4<sup>+</sup> T cell epitope within the M protein of a neurotropic coronavirus. *Virology* 208, 173–179.
- Yandava, B.D., Billingham, L.L., Snyder, E.Y., 1999. “Global” cell replacement is feasible via neural stem cell transplantation: evidence from the dysmyelinated shiverer mouse brain. *Proc. Natl. Acad. Sci. U. S. A.* 96, 7029–7034.