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Mechanisms of Rejection and Repair Following Neural Precursor Cell Transplantation in a Viral Model of Multiple Sclerosis

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biology

by

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DEDICATION

To

my parents and grandparents

for a lifetime of encouragement and support;

to my beautiful wife, Sheri

for her limitless love and inspiration;

and

to my children.

Your father did this for you.

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LIST OF ABBREVIATIONS

APC	antigen presenting cell
BBB	blood brain barrier
CD	cluster of differentiation
CFA	complete Freund's adjuvant
CMV	cytomegalovirus
CNS	central nervous system
CTL	cytotoxic T lymphocyte
DMT	disease modifying therapy
DNA	deoxyribonucleic acid
EAE	experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
ELR	glutamic acid-leucine-arginine
ESC	embryonic stem cell
FDA	Food & Drug Administration
GFP	green fluorescent protein
H&E	hematoxylin and eosin
HHV	human herpesvirus
i.c.	intracranial
IFN	interferon
IL	interleukin
i.p.	intraperitoneal
iPSC	induced pluripotent stem cell

ISF	interstitial fluid
i.v.	intravenous
JCV	John Cunningham Virus
JHMV	John Howard Mueller virus
LCMV	lymphocytic choriomeningitis virus
LFB	luxol fast blue
MBP	myelin basic protein
MHC	major histocompatibility complex
MHV	mouse hepatitis virus
MMP	matrix metalloproteinase
MOG	myelin oligodendrocyte protein
MS	multiple sclerosis
NK	natural killer
NPC	neural precursor cell
OPC	oligodendrocyte precursor cell
p.i.	post-infection
PLP	proteolipid protein
p.t.	post-transplant
RNA	ribonucleic acid
RRMS	relapsing-remitting multiple sclerosis
SPMS	secondary progressive multiple sclerosis
TGF	transforming growth factor
Th	T helper

TMEV	Theiler's murine encephalomyelitis virus
TNF	tumor necrosis factor
Treg	regulatory T cell

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ABSTRACT OF THE DISSERTATION

Mechanisms of Rejection and Repair Following Neural Precursor Cell Transplantation in a Viral Model of Multiple Sclerosis

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University of California, Irvine, 2015

Professor Craig Michael Walsh, Chair

Multiple sclerosis (MS) is a chronic autoimmune demyelinating disease for which there is no cure. Compelling evidence from animal models of immune-mediated demyelination suggests transplanted neural precursor cells (NPCs) dampen neuroinflammation and promote remyelination, resulting in improved clinical outcome. However, much of the work evaluating NPC transplantation has been conducted using grafts from genetically identical donors or immune suppressed subjects. From a clinical perspective, NPCs are most likely to be genetically mismatched (allogeneic), and systemic immune suppression increases the risk of peripheral infection for the host. Therefore, when considering NPC transplantation for MS, the potential for engrafted cells to elicit an immune response, the consequences of engraftment in an environment with ongoing inflammation, and the susceptibility of NPCs to infection must be evaluated.

The focus of this dissertation is to elucidate mechanisms of immune rejection and repair following transplantation of NPCs in a virus-induced model of neuroinflammatory demyelination. Inoculation of susceptible mice with the neuroattenuated JHM variant of

mouse hepatitis virus (MHV) induces acute encephalomyelitis followed by chronic immune-mediated demyelination that recapitulates histopathological and clinical features of MS. Using this well-accepted model, we demonstrate that allogeneic NPCs are antigenic and are rejected following transplantation. Rejection is correlated with expression of major histocompatibility complex (MHC) and co-stimulatory molecules by NPCs, as well as increased CD4+ and CD8+ T cell activity *in vitro* and *in vivo* (**Chapter 2**). Additionally, allogeneic NPCs are lysed by natural killer (NK) cells following binding of retinoic acid early inducible-1 (RAE-1) to the NK-activating receptor NKG2D. NK cells localize with allografted NPCs and NK cell infiltration is correlated with reduced graft survival. Furthermore, NPCs can be infected by MHV, which elicits destruction via NK cell-mediated mechanisms (**Chapter 3**). We provide evidence that CD4+ and CD8+ T cells participate in suppression of virus replication in MHV-infected NPCs via secretion of interferon-gamma (IFN-γ) and direct cytolysis (**Chapter 4**). Lastly, induced pluripotent stem cells (iPSCs) represent an unlimited source of NPCs that are genetically identical to the host. We demonstrate that NPCs derived from human iPSCs promote focal remyelination concomitant with suppressed CD4+ T cell infiltration in the CNS dependent on increased regulatory T cell (Treg) activity (**Chapter 5**).

CHAPTER ONE

Mouse hepatitis virus-induced demyelination as a pre-clinical model for evaluating neural precursor cell therapy

1.1 Multiple Sclerosis

Multiple sclerosis (MS) is a chronic autoimmune disease characterized by the development of inflammatory demyelinating lesions in the brain and spinal cord (1). As a result of disruption of myelinated tracks in the central nervous system (CNS), patients with MS typically develop a variety of deficits in motor, sensory, visual, and autonomic systems, including muscle weakness and fatigue, vision loss, loss of balance, and emotional and cognitive decline (2). Recent estimates suggest that more than 400,000 individuals in the United States are afflicted with MS, with approximately 2.5 million cases worldwide (3). In contrast to many other neurodegenerative diseases, symptoms manifest between the ages of 16 and 45, making it the leading cause of disability in young women and the second leading cause of disability in young men (1, 4, 5). The majority of patients diagnosed with MS initially present with relapsing-remitting MS (RRMS), a stage of the disease characterized by transient inflammation and compensatory remyelination resulting in a cycle of neurologic dysfunction and recovery (5, 6). However, endogenous mechanisms of myelin repair are not sustained, and within 25 years of diagnosis 90% of patients will progress to secondary progressive MS, a stage of irreversible neurological decline and accumulation of disability (1, 7).

MS is a disease of complex etiology with a variety of genetic and environmental factors contributing to onset and progression of related sequelae. Genome-wide association studies have confirmed that susceptibility to MS is linked to polymorphisms in the major histocompatibility complex (MHC) gene on chromosome 6, with the HLA-DR and HLA-DQ alleles of the MHC class II gene conferring the greatest degree of risk (8-10). Furthermore, complete genome screens of families with multiple cases of MS and transcriptome analysis

of MS lesion materials support a contributory role for immunoglobulin, osteopontin, and genes involved in the T-helper (Th) cell differentiation pathway (11, 12). However, with the possible exception of HLA-DR, the calculated risk attributable to a single gene is weak-to-modest, and the functional consequences of polymorphisms at candidate susceptibility genes remain to be elucidated. Importantly, a 70% discordance rate amongst monozygotic twins demonstrates genetic susceptibility alone cannot definitively explain the cause of MS, suggesting a role for environmental factors as triggers for disease (13). For example, the global distribution of MS is lowest in the tropics, increases north or south of the equator, and migration from high-risk to low-risk regions is associated with reduced susceptibility (5). This is perhaps associated with the observation that reduced exposure to sunlight and vitamin D deficiency has been linked to higher incidence of MS (14). Microbial infection has also been proposed as an environmental contributor to the development of MS. Patients with MS report being report being infected with measles, mumps, rubella, and Epstein-Barr virus (EBV) at later ages, and antigens expressed by EBV mimic components of myelin protein (15, 16). Furthermore, patients with MS have a higher prevalence of infection with human herpesvirus-6 (HHV-6) and a correlation between HHV-6 reactivation and disease activity has been established (17, 18). Still, a causal relationship between environmental factors such as viral infection and the development of MS remains to be clarified and our understanding of MS etiology remains incomplete.

Although the underlying cause of MS is enigmatic (19, 20), the prevailing hypothesis is that activation of autoreactive T cells in the periphery and subsequent migration of lymphocytes through a compromised blood brain barrier leads to the destruction of myelin and oligodendrocytes, the cell type responsible for myelinating the axon portion of neurons

in the CNS. Indeed, CD4+ and CD8+ T cells specific for the myelin components proteolipid protein (PLP), myelin oligodendroglial glycoprotein (MOG), and myelin basic protein (MBP) are enriched in the peripheral blood of patients with MS, and the majority of lymphocytes in early MS lesions are CD4+ T cells (6, 21, 22). Within established plaques, macrophages and microglia constitute the majority of inflammatory cell types and are responsible for myelin phagocytosis, antigen presentation to T cells, and secretion of pro-inflammatory cytokines that drive T cell activity and contribute to the inhibition of remyelination by oligodendrocytes (23, 24). Additionally, immunoglobulins with specificity to myelin proteins have been eluted from MS brain plaques, demonstrating a role for B cells in MS pathology (25, 26). Cumulatively, the concerted actions of innate and adaptive immune components drive demyelination, resulting in axonal degeneration and progression of MS-related symptoms.

The underlying immune component of MS has been a target of therapeutic strategies for years, and animal models of demyelination have led to the approval of several immune-modulatory agents for treatment of MS (27, 28). Interferon-beta (IFN-β; Avonex/Rebif, Betaferon) and glatiramer acetate (Copaxone) have been used as first line disease-modifying therapies (DMTs) for over a decade, and administration of these DMTs is associated with a reduction in relapses and number of active brains lesions in patients with MS (1, 29). The interferons function mainly by suppressing proliferation of effector T cells, reducing the amount or activity of pro-inflammatory cytokines, and augmenting the function of suppressor T cells (30). Glatiramer acetate may block presentation of myelin antigens, thereby preventing activation of antigen-specific T cells (31). First-line therapies for MS carry significant side effects ranging from reactions at the injection site and flu-like

symptoms to cardiac failure, anaphylaxis, and mood disorders. Furthermore, patients may develop neutralizing antibodies against IFN-β and glatiramer acetate. DMTs for treating patients who have a sub-optimal response to first-line therapies, or who have more aggressive forms of RRMS, include natalizumab (Tysabri) and fingolimod (FTY720; Gilenya). Natalizumab is a recombinant monoclonal antibody to alpha-4 integrins that inhibits leukocyte migration to the CNS (32). FTY720, the first approved oral treatment for RRMS, is a sphingosine 1-phosphate receptor modulator that prevents lymphocyte egress from the lymph nodes (33). Compared to IFN-β and glatiramer acetate, both natalizumab and FTY720 demonstrate greater efficacy in regards to halting progression of disability, reducing relapse rate, and preventing development of new lesions. However, about 1 in 600 patients treated with natalizumab develop progressive multifocal leukoencephalopathy (PML) as a result of brain infection with John Cunningham virus (JCV), and longer treatment durations or prior immunosuppressant use increases risk of PML (34, 35). The long-term safety of FTY720 remains to be determined, but known side-effects include bronchitis, lymphopenia, bradycardia, liver function abnormalities, macular edema, and increased risk of infections (33). Patients with the most aggressive and unresponsive forms of RRMS may resort to immunosuppression with cyclophosphamide or mitoxantrone, or high dose chemotherapy followed by autologous hematopoietic stem cell transplantation, but those strategies are not well-tolerated and carry inherent risk for serious complications.

Current DMTs approved by the Food and Drug Aministration (FDA) for the treatment of MS are immune modulatory in nature with presumed, but poorly defined, mechanisms of action which carry adverse side effects. Moreover, these therapeutic

strategies are designed to limit damage to the CNS during or following acute episodes of inflammation and do not directly promote remyelination. Patients suffering from progressive forms of MS in which compensatory remyelination has failed and severe axonal degeneration is prevalent are unresponsive to available treatments. Thus, there is an unmet clinical need for an approach that addresses inflammatory cell infiltration while promoting long-term remyelination.

1.2 Animal models of immune-mediated demyelination

Animal models of neuroinflammatory demyelination have been paramount to the understanding of the pathogenesis of human MS. The majority of experimental MS therapies currently in phase II and phase III trials were first evaluated within the context of the prototypic MS model experimental allergic encephalomyelitis (EAE). EAE is a CD4+ T cell-mediated disease that can be induced in a variety of species and strains, but most commonly involves injecting mice with an emulsion of MBP, PLP, or MOG in complete Freund's adjuvant. Peripheral immunization with myelin peptides disrupts tolerance and leads to the activation of autoreactive CD4+ T cells belonging to the Th1 and Th17 subclasses in secondary lymphoid organs (36). Once myelin-specific T cells infiltrate the CNS, presentation of myelin antigens by resident antigen presenting cells (APCs) leads to T cell reactivation and subsequent secretion of pro-inflammatory cytokines including IFN-γ, IL-17, granulocyte-macrophage colony-stimulating factor (GM-CSF), and tumor necrosis factor alpha (TNF-α). Cytokine production results in damage to the CNS and recruitment of non-specific effector cells such as γδ T cells, monocytes, macrophages, and neutrophils (37). White matter damage carried out by infiltrating T cells, macrophages, and B cells culminates in formation of lesions in the brain and spinal cord, as well as ascending limb paralysis reminiscent of human MS. The extent of demyelination and severity of paralysis can be altered to mimic either a relapsing-remitting course or chronic progressive disability by manipulating the myelin antigen and/or rodent strain employed (37).

The clinical and histopathological similarities between EAE and human MS have led to wide use of EAE as a pre-clinical model for drug development. However, this model is based on the premise that MS is a primary autoimmune inflammatory disease. Studies of

neuropathology in MS lesions raise the question of whether or not oligodendroglial apoptosis and myelin degeneration precede inflammation (19, 20). Proponents of the oligodendrogliopathy hypothesis for MS contend that inflammation is a secondary response to demyelination to facilitate clearing of myelin debris. In addition, EAE requires an immunization step to be induced, whereas sensitization to myelin antigens is not artificially induced in humans. Moreover, the inducing antigens are well-defined in EAE, while a causative antigen has not been identified in MS (36). Importantly, more than 100 compounds have shown to significantly alleviate EAE-related symptoms in animal models, yet only 8 FDA-approved therapeutics are currently available for patients with MS, each of which is only modestly effective at reducing relapse rates (38). Even in the case of the most potent licensed DMT, natalizumab, EAE could not be used to predict opportunistic infection due to reactivation of latent JCV. Discordance between the pathogenesis of EAE and human MS has led to minimal translational success of candidate therapeutics. It stands to reason that treatment strategies demonstrating a high degree of concordance across multiple models of neuroinflammatory demyelination will have a higher likelihood of clinical success.

Virus-induced models of MS are of particular interest due to the link between infectious agents and higher incidence of MS (39). Proposed contributions of viral infection to onset of MS include death of oligodendrocytes as a result of virus replication, toxic effects of cytokine release resulting from infection in the CNS, and molecular mimicry between viral antigens and autoantigens (40, 41). Indeed, JCV and measles virus are known to trigger autoimmune reactions leading to the demyelinating diseases PML and subacute sclerosing pancephalitis (SSPE), respectively. Furthermore, EBV and HHV-6 appear to be

prevalent in the CNS tissues or spinal fluid of patients with MS (42). Serological testing and immunohistochemistry has also detected coronavirus RNA and antigen in patients with acute disseminated encephalomyelitis, as well as relapsing-remitting and progressive MS (43, 44). For these reasons, inoculation of animals with Semliki Forest virus (SFV), Theiler's murine encephalomyelitis virus (TMEV), or mouse hepatitis virus (MHV) has been used to probe mechanisms of acute and chronic demyelination. Mice infected intraperitoneally (i.p.) with the alpha-togavirus SFV develop acute encephalomyelitis. Following viral clearance, immune-mediated demyelination occurs in the cerebellum, brainstem and corpus collosal white matter as a result of microglial activation, T cell infiltration, and the production of virus and myelin specific antibodies (45-47). Remyelination occurs by day 35 post-infection (p.i.), at which point tissues appear normal. In contrast, intracranial (i.c.) infection with TMEV, a member of the *Picornaviridae* family, leads to an acute disease phase followed by chronic progressive demyelination similar to secondary progressive MS (48). Paralysis during early infection involves little-to-no inflammation and is likely due to cytolysis of neurons after TMEV replication (48). Chronic progressive demyelination is characterized by an increased Th1 response and is maintained by persistent infection in macrophages, microglia, and astrocytes (49, 50). APCs from TMEV-infected mice not only present viral epitopes, but also PLP epitopes, providing evidence for the development of an autoimmune demyelinating disease supported by a molecular mimicry mechanism (48, 51). Another widely accepted viral model of MS involves i.c. inoculation with MHV. Infection with neurotropic strains of MHV triggers acute encephalomyelitis, followed by demyelination in which CD4+ and CD8+ T cell infiltration and macrophage activation in the CNS is required (52). Chronic demyelination resulting from MHV infection recapitulates

much of the immunopathology observed in active MS lesions and serves as a useful pre-clinical model for developing novel treatment strategies against MS.

1.3 Pathogenesis of MHV-induced disease

One of the most well-studied members of the *Coronaviridae* family is mouse hepatitis virus (MHV), a common pathogen of laboratory rodents associated with a variety of respiratory, gastrointestinal, and neurological diseases (53). The 32-kilobase MHV genome encodes four major structural proteins: spike (S), envelope (E), membrane (M), and nucleopcapsid (N), of which membrane protein, nucleocapsid protein, and envelope protein are required for the assembly of new virions (54). Binding of the spike glycoprotein to the canonical MHV receptor carcinoembryonic antigen-related cell adhesion molecule-1 (CEACAM-1) is required to initiate successful infection (55, 56). Viral entry is accomplished via fusion with the surface membrane of the host cell or through endocytosis, and virions are subsequently assembled within intracellular vesicles. Fusion of virus-containing vesicles to the plasma membrane leads to the release of virions, at which point MHV can infect other cells or replicate in the parent cell (57).

Numerous factors determine pathogenesis and immune response to MHV infection, including virus strain, route of inoculation, and host's age and genetic background (57). Approximately 25 strains of MHV have been described, with several isolates demonstrating tropism for neural cell types (53). Of particular interest, the John Howard Mueller serotype of MHV (JHMV) was originally isolated from the brain of a mouse that developed spontaneous hind limb paralysis as a result of infection (48). Intracranial (i.c.) inoculation of susceptible immunocompetent mice with J2.2v-1, the neutralizing monoclonal antibody-resistant variant of JHMV, leads to virus replication in ependymal cells lining the cerebral ventricles (57, 58). Dissemination of virus through the grey and white matter is accomplished via infection of oligodendrocytes, astrocytes, and microglia and leads to

acute encephalitis, followed by chronic demyelination and mild-to-severe hind limb paralysis in the host. Despite T cell-mediated clearance of replication-competent virions by 10-14 days p.i., viral antigen and RNA persist in the CNS of infected animals (57). During this latent period of infection, humoral immunity is largely responsible for prevention of viral reactivation, but neutralizing virus-specific antibodies are not believed to play a role in promoting demyelination (59, 60).

MHV-induced disease can be divided into two distinct phases: (1) acute encephalomyelitis and (2) chronic demyelination. Initial MHV infection rapidly triggers secretion of ELR+ chemoattractants such as CXCL1 and CXCL2 by resident CNS cell types, leading to infiltration of the brain and spinal cord by polymorphonuclear neutrophils within 24 hours p.i. (61, 62). Recruited neutrophils and monocytes/macrophages do not demonstrate direct antiviral activity but participate in degradation of the basal lamina and extracellular matrix of the BBB via release of matrix metalloproteinases (63-65). Permeabilization of the BBB allows migration of natural killer (NK) cells, virus-specific T cells, and B cells to the CNS. Within 48 hours of inoculation, pro-inflammatory cytokines including IL-1α, IL-1β, IL-6, IL-12, and TNF-α are detected at high levels in the brain and spinal cord of infected host (66, 67). Type I interferons are also in high abundance shortly following MHV infection and act to suppress viral replication via modulation of innate and adaptive immune components through MHC class I expression (68-70).

The major mediators of viral clearance during the acute phase of MHV-induced disease are effector T cells. Virus-specific CD4+ T cells conform to the Th1 subtype and are responsible for promotion of CD8+ T cell expansion in the peripheral lymphatics, facilitating CD8+ T cell access into the CNS parenchyma, and limiting CD8+ T cell activation-

induced apoptosis (71). Additionally, CD4+ T cells exert direct antiviral activity through secretion of perforin and IFN-γ, the latter of which also induces expression of MHC class I/II on infected astrocytes, oligodendrocytes, and microglia (71-73). Virus-specific CD8+ T cells are the main cytolytic effector cell in the CNS and carry out lysis of infected astrocytes, macrophages, and microglia through production of granzyme B and perforin. Furthermore, secretion of IFN-γ by CD8+ T cells is critical for suppression of MHV replication in oligodendrocytes (73-75). This vigorous antiviral response reduces MHV titers below detectable levels (as evaluated by plaque assay) by 2 weeks p.i. and is associated with a decline in the number of infiltrating lymphocytes. However, viral RNA and antigens persist in the CNS despite virus clearance, resulting in constitutive activation of glial cells and retention of effector T cells long after infection (76).

Persistence of non-infectious virus and development of inflammatory demyelinating lesions are hallmarks of the chronic phase of MHV-induced disease. Survivors of acute encephalomyelitis develop disturbed gait and mild-to-severe hind limb paralysis as a result of ascending white matter damage in the spinal cord. Analysis of CNS tissue from chronically infected mice demonstrates myelin degradation is concomitant with the presence of viral antigen and inflammatory infiltrates (58). During this phase, virus-specific T cells and macrophages are the main perpetuators of demyelination. Adoptive transfer of splenoctyes from immunocompetent MHV-infected mice into inoculated immunodeficient hosts results in demyelination (77). Furthermore, mice deficient in CD4+ T cells, CD8+ T cells, or macrophage trafficking demonstrate abrogated or reduced white matter damage independent of viral clearance (78, 79). Moreover, adoptive transfer of CD4+ or CD8+ T cells into MHV-infected RAG1$^{-/-}$ mice, which lack functional T cells and B cells, is sufficient

to initiate and amplify disease (78, 80, 81). Proposed mechanisms by which virus-specific T cells mediate demyelination during chronic MHV infection include secretion of IFN-γ and TNF-α (76, 82). Additional evidence suggests CD4+ T cells can enhance demyelination via production of the chemokine CCL5, which sequesters macrophages (78, 83). Indeed, macrophages are detected in areas of white matter damage during chronic MHV infection, and ultrastructural analysis has captured macrophages stripping and engulfing myelin (84, 85). Furthermore, neutralizing CCL5 or deleting its receptor in the germ line reduces macrophage infiltration to the CNS and dampens demyelination following infection (83, 86).

Oligodendrocytes represent an important reservoir for latent virus during chronic infection, but necroptosis and apoptosis of oligodendroglia are not considered primary mechanisms of demyelination (85, 87). Furthermore, no role for complement or endogenous antibody-mediated demyelination has been confirmed, though exogenous autoantibodies can enhance demyelination independent of complement (88, 89). Unlike EAE, TMEV, and human MS, epitope spreading and the emergence of autoreactive T cells specific for myelin epitopes has not been observed in mice chronically infected with MHV, confirming that disease progression is driven mostly by an antiviral response (82). It should be noted that adoptive transfer of JHMV-specific T cells from infected rats to naïve recipients induces demyelination, and the A59 strain of MHV has been observed to activate autoreactive T cells specific for MBP in mice (90, 91). However, within the context of those models and methods, the contribution of autoreactive T cells to demyelination remains ambiguous.

1.4 Neural precursor cell transplantation

Transplantation of neural tissue for the treatment of neurodegenerative disease has been under investigation since the late 19th century. Early studies typically involved transfer of homogenized whole brain into a donor's brain and provided little evidence to support consequences of the graft (92). In the 1980's, more systematic approaches to the grafting of fetal ventral mesencephalic tissue as a cell replacement therapy for Parkinson's disease (PD) led to the approval of several clinical trials in humans (93). Shortly thereafter, a number of studies demonstrated survival and efficacy of transplanted fetal CNS cells in patients with PD, thereby providing a springboard for transplantation of neural grafts for nearly every neurological disorder in which endogenous mechanisms to repair tissue damage and rescue lost function have failed (92, 94).

Since the inaugural neural graft experiments were performed, neural precursor cells (NPCs) have been identified as a major source of plasticity in the brain and are now regarded as an integral component of CNS repair. Lineage fate mapping of NPCs within the subventricular zone of lateral ventricles and subgranular zone of the hippocampus demonstrated the ability of these cells to differentiate into neurons, astrocytes, and oligodendrocytes throughout development (95, 96). Moreover, adult quiescent NPCs have been shown to proliferate, migrate, and differentiate in response to acute CNS damage, such as with spinal cord injury, stroke, and inflammatory demyelination (97-99). These findings have led to extensive evaluation of NPC transplantation as a treatment strategy for numerous neurological disorders. For example, NPCs grafted into rodent and primate models of Huntington's disease differentiate into mature striatal neurons, which is associated with rescue of motor skills (100-102). Injection of NPCs into rodents with

pathology that recapitulates aspects of Alzheimer's disease improves cognition, mediated mostly by increased production of brain derived neutrotrophic factor (BDNF) and endogenous synaptogenesis (103, 104). Furthermore, NPCs delivered to the CNS of spinal cord-injured rodents integrate with the host synaptic network following differentiation, induce remyelination, and inhibit the recruitment of proinflammatory macrophages at the lesion site (105, 106). These finding suggest that NPCs attenuate neurodegeneration and associated neurologic dysfunction through a combination of cell replacement and enhanced trophic support. Ultimately, the most convincing evidence for NPC transplantation will come from human patients with neurological disorders, and clinical trials testing safety and efficacy of NPC grafts in patients with chronic spinal cord injury, amyotrophic lateral sclerosis, and Pelizaeus-Merzbacher disease are currently underway (107, 108).

MS is an attractive target for cell-based therapeutic strategies. Current FDA-approved DMTs are immunomodulatory and do not address chronic demyelination in patients with progressive forms of the disease. On the other hand, evidence from pre-clinical models of MS suggests transplantation of NPCs may induce remyelination and dampen neuroinflammation, resulting in improvements in motor skills. For example, Pluchino et al. (2003) demonstrated that adult murine NPCs injected i.c. or intravenously (i.v.) into EAE mice migrated to areas of white matter damage, differentiated to oligodendrocytes, and myelinated host axons. Interestingly, only a small percentage of oligodendrocyte progenitor cells (OPCs) at sites of demyelination were donor-derived, indicating that NPCs also enhanced endogenous OPC activity (109). Importantly, the majority of transplanted mice experienced near-complete recovery from motor deficits. These findings were later recapitulated in non-human primates afflicted with EAE using

human fetal-derived NPCs (110). Within the context of MHV-induced demyelination, intraspinal injection of syngeneic murine NPCs leads to migration of engrafted cells to lesions, remyelination, and axonal sparing associated with improved clinical outcome in transplanted mice (111). Migration of NPCs to areas of demyelination and maturation of endogenous OPCs in MHV-infected mice is dependent on the CXCL12-CXCR4 chemokine axis, and differentiation of transplanted NPCs to the oligodendrocyte lineage requires expression of oligodendrocyte transcription factor 1 (Olig1) (112-114). Moreover, resolution of MHV-related pathology following adult NPC engraftment is independent of a dampened immune response in the CNS (115).

Early transplant studies in models of MS suggested a cell replacement role whereby donor NPCs differentiate into myelinating oligodendrocytes or enhance host oligodendrogenesis at sites of demyelination. However, potent immune regulatory properties have also been observed in NPCs. Intraventricular transplantation of NPCs during the acute inflammatory stage of EAE reduced the number of perivascular immune infiltrates, which correlated with attenuated disease pathology in rats (116). In mice, NPCs delivered systemically during acute EAE retained their undifferentiated phenotype and suppressed encephalitogenic T cell activity, resulting in reduced demyelination and disability (117, 118). Similar immune regulatory phenomena have been observed in EAE-afflicted mice and marmosets following transplantation with human NPCs (110, 119). A novel immunomodulatory function of human NPCs (hNPCs) that results in myelin sparing and improved motor skills was recently demonstrated in the MHV model of neuroinflammatory demyelination (120). Following intraspinal injection, increased numbers of suppressive regulatory T cells (Tregs) are detected in the CNS of hNPC

transplanted mice. Treg induction is dependent on secretion of transforming growth factor beta (TGF-β) by transplanted hNPCs, and *in vivo* ablation of Tregs abrogates immune suppression and concomitant remyelination. Moreover, the human neural graft was rejected within 8 days post-transplant (p.t.), yet clinical recovery was sustained to at least 6 months. To date, this is the only demonstration of the therapeutic benefit of human NPCs in a virus-induced model of demyelination.

1.5 Allogeneic transplantation in the CNS

During adulthood, NPCs are located in discrete regions of the brain with relative paucity, making autologous transplantation, or transplantation of cells derived from the patient, an implausible strategy. Indeed, the precedent set by the majority of clinical trials is to derive NPCs from donated fetal CNS tissue (121). However, it has been known since the initial studies of skin graft rejection that genetically mismatched (e.g. allogeneic) tissue is not well-tolerated by the host, and allografts are ultimately rejected (122, 123). Decades of research in the field of transplantation immunology has revealed that polymorphisms in MHC class I and II molecules are the most important series of antigens involved in allogeneic rejection (124). The predominant role of MHC expression is to provide T cells with cognate peptides from foreign pathogens, such as during a normal immune response to infection. However, alloreactive CD4+ and CD8+ T cells can directly detect polymorphisms in MHC expressed on transplanted tissue independent of the peptide being presented, resulting in T cell activation and proliferation rapidly after transplant (125, 126). As the graft is identified and destroyed, the host's APCs present alloantigens bound to MHC complexes to T cells in regional lymphoid organs, enhancing T cell trafficking to the donor tissue and exacerbating rejection (127). Production of pro-inflammatory cytokines (i.e. IL-2, IL-4, IFN-γ), direct lysis by cytotoxic T lymphocytes (CTLs), and alloantibody production by B cells culminate in complete destruction of donor tissue (128).

Current attempts to dampen the cascade of inflammatory events leading to rejection include matching the MHC haplotype of donor and host, and life-long systemic immunosuppression. Finding an MHC-matched donor can be difficult since it is one of the most polymorphic genes known; hundreds of alleles have been identified at the major

human MHC (human leukocyte antigen; HLA) loci (124). Furthermore, immunosuppression is required in spite of matching due to polymorphisms in genes not encoded in the MHC (129). Systemic immunosuppression using drugs that inhibit T cell activation, restrict lymphocyte migration, or trigger apoptotic pathways in immune cells is effective for promoting tolerance to allografts (124). Unfortunately, these drugs are also associated with toxic side effects and increased susceptibility to opportunistic infection. Moreover, chronic rejection and poor long-term survival of allografted tissue in spite of immunosuppressant agents remains unresolved (129).

During his seminal studies, Sir Peter Medawar made the additional observation that histoincompatible grafts survived longer when transplanted into the brain versus on to the skin (130). Subsequent evidence has led to a more complete understanding of the immune privileged status of the CNS. First, the BBB impedes diffusion of cellular and molecular components from the periphery. This shielding property is mainly attributed to a lack of expression of adhesion molecules required by leukocytes, as well as sealing at the margins of endothelial cells by tight junctions (131). As a result of the relative impermeability of the BBB, T cells, macrophages and dendritic cells are normally detected in the CNS at very low levels (132). Second, low expression of MHC molecules is observed in the CNS. Neurons and oligodendrocytes express little-to-no MHC I, and MHC II expression is restricted to microglia (133). Evidence suggests that electrical activity of neurons may suppress MHC on surrounding cells, and neurotrophins such as nerve growth factor (NGF), neurotrophin-3 (NT3), and BDNF have been shown to dampen MHC and co-stimulatory molecule expression (134, 135). Lastly, the CNS lacks lymphatic vasculature and, although there are well defined pathways for drainage of interstitial fluid (ISF) and cerebrospinal fluid (CSF), a

cellular route for antigen delivery from the CNS to draining lymphoid organs remains to be characterized (136, 137).

Despite being immunologically privileged, the CNS is not entirely beyond the reach of the immune system. Microglia, phagocytic residents of the brain and spinal cord, are known to express molecules involved in T cell engagement, including MHC I/II, CD80, CD86, CD40, CD11a, CD54 and CD58, and are an important mediator to the adaptive immune system (138). During TMEV and MHV infection of the CNS, for example, microglia engage T cells, secrete pro-inflammatory cytokines, and produce chemoattractants that recruit additional immune cells to the CNS (139, 140). The inflammatory milieu that results from infection or injury also compromises immune privilege independent of microglial activation. Certain pro-inflammatory cytokines, particularly IFN-γ and TNF-α, increase permeability of the BBB and enhance neural entry of leukocytes regardless of whether they were produced in the CNS or in the periphery (141). Moreover, there are examples of MHC expression by neurons, oligodendrocytes, and astrocytes in response to inflammatory triggers (139, 142). Clearly, immunity is not entirely muted in the CNS, and a robust immune response can be solicited with the appropriate stimuli, such as during foreign antigen presentation by resident cells of the CNS to inflammatory T cells.

Transplantable NPCs are typically derived from the immune privileged CNS, and there has been a long-standing presumption that they might exhibit reduced immunogenicity and be well-tolerated by MHC-mismatched recipients. Indeed, a number of early reports documented low or absent expression of MHC I/II and co-stimulatory molecules CD80, CD86, and CD40 by NPCs *in vitro* (110, 117, 143, 144). Moreover, NPC allografts did not upregulate MHC following transplantation and exhibited prolonged

survival in healthy and brain contused rodents, even when immune suppression was omitted or withdrawn (143, 145, 146). However, contradictory findings raise the question of whether or not transplanted NPCs are, in fact, immunoprivileged. For example, long-term expansion *ex vivo* and exposure to IFN-γ and TNF-α have been shown to induce upregulation of MHC I/II (147, 148). Furthermore, alloreactive T cells proliferate in response to MHC-expressing NPCs and target them for destruction (144, 147). Additionally, transplantation of allogeneic NPCs in a model of spinal cord injury resulted in CD8+ T cell infiltration into the CNS and NPC rejection (149). MHC mismatch can also lead to deficits in lineage commitment by NPCs; allogeneic NPCs transplanted into the hippocampus of mice experienced a 90% reduction in neuronal differentiation potential compared to MHC-matched isograft controls that was concomitant with T cell accumulation at engraftment sites and impaired allograft survival (150). Cumulatively, contradictory perspectives on the immunogenic potential of allogeneic NPCs warrant thorough investigation of the consequences of transplantation in animal models of neurological disorders. While it appears that allogeneic NPCs may not be antigenic under steady-state conditions and in the absence of pro-inflammatory cues, many of the neurodegenerative diseases for which NPCs are proposed as a treatment involve an immunological component that may affect MHC and costimulatory molecule expression by NPCs, resulting in graft destruction and loss of therapeutic benefit.

1.6 Pluripotent sources of NPCs

One caveat of previous studies evaluating human NPC transplantation is the use of fetal-derived CNS tissue. Fetal NPCs are limited by the relative dearth of donor samples, and, as with solid organ transplantation, finding HLA-matched material for transplantation can be difficult. Moreover, the use of aborted fetal tissue carries intrinsic legal and ethical issues that require careful consideration. An ideal source of NPCs would be well-characterized in regard to the donor's genetic background, be able to be cryogenically stored and recovered as-needed, and yield cell numbers sufficient for transplantation.

Nearly two decades ago, Thomson et al. reported the first isolation of pluripotent human embryonic stem cells (hESCs) from the inner cell mass of discarded blastocysts (151). Remarkably, hESCs could be propagated for dozens of generations while maintaining their ability to differentiate into any cell of three major tissue layers (endoderm, mesoderm, ectoderm) *in vitro*. In addition, when transplanted into immune privileged sites in rodents, hESCs formed teratomas, solid tumors representing endodermal, mesodermal, and ectodermal differentiation (151). Since the initial derivation of hESCs, efficient methods to perform specification to the neuroectodermal lineage have been developed. Numerous groups report generating homogenous populations of NPCs en bloc using antagonists of bone morphogenic proteins (BMP) and Acitivin/Nodal signaling (152). These hESC-derived progeny commit to terminal neural cell types and express markers characteristic of neural stem and progenitor cells in the CNS, including sex determining region Y-box 1 (Sox1), Sox2, Nestin, and paired box protein-6 (Pax6).

The ability of hESCs to reproducibly generate unlimited numbers of NPCs provides a promising cell source for regenerative medicine applications. Indeed, studies in animal

models of neurodegenerative disorders affirm the therapeutic potential hESC-NPCs. For example, hESC-NPCs were shown to foster behavioral improvement following transplantation into the substantia nigra of Parkinsonian rats (153). Importantly, the graft was well-tolerated and did not result in teratoma formation. Similarly, NPCs derived from two separate hESC lines differentiated and integrated into the host synaptic network of rats with complete spinal transections, resulting in significant functional improvement (154). In the EAE-based model of MS, hESC-NPCs are neuroprotective and exert immunomodulatory effects similar to those reported by fetal neural grafts (119). More recently, our group demonstrated that intraspinal injection of hESC-NPCs results in sustained clinical recovery concomitant with remyelination and dampened neuroinflammation in mice suffering from MHV-induced demyelination (120).

Pre-clinical observations suggest transplantation of hESC-derived NPCs may be a viable therapeutic strategy for neurological diseases, including MS, and clinical trials utilizing neural lineage cells produced from hESCs have been initiated (155, 156). However, similar to donated fetal tissue, the use of hESCs as a biological therapeutic is inherently controversial since isolation results in the destruction of the embryo. Furthermore, although a complete genetic profile can be compiled for every hESC line, long-term immune suppression is still required. On the other hand, seminal research in the laboratory of Shinya Yamanaka demonstrated that pluripotent stem cells could be induced from adult somatic cell types, such as dermal fibroblasts, using retroviruses expressing four transcription factors: octamer-binding transcription factor 4 (Oct4), Sox2, Kruppel-like factor 4 (Klf4), and c-Myc (157, 158). Human induced pluripotent stem cells (hiPSCs) faithfully recapitulate differentiation and proliferation potential observed in hESCs.

Moreover, hiPSCs maintain the genetic background of the donor and may not be immunogenic, raising the possibility of autologous transplantation (159, 160). Animal models of ischemic stroke, ALS, and Parkinson's disease all report therapeutic benefit of NPCs derived from iPSCs (iPSC-NPC), and neuroprotective effects of iPSC-NPCs have been observed in EAE mice (161-164). However, variability in NPC lineage commitment exists between patient cell lines, and at least one study reports a lack of functional improvement when hiPSC-NPCs were compared to hESC-NPCs in spinal cord-injured rats (165-167). Overall, evidence supporting iPSC-NPC transplantation for resolving CNS disorders is still in its infancy, especially with regard to MS, and a thorough investigation of the consequences of hiPSC transplantation will be critical before therapies are transitioned from the bench to the bedside.

1.7 Summary

To date, the majority of experimental treatments developed using EAE, the prototypic autoimmune model of MS, have proven to be ineffective in patients. Furthermore, patients with progressive forms of the disease are unresponsive to even the most potent FDA-approved therapeutics. This emphasizes a disparity between pre-clinical animal models and human MS, and highlights an unmet clinical need for a treatment strategy that addresses both neuroinflammation and chronic demyelination. Evidence from transplantation studies under syngeneic or immunosuppressed conditions suggests NPCs may address the latter issue. However, contrary to early presumptions, neural allografts may be antigenic, particularly in the inflamed CNS. Additionally, neurotropic viruses, including those that elicit myelin degeneration, are postulated to contribute to the onset and progression of MS in humans, and the consequences of transplanting NPCs during persistent viral infection are unclear.

Infection of susceptible mice with the neuroattenuated JHM strain of MHV results in chronic immune-mediated demyelination that recapitulates histopathological and clinical features of human MS. Studies in the MHV model have provided valuable insight regarding the therapeutic potential of murine NPC isografts. However, in practice, NPCs are unlikely to come from a genetically identical donor. Therefore, examination of host immune response during allogeneic and xenogeneic NPC transplantation provides a more relevant perspective.

Chapter 2 demonstrates a role for the adaptive immune system in rejection of allogeneic NPCs. NPCs isolated from post-natal day 1 C57BL/6 mice constitutively expressed costimulatory molecules CD80 and CD86 and could be induced to express MHC

I/II when exposed to IFN-γ or media conditioned with cytokines secreted by MHC-mismatched T cells. *In vivo*, intraspinal transplantation of allogeneic NPCs into BALB/c mice previously inoculated with MHV led to rejection and was correlated with increased T cell infiltration in the CNS. Importantly, injection of mice with T cell ablating antibodies prior to transplantation increased allogeneic NPC survival. These data support the notion that allogeneic NPCs are antigenic, and provide evidence of an important role for the adaptive immune system, particularly T cells, in the rapid rejection of allograft.

Chapter 3 describes a role for the innate immune system in rejection of allogeneic NPCs. Allogeneic NPCs were found to express retinoic acid early precursor transcript-1 (RAE-1), a ligand for the NK cell-activating receptor NKG2D, and RAE1+ NPCs, but not RAE1- NPCs, were directly lysed by NK cells *in vitro*. NK cells were observed to co-localize with allografted NPCs *in vivo* and administration of an inhibitory antibody raised against NKG2D increased survival of the graft. Allogeneic NPCs were also infected by MHV following engraftment into inoculated mice, and *in vitro* MHV infection of NPCs led to enhanced NK cell-mediated lysis. This provides evidence for allorecognition by NK-cells mediated through the RAE-1/NKG2D signaling axis and clarifies the involvement of innate immunity in NPC rejection.

Chapter 4 is an *in vitro* analysis of the consequences of MHV infection in transplantable NPCs. NPCs are shown to support MHV replication in a CEACAM-1 dependent manner. Infection leads to a reduction in IFN-γ-inducible MHC II expression and ultimately increased cell death. The inflammatory milieu produced by CD4+ T cells inhibited virus production, and CD8+ T cells lysed NPCs expressing viral antigens. This

provides evidence that NPCs can support productive neurotropic virus infection and implicates T cells as important regulators of MHV infection in NPCs.

Chapter 5 evaluates the therapeutic potential of hiPSC-NPCs in mice with chronic demyelination as a result of MHV infection. Previously, intraspinally transplanted hESC-NPCs were shown to dampen neuroinflammation, induce remyelination, and promote sustained recovery in MHV-infected mice. The therapeutic benefit of hESC-NPCs was associated with elevated Treg numbers in the CNS. In contrast, transplantation of hiPSC-NPCs induced only focal remyelination, and suppression of immune cell infiltration was limited to CD4+ T cells. Tregs were induced by hiPSC-NPCs *in vitro* and were detected in the draining cervical lymph nodes at early time points post-transplant, but significant clinical recovery was not observed. This chapter proposes that NPCs generated from pluripotent sources do not have equivalent therapeutic value when compared to hESC-NPCs in the context of MHV-induced disease.

Cumulatively, these studies provide novel insights into the dynamic immunological interactions between the inflamed CNS and transplanted NPCs after infection with a demyelinating virus. Antigenicity of NPCs, the capacity of NPCs to act as viral reservoirs, and the ability of NPCs to exert immune regulatory bystander effects should be taken into consideration when evaluating NPC engraftment as a therapeutic intervention for MS.

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CHAPTER TWO

MHC mismatch results in neural progenitor cell rejection following spinal cord transplantation in a model of viral-induced demyelination

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Abstract

Transplantation of syngeneic neural progenitor cells (NPCs) into mice persistently infected with the JHM strain of mouse hepatitis virus (JHMV) results in enhanced differentiation into oligodendrocyte progenitor cells (OPCs) that is associated with remyelination, axonal sparing, and clinical improvement. Whether allogeneic NPCs are tolerated or induce immune-mediated rejection is controversial and poorly defined under neuroinflammatory demyelinating conditions. We have used the JHMV-induced demyelination model to evaluate the antigenicity of transplanted allogeneic NPCs within the central nervous system (CNS) of mice with established immune-mediated demyelination. Cultured NPCs constitutively expressed the co-stimulatory molecules CD80/CD86 and IFN-γ treatment induced expression of MHC class I and II antigens. Injection of allogeneic C57BL/6 NPCs (H-2^b background) led to a delayed type hypersensitivity (DTH) response in Balb/c (H-2^d background) associated with T cell proliferation and IFN-γ secretion following co-culture with allogeneic NPCs. Transplantation of MHC-mismatched NPCs into JHMV-infected mice resulted in increased transcripts encoding the T cell chemoattractant chemokines CXCL9 and CXCL10 that correlated with increased T cell infiltration that was associated with NPC rejection. Treatment of MHC-mismatched mice with T cell subset-specific depleting antibodies increased survival of allogeneic NPCs without affecting commitment to an oligodendrocyte lineage. Collectively, these results show that allogeneic NPCs are antigenic and T cells contribute to rejection following transplantation into an inflamed CNS suggesting that immunomodulatory treatments may be necessary to prolong survival of allogeneic cells.

2.1 Introduction

Multiple sclerosis (MS) is the most common cause of neurological disability in young adults (1). The etiology of MS is thought to be multi-factorial including genetic, and environmental factors that may lead to initiation, maintenance and/or progression of disease (2). For example, viral infection has long been considered a potential triggering mechanism involved in demyelination and numerous human viral pathogens have been suggested to be involved in eliciting myelin-reactive lymphocytes and/or antibodies that subsequently infiltrate the central nervous system (CNS) and damage the myelin sheath (3-6). Therefore, viral models of demyelination are clearly relevant and have provided important insight into mechanisms associated with disease initiation, neuroinflammation, demyelination, and remyelination. An important clinical aspect related to the pathogenesis of MS is the eventual remyelination failure in chronic demyelinated plaques by endogenous oligodendrocyte progenitor cells (OPCs) (7-9). With this in mind, cell-based therapies using neural progenitor cells (NPCs) have emerged as a potentially viable approach for promoting remyelination (10, 11). Our laboratory has recently demonstrated that transplantation of syngeneic mouse NPCs into mice persistently infected with the neurotropic JHM strain of mouse hepatitis virus (JHMV) is well tolerated and is associated with axonal sparing accompanied by extensive remyelination while not significantly dampening either neuroinflammation or T cell responses (12, 13). Evident from this work is the ability of engrafted NPCs to i) migrate to and colonize regions of demyelination by responding to the chemokine ligand CXCL12 (14) ii) preferentially differentiate into cells of an oligodendrocyte linage (14, 15), and iii) promote axonal sparing and remyelination (15).

NPC transplantation offers a promising therapeutic approach for promoting remyelination in patients with demyelinating disease such as MS. However, similar to solid-organ transplantation, donor specific allogeneic responses are likely to occur that may require life-long immunosuppression that elevates susceptibility to opportunistic infections and tumors. Therefore, an important and clinically relevant question related to stem cell therapies revolves around the allograft rejection of implanted allogeneic stem cells as they may not be "self-derived." This is particularly important when considering cellular transplantation for treatment of chronic neurodegenerative diseases as ongoing debate has centered on whether MHC matching is critical for successful engraftment into the CNS. Compelling evidence argues that unmatched grafts are well-tolerated within the CNS due to muted immunogenicity of NPCs and clinical studies supporting that transplantation of allogeneic NPCs results in prolonged survival (16-18). However, the immunoprivileged status of NPCs has recently been questioned (19) and more recent studies argue that MHC mismatching diminishes survival of NPCs and mutes endogenous neurogenesis, and this is associated with innate immune responses (20). Moreover, transplantation of allogeneic NPCs in a model of spinal cord injury results in activation of the immune system and NPC rejection (21). These findings indicate that in order for long-term engraftment of NPCs to be efficacious the use of immunomodulatory agents must be considered. With this in mind, it is imperative to determine if allogeneic NPCs are antigenic following transplantation into an environment with established inflammatory demyelinating disease. To this end, we provide evidence that allogeneic NPCs are recognized as foreign and infiltrating lymphocytes contribute to rejection following transplantation into JHMV-infected mice with established demyelination.

2.2 Materials and Methods

Animals and virus

Age-matched (5-7wk) C57BL/6 (H-2^b, National Cancer Institute (NCI), Frederick, MD) and Balb/c (H-2^d, NCI) mice were infected intracranially (i.c.) with 150 (C57BL/6) or 15,000 (Balb/c) plaque forming units (PFU) of MHV strain J2.2v-1 (JHMV) in 30 µl sterile HBSS for transplantation studies or intraperitoneally (i.p.) with $2.50x10^5$ PFU of the DM strain of JHMV suspended in 200 µl sterile HBSS for T cell conditioned media (CM) preparation (14). Mice were sacrificed at various days post infection (p.i.) and spinal cords were removed and processed for analysis. All experiments were approved by the University of California, Irvine Institutional Animal Care and Use Committee.

Cell culture, reagents, and transplantation

Enhanced Green Fluorescent Protein expressing NPCs (GFP-NPCs), derived from C57BL/6 mice, were cultured as previously described (14). Undifferentiated GFP-NPCs were transplanted (2.5×10^5 in 2.5 µl HBSS/mouse) at spinal cord T9 at day 14 post-infection (p.i.) into C57BL/6 (syngeneic) and Balb/c (allogeneic) mice. As previously published, this time point for transplantation was chosen since virus and inflammation has waned while demyelination has peaked (14). As a sham control, virally-infected mice were transplanted with HBSS alone (15). Recombinant mouse IFN-γ was purchased from Cell Sciences (Canton, MA).

Mixed Lymphocyte Reaction (MLR)

An MLR is an *in vitro* method for assaying T cell proliferation in response to alloantigen. Cells were isolated from 4-5 pooled spleens harvested from naive and infected mice at day 21 p.t. Responding T cell populations were purified by negative selection using a pan T cell isolation kit (Miltenyi Biotec, Auburn, CA) with an MS column, composed of ferromagnetic spheres capable of separating magnetically labeled and unlabeled cells when placed in the column attached to a magnet (Miltenyi Biotec). The loaded column was washed 5x with 3mL HBSS to remove and collect all unlabeled cells. Cells were further separated into CD4+ and CD8+ populations by staining with PE-conjugated CD4 (BD Biosciences, clone GK1.5) and PE-Cy7-conjugated CD8 (eBioscience, clone 53-6.6) antibodies and sorting with a FACSAria (BD Biosciences, Franklin Lakes, NJ). Aliquots of enriched T cells were stained for 8 min at room temperature with 5μm eFluor670 (EF670) labeling dye (eBioscience), which has an excitation of 647nm and is detected with an APC filter. As cells divide, dye expression is dampened by being evenly distributed into daughter cells. After staining, cells were immediately washed 2x with PBS + 2% fetal calf serum. Stimulating populations of cells included control splenocytes and NPCs that were treated with 50μg/ml mitomycin C (AG Scientific, San Diego, CA) for 30 min at 37°C. Cells were washed 5x with 15mL HBSS. $1x10^5$ stimulators and $1x10^5$ responders were plated together in a round bottom 96-well plate in the presence or absence of recombinant mouse IFN-γ (100U/ml). Co-cultures of cells were incubated for 5 days and number of dividing responder T cells isolated from spleens of Balb/c mice at day 21 p.t. was compared to number of dividing responder T cells isolated from spleens of naive Balb/c mice for statistical significance.

Enzyme linked immunosorbent spot (ELISPOT) assay

An ELISPOT assay captures secreted proteins on a specific antibody-coated microplate and can be used to determine memory T cell activation by detecting IFN-γ secretion. The frequency of alloreactive T cells was assessed by performing a 48 h MLR with purified T cells in 96 well Multiscreen-IP plates (Millipore, Billerica, MA). Responder T cells were purified from the spleens of naive Balb/c mice and Balb/c mice at day 21 p.t. by MACS sorting as described above. Stimulator cell populations were C57BL/6 splenocytes or GFP-NPCs. Stimulators and responders were incubated as previously described above with a minimum of 6 wells per experimental condition. Briefly, plates were coated with 4μg/ml IFN-γ capture antibody (eBioscience, Clone AN-18) prior to MLR. Plates were washed with 0.01% Tween 20/PBS, followed by a 2 h incubation at 37° C with 0.5μg/ml biotinylated IFN-γ detection antibody (eBioscience, clone R4-6A2). Plates were washed with PBS/Tween 20 and incubated with 1:1000 Streptavidin AP (Invitrogen, Carlsbad, CA) for 45 minutes at room temperature. Plates were washed with PBS only, and then incubated with 100μl per well of BCIP/NBT (Sigma, St. Louis, MO) until spots developed. Spots were counted using a dissection microscope and Repeated Measures ANOVA was performed for statistical analysis.

Delayed Type Hypersensitivity (DTH) Assay

A DTH assay was employed to determine if memory T cells were present in an antigen-sensitized animal. Naïve Balb/c or C57BL/6 mice were sensitized with a subcutaneous flank injection of C57BL/6 splenocytes ($50x10^6$ cells) or GFP-NPCs ($15x10^6$ cells) and 8 days later were challenged with a footpad injection of either $10x10^6$ C57BL/6 splenocytes or $2.5x10^6$ NPCs. Footpad swelling was measured at defined times post-footpad injection

with a digital micrometer and measurements were normalized to measurements from sham-sensitized mice. Alternatively, JHMV-infected Balb/c mice at day 21 p.t. with GFP-NPCs were challenged with a footpad injection of either $10x10^6$ allogeneic splenocytes or $2.5x10^6$ allogeneic NPCs and footpad swelling determined.

Histopathology

Animals were euthanized by inhalation of halothane (Sigma, St. Louis, MO) and fixed by cardiac profusion. The spinal cord was extracted and processed for OCT and resin embedded sections as previously described (12). The number of GFP-positive cells was counted on at least two sections 80μm apart from each tissue block for each animal. Counts from experimental mice were averaged and data presented as average$\pm$SD. For immunofluorescent staining, rat-anti-platelet derived growth factor-α (PDGFRα, 1.67μg/ml; eBioscience, San Diego, CA) was used. Secondary antibody used for visualization was Alexa 594 goat anti-rat (Invitrogen). DAPI Fluoromount-G (Southern Biotech, Birmingham, AL) was used to visualize nuclei.

T cell conditioned media (CM).

C57BL/6 mice were infected with an i.p. injection of $2.5x10^5$ PFU of DM virus. At day 8 p.i., CD4 and CD8 T cells were isolated from spleen by negative selection followed by FACS to enrich for T cell subsets. Antigen presenting cells (APCs) were isolated by collecting the column bound non-T cells. Enriched APCs were treated with 50μg/ml mitomycin-C (AG Scientific) and $35x10^6$ APCs + 5μm CD4 specific [membrane (M) glycoprotein spanning amino acid residues 133-147 (M133-147), Bio-Synthesis, Lewisville, TX] or 5μm CD8

specific [spike (S) glycoprotein spanning amino acid residues 510-518 (S510-518), Bio-Synthesis] viral peptide were co-cultured with $35x10^6$ CD4+ or CD8+ T cells in a 25mm culture dish with 10ml GFP-NPC media for 48 h. Following incubation, CD4 and CD8 T cell CM was administered to GFP-NPCs for 18, 24, or 42 h.

Quantitative real-time PCR

Total RNA was extracted from homogenized spinal cord of JHMV infected, NPC or sham transplanted C57BL/6 and Balb/c mice at days 1, 8, and 21 p.t. and cDNA was generated as previously described (22). Quantitative real-time Taqman analysis for HPRT and eGFP with previously described primers and probes (23, 24) was performed using a BioRad (Hercules, CA) iCycler instrument according to the manufacturer's instructions. Expression of eGFP was normalized to HPRT. Primers were purchased from Invitrogen and the probe was purchased from Integrated DNA Technologies (Coralville, IA). Bio-Rad iQ Supermix was used for the reactions. Data were analyzed using the Bio-Rad iCycler iQ version 3.0a software and quantified using the relative expression software tool, version 2 (25). The real-time SYBR green analysis for GAPDH, CD4 and CD8 was performed using previously described primers (26) with a BioRad SYBR green kit on a BioRad iCycler. The primers for CXCL9, CXCL10, and IFN-γ are: CXCL9 forward: TTT TCC TTT TGG GCA TCA TCT T, CXCL9 reverse: AGC ATC GTG CAT TCC TTA TCA CT; CXCL10 forward: TCA GCA CCA TGA ACC CAA G, CXCL10 reverse: CTA TGG CCC TCA TTC TCA CTG; IFN-γ forward: CTT TGG ACC CTC TGA CTT GAG, IFN-γ reverse: TCA ATG ACT GTG CCG TGG. CD4, CD8, CXCL9, CXCL10, and IFN-γ mRNA expression was normalized to GAPDH mRNA expression.

Lymphocyte enrichment from spinal cord

Cells were isolated from spinal cords from experimental mice as previously described (27-30). Briefly, spinal cords were removed from PBS-perfused mice and isolated tissue was ground with the rubber end of a 5mL syringe plunger in a 100mm petri dish, followed by vigorous trituration in 7mL DMEM medium. Single cell suspensions were centrifuged for 30 min at 1200 x *g* at 4°C over a discontinuous percoll gradient at which point percoll and lipid layers were removed. Isolated cells were filtered, washed with 15mL DMEM, centrifuged at 1000 x *g* at 4°C, counted, and prepared for flow cytometry (see below). Cells isolated from spleens were used as positive controls.

Flow Cytometry

Lymphocytes isolated from the spinal cord were immunophenotyped with fluorescent antibodies (1:200) for the following cell surface markers: PE-conjugated CD4 (GK1.5; BD Biosciences) and PE-Cy7-conjugated CD8 (Ly-2; BD Biosciences). Appropriate isotype controls were used for each antibody. Cells were run on a FACStar flow cytometer (BD Biosciences) or LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star, OR). NPCs were trypsinized with 0.05% trypsin (Invitrogen) and immunophenotyped with fluorescent antibodies (1:200) specific for the following cell surface markers: PE-conjugated MHC class I (eBioscience), MHC class II (BD Biosciences), CD80 (eBioscience) and CD86 (eBioscience). All cells for flow cytometry were FC blocked with anti-CD16/32 (1:200; BD Biosciences) for 20 min at 4°C.

Antibody Treatment

JHMV-infected mice were intraperitoneally (i.p.) treated with 100μg /mouse of either a depleting monoclonal antibody for CD4 [GK1.5, American Type Culture Collection (ATCC, Rockville, MD) TIB 207] or CD8 (Ly-2.2, 2.43, ATCC TIB 210), or control Rat IgG (Sigma) in 150μl sterile saline at days -1, 1, 3, 5, 12, and 19 p.t. The initial time point was chosen to ensure depletion occurs after viral clearance but begins prior to NPC transplantation. Delivery of antibody every other day allowed for efficient depletion of cells which was then switched to once a week for maintenance of cell depletion. These time points followed previously published guidelines (31). Various concentrations were tested and sufficient depletion as determined by flow analysis of spleen was achieved at 100μg/mouse/time point.

Statistical Analysis

Statistical analysis was carried out by student's T-test, one-way Anova, or Repeated Measures ANOVA and $p \leq 0.05$ was considered significant.

2.3 Results

NPCs express MHC class I and II in response to IFN-γ treatment

Cultured GFP-NPCs (derived from C57BL/6 mice, H-2^b background) were treated with either medium alone or IFN-γ (100U/ml) and expression of MHC class I and II antigens were measured by flow cytometry. Expression of both MHC class I and II antigens increased over time as determined by staining at 18, 24, and 42 hours post-treatment when compared to medium only-treated cultures (**Figure 2.1**). Medium only-treated GFP-NPCs expressed <1% MHC class I and II. The kinetics of expression in response to IFN-γ treatment were different between the two surface antigens with 89.9±0.7% of cells expressing MHC class I by 18 h and ultimately peaking to 99.5±0.2% at 42 h (**Figures 2.1A and B**). In contrast, 2.2±0.9% of IFN-γ-treated cells expressed detectable MHC class II by 18 h and this reached 21.4±0.6% by 42 h (**Figures 2.1A and B**). These data demonstrate that while cultured NPCs are capable of expressing both MHC class I and II, expression of MHC class I is markedly more rapid and robust when compared to MHC class II. Examination of CD80 and CD86 expression revealed that cultured NPCs constitutively expressed co-stimulatory molecules and exposure to IFN-γ did not augment expression of either of these molecules (**Figures 2.1C and D**). We next evaluated whether allogeneic NPCs could evoke a T cell-mediated response. Balb/c mice (H-2^d background) were immunized with splenocytes derived from C57BL/6 mice (H-2^b background) and challenged via footpad injection (8 days post-immunization, p.i.) with either NPCs or splenocytes derived from either C57BL/6 mice or Balb/c mice. Within 24 h post-challenge, immunized Balb/c mice exposed to MHC-mismatched NPCs or splenocytes displayed increased footpad swelling with splenocyte challenged mice displaying greater swelling

(0.36±0.12mm, $p<0.01$, n=3) as compared to NPC challenged mice (0.18±0.04mm, $p<0.05$, n=3). By 48 h, swelling had decreased in splenocyte-challenged mice yet increased in NPC-challenged animals (0.31±0.02mm, $p<0.01$), indicating that although the memory response following splenocyte immunization was stronger against splenocytes than NPCs, NPCs were capable of inducing a memory T cell response (**Figure 2.1E**).

NPCs express MHC class I and II in response to treatment with conditioned media (CM) from antigen-sensitized T cells

CD4+ and CD8+ T cells were isolated from the spleens of C57BL/6 mice at 8 days p.i. with JHMV. Enriched T cell subsets were co-cultured with antigen presenting cells (APCs) from uninfected C57BL/6 mice pulsed with viral peptides corresponding to the CD4 specific epitope within the membrane (M) glycoprotein spanning amino acid residues 133-147 (M133-147) or CD8 specific epitope within the spike (S) glycoprotein spanning amino acid residues 510-518 (S510-518) in order to stimulate virus-specific T cells. After 48 h, media was collected and cultured GFP-NPCs were treated for defined periods of time and expression of MHC class I and II measured by flow cytometry. Similar to IFN-γ treatment, CM from antigen sensitized CD4 and CD8 T cells induced MHC class I expression on cultured GFP-NPCs at 18, 24, and 42 h (**Figures 2.2A and B**). In addition, there was a significant increase in the frequency of MHC class II-positive NPCs following treatment with CM from viral peptide stimulated populations of CD4+ and CD8+ T cells relative to IFN-γ treatment (**Figures 2.2A and B**) . While MHC class I expression was not appreciably increased on GFP-NPCs following incubation with CD4+ and CD8+ T cell conditioned media relative to IFN-γ, MFI analysis determined that the level of MHC class I expression per cell

was increased approximately 2-fold compared to IFN-γ treatment, although this diminishes after prolonged exposure to IFN-γ (**Figures 2.2C and D**). Conversely, while MHC class II was expressed a greater frequency of GFP-NPCs following incubation with CD4+ and CD8+ T cell CM, the amount of MHC class II per cell was not appreciably increased. Although the MFI of MHC class II increased at the later time points relative to IFN-γ this was likely due to increased number of cells expressing MHC class II, as shown by the histogram in **Figure 2.2C**. These data demonstrate that antigen sensitized CD4+ and CD8+ T cells are capable of inducing MHC class I and MHC II expression on NPCs and the overall increased expression represents the presence of additional proinflammatory cytokines working synergistically with IFN-γ being secreted from virus-specific T cells.

Allogeneic NPCs are rejected following transplantation into JHMV-infected mice

Infection of susceptible mice with JHMV induces an acute encephalomyelitis followed by an immune-mediated demyelinating disease that results in clinical and histological disease with similarities to the human demyelinating disease MS (27, 32-35). C57BL/6 (H-2^b background) and Balb/c (H-2^d background) mice were infected i.c. with JHMV and subsequently transplanted by intraspinal injection with GFP-NPCs (H-2^b background) at day 14 p.i. which represents a time in which demyelination is established (14, 15, 36, 37). Recipient mice were not treated with any immunomodulatory drugs and animals were sacrificed at 8 days and 3 weeks p.t. Survival of engrafted cells was determined by visualizing expression of GFP from transplanted cells (14) and PCR amplification of GFP mRNA transcripts. Syngeneic transplant of GFP-NPCs into infected C57BL/6 mice resulted in extensive migration along the spinal cord both rostral and caudal to implantation site

and preferentially colonized within areas of white matter damage (**Figure 2.3A**). Conversely, allogeneic GFP-NPCs, transplanted into JHMV-infected Balb/c mice, were detected at day 8 p.t. but not at day 21 p.t. (**Figure 2.3B**). Quantification of transplanted cells, as determined by counting GFP-positive cells in defined areas rostral and caudal to the implantation site, revealed a significant difference in the number of GFP-NPCs present in the spinal cords of syngeneic transplanted mice compared to allogeneic transplanted mice at day 21 p.t. (**Figure 2.3C**). Further, determination of GFP expression by mRNA by quantitative PCR revealed a >180-fold decrease in signal intensity in allogeneic transplant at day 21 p.t. relative to day 1 p.t. as compared to syngeneic recipients in which there was an ~9-fold increase ($p \leq 0.001$) in GFP transcript levels at day 21 p.t. relative to day 1 p.t., consistent with earlier findings that engrafted cells proliferate (**Figure 2.3D**)(14).

Elevated T cell inflammation into the spinal cords of allogeneic recipients

Spinal cords from mice receiving either syngeneic or allogeneic NPCs were isolated and lymphocyte infiltration was determined. By day 8 p.t., both CD4+ ($p<0.05$) and CD8+ T cell infiltration was elevated in comparison to non-transplanted JHMV-infected mice as determined by flow cytometric analysis (**Figure 2.4A)**. There was not a significant increase of either CD4 or CD8 T cells following syngeneic transplant (**data not shown**). We next determined if transcripts associated with T cell infiltration were elevated in allogeneic transplanted mice compared to non-transplanted mice. At day 1 p.t. transcripts for the T cell chemoattractant CXCL10 **(Figure 2.4B)** and day 8 p.t. the chemokines CXCL9 and CXCL10 and the cytokine IFN-□ **(Figure 2.4C)** were significantly increased ($p<0.05$) in the allogeneically transplanted spinal cord relative to sham transplant. There was no

significant difference in CXCL9, CXCL10, or IFN-γ following syngeneic transplant (**data not shown**).

T cells are sensitized to allogeneic NPCs

We next determined if T cells isolated from transplanted mice were sensitized to allogeneic NPCs by measuring T cell proliferation in response to NPC co-culture through us of an MLR assay. To ensure T cells proliferation was not caused by potential contamination of APCs during the isolation, T cells were enriched from the spleens of recipient mice and subsequently sorted by FACS to isolate specific CD4+ and CD8+ T cell populations (>99% purity, **Figure 2.5A**). Co-cultures of T cell subsets from mice at day 21 p.t. mice showed increased CD4+ T cell proliferation in response to exposure to either MHC-mismatched splenocytes as well as NPCs treated in medium alone or IFN-γ as compared to CD4+ T cells from naïve non-transplanted Balb/c mice (**Figure 2.5B**). Notably, IFN-γ treatment did not significantly alter T cell proliferative response. Although CD8+ T cells exhibited a similar response to MHC-mismatched splenocytes as CD4+ T cells, there was a comparatively muted response to NPCs (**Figure 2.5B**). Further, ELISPOT analysis revealed increased ($p<0.05$) numbers of IFN-γ-producing T cells following co-culture with either C57BL/6 splenocytes or NPCs used as stimulators compared to T cells obtained from naive non-transplanted mice (**Figure 2.5C**). We next tested whether there was an antigen-recall response to transplanted allogeneic NPCs *in vivo* by measuring a DTH response. Naive non-transplanted Balb/c mice or GFP-NPC-transplanted Balb/c mice received a footpad injection of GFP-NPCs at day 21 p.t. The majority of injected GFP-NPC transplanted mice (75%; 6 out of 8) exhibited increased footpad swelling relative to naive non-transplanted

controls by 24 h post footpad injection, and this increased to ~90% of mice responding by 48 h following injection. At 24 h, the footpad swelling in mice that received intraspinal NPCs (0.42±0.12mm) was significantly ($p<0.01$) greater than swelling in naive non-transplanted control mice (0.18±0.10mm, **Figure 2.5D**). As controls, naïve Balb/c or C57BL/6 mice received a subcutaneous injection of GFP-NPCs and 8 days later challenged with GFP-NPCs and footpad swelling measured at days 1, 2, and 4 post-injection. Challenge of Balb/c mice injected with allogeneic GFP-NPCs resulted in a significant ($p<0.05$) increase in footpad swelling at days 2 and 4 post-challenge compared to syngeneic confirming that GFP-NPCs were antigenic within the context of allogeneic injection whereas syngeneic injection elicits no immune response (**data not shown**).

T cell depletion increases survival of allogeneic NPCs

As an additional test to confirm the importance of T cells in contributing to rejection of MHC-mismatched NPCs, JHMV-infected Balb/c mice were transplanted with GFP-NPCs, treated with depleting monoclonal antibodies specific for either CD4+ or CD8+ T lymphocytes, and allogeneic cell survival determined at 3 weeks p.t. Treatment with antibodies specific for either T cell subset resulted in >98% depletion of both CD4+ and CD8+ T cells within the periphery (**Figure 2.6A, top panel**). However, within the spinal cord, antibody treatment only resulted in ~45% reduction in CD4+ and CD8+ T cell levels (**Figure 2.6A, bottom panel**). This most likely reflects limited penetration of monoclonal antibodies into the CNS and/or muted efficacy in depleting T cells within the CNS. Nonetheless, T cell depletion resulted in a significant increase in numbers of allogeneic NPCs when compared to allogeneic recipients treated with control antibody (**Figure 2.6C**).

Depletion of CD4+ T cells had the greatest effect on allogeneic NPC survival when compared to animals treated with anti-CD8+ T cells (**Figure 2.6C**). In recipients of allogeneic NPCs treated with anti-CD4, surviving NPCs accumulated within white matter tracts in which demyelinating lesions are present whereas anti-CD8 treatment restricted migration of cells which retained primarily the dorsal funiculus (**Figure 2.6B**). These observations implicate T cells as important in contributing to rejection of allogeneic NPCs. Finally, surviving allogeneic GFP-NPCs from transplanted mice treated with either anti-CD4 or anti-CD8 were capable of differentiating into PDGFRα+ oligodendrocytes, similar GFP-NPCs in a syngeneic transplant without treatment. This demonstrates that the differentiation fate of allogeneically transplanted NPCs are not affected by the antibody treatment (**Figure 2.6D)**.

2.4 Discussion

The use of neural stem/progenitor cells for treatment of human neurologic diseases is recognized as a clinically viable approach for reducing disease severity and promoting recovery. Indeed, human neural stem cells (NSCs) are currently approved for use in clinical trials for improving function in neuronal ceroid lipofuscinosis, a fatal neurodegenerative disorder in children as well as Pelizaeus-Merzbacher Disease (PMD), a fatal myelination disorder (www.stemcellsinc.com). Additionally, use of NPCs has been shown to restore cognition following transplantation into the CNS of mice with neuropathological conditions similar to those observed in patients with Alzheimer's Disease (AD)(38) and ameliorates radiation-induced cognitive dysfunction in rats (39, 40). NPCs are attractive to use for treating a broad array of human neurologic disease conditions based on evidence that NPCs are capable of differentiating into distinct glial lineages and neurons as well as promoting neurogenesis (15, 41-43). Further, ongoing clinical trials and experimental models of neurologic disease indicate there is no evidence of tumor or non-neural tissue formation that further strengthens the relevance of using these cells for disease treatment (44-46). Therefore, the potential for using NPCs for restoring functional and behavioral deficits arising from loss or damage of host CNS cells holds great promise and clinical interest. A potentially critical aspect in better understanding the biology of NPC transplantation relates to the antigenicity of these cells as well as their ability to modulate the host's immune response. Highlighting the importance of this area of research are conflicting reports indicating that NPCs are immunologically inert and well-tolerated following transplantation into MHC-mismatched animals (16) while other reports demonstrate that

both innate and adaptive arms of the immune system are activated and participate in killing allogeneic NPCs (20, 21).

Our previous studies have convincingly demonstrated that intraspinal transplantation of syngeneic NPCs into mice persistently infected with JHMV results in migration from the site of transplant with selective colonization of areas of white matter damage, enhanced differentiation into oligodendroglia accompanied by axonal sparing and remyelination (14, 15, 36). Based upon this body of work, it is clear that transplanted cells were critical in contributing to the improved histopathological outcome (14, 47). Therefore, understanding whether allogeneic NPCs survive following transplantation into JHMV-infected mice is important with regards to improved clinical and histologic outcome within the context of this model but also with regards to other models in which NPC transplantation has been shown to be beneficial. Our findings clearly demonstrate that MHC mismatched NPCs are not immunoprivileged but rather antigenic following transplantation into JHMV-infected mice with established neuroinflammatory-mediated demyelination. Evidence is provided that supports an important role for T cells in contributing to rejection as i) NPCs evoke a DTH response following footpad injection into transplanted mice, ii) co-culture of NPCs with T cells isolated from transplanted mice results in T cell proliferation and IFN-γ secretion, and iii) depletion of T cell subsets increases survival of allogeneic NPCs.

Our data demonstrating that treatment of cultured NPCs with IFN-γ increases expression of both MHC class I and II is consistent with earlier studies indicating cultured NPCs are capable of expressing these molecules in response to IFN-γ treatment (16, 20, 48). Similarly, we show that cultured NPCs do not constitutively express either MHC I and

II but detectable levels are present only in response to treatment with IFN-γ. Further, exposure of cultured NPCs with CM obtained following stimulation of virus-specific T cells not only increased the overall frequency of both MHC class I and II on NPCs but also enhanced expression on a per cell basis. These findings reflect that NPCs are sensitive to inflammatory cytokines that subsequently increase surface expression of MHC antigens. Lack of constitutive MHC expression on NPCs is consistent with other studies reporting that both embryonic stem cells (mouse and human) and induced pluripotent stem cells do not express MHC antigens (49, 50). Muted expression of MHC antigens on stem/progenitor cells has been suggested as one potential mechanism contributing to prolonged survival following transplantation into an MHC incompatible host as it would limit recognition by inflammatory T cells. We also report that co-stimulatory molecules CD80 and CD86 are constitutively expressed on NPCs and expression is not modulated following exposure to IFN-γ *in-vitro.* This is in contrast to previous reports that show that although mouse NPCs express CD80 and CD86, these co-stimulatory molecules are upregulated following IFN-γ treatment or under pathological conditions (51, 52). Collectively, these findings highlight that the environmental inflammatory cytokine milieu may be critical with regards to influencing expression of surface antigens on allogeneic NPCs that allow for detection by activated immune cells.

Our findings are in contrast with earlier studies (16, 21) indicating that NPCs possess inherent immune privilege and are capable of prolonged survival in an unsensitized host. Indeed, our findings are more consistent with Chen et al (20) indicating that allogeneic NPCs exhibit limited survival in response to transplantation into the CNS although innate, rather than adaptive, immune responses were responsible for NPC killing

in their model system. However, caution must exercised when considering these studies collectively. Both Hori et al (16) and Chen et al (20) used normal recipient mice in which no inflammatory disease conditions were present prior to transplantation.

The presence of inflammatory cytokines, such as IFN-γ within diseased tissue may be a relevant factor in contributing to allogeneic NPC survival. Exposure of allogeneic NPCs to proinflammatory cytokines upon transplant could presumably increase MHC antigen expression leading to recognition by infiltrating T cells. Consistent with this possibility is our data demonstrating that allogeneic NPCs are quickly rejected following transplantation into spinal cords of JHMV-demyelinated spinal cord, an environment where activated T cells secreting IFN-γ are readily present. Moreover, we demonstrate that NPCs are sensitive to exposure to conditioned media obtained from JHMV-specific T cells stimulated with defined viral antigens and this increases expression of MHC class I and II antigens.

Based on our results, both CD4+ and CD8+ T cells contribute to rejection as depletion of either subset increased survival of engrafted cells. However, greater numbers of inflammatory CD4+ T cells were present within the spinal cords of allogeneic NPC recipients when compared to infiltrating CD8+ T cells. Further, treatment with anti-CD4+ T cell subset depleting antibodies resulted in enhanced survival of allogeneic NPCs when compared to animals treated with anti-CD8+ T cell subset antibodies. These data support a recent report demonstrating a more important role for CD4+ T cells in immune-mediated rejection of hESC xenografts (50). Nevertheless, T cells are clearly sensitized to allogeneic NPCs following injection into MHC mismatched JHMV-infected mice as demonstrated by increased proliferation, detectable DTH responses, and IFN-γ secretion by T cells. It is important to emphasize that administration of T cell specific depleting antibodies did not

completely eliminate T cells within the CNS although these cells were efficiently depleted from the periphery.

Mechanisms by which T cells participate in killing NPCs include direct recognition of surface-bound antigen(s) that are either constitutively expressed or induced upon transplantation. Therefore, increased expression of MHC antigens on transplanted allogeneic NPCs would represent a potential target for activated T cells present within the spinal cords of persistently-infected mice. In addition, indirect recognition (*e.g.* phagocytic engulfment of dead/dying NPCs) and subsequent presentation of antigens represents an additional scenario by which T cells become sensitized to alloantigens. This is not an unreasonable scenario as many NPCs will die during injection into the spinal cord and these cells are presumably removed by activated inflammatory macrophages that could then migrate to secondary lymphatic tissue and present novel antigens to naïve lymphocytes. Alternatively, alloantigens could be shed following transplantation and presented by local APCs following phagocytic uptake. At this time, we have not distinguished whether direct and/or indirect recognition of NPC antigens is occurring in response to NPC transplantation into JHMV-infected mice. However, indirect recognition may favor a more important role for CD4+ T cells in contributing to rejection and this is consistent with our findings. Interestingly, we also demonstrate NPCs that survive following depletion of T cell subsets exhibit normal differentiation into oligodendrocyte progenitor cells (OPCs) similar to syngeneic NPC recipients. These findings argue that the local microenvironmental niche remains capable of providing necessary growth factors required for fate commitment to the oligodendroglia lineage.

Although T cells are clearly important in killing allogeneic NPC transplants, it does not rule out the possibility that cellular components of the innate immune response are also contributing to rejection. For example, NK cells have been shown to be involved in numerous models of allograft rejection including bone marrow, skin, and cornea allografts (53-55). The observed result that not every allogeneic NPC transplanted mouse yielded DTH swelling or IFN-γ production in response to allogeneic NPC or splenocyte stimulators could be due to NK cell-mediated rejection, thus limiting memory T cell formation. As recently illustrated by Palmer and colleagues (20), transplantation of allogeneic NPCs into the hippocampus of normal mice resulted in a dramatic reduction in survival and this was the result of innate immune responses directed to transplanted cells. Induction of immune suppression through treatment with cyclosporine A did not increase allogeneic NPC survival yet treatment with nonsteroidal anti-inflammatory drugs protected NPCs from destruction (20). These findings clearly illustrate the importance of innate immune responses in participating in allograft destruction and further highlight the importance of the local microenvironment in allogeneic NPC transplantation as the lingering effects of inflammation at the transplant site are likely to result in killing of allografts through both innate and adaptive immune responses.

In conclusion, our findings provide evidence that allogeneic NPCs are not immune privileged upon transplantation into an established inflammatory environment. Rejection is rapid and involves T cell responses directed against NPC alloantigens, although CD4+ T cells are a more important mediator of immune-mediated rejection. In consideration of using NPCs for treating chronic neuroinflammatory diseases such as MS, the use of

immunomodulatory drugs should be considered in order to increase survival of allografts in order to improve clinical outcome.

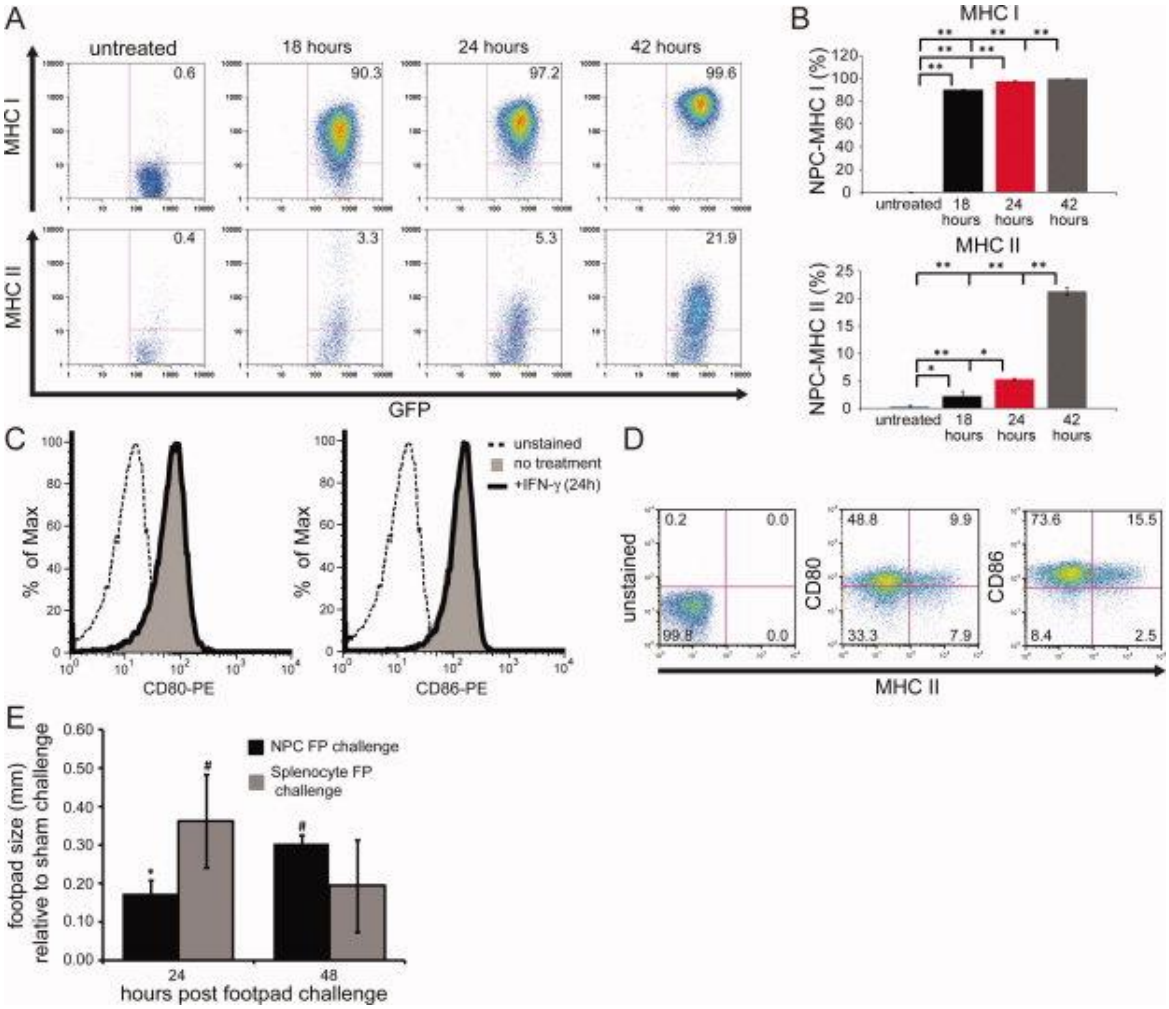

Figure 2.1: GFP-NPCs express MHC antigens and are antigenic. Cultured GFP-NPCs were treated with IFN-γ (100U/ml) for defined periods of time and MHC class I and II as well as CD80/86 determined by flow cytometry. (**A**) Representative flow analysis for MHC class I and II shown and (**B**) quantification of both class I and II induction. Data represents three independent experiments and data is shown as average±SD. (**C**) Representative histogram for CD80/86 staining show that IFN-γ does not increase expression of co-stimulatory antigens. (**D**) Dual expression of MHC class II and CD80/86 on IFN-γ-treated (24 h) GFP-NPCs. (**E**) Balb/c mice sensitized with subcutaneous injection of C57BL/6 splenocytes were challenged with either GFP-NPCs or C57BL/6 splenocytes and footpad swelling determined at 24 and 48 h post-injection. Footpad swelling in experimental mice was compared to unsensitized but footpad challenged mice. Data presented represents three independent experiments and data is presented as average±SD; * $p<0.05$, # $p<0.01$, ** $p<0.001$.

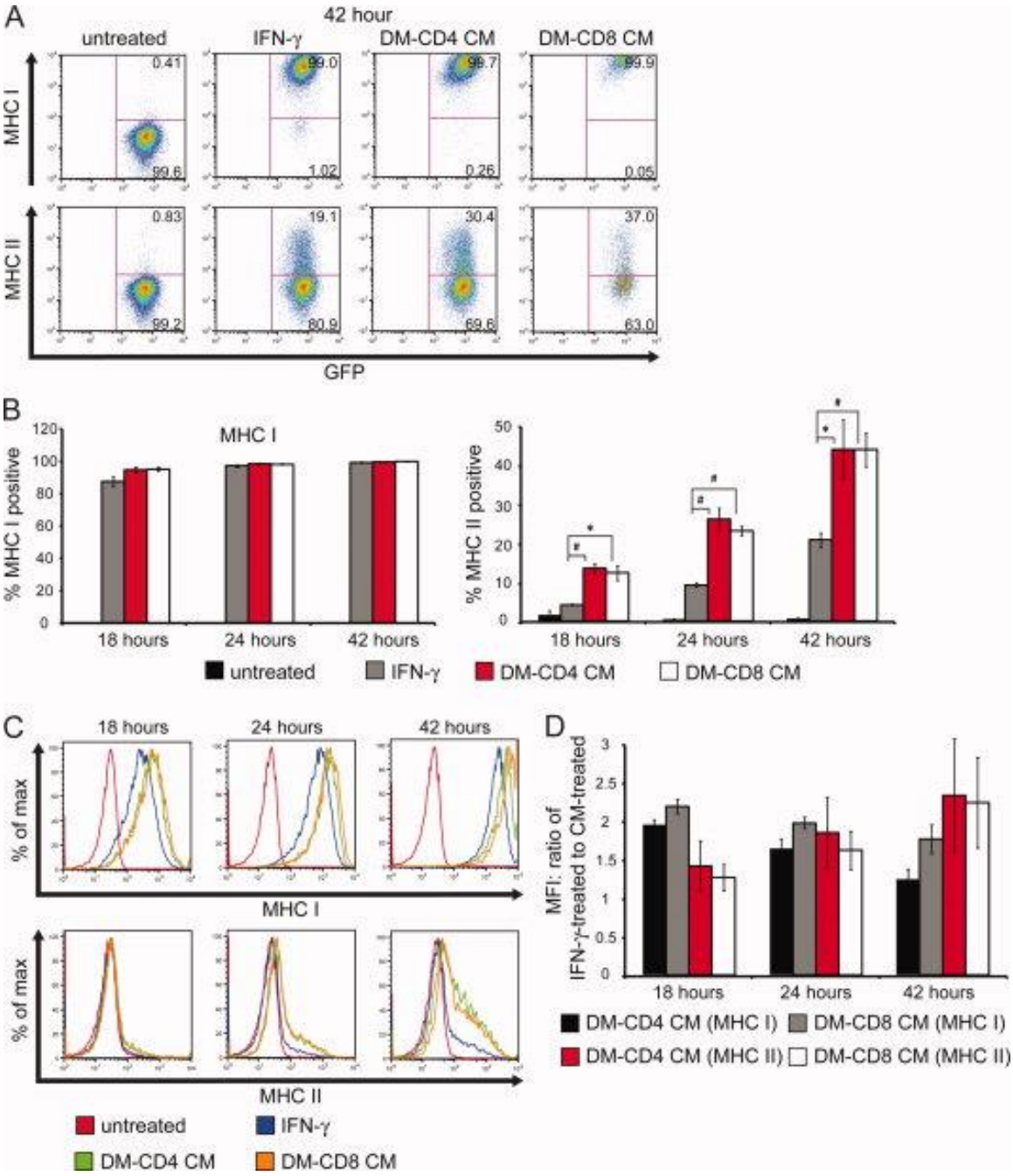

Figure 2.2: NPCs express MHC class and II in response to treatment with conditioned media from antigen sensitized T cells. Conditioned media (CM) was collected following exposure of T cells isolated from JHMV-immunized mice with defined CD4+ (M133-147) and CD8+ (S510-518) viral peptides for 48 h. Cultured GFP-NPCs were treated with either CD4 CM, CD8 CM, IFN-γ (100U/ml) or control media (untreated) for 18, 24, or 42 h and expression of MHC class I and II determined by flow cytometry. (**A**) Representative flow analysis at one time point (42 h) and (**B**) quantification of MHC I and II at 18, 24, and 42 h. Statistics are calculated for MHC expression on GFP-NPCs following treatment with CD4 or CD8 CM relative to treatment with IFN-γ; *p<0.05, #p<0.01. (**C**) Representative histogram

of MHC class I and II expression at 18, 24, and 42 h. (D) The MFI for MHC I and II expression on GFP-NPCs following incubation with CD4 or CD8 CM was determined and divided by the MFI for MHC I and II following IFN-γ treatment to determine the fold increase in MFI.

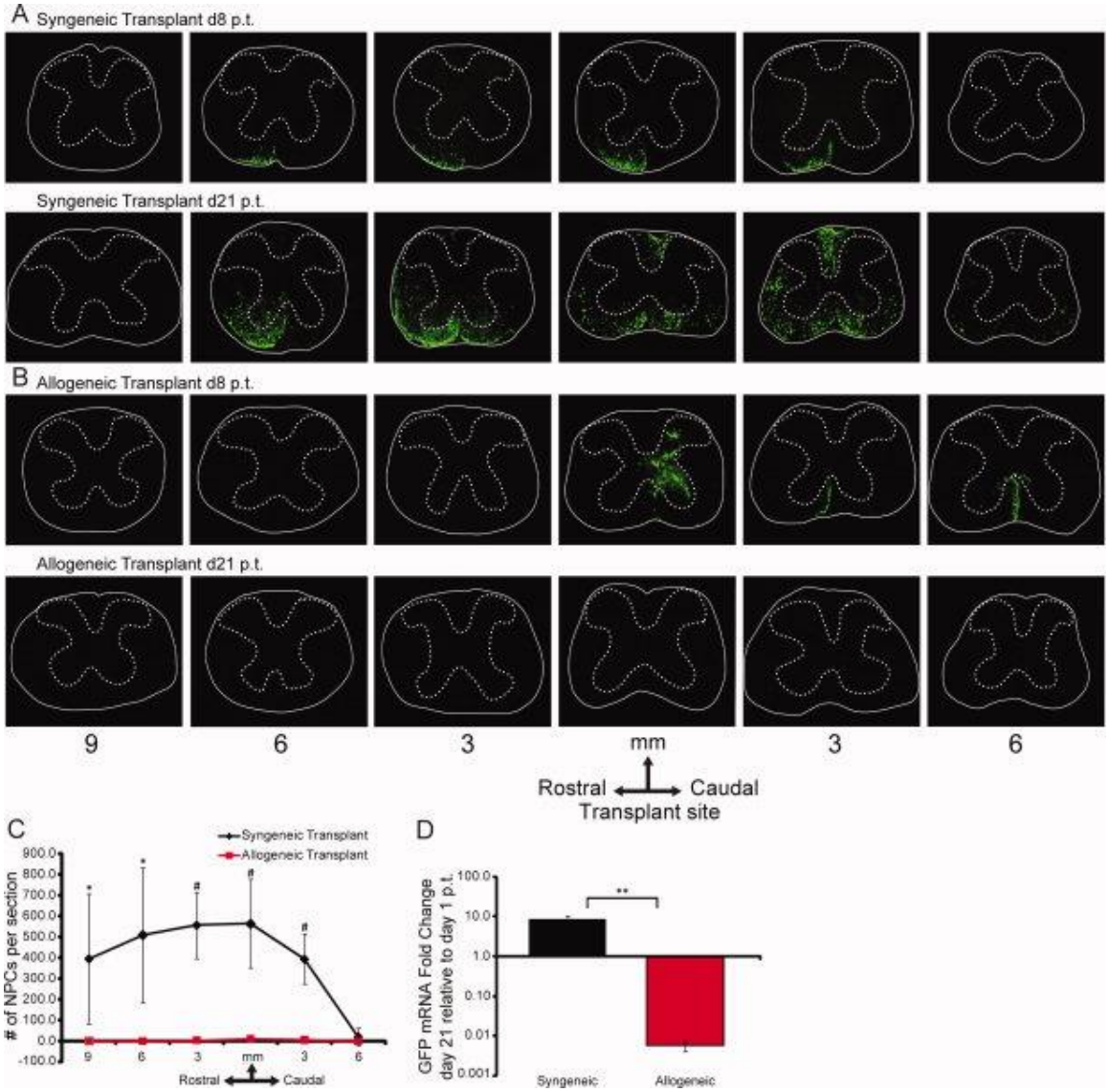

Figure 2.3: Transplanted allogeneic GFP-NPCs are rejected following intraspinal injection into JHMV-infected mice. (A, B) Representative coronal spinal cord sections obtained at defined locations rostral and caudal to the transplantation site from JHMV-infected mice receiving either syngeneic (A) or allogeneic (B) NPCs. Experimental mice were sacrificed at either 8 or 21 days p.t. and migration/survival of transplanted cells evaluated by visualization of GFP-expression from transplanted cells. (C) At day 21 p.t. migration/survival of transplanted was cells enumerated. Dual-positive DAPI and GFP NPCs were counted in coronal sections 9mm rostral and 6mm caudal to transplant site at 3mm intervals from allogeneically transplanted (n=6) and syngeneically transplanted (n=7) mice. Arrow indicates transplantation site. Increased numbers (* $p<0.05$; # $p<0.01$) of GFP-NPCs were present within the spinal cords of syngeneic recipients compared to allogeneic. (D) GFP expression (determined by quantitative RT-PCR) is increased (** $p<0.001$) within the spinal cords of mice following syngeneic versus allogeneic transplant. The fold change of GFP expression was determined at d21 p.t. relative to d1 p.t. in

syngeneically transplanted (day 1, n=3, d21: n=4) and allogeneically transplanted (day 1, n=3, d21: n=3) mice .

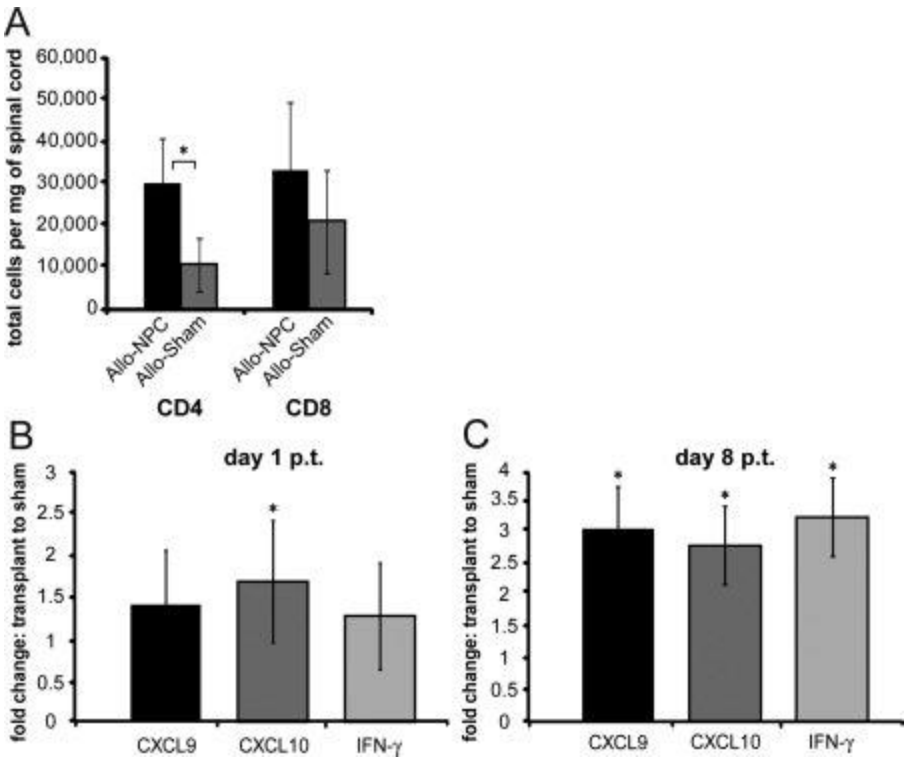

Figure 2.4: Increased CD4+ T cells in spinal cord of allogeneically transplanted mice. A) Flow cytometric analysis was performed on mononuclear cells isolated from spinal cord (9mm rostral and caudal to transplant site was used) of mice 8 days following allogeneic transplantation of NPCs or sham transplantation. Two spinal cords were pooled for each cell isolation. Cells were stained with PE-conjugated CD4 and PE-Cy7-conjugated CD8. The number of CD4+ and CD8+ T cells was determined in each NPC transplanted and sham transplanted group and normalized to mg of isolated spinal cord. B,C) CXCL9, CXCL10, and IFN-γ mRNA from spinal cords of mice allogeneically transplanted with GFP-NPC (n=9) or sham transplanted (n=7) at day 1 p.t. (B) and day 8 p.t. (C) was analyzed by qRT-PCR. C_t values were normalized to GAPDH and the ratio of each mRNA from allogeneic transplant to sham transplant at 8 p.t. was determined ($\Delta\Delta C_t$). ΔC_t for allogeneic transplant compared to ΔC_t for sham transplant was used for calculating statistical significance, *$p<0.05$. Standard error is presented as $\Delta\Delta C_t$.

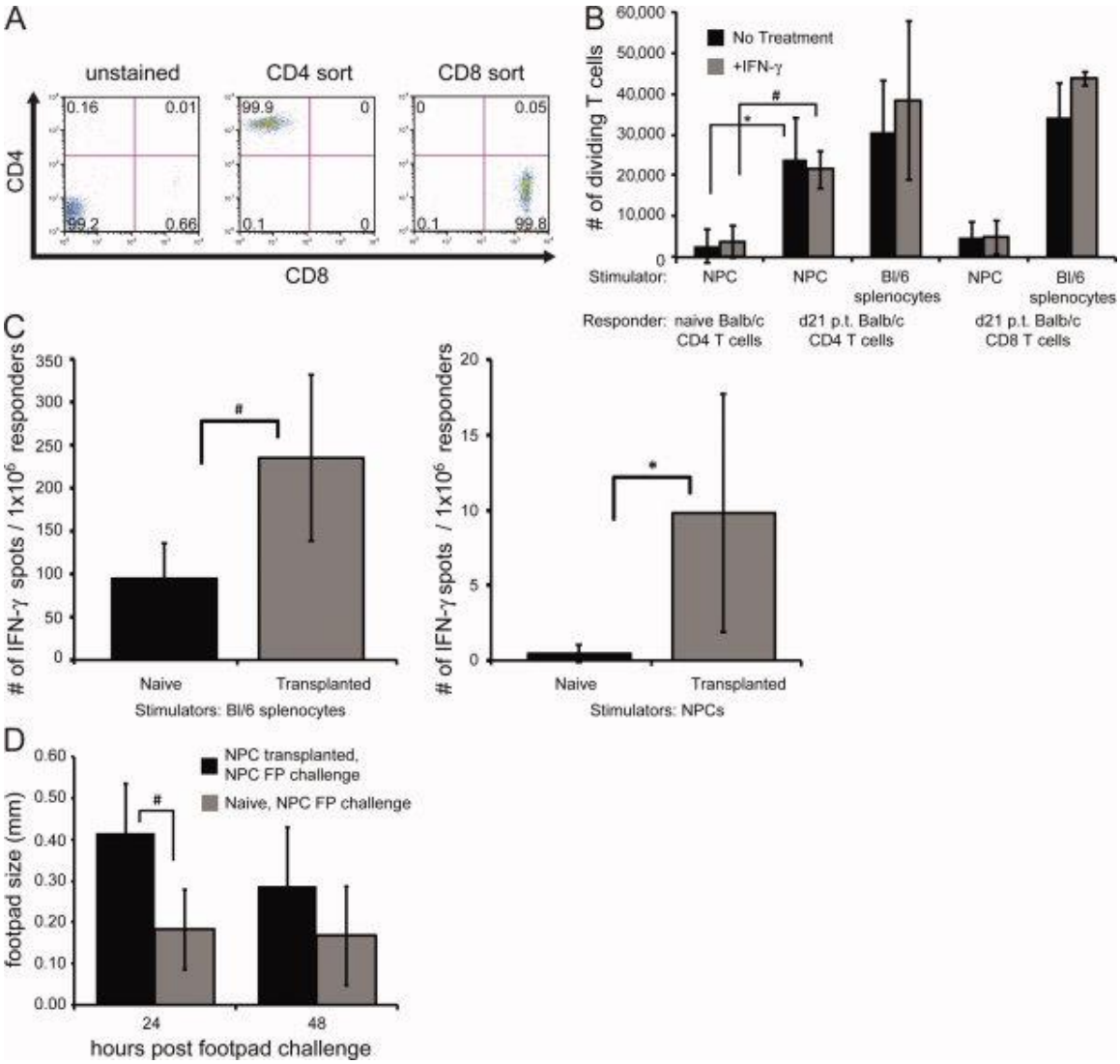

Figure 2.5: Allogeneic GFP-NPCs elicit T cell response. (A) Representative flow cytometric analysis revealing >99% purity of FACS sorted CD4+ and CD8+ T cells from spleens of mice transplanted with allogeneic NPCs at day 21 p.t. (B) Purified T cells from (*A*) were co-cultured with GFP-NPCs and splenocytes treated with either medium or IFN-γ (100U/ml) and proliferation determined by EF670 dye expression. Statistical significance was determined by comparing the number of dividing T cells from Balb/c mice at day 21 p.t. with the number of dividing T cells from naive non-transplanted Balb/c mice (* $p<0.05$, # $p<0.01$). (C) IFN-γ production from T cells isolated from spleens of mice receiving either allogeneic NPCs or naïve mice was determined by ELISPOT following exposure to GFP-NPCs or C57BL/6 splenocytes. (D) Footpad swelling was determined at 24 and 48 h post GFP-NPC footpad injection (d21 p.t.) in naive Balb/c mice and Balb/c mice that received intraspinal transplant of GFP-NPCs.

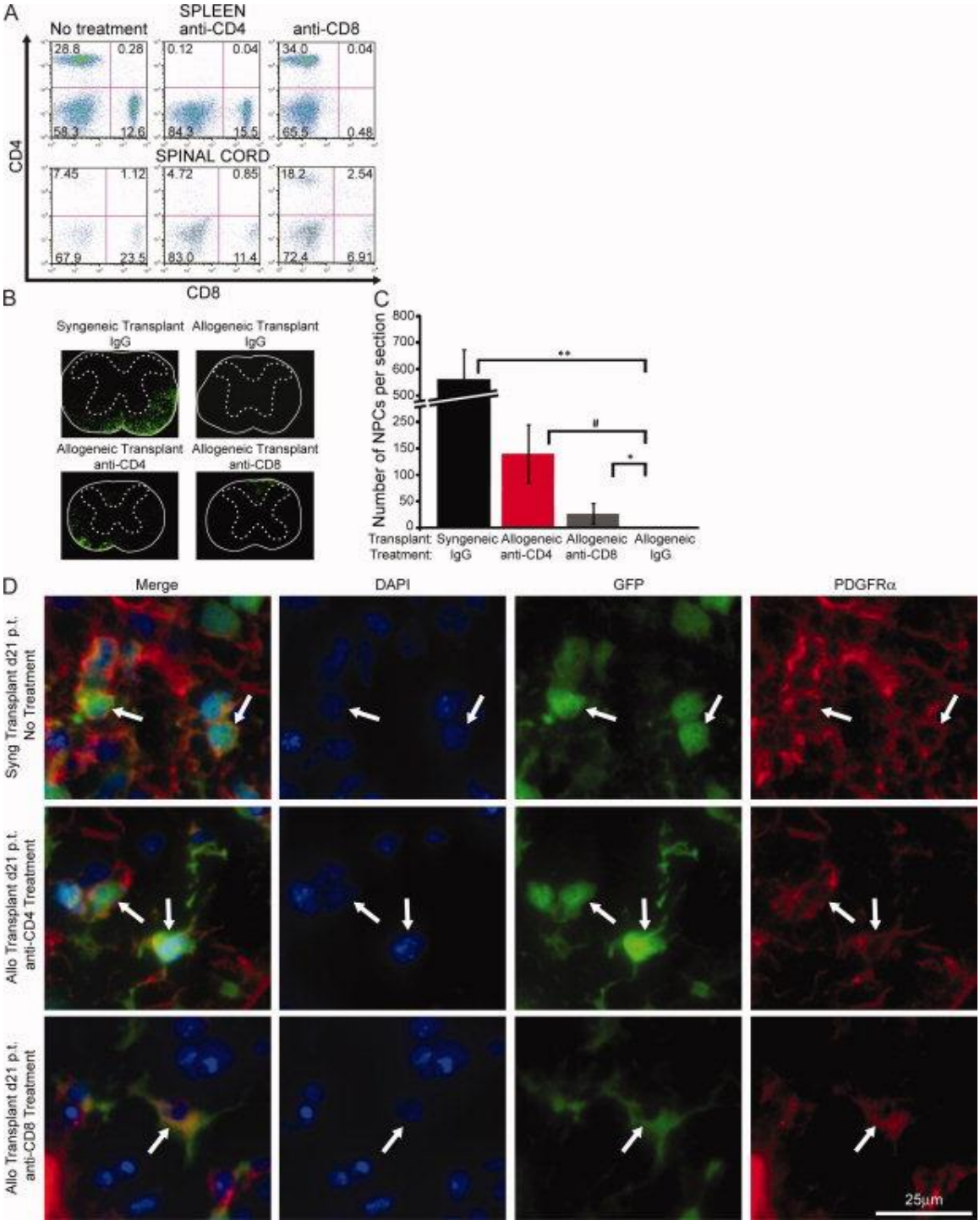

Figure 2.6: Depleting T cells increases survival of allogeneic GFP-NPCs within the spinal cords of JHMV-infected mice. JHMV infected mice transplanted with either syngeneic and allogeneic GFP-NPCs were treated with anti-CD4 (GK1.5, n=5), anti-CD8 (Ly2.2, n=3), or isogenic IgG control (n=4) at d -1, 1, 3, 5, 12, and 19 p.t. and mice sacrificed at d21 p.t. Presence of NPCs was determined by immunofluorescence imaging of GFP expression. (A) Representative flow analysis of the spleen (top panel) and spinal cord

(bottom panel) reveals the presence of CD4+ and CD8+ T cells following anti-CD4 or anti-CD8 treatment. (B) Representative spinal cord sections from transplanted mice treated with either CD4 or CD8 depleting antibodies showing the presence of GFP-NPCs. (C) Quantification of GFP-NPCs in transplanted mice treated with T cell depleting antibodies and numbers of GFP-NPCs determined along the length of the spinal cord both rostral and caudal to implantation site. Depletion of either CD4+ or CD8+ T cell subsets increases survival (* $p<0.05$, # $p<0.01$, ** $p<0.001$) in mice transplanted with allogeneic NPCs compared to mice transplanted with syngeneic NPCs. (D) Representative immunofluorescence 40x images showing PDGFRα+ (red) GFP-NPCs (green), with DAPI stained nuclei (blue), at day 21 p.t. of syngeneically transplanted mice without antibody treatment or allogeneically transplanted mice with anti-CD4 or anti-CD8 treatment.

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CHAPTER THREE

Activating receptor NKG2D targets RAE-1-expressing allogeneic neural precursor cells in a viral model of multiple sclerosis

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Abstract

Transplantation of major histocompatibility complex (MHC)-mismatched mouse neural precursor cells (NPCs) into mice persistently infected with the neurotropic JHM strain of mouse hepatitis virus (JHMV) results in rapid rejection that is mediated, in part, by T cells. However, the contribution of the innate immune response to allograft rejection in a model of viral-induced neurological disease has not been well defined. Herein, we demonstrate that the natural killer (NK) cell-expressing activating receptor NKG2D participates in transplanted allogeneic NPC rejection in mice persistently infected with JHMV. Cultured NPCs derived from C57BL/6 (H-2^b) mice express the NKG2D ligand retinoic acid early precursor transcript (RAE)-1 but expression was dramatically reduced upon differentiation into either glia or neurons. RAE-1^+ NPCs were susceptible to NK cell-mediated killing whereas RAE-1^- cells were resistant to lysis. Transplantation of C57BL/6-derived NPCs into JHMV-infected BALB/c (H-2^d) mice resulted in infiltration of NKG2D$^+$CD49b$^+$ NK cells and treatment with blocking antibody specific for NKG2D increased survival of allogeneic NPCs. Further, transplantation of differentiated RAE-1^- allogeneic NPCs into JHMV-infected BALB/c mice resulted in enhanced survival, highlighting a role for the NKG2D:RAE-1 signaling axis in allograft rejection. We also demonstrate that transplantation of allogeneic NPCs into JHMV-infected mice resulted in infection of the transplanted cells suggesting that these cells may be targets for infection. Viral infection of cultured cells increased RAE-1 expression, resulting in enhanced NK cell-mediated killing through NKG2D recognition. Collectively, these results show that in a model of viral-induced demyelination, NK cells contribute to rejection of allogeneic NPCs through an NKG2D signaling pathway.

3.1 Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) involving immune responses directed against self-antigens within the CNS resulting in neuroinflammation and demyelination (1, 2). Ultimately, myelin and axonal loss culminates in extensive disability through defects in neurological function (3-6). Although myelin repair can occur during the course of the disease, it is often transient and not sustained (7, 8). Therefore, an important unmet clinical need for MS patients is an effective method to induce sustained remyelination while limiting disease progression and ongoing demyelination (9, 10). In recent years, considerable effort has focused on cell replacement therapies through use of neural precursor cells (NPCs) to promote remyelination. Indeed, in animal models of autoimmune neuroinflammatory demyelination there is evidence that transplantation of NPCs results in improved clinical outcome accompanied by reduced neuroinflammation and myelin repair (11-15).

Using a viral model of demyelination, we have demonstrated that intraspinal transplantation of mouse NPCs into animals with established demyelination results in improved motor skills along with limited spread of demyelination accompanied by axonal sparing and remyelination (16) Intracranial infection with the neuroadapted JHM strain of mouse hepatitis virus (JHMV) results in an acute encephalomyelitis followed by chronic immune-mediated demyelinating disease similar clinically and histologically to the human demyelinating disease multiple sclerosis (MS) (17-19) While the etiology of MS is unknown, both genetic factors as well as environmental influences *e.g.* viral infection have long been considered important in triggering disease (20-23). Therefore, defining mechanisms contributing to demyelination as well as remyelination in animals in which

disease is initiated by a persistent infection with a neurotropic virus is clinically relevant. With this in mind, we have shown that following intraspinal injection of syngeneic NPCs into JHMV-infected mice, transplanted cells are well-tolerated, preferentially differentiate into cells of an oligodendrocyte lineage, and selectively colonize areas of white matter damage within the spinal cord (16, 24).

While the findings from our transplantation studies emphasize the therapeutic potential of NPCs in ameliorating disease in JHMV-infected mice, the majority of transplantation studies have utilized syngeneic NPCs for CNS engraftment and do not address the important issue of whether MHC-mismatched NPCs are recognized as foreign by the host immune system and subsequently rejected. Evidence argues that unmatched grafts are well-tolerated within the CNS due to muted immunogenicity of NPCs and clinical studies supporting that transplantation of allogeneic NPCs results in prolonged survival (25-27). However, the immunoprivileged status of NPCs has recently been questioned (28) and more recent studies argue that allogeneic NPCs exhibit diminished survival upon transplantation (29-31). Our recent studies demonstrate that transplantation of MHC-mismatched NPCs into the CNS of mice infected with the JHM strain of mouse hepatitis virus (JHMV) results in rapid rejection in which T cells participate in recognition and rejection of allogeneic cells(32). These findings, along with others (33-35), indicate an important role for T cells in contributing to rejection of foreign cells following CNS injection and argue for sustained immunosuppression using drugs targeting T cells. More recently, Palmer and colleagues (36) have provided compelling evidence indicating an important role for the innate immune system, *e.g.* natural killer (NK) cells, in recognizing and killing allogeneic NPCs via NKG2D signaling upon injection into the CNS. These studies build upon

a growing literature illustrating the importance of the innate immune system in contributing to allograft rejection (34, 37, 38).

Retinoic acid early precursor transcript (RAE)-1 is the ligand for the NK cell activating receptor NKG2D. Numerous studies have highlighted a functional role for RAE-1 as a target for NKG2D recognition and killing of virally-infected cells and tumor cells as well as contributing to allograft rejection (39-44) RAE-1 is expressed on NPCs and is thought to be important in regulating proliferation suggesting a non-immune functional role during development (45). In this study, we demonstrate that transplantation of allogeneic NPCs into the spinal cords of mice persistently infected with JHMV results in rapid rejection that is mediated, in part, through an NKG2D-dependent pathway. Further, JHMV infection of cultured NPCs increases expression of RAE-1 and these cells are susceptible to NK cell-mediated lysis that is enhanced upon NKG2D recognition. These findings support a role for NKG2D signaling in allograft rejection as well as killing virally infected NPCs in a model of viral-induced demyelination.

3.2 Material & Methods

Animals and Virus

Age-matched (5-7wk) C57BL/6 (H-2^b for syngeneic transplants, National Cancer Institute (NCI, Frederick, MD), BALB/c (H-2^d for allogeneic transplants, NCI), and SCID/NCr (H-2^d for allogeneic transplant, NCI) mice were infected intracranially (i.c.) with 150 (C57BL/6), 15,000 (BALB/c), or 2,000 (SCID/NCr) plaque forming units (PFU) of mouse hepatitis virus (MHV) strain J2.2v-1 (JHMV) in 30 µl sterile HBSS (24). SCID/NCr are immunodeficient for B and T lymphocytes but have normal numbers of NK cells, macrophages, and granulocytes. Mice were sacrificed at defined times post-infection (p.i.) by either perfusion with 1x PBS or 4% paraformaldehyde in PBS and spinal cords were removed and processed for analysis. All animal experiments were approved by the University of California, Irvine Institutional Animal Care and Use Committee.

Cell culture, transplantation, and reagents

Enhanced Green Fluorescent Protein expressing NPCs (eGFP-NPCs) were cultured in the absence of growth matrix in NPC media containing DMEM/F12+glutamax (Gibco), 10ng/ml ciproflox (Cellgro), 50ng/ml gentamicin (Sigma), 1x fungizone (Invitrogen), 1x Penicillin/streptomycin (Gibco), 1x N2 (Gibco), and 20ng/ml human EGF (Invitrogen). Cells were passaged with 0.05% trypsin for 30 sec, followed by quenching with cold NPC media. Undifferentiated or differentiated eGFP-NPCs were transplanted (2.5 x 10^5 in 2.5 µl Hank's balanced salt solution (HBSS)/mouse) at spinal cord T9 at day 14 p.i. into C57BL/6 (syngeneic) and BALB/c (allogeneic) mice or at day 7 p.i. into SCID/NCr mice. As a sham control, virally-infected mice were transplanted with HBSS alone (vehicle only) (16). eGFP-

NPC media consisted of DMEM/F12 with glutamax (1x, Gibco, cat# 10565-018), ciproflox (100 μg/ml, Cellgro), gentamicin (50μg/ml, Sigma), fungizone (2.5 μg/ml, Gibco), penicillin/streptomycin (1000 U/ml, Gibco), and N2 (1x, Gibco). The addition of human epidermal growth factor (hEGF) is necessary to maintain undifferentiated NPCs. eGFP-NPCs were differentiated by culturing on matrigel (1:25, BD Biosciences)-coated plates for 5 days in eGFP-NPC media (as previously described (16)) in the absence of hEGF (20 ng/ml, Sigma). Media were changed every other day. Recombinant mouse IFN-γ was purchased from Cell Sciences (Canton, MA). eGFP-NPCs were infected with JHMV (0.1 moi) for 24 hr at which point media were replaced and cells used for experimentation. YAC-1 cells, used as a positive control for NK cell-mediated lysis, were grown in RPMI-1640 medium with 10% fetal bovine serum, glutamax (1x), and penicillin/streptomycin (1000 U/ml).

Flow cytometry

Lymphocytes were isolated from the spinal cord (9 mm rostral and 9 mm caudal to the transplant site) of C57BL/6 and BALB/c mice on day 8 following transplantation with eGFP-NPCs or vehicle only using a discontinuous Percoll gradient as previously described (32, 46-49). Following block of FC receptors with anti-CD16 + CD32 mAb (clone 2.4G2; BD Biosciences) for 20 min at 4°C, cells were stained using the following mAbs: PerCp or PE/Cy5-conjugated anti-CD3e (BD Biosciences), PE or APC-conjugated anti-CD49b (BD Biosciences), and PE or APC-conjugated anti-NKG2D (eBioscience). Cultured eGFP-NPCs were trypsinized with 0.05% trypsin (Invitrogen), blocked with anti-CD16 + CD32 mAb as described above, and stained with either PE-conjugated anti-MHC class I (eBioscience),

anti-MHC class II (BD Biosciences), anti-pan RAE-1 (R&D Systems), or APC-conjugated anti-CD133 (Biolegend). Cells were analyzed using a FACStar flow cytometer (BD Biosciences) or LSRII flow cytometer (BD Biosciences) with FlowJo software (Tree Star, OR). All data are shown as percentage of gated single (forward scatter height versus forward scatter area) live (forward scatter versus side scatter) $eGFP^+$ cells. Appropriate isotype-matched control Ig's were used for each antibody. eGFP-NPCs were stained for RAE-1 as described above and $eGFP^+RAE\text{-}1^+$ or $eGFP^+RAE\text{-}1^-$ cells were sorted using a FACS Aria III (BD Biosciences). Sorted $RAE\text{-}1^+$ and $RAE\text{-}1^-$ eGFP-NPCs were plated in eGFP-NPC media.

NK cell isolation

NK cells were isolated from the blood of BALB/c mice using an EasySep mouse NK cell enrichment kit (Stem Cell Technologies, Vancouver, BC). Briefly, red blood cells were lysed by treatment (twice) with 2ml ACK buffer (0.15M NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA in double distilled H_20) for 90 sec at room temperature (RT). Following the final wash, cells were resuspended at $1x10^8$ cells/ml in EasySep buffer (1X PBS + 2% fetal bovine serum + 1mM EDTA) in a 14 ml polystyrene tube. Fifty µl/ml EasySep negative selection mouse NK cell enrichment cocktail was added for 15 min, followed by 200 µl/ml EasySep biotin selection cocktail for 15 min, followed by 200 µl/ml EasySep D magnetic particles for 10 min. All incubations were done at RT. Cell suspension was brought to a volume of 5 ml with EasySep buffer and placed in "the big easy" EasySep magnet for 5 min at RT. Following incubation, non-labeled cells were transferred to a new tube and counted. NK cell ($CD3^-$ $CD49b^+$) purity was determined to be >90% as determined by flow cytometry.

Non-Radioactive Cytotoxicity Assay

Cytotoxicity was determined by readout of lactase dehydrogenase (LDH) released from dying cells using a CytoTox 96 non-radioactive cytotoxicity kit (Promega, Madison, WI). eGFP-NPCs (target, T) were plated in 100 µl at $2x10^4$ cells per well in 96-well flat-bottom plates and allowed to adhere prior to the addition of NK cells (effector, E) at 20:1, 10:1, and 5:1 E:T ratio in 100 µl. Cells were incubated for 4.5 hr at 37° C, plates were centrifuged at 250 x g for 3 min and 50 µl from each well was transferred to a corresponding well of another 96-well flat-bottom plate. Fifty µl of substrate mix was added to each well, the plate was incubated for 30 min at RT in the dark, and then 50 µl of stop solution was added to each well. Absorbance was recorded for each well at 490 nm using a Synergy HT plate reader (BioTek; Winooski, VT). Control wells included NK cells only to determine spontaneous effector LDH release, eGFP-NPCs only to determine spontaneous target LDH release, eGFP-NPCs plus lysis solution (Promega) to determine maximum lysis, eGFP-NPC media only for background control, and eGFP-NPC media plus lysis solution for volume correction control. Forty-five minutes prior to harvesting supernatant, 20µl of lysis solution was added to maximum lysis and volume correction control wells. Percent cytotoxicity was calculated as:

$$\frac{\text{NK cell-mediated NPC lysis - NK cell spontaneous - NPC spontaneous}}{\text{NPC maximum - NPC spontaneous}} \times 100$$

Histopathology

Animals were euthanized by inhalation of halothane (Sigma) and fixed by cardiac perfusion. Spinal cords were extracted and processed for OCT sections as previously

described (16). The number of eGFP-positive cells was counted and data are presented as average±SEM. For immunofluorescent staining, we used mouse-anti-JHMV (specific for the nucleocapsid (N) protein, kindly provided by Dr. Stanley Perlman, University of Iowa), rat-anti-CD49b (DX5), and rat-anti-NKG2D (CX5). Alexa 594-conjugated secondary antibodies used were: goat anti-mouse (detection of anti-N; Invitrogen), goat anti-rat IgM (for anti-CD49b; Invitrogen), and goat anti-rat IgG (for anti-NKG2D; Invitrogen). DAPI Fluoromount-G (Southern Biotech, Birmingham, AL) was used to visualize nuclei. Images were taken on an Eclipse Ti inverted microscope (Nikon, Melville, NY).

Antibody Treatment

JHMV-infected mice were intraperitoneally (i.p.) treated with 100 μg /mouse of anti-NKG2D (CX5), or control Rat IgG (Sigma) in 300μl sterile HBSS at days -1, 1, 3, 5, 12, and 19 post-transplantation (p.t.) for BALB/c mice sacrificed at day 21 p.t. and at days -1, 1, 3, and 5 p.t. for SCID/NCr and BALB/c mice sacrificed at day 7 p.t.

Statistical Analysis

Statistical analysis was performed using an unpaired or paired Student's *t* test and $p \leq 0.05$ was considered significant.

3.3 Results

NK cells target RAE-1$^+$ NPCs

Expression of the NKG2D ligand RAE-1 on cultured NPCs expressing eGFP (eGFP-NPCs, derived from mice on the C57BL/6 background) was determined by flow cytometric analysis. We first determined that 85.1±1.6% of eGFP-NPCs express CD133 (**Figure 3.1A**). Subsequently, gating on the eGFP$^+$CD133$^+$ cells revealed 64.3±0.7% of dual-positive cells expressed RAE-1 while 48.3±2.0% of eGFP$^+$CD133$^-$ cells expressed RAE-1 (**Figure 3.1A**). Although we did not phenotype the CD133$^+$RAE-1$^-$ cells, we believe this population most likely represents NPCs in varying states of either proliferation and/or differentiation. Similarly, we believe the eGFP$^+$CD133$^-$ population represents cells undergoing differentiation. We next demonstrated that *in vitro* differentiation of eGFP-NPCs into glial-enriched culture (16) resulted in dramatically diminished expression of RAE-1 (<1% when compared to undifferentiated cultures), suggesting that RAE-1 is restricted to undifferentiated NPCs (**Figures 3.1B and C**). We have previously shown that treatment of cultured NPCs with the proinflammatory cytokine IFN-γ increases expression of MHC class I and II (32); however, exposure to IFN-γ (100U/ml) resulted in an average 5.4±1.5% reduction in RAE-1 expression compared to untreated cells ($p<0.05$) (**Figures 3.1B and D**). We did not detect expression of the NK cell-activating minor histocompatibility antigen H60 on either undifferentiated or differentiated NPCs (**data not shown**).

We next tested whether NK cells could lyse cultured eGFP-NPCs using an *in vitro* cytolytic killing assay. Primary NK cells (CD3$^-$CD49b$^+$) were isolated from the blood of BALB/c mice to greater than 90% purity **(data not shown**). Cultured NPCs were sorted into RAE-1$^+$ and RAE-1$^-$ populations (**Figure 3.2A**) and cultured with NK cells. Enriched NK

cells killed allogeneic RAE-1$^+$ NPCs over a range of effector-to-target ratios similar to NK cell-mediated killing of YAC-1 cells that were used as a positive control (**Figure 3.2B**). Enriched NK cells from C57BL6 mice recognized and killed syngeneic NPCs at low levels (**data not shown**). These findings indicate that RAE-1 expression by NPCs is regulated, in part, by the differentiation fate of cells and that RAE-1 expression on allogeneic NPCs allows for recognition by NK cells that participate in allograft rejection.

Antibody blockade of NKG2D increases survival of allogeneic NPCs

To examine if NK cells contribute to allograft rejection, JHMV-infected BALB/c mice were intraspinally transplanted with either C57BL/6-derived eGFP-NPCs or HBSS (vehicle control) at day 14 p.i. that represents a time in which persistent virus is present within the CNS and demyelination is established (24, 32). In addition, JHMV-infected C57BL/6 mice were transplanted with syngeneic eGFP-NPCs via intraspinal injection at day 14 p.i. Experimental mice were sacrificed at day 8 post-transplantation (p.t.), and infiltrating lymphocytes were isolated from a defined area of the spinal cord, 9 mm rostral and caudal to the transplantation site, and were immunophenotyped by flow cytometry. Transplantation of allogeneic eGFP-NPCs into infected BALB/c mice resulted in a significant ($p<0.01$) increase in the number of CD3$^-$CD49b$^+$NKG2D$^+$ NK cells migrating into the spinal cord of mice compared to infected C57BL/6 mice receiving syngeneic eGFP-NPCs (**Figures 3.3A and B**). Importantly, allogeneic and syngeneic transplants were normalized to vehicle only transplant controls to account for NK cell infiltration into the spinal cord as a result of JHMV infection or due to trauma from needle injection.

To determine if NKG2D$^+$ NK cells were recruited to the site of allogeneic transplant in the absence of viral infection, non-infected mice were transplanted with allogeneic eGFP-NPCs or vehicle alone. eGFP-NPCs were transplanted into the spinal cord of non-infected Balb/c mice, spinal cords were removed at day 8 p.t., and NK cell infiltration was determined. Transplantation of allogeneic eGFP-NPCs into non-infected Balb/c mice resulted in an increase in the percentage of CD3$^-$CD49b$^+$ NK cells (**Supp. Figure 3.1A**) as well as the percentage of NKG2D$^+$ NK cells within the spinal cord compared to vehicle only-transplanted mice (**Supp. Figures 3.S1A and B**).

To test the role of NKG2D signaling in allograft rejection, allogeneic recipients were treated with either the non-depleting, neutralizing anti-NKG2D mAb or an isotype-matched control antibody. As an additional control, syngeneic recipients received isotype control antibody. Administration of isotype control antibody to syngeneic recipients did not affect either transplanted eGFP-NPC migration rostral or caudal to implantation site or colonization of white matter tracts (**Figures 3.4A and D**) whereas eGFP-NPCs were not detected within the spinal cords of allogeneic recipients treated with control antibody (**Figures 3.4B and D**). Treatment of infected mice receiving allogeneic eGFP-NPCs with a blocking antibody specific to NKG2D resulted in graft survival at day 21 p.t. in 4 of 5 mice **(Figures 3.4C and D)**. There was a significant ($p<0.001$) increase in the frequency of surviving cells (17.7% at transplant site) when compared to allogeneic transplants receiving injections with an isotype control antibody, in which 0 of 4 mice had a surviving graft (0%) (**Figure 3.4D**). Survival of allogeneic NPCs in mice treated with anti-NKG2D antibody remained lower when compared to mice receiving syngeneic NPCs (**Figures 3.4C and D**).

Glial differentiation of allogeneic NPCs increases survival following transplantation

Our findings argue that expression of RAE-1 by NPCs renders these cells susceptible to NKG2D recognition and subsequent lysis of allografts by infiltrating NK cells. Additionally, upon differentiation of cultured NPCs, RAE-1 expression was dramatically reduced resulting in limited NK cell killing of allogeneic cells by NK cells and this further supports the notion that NK cells recognize and kill allogeneic NPCs through NKG2D recognition of RAE-1. As an additional test, we transplanted either eGFP-NPCs that constitutively express RAE-1 or differentiated eGFP-NPCs in which RAE-1 expression is greatly reduced into JHMV-infected mice. Consistent with our earlier findings (16), differentiation of eGFP-NPCs resulted in ~80% of cells expressing oligendroglia markers NG2 and PDGFRα (**data not shown**). Initially, we transplanted undifferentiated and differentiated eGFP-NPCs into JHMV-infected C57BL/6 mice, representing a syngeneic transplant, in order to compare migration of the two cell populations. Examination of coronal sections of spinal cords at day 21 p.t. indicated similar numbers of eGFP-NPCs present within the white matter tracts of animals receiving undifferentiated NPCs compared to differentiated cells (**Figures 3.5A and B**). Surviving grafts were found in 100% of C57BL/6 mice that received either undifferentiated (n=13) or differentiated (n=12) NPC syngeneic eGFP-NPCs. Quantification of cell numbers within transplanted mice revealed similar numbers of differentiated cells compared to undifferentiated cells and cell migration rostral and caudal to the implantation site was almost identical (**Figure 3.5C**). Consistent with our earlier studies (32), allogeneic undifferentiated eGFP-NPCs were rejected below the level of detection by day 21 p.t. (**Figures 3.6A and C**), whereas eGFP-NPCs that were differentiated prior to

transplant were found in 50% (6 of 12) of allogeneically transplanted mice (**Figures 3.6B and C**). Quantification of transplanted cells demonstrated increased numbers ($p<0.01$) of differentiated allogeneic eGFP-NPCs within spinal cords as compared to undifferentiated allogeneic eGFP-NPCs and surviving cells migrated rostral and caudal to the implantation site (**Figure 3.6C**). Immunohistochemical staining at day 8 p.t. for the NK cell marker CD49b (50) demonstrated accumulation of these cells in areas in which undifferentiated cells are present while CD49b staining was not detected around differentiated cells (**Figure 3.6D**). These findings provide further support for RAE-1 expression on undifferentiated NPCs as a target for NK cell recognition and killing of allografts.

Elevated RAE-1 expression by NPCs in response to JHMV infection

NK cells exhibit a rapid response following viral infection and participate in initiating an effective anti-viral immune response, as well as directly eliminating infected cells (51-53). As we are implanting NPCs into the CNS of mice infected with a neurotropic virus, it is possible that transplanted cells may become infected with virus and serve as targets for immune recognition and destruction. In support of this possibility, NPCs are susceptible to infection with neurotropic coxsackie virus (54-56) and we have previously shown that differentiated NPCs support replication of JHMV (57). To determine if JHMV is capable of infecting NPCs, we first infected SCID mice, which lack T and B lymphocytes but retain functional NK cells, with JHMV and subsequently transplanted the mice with eGFP-NPCs at day 7 p.i. Our rationale for using SCID mice for these studies is that virus-specific T lymphocytes recruited to the CNS following JHMV infection control viral spread and we wished to avoid an adaptive immune response specific for virus (47, 58, 59). As shown in

Figure 3.7A, JHMV antigen is readily detectable within transplanted eGFP-NPCs as determined by immunohistochemical staining. Approximately 50% of surviving NPCs at day 7 p.t. were infected with JHMV (**data not shown**). We next tested whether JHMV infection of eGFP-NPCs increases susceptibility to NK cell-mediated death through a RAE-1 signaling pathway. Following 24 hr infection of cultured eGFP-NPCs with JHMV, expression of RAE-1 significantly ($p<0.01$) increased from 44.4±3.2% of non-infected NPCs to **7**5.4±3.4% of the JHMV-infected NPCs, representing an ~2-fold increase in expression of RAE-1 ($p<0.01$) compared to non-infected cells (**Figures 3.7B and C**). To test whether increased RAE-1 expression following JHMV infection increased susceptibility to NK cell-mediated lysis, a cytotoxicity assay was performed. Infected (24 hr) and non-infected C57BL/6-derived NPCs were co-cultured with allogeneic BALB/c NK cells and target cell lysis was determined. NK cell-mediated killing of eGFP-NPCs was significantly ($p<0.05$) increased in infected cells compared to non-infected cells at an effector to target (E:T) ratio of 20:1 and killing diminished with decreased E:T ratios (**Figure 3.7D**). Inclusion of anti-NKG2D blocking antibody (20 μg/ml) resulted in diminished NK cell-mediated killing of allogeneic JHMV-infected eGFP-NPCs that trended down with decreasing E:T ratios (**Figure 3.7E**). A similar increase in susceptibility was observed when JHMV-infected NPCs were co-cultured with syngeneic C57BL/6 NK cells; approximately 40% of JHMV-infected NPCs were lysed by syngeneic NK cells at an E:T ratio of 20:1, compared to <10% of non-infected NPCs (**data not shown**).

3.4 Discussion

NPCs have emerged as a viable replacement therapy for demyelinating disease, such as MS (9, 10). Preclinical animal models of MS have convincingly shown the ability of transplanted NPCs to improve clinical outcome that is associated with enhanced remyelination of axons. The ability of NPCs to preferentially differentiate into either glial lineage cells or neurons is considered important within the context of regaining motor function through investment of new myelin as well as potential immunomodulatory activity (9, 16, 24). While originally believed to be immunologically inert, we have recently demonstrated that transplantation of allogeneic NPCs into the spinal cords of JHMV-infected mice results in rejection that is mediated, in part, by T lymphocytes (32). Although cultured NPCs do not constitutively express high levels of MHC class I or II, upon exposure to the proinflammatory cytokine IFN-γ expression of both of these molecules increases dramatically (29, 32, 60) suggesting these molecules aid in immune recognition and destruction. In addition, Palmer and colleagues (36) have recently demonstrated an important role for NK cells in recognizing and killing allogeneic NPCs through NKG2D recognition of RAE-1 that is expressed on the surface of NPCs. Further evidence for the involvement of the innate immune response in rejection of NPCs comes from a recent study of human NPCs, which showed a robust innate immune response directed to human NPCs transplanted into the CNS (61). Therefore, a better understanding of how the innate immune response contributes to rejection of allografts within the context of the JHMV model of demyelination is merited and is the focus of this report. Our findings support and extend recent studies examining the role of the innate immune response in recognizing and killing transplanted allogeneic NPCs (36). Phillips *et al*. elegantly demonstrated improved

survival of allogeneic NPCs following transplantation into the CNS of mice lacking NKG2D (*Klrk1-/-* mice) (36). Our findings are consistent with these results by showing that cultured RAE-1-expressing NPCs facilitate recognition and killing by NK cells through an NKG2D-dependent pathway and blocking NGK2D dampened NK cell-mediated lysis of cultured NPCs. Importantly, using the JHMV model of neuroinflammatory demyelination, we show that administration of anti-NKG2D antibody increased survival of transplanted allogeneic NPCs. Blocking NKG2D did not result in 100% survival of allografts highlighting the importance of infiltrating T lymphocytes or other cells in killing (32). It is possible that NKG2D signaling on T cells may also contribute to recognition and killing of allogeneic NPCs. NKG2D functioning as a co-stimulatory molecule on T cells has been implicated in allograft rejection (41) although the majority of studies emphasize either a direct role for NKG2D killing via NK cells (39, 40, 44) or indirectly through T cell activation (62). Furthermore, we have previously shown a role for NKG2D in enhancing $CD8^+$ T cell-mediated lysis in response to JHMV infection of the CNS (63). Also, it is possible some cell loss following transplantation can be a result of failed engraftment due to the injection, or rejection from other cells besides T cells or NK cells. Nonetheless, our findings demonstrating that killing of undifferentiated allogeneic NPCs is muted in the absence of RAE-1 as well as blocking NKG2D signaling diminishes NK-mediated lysis supports a role for NKG2D-mediated lysis of transplanted allogeneic cells by infiltrating NK cells. Furthermore, these results build upon previous studies showing that NKG2D is involved in non-CNS transplant allograft transplant rejection (44).

Our results support the hypothesis that regulation of RAE-1 expression by allogeneic NPCs is important in increasing survival. Treatment of cultured NPCs with the

proinflammatory cytokine IFN-γ resulted in a marginal reduction in RAE-1 expression which is in contrast to the dramatic increase in both MHC class I and II following IFN-γ exposure (29, 32, 60). Ultimately, IFN-γ-treatment of NPCs did not diminish NK-mediated lysis, indicating that sufficient surface levels of RAE-1 remain thus allowing recognition by NK cells. Whether this is the result of compensation by other NKG2D ligands such as MULT-1, which is weakly expressed on NPCs (36), is not know at this time.

Our data indicate that upon differentiation of cultured NPCs, RAE-1 expression dramatically declines to almost undetectable levels as determined by flow cytometry. Following differentiation of cultured NPCs, the majority (~80%) of cells are $GalC^+$ and $NG2^+$ oligodendroglia with remaining populations comprised of GFAP-positive astrocytes and Tuj1+ neurons (16). Our results demonstrate that transplantation of allogeneic differentiated NPCs lacking RAE-1 results in enhanced survival when compared to transplantation of RAE-1^+ undifferentiated NPCs. In addition, differentiated cells are able to successfully migrate and colonize areas of white matter damage in a manner similar to undifferentiated cells (16, 24). Within the context of our model of viral-induced demyelination, we propose that following allogeneic transplantation of NPCs into the spinal cord JHMV-infected mice, these cells are likely first targeted by NK cells as we observed increased numbers of infiltrating NK cells within the spinal cords of allograft recipients that was associated with a rapid reduction in numbers of transplanted cells. Over time, surviving cells differentiate and this is accompanied by diminished RAE-1 expression that limits NK cell-mediated rejection. However, inflammatory T cells presumably recognize allogeneic transplanted NPCs through an MHC-mediated pathway following exposure to IFN-γ culminating in complete rejection. Collectively, our *in vitro* and *in vivo* data argue

that reducing RAE-1 expression on allogeneic stem cell populations may increase survival and improve both motor skills and histology outcomes in preclinical animal models of MS.

In addition to viral pathogens being an environmental factor associated with MS, there are numerous viruses capable of persisting within the CNS, including JC virus and Epstein-Barr virus (64-66). In the absence of immune surveillance, *e.g.* as a result of immunosuppressive therapy necessary for transplantation, viral recrudescence is a valid concern (67). A clinically relevant example is the development of progressive multifocal leukoencephalopathy (PML) due to JC virus in MS patients resulting from administration of natalizumab which impairs T cell infiltration into the CNS. This leads to the question of whether NPCs transplanted to treat demyelinating diseases would be susceptible to viral infection. *In vitro*, RAE-1 expression was elevated in response to JHMV infection, and this enhanced NKG2D-dependent NK cell-mediated death of allogeneic NPCs, demonstrating that RAE-1 and NKG2D are mediators of NK cell lysis activity following JHMV infection. NPCs transplanted into immune-deficient mice succumb to infection; although, it is not clear whether virally infected transplanted NPCs die as a result of viral-mediated or NK cell-mediated lysis; *in vitro,* ~20% of NPCs initially die following JHMV infection (68). While some studies have examined the susceptibility of NPCs to various neurotropic viruses, reports are limited with regards to how viral infection of allogeneic NPCs following transplant affects immune recognition and survival. Recently, Basu and colleagues (69) determined that Japanese Encephalitis Viral infection of NPCs alters their immunogenicity, resulting in recognition by allogeneic T cells, and subsequent T cell proliferation. We believe our findings, in conjunction with others, highlights the need for additional

information regarding the immunogeneic affect of viral infection of NPCs within the context of allogeneic transplant.

3.5 Conclusion

Our findings demonstrate that in addition to T cell suppression, modulation of NKG2D-RAE-1 signaling may be necessary for long-term survival of allografts transplanted into the CNS. Possible mechanisms include elimination of NK cells, blocking NK cell activation, or limiting RAE-1 expression on transplanted allogeneic NPCs. However, the consequences of these potential therapeutic interventions must be considered within the context of possible viral infection of transplanted NPCs and the consequences on optimal host defense.

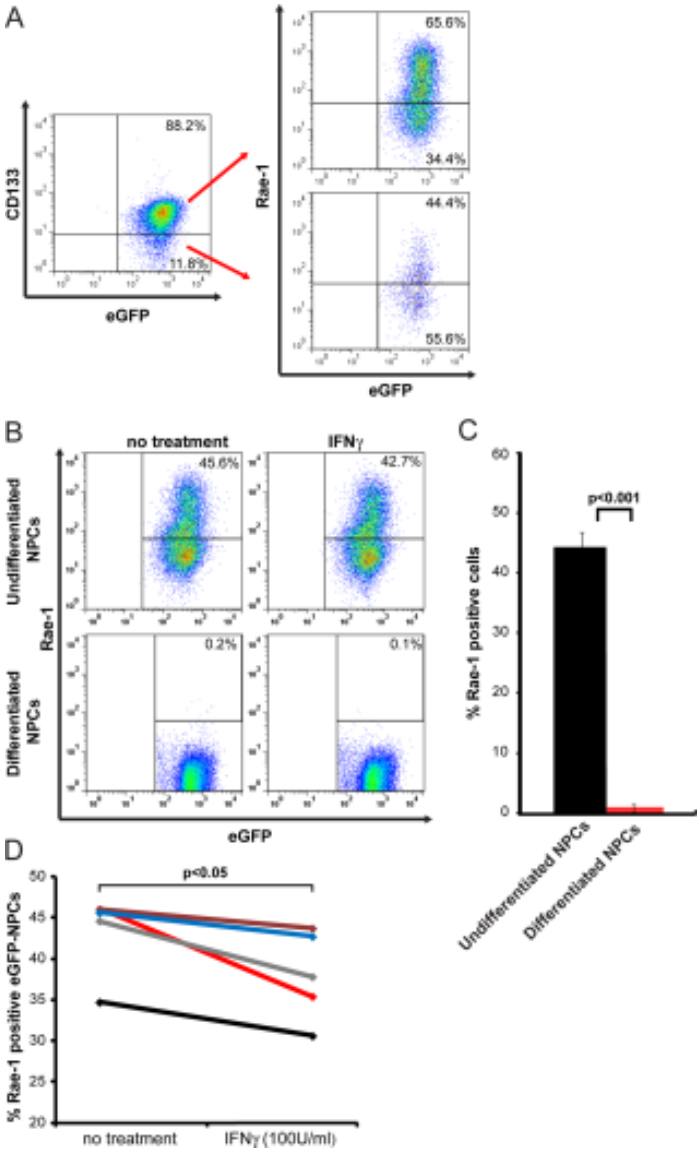


Figure 3.1: RAE-1 expression on cultured NPCs. (A) Representative dot blot showing staining for the NPC marker CD133 and eGFP; 85.1±1.6 % of cultured eGFP-NPCs expressed CD133. Subsequent staining for RAE-1 on $eGFP^{+}CD133^{+}$ and $eGFP^{+}CD133^{-}$ revealed 64.3±0.7% of dual-positive cells expressed RAE-1 while 48.3±2.0% of $eGFP^{+}CD133^{-}$ cells expressed RAE-1. (B,C) Differentiated and undifferentiated cultured eGFP-NPCs were treated with IFN-γ (100 U/ml) for 24 hr and RAE-1 expression was determined by flow cytometry. (**B**) Representative flow analysis for RAE-1 expression on IFN-γ-treated or non-treated differentiated and undifferentiated eGFP-NPCs is shown. (**C**) Quantification of RAE-1 expression on differentiated and undifferentiated non-treated eGFP-NPCs. Data represents five independent experiments and data is shown as average±SEM; $p<0.05$. (**D**) Quantification of RAE-1 expression on non-treated and IFN-γ-treated undifferentiated eGFP-NPCs. Paired data from five independent experiments showing decreased RAE-1 expression following IFN-γ treatment. The average decrease from all experiments is 12.3±3.3% SEM; each line represents an individual experiment; $p<0.05$.

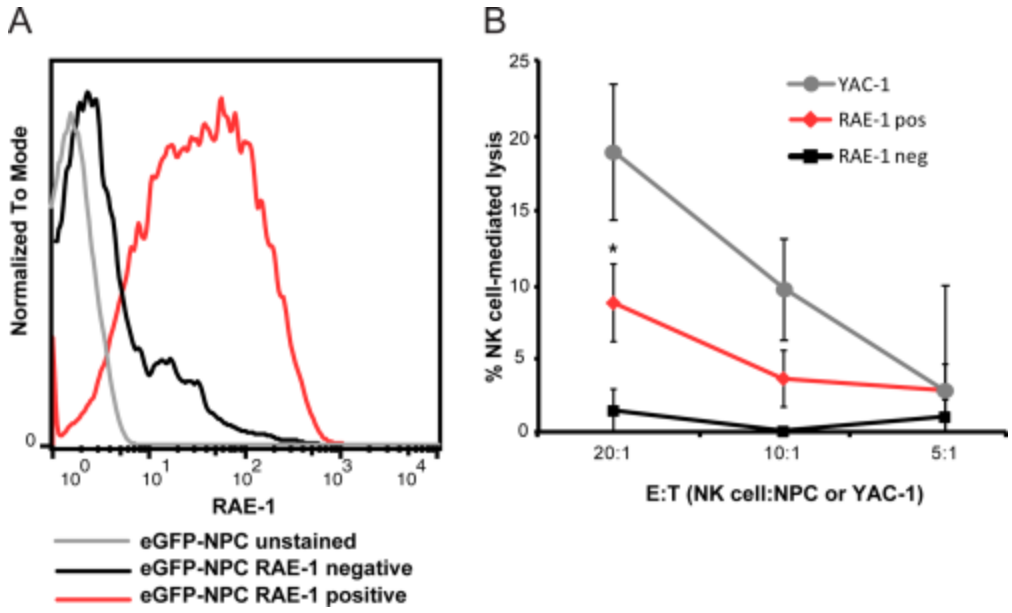


Figure 3.2: NK cell lysis of RAE-1$^+$ NPCs. (**A**) Representative histogram depicting RAE-1$^+$ (red line) and RAE-1$^-$ (black line) eGFP-NPCs sorted by FACS. eGFP-NPCs stained with isotype control antibody are indicated by grey line. (B) RAE-1$^+$ (red line) eGFP-NPCs, RAE-1$^-$ (black line) eGFP-NPCs, or YAC-1 (grey line) cells (control for NK cell lysis) were cultured with allogeneic NK cells in an LDH assay and the percentage of NK cell-mediated lysis at three different E:T ratios is shown. Data represent three independent experiments; *$p<0.05$, unpaired student's T test between RAE-1$^+$ and RAE-1$^-$ eGFP-NPCs.

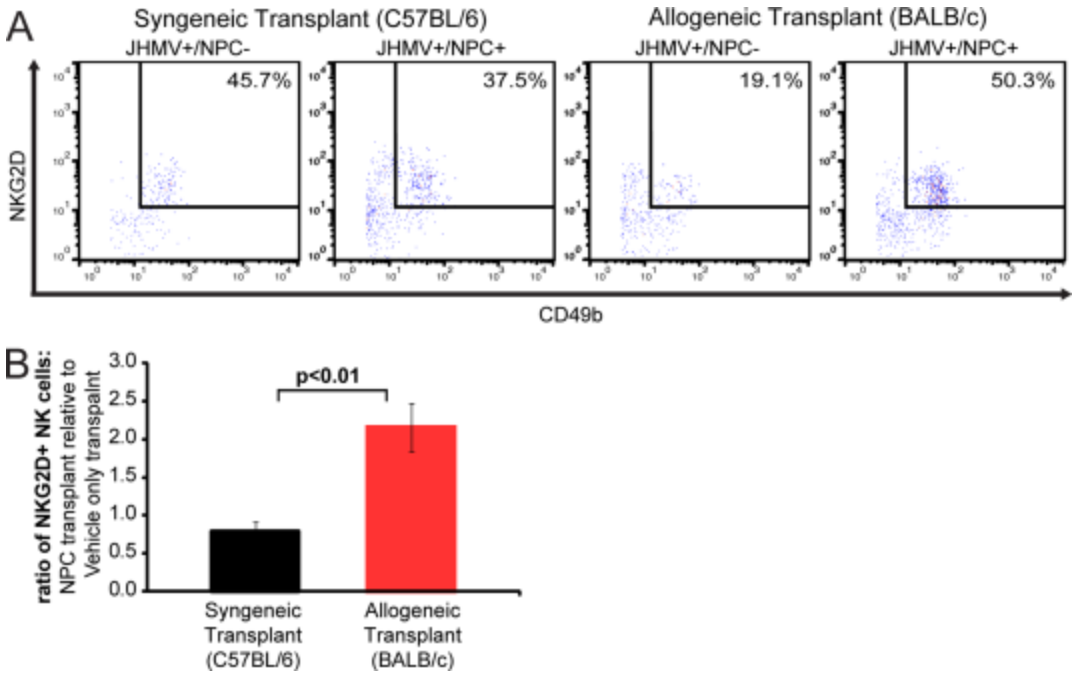


Figure 3.3: NK cell infiltration into spinal cords following allogeneic NPC transplantation. Vehicle only or eGFP-NPCs were transplanted into JHMV-infected C57BL/6 (syngeneic transplant) and JHMV-infected BALB/c (allogeneic transplant) mice on day 14 post-JHMV-infection. (**A**) Mice were sacrificed at day 8 p.t. and the frequency of $NKG2D^+$ NK cells among total lymphocytes in the spinal cord (9mm rostral and caudal to transplant site) of recipient mice was determined by flow cytometry. Representative flow analysis of $CD3^-CD49b^+NKG2D^+$ NK cells in syngeneic and allogeneic eGFP-NPC transplanted, and vehicle only transplanted mice is shown. (**B**) Quantification of NK cells in allogeneic and syngeneic transplanted mice normalized to vehicle only transplant. Data is presented as average$\pm$SEM and is 1 of 2 representative experiments with a minimum of 3 mice per group; $p<0.01$.

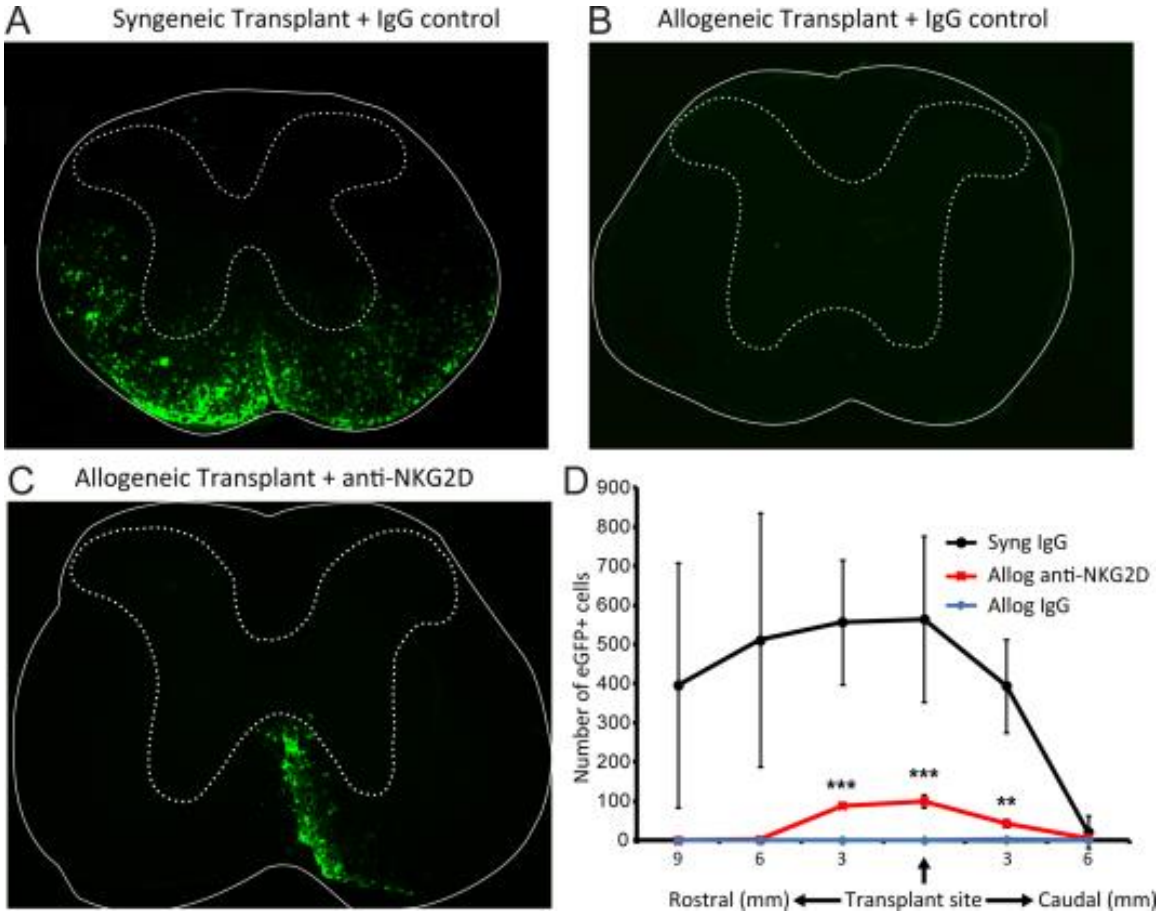


Figure 3.4: Blocking NKG2D increases survival of transplanted allogeneic NPCs. Representative coronal spinal cord sections of the transplant site from JHMV-infected mice receiving either syngeneic eGFP-NPCs treated with IgG control antibody (**A**), allogeneic eGFP-NPCs plus anti-NKG2D (**B**) or IgG control antibody (**C**). Experimental mice were sacrificed at day 21 p.t. and migration/survival of transplanted cells was evaluated by visualization of eGFP-expression from transplanted cells. (**D**) Dual-positive DAPI and eGFP-NPCs were counted in coronal sections (9 mm rostral and 6 mm caudal to transplant site at 3 mm intervals) from mice syngeneically transplanted treated with an IgG control antibody (n=5), allogeneically transplanted treated with anti-NKG2D (n=5), and allogeneically transplanted treated with an IgG control antibody (n=4). Increased numbers of eGFP-NPCs (**p<0.01, ***p<0.001) were present within the spinal cords of allogeneically transplanted mice treated with anti-NKG2D antibody compared to allogeneically transplanted mice treated with an IgG control antibody. 100% (5/5) syngeneically transplanted mice treated with an IgG control antibody, 80% (4/5) allogeneically transplanted mice treated with anti-NKG2D, and 0% (0/4) allogeneically transplanted treated with an IgG control antibody had a surviving graft at day 21 p.t.

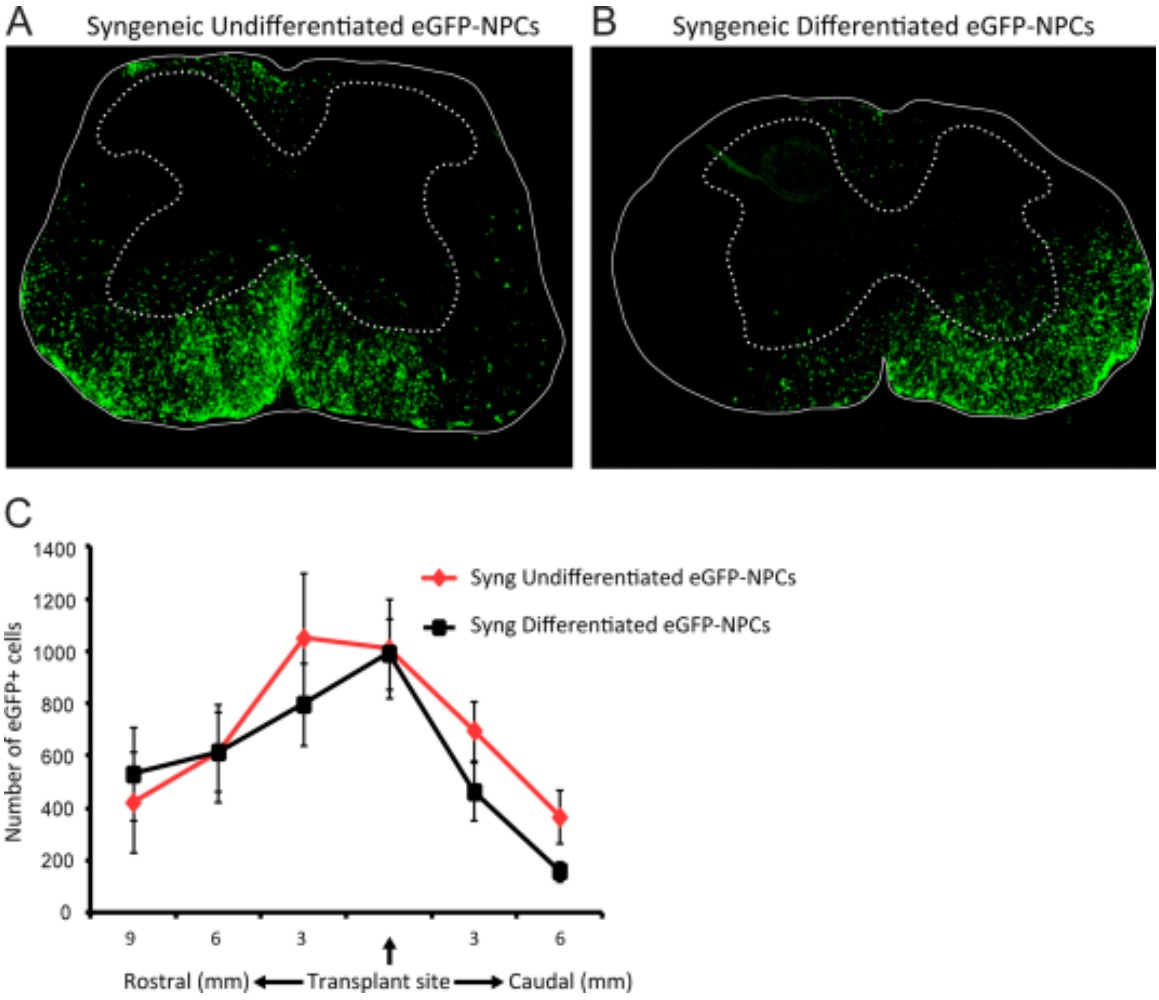


Figure 3.5: Differentiated NPCs migrate following transplantation. Undifferentiated and differentiated eGFP-NPCs were transplanted into C57BL/6 mice (syngeneic transplant) on day 14 post-JHMV-infection. Representative coronal spinal cord sections of the transplant site from JHMV-infected mice receiving syngeneic undifferentiated eGFP-NPCs (n=13; **A**) or syngeneic differentiated eGFP-NPCs (n=12; **B**). Experimental mice were sacrificed at day 21 p.t. and migration and/or survival of transplanted cells evaluated by visualization of eGFP-expression from transplanted cells. (**C**) eGFP-NPCs were counted in coronal sections (9 mm rostral and 6 mm caudal to transplant site at 3 mm intervals) from mice syngeneically transplanted with undifferentiated (n=11) or differentiated (n=11) eGFP-NPCs. There was no significant difference between the numbers of undifferentiated or differentiated eGFP-NPCs.

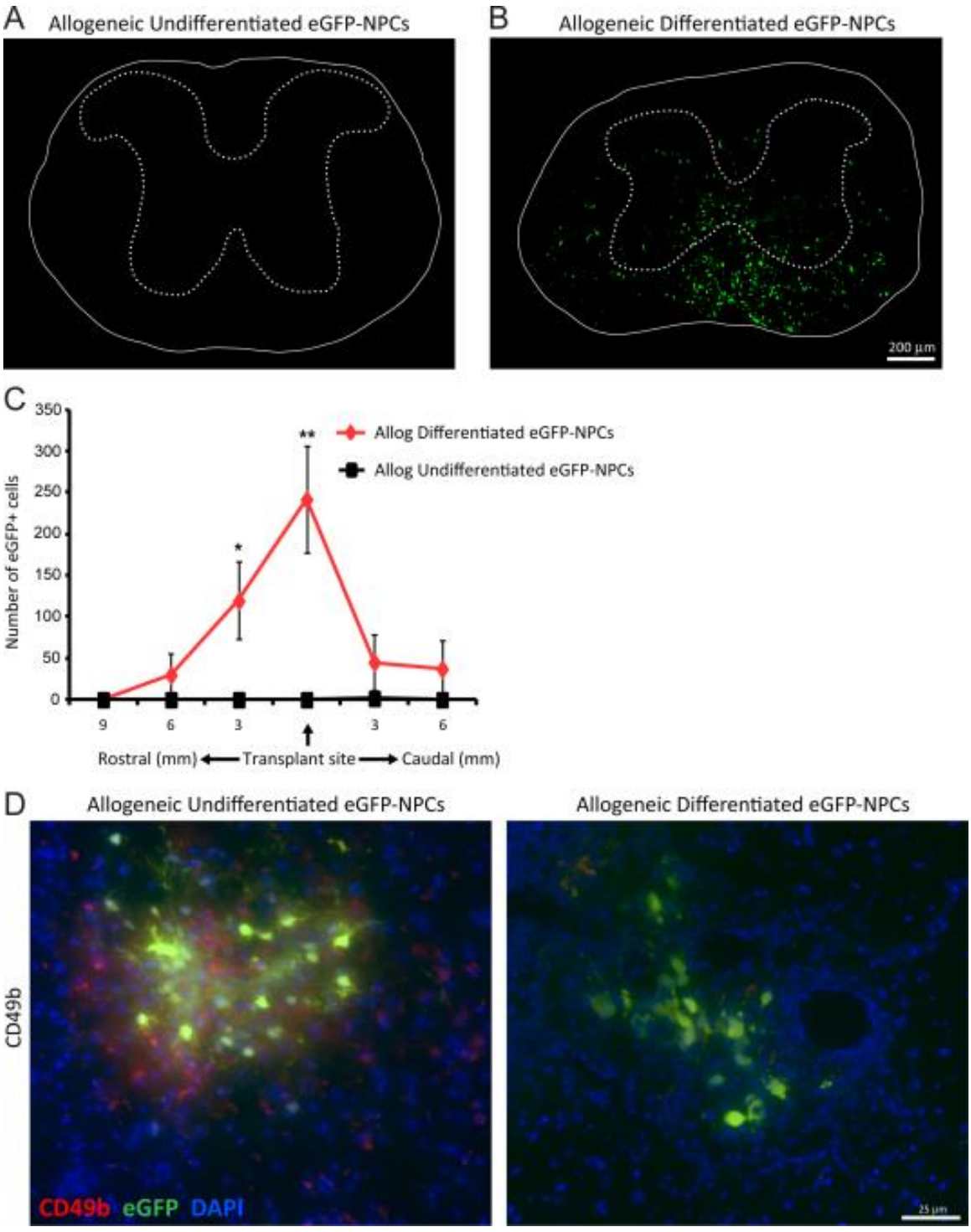


Figure 3.6: Transplanted allogeneic differentiated NPCs display increased survival following transplantation. Undifferentiated and differentiated eGFP-NPCs were transplanted into BALB/c mice (allogeneic transplant) on day 14 post-JHMV-infection. Representative coronal spinal cord sections of the transplant site from JHMV-infected mice receiving allogeneic undifferentiated eGFP-NPCs (**A**) or allogeneic differentiated eGFP-NPCs (**B**). Experimental mice were sacrificed at day 21 p.t. and migration and/or survival

of transplanted cells evaluated by visualization of eGFP-expression from transplanted cells. (**C**) eGFP-NPCs were counted in coronal sections 9mm rostral and 6mm caudal to transplant site at 3mm intervals from mice transplanted with undifferentiated (n=4) or differentiated (n=6) eGFP-NPCs. Increased numbers of eGFP-NPCs (*$p<0.05$, **$p=0.01$) were present within the spinal cords of mice transplanted with differentiated eGFP-NPCs compared to undifferentiated allogeneic NPCs. (**D**) Representative immunofluorescence images showing $CD49b^+$ NK cells (red) and eGFP-NPCs (green) with DAPI-stained nuclei (blue) at day 8 p.t. in coronal sections of spinal cords from mice transplanted with undifferentiated and differentiated allogeneic eGFP-NPCs.

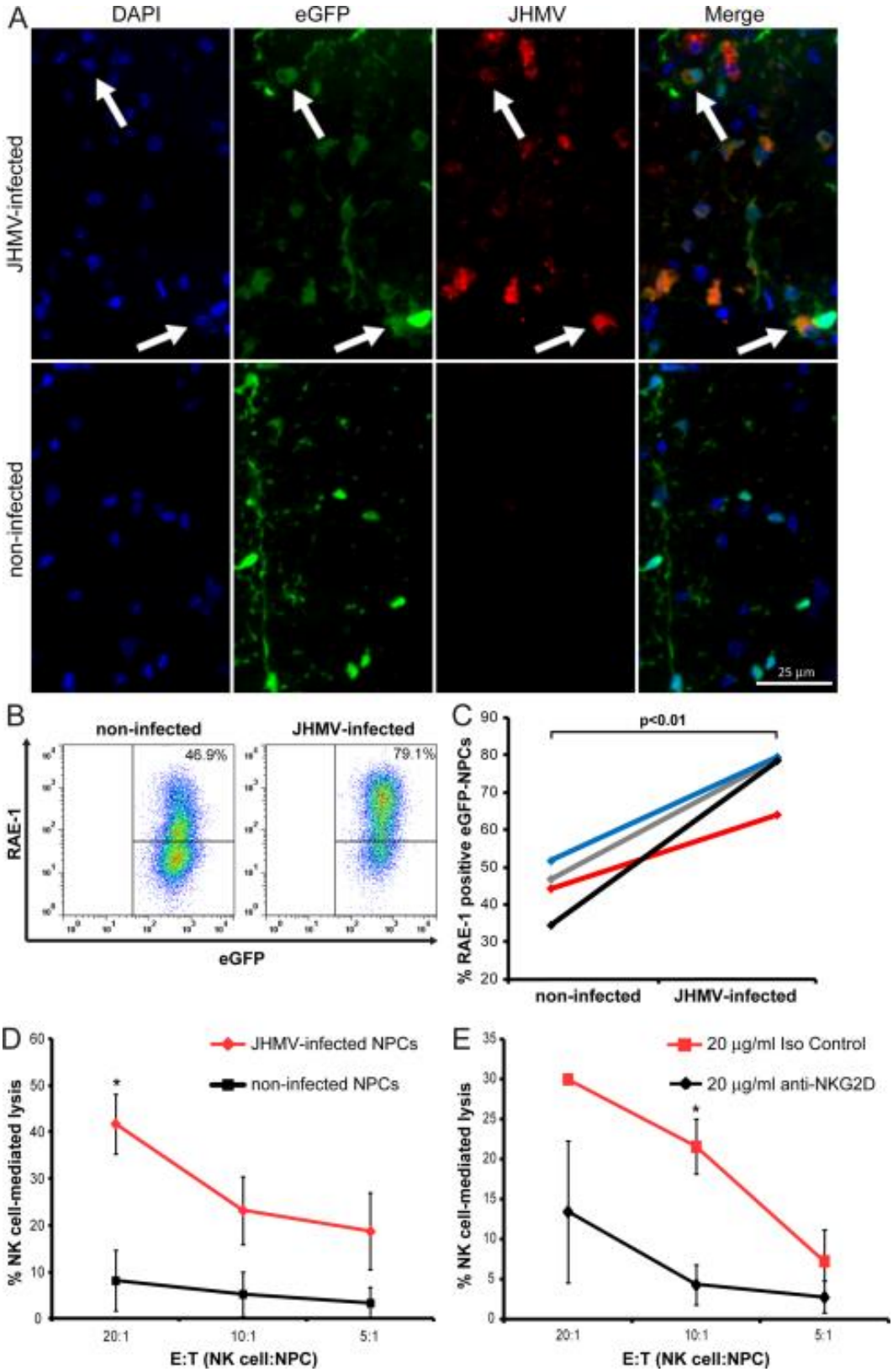
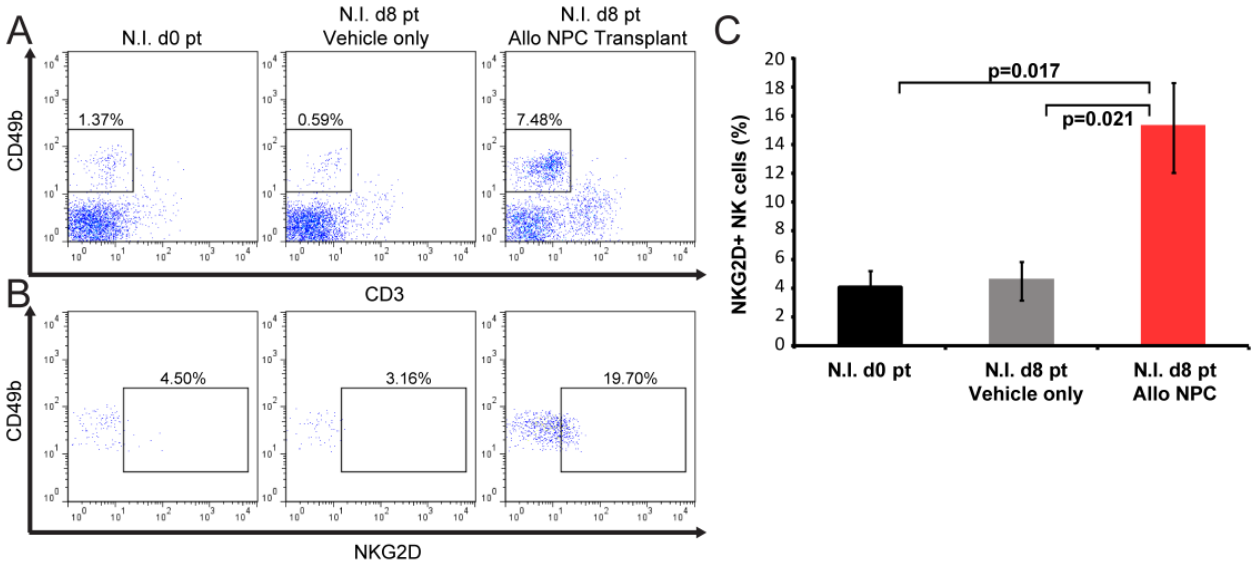


Figure 3.7. JHMV-infection increases RAE-1 expression on NPCs and elevates susceptibility to NK cell mediated lysis. (**A**) Representative immunofluorescence images

revealing co-localization (white arrows) of JHMV (spike protein; red) with eGFP (green) and DAPI-stained nuclei (blue) at day 7 p.t. in coronal sections of spinal cords from JHMV-infected (top panels) and non-infected (bottom panels) SCID mice transplanted with allogeneic eGFP-NPCs. (**B**) Cultured eGFP-NPCs were infected with JHMV (0.1 moi) for 24 hr and RAE-1 expression determined by flow cytometry. Representative flow analysis for RAE-1 expression on non-infected or JHMV-infected eGFP-NPCs is shown. (**C**) Paired data from four independent experiments showing increased ($p<0.01$) RAE-1 expression following JHMV infection. Each line represents and individual experiment. (**D**) Non-infected (black line) and JHMV-infected (red line) eGFP-NPCs were cultured with allogeneic NK cells in an LDH assay and the percentage of NK cell-mediated lysis at three different E:T ratios is shown. Data represent five independent experiments; *$p<0.05$. (**E**) JHMV-infected eGFP-NPCs were cultured with allogeneic NK cells plus 20 μg/ml anti-NKG2D (black line) or 20 μg/ml isotype-matched control Ig (red line) in an LDH assay and the percentage of NK cell-mediated lysis at three different E:T ratios is shown. Data represents three independent experiments; *$p<0.05$.



Supplementary Figure 3.S1: NK cell infiltration into spinal cords of non-infected mice following allogeneic NPC transplantation. Non-infected (N.I.) mice were transplanted with eGFP-NPCs or vehicle only at day 14 p.i. (**A,B**) Mice were sacrificed at days 0 and 8 p.t. and the frequency of NKG2D$^+$ NK cells among total lymphocytes in the spinal cord (9mm rostral and caudal to transplant site) of recipient mice was determined by flow cytometry. Representative flow analysis of CD3$^-$CD49b$^+$ NK cells (% of total live cells, **A**), and NKG2D$^+$ NK cells (% of CD3$^-$CD49b$^+$ cells, **B**) in allogeneic eGFP-NPC- and vehicle only-transplanted mice are shown. (**C**) Quantification of percentage of NKG2D$^+$ NK cells in spinal cords of allogeneic eGFP-NPC- and vehicle only-transplanted mice. Data is presented as average$\pm$SEM with 4 mice per group; $p<0.05$.

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CHAPTER FOUR

T cell mediated suppression of neurotropic coronavirus replication in neural precursor cells

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Abstract

Neural precursor cells (NPCs) are the subject of intense investigation for their potential to treat neurodegenerative disorders, yet the consequences of neuroinvasive virus infection of NPCs remain unclear. This study demonstrates that NPCs support replication following infection by the neurotropic JHM strain of mouse hepatitis virus (JHMV). JHMV infection leads to increased cell death and dampens IFN-γ-induced MHC class II expression. Importantly, cytokines secreted by CD4+ T cells inhibit JHMV replication in NPCs, and CD8+ T cells specifically target viral peptide-pulsed NPCs for lysis. Furthermore, treatment with IFN-γ inhibits JHMV replication in a dose-dependent manner. Together, these findings suggest that T cells play a critical role in controlling replication of a neurotropic virus in NPCs, a finding which has important implications when considering immune modulation for NPC-based therapies for treatment of human neurologic diseases.

4.1 Introduction

Transplantation of multipotent neural precursor cells (NPCs) is emerging as a feasible therapeutic strategy for the treatment of a variety of neurological disorders. Recent studies have demonstrated both short and long-term clinical benefits following NPC engraftment within the context of rodent models of Alzheimer's disease, Parkinson's disease, Huntington's disease, and acute spinal cord injury (1-4). Furthermore, in murine and non-human primate models of the neuroinflammatory disease multiple sclerosis (MS) the ability of human NPCs to function as modulators of the immune system in addition to replacing lost or damaged neural cell populations has been suggested (5-7). However, despite the clinical and histological benefits of NPC transplantation in pre-clinical animal models of neurologic disease, there is limited evidence addressing the capacity of neural grafts to act as reservoirs for viral replication. Studies using the non-polio enterovirus coxsackievirus B (CVB) demonstrate the ability of CVB to preferentially replicate in murine NPCs (8). The ensuing carrier-state infection results in increased cell death and impaired differentiation potential *in vitro*, as well as inflammation, microgliosis, and a variety of CNS developmental defects *in vivo* (8, 9). Intracerebral infection of neonates with murine cytomegalovirus (MCMV) results in the loss of neural stem cells and their neuronal progeny, as well as a decrease in the production of neurotrophins imperative to normal brain development (10). Borna disease virus (BDV) infection of human fetal human NPCs results in cell death upon differentiation and impaired neurogenesis (11). Thus, the role of neural stem and progenitors as targets for a variety of neuroinvasive viruses is evident, while the consequences of infection within the context of cellular therapy remain to be elucidated.

Complicating NPC-based therapies is the controversial issue of antigenicity of transplanted cells and immune-mediated recognition. A growing body of evidence suggests NPCs are not immunoprivileged, as has previously been reported (12). Indeed, we have shown that NPCs derived from post-natal C57BL/6 brains express the co-stimulatory molecules CD80 and CD86 and up-regulate major histocompatibility complex (MHC) molecules in response to the pro-inflammatory cytokine interferon gamma (IFN-γ) (13). Furthermore, allogeneic NPCs are rapidly rejected via a T cell mediated mechanism following intraspinal transplantation into MHC-mismatched recipients (13). Similarly, human NPCs have the capacity to express MHC I and II and induce T cell proliferation (14).The apparent antigenicity of NPCs suggests successful engraftment may require the use of immunomodulatory agents and lifelong suppression of the immune system, as with solid organ transplants. However, an unintended consequence of immune suppression is the potential for latent viruses to become activated, or for uncontrolled viral replication to occur following opportunistic infection (15-18). Therefore, it is imperative to understand the consequences of neurotropic virus infection of NPCs as cell-replacement therapies continue to move into the clinic (19, 20).

In this study, we demonstrate that cultured murine NPCs are infected by the neurotropic JHM strain of mouse hepatitis virus (JHMV), which induces acute encephalomyelitis and chronic demyelination when injected intracranially into immunocompetent mice. JHMV-infected NPCs support replication that ultimately results in increased cell death over time. Importantly, CD8+ T cells kill NPCs pulsed with viral-peptides, and JHMV replication in NPCs was suppressed, in part, by IFN-γ secreted from virus-specific CD4+ T cells.

4.2 Results

NPCs express the MHV receptor CEACAM1a and are infected by JHMV

JHMV is a neurotropic coronavirus with relatively restricted tropism for glial cells through recognition and binding to the receptor carcinoembryonic antigen-cell adhesion molecule 1a (CEACAM1a) (21, 22). CEACAM1a expression in mouse tissues is widespread and can be detected on the surface of a variety of epithelial cells in the gastrointestinal, respiratory, and reproductive tracts, as well as on small vascular endothelia and hematopoietic cells (23). However, CEACAM1a expression is not ubiquitous, and although it is known to be located at the surface of resident cells of the CNS including glia, expression by neural stem or progenitor cells has not been evaluated. To determine if NPCs derived from C57BL/6 transgenic mice engineered to express GFP (GFP-NPCs) express CEACAM1a, mRNA was isolated from cultured NPCs and receptor expression was evaluated by PCR. Using CEACAM1a-specific primers, PCR amplicons were detected in NPCs, as well as mixed splenocytes from C57BL/6 mice acting as controls (**Figure 4.1A**), and nucleotide sequencing confirmed homology with the specified region of the gene (**data not shown**). Furthermore, cell surface expression of CEACAM1a was confirmed with more than 90% of NPCs expressing the receptor as determined via flow cytometric analysis (**Figure 4.1B**).

We next infected Sox2+ GFP-expressing NPCs with JHMV to assess susceptibility to infection. Infected NPC cultures were fixed 72 hours post-infection (p.i.) and stained with an antibody specific for the carboxyl terminus of the JHMV nucleocapsid (N) protein and subsequently imaged using fluorescence microscopy. Compared to non-infected NPCs that form a confluent monolayer when grown in tissue culture-treated, matrigel-coated vessels,

Sox2+ NPCs infected at a multiplicity of infection (m.o.i.) of 0.1 displayed JHMV-specific syncytia formation by 72 hours post-infection (**Figure 4.2A**). Correspondingly, increasing viral titers were detected when plaque forming unit (PFU) assays were performed on supernatants harvested from JHMV-infected NPC cultures at 24, 48, and 72 hours p.i. (**Figure 4.2B**). Furthermore, determination of lactate dehydrogenase (LDH) released into the supernatants of infected cultures at defined times p.i. revealed increased NPC death over time, ranging from 10.1% ± 1.5% at 24 hours p.i., increasing to 29.7% ± 3.7% at 48 hours p.i., and peaking at 35.4% ± 3.6% by 72 hours (**Figure 4.2C**). As JHMV replication has been reported to occur via CEACAM1a-dependent and independent mechanisms (24), we performed a monoclonal antibody blockade to determine the role of CEACAM1a in the spread of JHMV infection in cultured NPCs (**Figure 4.2D**). By 72 hours p.i., significant ($p < 0.05$) inhibition of viral replication was observed in anti-CEACAM1a-treated cells ($4.4x10^3 \pm 1.4x10^3$ PFU/mL, n=3) when compared to non-treated, JHMV-infected NPCs ($2.6x10^5 \pm 5.4x10^4$ PFU/mL).

MHC class I and II expression by NPCs in response to JHMV infection

Under normal culture conditions, expression of MHC class I and II is undetectable on NPCs, yet MHC expression can be induced by treatment with IFN-γ (13, 25). To investigate if JHMV infection alters MHC class I and/or II expression on NPCs, we compared surface expression levels of these molecules on non-infected and infected cells in the absence or presence of 100 U/ml IFN-γ. Our findings indicated ≤1% of NPCs were found to be positive for MHC class I (**Figures 4.3A and C**) or II (**Figures 4.3B and C**) within non-infected, non-IFN-γ-treated groups. JHMV infection did not increase either MHC class I or II (0.7% ± 0.2%

and 1.3% ± 0.5%, respectively) in medium-treated NPC cultures (**Figures 4.3A and C**). Treatment with IFN-γ (100 U/ml) for 24 hours dramatically increased levels of MHC class I (97% ± 0.7%) on non-infected NPCs, whereas only 32.9% ± 3.7% of non-infected, IFN-γ-treated NPCs expressed MHC class II (**Figures 4.3A and C**). IFN-γ treatment of JHMV-infected NPCs did not substantially increase MHC class I expression (97% ± 0.8%) in comparison to non-infected IFN-γ-treated cultures (97% ± 0.7%; **Figures 4.3A-C**). However, MHC class II was detected on a significantly ($p < 0.05$) lower fraction (24.5% ± 2.6%, n=3) of infected, IFN-γ-treated NPCs compared to non-infected, IFN-γ-treated NPCs (32.9% ± 2.1%, n=3) (**Figure 4.3C**). Furthermore, MHC class II could not be detected on the majority of JHMV-infected NPCs as determined by dual staining for viral antigen and MHC class II (**Figure 4.3D**).

Control of JHMV replication in infected NPCs

CD8+ and CD4+ T cells are pivotal in controlling JHMV replication within the infected CNS (26, 27). Virus-specific effector CD8+ T cells help control replication in infected astrocytes and microglia through cytolytic activity (28). In addition to secreting IFN-γ that limits viral replication in oligodendrocytes, CD8+ T cells carry out perforin-dependent cytolysis of astrocytes and microglia (27, 28). We co-cultured virus-specific CTLs at diminishing effector-to-target (E:T) ratios with NPCs pulsed with the immunodominant CD8 peptide specific for JHMV spike (S) glycoprotein spanning amino acids 510-518 (S510-518), and treated with IFN-γ to induce MHC class I expression. Subsequently, LDH released in the supernatants was evaluated to quantify CTL-mediated NPC lysis; RMA-S cells, a murine lymphoma cell line that presents viral peptides to CTLs in an MHC class I dependent

manner, were used as a positive control (29). NPCs pulsed with S510-518 peptide were specifically lysed by virus-specific CTLs at an E:T ratio of 10 to 1 ($p < 0.05$, n=3), indicating that virus-specific CD8+ T cells are capable of recognizing and directly killing JHMV-infected NPCs *in vitro* (**Figure 4.4**). Importantly, this cytolytic effect waned as the E:T to target ratio declined. CD4+ T cells have both indirect and direct antiviral roles during acute JHMV-induced encephalomyelitis, which include inducing the effector functions of virus-specific CTLs, along with IFN-γ secretion (30, 31). To evaluate if virus-sensitized CD4+ T cells can control replication of JHMV-infected NPCs, enriched populations of CD4+ T cells were isolated from C57BL/6 mice immunized against the DM variant of JHMV. Subsequently, splenocytes were isolated from JHMV-immunized and naïve mice, and magnetically-assisted negative selection was performed to purify the respective T cell populations. NPC media was conditioned with CD4+ T cell cytokines for 48 hours and then added to JHMV-infected NPCs. Supernatants from either naïve or virus-specific CD4+ T cells suppressed viral replication in NPCs at 24 and 48 hours post-infection, with the most significant inhibitory effects observed in groups treated with media enriched with virus-specific CD4+ T cell cytokines (**Figure 4.5A**). However, while the suppressive effects of naïve T cell media appeared to wane by 72 hours p.i. ($4.7x10^5 \pm 2x10^5$ PFU/mL), supernatants from NPCs treated with virus-specific CD4+ T cell conditioned media maintained low viral titers ($6.8x10^3 \pm 6.3x10^3$ PFU/mL) in comparison to non-treated controls ($2.6x10^5 \pm 5.4x10^4$ PFU/mL; **Figure 4.5A**). T cell derived IFN-γ is critical in controlling JHMV replication in the CNS (28, 32). Furthermore, treatment with IFN-γ specifically inhibits JHMV replication in oligodendrocyte progenitors (OPCs) derived from C57BL/6 NPCs, and inhibition of IFN-γ signaling in oligodendrocytes is associated with

increased viral loads and mortality (33, 34). We evaluated levels of IFN-γ in naïve-versus-DM specific CD4+ T cell conditioned media by enzyme-linked immunosorbent assay (ELISA); absorbance values from media conditioned with DM-CD4+ T cells were increased ~60-fold when compared to naïve T cell conditioned media ($p < 0.0001$; **Figure 4.5B**). We subsequently treated JHMV-infected NPCs with varying amounts of mouse recombinant IFN-γ for 24 hours and determined its effects on viral titers. NPCs treated with 1 or 10 U/ml IFN-γ maintained high JHMV titers ($1.6x10^5 \pm 6.4x10^4$ PFU/mL and $1.3x10^5 \pm 7.8x10^4$, respectively) in relation to non-treated groups ($1.1x10^5 \pm 6.6x10^4$ PFU/mL; **Figure 4.5C**). However, JHMV replication in NPCs was reduced in cultures treated with 50 or 100 U/ml IFN-γ ($4.3x10^4 \pm 2x10^4$ PFU/mL and $4.1x10^4 \pm 2.3x10^4$ PFU/mL, respectively; **Figure 4.5C**). We next performed a 72-hour time course to further probe the effects of IFN-γ (100U/mL) on JHMV-infected NPCs. A reduction from $2.8x10^5 \pm 3.4x10^4$ PFU/mL to $9.2x10^4 \pm 4.9x10^4$ PFU/mL was observed in IFN-γ-treated cultures by 48 hours post-treatment when compared to non-treated groups ($p < 0.05$, $n=3$), and JHMV levels were reduced to $2.3x10^4 \pm 3.3x10^3$ in IFN-γ treated cultures, versus $5.2x10^5 \pm 1.6x10^5$ in non-treated cultures, by 72 hours post-treatment ($p < 0.05$) (**Figure 4.5D**). We previously showed that multiple pro-inflammatory cytokines secreted by DM-specific T cells have synergistic effects with IFN-γ (13). To confirm the role of IFN-γ as the major cytokine contributing to suppression of JHMV replication in infected NPC cultures, monoclonal antibody blockade against the IFN-γ receptor was performed on NPCs before and during treatment with virus-specific CD4+ T cell enriched media. As expected, by 48 hours p.t. JHMV levels were significantly ($p < 0.01$) reduced in conditioned media treated cultures compared to NPCs grown in non-conditioned media ($1.3x10^5 \pm 1.8x10^4$ and $3.1x10^5 \pm 2.4x10^4$ PFU/mL, respectively; **Figure**

4.5E). However, treatment with anti-IFN-γ receptor resulted in higher ($p < 0.05$) viral titers ($5.4x10^5 \pm 1.4x10^5$) compared to CD4+ T cell media treated cultures, thereby confirming the pivotal role of IFN-γ in CD4+ T cell mediated suppression of JHMV in NPCs. We have previously shown that IFN-γ treatment of JHMV-infected OPCs increases IFN-α/β secretion, and treatment with IFN-β suppresses JHMV replication (33). Type I interferon (IFN-β) levels in JHMV-infected, IFN-γ treated NPC supernatants were assessed by ELISA and IFN-β was not detected above background levels (**data not shown**).

Effects of IFN-γ treatment on CEACAM1a and JHMV structural proteins

We evaluated the expression of the JHMV receptor CEACAM1a on NPCs following treatment with 100 U/mL IFN-γ and did not observe a change in the frequency of CECAM1a+ NPCs between treated and non-treated groups at 24 hours p.t. (**Figures 4.6A and B**). However, by 48 hours p.t. the frequency of CEACAM1a+ NPCs decreased from 86.1% ± 1.8% in non-treated NPCs to 62.4% ± 2.5% in IFN-γ treated cultures, and by 72 hours p.t. 61.9% ± 3.5% IFN-γ treated NPCs expressed CEACAM1a (compared to 73.3% ± 0.5%), indicating that IFN-γ dampens JHMV receptor expression by NPCs. The JHMV membrane (M) glycoprotein is critical in virus assembly (35). To determine if M transcripts were decreased following IFN-γ treatment, gene-specific quantitative PCR (qPCR) was performed on total RNA extracts from JHMV-infected NPCs and M transcript levels were normalized to β-actin. M expression was significantly reduced in IFN-γ treated NPCs compared to non-treated NPCs at 48 and 72 hours p.t. ($p < 0.05$; **Figure 4.6C**). These findings suggest that the IFN-γ-induced inhibitory effect on JHMV replication within NPCs is related to both muted expression of CEACAM-1a as well as inhibiting viral RNA synthesis.

4.3 Discussion

This study demonstrates that NPCs derived from the brains of post-natal C57BL/6-GFP mice express the JHMV receptor, CEACAM1a, and support viral replication following CEACAM1a-dependent infection. Additionally, JHMV infection of cultured NPCs induces cytopathic effects over time as evidenced by syncytia formation and elevated LDH levels. Within the context of JHMV infection of the CNS, these findings demonstrate that resident NPCs present within defined anatomical niches may be susceptible to viral infection. Moreover, we have previously shown that intraspinal transplantation of NPCs into mice persistently infected with JHMV results in clinical recovery associated with remyelination (36, 37). Data presented within this report argues that transplanted NPCs may be susceptible to JHMV infection, a finding that highlights important clinical implications for emerging therapies utilizing NPCs to treat human neurologic disease as engrafted cells may be susceptible to infection by persistent neurotropic viruses.

JHMV infection has previously been shown to inhibit constitutive expression of MHC class I in mouse primary astrocyte cultures and to block IFN-γ-induced MHC class II expression on murine cerebral endothelial cells (38, 39). Here, we show that JHMV does not significantly affect MHC class I or II expression following infection of cultured NPCs in the absence of IFN-γ. However, IFN-γ-induced expression of MHC class II was reduced following JHMV infection. MHC expression plays an important role in immune surveillance during viral infection, and control of JHMV replication within the CNS requires antigen recognition by MHC class I and class II restricted CD8+ and CD4+ T cells (26-28). Impaired expression of MHC class II following IFN-γ-treatment of infected NPCs may be a mechanism employed to subvert detection by infiltrating virus-specific CD4+ T cells. Nonetheless,

conditioned media from virus-specific CD4+ T cells was able to suppress JHMV replication within NPCs, likely due to the effects of IFN-γ. Supporting this notion, treatment of infected NPCs with recombinant mouse IFN-γ had a dose-dependent inhibitory effect on virus replication, and blocking IFN-γ receptor abrogated the observed suppressive effects. IFN-γ treatment resulted in fewer CEACAM1a-expressing NPCs with a concomitant decrease in JHMV membrane glycoprotein transcripts, suggestive of viral entry inhibition and reduced virion assembly. We also observed that NPCs pulsed with the CD8-specific viral peptide S510-518 were detected and killed by virus-specific CD8+ T cells, indicating that virally-infected NPCs may be targeted for lysis by CTLs infiltrating into the CNS in response to infection. Collectively, our findings argue that T cells are important for controlling viral replication within NPCs through both cytolytic activity and IFN-γ secretion.

Lineage fate mapping of neural stem/precursor cells residing within the subventricular zone of lateral ventricles and subgranular zone of the hippocampus demonstrate the ability of these cells to differentiate into neurons and glia throughout development (40, 41). Furthermore, endogenous NPCs have been shown to proliferate, migrate, and differentiate in response to acute CNS inflammatory events, such as with spinal cord injury, stroke, and experimental models of chronic inflammatory demyelinating disorders (42-44). Though viewed as a glial tropic virus, this study highlights the potential for NPCs to serve as a reservoir for JHMV infection and replication. CTL-mediated lysis of JHMV-infected NPCs may be detrimental to NPC-mediated repair during CNS inflammation, and a loss of NPCs destined to become oligodendrocytes could contribute to limited remyelination observed in the JHMV-infected CNS. Additionally, our findings have clinical relevance, as NPCs are currently being employed in clinical trials for spinal cord injury as

well as for treating Pelizaeus-Merzbacher disease, a genetic disorder that affects the growth of the myelin sheath (19, 45). As NPCs used for clinical trials are unlikely to be "self-derived", they would be subject to immune recognition and potential destruction by both innate and adaptive immune responses, necessitating long-term immune suppression to prevent graft rejection (13, 25, 46). Several classes of immunosuppressive drugs used during transplantation, including calcineurin inhibitors *i.e.* cyclosporine and FK506, inhibit the activation and/or proliferation of T cells. Such immunosuppressive drugs would foster an environment whereby opportunistic infection or reactivation of latent virus might occur. This raises the possibility that transplanted NPCs may be subject to infection and, in the absence of adequate immune surveillance of the CNS, could lead to damage/death of engrafted cells. With this in mind, careful consideration should be given to potential viral infection when contemplating NPC grafting for treating neurological disease.

4.4 Materials & Methods

Virus

The JHM strain of mouse hepatitis virus (J2.2v-1) was added to NPC cultures at a multiplicity of infection (MOI) of 0.1 PFU/cell. Virus was allowed to absorb overnight (16-18 hours) before media was replaced. Supernatants of infected cultures were collected at defined times p.i. and viral titers were determined using the DBT astrocytoma cell line as previously described (47).

Neural precursor cell culture and reagents

NPCs derived from the striatum of postnatal day 1 transgenic C57BL/6 mice expressing enhanced green fluorescent protein (GFP) were cultured as previously described (37). NPC media consisted of DMEM/F12 with Glutamax (Gibco), N2 supplement (1X, Gibco), ciprofloxacin hydrochloride (100 μg/mL, Cellgro), gentamicin (50 μg/mL, Sigma-Aldrich), Fungizone (2.5 μg/mL, Gibco), penicillin/streptomycin (1000 U/mL, Gibco), and human epidermal growth factor (20 ng/mL, Sigma-Aldrich). Recombinant mouse IFN-γ was purchased from Cell Sciences. For studies involving blockade of CEACAM-1a, NPCs were infected overnight and monoclonal antibody CC1 (eBiosciences) was subsequently added at a concentration of 1 μg/mL. Media was harvested 72 hours p.i. and plaque assay performed to determine viral titers. Experimental blockade of IFN-γ receptor was performed using JHMV-infected NPCs incubated with 250 nM (final) anti-mouse CD119 (IFN gamma receptor 1; eBiosciences) or 250 nM purified rabbit IgG (control; BD Pharmigen) for one hour before media was replaced with non-conditioned or CD4+ T cell conditioned media

+/- anti mouse CD119 or rabbit IgG. Supernatants were harvested 48 hours post-treatment and viral titers determined.

Flow cytometric analysis

Cultured NPCs were dissociated using 0.05% trypsin-EDTA and suspended in PBS containing 0.5% BSA and 2 mM EDTA (Invitrogen). Cells were subsequently treated with blocking antibody (purified rat IgG_{2b} anti-mouse CD16/CD32 monoclonal antibody, 1:200; BD Biosciences) for 20 minutes at 4°C before being incubated with antibodies specific for CEACAM1a (APC-conjugated, 0.06 μg/test, eBioscience), MHC class I (PE-conjugated, 1:200, eBioscience), or MHC class II (PE-conjugated, 1:200, BD Biosciences), for 20-30 minutes. In experiments where FACS analysis of JHMV was performed, NPCs were fixed with 4% paraformaldhyde for 15 minutes before being permeabilized using BD Perm/Wash buffer (BD Biosciences). The anti-JHMV mAb J.3.3 specific for the carboxyl terminus of the viral nucleocapsid (N) protein was conjugated to Alexa Fluor 647 using the APEX labeling system (Life Technologies) and used at a final concentration of 1.5 ng/mL. Detection of fluorescence was performed using a LSR II flow cytometer (BD Biosciences) and analysis of FACS data was performed with FlowJo software (Tree Star).

RNA Isolation, reverse transcription, and polymerase chain reaction

Total RNA was isolated from C57BL/6 splenocytes and NPCs using TRIzol reagent (Invitrogen) and purified by phenol-chloroform extraction. cDNA was reverse transcribed from RNA according to manufacturer's instructions using the SuperScript III First-Strand Synthesis system (Invitrogen) and random hexamers. Standard PCR for CEACAM1a

expression was performed with an Eppendorf Mastercycler using the Platinum Taq DNA polymerase kit (Invitrogen) and the following primers purchased from Integrated DNA Technologies: TTCCCTGGGGAGGACTACTG (forward primer) and TGTATGCTTGCC CCGTGAAAT (reverse primer). Gene products were run alongside a 1 Kb Plus DNA ladder (Invitrogen) on a 1% agarose gel containing ethidium bromide before being imaged using the Bio-Rad GelDoc system. For quantitative RT-PCR experiments, primers specific for the JHMV membrane protein (forward: CGAGCCGTAGCATGTTTATCTA; reverse: CGCATACACGCAATTGAACATA) were designed using PrimerQuest software (Integrated DNA Technologies, Inc.). SYBR Green Real-Time PCR Master Mix (Life Technologies) was used according to manufacturer's specifications and RT-PCR was performed using the Applied Biosystem ViiA 7 Real-Time PCR System. C_t values of M protein transcripts were normalized to β-actin C_t values (forward: GGCCCAGAGCAAGAGAGGTATCC; reverse: ACGCACGATTTCCCTCTCAGC) and compared using the $\Delta\Delta C_t$ method.

Immunofluorescence

To evaluate JHMV infection of cultured NPCs, cells were dissociated and plated on slides or cover slips coated with reduced growth factor Matrigel (BD Biosciences). NPCs were infected with JHMV overnight and fixed 72 hours p.i. with 4% paraformaldehyde for 10 minutes at room temperature. Immunofluorescence staining was performed as previously described (33) using the anti-JHMV mAb J.3.3 (1:20 dilution) specific for the carboxyl terminus of the viral nucleocapsid (N) protein and the Alexa Fluor 405 goat anti-mouse IgG_1 secondary antibody (Life Technologies), as well as rabbit monoclonal anti-Sox2

(Epitomics) and Alexa Fluor 568 goat anti-rabbit IgG_1 secondary antibody (Life Technologies) . Slides were imaged using a Nikon Eclipse Ti inverted microscope.

JHMV-induced cell death assay

NPC death due to JHMV infection was evaluated at 24, 48, and 72 hours p.i. by measuring lactate dehydrogenase released by lysed cells according to manufacturer's recommendations using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega). Briefly, spontaneous and virus-induced LDH levels were determined using the following formula: % Lysis = (Experimental LDH release) / (Maximum LDH release). LDH levels from JHMV-infected cultures were then normalized to spontaneously released LDH and expressed as cell death due to infection (%).

CD4+ T cell isolation for NPC media conditioning

C57BL/6 mice were infected with an i.p. injection of 2.5×10^5 PFU of a demyelinating (DM) variant of JHMV. On day 8 p.i., CD4+ T cells were isolated from spleens by negative selection according to manufacturer's specifications using the EasySep Mouse CD4+ T cell Isolation kit (Stemcell Technologies). Briefly, red blood cell depleted splenocytes were suspended at a concentration of 1×10^8 cells/mL in PBS + 2% FBS with 1 mM EDTA. Normal rat serum was added at the appropriate concentration and cells were incubated with a cocktail containing a combination of biotinylated monoclonal antibodies directed against CD8a, CD11b, CD11c, CD19, CD45R/B220, CD49b, TCRγ/δ and TER119, for 10 minutes. Subsequently, a suspension of streptavidin-coated magnetic particles in PBS was added and incubated with the cells for 2.5 minutes; buffer was added to the appropriate volume, and

cells were incubated in the EasySep Magnet for 2.5 minutes to foster binding of magnetically-labeled unwanted cells to the tube walls before CD4+ T cells were poured off. To generate CD4+ T cell conditioned NPC media, the magnetically-labeled fraction following depletion of total T cells was collected using the EasySep Mouse T Cell Isolation kit (Stemcell technologies). This enriched fraction was treated with 50 μg/ml mitomycin-C (AG Scientific), and 35×10^6 cells were co-cultured with 35×10^6 CD4+ T cells in 10 mL NPC media containing 5 μm CD4-specific membrane (M) glycoprotein spanning amino acid residues 133–147 (M133-147, Bio-Synthesis) for 48 hours. CD4+ T cell conditioned media was administered to JHMV-infected NPCs and supernatants were harvested 24, 48, and 72 hours p.i. for determination of viral titers. Levels of IFN-γ in CD4+ T cell conditioned media were determined by ELISA using the Mouse IFN-γ DuoSet according to manufacturer's recommendations (R&D Systems). Interferon-beta levels in JHMV-infected NPC cultures were evaluated using the VeriKine Mouse Interferon Beta ELISA kit (PBL Assay Science). The animal protocols and procedures used for these studies were reviewed and approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

CD8+ T cell cytotoxicity assay

NPCs were seeded at a density of 20,000 cells/well in a flat-bottom 96-well format tissue culture plate (Corning Life Sciences) and pulsed overnight with 5 μM of the immunodominant CD8 peptide specific for MHV spike (S) glycoprotein spanning amino acids 510-518 (S510-518, Bio-Synthesis). NPCs were simultaneously treated overnight with 100 U/ml IFN-γ to induce MHC class I expression for the presentation of S510-518. CD8+ T cells isolated from DM-infected C57BL/6 mouse splenocytes (as mentioned for

CD4+ T cells) using the EasySep Mouse CD8+ T cell Isolation kit (Stemcell Technologies) were then plated with NPCs at effector-to-target (E:T) ratios ranging from 10:1 to 0.31:1. Co-cultures were incubated for 4 hours at 37°C in 5% CO_2 at a final volume of 200 µL/well. The amounts of lactate dehydrogenase released from lysed cells were determined using a CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega). The percentage of CTL-mediated lysis was determined as specified by the manufacturer's protocols. RMA-S cells pulsed overnight with 50 µM S510-518 were used as a positive control for cell lysis.

Statistical analysis

Statistical analysis was carried out using student's *t* test, one-way ANOVA, or repeated measures ANOVA and $p \leq 0.05$ was considered significant.

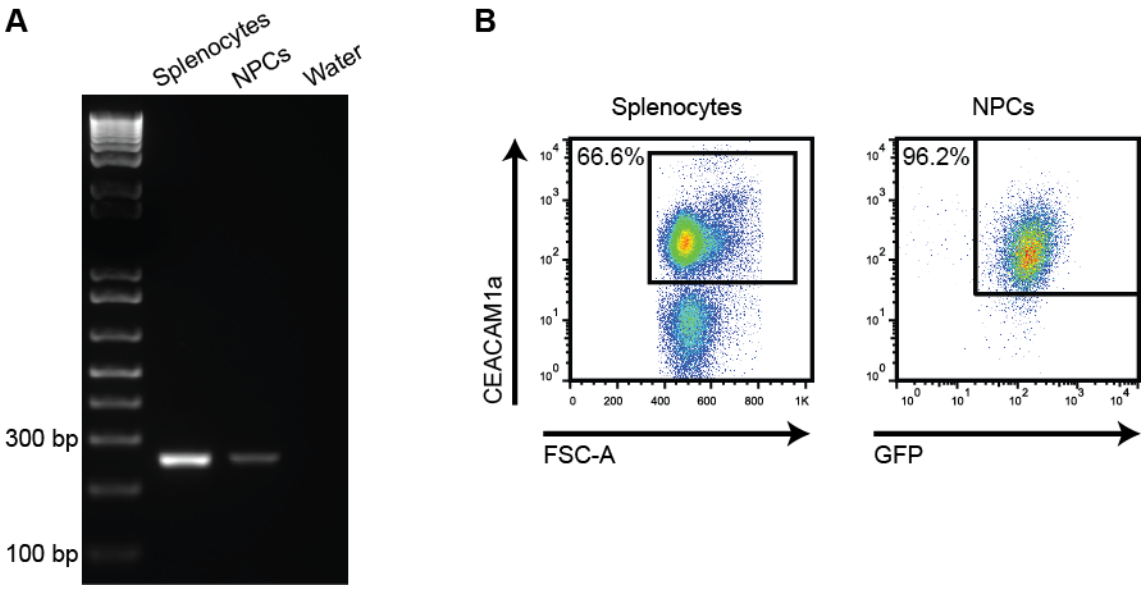

Figure 4.1. Cultured NPCs express CEACAM1a. (**A**) PCR amplification revealed that cultured C57BL/6 NPCs express transcripts specific for the JHMV receptor CEACAM1a. A 250 bp amplicon, specific for CEACAM1a, was amplified from cDNA generated from total RNA extracted from cultured NPCs; controls included water and splenocytes isolated from non-infected mice. (**B**) Representative dot plots showing CEACAM1a expression on the cell surface of C57BL/6 splenocytes (control) and cultured NPCs.

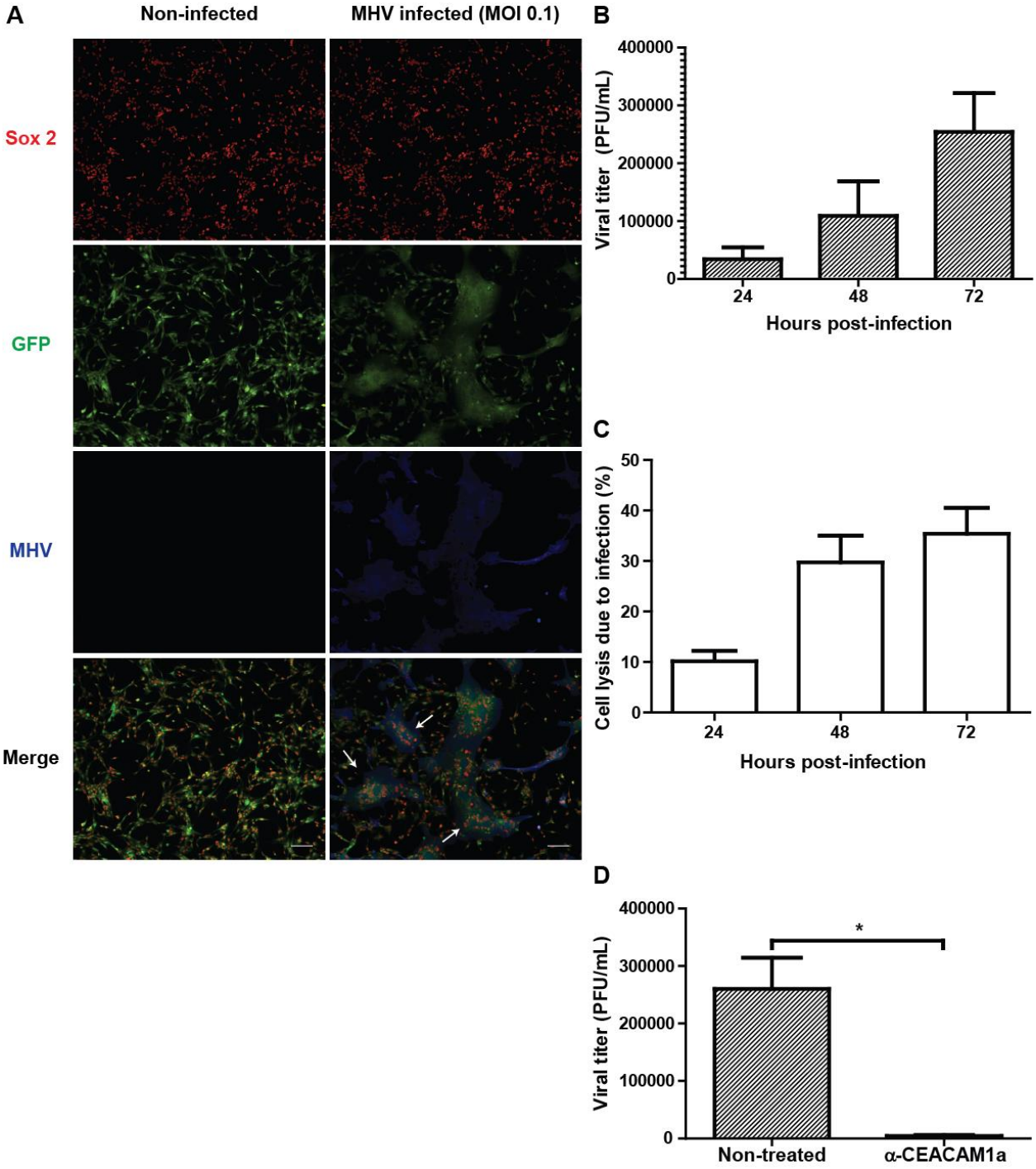

Figure 4.2. JHMV infection of cultured NPCs. (**A**) GFP-expressing NPCs were infected with JHMV (m.o.i. = 0.1) for 18 hours. After a 72 hour incubation, cells were fixed and stained with antibodies specific for Sox2 (red) and the JHMV nucleocapsid (N) protein (blue) and visualized by fluorescence microscopy (scale bar = 100 uM). Arrows indicate Sox2+, JHMV+ syncytia. (**B**) Supernatants were harvested from MHV-infected NPC cultures 24, 48, and 72 hours following infection and viral titers determined by plaque assay. (**C**)

LDH released by infected NPCs was detected in viral supernatants and normalized to LDH levels from non-infected NPC cultures to evaluate cell death due to MHV infection. (**D**) Monoclonal CC1 antibody blockade of CEACAM1a following JHMV infection resulted in significantly ($p < 0.05$) reduced viral titers in cultured NPCs compared to non-treated controls by 72 hours p.i. For panels *B-D*, data is presented as average ± SEM and represents 3 independent experiments.

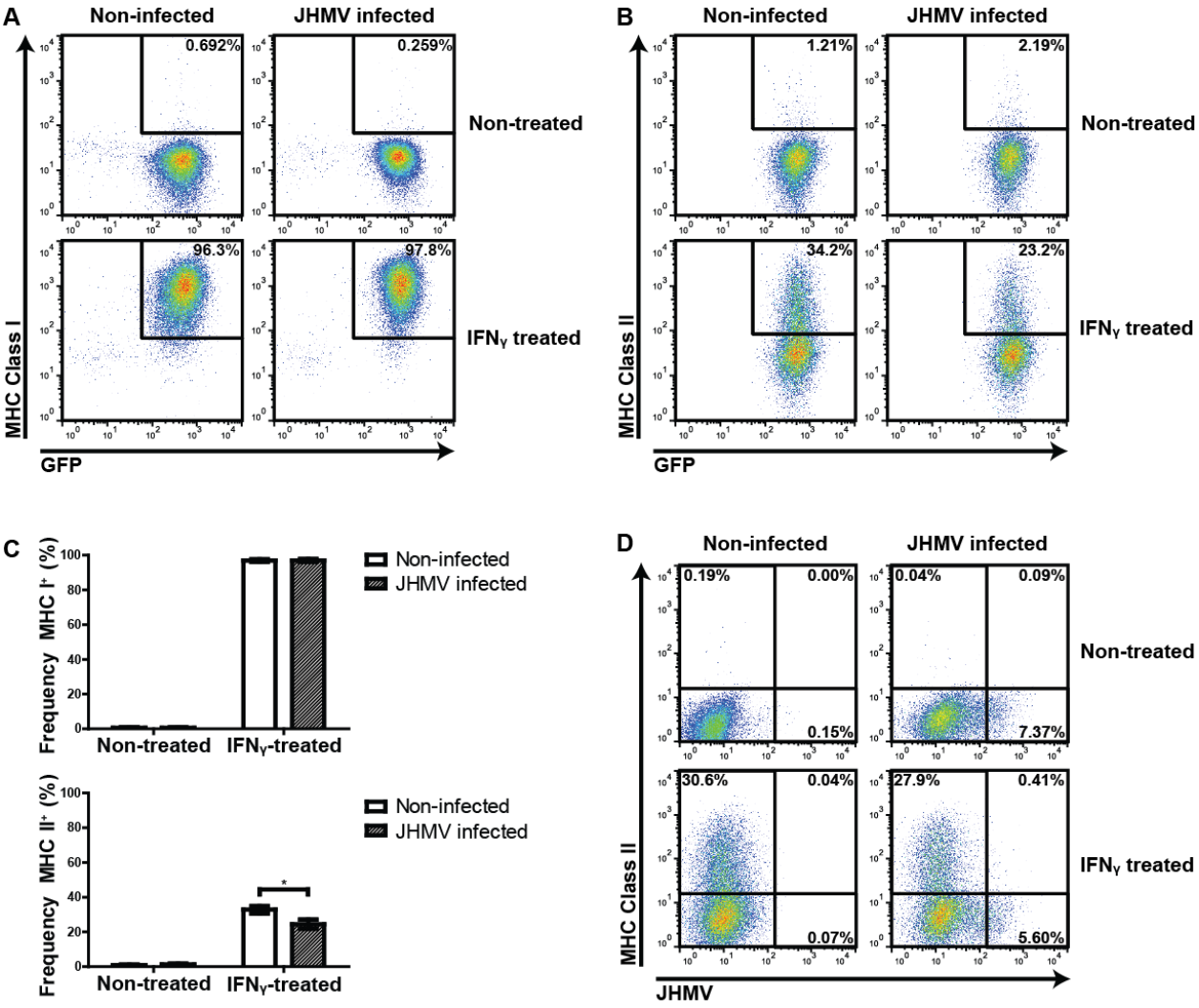

Figure 4.3. MHC expression by NPCs in response to JHMV infection. Cultured GFP NPCs were infected with JHMV (m.o.i.=0.1) for 18 hours and subsequently replenished with media alone or containing 100 U/ml IFN-γ. Cells were incubated for 24 h and MHC class I/II expression of non-infected and infected NPCs evaluated via flow cytometry. The frequency of NPCs expressing either MHC class I (**A**) or MHC class II (**B**) is shown in representative dot plots. (**C**) Quantification of the frequency of MHC I/II expression by infected or non-infected NPCs treated with media alone or in combination with IFN-γ. Data is presented as average ± SEM and represents 3 independent experiments (*$p < 0.05$).(**D**) Representative dot plots of MHC II-positive NPCs versus JHMV-positive NPCs.

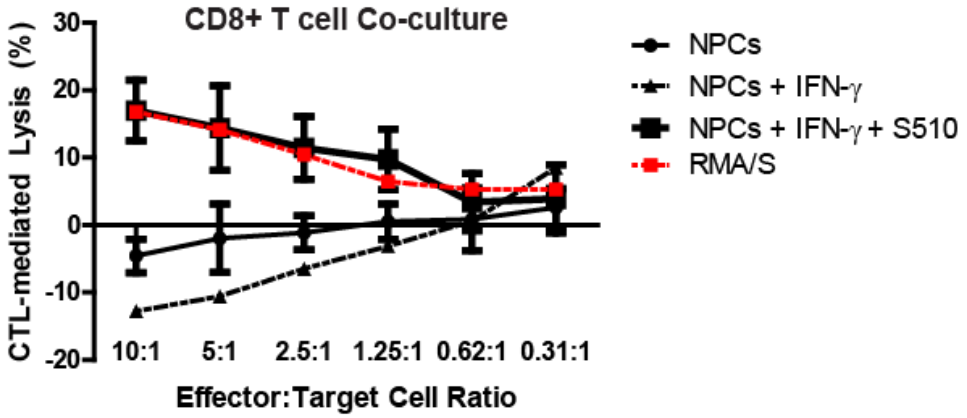

Figure 4.4. Virus-specific CD8+ T cells target S510-518 pulsed NPCs for lysis. CTLs were harvested from mice immunized with the DM variant of JHMV and co-cultured at varying effector:target ratios with S510-518 pulsed, IFN-γ-treated NPCs for 4 hours, and lactate dehydrogenase released into the supernatant was subsequently measured. Non-IFN-γ-treated RMA/S cells pulsed with 50μM S510-518 were used as a positive lysis control.

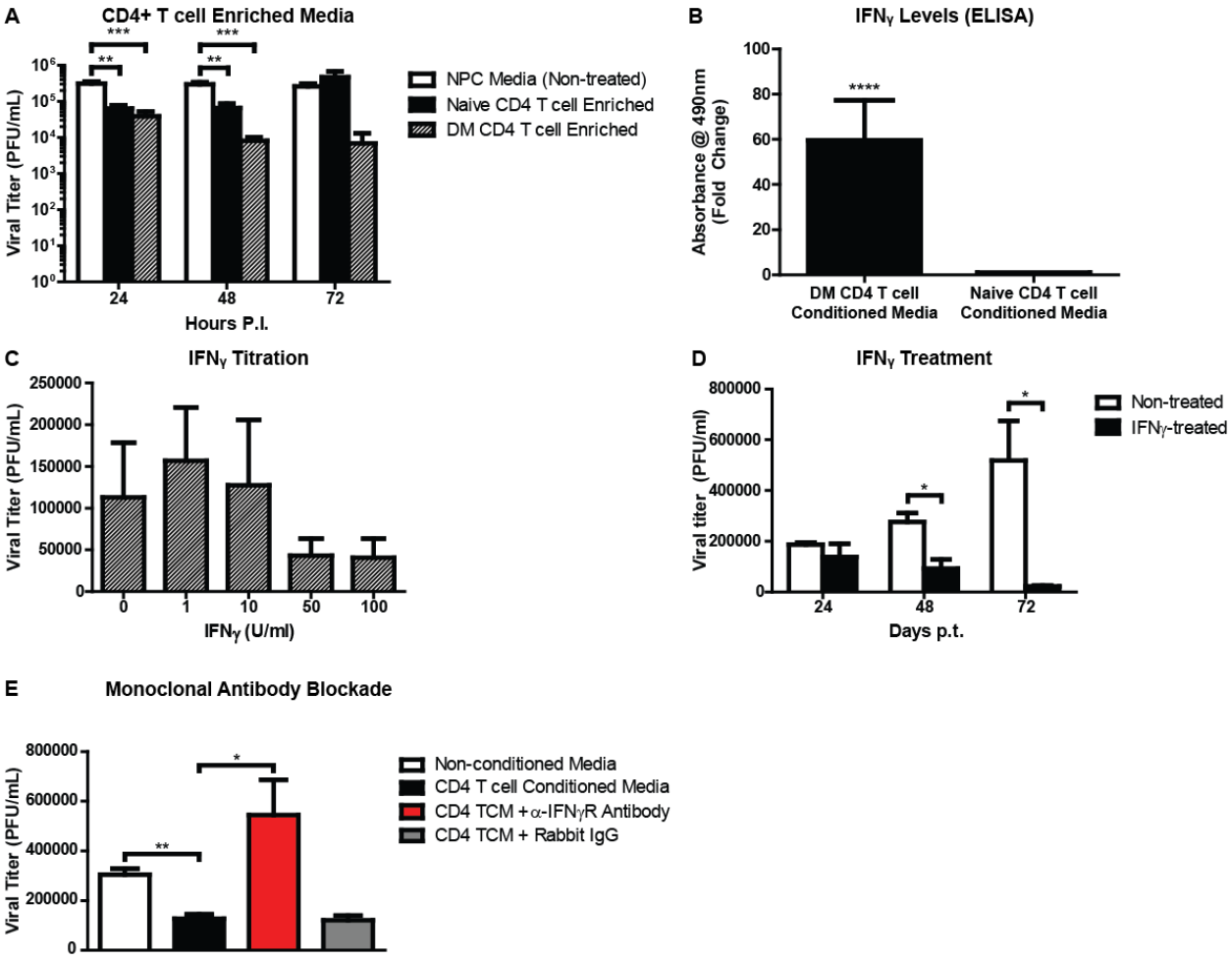

Figure 4.5. Virus-specific CD4+ T cell enriched media reduces viral titers via IFN-γ. (**A**) Media enriched with cytokines from naïve or CD4+ T cells significantly ($p < 0.01$ and $p < 0.001$, respectively) reduced viral titers in JHMV-infected NPCs at defined times p.i. compared to non-conditioned media. (**B**) Enzyme-linked immunosorbent assay for IFN-γ in naïve-versus-DM-specific CD4+ T cell supernatants. Absorbance values at 490nm were normalized to media alone and expressed as fold changes (DM absorbance / naïve absorbance). For statistical analysis, background-subtracted absorbance values were used (****$p < 0.0001$). (**C**) High doses of IFN-γ suppress JHMV replication in NPCs, and (**D**) treatment with 100 U/mL IFN-γ significantly inhibits virus replication at 72 hours p.t. (**E**) Monoclonal antibody blockade of IFN-γ receptor abrogated the virus-suppressive effects of DM CD4+ T cell conditioned media. For all panels, data is presented as average ± SEM and represents 3 independent experiments (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

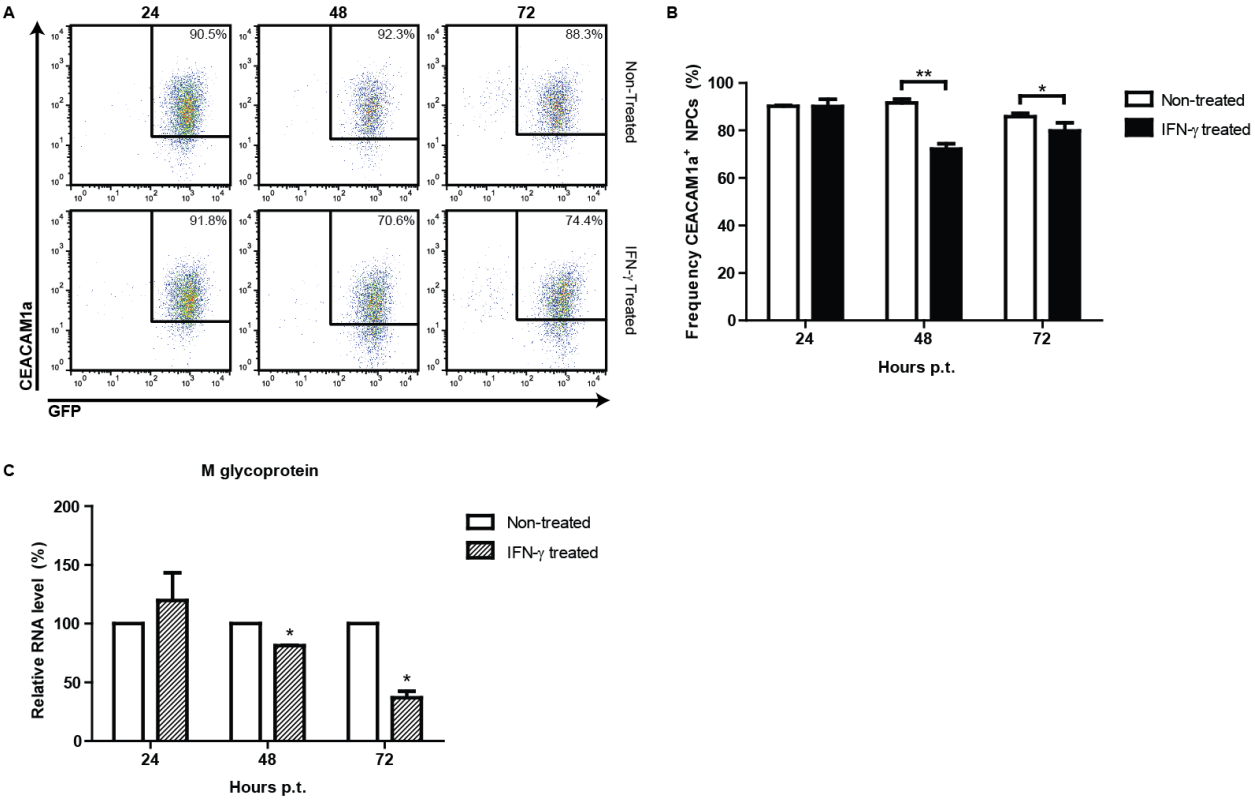

Figure 4.6. IFN-γ reduces JHMV receptor expression and JHMV transcripts in infected NPCs. (**A**) Representative dot plots of CEACAM1a-expressing GFP NPCs at 24, 48, and 72 hours post-IFN-γ-treatment. (**B**) Quantification of the frequency of CEACAM1a+ NPCs with or without IFN-γ treatment. (**C**) Relative percent expression of JHMV membrane (M) protein transcripts from infected, IFN-γ-treated NPCs compared to non-infected, non-treated NPCs. For panels B and C, data is presented as average ± SEM and represents 3 independent experiments (*$p \leq 0.05$). For panel C, ΔC_t values were used for statistical analysis.

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CHAPTER FIVE

Remyelination is correlated with regulatory T cell induction following human iPSC-derived neural precursor cell transplantation in a viral model of multiple sclerosis

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Abstract

We have recently described sustained clinical recovery associated with dampened neuroinflammation and remyelination following transplantation of neural precursor cells (NPCs) derived from human embryonic stem cells (hESCs) in a viral model of the human demyelinating disease multiple sclerosis. Induced pluripotent stem cells (iPSCs) are a preferable source of NPCs as they can be reprogrammed from adult somatic cell types, circumventing the destruction of embryos and permitting autologous transplantation. Here, we investigated the therapeutic potential of NPCs generated from human iPSCs in mice infected with the neurotropic JHM strain of mouse hepatitis virus (JHMV) that induces an immune-mediated demyelinating disease sharing clinical and histologic similarities to the human demyelinating disease multiple sclerosis (MS). JHMV-infected mice intraspinally transplanted with iPSC-derived NPCs (hiNPCs) exhibited decreased accumulation of CD4+ T cells in the central nervous system that was concomitant with reduced demyelination at the site of injection. Dampened neuroinflammation and remyelination was correlated with a transient increase in CD4+FOXP3+ regulatory T cells (Tregs) concentrated within the peripheral lymphatics. Importantly, pathological improvements were limited in comparison to our previous report on hESC-derived NPCs and did not result in significant clinical recovery. These findings highlight an intrinsic disparity in the therapeutic potential of NPCs generated from pluripotent stem cells.

5.1 Introduction

Multiple sclerosis (MS) is considered a chronic autoimmune disorder affecting the central nervous system (CNS) in which infiltration and accumulation of lymphocytes in the brain and spinal cord leads to demyelination followed by axonal degeneration. Early stages of the disease are characterized by transient inflammation and compensatory remyelination resulting in a cycle of descending neurologic dysfunction and limited recovery (1, 2). However, endogenous myelin repair is not sustainable and ultimately gives way to a stage of chronic neurodegeneration and progressive accumulation of disability. Current FDA-approved disease-modifying therapies (DMTs) target the immune component of MS and have demonstrated effectiveness in reducing relapse rates, although this is often not sustainable (3). However, the most commonly prescribed DMTs do not directly address white matter damage in the CNS and are consequently ineffective at treating advanced stages of MS. Therefore, an unmet need for a treatment strategy that addresses inflammatory cell infiltration while promoting long-term remyelination remains.

Neural precursor cells (NPCs) have emerged as a viable therapeutic approach for the treatment of a variety of neurological disorders. Previously, transplantation of NPCs was shown to attenuate disease pathology in animal models of Alzheimer's disease, Parkinson's disease, Huntington's disease, and spinal cord injury (4-8). Human NPCs have also been shown to differentiate into myelin-competent oligodendrocytes and directly remyelinate host axons when transplanted into the CNS of hypomyelinated mice (9). Importantly, in rodent and non-human primate models of MS where neuroinflammatory demyelination is triggered via immunization against myelin peptides, NPCs suppress encephalitogenic T cell activation and enhance endogenous myelin regeneration (10-13).

One caveat of previous studies is the use of NPCs isolated from fetal CNS tissue. The dearth and controversial nature of fetal-derived NPCs necessitates an alternative cell source that can be robustly expanded and characterized *in vitro*. We have recently shown that intraspinal injection of human embryonic stem cell (hESC) derived neural precursor cells promotes neurologic recovery in mice afflicted with virus-induced immune-mediated demyelination (14). Importantly, recovery was facilitated by the emergence of immunosuppressive regulatory T cells (Tregs) and was not dependent on long-term engraftment of human NPCs. Thus, human pluripotent stem cells may serve as an inexhaustible source from which to generate NPCs for the treatment of MS.

We and others have shown that CNS transplantation of major histocompatibility complex (MHC) mismatched NPCs results in recognition and destruction by inflammatory T cells and natural killer (NK) cells (15-17). Induced pluripotent stem cells (iPSCs) are an attractive alternative to hESCs since they are not acquired from a blastocyst-stage embryo and maintain the genetic background of the donor, which may circumvent the requirement for immunosuppressant drugs that leave patients vulnerable to infection and re-activation of dormant viruses. Herein, we demonstrate that NPCs generated from a human iPSC line (hiNPCs) are rapidly rejected following intraspinal transplantation into mice in which immune-mediated demyelination was initiated via inoculation with the neurotropic JHM strain of mouse hepatitis virus (JHMV). Although significant clinical recovery was not observed, transplanted mice exhibited dampened accumulation of CD4+ T cells in the CNS. Reduced T cell infiltration was correlated with focal remyelination and a transient increase in CD4+FOXP3+ Tregs in draining cervical lymph nodes (CLNs). Finally, hiNPCs induced conversion of conventional CD4+ T cells to Tregs *in vitro*, and *in vivo* ablation of Tregs

abrogated hiNPC-induced histopathological improvement. Combined with our previous work utilizing hESC-derived NPCs, these findings suggest variability in the therapeutic potential of pluripotent stem cell derived products within the context of neuroinflammatory demyelination.

5.2 Results

Neural precursor cells derived from human iPSCs are rapidly rejected following transplantation into mice persistently infected with JHMV

In order to evaluate graft survival and migration following intraspinal delivery of human NPCs, the feeder-free adapted HDF51iPS1 line of human fibroblast-derived iPSCs was transduced with lentivirus expressing the *Photinus pyralis* firefly luciferase gene. Following puromycin selection, iPSCs constitutively expressing luciferase produced detectable photons in response to D-luciferin *in vitro* (**Figure 5.1A**). Next, luciferase-expressing iPSCs were differentiated to the neural precursor cell (NPC) stage using a combination of the small molecule SB431542 and recombinant human Noggin, potent inhibitors of SMAD signaling, as previously described (18). Dual-inhibition of SMAD signaling results in heterogeneous cultures of neural stem and precursor cells, neural crest cells, and glial contaminants, and human iPSC exhibit variability in their potential to differentiate to NPCs (19, 20). Therefore, differentiated iPSCs were enriched for NPCs using a flow cytometric (FACS) based sorting strategy using the markers CD24, CD184, CD271, and CD44 (**Figure 5.1B**) (19, 21). Following isolation and recovery, the CD184+/CD24+/CD271-/CD44- population expressed high levels of the hallmark neural stem/precursor cell markers Nestin, Pax6, and Sox2 (Figure 1C, top row). Furthermore, upon growth factor withdrawal, CD184+/CD24+/CD271-/CD44- NPCs differentiated into Tuj1+ neurons, GFAP+ astrocytes, and NG2+ oligodendrocyte precursors, confirming multipotency (**Figure 5.1C**).

Mice intracranially inoculated with a neuroadapted JHM strain of mouse hepatitis virus (JHMV) develop inflammatory lesions in the white matter tracts of the brain and spinal cord, ultimately resulting in hind-limb paralysis (22). To evaluate clinical and

immunological consequences of transplanting multipotent human iPSC-derived NPCs (hiNPCs), hiNPCs were injected into the spinal cords of immunocompetent mice afflicted with chronic neuroinflammatory demyelination as a result of JHMV infection as previously described (23). *In vivo* bioluminescence (IVIS) imaging revealed that transplanted hiNPCs were present in the spinal cords of mice at day 1 post-transplant (p.t.). However, a marked reduction in luciferase activity was detected at day 4 p.t., and by day 8 p.t. hiNPCs were undetectable by IVIS imaging (**Figure 5.2A**). Histologic analysis of a human-specific cytoplasmic marker confirmed loss of hiNPCs in the spinal cords of transplanted mice, suggesting rejection of hiNPCs was complete by approximately 1 week p.t. (**Figure 5.2B**).

Demyelination and T cell infiltration is reduced in hiNPC-transplanted animals

We have previously shown that animals transplanted with NPCs derived from the WA09 line of human ESCs exhibit sustained clinical recovery associated with reduced neuroinflammation and demyelination (14). JHMV-infected animals that received an intraspinal transplant of CD184+/CD24+/CD271-/CD44- hiNPCs at day 14 p.i. did not exhibit clinical benefits as compared to animals transplanted with human dermal fibroblasts, or animals intraspinally injected with HBSS as a control by day 21 p.t. (**Supplementary Figure 5.S1**). Furthermore, FACS analysis of the 12 mm spinal cord region spanning the injection site revealed no differences between the frequencies of CD8+ T cells, virus-specific CD4+ and CD8+ T cells, and macrophages compared between HBSS, fibroblast, and hiNPC injected animals at day 21 p.t. (**Figures 5.3A,B and Supplementary Figure 5.S2**). However, there was a significant ($p<0.01$) reduction in CD4+ T cell frequency

was observed in hiNPC transplanted animals compared to controls, indicating dampened CD4+ T cell accumulation as a result of transient hiNPC engraftment (**Figures 5.3A and B**).

Luxol fast blue (LFB) staining of serially-sectioned spinal cord tissue revealed that hiNPC transplanted animals had reduced demyelination compared to fibroblast and HBSS injected controls (**Figure 5.4A**). Quantification of demyelination confirmed a significant ($p<0.01$) difference in demyelination at the transplant site of hiNPC-transplanted animals compared to controls (**Figure 5.4B**). However, the observed reduction in demyelination was not sustained along the rostro-caudal axis relative to the injection site, indicating that hiNPC sparing of myelin was focally restricted (**Figure 5.4C**). We further investigated the extent of remyelination in hiNPC-transplanted animals by performing transmission electron microscopy (TEM) of spinal cord sections and compared the axon diameters of neurons to total myelinated fiber diameters (14). Indeed, hiNPC transplanted mice had extensive signs of remyelination compared to HBSS or fibroblast injected animals, and *g*-ratio analysis confirmed a significant ($p<0.001$) difference in myelin status at the site of transplant (**Figures 5.4D and E**).

Regulatory T cells are induced as a result of hiNPC transplantation and are required for histopathological improvement

Regulatory T cells (Tregs) are a subset of suppressive T cells that are crucial to the development and maintenance of self-tolerance which have been shown to modulate autoimmune disease (24). JHMV-infected animals exhibit dampened neuroinflammation and attenuated disease pathology as a result of Treg adoptive transfer (25). We have previously shown that Tregs induced in the CNS following transplantation of hESC-derived

NPCs are critical for clinical and histologic improvement in mice persistently infected with JHMV (14). We evaluated the levels of CD4+FOXP3+ Tregs in the draining cervical lymph nodes (CLNs) and CNS of animals transplanted with hiNPCs at days 5, 7, and 21 p.t. Tregs were significantly increased in the CLNs, but not the CNS, of hiNPC-transplanted animals compared to fibroblast and HBSS injected controls at day 5 p.t. (**Figure 5.5A**). Increased Treg accumulation in the CLNs and spinal cord was not detected in hiNPC, fibroblast, or HBSS injected animals at days 7 and 21 p.t., suggesting a rapid and transient induction of Tregs in hiNPC-transplanted mice (**Figures 5.5B and C, and data not shown**). To determine if hiNPCs could directly convert T cells to a Treg phenotype, hiNPCs were co-cultured with T cells enriched from splenoctyes of non-infected mice. After 3 days, an increase in the frequency and number of CD4+FOXP3+ Tregs was observed in cultures when activated T cells were co-cultured with hiNPCs, but not when T cells were cultured in the absence of hiNPCs (**Figures 5.6A and B**). Furthermore, Treg induction was proportionate to the number of hiNPCs and was concomitant with a decrease in conventional CD4+FOXP3- T cells (**Figure 5.6C**). We previously demonstrated that *in vitro* Treg induction by hESC-NPCs was dependent on secretion of the pleiotropic cytokine transforming growth factor-beta (TGF-β) (14). However, compared to hESC-NPCs, secretion of TGF-β1 and TGF-β2 could not be detected in the culture media of hiNPCs (**Supplementary Figure 5.S3**).

In order to evaluate the requirement of Tregs for remyelination following hiNPC transplantation, mice were injected with a monoclonal antibody raised against CD25 at days -2, 0, and 2 pt. Treatment with anti-CD25 resulted in a decrease in the frequency of circulating Tregs by day 3 pt that was sustained out to day 21 pt, the day spinal cords were

collected for histology (**Figures 5.7A and B**). *In vivo* ablation of Tregs resulted in increased demyelination compared to non-treated hiNPC transplanted animals (**Figures 5.7C and D**).

5.3 Discussion

Neural precursor cells (NPCs) are rapidly transitioning to the clinic for the treatment of a variety of neurological disorders. Human trials testing safety and efficacy of NPC grafts in patients with chronic spinal cord injury, amyotrophic lateral sclerosis, and Pelizaeus-Merzbacher disease are currently being performed (26, 27). Given that current FDA-approved front-line therapies do not offer relief from symptoms for patients with progressive forms of disease, MS is an attractive target for cell based therapeutics. However, the variety of sources from which to procure NPCs, combined with the growing number of methods whereby NPCs can be generated *in vitro*, warrants thorough investigation of the therapeutic value of relevant cell types.

Human NPCs isolated from fetal CNS tissue have an extensive history of transplantation in animal models of neurodegenerative disease. Non-human primates afflicted with autoimmune encephalomyelitis (EAE) demonstrated improvements in disease pathology and clinical outcome when human fetal NPCs were injected systemically (11). However, long-term immune suppression via administration of the calcineurin inhibitor cyclosporine prolonged the survival of fetal NPCs *in vivo*. Evidence suggests allogeneic NPCs will be rapidly rejected following transplantation into immune competent animals with ongoing neuroinflammation (15), and both the immune-modulatory and trophic support functions of fetal NPCs have been shown to be limited temporally (28, 29). Moreover, the use of fetal-derived NPCs for transplantation is restricted by the availability of donor tissue.

Human pluripotent stem cells have the intrinsic capacity for unlimited self-renewal and for the production of differentiated cell types suitable for transplant therapy. NPCs

derived from hESCs ameliorate the clinical course of EAE by modulation of peripheral T cells in affected mice (10). However, as with fetal NPCs, the neuroprotective effect of transplanted hESC-NPCs in EAE was dependent on survival and migration of the xenograft. In contrast, we recently demonstrated that sustained clinical recovery could be achieved in spite of xenograft rejection following intraspinal injection of hESC-NPCs in a virus-induced model of neuroinflammatory demyelination (14). Suppression of lymphocyte infiltration was facilitated by local induction of regulatory T cells in a TGF-β-dependent fashion, contradicting previous findings that hESC-NPCs must migrate to the peripheral lymphatic system and inhibit dendritic cell maturation or T cell activation in the lymph nodes for histologic improvement to be observed.

Here, we report the first evaluation of the therapeutic potential of neural precursor cells derived from human iPSCs in a model of virus-induced immune-mediated demyelination. Similar to hESCs, hiPSCs have unlimited proliferative potential and generate NPCs *in vitro*. Additionally, hiPSCs need not be isolated from blastocyst stage embryos, and the movement to establish a global library of iPSCs compliant with clinical good manufacturing practices is underway (30, 31). However, though they follow the same transcriptional program as hESCs during neural conversion, hiPSCs exhibit variable propensity to differentiate, particularly to the neural precursor stage (20, 32). Therefore, we implemented a FACS-based strategy to enrich for a population of NPCs free of glial and neural crest contaminants. FACS-sorted NPCs expressed hallmark markers of neural stem and precursor cells and subsequently differentiated to the major CNS lineages *in vitro*. As with hESC-NPCs, hiNPCs were rapidly rejected following injection into the spinal cords of JHMV-infected mice, and no cellular migration to peripheral immune organs was observed.

In spite of rejection, CD4+ T cell infiltration was muted in the CNS of hiNPC-transplanted mice, which correlated with a transient induction of Tregs in the draining CLNs. Tregs, a T cell lineage essential to the establishment and maintenance of self-tolerance, have been shown to be protective in animal models of inflammatory and autoimmune disease, including EAE and JHMV-induced demyelination (25, 33). Furthermore, NPCs have been suggested to convert conventional and encephalitogenic T cells to a Treg phenotype (34, 35), and hESC-NPC induced recovery in JHMV-infected animals was dependent on local Treg induction (14). Indeed, hiNPCs induced conventional T cells to a Treg phenotype *in vitro*, and ablation of Tregs by monoclonal antibody blockade abrogated histopathological improvement in transplanted mice. Induction of Tregs and dampened accumulation of CD4+ T cells was accompanied by remyelination in the white matter tracts of hiNPC injected animals. Remyelination was evaluated at the point at which hiNPCs could no longer be detected in the CNS by immunohistochemical staining or *in vivo* imaging, suggesting that endogenous myelin regeneration, and not cell replacement by hiNPCs, was responsible for observed histologic improvement. Xenograft rejection has been proposed to foster limited remyelination following transplantation of human glial-restricted precursors in the JHMV model (36). In this study, we did not observe enhanced remyelination or a reduction in infiltrating lymphocytes in animals transplanted with human fetal fibroblasts, confirming the specificity of hiNPC-induced remyelination.

In contrast to our previous study utilizing hESC-derived NPCs (14), JHMV-infected mice transplanted with hiNPCs did not display significant improvements in clinical outcome. These data corroborate a recent study that demonstrated injection of human iPSC-derived neural stem cells (NSCs) did not recapitulate functional improvements

observed following CNS transplantation of hESC-derived NSCs in a rodent model of spinal cord injury (37). There are several hypotheses that may explain observed differences in clinical improvement in our model, and of particular interest is the production of transforming growth factor beta (TGF-β) by NPCs. TGF-β is a ubiquitous cytokine that exists in at least three isoforms: TGF-β1, TGF-β2, and TGF-β3. Generation of Tregs from CD4+ precursors requires stimulation of the TGF-β receptor, and TGF-β2 triggers Foxp3 expression in conventional CD4+ T cells (38). Using transcriptome analysis, Chen et al. (2014) observed upregulation of TGF-β1 and TGF-β2 by hESC-NPCs, and secreted TGF-β was detected in hESC-NPC supernates. Immunoassay of media collected from hiNPC cultures did not detect TGF-β1 or TGF-β2, which may explain the different spatial and temporal dynamics of Treg induction, and lack of functional improvement, in hiNPC-transplanted JHMV-infected mice. An additional pleiotropic cytokine that may contribute to immune modulation and repair in models of demyelination is leukemia inhibitory factor (LIF). LIF promotes Treg induction mainly by suppressing differentiation of conventional CD4+ T cells to a pro-inflammatory phenotype (39, 40). Moreover, LIF is secreted by transplanted murine NPCs and is proposed to be involved in NPC-induced neuroprotection in EAE (41, 42). Similar to TGF-β, human LIF could not be detected in hiNPC supernates (data not shown). Thus, it is possible that the methods used to generate and isolate hiNPCs in this study may have yielded a population of cells that lack the appropriate biomarkers to confer significant and sustained repair in JHMV-infected mice.

Cumulatively, this report highlights the need for rigorous characterization and selection of therapeutically valuable cell types derived from human pluripotent stem cells for the treatment of neurodegenerative disease. The ability to suppress neuroinflammation

via Treg induction and consequently induce endogenous remyelination makes NPCs an attractive candidate therapeutic for treating MS. However, the use of NPCs for cell based therapies is complicated by the growing number of methods to generate neuroectodermal-lineage cell types from hESCs and hiPSCs (43). When significant clinical improvement was observed following transplantation of hESC-NPCs into JHMV-infected mice, cells were selected for based on a definitive transcriptomic signature (14). Uniform methods for validating the genetic and functional phenotype of clinically relevant NPCs, as well as standardized protocols for driving pluripotent stem cells to disease-modifying NPCs, will be critical for future success of NPC transplantation in patients with neurologic disorders.

5.4 Materials and Methods

Animal Care and Infection

Age-matched male C57BL/6 mice (H-2b, National Cancer Institute) were infected intracranially (i.c.) with 150 plaque-forming units (p.f.u.) of the JHM (J2.2-V-1) strain of MHV in 30 µl HBSS. Mice were sacrificed via isofluorane inhalation at defined time points post-transplant for tissue harvesting and analysis. The animal protocols and procedures used for these studies were reviewed and approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

Derivation and Maintenance of hiNPCs

The human iPSC line HDF51iPS1 was reprogrammed from primary fetal fibroblasts by infection with retroviruses carrying expression vectors for Pou5f1, Sox2, Klf4, and c-Myc genes as previously described (44). hiPSCs were adapted to feeder-free conditions and maintained in Essential 8 medium (Gibco) on Geltrex-coated (Gibco) dishes before being differentiated to NPCs according to established methods (18). Briefly, feeder-free hiPSCs were dissociated using 0.5 mM EDTA (Gibco), transferred to ulta-low adherence 6-well plates, and cultured for 5 days in human ESC medium (DMEM/F12 + GlutaMax, 20% Knockout Serum Replacement, 1X non-essential amino acids, and 0.1 mM 2-mercaptoethanol; all from Gibco) supplemented with 500 ng/ml recombinant Noggin (R&D Systems) and 10 µM SB431542 (Tocris). On the fifth day of culture in hESC medium, hiPSCs formed embryoid body-like structures (EBs) and 20-50 EBs were transferred to each well of a Geltrex-coated 6-well dish. Increasing amounts of N2 medium (DMEM/F12 + GlutaMax, 1X N2 supplement [Gibco]) supplemented with 500 ng/ml Noggin and 10 µm SB431542

were added every other day for 14-16 days. Resulting columnar rosette structures were collected using Accutase (BD) and stained with anti-CD184, anti-CD24, anti-CD44, and anti-CD271 antibodies according to manufacturer specifications using the BD Stemflow Human Neural Cell Sorting Kit. Sorted hiNPCs were maintained in NPC medium (DMEM/F12 + GlutaMax, .5X N2, .5X B27 without vitamin A [Gibco], 20 ng/ml bFGF [Gibco]) on Geltrex-coated dishes and passaged using Accutase when cell density reached 80-90% confluence.

Characterization of hiNPCs

NPCs derived from the HDF51iPS1 line of iPSCs were seeded on Geltrex-coated slides, fixed with 4% paraformaldehyde, and stained with rat anti-Nestin (1:500; Millipore), rabbit anti-Sox2 (1:200; Epitomics), or rabbit anti-Pax 6 (1:50; BioLegend) before addition of respective secondary antibodies (goat anti-rabbit AlexaFluor 568 and goat anti-rat AlexaFluor 488; both from Invitrogen). For analysis of multipotency, hiNPCs were cultured in neuronal differentiation medium (Neurobasal, 1X B27, 1X GlutaMax; all Gibco), astrocyte differentiation medium (DMEM [Gibco], 1X N2, 1X GlutaMax, 1% FBS [Atlanta Biologicals]), or oligodendrocyte differentiation medium (Neurobasal, 1X B27, GlutaMax, 30 ng/ml T3 [Sigma]) for at least 14 days before being fixed with 4% paraformaldehyde and stained with mouse anti-beta-III-tubulin (1:500; abcam), rabbit anti-GFAP (1:200; Invitrogen), or rabbit anti-NG2 (1:200; Chemicon). Slides were cover-slipped and mounted using VectaShield Hard Set Mounting Medium with DAPI (Vector Labs), and all images were captured using a Nikon Eclipse Ti inverted microscope.

Transplantation of hiNPCs

Mice previously inoculated with JHMV were injected with 250,000 hiNPCs or human fetal fibroblasts resuspended in 2.5 µl HBSS, at the T9 vertebral level on day 14 post-infection (pi) as previously described (23). Some JHMV-infected animals received 2.5 µl HBSS intraspinally as vehicle control.

In vivo imaging of firefly luciferase activity

hiNPCs were transduced with the lentiviral vector pLenti CMV Puro LUC w168-1 (plasmid #17477; addgene) as previously described (14). Firefly luciferase expression was verified *in vitro* by addition of D-luciferin (Caliper Life Sciences) to culture wells and detection of chemiluminescence at 560 nm using a Bio-Rad Gel Doc system. For *in vivo* detection of chemiluminescence, hiNPC transplanted mice were injected with 250 mg/kg D-luciferin approximately ten minutes before imaging. Mice were then anesthetized using a sub-lethal dose of isofluorane delivered via inhalation. Bioluminescence due to firefly luciferase activity was captured using the IVIS Series Pre-clinical In Vivo Imaging System (PerkinElmer), and a pseudocolored image of bioluminescent intensity was overlaid onto a gray-scale photograph of the mice.

Flow Cytometric Analysis of Spinal Cords

Spinal cords or cervical lymph nodes were dissected into single-cell suspension and passed through a discontinuous Percoll gradient as previously described (45). Single-cell suspensions were filtered, washed, and counted before being blocked with anti-mouse CD16/32 (1:200; BD Biosciences). Cells were subsequently stained with anti-CD4 (FITC-conjugated GK1.5; BD Biosciences), anti-CD8 (PE-Cy7-conugated Ly-2; BD Biosciences),

anti-CD45 (APC-conjugated 30-F11; eBioscience), anti-F4/80 (FITC-conjugated Ci-A3-1; Serotech), anti-FOXP3 (EF660-conjugated FJK-16s; eBioscience) or PE-conjugated tetramers I-Ab/M133–147 and Db/S510–518 (8 μg/ml; National Institute of Allergy and Infectious Diseases MHC Tetramer Core Facility). Cells were processed using an LSR II flow cytometer (BD) and analyzed using FlowJo software (Tree Star).

Immunohistochemistry and analysis of histopathology

Animals were euthanized via inhalation of a lethal dose of isofluorane and cardiac perfusion with PBS was performed. Spinal cords were dissected and fixed overnight in 4% paraformaldehyde before being embedded in OCT compound. Frozen tissues were serially sectioned and stained with luxol fast blue (LFB) and counterstained with hematoxylin and eosin (H&E) to assess the severity of demyelination. The total area of the white matter was quantified and compared to the area of demyelinated regions using ImageJ software (National Institutes of Health). All demyelination measurements were performed independently by two investigators. For identification of engrafted hiNPCs, 6 um coronal spinal cord sections were stained with STEM121 (1:200; Clontech Laboratories) and a biotinylated secondary antibody (1:400; Vector Labs) before the ABC Elite staining system (Vector Labs) and diaminobenzidine (DAB; Vector Labs) were applied for visualization.

Transmission electron microscopy

Mice were sacrificed via inhalation of a lethal dose of isofluorane and cadiac perfusion was performed using 0.1 M cacodylate buffer containing 2% paraformaldehyde and 2% glutaraldehyde. Spinal cords were dissected and embedded in EPON epoxy resin before

being ultrasectioned, stained with uranyl acetate-lead citrate, and imaged using a transmission electron microscope according to standard protocols. G-ratios were determined by measuring axon diameter and comparing it to the total fiber diameter (axon diameter/total fiber diameter) using ImageJ software (National Institutes for Health). Measurements were performed independently by two investigators and at least 300 axons were measured per experimental group.

In vivo Treg ablation

JHMV-infected mice were injected intraperitoneally (i.p.) with 150 μg of a rat monoclonal antibody specific for CD25 (rat anti-mouse CD25, clone PC61.5) or control rat immunoglobulin G (Sigma) as previously described (14). Efficiency of anti-CD25 treatment in transplanted mice was determined by collection of peripheral blood from the peri-orbital sinus, followed by quantification of the frequency of circulating Tregs by FACS.

T cell – hiNPC co-culture for evaluation of Treg induction

Spleens were dissected from naïve age-matched C57BL/6 mice and total T cells were isolated using the EasySep Mouse T cell Isolation Kit (STEMCELL Technologies). Mitomycin C (Roche) treated hiNPCs were mixed with isolated T cells at defined proportions in round-bottom 96-well plates and incubated at 37°C, 5% CO2 for 3 days in 200 μl final volume of complete T cell medium (RPMI-1640 [Gibco], 1X GlutaMAX-1 [Gibco], 1X non-essential amino acids [Gibco], 100 U/ml penicillin [Gibco], 100 μg/ml streptomycin [Gibco], 1 mM sodium pyruvate [Life Gibco], 55 μM 2-mercaptomethanol [Gibco], and 10% FBS [Atlanta Biologicals]). In wells where activation of T cells was desired, Dynabeads Mouse T-

Activator CD3/CD28 beads (Life Technologies) were added at a concentration of 1 bead per T cell.

Enzyme-linked immunosorbent assays (ELISAs)

TGF-β1 and TGF-β2 production by cultured hESC-NPCs and hiNPCs was determined using human Quantikine ELISA Kits from R&D Systems in accordance with the manufacturer's specifications.

Statistics

Data were analyzed using Prism software (GraphPad). Unless otherwise noted, comparisons were performed using one-way analysis of variance, followed by post hoc analysis using Tukey's procedure or Dunnett's correction where appropriate.

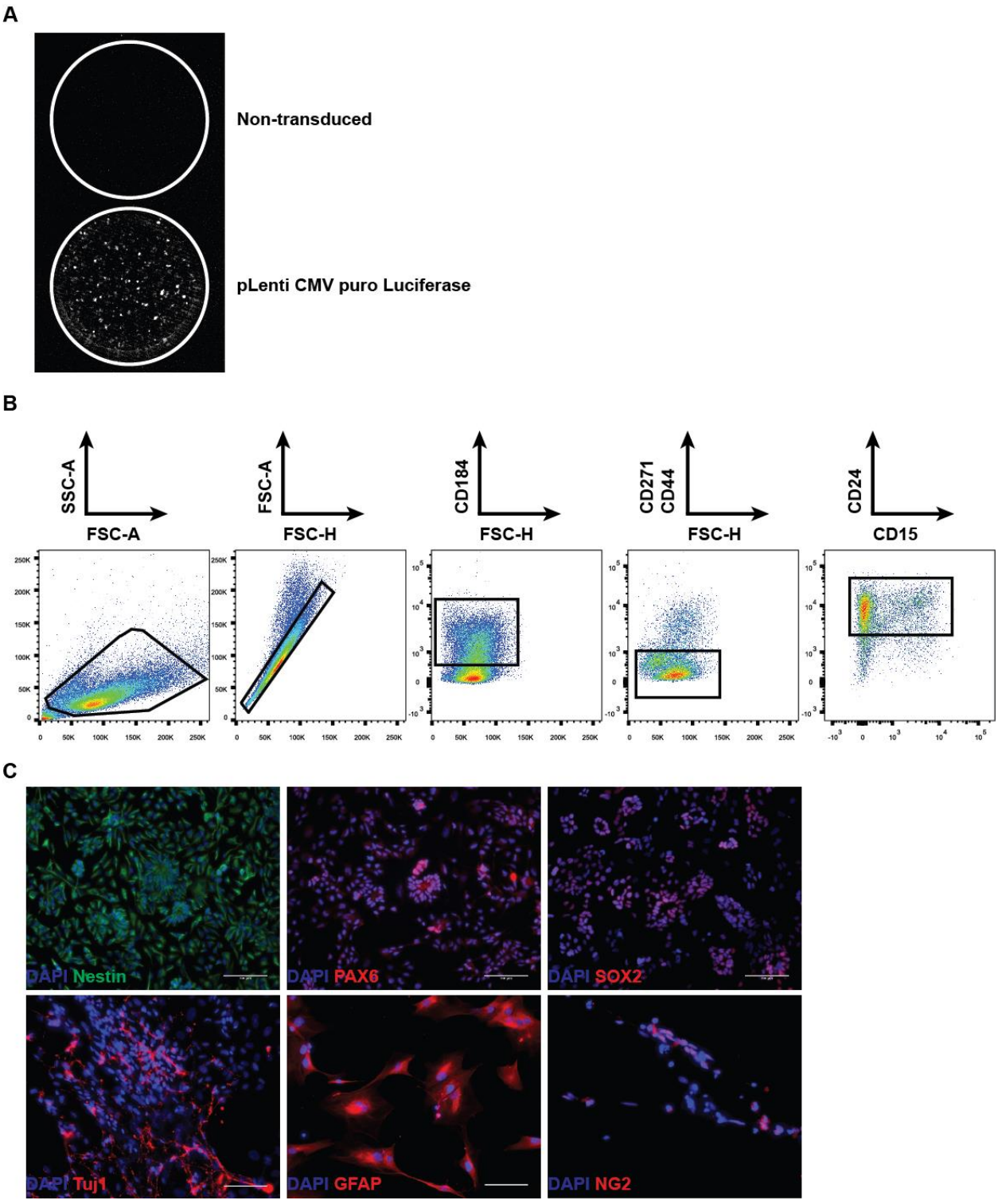


Figure 5.1: **Lentivirus-transduced human iPSCs differentiate to multipotent neural precursor cells.** (A) Representative micrographs of human iPSCs transduced with a lentiviral vector containing the *Photinus pyralis* luciferase gene and puromycin resistance cassette. Photon emission in response to D-luciferin could be detected in transduced puromycin-selected iPSCs (bottom) but not in non-transduced iPSCs (top) *in vitro*. (B) Representative FACS plots demonstrating CD184+/CD271-/CD44-/CD24+ gating scheme

for isolation of NPCs from differentiated iPSCs. (C) Immunofluorescence microscopy demonstrated FACS-sorted hiNPCs expressed markers characteristic of NPCs, including Nestin, Pax6, and Sox2 (top). hiNPCs could be further differentiated into Tuj1+ neurons, GFAP+ astrocytes, and NG2+ oligodendrocyte precursor cells (bottom). Scale bars = 100 μm.

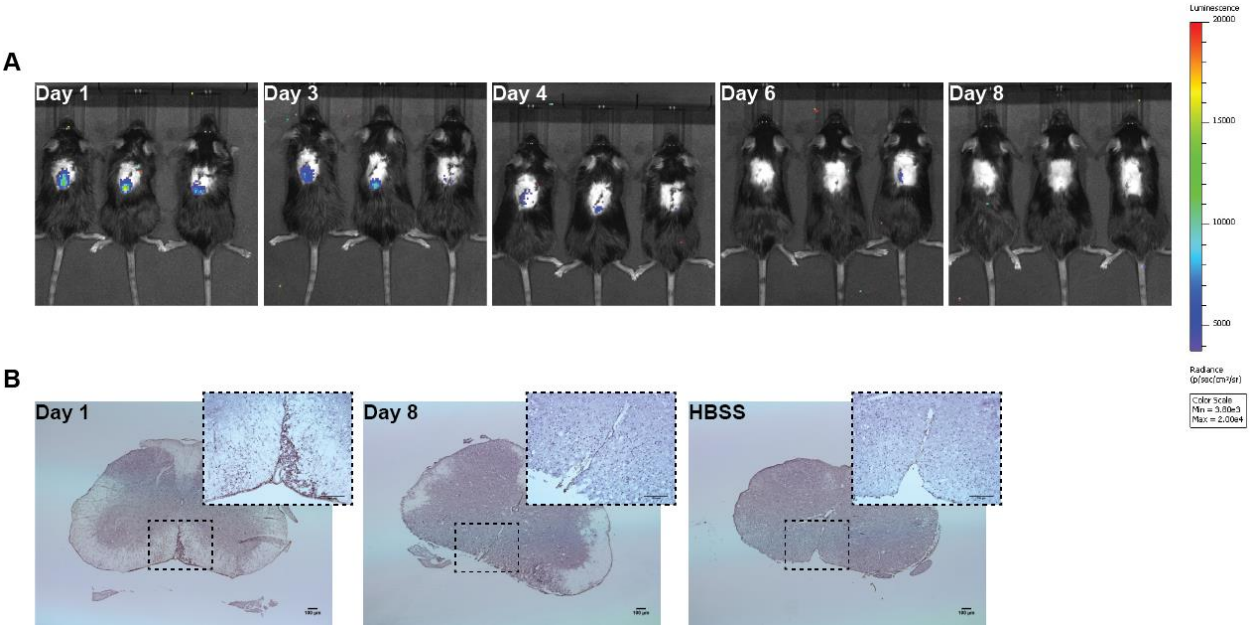


Figure 5.2: Human iPSC-derived NPCs are rapidly rejected following intraspinal transplantation in JHMV-infected mice. (A) *In vivo* bioluminescence imaging revealed hiNPCs could be detected in the spinal cords of transplanted animals as early as day 1 post-transplant (pt) and were undetectable by day 8 pt. (B) Representative brightfield images of coronal spinal cord sections from hiNPC transplanted mice stained with SC121, a monoclonal antibody specific for human cytoplasm. Human cells were detected in ventral white matter regions at day 1 pt but could be not be detected by day 8 p.t., confirming rejection of hiNPCs. Scale bars = 100 μm.

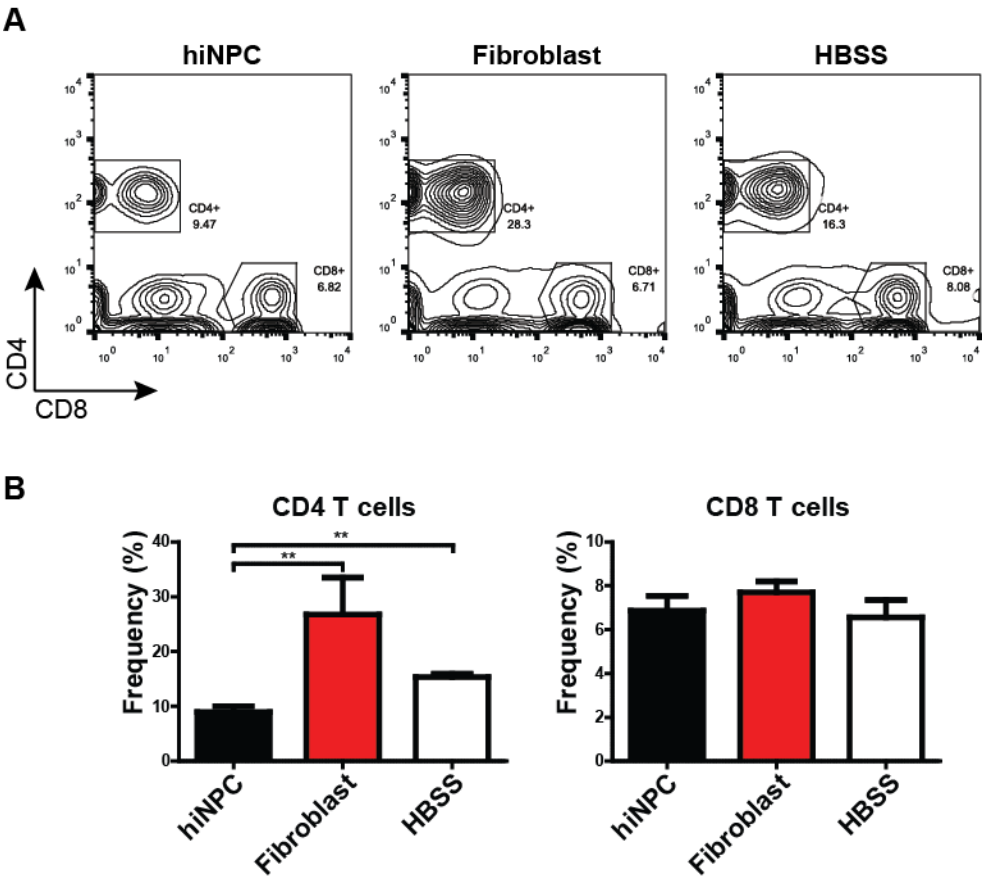


Figure 5.3: CD4 T cell accumulation is reduced in hiNPC-transplanted mice. (A) Representative FACS plots demonstrating the frequency of CD4+ and CD8+ cells in the spinal cords of hiNPC, human fibroblast, and HBSS-injected mice at day 21 pt. (B) Quantification of the frequency of CD4 and CD8 T cells. The frequency of CD4 T cells was significantly ($p < 0.01$) reduced in hiNPC-transplanted animals compared to animals injected with human fibroblasts or HBSS. Data represents two independent experiments with at least 4 animals per group. Error bars represent SEM.

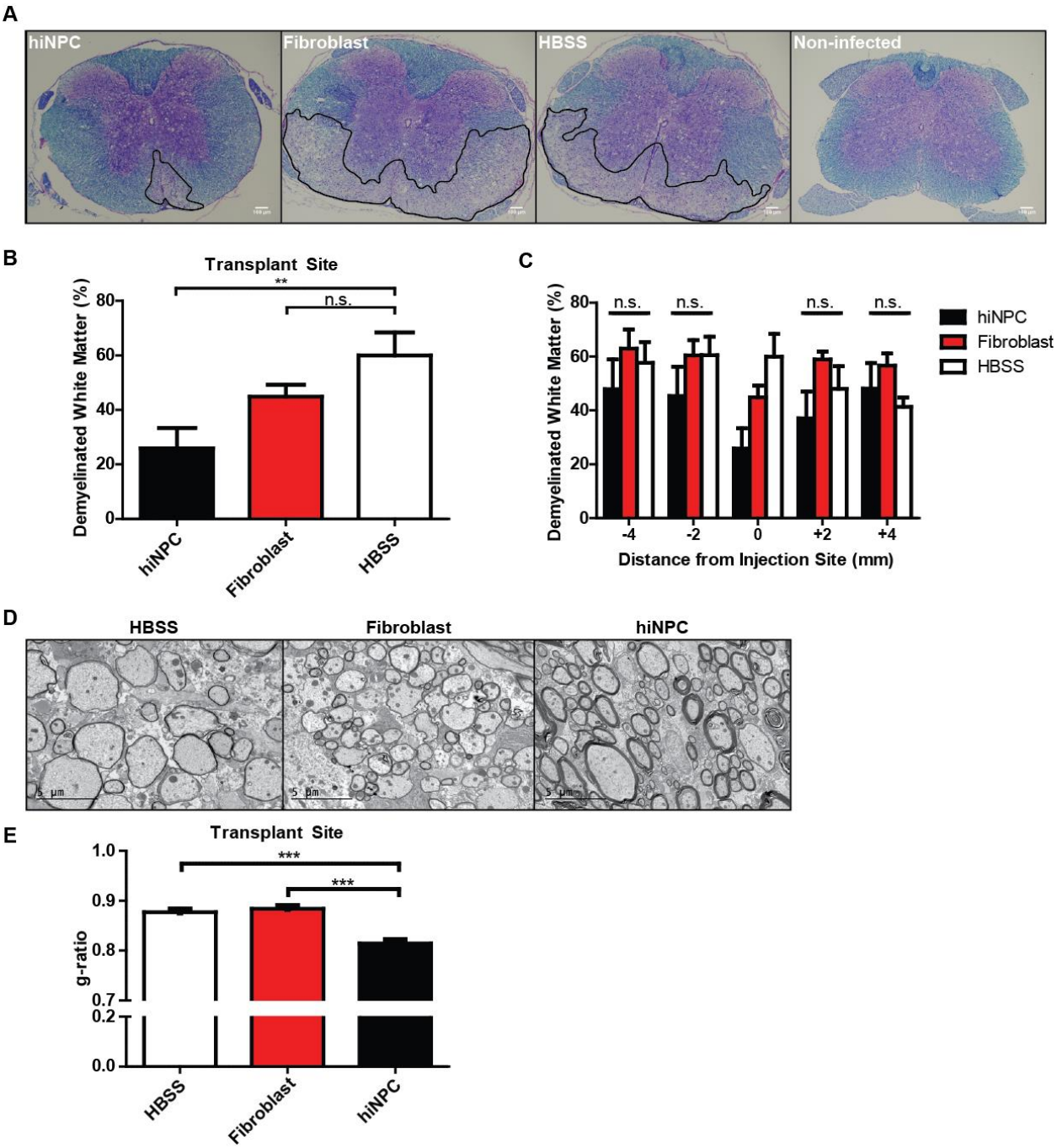


Figure 5.4: Focal remyelination in animals transplanted with hiNPCs. (A) Representative brightfield images of coronal spinal cord sections stained with luxol fast blue (LFB) and counter-stained with hemotoxylin and eosin (H&E). (B) Quantification of demyelination in the ventral white matter of hiNPC, fibroblast, and HBSS injected mice revealed significantly ($p < 0.01$) reduced demyelination at the injection site in the spinal cords of hiNPC-transplanted mice. (C) Quantification of demyelination in areas adjacent to the injection site revealed that reduced demyelination was not sustained along the rostrocaudal axis. (D) Representative electron micrographs of coronal spinal cord sections from HBSS, fibroblast, and hiNPC-injected mice. (E) Analysis of the ratio of the axon

diameter vs. total fiber diameter (*g*-ratio) confirmed enhanced remyelination at the transplant site of hiNPC-injected mice compared to controls ($p < 0.001$). For (B) and (C), data represents two independent experiments with at least 4 animals per group. For (E), at least 300 axons were measured per experimental group. All data is presented as average ± SEM.

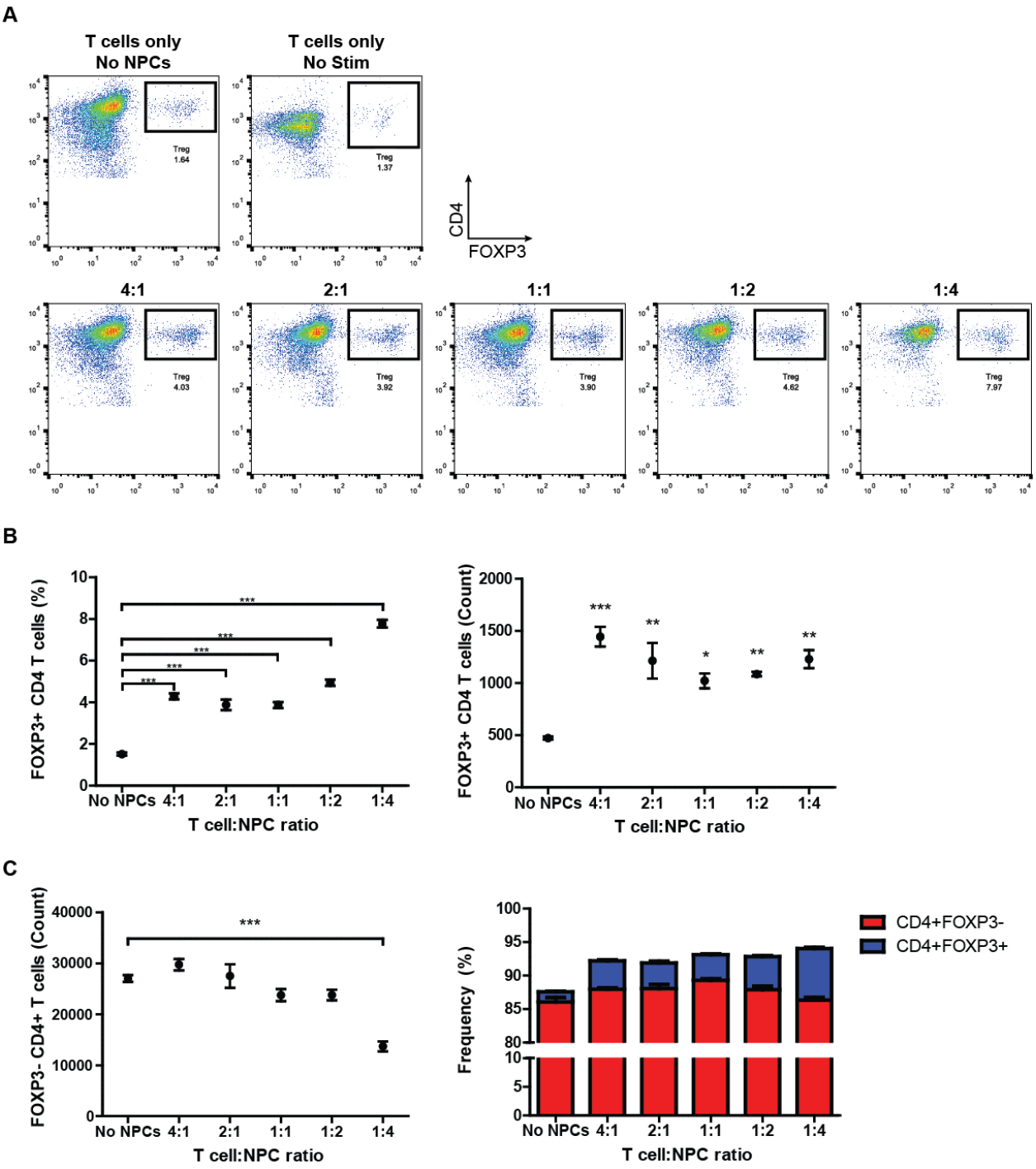


Figure 5.5: Regulatory T cells are increased in the draining cervical lymph nodes as a result of transient hiNPC engraftment. (A) Representative FACS plots of CD4+FOXP3+ cell analysis from the draining cervical lymph nodes (CLN; top) and spinal cords (bottom) of mice injected with hiNPCs, fibroblasts, or HBSS. (B) Quantification of the number of CD4+FOXP3+ Tregs demonstrated a significant ($p < 0.05$) increase in the CLNs of hiNPC transplanted mice compared to controls at day 5 p.t. (top). An increase in Tregs was not detected in the CLNs or spinal cords of hiNPC transplanted mice by day 7 p.t. (bottom).

Data represents two independent experiments with at least 4 animals per experimental group and is presented as average ± SEM.

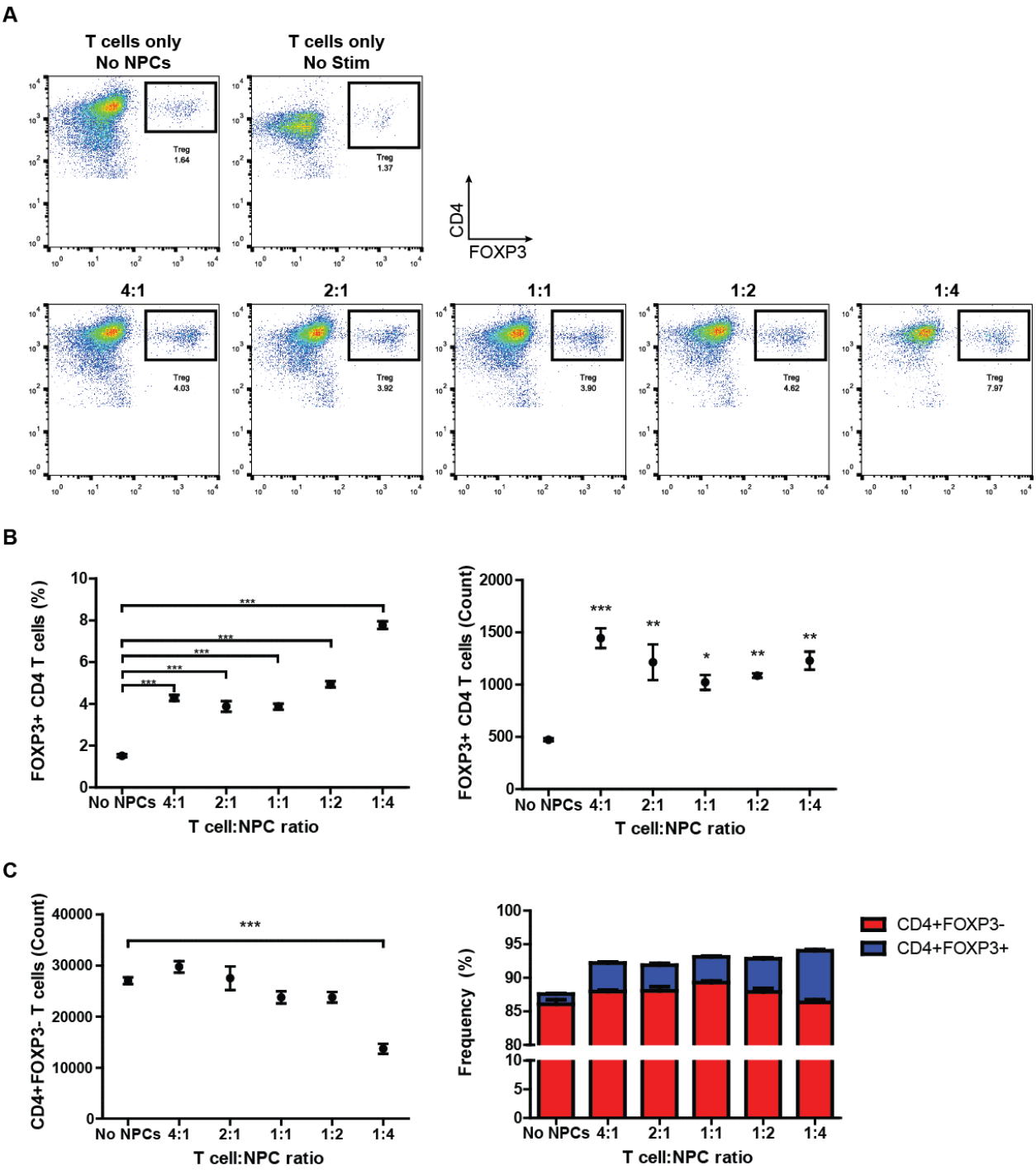


Figure 5.6: Human iPSC-derived NPCs directly induce Treg conversion *in vitro*. (A) Representative FACS plots of CD4+FOXP3+ Tregs from hiNPC-T cell co-cultures. T cells were mixed with hiNPCs at varying T cell-to-hiNPC ratios and activated in the presence of anti-CD3/anti-CD28 beads for three days. (B) The frequency and number of FOXP3-expressing CD4+ T cells was significantly increased in the presence of hiNPCs. (C) The observed increase in Tregs was correlated with a significant decrease in the number of conventional CD4+ T cells at a T cell-to-hiNPC ratio of 1:4. All data is presented as average ± SEM and represents 4 biological replicates; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

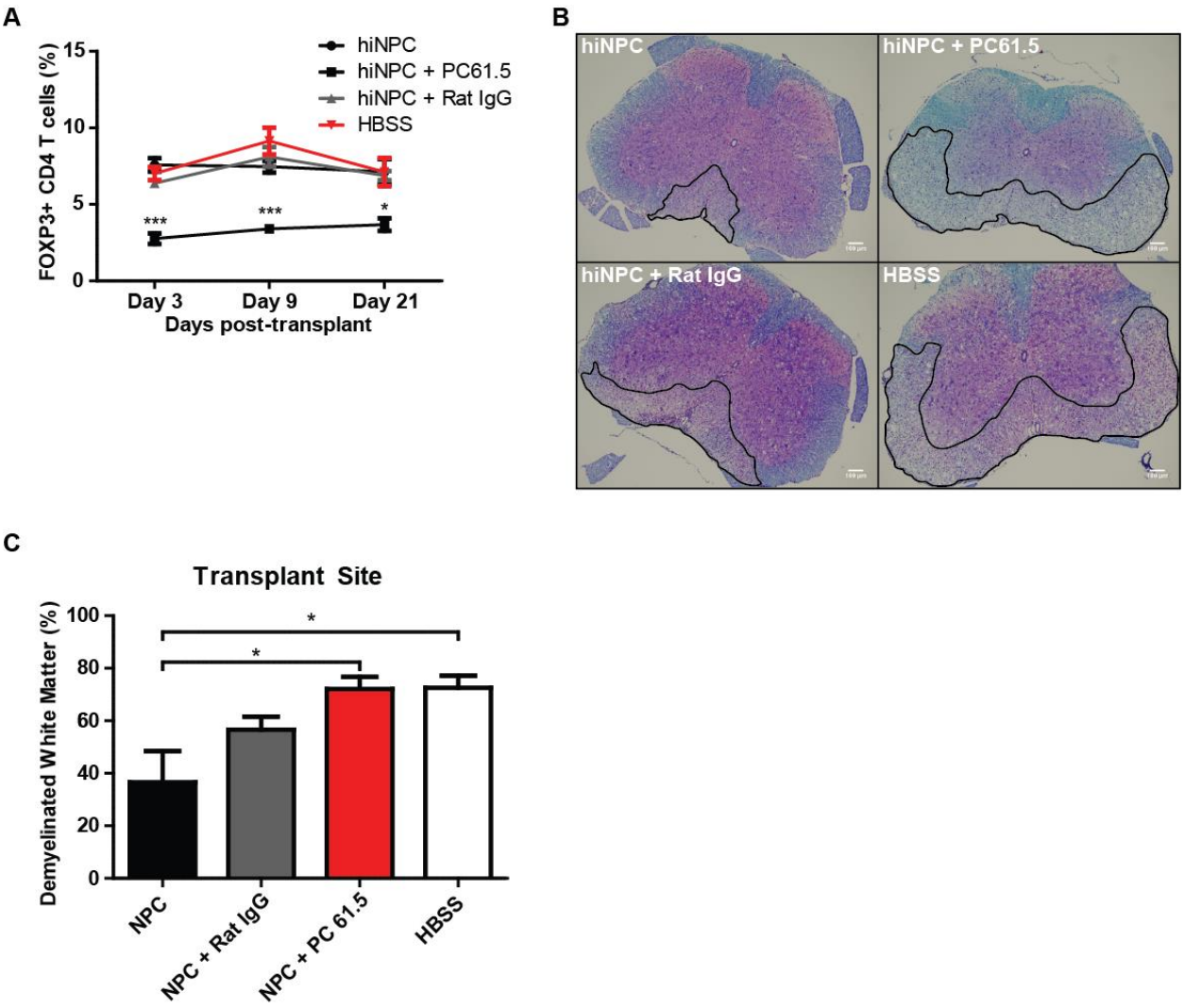
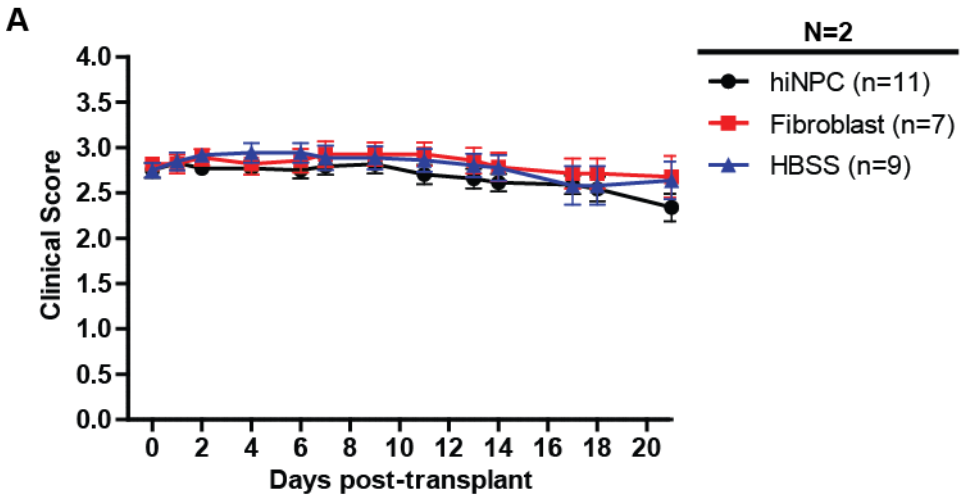
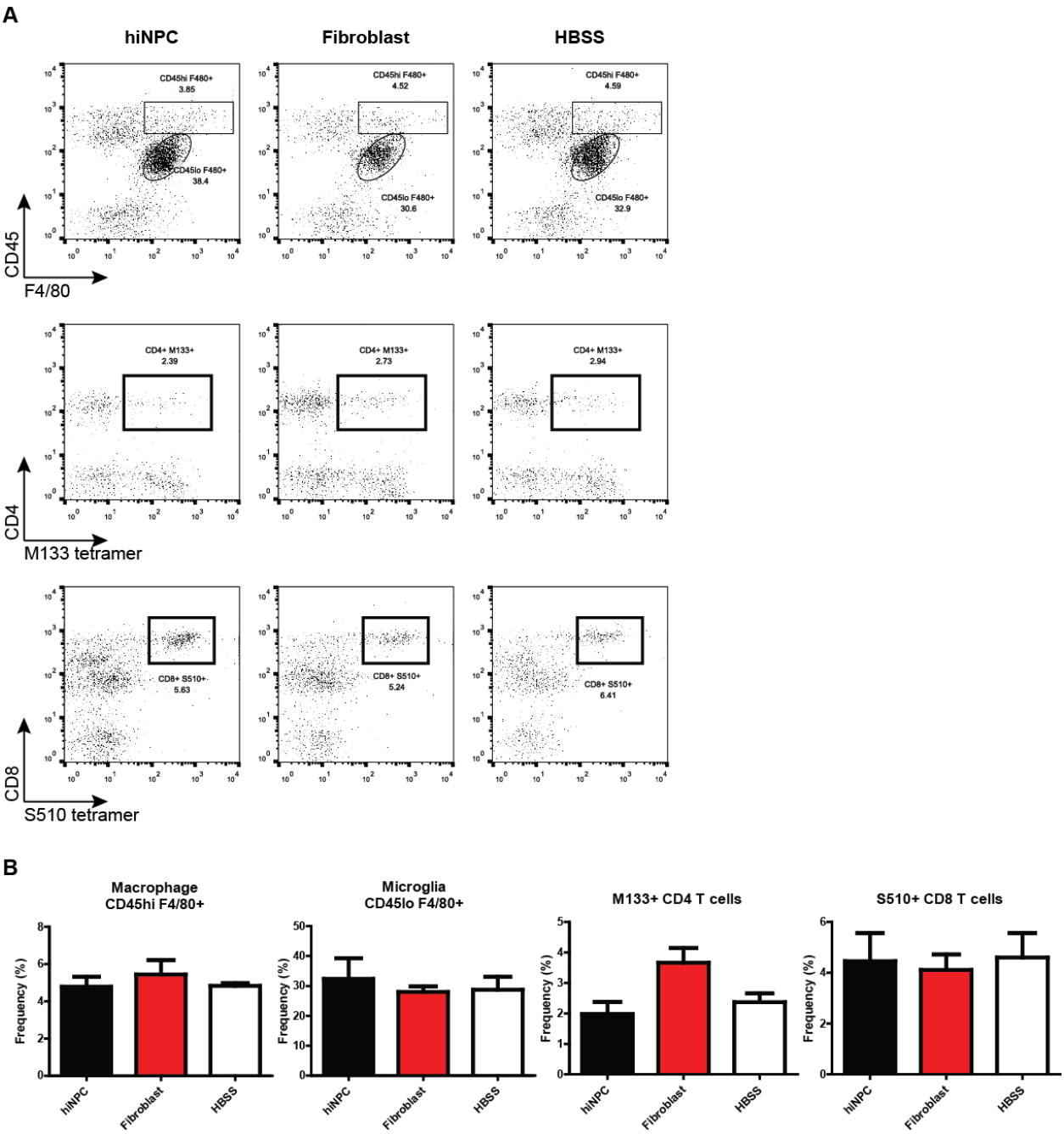


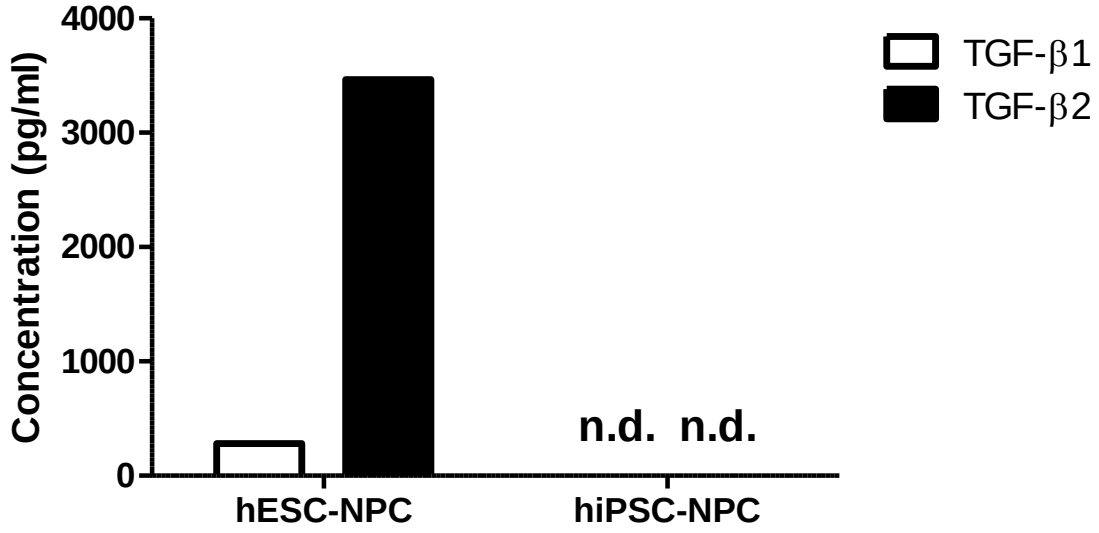
Figure 5.7: Regulatory T cells are necessary for hiNPC-induced myelin sparing. (A) Treatment of hiNPC-transplanted mice with PC61.5, a rat monoclonal antibody raised against CD25, resulted in a significant reduction in the frequency of circulating CD4+FOXP3+ cells that was sustained to day 21 p.t. (B) Representative spinal cord sections stained with LFB and H&E. Outlined areas highlight demyelination. (C) Quantification of white matter damage revealed PC61.5-treated hiNPC-transplanted mice did not have reduced demyelination when compared to non-treated hiNPC-transplanted mice. Data represents two independent experiments with at least 3 animals per group and is presented as the average ± SEM; *$p < 0.05$, ***$p < 0.001$.



Supplemental Figure 5.S1: Intraspinal delivery of hiNPCs does not promote neurologic recovery in mice persistently infected with JHMV. Graph of clinical scores of mice injected intraspinally with hiNPCs (black), human fibroblasts (red), and HBSS (blue) at defined time points post-transplant (p.t.). No improvement in locomotion was observed by day 21 p.t. Clinical evaluation was based on the following scoring system: 0, asymptomatic; 1, limp tail; 2, waddling gait with righting difficulty; 3, hind-limb weakness and extreme righting difficulty; 3.5, complete hind limb paralysis; and 4, death. Data represents two independent experiments and is presented as average ± SEM.



Supplemental Figure 5.S2: Spinal cord accumulation of macrophages, microglia, and virus-specific T cells is unaffected by hiNPC transplantation. (A) Representative FACS plots demonstrating gating strategies for macrophages (CD45hi, F4/80+), microglia (CD45lo, F4/80+), and T cells specific for the CD4 immunodominant epitope M133–147 or the CD8 immundominant epitope S510-518. (B) Quantification of the frequencies of infiltrating macrophages, microglia, M133-147+ CD4 T cells, and S510-518+ CD8 T cells reveals no difference between hiNPC, fibroblast, and HBSS injected animals. Data is presented as average ± SEM and represents 3 animals per treatment group.



Supplemental Figure 5.S3: Secreted TGF-β is detected in hESC-NPC, but not hiNPC, culture media. Representative enzyme linked immunosorbent assay (ELISA) results demonstrating levels of TGF-β1 and TGF-β2 in culture media collected from hESC-derived NPCs and hiPSC-derived NPCs. "n.d." = not detected.

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CHAPTER SIX

CONCLUSION

6.1 Summary & Significance

Transplantation of multipotent NPCs has long been suggested as a cell replacement therapy for neurodegenerative disease. Mounting evidence from models of inflammatory disorders suggests NPCs also possess immunomodulatory properties that are relevant for treatment of patients with MS (1). However, many previous studies utilized NPCs procured from genetically identical donors, a clinically implausible approach considering alleles at the MHC loci are some of the most polymorphic known in humans. While NPCs are not consistently observed to express MHC and co-stimulatory molecules implicated in immune surveillance, and while some groups demonstrate prolonged survival of allogeneic NPCs in the absence of immune suppression, a consensus on the antigenic potential of NPC allografts, particularly under inflammatory conditions, has not been reached (2). Systemic immune suppression reduces the potential for rejection by the host, but leaves the recipient susceptible to reactivation of latent viruses and infection from the environment.

Our studies reaffirm a lack of MHC I/II expression on post-natal SVZ-derived NPCs under steady-state conditions *in vitro*, but demonstrate constitutive expression of T cell co-stimulatory molecules CD80/CD86 (**Chapter 2**) (3). Moreover, expression of MHC I/II could be induced in the presence of IFN-γ, a pro-inflammatory cytokine produced in high abundance in the CNS of mice persistently infected with JHMV, and MHC-expressing NPCs induced allogeneic T cell proliferation. Following intraspinal transplantation into the spinal cords of MHC-mismatched mice afflicted with chronic MHV-induced demyelination, NPCs were rapidly rejected concomitant with increased T cell infiltration in the CNS. One particularly important finding was that allograft survival could be prolonged by *in vivo* depletion of T cells, with ablation of CD4+ T cells providing the most benefit. This highlights

a prominent role for the adaptive immune system in allograft rejection, and infers indirect recognition of NPC antigens by T helper cells through MHC II during inflammatory demyelination. Since T cell infiltration in the CNS was not completely resolved by monoclonal antibody treatment, antibody blockade may not be a plausible solution for extending allograft survival from a clinical perspective. However, gene editing systems have been developed based on CRISPR/Cas9 that permit modification of even single nucleotides with minimal off-target effects (4). An interesting future study would investigate the consequences of genetic ablation of MHC II in NPCs, which may permit immune evasion and prolong allograft survival.

Despite efficient depletion of CD4+ T cells from the periphery, survival of allogeneic NPCs in MHV-infected mice did not reach levels observed in syngeneic settings, implying additional immune contributors to allograft rejection. Others have observed innate immune targeting of NPCs under non-inflammatory circumstance *in vitro* and *in vivo* (5, 6). Indeed, we demonstrated that NK cells could detect and lyse allogeneic NPCs following binding of the NK receptor NKG2D to RAE-1 expressed by NPCs (**Chapter 3**) (7). Moreover, NK cells were observed to co-localize with NPC allografts in MHV-infected mice, and inactivation of NK cells using an antibody raised against NKG2D prolonged graft survival. Cumulatively, this supports a role for the innate immune system in allogeneic rejection during immune-mediated demyelination. Notably, RAE-1 was not homogenously expressed by NPCs, and RAE-1$^-$ NPCs were not lysed by NK cells. In theory, an ideal candidate cell population for transplantation could be screened and isolated based on expression levels of activating receptors. An additional important finding was that allogeneic NPCs could be infected by MHV *in vivo*, and infection of NPCs *in vitro* enhanced NK cell-mediated lysis.

Immune suppression has been linked to the reactivation of latent viruses and increases susceptibility to infection. Our data imply that NPC allografts may act as an additional reservoir for virus infection after transplantation which may be detrimental to the survival of the graft.

We also determined that infection of NPCs by MHV was dependent on the expression of CEACAM1a, the canonical MHV receptor, and NPCs could support rapid propagation of neurotropic coronavirus *in vitro* (**Chapter 4**) (8). MHV infection dampened IFN-γ-induced expression of MHC II, perhaps as an immune evasion mechanism. Nonetheless, the inflammatory milieu secreted by CD4+ T cells and IFN-γ suppressed virus replication, and virus-specific CTLs targeted infected NPCs for lysis. T cell infiltration into the CNS is a prerequisite for the development of MHV-induced encephalomyelitis, and emergence of neurotropic viruses in patients with MS who are immune suppressed has been observed (9, 10). Thus, analyzing the ability of T cells to detect and destroy NPCs transplanted into mice during the acute phase of MHV-induced disease would provide valuable insight regarding the fate of engrafted cells undergoing viral infection. Presumably, T cells will promote lysis of transplanted NPCs similar to NK cells *in vivo*, reducing the survival of NPCs regardless of genetic background and impacting therapeutic potential.

Lastly, human ESC-derived NPCs have been shown to attenuate and ameliorate disease pathology in animal models of MS (11, 12). Moreover, we previously demonstrated that sustained clinical recovery is not dependent on survival of transplanted hESC-NPCs in the MHV model of neuroinflammatory demyelination (13). iPSCs have been proposed as a preferable source of transplantable NPCs since they maintain the genetic background of the

donor and may circumvent rejection. However, a disparity in the therapeutic potential of iPSC-derived NPCs relative to hESC-NPCs has been suggested. Here, we report localized remyelination concomitant with dampened CD4+ T cell infiltration and the emergence of Tregs in the draining cervical lymph nodes of MHV-infected mice. However, in contrast to our previous study utilizing hESC-NPCs, transplanted mice did not demonstrate significant improvement in motor skills (**Chapter 5**). We propose that production of TGF-β by human NPCs is required for induction of Tregs in the CNS and is likely the major contributor to clinical recovery. Since a discernible amount of heterogeneity exists among methods to generate NPCs from pluripotent cells (14), identification of biomarkers that correlate with therapeutic benefit will be critical for transitioning cell-based therapies to the clinic. Considering that LIF promotes remyelination in EAE mice transplantation with murine iPSC-NPCs (15), and observations that production of LIF by Tregs occurs in response to antigen (16), we cannot exclude a role for LIF in molecular events leading to remyelination in MHV-infected mice. Additionally, the ultimate goal of iPSC-based therapies is to perform successful autologous transplantation in patients, and the fate of iPSC-NPCs should be evaluated in the absence of immune rejection. Infecting immunodeficient mice with MHV leads to fatal viremia, but adoptive transfer of virus-specific T cells reduces mortality, and surviving animals develop demyelination similar to immune competent mice (17). Transferring T cells specific for MHV antigens into inoculated $RAG2^{-/-}\gamma C^{-/-}$ mice may provide a context in which the consequences of long-term iPSC-NPC engraftment can be determined.

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