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UNIVERSITY OF CALIFORNIA,
IRVINE

The role of sphingosine-1-phosphate in host defense and disease following infection with a neurotropic coronavirus

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

Caroline Ann Kerstin Blanc

Dissertation Committee:
Professor Thomas E. Lane, Chair
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Professor Craig M. Walsh

2014

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DEDICATION

To my loving parents, Marc-André and Charlotte Blanc

Brother Fredrik Blanc,

and

my friends and family

I am forever grateful for all your encouragements and support throughout this incredible journey

I love you all

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LIST OF ABREVIATIONS

ATP	adenosine triphosphate
BBB	blood-brain-barrier
bHLH	basic helix-loop-helix
CCR7	C-C chemokine receptor type 7
CD	cluster of differentiation
CEACAM	carcinoembryonic antigen-related cell adhesion molecule
CNS	central nervous system
EAE	experimental autoimmune encephalomyelitis
EBV	epstein-barr virus
FDA	food and drug administration
FREEDOMS	*FTY720* Research Evaluating Effects of Daily Oral therapy in Multiple Sclerosis
GA	glatiramer acetate
GFP	green fluorescent protein
HDL	high-density lipoprotein
hESC	human embryonic stem cell
HHV-6	human herpes virus 6
HSV-1	herpes simplex virus 1
HTLV-1	human T-lymphotropic virus 1
i.c	intra-cerebral
IFN	interferon

IL	interleukin
JCV	John Cunningham virus
JHMV	John Howard Mueller virus
MBP	myelin basic protein
MHC	major histocompatibility complex
MHV	mouse hepatitis virus
MMP-9	matrix metalloproteinase 9
MS	multiple sclerosis
MSC	mesenchymal stem cells
NFkB	nuclear factor kappa-light-chain-enhancer of activated B cells
NGN2	neurogenin 2
Ngns	neurogenins
NK	natural killer
NPC	neural progenitor cell
OPC	oligodendrocyte precursor cell
p.i	post-infection
PLP	proteolipid protein
PML	progressive multifocal leukoencephalopathy
PMN	polymorphonuclear
RC	restricted vesicle
RNA	ribonucleic acid
RRMS	relapsing-remitting multiple sclerosis
S1P	sphingosine-1-phosphate

S1P1	sphingosine-1-phosphate receptor 1
S1P2	sphingosine-1-phosphate receptor 2
S1P3	sphingosine-1-phosphate receptor 3
S1P4	sphingosine-1-phosphate receptor 4
S1P5	sphingosine-1-phosphate receptor 5
SPHK1	sphingosine kinase 1
SPHK2	sphingosine kinase 2
SVZ	subventricular zone
Tcm	T central memory
Tem	T effector memory
Th17	T helper cell 17
TMEV	Theiler's murine encephalomyelitis
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TRANSFORMS	Trial assessing injectable interferon vs *FTY720* oral in RRMS
Tregs	T regulatory cells
VCAM-1	vascular cell adhesion molecule 1
YFP	yellow fluorescent protein

ACKNOWLEDGMENTS

First and foremost, I want to express my deepest gratitude to my mentor and friend Dr. Tom Lane for his exceptional guidance. Tom has undoubtedly been instrumental in my development as a scientist and his unwavering support has allowed me to reach my goals as a Graduate student in his laboratory.

I want to thank previous and current members of the Lane Laboratory for their advice and assistance. This includes Dr. Lu Chen, Dr. Laura Dickey, Patrick Duncker, Jon Grist, Dr. Kathy Held, Edna Hingco, Leslie Kirby, Dane Larsen, Brett Marro, Warren Plaisted, Michelle Tsukamoto, Dr. Jason Weinger and Colleen Worne. Their company will be greatly missed.

A special thanks to the members of my dissertation and advancement committee. This includes Dr. Mathew M. Blurton Jones, Dr. Melissa B. Lodoen, Dr. Irene M. Pedersen and Dr. Craig Walsh.

I want to thank the NIH for financial support through grant R01 NS041249 and the Hausman Family Foundation and Dawn Beattie for their generous donations. I thank Elsevier and the Journal of Neuroinflammation for permission to include previous published materials.

Finally, I would like to thank my family and my dearest friends Brett Marro, Adrien Semoroz, Nicole Hernandez-Semoroz, Hoda Emami and Anna-Karin Bjorklund for their constant support and encouragement. All of you never stopped believing in me.

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Blanc CA, Rosen H, Lane TE. FTY720 (fingolimod) modulates the severity of viral-induced encephalomyelitis and demyelination*. J. Neuroinflammation.* 2014; 11(1): 138.

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ABSTRACT OF THE DISSERTATION

The role of sphingosine-1-phosphate in host defense
and disease following infection with neurotropic coronavirus

By

Caroline Ann Kerstin Blanc

Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2014

Professor Thomas Edward Lane, Chair

Multiple sclerosis (MS) is a complex neurological disease that causes permanent disability in young adults. The cause of MS is still unknown, although both genetic and environmental factors are believed to play an important role in onset of disease. FTY720 is the first oral drug approved by the FDA to treat relapsing-remitting forms of MS presumably by inhibiting the egress of autoreactive T cells from the lymph nodes thereby preventing infiltration into the central nervous system (CNS). Treated-patients display a reduction in relapses, a decrease in new lesion formation and diminished brain volume loss shown by MRI.

We employ a viral model of demyelination to study various aspects related to the pathogenesis of MS. Intracerebral infection with the neurotropic JHM strain of mouse hepatitis virus (JHMV) results in an acute encephalomyelitis followed by chronic immune-mediated demyelination, that is similar both clinically and histologically to MS. The primary focus of research of this dissertation is to investigate whether FTY720 affects the host response and/or disease progression in response to JHMV infection of the CNS.

Administration of FTY720 to JHMV-infected mice resulted in increased mortality associated impaired ability to control CNS viral replication that correlated with dampened accumulation of virus-specific CD4+ and CD8+ T cells. Treatment with FTY720 did not affect proliferation, cytokine secretion, or cytolytic activity by virus-specific T cells. Administration of FTY720 resulted in diminished severity of JHMV-induced demyelination associated with reduced T cell accumulation within the CNS. These findings indicate that FTY720 mutes effective antiviral immune responses by limiting infiltration of virus-specific T cells within the CNS following infection with a neurotropic virus.

Engraftment of mouse neural progenitor cells (NPCs) into the CNS of JHMV-infected mice with established demyelination results in extensive remyelination and axonal sparing. FTY720 readily penetrates the CNS. Therefore, the effect of FTY720 treatment on the biology of NPC's following CNS transplantation into JHMV-infected mice was examined. FTY720 increased NPC migration and proliferation within the spinal cord but did not diminish neuroinflammation, decrease the severity of demyelination nor increase remyelination. Therefore, FTY720 does not enhance the therapeutic potential of NPCs in a model of chronic-induced demyelination.

CHAPTER ONE

Coronavirus-induced encephalomyelitis as a pre-clinical model for the human demyelinating disease Multiple Sclerosis

1.1 Multiple Sclerosis

Multiple sclerosis (MS) is a chronic disease of the central nervous system (CNS) characterized by multifocal regions of inflammation and myelin destruction [1]. Typically, MS patients are initially diagnosed with relapsing remitting multiple sclerosis (RRMS) defined by episodes of acute inflammation within patches of white matter leading to demyelination followed by variable periods of remission with near complete recovery from clinical disease [2, 3]. MS results in physical and cognitive impairment and can diminish the quality of life. While the cause of MS remains unknown, research suggests it is multifactorial and includes the genetic background of the individual as well as environmental influences *e.g.* microbial infection [1, 4-12]. Genes encoding proteins important in immune response such as major histocompatibility (MHC) and T cell receptor complex may be involved in genetic predisposition [1, 13]. In MS patients, autoreactive T cells specific for myelin proteins such as proteolipid protein of myelin (PLP) and myelin basic protein (MBP) are enriched in peripheral blood suggesting MS to be an autoimmune disease. Myelin is essential to maintain axonal conductivity and oligodendrocytes, the myelin secreting cells of the CNS are damaged or destroyed in MS patients. Perivascular infiltrates such as activated CD4+ and CD8+ T cells and macrophages located in active lesions release pro-inflammatory cytokines that results in dysregulation of oligodendrocytes [3, 14] Spontaneous remyelination, referred to as shadow plaques, can occur yet this process leads only to thin myelin sheaths insulating the axons and partial recovery [15-20]. However, remyelination is not sustained highlighting the paucity in continuous myelin investment in MS patients.

Nearly 80% of patients with RRMS will develop progressive forms of the disease within two decades following diagnosis, whereas approximately 15% of patients are initially diagnosed with progressive forms [21]. During progressive disease, expanding cortical lesions lead to severe neurological impairment including loss of motor function. The gradual worsening of symptoms is likely due to axonal degeneration yet components of the adaptive immune response do not seem to contribute to pathology [22-25]. Indeed, gray matter inflammation is associated with microglial activation and the presence of macrophages, while T cells and B cells reside in the border of pre-existing lesions [14, 26]. Moreover, remyelination of damaged areas by oligodendrocyte precursor cells (OPCs) fails to occur which severely reduces potential recovery [27, 28].

Using mouse models of demyelination, cellular and antigenic aspects of MS that potentially contribute to demyelination have been identified leading to the development of therapeutic treatment for patients. The majority of the Food and Drug Administration (FDA)-approved therapies display potent immunosuppressive properties by limiting leukocyte infiltration into the brain resulting in a reduction in relapse events [29]. First-line therapies include interferon-β (IFN-β) and glatiramer acetate (GA). These drugs are administered by either subcutaneous or intramuscular injections with adverse effects ranging from influenza-like symptoms to injection-site reactions [30]. IFN-β reduces T cell activation and displays anti-viral effects as well as induces apoptosis of autoreactive T cells. GA is a polymer of amino acids that mimic myelin basic protein presumably shifting immune responses from pro-inflammatory to anti-inflammatory [31]. A second-line therapy, Natalizumab is administered intravenously to MS patients that have aggressive forms of RRMS and in which first line of defense treatment fail to reduce relapse rates [32].

Natalizumab is a humanized monoclonal antibody targeting α4 subunit of the integrin α4β1 on lymphocytes that reduces leukocyte migration into the central nervous system by preventing binding to vascular cell adhesion molecule (VCAM-1). It has higher efficacy compared to other drugs but its potential serious adverse effects include allergic reactions, liver disorders, and more importantly, the potential of patients to develop progressive multifocal leukoemcephalopathy (PML), a rare disease which can be fatal and is the result of an infection of oligodendrocytes in the CNS with the human polyoma virus JC (JCV). A third-line therapy, the cytotoxic agent mitoxantrone is only used in severe forms of MS due to its high toxicity and is administered by i.v. injections. It acts through inhibition of type II topoisomerase and disrupts DNA synthesis. A more convenient route of drug delivery for MS patients is orally, which increases convenience as well as compliance and drug discovery research has recently been focusing on this mode of delivery. FTY720 is the first FDA-approved oral drug for MS patients. FTY720 is a sphingosine-1-phosphate analogue that binds to S1P1 expressed on lymphocytes thereby preventing their egress from lymph nodes and consequently preventing their infiltration in the CNS. Moreover, FTY720 seems to act directly on resident cells of the CNS and may reduce neurodegeneration. Teriflunomide, a pyrimidine synthesis inhibitor, reduces autoreactive T and B cells and is also administered orally. Finally, dimethyl fumarate, another oral drug for MS patients, modulates immune cell responses, however the effector mechanisms remain undefined [30, 31].

Most of the therapeutic strategies used to treat MS patients act upon the immune system to limit damage in the CNS but there are currently no effective treatments to enhance remyelination and subsequently no treatment for the progressive forms of MS.

Stem cell transplants offers hope in this regard and clinical investigations have begun with the intravenous injection of autologous bone-marrow-derived mesenchymal stem cells into patients with progressive MS. To date, treated patients have exhibited no serious side effects and reported improved visual acuity suggesting diminished severity of optic neuritis [33, 34].

1.2 Viruses and Multiple Sclerosis

The mechanism(s) by which the auto-reactive T cells are activated and how they escape thymic surveillance is poorly understood [13]. Reports of a 70% discordance rate among monozygotic twins suggest that other environmental factors could be the underlying cause of MS. Those factors include microbial infections including viruses [35]. Research has been made in attempt to identify an etiologic agent that is responsible for MS. However, these attempts were unsuccessful, as no single microbe has been compellingly demonstrated as the cause of disease. In some studies, upper respiratory tract infections lead to an increased risk of onset and/or exacerbation of clinical symptoms of MS perhaps due to increased IFNγ secretion by activated lymphocytes [36, 37]. Viruses including herpes simplex virus type-1 (HSV-1), measles, human T cell leukemia virus type-1 (HTLV-1), human coronaviruses, human herpesvirus-6 (HHV-6), and Epstein Bar Virus (EBV) have been associated with MS but a clear causal connection between MS and a viral infection has yet to be proven [36-44].

Animal models that are similar both clinically and histologically to what is observed in MS patients are crucial in order to better understand the underlying pathological mechanisms contributing to MS. The prevalent rodent model of MS is experimental autoimmune encephalomyelitis (EAE), an autoimmune model of neuroinflammation and demyelination [45, 46]. In addition, viral models of demyelination are also important tools for studying the pathogenesis of disease. One of these models utilizes infection with Theiler's murine encephalomyelitis virus (TMEV) and this has provided important insight into immunopathological agent of demyelination [42, 44, 46, 47]. Another viral model of demyelination is intracranial infection with neurotropic strain of mouse hepatitis virus

(MHV); MHV infection of the CNS results in an acute encephalomyelitis followed by chronic phase of demyelination. Immune cells such as T cells and macrophages play an important role in white matter destruction and animals show similar clinical and histologic disease profiles as compared to MS patients [48-53].

As viral infection is considered to be a potential contributing cause of MS, the experimental infection with the neurotropic JHM strain of mouse hepatitis virus (JHMV) to induce immune-mediated demyelination offers not only an excellent model to study the immunopathological mechanism driving demyelination in MS patients but offers an opportunity to study novel therapeutic methods for promoting remyelination.

1.3 MHV-acute disease

MHV is a member of the *Coronaviridae* that is a ubiquitous group of positive-strand RNA viral pathogens of human and animals. Viral strain, inoculation route and mouse background dictates MHV pathogenesis [54]. MHV is comprised, in part, by three predominant structural proteins; the nucleocapsid (N, 60kDa), the membrane protein (M, 25kDa) and the spike glycoprotein (S, 180kDa) [54]. The primary MHV receptor is a murine carcinoembryonic antigen-related cell adhesion molecule (CEACAM). The MHV spike protein S binds to CEACAM inducing structural changes leading to membrane fusion and entry of virus into the host cell [55]. Upon entry into the host cell, MHV replicates in replication complexes (RCs) that are associated with cellular membranes [56]. The assembly of the virus takes place in the vesicles and virus is released through fusion of the vesicles with the host cell membrane [57].

MHV infection is associated with a wide spectrum of respiratory, gastrointestinal, and neurological diseases [54, 58, 59]. I.c. infection of susceptible strains of mice, such as C57BL/6, with the neuroattenuated variant MHV strain J2.2v-1 (resistant to neutralizing antibodies directed to the S glycoprotein) results in the dissemination of viral particles in the ventricles followed by infection of the ependymal cells. MHV can spread into the spinal cord through the cerebrospinal fluid and infect oligodendrocytes, astrocytes and microglia [60]. Inoculation with virus leads to an acute encephalomyelitis with oligodendrocytes, astrocytes, and microglia infection in the gray matter [61].

In response to MHV infection, resident cells of the CNS secrete cytokines and chemokines, such as CXCL1 and CXCL2. These chemokines are upregulated early following infection and attract polymorphonuclear (PMN) cells such as CXCR2+ neutrophils that

infiltrate the CNS within 24 hours. Neutrophils secrete matrix metalloproteinase-9 (MMP-9) allowing degradation of the blood-brain-barrier (BBB) [62, 63]. This permeabilization of the BBB by innate immune cells such as neutrophils, macrophages and natural killer cells [63-66] enables sensitized MHV-specific CD4+ and CD8+ T cell infiltration into the CNS [63, 66, 67]. During the acute phase of the disease, CD4+ T cells, CD8+ T cells, natural killer (NK) cells, B cells, and macrophages take part in the immune response [68, 69] resulting in secretion of pro-inflammatory factors such as IFN-α, IFN-β, IL-1α/β, IL-6, IL-12 and TNF-α [70, 71]. Both CD4+ and CD8+ T cells mediate clearance of the infectious virus [48, 68, 71, 72]. Importantly, Type I interferons *e.g* IFN-α/β play a crucial protective role against MHV infection. Indeed, type I interferon treatment restricts dissemination of virus and mice deficient in the IFN-α/β receptor display higher mortality as well as elevated viral load in the CNS [73-75].

CD4+ T cells are critical for CD8+ T cell maintenance however the mechanism(s) are not clearly defined [76]. CD8+ T cells, the primary cytolytic effector cell in the CNS, eliminate virus from infected astrocytes and microglia through a granzyme B and perforin-dependent mechanism and from infected oligodendrocytes via IFN-γ which also induces upregulation of MHC class II expression on microglia and MHC class I on infected astrocytes and microglia [77, 78]. Fas/FasL and TNFR signaling on infected cells do not appear to contribute to viral clearance [79]. Depletion of CD4+ T cells modifies CD8+ T cell-mediated control of viral replication within the CNS due to a reduction of IFN-γ expression as well as an increase in CD8+ T cell apoptosis [80].

Regulatory T cells (Tregs) are essential not only for the suppression of pro-inflammatory immune responses and preventing activation of autoreactive immune cells

but also for the clearance of viral pathogens by facilitating immune cell entry into inflamed tissue [81]. Indeed, Tregs do not solely recognize self-antigens but also recognize viral epitopes [82]. Tregs play important roles during both acute and chronic MHV-infection; depletion of Tregs in mice infected with MHV show higher mortality and this suggests that Tregs dampen the disease without impacting viral clearance [83]. Importantly, MHV specific CD4+T regulatory cells are present in the CNS where they ameliorate encephalomyelitis and play a role in muting the proliferation of virus specific CD4+ T cell through IL-10 secretion in the draining cervical lymph nodes but do not prevent their activation [84]. Finally, studies have shown that adoptively transferred Tregs to MHV-infected mice limits clinical disease severity by attenuating neuroinflammation leading to a decrease in demyelination [85].

1.4 MHV-induced chronic disease

The chronic phase of the disease is characterized by MHV persistence in white matter tracts leading to demyelination that is associated with areas of viral RNA/antigen and this can last up to one year post-infection (p.i.) [53]. Analysis of spinal cords from mice with established MHV-induced demyelination reveals that oligodendrocyte dysfunction and myelin integrity loss in the white matter tracts is correlated with the presence of inflammatory leukocytes as well as presentation of viral antigen through MHC-class I and MHC-class II and not by apoptosis and/or necrosis of mature oligodendrocytes [50, 86, 87]. Immune infiltration into the CNS diminishes two weeks p.i., however virus specific T cells and macrophages persist in the CNS. Demyelination is not due to activation of myelin specific T cells through epitope spreading but due to virus-specific T cells. Both CD4+ and CD8+ T cells play an important role in demyelination by secreting inflammatory cytokines such as IFN-γ and TNF-α in the brain and spinal cord following viral clearance [88-90]. Furthermore, macrophages localized within white matter areas of demyelination are shown stripping and engulfing myelin [91] and thus play an important role in demyelination [50]. Although neutralizing virus-specific antibody is considered important in suppressing viral recrudescence by limiting virus replication to oligodendrocytes [92, 93], it does not appear to have a prominent role in promoting demyelination [92, 93], as neutralizing anti-MHV antibodies are not detected in early MHV rather after infectious virus has been eliminated from the CNS [94, 95].

The scarcity of infectious viral particles present in the CNS during chronic disease suggests that demyelination is not amplified by productive infection of glial cells.

Rather, the presence of viral RNA quasispecies with mutants and deletion variants in the CNS results in chronic inflammation and demyelination in persistently infected mice [96-98]. Axonopathy within the white matter tracts of the spinal cord was originally suggested to occur in conjunction with demyelination. However, axonal degeneration might take place prior to immune-mediated demyelination in MS patients [99, 100]. For years, MHV was believed to induce demyelination without causing axonal loss; however, studies have shown that the MHV-A59 strain causes axonal damage within the optic nerve [101]. In addition, axonopathy was detected not only in areas of demyelination but also in areas where no myelin damage was observed in mice infected with MHV. The use of *RAG-/-* mice, which lack functional B and T cells, demonstrated the requirement of T cells to induce demyelination and axonal damage [99]. Furthermore, MHV-infected Thy1-YFP mice, that express yellow fluorescent protein (YFP) from medium-to-large caliber axons, display extensive axonal damage. Importantly, axonal loss was noticed earlier than expected during MHV-induced disease [102]. These findings argue that axonal degeneration occurs prior to immune-mediated demyelination [102] and that axonopathy may be important in contributing to white matter damage and myelin loss. Viral infection of neurons as well as viral protein transport could take part in axonal loss however, this is not well characterized [100].

1.5 Progenitor cell transplantation and remyelination

FDA-approved therapies for MS are designed to reduce neuroinflammation and ultimately limit disease progression [103]. However, these approaches often do not address the neurodegenerative phase of disease. Spontaneous remyelination can occur in the adult human brain and this is characterized as shadow plaques where regions of white matter are undergoing remyelination [19]. Ultimately, remyelination is neither extensive nor sustained and two potential mechanisms have been suggested to explain this failure in MS. First, the recruitment of OPCs to areas of demyelination could be impaired and second, the differentiation of OPCs into mature myelin-producing oligodendrocytes could be inhibited [104]. Nevertheless, in both scenarios signaling by growth factors, cytokines/chemokines, and extracellular matrix molecules are possibly contributing to an environment unfavorable for enhancing remyelination.

Stem cells and neural precursors can be differentiated to oligodendrocytes and are appealing sources for the generation of remyelination-competent cells [105, 106]. Animal models have been crucial in identifying methods for promoting remyelination and studies have shown that stem cell transplantation induce remyelination in animal models of acute demyelination [107]. Studies have shown that transplantation of embryonic stem cell-derived NPCs into myelin-deficient shiverer mice lead to migration of the cells in the spinal cord associated with differentiation into astrocytes and oligodendrocytes followed by remyelination of axons [106]. Also, transplantation of human embryonic stem cell (hESC) derived oligodendrocytes progenitor cells into myelin-deficient shiverer mice showed similar results [108].

Our laboratory has demonstrated that engraftment of hESC-derived oligodendrocytes into MHV-infected mice results in focal remyelination however, engrafted cells were not detected following 2 weeks post transplantation. Remyelination may be the result of enhanced inflammation surrounding the hESC-OPC xenograft resulting in activation of endogenous OPC's [109]. These findings highlight the importance of the model system and cell type used to assess the therapeutic potential with regards to remyelination [110]. In EAE, recovery from disease and reduction in demyelination severity was reported following transplantation of adult neurospheres [111]. In addition, transplantation of stromal bone marrow cells into demyelinated rat spinal cord resulted in remyelination [112].

It is necessary to understand the environmental conditions that allow remyelination by engrafted cells to conclude whether transplantation can be a potential treatment for human demyelinating diseases. In our MHV model of viral-induced demyelination, an (i.c.) injection of virus results in chronic neuroinflammation and demyelination. Intraspinal engraftment of mouse NPCs into MHV-infected mice led to an extensive migration of transplanted cells to sites of lesions, remyelination, axonal sparing, and clinical improvement [113]. Similarly, engrafted NPCs expressing green fluorescent protein (GFP-NPCs) migrated to sites of demyelination and colonized the ventral and lateral funiculus of white matter tracts of the spinal cord. Migration of NPCs was dependent, in part, to CXCR4 receptor expression that responds to a CXCL12 chemokine gradient that is enriched in white matter tracts [114]. Importantly, engraftment of NPCs in the spinal cord did not decrease leukocyte infiltration into the CNS or cytokine secretion suggesting that NPCs directly affect remyelination [115].

To conclude, transplanted-mediated remyelination can occur in an environment in which chronic neuroinflammation and demyelination is triggered by infection with a neurotropic virus. To date, these are the only studies that have shown the therapeutic benefit of engrafted NPCs using a model of viral-induced demyelination.

1.6 Lineage-fate commitment of NPCs and remyelination

Within NPCs, different classes of transcription factors determine the neuron versus glial lineage commitment. The basic helix-loop helix transcription factors (bHLH) encoded by *Olig1* and *Olig2* genes promote glial differentiation and Neurogenins (Ngns) and Mash1 promote neuronal differentiation. Olig1 and Olig2 are essential for important processes of CNS development such as oligodendrocyte formation [116, 117]. The importance of these genes was demonstrated via generation of null mutant mice specific for *Olig1* and *Olig2* [117-119]. *Olig1*-deficient mice are viable and exhibit only slight alterations in oligodendrocyte maturation, however, *Olig2*-deficient mice are embryonically lethal and do not survive past the fetal development stage [117]. Olig1 plays an important role for oligodendrocyte maturation, remyelination [120] and post-natal myelination [121] whereas Olig2 is important for generation of motor neurons and oligodendrocytes. The balance between Olig2 and neurogenins 2 (NGN2) that are co-expressed in polymorphonuclear cells (pMN) dictates the differentiation fate of these cells into either oligodendrocytes or motor neurons [122]. Importantly, in the context of demyelination in the adult CNS, Olig1 is crucial for remyelination, as investment of new myelin in spinal cord and brain is impaired in *Olig1* deficient mice compared to wild-type mice with failure of NPCs to differentiate into myelin basic protein (MBP) expressing oligodendrocytes [120]. However, recruitment of NPCs to site of demyelination was not impacted [120]. Forced retroviral expression of *Olig1* and *Olig2* in subventricular zone (SVZ) neural stem cells under inflammatory conditions resulted in oligodendrocte differentiation [123]. The role of *Olig1* in remyelination by oligodendrocytes during chronic viral infection was also demonstrated following spinal cord engraftment of wild-type NPCs and *Olig1-/-* NPCs into

MHV infected mice with established demyelination. Remyelination was significantly diminished in *Olig1-/-* NPCs recipients compared to mice that received wild-type NPCs. In addition, *Olig1 -/-* NPCs differentiated into astrocytes while WT NPCs preferentially differentiated into oligodendrocytes demonstrating the importance of *Olig1* for oligodendroglial lineage commitment [124].

1.7 Sphingosine-1-phosphate

Sphingosine-1- phosphate (S1P) is a lipid that plays a key role in immune cell trafficking and is important for normal embryonic development and vascular system development [125, 126]. S1P is synthesized from the sphingolipids that are located in the cell membrane and form lipid rafts. An enzymatic cascade involving sphingomyelinase and ceramidase leads to conversion of sphingomyelin to ceramide and ultimately to sphingosine. There are two ubiquitously distributed sphingosine kinases (SPHK1 and SPHK2) that phosphorylate sphingosine into S1P and are important for homeostatis of circulating S1P. SPHK1, present predominantly in lungs and spleen, upregulates NFkB during stress responses and controls S1P both intracellularly an exrtacellularly. SPHK2 is present predominantly in the heart, brain and liver, can localize in the nucleus, and induce apoptosis [125, 126]. The levels of S1P are controlled by the activity of enzymes related to the sphingolipid metabolic pathway and S1P lyase and phosphatases are responsible for S1P catabolism. S1P levels are present in low micromolar concentrations in most tissues, however S1P concentration is increased within hundred-nanomolar range in the lymph and blood due to erythrocyte production. S1P is either free, bound to serum albumin or high-density lipoprotein (HDL). Due to its amphiphilic nature, S1P cannot cross membranes easily and requires the ATP-binding cassette (ABC) family of transporters [125].

S1P binds to G-protein-coupled receptor S1P1-S1P5 [30, 125]. The expression pattern of S1PRs are important in diverse biological functions such as leukocyte circulation, morphological changes including cell-cell aggregation or cytoskeleton rearrangement, endothelial cell function and neural cell proliferation [32, 127]. S1P1, although mainly

expressed by immune cells, is also secreted by endothelial cells, glial cells and smooth muscles cells and plays an important role in immune cell trafficking, neurogenesis and angiogenesis, respectively [30]. The importance of S1P signaling in numerous biological processes is emphasized in that deletion of S1P1 is embryonically lethal at E13.5 [126]. S1P2 is important for neuronal excitability and proper function of the auditory and vestibular systems, S1P3 seems to be important for cardiovascular functions, S1P4 importance remains unknown but is expressed in lymphoid and hematopoietic tissues and S1P5 is expressed primarily on oligodendrocytes [30].

Immune cell homing to lymphoid organs as well as their exit into blood and lymph is regulated by S1P signaling. The higher concentration in vascular compartments drives the egress of immune cells from lymphoid tissues where S1P levels are low. When inflammation occurs, S1P concentration rises in the tissues shifting the immune cell trafficking and allowing appropriate localization of immune cells in lymphoid and non-lymphoid tissues [125]. In the presence of high levels of S1P, S1P1 is down regulated and can be recycled to the cell surface. In addition, CD69 an immune cell activation marker linked with S1P1 expression and down-regulates expression [125]. In the lymph nodes, naïve T cells express S1P1 however, when they encounter their cognate antigen presented by an antigen presenting cell such as a dendritic cell, naïve T cells become activated, central memory T cells (TCM) are reactivated and they downregulate S1P1. After clonal expansion, activated antigen-specific T cells up-regulate S1P1 and exit lymph nodes to migrate to the inflamed tissues through the S1P gradient [32, 128]

1.8 S1P and Multiple Sclerosis

S1P receptors have been associated with MS pathology as they play key roles in inflammation and repair. MS patients display altered sphingolipid metabolism and disrupted myelin lipid distribution [32, 129]. Moreover, S1P signaling might be disrupted in MS with decreased levels of sphingomyelin in the white matter but a higher level of S1P in the cerebrospinal fluid. Astrocytes display an increase in expression of S1P1 and S1P3 in active and chronic MS lesions [130, 131]. Interestingly, antibodies against sphingomyelin have been found in serum and CSF of patients with MS [129]. S1P5, mostly expressed on oligodendrocytes, is been thought to be involved in axonal degeneration and demyelination [129] and S1P5 was shown to be reduced in demyelinated lesions [132]. Therefore, clinical evidence points to modulation of S1PRs within MS lesions. In addition, recent studies have demonstrated that S1P2 expression was enhanced in CNS lesions in both EAE female mice as well as female MS patients. S1P2 is important for regulation of BBB permeabilization through disruption of adherens junctions and blockade of S1P2 signaling decreased EAE disease severity [133]. This is an interesting finding as women have a higher risk in developing MS compared to men. Collectively, studies derived from MS patients and pre-clinical mouse models indicate that S1P receptors could serve as therapeutic targets for MS, as they seem to play an important role in MS pathology. Indeed, Novartis developed FTY720, an oral drug that binds to S1P receptors and that showed decrease relapses and disease progression.

1.9 FTY720

FTY720, also called fingolimod/ Gilenya, is an oral drug approved by the FDA in September 2010 for treatment of patients with relapsing MS [134-138]. It was originally synthesized by Novartis in 1992 through chemical modification of myriocin, a metabolite of the parasitic fungus *Isaria sinclairii* [139]. FTY720 is an immunomodulating drug that has shown to reduce not only acute relapses but also new lesion formation as well as disability progression, brain volume loss and BBB leakiness in MS patients. Approximately 70% of MS patients exhibited no relapses after three years of treatments [125]. It's efficacy was validated in two randomized, double-blind, phase 3 clinical trials: a placebo-controlled trial FREEDOMS (FTY720 Research Evaluating Effects of Daily Oral Therapy in Multiple Sclerosis), and TRANSFORMS (Trial Assessing Injectable Interferon versus FTY720 Oral in Relapsing-Remitting Sclerosis) a comparison between FTY720 and IFN-β trial [140]. Common side effects of FTY720 are flu-like symptoms, nasopharyngitis, liver damage and back pain. Severe side effects include bradycardia, blood pressure increase, macular edema that can lead to visual loss, and importantly, viral infections such as varizella zoster [31]. In addition, during Phase II clinical trials, two patients receiving the highest non -approved dose of FTY720 died from varizella zoster infection and herpes simplex virus encephalitis [31].

The mechanism of how FTY720 functions are not yet defined. However, FTY720 is phosphorylated by sphingosine kinase (SPHK) generating the active form FTY720P that is an S1P receptor modulator. FTY720 displays a similar structural homology with sphingosine, a metabolite of sphingomyelin present in the cell membrane. FTY720 binds to

four of the five S1P receptors: S1P1, S1P3, S1P4 and S1P5. The suggested mechanism by which FTY720P functions includes inhibiting egress of lymphocytes from lymph nodes and mature thymocytes from the thymus [30, 32, 125] resulting in a dampening of pathogenic T cell infiltration into the CNS. FTY720 down-regulates and degrades S1P1 acting as a functional antagonist [32]. S1P1 is not important for binding and rolling on endothelial cells but for diapedesis. Furthermore, FTY720 displays antagonistic roles with CC-chemokine receptor 7 (CCR7) resulting in the retention of $CCR7^{+}$ naïve T cells and CCR7+ central memory T cells (Tcm) in lymph nodes but not preventing $CCR7^{-}$ effector memory cells (Tem) from undergoing egress. While FTY720 affects both CD4+ and CD8+ T cells, treatment appears to exert a greater effect on CD4+ T cell. In MS patients, studies have shown that autoreactive T cells are composed of mainly Th17 Tcm and FTY720 has shown to decrease Th17 cells in the blood in MS patients. Importantly, FTY720 does not appear to affect Tem, which do not express homing markers for lymph nodes such as CD62L and CCR7. Rather, Tem are important for rapid suppression of locally invading pathogens. These findings argue that immune surveillance and the ability to mount memory immune response in MS patients receiving FTY720 treatment is not negatively impacted. However, by retaining activated T cells and Tcm in lymphoid organs, immune responses can potentially be diminished leading to increase in susceptibility to viral infections [141]. Indeed, recent clinical studies have detailed emergence of herpes zoster and associated neurologic complications in MS patients during FTY720 treatment [142, 143]. Immunosuppression may arise in response to FTY720 treatment resulting in re-emergence of persistent viruses. Interestingly, FTY720 has been shown to impair CD8-T-cell function through inhibition of cytosolic phospholipase A2 and increasing mortality in a murine

influenza model [144]. Moreover, studies have demonstrated that treatment with FTY720 or other S1P1 agonists affects cytokine production as well as disease outcome in mice infected with influenza virus, indicating the immunomodulatory effects of such treatment [145, 146]. In contrast, administration of FTY720 to mice infected with lymphocytic choriomeningitis did not ameliorate persistence advocating that the virus and sites of infection may dictate the outcome following treatment [146]. Pachner and colleagues [147] showed that FTY720 treatment had no effect on clinical disease progression or viral load within the CNS using the TMEV model of demyelination. However, these findings are in contrast with studies using EAE, in which FTY720 treatment reduced clinical disease severity accompanied by limited infiltration of immune cells into the CNS [148-152].

Most of the current treatment strategies for MS target immune cells in the periphery but do not act directly in the CNS to promote neuroprotection and repair. As indicated earlier, FTY720, due to its lipophilic nature, penetrates the BBB and readily enters the CNS parenchyma. Furthermore, FTY720P is detected *in situ* suggesting that it may influence the biology of resident cells of the CNS by impacting neuroregeneration and endogenous repair mechanisms. Upon phosphorylation, FTY720 preferentially localizes and accumulates in the white matter where damage is present. In addition, FTY720 levels are higher in CNS tissues than in the blood [153]. FTY720 has been shown to improve disease severity in EAE and recovery was associated with dampening of neuroinflammation and associated with improved motor skills [148, 149, 154]. These results argue that FTY720 could have effects other than modulation of lymphocyte trafficking, such as potentially acting on glial cells to trigger neuroregeneration. Supporting this are studies by Antel and colleagues [155] demonstrated that FTY720 enhances remyelination following demyelination of

organotypic cerebellar slices. These findings indicated that FTY720 treatment following chemical-induced demyelination promoted remyelination in correlation with the presence of OPCs and mature oligodendrocytes [155].

Glial cells and neurons express S1P receptors (S1P1 to S1P5); however, each cell type can display different combinations of these receptors [134, 138]. In addition, receptor activation can have different effects on differentiation, migration, and survival of astrocytes, microglia, and oligodendrocytes [154, 156, 157]. Astrocytes are the most predominant cells of the CNS and perform diverse functions. They are responsible for the glial scaring at sites of demyelination, which is thought to prevent remyelination from occurring but astrocytes can also promote oligodendrocyte migration, differentiation and proliferation [130]. In the presence of FTY720, human cultured astrocytes decreased inflammatory cytokines production [131]. In addition, EAE severity was attenuated in mice in which S1P1 is selectively ablated on astrocytes suggesting that that lymphocyte depletion is not the sole reason for FTY720 efficacy in EAE [154].

The potential effects of FTY720 on neurodegeneration has been studied in *in vitro* models as well as animal models of demyelination, however the influence of S1P in controlling NPC responses remain not well characterized [134, 135]. NPCs have been shown to express S1P receptors and signaling through these receptors influences proliferation associated with morphological changes and differentiation [127]. In addition, NPCs employ S1P receptors for migration toward a site of spinal cord injury [158].

We have previously determined that cultured NPCs utilize the chemokine receptor CXCR4 to migrate in response the ligand CXCL12 [114]. Moreover, transplanted NPCs also express CXCR4 and CXCL12 is preferentially expressed within areas of demyelination in

MHV-infected mice [114]. S1P has been shown to be involved in stimulating the activation of CXCR4 via S1P3 receptor [159]. However, the impact of FTY720 treatment on the biology of NPCs either *in vitro* or in models of immune-mediated demyelination have not been well defined and is a focus of this thesis.

1.10 Summary

A well-accepted model of viral-induced demyelination is utilized to investigate mechanisms associated with FTY720 treatment during acute encephalomyelitis as well as its influence on NPC biology. The results presented in this dissertation are novel as majority of published studies focusing upon the therapeutic benefit of FTY720 have been performed in autoimmune animal models such as EAE or toxin injections. Our viral model of demyelination relies upon MHV infection of the CNS resulting in an acute encephalomyelitis with extensive neuroinflammation leading to myelin destruction associated with diminished motor skills. This is clinically and histopathologically similar to MS. Notably, viral infection of the CNS can potentially initiate demyelination and it has been suggested that viral pathogens can activate autoreactive lymphocytes that destroy myelin sheath following CNS infiltration. Moreover, it is well known that neurotropic viruses can be persistent in the CNS. For these reasons, MHV is a relevant model to study MS.

FTY720 is the first orally administered drug approved for MS and has been shown to reduce the rate of relapses in relapsing-remitting multiple sclerosis presumably through inducing lymphocyte sequestration in secondary lymphoid tissue. As FTY720 triggers lymphopenia, MS patients treated with FTY720 could have increased susceptibility to viral infections, therefore, we investigated the immune response to MHV infection during FTY720 treatment. Importantly, FTY720 crosses the BBB and enters the CNS indicating a potential effect on resident cells of the CNS. We focus on an important and clinically relevant issue regarding how FTY720 affects NPCs biology. As stem cell transplantation therapies are being considered for MS patients, these experiments will provide valuable information on how FTY720 can potentially induce migration of NPCs thus contributing in

enhancing remyelination. In summary, these studies demonstrate how FTY720 treatment can potentially affect a proper immune response against viral pathogens by preventing effector T cell egress from lymph nodes thereby limiting their access to inflamed tissues. However these studies also demonstrate the beneficial effect of FTY720 on migration of transplanted NPCs during the chronic phase of MHV disease.

Chapter 2 illustrates how FTY720 treatment increases severity of MHV disease characterized by high mortality and viral load in the brain and spinal cord. We show that this is associated to not only a decrease in infiltration of virus-specific CD4+ and CD8+ T cells into the CNS but also due to trapping of lymphocytes in the draining cervical lymph nodes. Moreover, FTY720 does not impact T cell function, as proliferation and cytokine production is not altered. This suggests that FTY20 increases severity of disease by affecting T cell trafficking into the CNS.

Chapter 3 describes the effect of FTY720 on NPC biology in terms of migration, differentiation and remyelination following transplantation into MHV-infected mice with established disease. We show that FTY720 enhances migration and proliferation of GFP-labeled NPCs engrafted into the spinal cord of mice with established demyelination without affecting CXCR4 expression. However, FTY720 does not affect differentiation of NPCs, the severity of demyelination and does not induce remyelination.

Chapter 4 details the role of *Olig1* in remyelination by oligodendrocytes during chronic viral infection. Specially, we have reported that engraftment of wild type NPCs and *Olig1*-/-NPCs into MHV infected mice and with established demyelination lead to migration throughout the white matter tracts of the spinal cord. However, remyelination was

significantly diminished in *Olig1-/-* NPCs recipients compared to mice that received wild-type NPCs. Moreover, *Olig1 -/-* NPCs differentiate into astrocytes while WT NPCs preferentially differentiate into oligodendrocytes demonstrating the importance of Olig1 for oligodendroglial lineage commitment.

1.10 References

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CHAPTER TWO

FTY720 (fingolimod) modulates the severity of viral-induced encephalomyelitis and demyelination

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Abstract

Background

FTY720 (fingolimod) is the first oral drug approved by the Food and Drug Administration for treatment of patients with the relapsing-remitting form of the human demyelinating disease multiple sclerosis. Evidence suggests that the therapeutic benefit of FTY720 occurs by preventing the egress of lymphocytes from lymph nodes thereby inhibiting the infiltration of disease-causing lymphocytes into the central nervous system (CNS). We hypothesized that FTY720 treatment would affect lymphocyte migration to the CNS and influence disease severity in a mouse model of viral-induced neurologic disease.

Methods

Mice were infected intracranially with the neurotropic JHM strain of mouse hepatitis virus. Infected animals were treated with increasing doses (1, 3 and 10 mg/kg) of FTY720 and morbidity and mortality recorded. Infiltration of inflammatory virus-specific T cells (tetramer staining) into the CNS of FTY720-treated mice was determined using flow cytometry. The effects of FTY720 treatment on virus-specific T cell proliferation, cytokine production and cytolytic activity were also determined. The severity of neuroinflammation and demyelination in FTY720-treated mice was examined by flow cytometry and histopathologically, respectively, in the spinal cords of the mice.

Results

Administration of FTY720 to JHMV-infected mice resulted in increased clinical disease severity and mortality. These results correlated with impaired ability to control viral replication ($P < 0.05$) within the CNS at days 7 and 14 post-infection, which was associated with diminished accumulation of virus-specific CD4+ and CD8+ T cells ($P < 0.05$) into the CNS. Reduced neuroinflammation in FTY720-treated mice correlated with increased retention of T lymphocytes within draining cervical lymph nodes ($P < 0.05$). Treatment with FTY720 did not affect virus-specific T cell proliferation, expression of IFN-γ, TNF-α or cytolytic activity. FTY720-treated mice exhibited a reduction in the severity of demyelination associated with dampened neuroinflammation.

Conclusion

These findings indicate that FTY720 mutes effective anti-viral immune responses through impacting migration and accumulation of virus-specific T cells within the CNS during acute viral-induced encephalomyelitis. FTY720 treatment reduces the severity of neuroinflammatory-mediated demyelination by restricting the access of disease-causing lymphocytes into the CNS but is not associated with viral recrudescence in this model.

Keywords: FTY720, S1P receptor, virus, central nervous system, T lymphocytes, demyelination

2.1 Background

Multiple sclerosis (MS) is a neurodegenerative inflammatory disease of the CNS that leads to demyelination and progressive neurological disability [1, 2]. FTY720, also called Gilenya/Fingolimod, is an oral drug recently approved by the FDA for treatment of patients with the relapsing-remitting form of MS [3-8]. FTY720 is an immunomodulatory drug that has shown to reduce both acute relapses but also new lesion formation as well as disability progression and brain volume loss in MS patients [9]. The mechanism of how FTY720 functions are not yet defined; however, the phosphorylated active form of FTY720 (FTY720P) is an S1P receptor modulator that inhibits egress of lymphocytes from lymph nodes [9-11]. It is thought that this leads to a dampening of autoreactive T cells specific for myelin antigens infiltrating into the CNS. Importantly, FTY720, due to its lipophilic nature, penetrates the blood-brain-barrier and readily enters the CNS parenchyma [9]. Furthermore, FTY720P is detected in situ suggesting that it may influence the biology of resident cells of the CNS [9] .

FTY720 has been shown to improve disease severity in experimental autoimmune encephalomyelitis (EAE), an autoimmune model of neuroinflammation and demyelination commonly used as a model for MS [12-14]. Indeed, therapeutic administration of FTY720 in EAE models is associated with reduced neuroinflammation and improved motor skills [12-15]. In addition to EAE, viral models of demyelination are also relevant tools for studying the pathogenesis of neuroinflammatory-mediated demyelination. For example, infection of susceptible mice with the neurotropic JHM strain of mouse hepatitis virus (JHMV) results in an acute encephalomyelitis followed by chronic demyelination. Similar

to MS, components of the immune system, such as T cells and macrophages, are important contributors to white matter destruction [16-18]. Moreover, JHMV-infected mice undergoing chronic demyelination show similar clinical and histologic disease profiles as compared to MS patients [19-21]. As viruses are considered to be a contributing cause of MS [22-35], JHMV infection of the CNS offers not only an excellent model to study the immunopathological mechanism driving demyelination in MS patients but also can provide insight into effects of MS therapeutics within the context of viral-induced demyelination. We have evaluated the effects of FTY720 on both host defense and disease progression in JHMV-infected mice. Our findings reveal that FTY720 treatment resulted in increased mortality associated with impaired ability to control viral replication. FTY720 did not alter anti-viral effects of T cells *e.g.* cytokine secretion or cytolytic activity but affected lymphocyte egress from draining cervical lymph nodes and accumulation of virus-specific T cells within the CNS. Therefore, FTY720 treatment mutes effective anti-viral immune responses following infection with a neurotropic virus by dampening trafficking of virus-specific T cells to the CNS. Conversely, administration of FTY720 to JHMV-infected mice reduced the severity of demyelination by limiting infiltration of inflammatory T cells into the CNS.

2.2 Methods

Virus and Mice

Age-matched (5-7 week) S1P1 eGFP knock-in mice (C57BL/6 background) [36] and C57BL/6 mice were anesthetized by intra-peritoneal injection (i.p.) with 150ml of a mixture of ketamine (Western Medical Supply, Arcadia, CA, USA) and xylazine (Phoenix Pharmaceutical, Saint Joseph, MO, USA) in Hank's balanced salt solution (HBSS). Mice were injected intra-cranially (i.c.) with 150 plaque forming units (PFU) of JHMV (strain V2.2-1) suspended in 30ul saline [37]. Clinical severity was assessed using a previously described 4-point scoring scale [16]. FTY720 (2-amino-2- [2-(4-octylphenyl) ethyl]-1,3-propanediol, hydrochloride) and FTY720P (2-amino-2[2-(4-octylphenyl) ethyl]-1,3-propanediol, mono dihydrogen phosphate ester) were purchased from Cayman Chemical Co, (Ann Arbor, MI). Administration of FTY720 or vehicle was performed by daily intra-peritoneal injections of 100ul starting at day 5 post-infection. For analysis of viral titers, mice were sacrificed at defined time points, one half of each brains were removed as well as spinal cords and homogenized and used in a plaque assay performed using DBT mouse astrocytoma cell line [38]. Experiments for all animal studies were reviewed and approved by the University of Utah and the University of California, Irvine Institutional Animal Care and Use Committee.

Cell isolation and flow cytometry

Immunophenotyping of the cellular infiltrate present within cervical lymph nodes, brains and spinal cords of infected mice was accomplished by homogenizing isolated tissue and generating a single-cell suspension for analysis by flow cytometry as previously described [39-41]. In brief, isolated cells were Fc blocked with anti-CD16/32 1:200. The following

antibodies were used for immunophenotyping: APC-conjugated rat anti-mouse B220 for B cells; APC-conjugated rat anti-mouse CD4 for CD4 T cells: PE-conjugated rat anti-mouse CD8 and APC-conjugated rat anti-mouse CD8 for CD8 T cells; PE-conjugated rat anti-mouse IFN-g for intracellular cytokine staining; PE-Cy7-conjugated rat anti-mouse CD45, APC-conjugated rat anti-mouse CD19, PE-conjugated rat anti-mouse CD138 for antibody secreting cells, M133-147 tetramer-PE for virus specific CD4+ T cells and S510-518 tetramer-PE for virus specific CD8 + T cells. Cell isolates for IFN-g intracellular staining were cultured in 200ul RPMI-1640 supplemented with 10% FBS, L-glutamine, penicillin-streptomycin and stimulated *ex vivo* with the immunodominant CD4 epitope (M133-147) or the immunodominant CD8 (S510-518) and Golgi stop for 6 hours followed by intracellular staining [42, 43]. The cells were then fixed and permeabilized by using a BD cytofix/cytoperm plus kit and then stained for intracellular IFN-g for 30min at 4°C [44]. Immunophenotyping of lymphocytes was performed following red blood cell lysis on blood samples collected with heparin-coated syringes by heart puncture from S1P1 eGFP knock in mice. Cells were then Fc blocked with anti-CD16/32 1:200 and stained with PE-conjugated rat anti-mouse CD3. Samples were then analyzed on a BD LSR II flow cytometer.

Proliferation assay

Splenocytes were isolated from mice at day 8 following i.p. infection with 2.5×10^5 PFU of the DM strain of MHV (MHV-DM) and enriched populations of CD4+ and CD8 + T cells isolated according to the manufacturer's instructions (CD4 and CD8 Isolation kits, Miltenyi Biotec) were labeled with the fluorescent dye, carboxyfluorescein diacetate succinimidyl ester (CFSE), (Life Technologies, Grand Island, NY, USA) at 2.5 μM final concentration.

$1x10^6$ total cells/well were incubated with FTY720P 100nM or vehicle and stimulated with 5 μM final peptide concentration of $CD4^+$ T cell immunodominant epitope M133-147, $CD8^+$ T cell subdominant epitope S510-518, or non-specific OVA control, and cultured for 72 hours at 37°C, 5% CO_2 in complete media. Cells were then washed and Fc receptor blocked with 1xPBS containing 1% BSA and a 1:200 dilution of rat anti-mouse CD16/32 antibody (Pharmingen, San Jose, CA, USA). Next, cells were stained for surface antigens using APC-conjugated rat anti-mouse CD4 and CD8 (Pharmingen, San Jose, CA, USA), according to the viral peptide stimulation condition, for 45 min at 4°C. Cells were analyzed and data assessed as described above.

CTL assay

Spleen-derived CD8+ T cells were analyzed for lytic activity at day 8 following i.p. infection of C57BL/6 mice with $2.5x10^5$ PFU of MHV-DM. A CD8+ T cell-enriched population of cells was obtained via negative selection through use of a magnetically labeled antibody specific for the CD8 antigen followed by passage over a magnetic column (Miltenyi Biotec, Auburn, CA) [45]. Numbers of S510-518-specific CD8+ T cells were determined by tetramer staining and these cells were used as the effector population. RMA-S cells, a murine lymphoma cell line that presents viral peptides to cytotoxic T lymphocytes (CTL), were cultured at a density of 10,000 per well in flat-bottomed 96 well format tissue culture plate (Corning Life Sciences, Tewksbury, MA, USA) 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, Lewisville, TX, USA). CD8 T-cells, exposed to either FTY720P (100 nM) or vehicle alone, were then plated with RMA-S cells at effector-

to-target (E: T) ratios ranging from 20:1 to 2.5:1. 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, Madison, WI, USA). The percentage of CTL-mediated lysis was determined as specified by the manufacturer's protocols.

Cytokine production

Spleen-derived CD4 and CD8+ T cells from MHV-DM infected mice [39] were analyzed for cytokine secretion. CD4 + and CD8+ T cells were isolated as described above using an isolation kit according to manufacturer's instructions (Miltenyi Biotec). $1x10^6$ T cells per wells on a round bottom 96 well plate were incubated for 48 hours at 37°C in 5% CO_2 in the presence of FTY720P 100nM or vehicle. Supernatants were then collected and an ELISA was performed for the following cytokines: IFNγ and TNFα. Samples were run in triplicates in accordance to the manufacturer's directions (R&D Systems, Minneapolis, MN, USA).

Histology

Clinical severity was assessed using a previously described 4-point scoring scale [16]. Spinal cords were isolated at defined time points and fixed overnight with 4% paraformaldehyde at 4°C. Spinal cords were separated into twelve coronal sections, cryoprotected in 20% sucrose and embedded in optimum cutting temperature (O.C.T) formulation (VWR, Radnor, PA, USA) [46]. Eight-micron thick coronal sections were cut and sections were stained with luxol fast blue (LFB). Areas of total white matter and demyelinated white matter were determined with Image J Software. Demyelination was

scored as a percent of total demyelination along the entire length of the spinal cord. A hematoxylin and eosin stain was performed to determine the extent of inflammation. Spinal cord sections were scored using a 4-point scale to assess neuroinflammation [16].

2.3 Results

FTY720 treatment of JHMV-infected mice increases clinical disease severity and impairs control of viral replication

S1P1 eGFP knock-in mice C57BL/6 mice [36] were infected intracranially (i.c.) with JHMV (150 PFU) and subsequently treated with increasing concentrations (1, 3, or 10 mg/kg) of FTY720 via intraperitoneal (i.p.) injection administered daily starting at day 5 post-infection (p.i.); mice were scored daily until day 21 p.i. FTY720 treatment resulted in increased severity of clinical disease with the greatest effects occurring at 3 mg/kg and 10 mg/kg ($p<0.05$) doses compared to vehicle-treated mice (**Figure 2.1A**). In accordance with clinical data, FTY720-treated mice exhibited increased mortality in a dose-dependent manner (**Figure 2.1B**). By day 21 p.i., <30% of mice treated with 10 mg/kg FTY720 survived while mice treated with either 3 mg/kg or 1 mg/kg exhibited 40% and ~60% survival, respectively (**Figure 2.1B**). Based on the morbidity and mortality data, 3 mg/kg FTY720 was used for subsequent *in vivo* studies. FTY720 treatment resulted in increased viral burden within the brain and spinal cord as determined by plaque titer at days 7 and 14 ($p\leq0.05$) when compared to vehicle-treated control mice (**Figure 2.1C and D**). However, at later times p.i. the majority of mice treated with FTY720 had reduced viral titers below the level of detection (~100 PFU/g) within the brain and spinal cord (**Figure 2.1C and D**). These findings indicated that the increase in mortality following administration of FTY720 correlated with impaired ability to control viral replication within the CNS and argues that either trafficking of virus-specific lymphocytes is impaired and/or anti-viral effector functions is negatively affected.

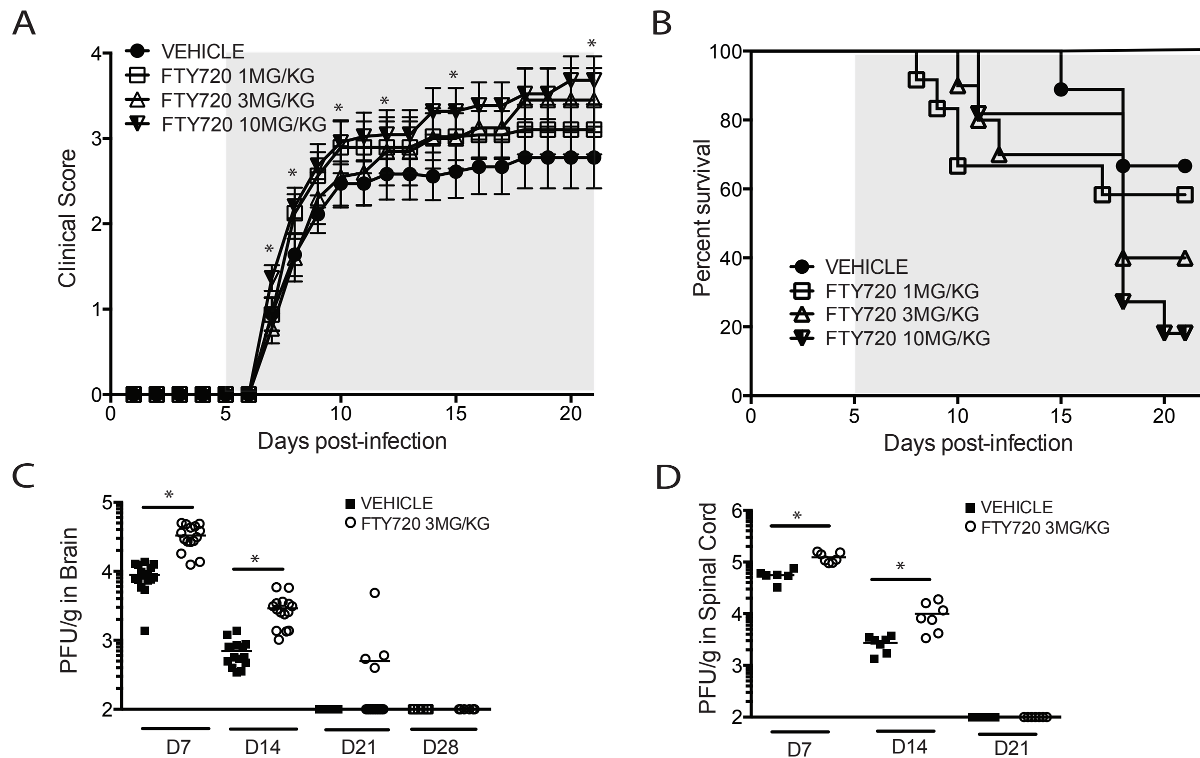


Figure 2.1. FTY720 treatment increases clinical disease severity, mortality and affects viral clearance from the CNS. (**A**) S1P1 eGFP knock-in mice C57BL/6 were infected i.c. with JHMV (150 PFU) and treated daily beginning at day 5 p.i. (shaded area) with either FTY720 (1, 3, and 10 mg/kg, n=10/group) or vehicle control by i.p. injection. Infected mice showed increased clinical disease severity at all FTY720 concentrations used with 10 mg/ml (*p<0.05) having the greatest effect when compared to vehicle-treated control mice. Data is presented as average±SEM and represents a minimum of two independent experiments with a minimum of 5 mice/group. (**B**) FTY720 treatment (beginning at day 5 p.i., shaded area) results in a dose-dependent increase in mortality when compared to vehicle-treated mice. Data is representative of two independent experiments with a minimum of 5 mice/experimental group. Viral titers within the brain (**C**) and spinal cord (**D**) in JHMV-infected mice treated with either FTY720 (3 mg/kg) or vehicle (beginning at day 5 p.i.) were determined at days 7, 14, and 21 p.i. Data points represent individual mice and bars indicate average. CNS viral titers represent two independent experiments. *P <0.05

T cell anti-viral effector function and FTY720 treatment

T cell responses including proliferation, secretion of IFN-g and CTL activity are critical in controlling JHMV replication within the CNS [47-53]. FTY720P treatment (100 nM) had no appreciable affect in dampening proliferation of either CD8+ T cells specific for the immunodominant epitope for the spike (S) glycoprotein spanning amino acid residues 510-518 (S510-518) [42] or CD4+ T cells recognizing the matrix (M) glycoprotein peptide 133-147 (M133-147) [43] (**Figure 2.2A and B**). Lymphocytes were isolated from spleens of JHMV-DM infected mice day 8 p.i., pulsed with either M133-147 or S510-518 peptides and treated with FTY720P (100nM) to determine if cytokine secretion was affected. FTY720 treatment did not affect secretion of either IFN-g or TNF-a as compared to vehicle-treated cultures (**Figure 2.2C**). Finally, FTY720 treatment of CD8+ T cells did not affect lytic activity when compared to controls (**Figure 2.2D**). These findings argue that FTY720 treatment does not dampen antiviral T cell effector functions.

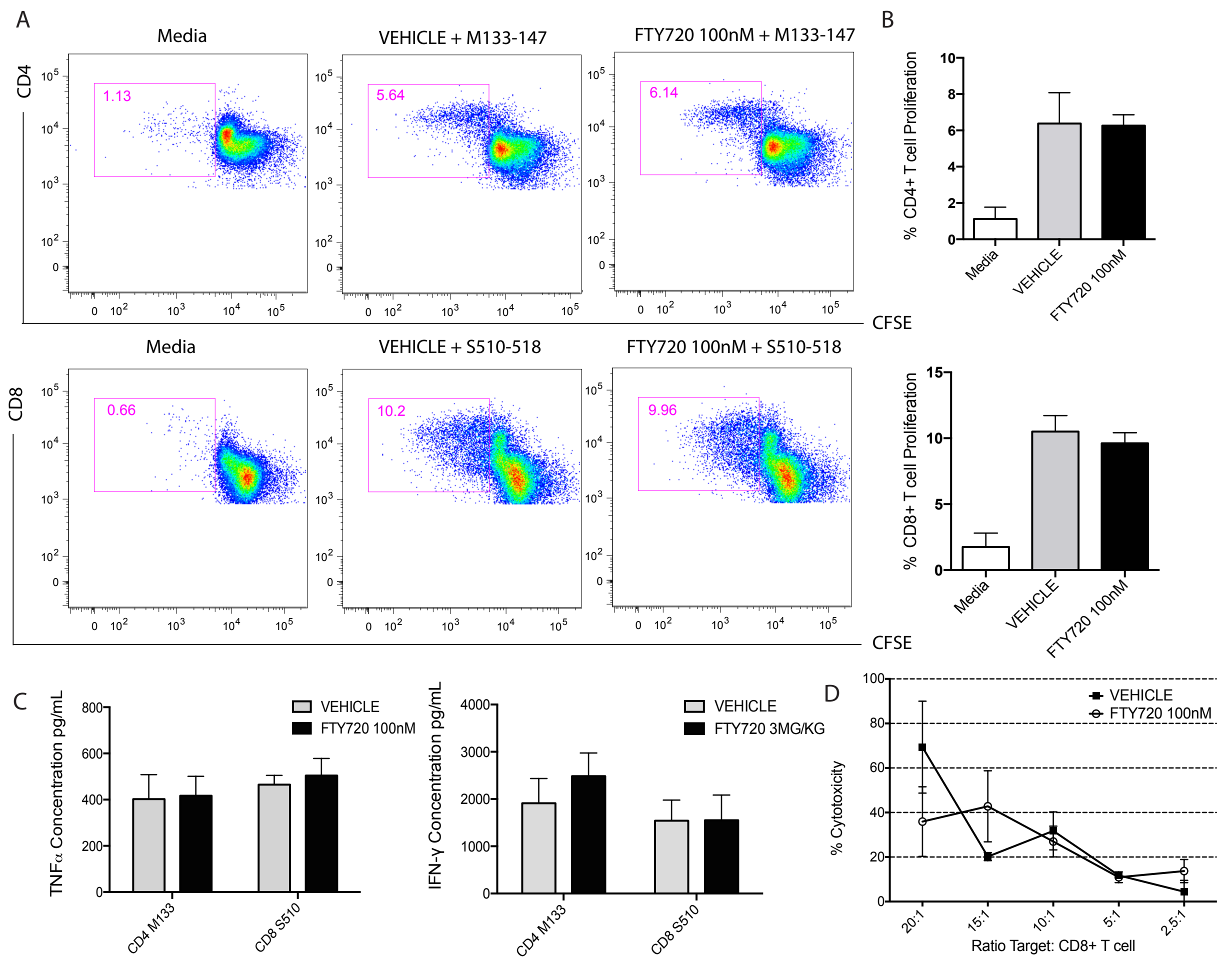


Figure 2.2. FTY720 treatment does not impair T cell anti-viral effector function. Representative flow data **(A)** showing proliferation (CFSE staining) of virus specific CD4+ and CD8+ T cells in response to treatment with FTY720P (100 nM). (**B**) Exposure to FTY720 does not affect proliferation when compared to vehicle-treated controls. Data is presented as average+SEM and represents two independent experiments. (**C**) Cytokine production by CD4+ and CD8+ T cells obtained from JHMV-infected mice and subsequently stimulated with either [Matrix (M) glycoprotein 133-144, CD4 immunodominant epitope] or [Spike (S) glycoprotein 510-518, CD8 immunodominant epitope] and incubated with or without FTY720P (100nM) for 48 hours at which point supernatants were collected and indicated cytokine levels determined by ELISA. (**D**) Lactate dehydrogenase (LDH) assay showing target cell (RMA-S cells pulsed with 5mM S510-518 peptide) lysis by CD8+ T cells pre-incubated with FTY720P 100nM or vehicle. LDH release was determined after 4 hr. incubation at 37ºC; data is representative of two independent experiments and presented as average±SEM.

FTY720 treatment impairs T cell trafficking into the CNS

We next determined if FTY720 affected S1P1 expression on circulating lymphocytes in JHMV-infected mice. Administration of FTY720 reduced ($p<0.001$) S1P1 on circulating CD3+ lymphocytes as compared to vehicle treated controls at day 7 p.i. (**Figure 2.3A and B**). Examination of T cell infiltration into the CNS of FTY720-treated mice infected with virus indicated reduced frequency of CD4+ T cells ($p<0.05$) at day 7 p.i. although CD4+ T cell trafficking was not affected at days 14 and 21 p.i (**Figure 2.3C and D**). Further, FTY720 treatment did not affect CD8+ T cell infiltration into the CNS at days 7, 14 and 21 p.i. (**Figure 2.3C and E**).

Infiltration of virus-specific CD4+ and CD8+ T cells, as determined by intracellular IFN-g staining in response to treatment with immunodominant CD4+ and CD8+ viral epitopes [42, 43], is diminished at days 7 ($p<0.01$) and 14 ($p<0.05$) following FTY720 treatment in comparison to vehicle-treated mice (**Figure 2.4A and B**). By day 21 p.i., infiltration of virus-specific CD4+ T cells, but not virus-specific CD8+ T cells, was also reduced ($p<0.05$) in FTY720 treated mice compared to control animals (**Figure 2.4B**). Infiltration of antibody secreting cells (ASCs, $CD45^{-}CD19^{low}$ $CD138^{+}$) into the CNS of JHMV-infected mice was not reduced at either days 7, 14, or 21 p.i. following FTY720 treatment compared to control mice (**Figure 2.4C and D**). These findings indicate that administration of FTY720 negatively impacts recruitment of T lymphocytes into the CNS in response to JHMV infection.

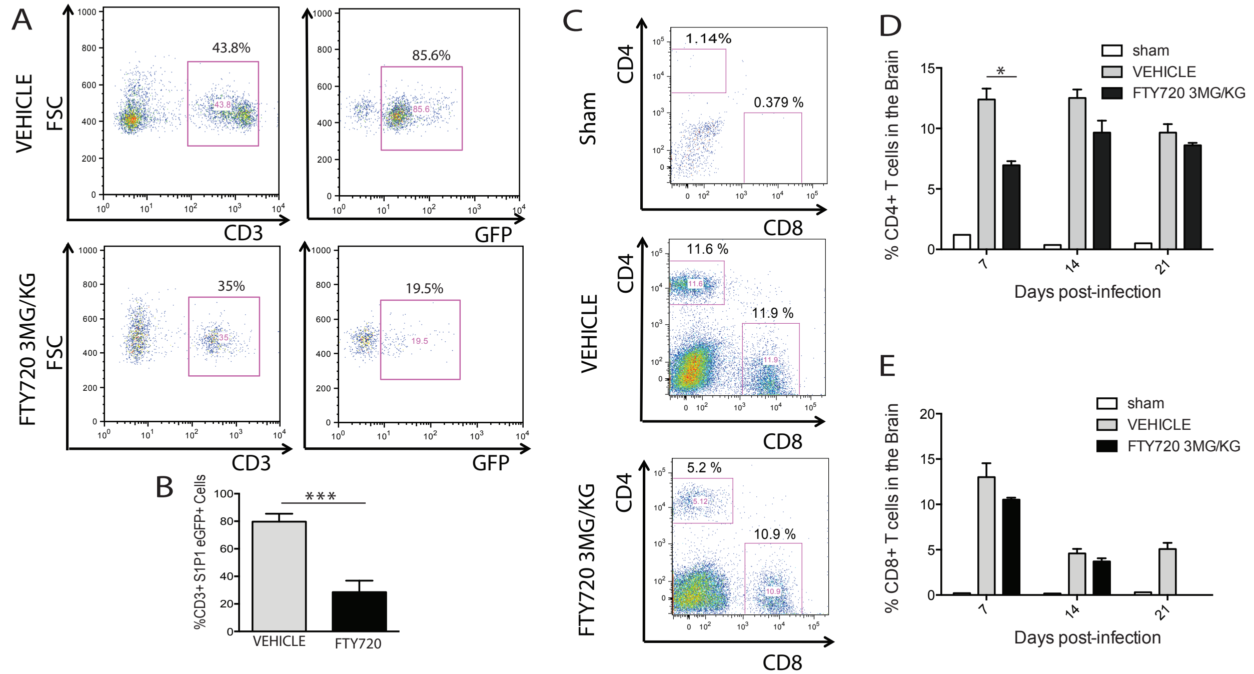


Figure 2.3. FTY720 treatment reduces T cell infiltration into the CNS of JHMV-infected mice. S1P1 eGFP mice were i.c. infected with JHMV (150 PFU) and treated daily with FTY720 (3 mg/kg) starting on day 5 p.i. **(A)** Representative flow data and **(B)** frequencies of CD3+ T cells showing reduced expression of S1P1 (as determined by eGFP expression) in blood day 7 p.i.. **(C)** Representative flow data showing CD4+ and CD8+ T cell accumulation within the CNS of sham-infected and JHMV-infected eGFP S1P1 mice treated daily with vehicle or FTY720 (3mg/kg) starting at day 5 p.i. Frequencies of CD4+ (**D**) and CD8+ T cells (**E**) present within the brains of either vehicle or FTY720-treated infected mice as well as sham-infected controls at defined times p.i. Data in panel B, D and E is presented as average+SEM and represents 3 independent experiments with a minimum of 5 mice/group. *P <0.05, *** P <0.001

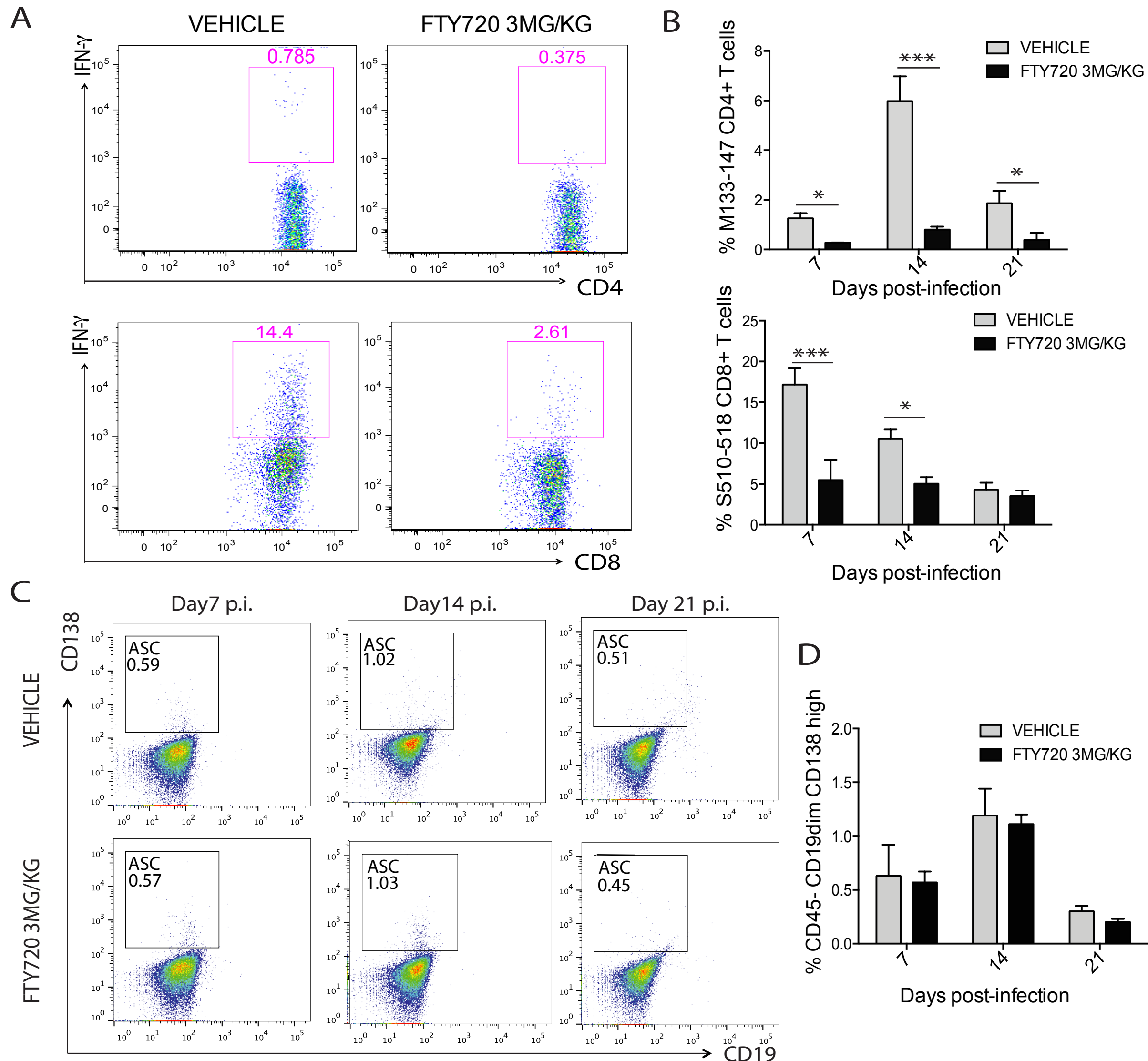


Figure 2.4. FTY720 treatment affects virus-specific T cell infiltration into the CNS. S1P1 eGFP mice were i.c. infected with JHMV (150 PFU) and treated daily with FTY720 (3 mg/kg) starting on day 5 p.i. (**A**) Representative flow staining showing intracellular IFN-γ staining by CNS infiltrating T cells following exposure to CD4+ T cell epitope [M133-144] and CD8+ T cell epitope [S510-518] at day 7 p.i. in mice treated with either FTY720 (3 mg/kg) or vehicle control (**B**) Reduced frequency of virus-specific IFN-γ producing CD4+ and CD8+ T cells into the CNS following FTY720 treatment at defined times p.i. (**C, D**) Similar frequencies of infiltrating ASCs ($CD45^{-}CD19^{low}$ $CD138^{+}$) into the brains of vehicle and FTY720 treated mice. Data in B and D is presented as average±SEM and represents a minimum of two independent experiments with at least 5 mice/experimental group. **P* <0.05, *** *P* <0.001

FTY720 restricts lymphocyte egress from draining cervical lymph nodes

As an S1P1 functional antagonist, FTY720 disrupts the S1P gradient in lymph nodes thereby trapping lymphocytes [4, 5]. To establish if this occurs during ongoing neuroinflammation in response to infection with neurotropic JHMV, draining cervical lymph nodes (dCLN) were examined at defined times p.i. following treatment with FTY720. Administration of FTY720 revealed an increase in size and weights of dCLN in FTY720-treated mice compared to control mice at day 7 p.i. (**Figure 2.5A and B**). Immunophenotyping lymphocytes in the dCLN by flow cytometry revealed an increased frequency of B220+ B cells (**Figure 2.5C**), CD4 + T cells (**Figure 2.5D**) and CD8+ T cells (**Figure 2.5E**) in mice treated with FTY720 compared to control indicating increased retention of lymphocytes in lymphatic tissue in response to S1P antagonism.

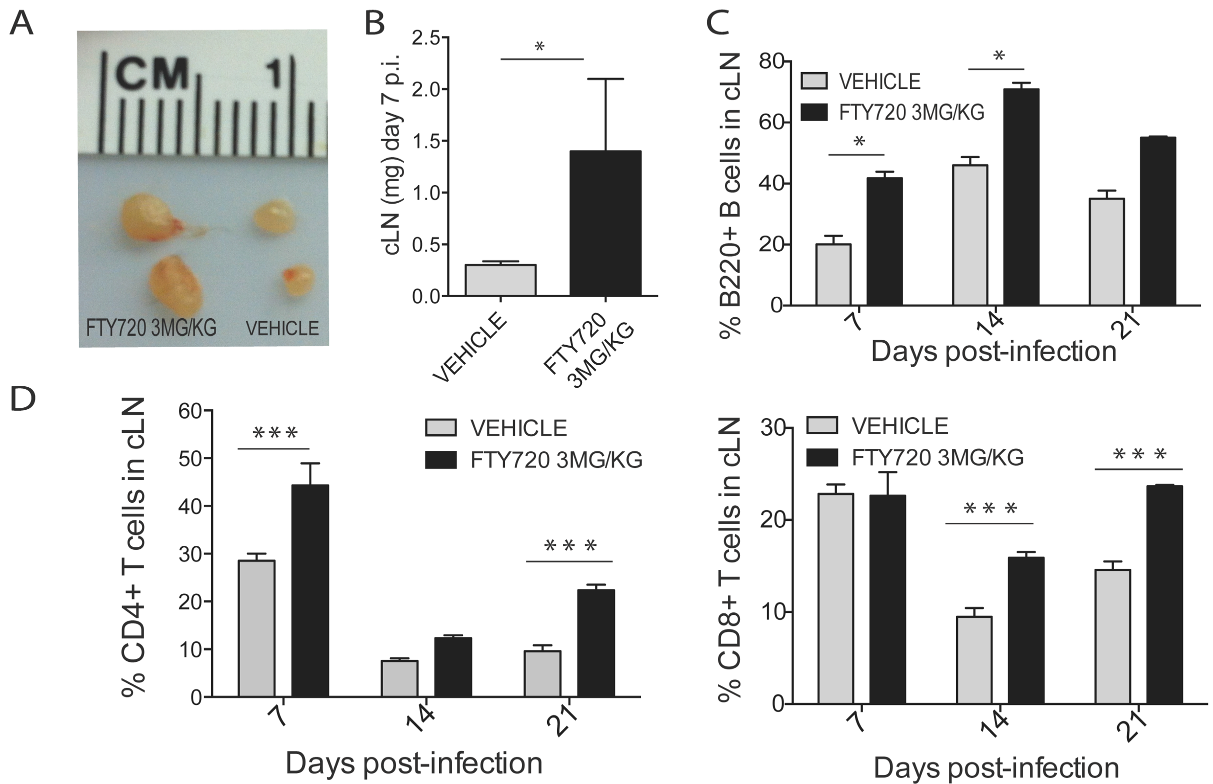
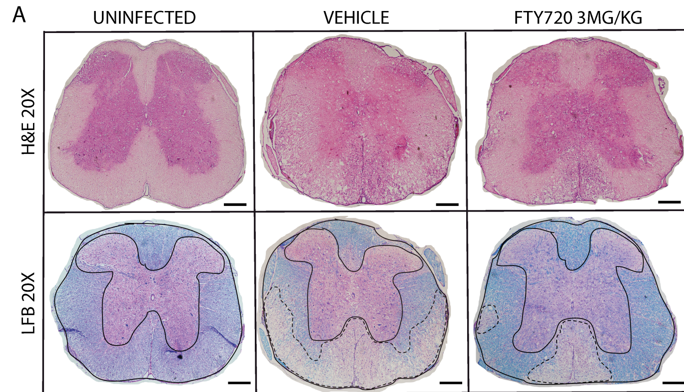


Figure 2.5. Increased lymphocyte retention in dCLN following FTY720 treatment of JHMV-infected mice. S1P1 eGFP mice were i.c. infected with JHMV (150 PFU) and treated daily with FTY720 (3 mg/kg) beginning on day 5 p.i. On days 7, 14, and 21 p.i., dCLNs were isolated to examine size, weight, and immunphenotype lymphocyte populations by flow cytometry. (**A**) Representative image depicting the increase in size of dCLN's obtained from FTY720-treated mice compared to vehicle control treated mouse at day 7 p.i. (**B**) Average weight of dCLNs is increased in response to FTY720 treatment compared to vehicle (p<0.05). Treatment with FTY720 resulted in increased retention of B220+ B cells (**C**), CD4+ T cells **(D)** and CD8+ T cells (**E**) at defined times p.i. Data in panels B-E represent average±SEM obtained from two to three independent experiments with a minimum of 4 mice/group. *P <0.05, *** P <0.001

FTY720 diminishes the severity of demyelination in JHMV-infected mice

To investigate the potential effect of FTY720 on spinal cord neuroinflammation and demyelination in JHMV-infected mice, an evaluation of the severity of white matter damage was performed at days 21 p.i. Spinal cord inflammation was reduced ($p<0.05$) within spinal cords at day 21 p.i. as assessed by H&E staining (**Figure 2.6A and B**). Moreover, Luxol fast blue (LFB) staining revealed a significant ($p<0.05$) reduction in demyelination in response to FTY720 treatment when compared to control mice (**Figure 2.6A and B**). Immunophenotyping of infiltrating lymphocytes in the spinal cord day 21 p.i. revealed a decrease in CD8+ T cell as well as CD4+ T cell percentage ($p <0.05$) in FTY720 treated mice compared to vehicle treated mice (**Figure 2.6C and D**). This suggests that reduction in severity of demyelination following FTY720 treatment is the result of a decrease in infiltration of inflammatory cells into the spinal cord.


A
UNINFECTED
VEHICLE
FTY720 3MG/KG
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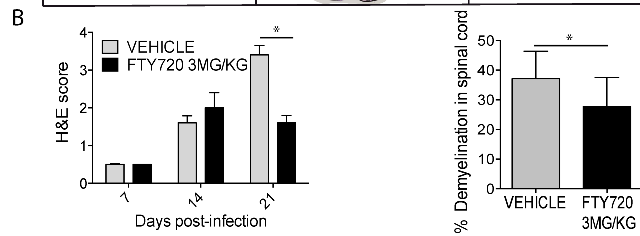
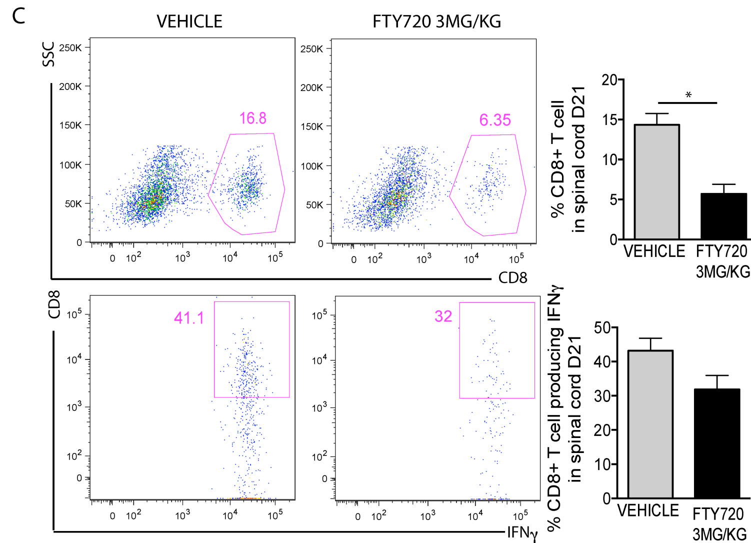
LFB 20X


B
VEHICLE
FTY720 3MG/KG
H&E score
Days post-infection
% Demyelination in spinal cord
VEHICLE
FTY720 3MG/KG


C
VEHICLE
FTY720 3MG/KG
SSC
CD8
16.8
6.35
41.1
32
IFNγ
% CD8+ T cell in spinal cord D21
% CD8+ T cell producing IFNγ in spinal cord D21
VEHICLE
FTY720 3MG/KG

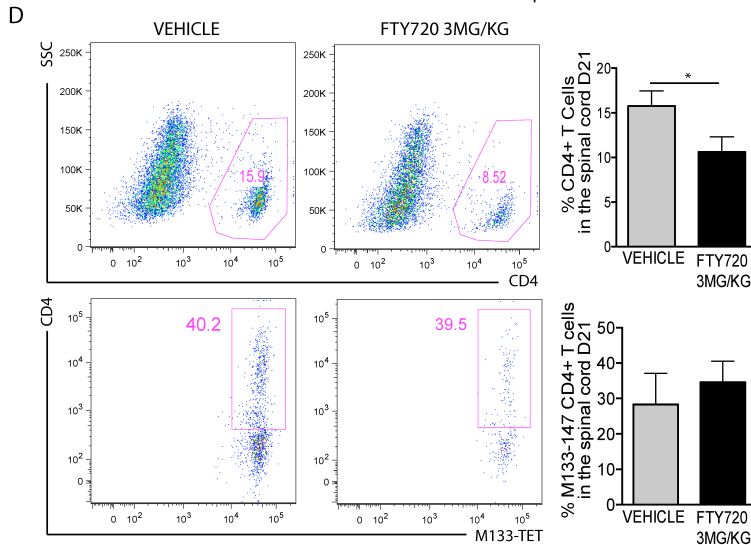

D
VEHICLE
FTY720 3MG/KG
SSC
CD4
15.9
8.52
40.2
39.5
M133-TET
% CD4+ T Cells in the spinal cord D21
% M133-147 CD4+ T cells in the spinal cord D21
VEHICLE
FTY720 3MG/KG

Figure 2.6. FTY720 treatment reduces the severity of JHMV-induced demyelination. (**A**) Representative LFB and H&E -stained spinal cord images showing an overall reduction in the severity of inflammation and demyelination within white matter tracts (hashed lines) in JHMV-infected eGFP S1P1 mice treated with FTY720 (3mg/kg) compared to vehicle control-treated mice at day 21 p.i. (**B**) FTY720 treatment results in reduced neuroinflammation ($p<0.05$) and demyelination ($p<0.05$) when compared to mice treated with vehicle control. Flow analysis of spinal cords demonstrates reduced entry of both CD8+ (**C**) and CD4+ T cells (**D**) while infiltration of virus-specific T cells was not affected. Data in panel B represent two independent experiments with a minimum of 10-mice/ group. Data in panels C and D are presented as average+SEM and represent two independent experiments with a minimum of 5 mice/group. *P <0.05. The scale bar in panel A represents 200µM.

2.4 Discussion

FTY720/Fingolimod was the first oral treatment approved by the FDA for relapsing forms of MS [10, 54, 55]. Numerous clinical trials highlighted FTY720 efficacy as demonstrated by benefits on relapses and MRI lesions [9]. In addition, disability progression was impacted and there was a reduction in brain volume loss in MS patients in response to treatment [56, 57]. Although the mechanisms by which FTY720 exerts a protective effect are not defined, it is generally accepted that the main mode of action is via an immunomodulatory effect by restricting lymphocyte circulation from lymph nodes to the CNS. Dampened accumulation of activated lymphocytes into the CNS in response to FTY720 treatment most likely accounts for reduced MRI lesion activity and this is supported in preclinical animal studies using EAE [14, 15, 58]. Whether the reduction in brain volume loss is also dependent upon reduced neuroinflammation or a direct neuroprotective effect has not been determined. Recent evidence argues that FTY720 exerts a neuroprotective effect as animals in which the receptor S1P1 is selectively ablated on astrocytes are resistant to the protective effects of FTY720 treatment following induction of EAE although S1P1 remains expressed on circulating lymphocytes [12, 59]. However, more recent studies by Cahalan et al. indicate that S1P1 antagonism reverses EAE without acting on S1P1 expressed within the CNS supporting the notion that restricting lymphocyte egress from lymphatic tissue is sufficient to diminish disease severity [60]. Moreover, the maintenance of egress inhibition is not required for efficacy in EAE if brain levels of agonist are maintained in steady state. Full efficacy is achieved with inhibition of egress for only 30% of the 24-hour dosing interval with complete recovery of circulating lymphocytes. Direct effects within CNS were demonstrated on neurons, astrocytes, blood-

brain-barrier and on the inhibition of migration of lymphocytes from perivascular cuffs into the parenchyma [61].

We chose a model of viral-induced neurologic disease to determine if FTY720 treatment affects host defense and disease progression. Our rationale for using the JHMV model of acute encephalomyelitis and demyelination to assess the therapeutic benefit of FTY720 is based on the fact that the overwhelming majority of preclinical animal model examining FTY720 mode of action is derived from EAE yet how this drug affects models of viral induced CNS disease are not well characterized. In addition, viral infections have long been thought to have a role in either initiating or contributing to relapse in MS patients [22-24, 31-34, 62]. Understanding how treatment with FTY720 influences outcomes in response to viral infection is highlighted by recent clinical studies detailing emergence of herpes zoster and associated neurologic complications in MS patients during FTY720 treatment [63, 64]. These findings suggest immunosuppression may arise in response to FTY720 treatment resulting in re-emergence of persistent viruses. However, administration of FTY720 to LCMV-infected mice did not ameliorate persistence, indicating that the outcome may be dictated, in part, by virus and sites of infection [65] Related to these observations are studies demonstrating that treatment with FTY720 or other S1P1 agonists dramatically affects cytokine production and disease outcome in mice infected with influenza virus indicating immunomodulatory effects of such treatment [66]. With regards to viral models of demyelination, Pachner and colleagues [67] demonstrated that administration of FTY720 had no effect on clinical disease progression or viral load within the CNS using the TMEV model of demyelination. These findings are in contrast with

findings using EAE in which FTY720 treatment reduced clinical disease severity accompanied by limited infiltration of immune cells into the CNS [13-15, 68, 69].

Our findings show that FTY720 treatment in JHMV infected mice increased clinical disease severity as well as mortality. Importantly, these findings correlate with impaired ability to control viral replication within the CNS. Muted host defense resulting from S1P receptor antagonism was not the result of dampened anti-viral T cell effector responses *e.g.* proliferation, cytokine secretion or CTL activity, but rather an inability of lymphocytes to migrate and accumulate within the CNS. Indeed, administration of FTY720 increased retention of T and B-lymphocytes within the dCLN consistent with earlier reports that blocking S1P receptors disrupts lymphocyte egress from secondary lymphatic tissue [70, 71]. Although viral titers were elevated within the CNS of FTY720 treated mice, surviving mice were able to reduce viral below the level of detection and this lasted out to day 28 p.i. arguing that viral recrudescence does not occur in this model.

Administration of FTY720 either prophylactically or therapeutically in models of EAE results in reduced lymphocyte penetration into the CNS associated with a reduction in the severity of demyelination [14, 15, 58]. Similarly, our results show that the effects of FTY720 treatment on CNS inflammation in JHMV-infected mice correlates with a reduction in the severity of spinal cord demyelination. Examination of the posterior funiculus and lateral white matter columns of FTY720 treated mice compared to control showed an overall reduction in lesion size demonstrating that in addition to reducing myelin damage in EAE FTY720 is also effective in limiting the spread of demyelination in a viral model of MS. FTY720 has also been shown to prevent axonal damage in EAE [58]. With regards to remyelination, use of FTY720 alone in combination with other drugs in EAE or cerebellar

slice culture has been shown to augment remyelination supporting a regenerative potential [72, 73]. These findings support the notion that FTY720 may act directly upon resident cells of the CNS promoting protection and repair. This is supported by elegant studies from Chun and colleagues [12] that showed attenuation in EAE and a loss of FTY720 efficacy in conditional null mouse mutants lacking S1P1 on astrocytes. These findings highlight that FTY720-mediated protection in EAE occurs via a nonimmunological mechanism and suggest targeting S1P signaling within the CNS may be the relevant targets for recovery in both EAE and MS patients. Whether extensive axonal sparing and/or remyelination is occurring following FTY720 administration to JHMV-infected mice is not known at this time and the area of ongoing work.

Conclusion

In this study we demonstrate that FTY720 mutes effective antiviral immune responses by preventing migration and accumulation of virus-specific T cells within the CNS during acute viral-induced encephalomyelitis. FTY720 treatment reduces the severity of neuroinflammatory-mediated demyelination by limiting T cell egress from lymph nodes thereby reducing lymphocyte infiltration into the CNS. FTY720 did not alter anti-viral effects of T cells *e.g.* cytokine secretion or cytolytic activity.

Abbreviations

CNS: Central nervous system; LDH: Lactate dehydrogenase; CFSE: Carboxyfluorescein succinimidyl ester; dCLN: Draining cervical lymph nodes; LFB: Luxol-fast-blue; IFNγ: Interferon-gamma; TNFα: Tumor necrosis factor alpha; S1P: Sphingosine-1-phosphate; FTY720P: FTY720-phosphate; EAE: Experimental autoimmune encephalomyelitis; PFU: Plaque forming units; MRI: Magnetic resonance imaging; i.p.: Intra-peritoneal; i.c.: Intra-cerebral; p.i.: Post-infection; LCMV: Lymphocytic choriomeningitis; TMEV: Theiler's murine encephalomyelitis virus; ELISA: Enzyme-linked immunosorbent assay; H&E: Hematoxylin and eosin; FBS: Fetal bovine serum; CTL: Cytotoxic T lymphocyte; MHV: mouse hepatitis virus.

Competing interest

CAB and TEL declare they have no competing interests. H.R. is a cofounder and member of the scientific advisory board for Receptos, Inc.

Authors' contributions

CAB designed, conducted, experiments, analyzed and interpreted the data, and wrote the manuscript. TEL designed research, analyzed and interpreted data and wrote the manuscript. HR assisted in data interpretation and wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgments

The authors wish to acknowledge the excellent technical assistance of Edna Hingco and Colleen Worne. This work was supported, in part, by NIH grant R01 NS041249 and generous support from the Hausman Family Foundation and Dawn Beattie.

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CHAPTER THREE

S1P receptor antagonism enhances proliferation of engrafted neural progenitor cells in a model of viral-induced demyelination

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Abstract

The effect of S1P receptor antagonism on the biology of mouse neural progenitor cells (NPCs) following transplantation in a viral model of demyelination was examined. Intracerebral infection with the neurotropic JHM strain of mouse hepatitis virus (JHMV) results in an acute encephalomyelitis followed by demyelination similar in pathology to the human demyelinating disease multiple sclerosis (MS). We have previously reported that intraspinal transplantation of mouse NPCs into JHMV-infected animals resulted in selective colonization of demyelinated lesions, preferential differentiation into oligodendroglia accompanied by axonal preservation, and increased remyelination. Cultured NPCs express transcripts specific for S1P receptors S1P1, S1P2, S1P3, S1P4, and S1P5. Treatment of cultured NPCs with FTY720, an S1P functional antagonist, increased MAP kinase phosphorylation as well as enhanced migration in response to the chemokine CXCL12. Administration of FTY720 to JHMV-infected mice resulted in elevated proliferation of transplanted NPCs following spinal cord engraftment and positional migration as well as accumulation of NPCs into demyelinated white matter tracts was not impaired. Neither differentiation of NPCs into oligodendroglia nor remyelination was affected by FTY720 treatment. Administration of FTY720 to NPC- transplanted mice did not attenuate infiltration of inflammatory CD4+ or CD8+ T cells into the CNS and neither clinical disease or the severity of demyelination was improved. These findings argue that FTY720 treatment selectively increases NPC proliferation yet does not improve either clinical outcome or enhance remyelination following transplantation into animals in which immune-mediated demyelination is initiated by viral infection of the CNS.

3.1 Introduction

Intracranial infection with the neurotropic JHM strain of mouse hepatitis virus (JHMV) results in an acute encephalomyelitis followed by chronic demyelination characterized by viral persistence within the CNS, axonal damage, and demyelination [1-9]. Previous studies from our laboratory have employed the JHMV model of neuroinflammatory-mediated demyelination to evaluate the therapeutic benefit of mouse neural progenitor cell (NPC) engraftment on remyelination of demyelinated axons. Transplantation of mouse NPCs into the spinal cords of JHMV-infected mice results in extensive migration and colonization of areas of white matter damage and preferential differentiation into oligodendroglia [10-12]. Engrafted NPCs physically engage damaged axons and this ultimately leads to increased axonal integrity that correlates with remyelination [13]. These findings, along with others [10, 11, 14, 15] argue that engraftment of NPCs may provide an important unmet clinical need for treatment of the human demyelinating diseases including multiple sclerosis (MS) by facilitating sustained remyelination that can restore motor function and ameliorate clinical symptoms.

Following engraftment of NPCs into the spinal cords of JHMV-infected mice, transplanted cells migrate both rostral and caudal to the implantation site [10]. The chemokine ligand CXCL12 is enriched within areas of demyelination and transplanted NPCs express the signaling receptor CXCR4 resulting in colonization of areas of white matter damage; blocking CXCR4 signaling upon NPC transplantation impaired NPC migration arguing for an important role for this chemokine signaling pathway in contributing to repair by mediating trafficking to sites of myelin damage [11]. However, the molecular mechanisms governing the positional migration of NPCs are likely complex and consist of

additional soluble factors that impact the ability of NPCs to effectively congregate within areas of white matter pathology.

Among potential molecules that may influence migration is the lysophospholipid sphingosine-1-phosphate (S1P) that is well documented in controlling proliferation and migration of numerous cell types [16-19]. Although the importance of S1P signaling in controlling lymphocyte homing and egress from lymphatic tissues is well documented [20-22], increasing evidence indicates a functional role within the CNS as glia and neurons express different combinations of specific signaling receptors S1P1, S1P2, S1P3, S1P4, and S1P5 [23, 24]. Activation of these receptors yields different effects on migration and survival of astrocytes, microglia, and oligodendrocytes [25-27]. In addition, NPCs express S1P receptors and signaling has previously been reported to influence *in vitro* differentiation [28]. Moreover, Kimura and colleagues [29] demonstrated an important role for S1P signaling in controlling migration of transplanted NPCs to injury site in a model of spinal cord injury.

The present study examines the functional role of S1P signaling following NPC transplantation into the spinal cords of JHMV-infected mice. Our findings reveal that treatment of cultured NPCs with FTY720, an antagonist for receptors S1P1, S1P3, S1P4, and S1P5 leads to an active signaling response as determined by phosphorylation of MAP-kinase and increased proliferation yet does not influence lineage fate commitment. FTY720 treatment of JHMV-infected mice transplanted with NPCs revealed increased numbers of NPCs compared to vehicle-treated control animals. FTY720 treatment did not affect accumulation of T cells or macrophages within the CNS. Our findings reveal that administration of FTY720 does not augment the effects of NPCs on influencing either the

severity of demyelination or remyelination but enhances proliferation upon engraftment in JHMV-infected mice.

3.2 Materials and Methods

Mice and Virus

Age-matched (5 to 7 weeks) S1P1 eGFP knock-in mice (C57BL/6 background) [30] and C57BL/6 mice were anesthetized with an intraperitoneal (i.p.) injection of 150 μl of a mixture of ketamine (Western Medical Supply, Arcadia, CA, USA) and xylazine (Phoenix Pharmaceutical, Saint Joseph, MO, USA) in Hank's balanced salt solution. Mice were injected intracranially (i.c.) with 150 plaque forming units (PFU) of JHMV (strain V2.2-1) suspended in 30 μl saline [11]. Clinical severity was assessed using a previously described four-point scoring scale [31]. FTY720 (2-amino-2- [2-(4-octylphenyl) ethyl]-1,3-propanediol, hydrochloride) and FTY720P (2-amino-2 [2-(4-octylphenyl) ethyl]-1,3-propanediol, mono dihydrogen phosphate ester) were purchased from Cayman Chemical Co (Ann Arbor, MI, USA). Administration of FTY720 or the vehicle was performed by daily i.p. injections of 100 μl starting at day 13 post-infection (p.i.). Experiments for all animal studies were reviewed and approved by the University of Utah and the University of California, Irvine Institutional Animal Care and Use Committee's.

Neural progenitor cell (NPC) isolation and culture

Neurosphere cultures were prepared from *eGFP*-S1P1 knock in mice as previously described from the brains of perinatal animals [10, 32]. Briefly, dissected striata were razor minced and triturated in 0.05% Trypsin for 10 min followed by anti-trypsin to inactivate the digestion. Single cells were resuspended in DMEM: F12 (Invitrogen) supplemented with B27 (Invitrogen), Insulin–Transferrin–Selenium–X (Invitrogen), Penicillin–Streptomycin (Invitrogen), 40 ng/ml T3 (Sigma-T67407), and 20 ng/ml human

recombinant EGF (Sigma-E9644). Cells were cultured for 6 days with replacement of media every other day; mature neurospheres were then isolated and visualized for GFP expression.

PCR

Total RNA was extracted from neurospheres, DNase treated, and purified via phenol-chloroform extraction. cDNA was synthesized with a reverse transcription kit superscript VILO (Invitrogen) according to the manufacturer's instructions. The following primers were used to identify S1P1-S1P5 mRNA expression by neurospheres: S1P1 F-TTTCCATCGCCATCCTCTAC, S1P1 R-GCAGGCAATGAAGACACTCA; S1P2 F-TCTCAGGGCATGTCACTCTG, S1P2 R-CAGCTTTTGTCACTGCCGTA; S1P3 F-GTGTGTTCATTGCCTGTTGG, S1P3 R-TTGACTAGACAGCCGCACAC; S1P4 F-GGCTACTGGCAGCTATCCTG, S1P4 R-AAGGCCACCAAGATCATCAG; S1P5 F-GATCCCTTCCTGGGTCTAGC, S1P5R-TAGAGCTGCGATCCAAGGTT. Primers were purchased from Invitrogen. Sequencing of PCR amplicons confirmed primer specificity.

Western blot

Neural progenitor cells were plated on matrigel coated 6-well plates and treated with either FTY720 phosphate 100nM or vehicle for 5 min, 30 min, 1h, 2h and 4h. The cells were then lysed using RIPA buffer (50mM Tris-HCL pH 7.4, 175 mM NaCl, 5mM EDTA, 1% NP-40, 0.1% SDS, 0.5% DOC) supplemented in protease and phosphatase inhibitors (Roche). P44/42 Map kinase (1:2,000; Cell Signaling) and phosphorylated p44/42 map kinase (1:15,000; Cell Signaling) were detected by western blot using HRP conjugated secondary

antibodies (1:25,000; Jackson ImmunoResearch Laboratory, West Grove, PA) were used for detection and exposed to Supersignal Wet-Femto chemiluminescent reagent (Pierce, Rockford, IL).

NPC differentiation

To assess differentiation potential, NPCs expressing GFP [11, 13] were grown on matrigel coated chamber slides with epidermal growth factor EGF for 24 hours. Growth factor was then removed and NPCs were allowed to differentiate for up to 7 days. Cells were treated daily with FTY720 phosphate 100nM or vehicle. Cells were then fixed in 4% paraformaldehyde for 20min at room temperature. Immunofluorescence was performed using established protocols: rabbit anti-mouse Olig 2 (1:200; Millipore), rabbit anti-mouse GFAP (1:1,000; Invitrogen), mouse anti-mouse Map2 (1:750; Sigma), rabbit anti-mouse Ki67 (1:300; Abcam); Alexa-594 anti-rabbit (1:500: Invitrogen) or anti-mouse (1:1,000: Invitrogen) was used as secondary antibodies. Samples were then washed in PBS and coverslip was mounted with DAPI Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA). The percent of immunopositive cells for each stain was determined by dividing the total number of immunopositive cells by the total number of DAPI positive cells.

NPC transplantation

Transplantation was performed on day 14 p.i. with JHMV where mice develop demyelination [2, 33]. Anaesthetized mice with an intra-peritoneal (i.p.) injection of 150 µl of a mixture of ketamine (Western Medical Supply, Arcadia, CA, USA) and xylazine (Phoenix

Pharmaceutical, Saint Joseph, MO, USA) in Hank's balanced salt solution received a laminectomy at T9 and 250,000 NPCs resuspended in 2.5ul solution were injected into the spinal cord using a 10ul Hamilton syringe as previously described [10, 34-36]. Recipient mice also received daily i.p. injections of FTY720 3mg/kg or vehicle starting at day 13 p.i.

Chemotaxis

In vitro chemotaxis assays were performed using Neuro Probe ChemoTx system according to the manufacturer's protocol. Briefly, GFP-NPCs treated with FTY720 100nM, 10nM and 1uM or vehicle were allowed to migrate in response to recombinant mouse CXCL2 (Peprotech). Migration following 16h of culture was assessed by a fluorescent microplate reader according to manufacturer specifications.

Histology

Spinal cords were isolated at defined time points and fixed overnight with 4% paraformaldehyde at 4°C. Individual spinal cords were sectioned and twelve 1mm coronal sections, cryoprotected in 20% sucrose and embedded in (O.C.T) (VWR, Radnor, PA, USA) [46]. Eight-μm-thick coronal sections were cut and sections were stained with luxol fast blue (LFB). Areas of total white matter and demyelinated white matter were determined with Image J Software [37]. Demyelination was scored as a percentage of total demyelination along the entire length of the spinal cord. The total numbers of GFP-positive cells was determined in each of the twelve spinal cord sections surrounding the transplant site by counting GFP-positive cells co-localized with DAPI-positive nuclei.

EM

Mice were perfused with 0.1 M cacodylate buffer containing 2% paraformaldehyde/2% glutaraldehyde and spinal cords were isolated, embedded in Epon epoxy resin. Serial ultrathin sections were stained with uranyl acetate-lead citrate and analyzed as previously described. Images at 1,200× magnification were analyzed for *g*-ratio using ImageJ software [38]. A minimum of 150 axons was analyzed per mice (n=3) and the *g*-ratio was calculated by diving the axon diameter by the total fiber diameter.

Immunofluorescence

Immunophenotyping of glial cells within spinal cord sections was performed by blocking fixed samples in 10% normal goat serum (NGS, Jackson ImmunoResearch, West Grove, PA) and 1% bovine-serum-albumin (BSA) for one hour at room temperature. This was followed by an overnight incubation at 4°C with the following primary antibodies in 1% BSA: rabbit anti-mouse GST-π (1:1,000; MBL, International corporation), rabbit anti-mouse GFAP (1:1,000; Invitrogen), and rabbit anti-mouse Ki67 (Abcam). Samples were then washed in PBS and incubated for one hour at room temperature with Alexa fluorescent-conjugated secondary antibodies (goat anti-rabbit Alexa 594 or goat anti-mouse Alexa 594; 1:1000 in PBS, Invitrogen). Samples were then washed in PBS and cover slip mounted using DAPI Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA). The percent of immunopositive cells for each stain was determined by dividing the total number of immunopositive cells by the total number of DAPI positive cells.

Cell isolation and Flow Cytometry

Brain, spinals cords and blood were isolated day 21 or 28 p.i. from infected mice treated with FTY720 (3mg/kg) or vehicle starting at day 13 p.i. and transplanted with GFP-labeled NPCs. Using previously described protocols [37], tissues were then homogenized and immunophenotyped with the following antibodies: rat-anti mouse CD4-APC (1:50: Biolegend), rat-anti mouse CD8-APC (1:50; Biolegend), S510-518 tetramer-PE (1:300; NIH), M133-147 tetramer-PE (1:150; NIH). Blood was collected by cardiac heart puncture and cells were stained with rat-anti mouse CD4-APC and CD8-PE following red blood cell lysis. Samples were analyzed using a BD-Fortessa Flow Cytometer.

3.3 Results

FTY720 treatment activates cultured NPCs

FTY720 targets members of the S1P receptor family whose natural ligand is S1P. Previous studies have demonstrated that FTY720 preferentially binds S1P1, S1P4, and S1P5 and with lower affinity for S1P3 but does not bind to S1P2 [20]. We tested whether mouse NPCs expressed S1P receptors and if FTY720 treatment affected defined responses. Neurospheres were isolated from the sub-ventricular zone of day 1 old eGFP-S1P1 knock-in mice [10, 32] and immunofluorescence confirmed that NPCs express S1P1 as evidenced by GFP expression (**Figure 3.1A**). Subsequent analysis of additional S1P receptor expression by NPCs at the mRNA level demonstrated expression of transcripts specific for all five defined S1P receptors (**Figure 3.1B**). Previous studies have demonstrated that FTY720 treatment activates a number of intracellular signaling cascades including phosphorylation of MAP kinase [39, 40]. Treatment of cultured NPCs with the activated phosphorylated form of FTY720 (FTY720-P, 100 nM) resulted in phosphorylation of MAP kinase in a time-dependent manner indicating receptor binding and activation (**Figures 3.1C and D**). These findings support earlier studies [16, 41] demonstrating S1P receptor expression by NPCs and indicates that FTY720 treatment initiates activation of intracellular signaling pathways.

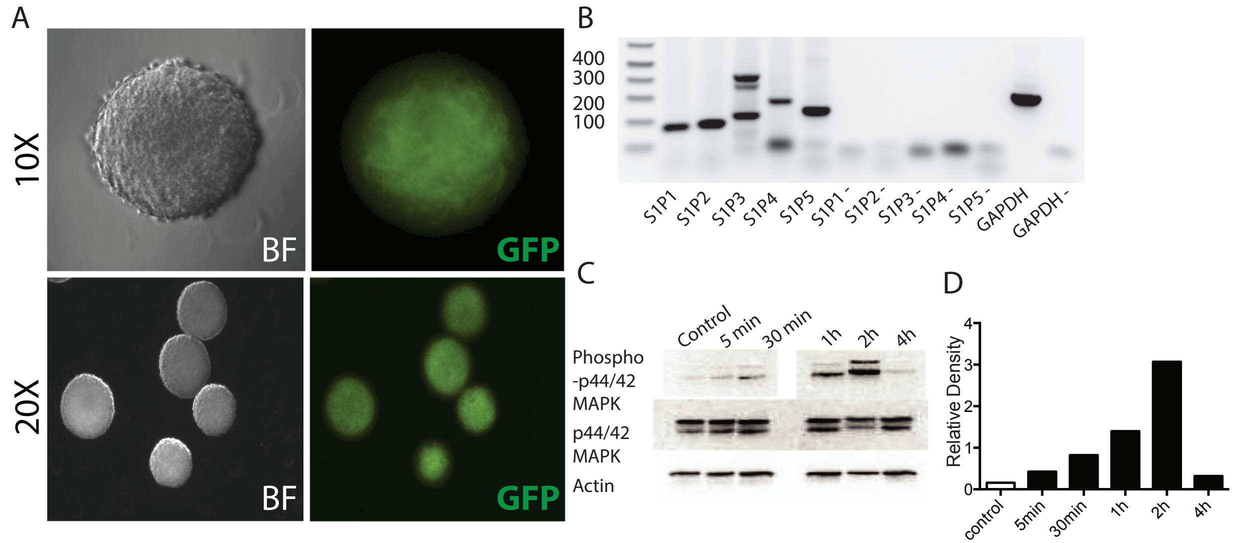
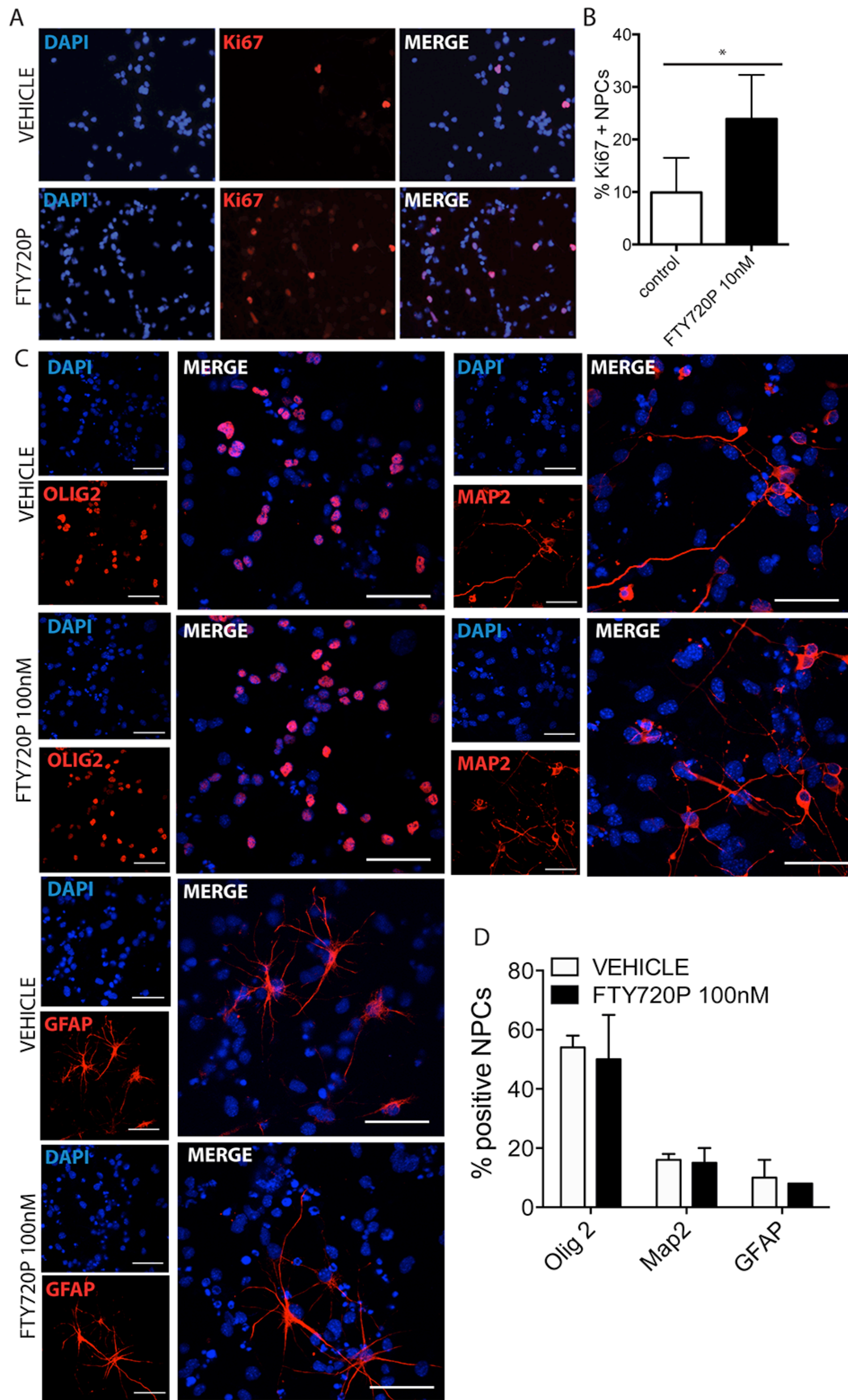


Figure 3.1. FTY720 treatment activates cultured NPCs. Neurospheres were isolated from the subventricular zone (SVZ) of S1P1 eGFP neonatal pups. **(A)** Representative immunofluorescence images confirmed that neurospheres express S1P1 as evidenced by GFP expression. **(B)** Analysis of S1P receptor expression by NPCs at the mRNA level demonstrated expression of transcripts specific for S1P1-S1P5; sequence of amplicons confirmed primer specificity. **(C-D)** Treatment of cultured NPCs with FTY720P (100 nM) resulted in phosphorylation of MAP kinase in a time-dependent manner indicating receptor binding and activation as determined by western blotting.

FTY720 promotes NPC proliferation but does not affect differentiation

We next tested whether exposure of cultured NPCs to FTY720 influenced proliferation as recent studies have implicated S1P receptor antagonism in enhancing glia proliferation [16]. Treatment of NPC cultures with FTY720-P (10 nM) for 48 h resulted in increased proliferation ($p<0.05$) as determined by Ki67 staining when compared to vehicle control-treated cultures (**Figures 3.2A and B**). Under defined conditions, cultured NPCs will preferentially differentiate into oligodendroglia although astrocytes and neurons are detected [10, 11]. Exposure of FTY720-P (100 nM) to NPCs did not influence lineage fate commitment as we detected similar frequencies of oligodendroglia (Olig2-positive), astrocytes (GFAP), and neurons (Map2) when compared to vehicle-control treated cultures (**Figures 3.2C and D**). Therefore, S1P receptor antagonism enhances NPC proliferation yet does not affect neural differentiation.


A
B
DAPI
Ki67
MERGE
VEHICLE
FTY720P
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control
FTY720P 10nM
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MAP2
FTY720P 100nM
GFAP
D
VEHICLE
FTY720P 100nM
% positive NPCs
Olig 2
Map2
GFAP

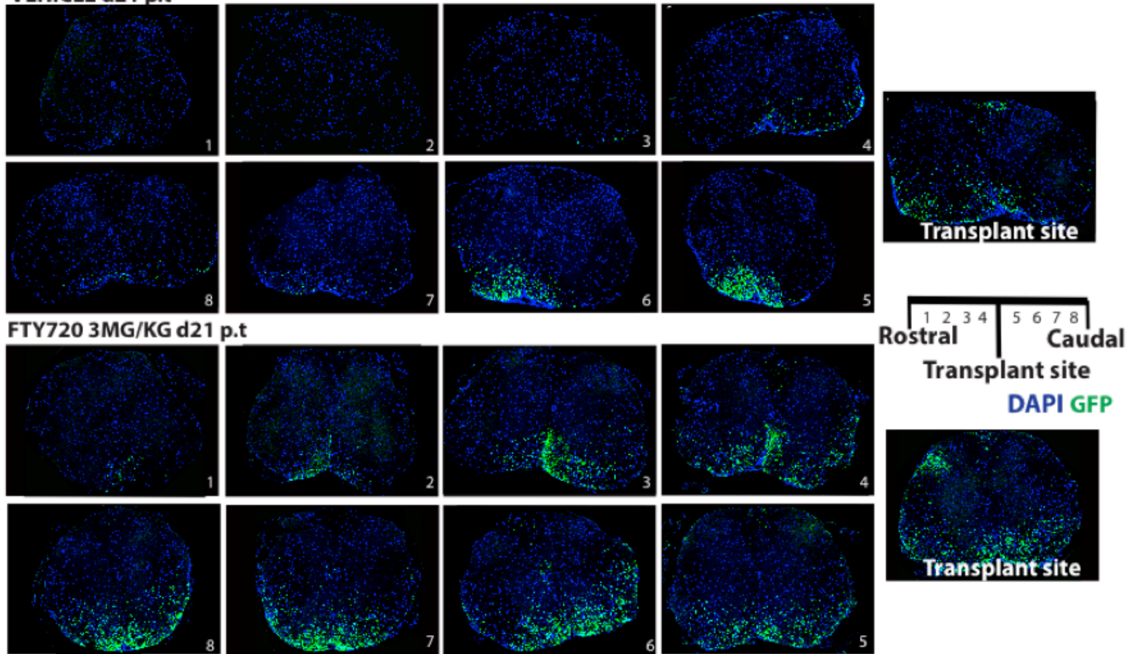
Figure 3.2. FTY720 promotes NPC proliferation but does not affect differentiation. Treatment of NPC cultures with FTY720-P (10 nM) for 48 h resulted in increased proliferation as determined by **(A)** Ki67 staining when compared to vehicle control-treated cultures. **(B)** Quantification of Ki67 staining confirmed FTY720-mediated enhancement of NPC proliferation. Results were derived from 2 independent experiments with cell counts obtained using 5 images per chamber well/group; data is presented as average$\pm$SEM **(C-D)** Exposure of FTY720-P (100 nM) to cultured NPCs did not influence lineage fate commitment as similar frequencies of oligodendroglia (Olig2), astrocytes (GFAP), and neurons (Map2) were detected when compared to vehicle-control treated cultures. Data were derived from three independent experiments and presented as average$\pm$SEM; * $p<0.05$.

FTY720 treatment enhances migration of engrafted GFP-NPCs

To test if FTY720 affects migration of engrafted NPCs, FTY720 treatment [3mg/kg, daily via intraperitoneal (i.p.) injection] was initiated at day 13 post-infection (p.i.) with JHMV and GFP-expressing NPCs (GFP-NPCs) were transplanted into the spinal cords at day 14 p.i. [11,13]. Control groups consisted of JHMV-infected mice transplanted with GFP-NPCs and treated with vehicle only as well as JHMV-infected mice treated with vehicle. Experimental mice were sacrificed at 3 weeks post-transplant (p.t.) and spinal cords were removed to assess migration of GFP-NPCs. In both FTY720 and vehicle-treated control mice, transplantation of GFP-NPCs resulted in extensive migration both rostral and caudal from the implantation site and colonization of areas of demyelination within the ventral funiculus and lateral white matter columns (**Figures 3.3A and B**). Quantification of GFP-NPCs in defined spinal cord sections rostral and caudal to the implantation site indicated a significant ($p<0.05$) increase in numbers of GFP-NPCs in FTY720-treated animals when compared to vehicle-treated mice (**Figure 3.3C**). Furthermore, increased proliferation in the presence of FTY720 was further confirmed by Ki67 staining of transplanted GFP-NPCs throughout the spinal cord at day 14 p.t. (**Figure 3.3D and E**).

These findings advocate for a role in S1P receptor antagonism in controlling either migration and/or proliferation of NPCs engrafted into the spinal cords. We have previously shown that CXCL12 is critical in controlling the positional migration of engrafted NPCs by signaling through CXCR4 expressed upon the surface of NPCs [11]. Further, Kimura et al. [42] have reported that FTY720 treatment promotes migration of human CD34+ hematopoietic progenitor cells by enhancing CXCR4 function on these cells. Cultured NPCs were treated with increasing concentrations of FTY720-P (10 nM, 100 nM, and 1mM) and

in vitro migration in response to recombinant mouse CXCL12 (200ng/ml) resulted in a dose-dependent migration response (**Figure 3.4A**). Flow analysis revealed that FTY720 treatment did not affect surface expression of CXCR4 at any concentration tested (**Figures 3.4B and C**). Therefore, administration of FTY720 enhances migration of NPCs potentially by enhancing CXCR4 function consistent with Kimura et al. [42].


A VEHICLE d21 p.t
Transplant site
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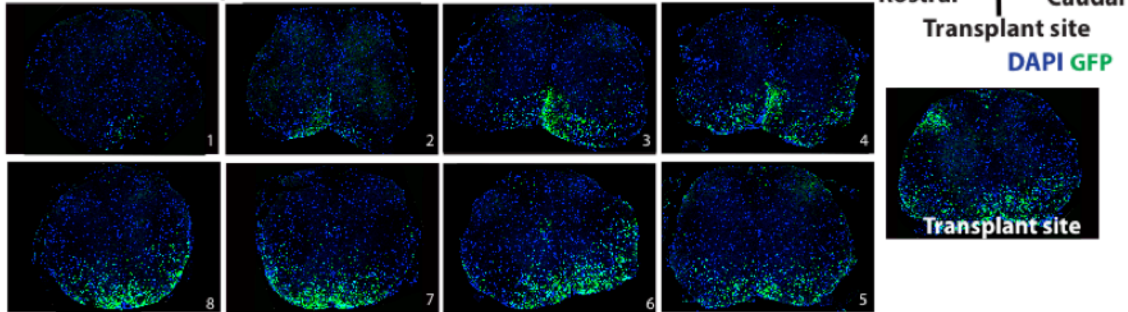
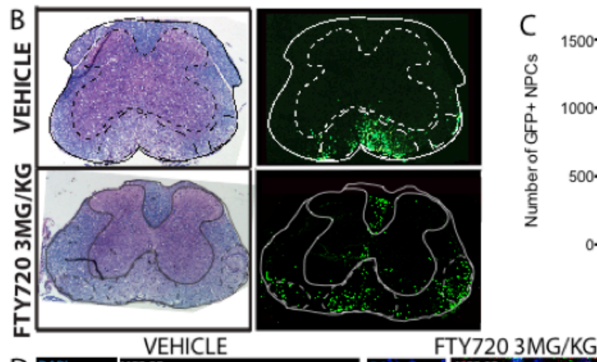
Transplant site
DAPI GFP
FTY720 3MG/KG d21 p.t
Transplant site

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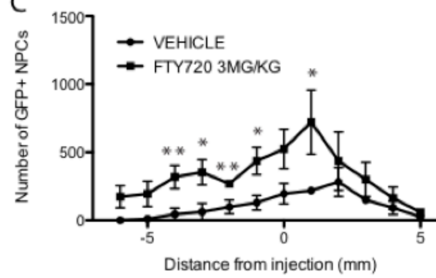
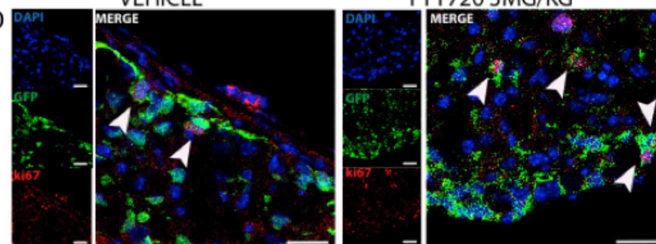

C
VEHICLE
FTY720 3MG/KG
Number of GFP+ NPCs
Distance from injection (mm)


D
VEHICLE
FTY720 3MG/KG
DAPI
GFP
ki67
MERGE

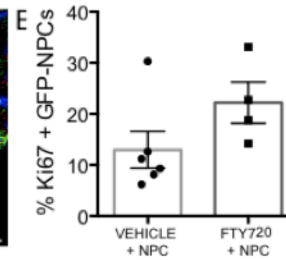

E
% Ki67 + GFP-NPCs
VEHICLE + NPC
FTY720 + NPC

Figure 3.3. FTY720 treatment enhances migration of engrafted GFP-NPCs. JHMV-infected mice were treated with FTY720 treatment [3mg/kg, daily via intraperitoneal (i.p.) injection] or vehicle control beginning at day 13 p.i.; GFP-expressing NPCs (GFP-NPCs) were transplanted into the spinal cords at day 14 p.i. and migration of transplanted cells rostral and caudal to the implantation site was assessed 3 weeks p.t. **(A)** In control and FTY720-treated mice, transplanted GFP-NPCs migrated both rostral and caudal from the implantation site and colonization of areas of demyelination **(B)** within the ventral funiculus and lateral white matter columns. **(C)** Quantification of GFP-NPC cell numbers at defined spinal cord sections rostral and caudal to the implantation site in vehicle control and FTY720-treated animals. **(D)** Representative images depicting Ki67 staining by transplanted GFP-NPCs in vehicle control and FTY720-treated mice. **(E)** Quantification of GFP-NPCs expressing Ki67. FTY720 treatment resulted in a significant (*$p<0.05$) increase in dual-positive cells as compared to vehicle control animals. Results represent a minimum of 2 independent experiments with a minimum 4 mice/group; data is presented as average$\pm$SEM.

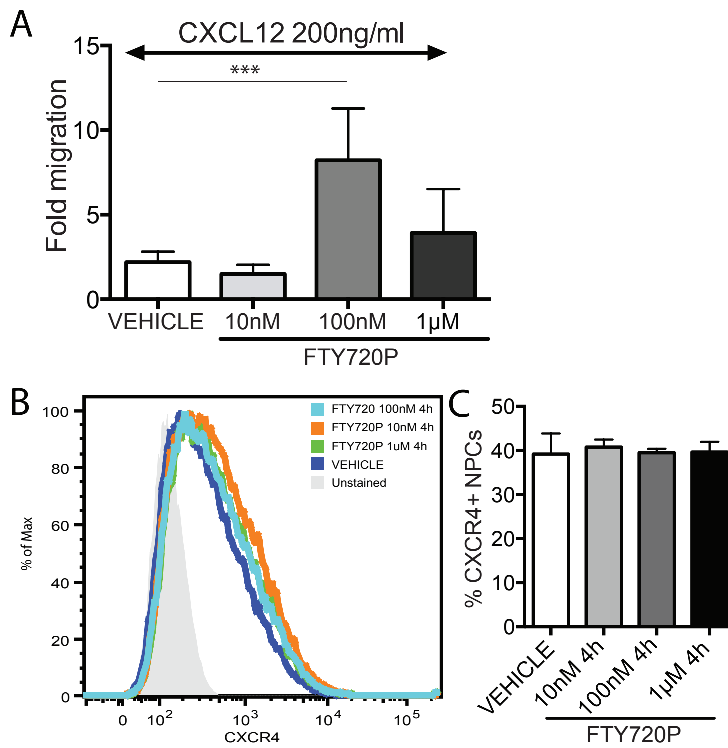
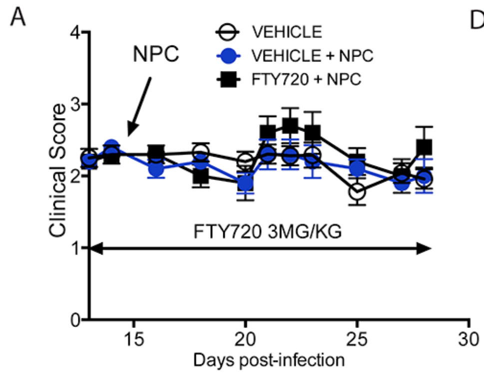
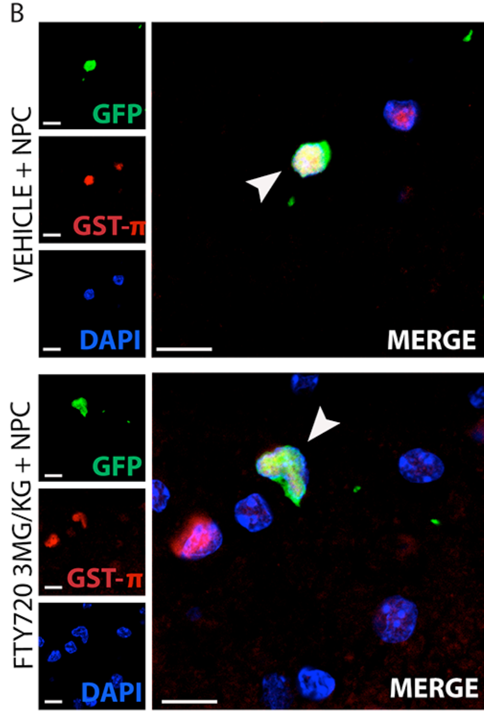


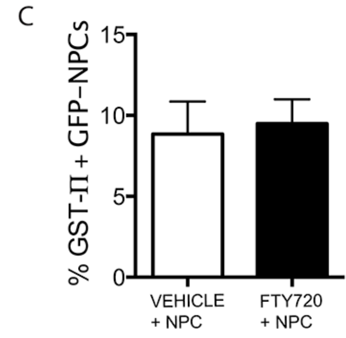
Figure 3.4. FTY720 does not affect CXCR4 expression by NPCs. (A) Cultured NPCs were treated with increasing concentrations of FTY720-P (10 nM, 100 nM, and 1μM) and *in vitro* migration in response to recombinant mouse CXCL12 (200ng/ml) resulted in a dose-dependent migration response Representative flow analysis **(B)** revealed that FTY720 treatment did not affect surface expression of CXCR4 by NPCs at defined concentration and this was confirmed by quantification of MFI of CXCR4 expression **(C)** of cultured cells under experimental conditions. Data in pane A is representative of two independent experiments and presented as average±SEM; *** $p<0.001$. Data in panel B represents three independent experiments and presented as average±SEM.

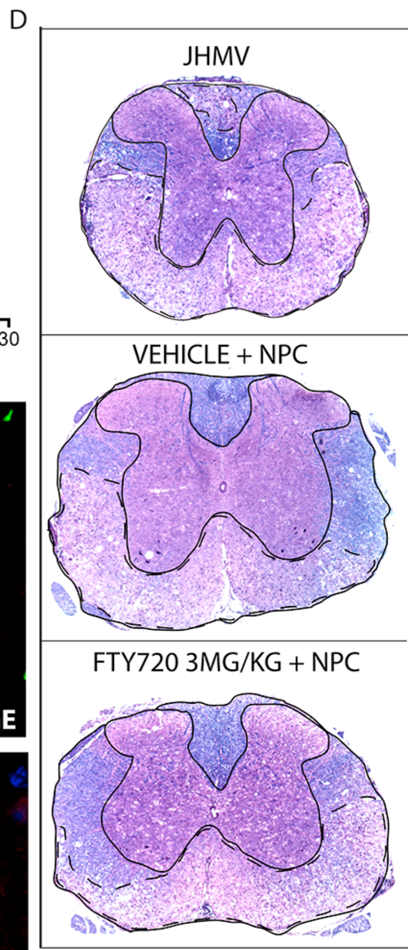
FTY720 effects on NPCs following transplantation into JHMV infected mice

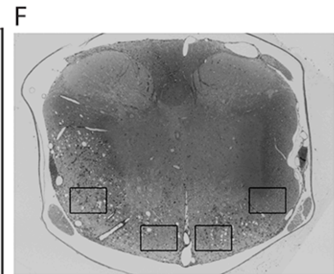
Clinical scoring of mice infected with JHMV and transplanted with GFP-NPCs at day 14 p.i. and treated daily with FTY720 or vehicle starting at day 13 p.i. was investigated. JHMV-infected mice treated with vehicle and not transplanted with NPC served as an additional control. There were no differences in clinical disease observed in any experimental groups (**Figure 3.5A**). We next examined if FTY720 treatment influenced the ability of engrafted NPCs to differentiate into oligodendroglia as our previous studies have shown that the majority of transplanted cells preferentially differentiate into these cells (OPCs) [10, 11]. By 3 weeks p.t. FTY720 did not affect lineage fate commitment of NPCs as similar frequencies of GST-π positive cells (a marker for mature myelin-producing oligodendrocytes) were observed in FTY720 or vehicle treated mice (**Figures 3.5B and C**). The severity of spinal cord demyelination in transplanted mice treated with FTY720 was examined by staining serial coronal sections emanating rostral and caudal to the implantation site with luxol fast blue (LFB) and quantifying the percentage of white matter damage [37, 38]. By day 14 p.t., the severity of demyelination was similar in transplanted mice treated with FTY720 when compared to control animals (**Figures 3.5D and E**). Remyelination was also investigated by EM analysis of spinal cord with assessment of *g*-ratio that represents the ratio of the inner axonal diameter to the total outer fiber diameter with a higher ratio signifying less myelination. Regions of spinal cord ventral and lateral white matter tracts of NPC transplanted mice treated with FTY720, transplanted mice treated with vehicle or mice treated with vehicle alone were analyzed (**Figure 3.5F**). Analysis showed no difference in remyelination in any experimental groups of mice (**Figures 3.5G and H**).

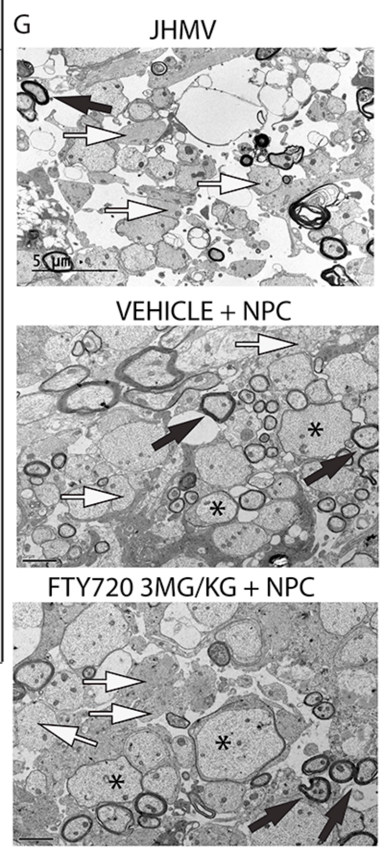

A
NPC
VEHICLE
VEHICLE + NPC
FTY720 + NPC
Clinical Score
FTY720 3MG/KG
Days post-infection

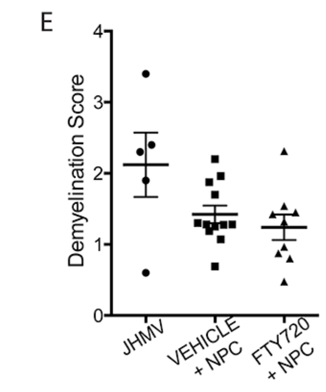

B
VEHICLE + NPC
GFP
GST-π
DAPI
MERGE
FTY720 3MG/KG + NPC
GFP
GST-π
DAPI
MERGE


C
% GST-Π + GFP-NPCs
VEHICLE + NPC
FTY720 + NPC


D
JHMV
VEHICLE + NPC
FTY720 3MG/KG + NPC


F


G
JHMV
5 μm
VEHICLE + NPC
FTY720 3MG/KG + NPC


E
Demyelination Score
JHMV
VEHICLE + NPC
FTY720 + NPC

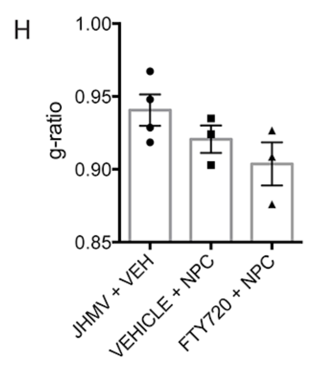

H
g-ratio
JHMV + VEH
VEHICLE + NPC
FTY720 + NPC

Figure 3.5. FTY720 does not decrease the severity of demyelination nor affect remyelination. **(A)** Mice were infected with 150PFU of JHMV and transplanted with GFP-NPCs at day 14 p.i. and treated with either FTY720 (3mg/kg) or vehicle control starting at day 13 p.i. In addition, JHMV-infected mice treated with vehicle alone served as an additional control. FTY720 did not influence the severity of clinical disease out to 21 days p.t.; data is derived from 3 independent experiments with a minimum 4 mice/experimental group **(B)** Representative immunofluorescent GST-π stained spinal cords isolated at day 14 p.t from JHMV -infected mice, engrafted with GFP-NPCs at day 14 p.i. and treated with either FTY720 or control at day 13 p.i. Scale bar represents 50 μm. **(C)** Frequencies of GFP+ mature oligodendrocytes in GFP-NPC transplanted mice treated with either FTY720 or vehicle; results represent 2 independent experiments with a minimum of four mice per experimental group and twelve spinal cord sections per mouse were counted to determine the frequency of transplanted GFP-NPCs that differentiated into GST- π positive cells. **(D)** Representative LFB stained spinal cord sections from NPC- transplanted mice treated with either FTY720, control vehicle or non-transplanted mice treated with control vehicle at day 14 p.t.. **(E)** Quantification of demyelination indicated no differences in the severity of white matter damage in experimental groups of mice. Data represents 2 experiments with a minimum of 5 mice per experimental group. By day 14 p.t., the severity of demyelination was similar in transplanted mice treated with FTY720 when compared to control. **(F)** Representative transverse spinal cord section; boxed areas indicates the regions of remyelination analysis. **(G)** Representative EM images (1200X) of spinal cords of mice transplanted with NPC and treated with FTY720 or vehicle. Black arrow = myelinated axon; white arrow = demyelinated axon; asterix = remyelinated axon; scale bar represents 2μm **(H)** EM analysis of spinal cords of mice transplanted with NPC and treated with FTY720 or vehicle. EM analysis represents 1 experiments with n=3 per group. A minimum of 150 axons per mouse was analyzed. Data in panel C, E and H is presented as average±SEM.

Treatment with FTY720 does not impact neuroinflammation in transplanted mice treated with FTY720

We have previously determined that FTY720 treatment of JHMV-infected mice during acute disease results in increased mortality and limited infiltration of T cells into the CNS that correlated with impaired ability to control viral replication within the CNS ([37] **Chapter 2**). We next examined whether S1P receptor antagonism affected T cell infiltration into the CNS of experimental mice. Flow analysis indicated no differences in infiltration of either total CD4+ or CD8+ T cells or virus-specific CD4+ or CD8+ T cells in either the brain (**Figures 3.6 A and C**) or spinal cord (**Figures 3.6 B and D**). We confirmed biologic activity of FTY720 during chronic disease by examining levels of circulating T cells within the blood. FTY720 significantly ($p<0.05$) diminished the frequency of both CD4+ (**Figures 3.7A**) and CD8+ (**Figures 3.7C**) T cells within the blood as compared to control mice. In addition, using S1P1 eGFP knock-in mice we determined that surface expression of S1P1, measured by eGFP expression, was also decreased ($p<0.05$) on circulating CD4+ (**Figures 3.7B**) and CD8+ T cells (**Figures 3.7D**) in FTY720-treated mice when compared to control animals. Therefore, FTY720 treatment of JHMV-infected mice functions as we have previously shown ([37] **Chapter 2**) as expected by diminishing levels of circulating lymphocytes that correlates with decreased S1P receptor expression. These results indicate that FTY720 does not affect T cell migration into the CNS during chronic disease in animals transplanted with NPCs.

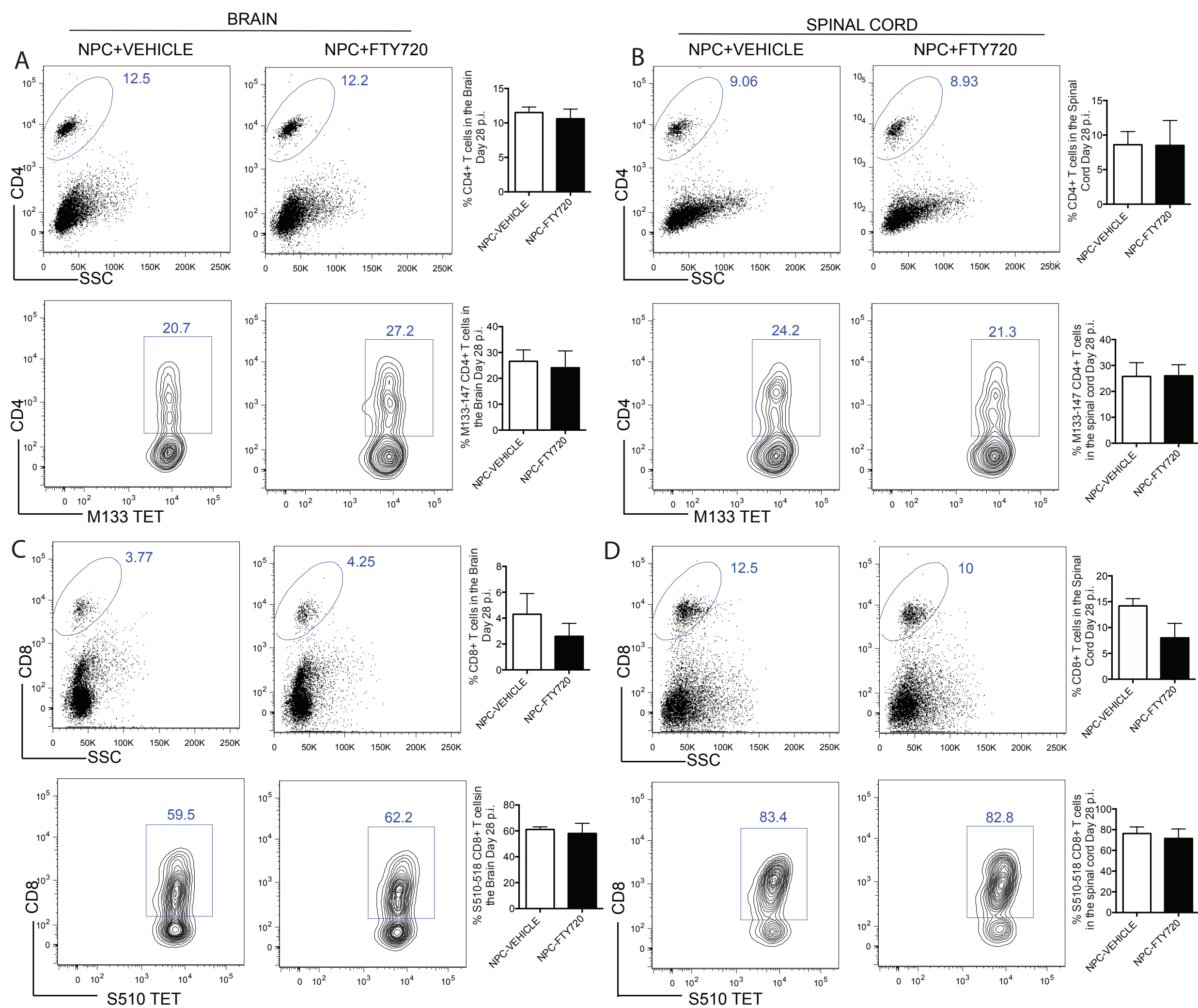


Figure 3.6 Treatment with FTY720 does not impact neuroinflammation in transplanted mice treated with FTY720. Flow analysis of spinal cords at 14 days p.t. revealed no significant differences in frequencies of either total and virus specific CD4+ T cell in **A)** brain **B)** spinal cords; total and virus specific CD8+ T cell in **C)** brain **D)** spinal cords in NPC transplanted mice treated with FTY720 compared to NPC-transplanted mice treated with vehicle alone. Results represents a minimum of 1 experiment with a minimum n=4 per group.

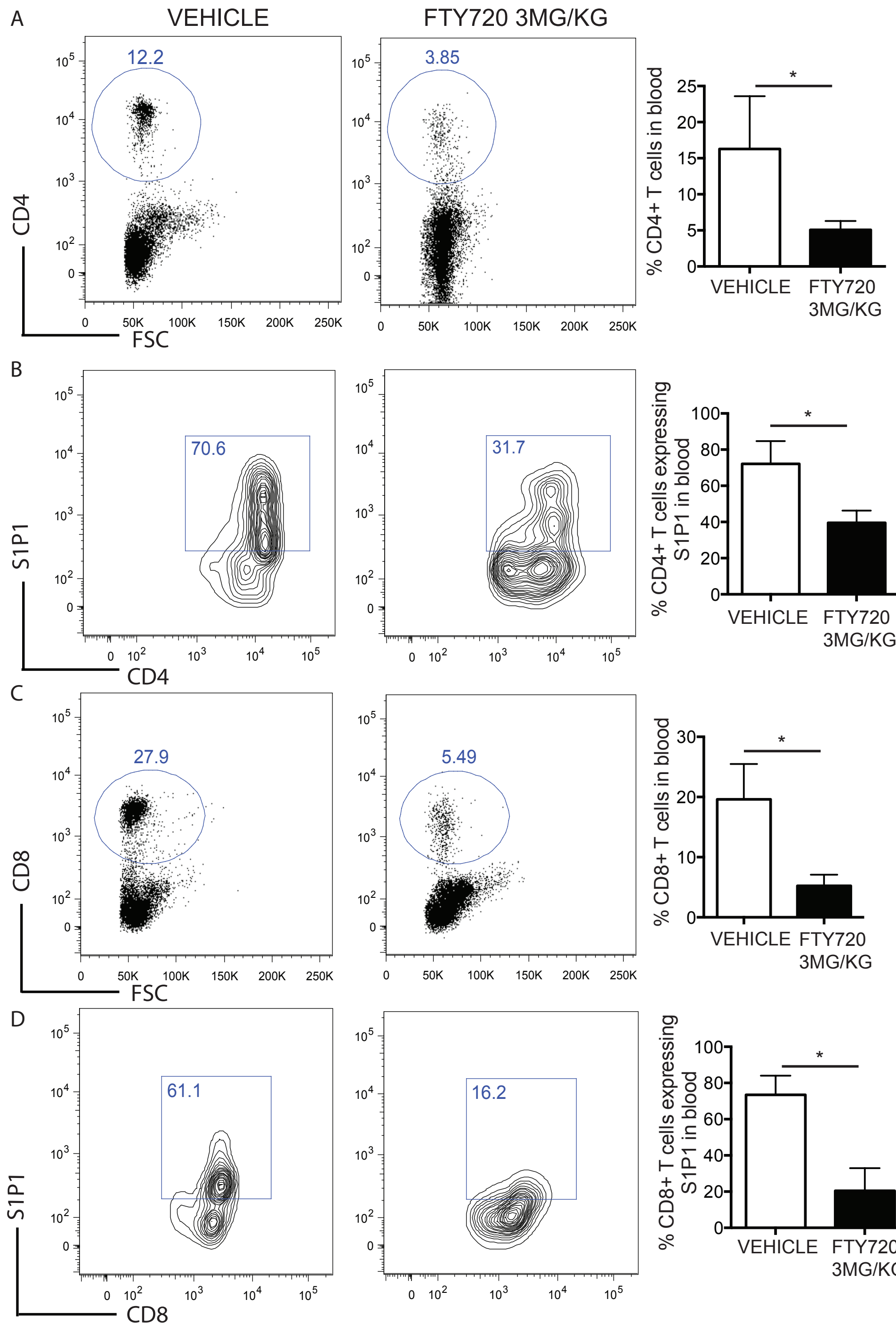


Figure 3.7. FTY720 induces lymphopenia and down-regulates S1P1 on T cells. Frequencies of CD4+ and CD8+ T cells in the blood day 7 p.t in S1P1 eGFP JHMV-infected mice treated daily with FTY720 or control starting at day 13 p.i. FTY720 significantly diminished the frequency of both **(A)** CD4+ and **(C)** CD8+ T cells and S1P1 expression measured by GFP expression on **(B)** CD4+ T cells and **(D)** CD8+ T cells. Results represents a minimum of 2 experiment with a minimum n=4 per group; bar graphs indicated are average$\pm$SEM. * $p<0.05$

3.4 Discussion

We have previously shown that FTY720 treatment of JHMV-infected mice results in increased mortality that is associated with impaired migration of virus-specific T cells into the CNS and elevated viral titers within the CNS ([37] **Chapter 2**). Dampened neuroinflammation correlated with increased cellularity of draining cervical lymph nodes consistent with previous reports indicating that S1P antagonism impairs lymphocyte egress from lymphatic tissue [20, 21, 43, 44]. Importantly, administration of FTY720 to JHMV-infected mice during acute disease was associated with diminished severity of demyelination. These findings argue that FTY720 treatment of JHMV-infected mice affects lymphocyte trafficking and accumulation within the CNS that impairs effective immune-mediated control of viral replication yet also limits the severity of white matter damage.

The use of stem cells for treatment of human demyelinating diseases such as MS to induce tissue repair offers an attractive therapy for promoting remyelination and potentially sustained clinical recovery [38, 45]. Following spinal cord engraftment of mouse NPCs into JHMV-infected mice, NPCs preferentially migrate to sites of demyelination by responding to the chemokine CXCL12 via expression of the receptor CXCR4 [11]. However, it is likely that other signaling cues are present within the microenvironment that participate in directing NPC migration. The S1P: S1P1 axis has been shown to be involved in NPCs migration to sites of damage in a model of spinal cord injury highlighting the importance of S1P receptors in mediating positional migration of NPCs [29]. In addition, treatment of mice with FTY720 augments CXCR4 signaling and potentiates migration of hematopoietic stem cells [42]. Moreover, FTY720 readily penetrates the CNS [20, 25, 41,

43, 44] arguing that it can modulate the biology of transplanted NPCs by binding to S1P receptors. Therefore, we investigated the effects of FTY720 treatment in conjunction with NPC therapy in a viral model of MS.

Here we show increased numbers of GFP-NPCs within white demyelinated white matter tracts of GFP-NPCs in JHMV-infected mice treated with FTY720 when compared to transplanted mice treated with vehicle control. However, FTY720 treatment did not affect differentiation into oligodendroglia nor augment remyelination indicating that the one pronounced biological effect was through influencing proliferation. These findings are consistent with our *in vitro* studies demonstrating that FTY720 treatment of cultured NPCs increased proliferation (**Figures 3.2A and B**). FTY720 treatment did not alter positional migration of transplanted NPCs as these cells efficiently congregated within areas of demyelination (**Figures 3.3A and B**). It is possible that in addition to increasing the proliferative capacity of NPCs, FTY720 also enhanced migration of transplanted NPCs. Supporting this are our *in vitro* experiments showing that FTY720 treatment of cultured NPCs increases migration in response to treatment with recombinant mouse CXCL12 and this was independent of elevated surface expression of CXCR4 on NPCs (**Figures 3.4A and B**). These findings indicate that a mechanism underlying enhanced NPC migration could involve either an effect on CXCR4 function and/or the downstream signaling cascade such as calcium mobilization or cytoskeleton rearrangement. Previous studies have shown that FTY720 activates the phosphorylation of CXCR4 through S1P3 activation followed by downstream cascade activation of Src kinase and JAK2 in progenitor cells [46] and affects CXCR4-mediated migration in hematopoietic stem cells following exposure to CXCL12 [42]. Future work focusing on defining the specific S1P receptor(s) involved in elevated CXCR4

function will be critical in order to better understand the molecular mechanisms governing how receptor agonists/antagonists influence NPC migration mediated by CXCR4.

Administration of FTY720 during acute JHMV-induced neurologic disease resulted in a reduction in the severity of demyelination that correlated with diminished infiltration of inflammatory T cells within the CNS ([37] **Chapter 2**). However, commencement of FTY720 treatment of mice transplanted with GFP-NPCs after demyelination and clinical disease were established did not improve clinical disease (**Figure 3.5A**) or diminish the severity of demyelination (**Figures 3.5D and E**) when compared to vehicle-treated control animals. FTY720 treatment of GFP-NPC-engrafted mice did not dampen T cell infiltration into the CNS (**Figures 3.6A-D**). Importantly, we have also determined that FTY720 treatment of JHMV-infected mice not transplanted with GFP-NPCs does not limit neuroinflammation nor demyelination (**not shown**). We confirmed that FTY720 was effective following treatment at later stages of disease as demonstrated by both a reduction in levels of circulating CD4+ and CD8+ T cells (**Figures 3.7A and C**) as well as reduced expression of S1P1 on these cells (**Figures 3.7B and D**). These findings argue that in a model of viral-induced demyelination the effects of S1P receptor antagonism on leukocyte trafficking and white matter damage may be dependent upon the stage of disease in which targeting drugs are administered.

Although FTY720 treatment of cultured NPCs resulted in activation, increased proliferation, and enhanced migration in response to CXCL12, there was effect on lineage fate commitment as similar frequencies of oligodendroglia, astrocytes, and neurons were observed compared to NPCs treated with control vehicle (**Figures 3.2C and D**). Similar frequencies of GFP-positive oligodendroglia expressing GST-π were detected within spinal

cords of mice GFP-NPC transplanted mice treated with either FTY720 or vehicle control (**Figure 3.5B and C**). Although there were increased numbers of GFP-NPCs within areas of demyelination, and presumably increased numbers of oligodendroglia, in FTY720-treated mice we did not observe any discernable increase in remyelination (**Figures 3.5G and H**). We have previously shown that engrafted NPCs can remyelinate demyelinated axons [13] and these current findings would argue that FTY720 does not increase the remyelination potential of engrafted NPCs. This is similar to previous studies that determined that FTY720 does not induce remyelination in either the cuprizone or lysophosphotidyl choline models of demyelination [47]. However, Miron et al. [48] have demonstrated that FTY720 treatment resulted in increased remyelination in organotypic cerebral slices where demyelination was induced by lysolecithin. The conflicting reports on the effects of FTY720 on remyelination highlight differences in model systems employed as well as emphasize the potential importance of selectively targeting specific receptors for promoting OPC maturation and myelin synthesis. For example, using a lysophosphotidyl choline-induced model of demyelination, administration of S1P5 agonist has been reported to have a greater effect on remyelination compared to S1P1 agonists [49]. FTY720 treatment of MS patients with the relapsing-remitting form of disease did reduce the risk if disability progression yet it is not clear if this is due to an increase in remyelination [50]. Additional studies in pre-clinical models of MS with more selective S1P receptor agonists/antagonists are required for a better understanding of the effects on both endogenous glial cells and transplanted NPCs on remyelination.

3.5 References

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CHAPTER FOUR

Olig1 function is required for remyelination potential of transplanted neural progenitor cells in a model of viral-induced demyelination

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Summary

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) resulting in cumulative neurologic deficits associated with progressive myelin loss. We have previously shown that transplantation of 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 and axonal sparing. The current study examines the contributions of the transcription factor Olig1 on NPC differentiation and remyelination. Under defined conditions, NPCs preferentially differentiate into oligodendroglia whereas NPCs isolated from Olig1-deficient (Olig1-/-) mice exhibit enhanced differentiation into astrocytes. Transplantation of Olig1-/- and Olig1+/+ NPCs into JHMV-infected mice resulted in similar cell survival, proliferation, and selective migration to areas of demyelination. However, only recipients of wild type NPCs exhibited extensive remyelination compared to mice receiving Olig1-/- NPCs. In vivo characterization of NPCs revealed that Olig1+/+ NPCs preferentially differentiated into NG2-positive OPCs and formed processes expressing myelin basic protein that encircled axons. In contrast, the majority of transplanted Olig1-/- NPCs differentiated into GFAP-positive cells consistent with the astrocyte lineage. These results indicate that exogenous NPCs contribute to improved clinical and histological outcome and this is associated with remyelination by this donor population. Further, these findings reveal that Olig1function is required for the remyelination potential of NPCs after transplant, through specification and/or maintenance of oligodendroglial identity.

4.1 Introduction

An important clinical aspect related to the pathogenesis of the human demyelinating disease multiple sclerosis is the eventual remyelination failure in chronic demyelinated plaques by endogenous neural progenitor cells (NPCs) that give rise to oligodendrocyte precursor cells (OPCs) [1]. Such failure in myelin regeneration could be due to multiple factors including inflammation, inhibitor molecules present in the lesion or age-related deficits in endogenous OPCs [2]. With this in mind, cell-based therapy using exogenous NPCs or OPCs have emerged as candidate therapies for promoting remyelination [3, 4]. Most studies have utilized either autoimmune models of neuroinflammatory-mediated demyelination, or chemical–induced gliotoxic demyelination to assess the remyelination potential of NPCs. While such models have advantages in capturing certain aspects of MS-like pathogenesis, they do not capture the full range of possible causative factors.

We have focused on a model of persistent viral infection that is correlative with chronic neuroinflammation and demyelination [5]. 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 [6-8]. Evident from this work is i) the ability of engrafted cells to migrate to regions of demyelination by responding to the chemokine ligand CXCL12 [9] and ii) preferential differentiation of transplanted NPCs into oligodendrocyte progenitor cells (OPCs) ([6, 8, 9]. The experiments described here address the functional contributions of exogenous NPCs to remyelination.

The genes Olig1 and Olig2 encode basic helix-loop helix transcription factors that are expressed in neural progenitor cells [10] and are required for fundamental processes of CNS development including oligodendrocyte formation [11]. Olig1 is especially involved in oligodendrocyte development as well as maturation [11]. Previous studies have shown the implication of Olig1 in differentiation and remyelination in toxin induced models of demyelination [12]. However these models do not take into account the potential effects of an inflammatory environment on NPCs. To this end, we have compared the remyelination and differentiation potential of competent and incompetent (Olig1-/-) NPCs post-transplant into JHMV-infected mice.

4.2 Materials and Methods

Mice and Virus

Five-week-old male C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD) and Olig1-/- male (Lu et al., 2002) mice (C57Bl/6 background) were bred in the UCI vivarium. For viral infection, mice were anesthetized by intraperitoneal (i.p.) injection of ketamine 80-100mg/kg (MP Biomedicals, OH) and xylazine 5-10 kg/mg (Phoenix Pharmaceutical, MO). Mice were infected i.c. with 200 plaque forming units (PFU) of JHMV (strain 2.2V-1) suspended in 30μl of sterile saline [31]. Timed pregnant C57BL/6 mice (P14) were purchased from the National Cancer Institute and perinatal pups were used for wild type NPC cultures. C57BL/6-Tg (CAG-EGFP) 10sbJ mice (expressing green fluorescent protein, GFP) were purchased from JAX (stock#003291) [11]. C57BL/6-Tg (CAG-EGFP) 10sbJ mice were bred with Olig1-/- mice to produce the GFP-positive Olig1-/- colony (confirmed by PCR genotyping). All experiments were reviewed and approved by the University of California, Irvine Institutional Animal Care and Use Committee (IACUC).

Neurosphere culture

Epidermal growth factor (EGF) responsive neurosphere cultures were prepared from either EGFP- Olig1+/+ or EGFP-Olig1-/- mice. Neurosphere cultures were prepared as previously described from the brains of perinatal animals [8, 32]. Briefly, dissected striata were razor minced and triturated in pre-warmed 0.05% Trypsin (Invitrogen) for 10 minutes. Trypsin digestion was halted with equal volume of 1X anti-trypsin (Invitrogen). Single cells were resuspended in DMEM: F12 (Invitrogen) supplemented with 1X B27 (Invitrogen), 1X Insulin-Transferrin-Selenium-X (Invitrogen), 1X Penicillin-Streptomycin

(Invitrogen), 40 ng/ml T3 (Sigma-T67407), and 20 ng/ml human recombinant EGF (Sigma-E9644) and cultured for 5-6 days. Culture supernatant was replaced with fresh media containing EGF on days 1, 3, and 5. After one week, mature neurospheres (100-200µm) were transferred to matrigel (BD Bioscience) coated flasks (thin coat method, 1:30 dilution). Within 24 hours individual cells had spread out from attached spheres and formed a monolayer. Following formation of a monolayer formed, cells were trypsinized and suspended in sterile saline for transplant experiment.

Differentiation of neurospheres

To assess differentiation potential, cells were grown on matrigel coated imaging slides for a total of 4 days, fixed in 4% paraformaldehyde (Fisher Scientific, Fair Lawn, NJ) for 20 min and immunofluorescence staining was performed using standard protocols. Imaging chambers were blocked with 10% normal goat serum (NGS) (Vector Laboratories, Burlingame, CA) for 1 hr at room temperature. Primary antibodies (polyclonal rabbit anti-GalC, Chemicon, 1:50 dilution in 10% NGS; polyclonal rabbit anti-GFAP, Invitrogen, 1:500 dilution in 10% NGS; polyclonal rabbit anti-NG2, Chemicon, 1:200 dilution in 10% NGS or blocking solution (negative control, 10% NGS in PBS) were applied to chambers overnight at 4°C. Slides were rinsed three times with PBS and fluorescent-conjugated secondary antibody (Alexa 594, goat anti-rabbit) was applied and incubated for 1 hour at room temperature. Slides were rinsed three times in PBS and mounted in vectashield (Vector Laboratories) with Dapi to visualize cell nuclei. Cell quantification was conducted using a Nikon Eclipse Ti microscope, 200x magnification. The percentage of immunopositive cells

was determined by dividing the total number of immunopositive cells by the total number of Dapi-positive cells in five images, multiplied by 100.

Transplantation

JHMV-infected mice develop demyelination associated with clinical disease 10-14 post-infection (p.i.) [31, 33]. Transplant experiments were performed on days 14 p.i., when replicating virus is reduced below detectable levels and there is evidence of demyelinating lesions. Clinical severity was assessed using a previously described 4-point scale [34]. Only animals that developed partial-to-complete hind limb paralysis were used for transplantation. For transplant experiments, recipient mice with comparable clinical disease received either GFP-Olig1+/+ or GFP-Olig1-/- NPCs, or vehicle control (sterile saline). Anaesthetized animals received laminectomy at T9-T10 to expose the spinal cord. Animals were then transplanted with 2.5μl of NPC (250,000 cells) or 2.5μl of sterile saline using a 10 μl Hamilton syringe (Hamilton) with a silicon-coated pulled glass tip affixed in a stereotactic arm as previously described [8, 35].

Histology

Transplanted recipient animals were euthanized at 21 and 35 days post-infection (p.i.) (7 and 21 days post-transplant) and tissue was fixed by intracardiac perfusion with 4% paraformaldehyde in PBS (pH 7.4). Intact spinal columns were removed and fixed overnight in 4% paraformaldehyde at 4°C. The bone was removed to expose the fixed spinal cord and the tissue 8 mm anterior and 8mm posterior from the injection site was divided into twelve tissue pieces (1mm). To evaluate GFP+ NPC migration and

differentiation in vivo, tissue sections were cryoprotected in 30% sucrose for 7 days and embedded in OCT (Tissue-Tek). Seven-micron thick transverse sections were cut and used for immunofluorescence staining or stained with luxol fast blue (LFB) in combination with H&E (hematoxylin and eosin) to determine the extent of demyelination. To evaluate the remyelination potential of transplanted NPC, even tissue pieces were processed and embedded in resin while odd tissue pieces were processed and embedded in OCT. For resin sectioning, tissue pieces were exposed to 1% Osmium tetroxide (Electron Microscopy Sciences), dehydrated in ascending alcohols, and embedded in Spurr resin (Electron Microscopy Sciences) according to standard protocols. Transverse semi-thin (1 μm) sections were cut from each block, stained with alkaline toluidine blue, cover slipped, and examined by light microscopy using an Olympus BX-60 microscope, 600x magnification. The myelination of axons was determined by assessing the thickness of the myelin sheath in relation to the axons diameter [36, 37]. Demyelinated axons, remyelinated axons and normally myelinated axons were counted within an area equal to 10% of the total area of demyelination. The quantitative assessment of remyelination was conducted throughout the region 8mm caudal and rostral to the transplant site. The number demyelinated axons, remyelinated axons, the total number of axons and the percent remyelinated axons was determined for each of the four regions on each tissue block, averaged, then averaged across animals within each group for each tissue block as previously described [8].

NPC migration

The total numbers of GFP-positive cells was determined in each of the twelve sampled locations surrounding the transplant site by counting all GFP-positive cells co-localized with Dapi-positive nuclei. Cell migration was represented in graphs of the number of GFP-positive cells versus distance from transplant site (mm), and the approximate number of GFP-positive NPC 21 days post-transplant was analyzed by area under the curve calculation performed in GraphPad Prism (GraphPad Software) [38].

Immunofluorescence

To assess in vivo differentiation of GFP-positive NPC 21 days after transplant, sections were dehydrated, washed in PBS to removed excess OCT, and blocked for 1 h at room temperature with 10% goat serum in PBS. Immunofluorescence staining was performed using standard protocols. The following primary antibodies were added overnight at 4°C: rabbit anti-MBP 1:200 (Chemicon, cat#AB980), rabbit anti-GST-p 1:1,000 (MBL, cat#311), rabbit anti-NG2 1:200 (Chemicon, cat#AB5320), rabbit anti-GFAP 1:1,1000 (Invitrogen, cat#18-0063), rabbit anti-NF-150 (Chemicon, cat#AB1991). Appropriate conjugated goat secondary antibodies were used for visualization (Invitrogen). Slides were mounted in vectashield (Vector Laboratories) with DAPI to visualize cell nuclei and to preserve fluorescence. Cell quantification was conducted using an Olympus BX-60 microscope, 200x magnification. The percentage of immunopositive cells was determined by dividing the number of immunopositive cells by the number of Dapi-positive nuclei, multiplied by 100.

Statistical analysis

All data is presented as average ± SEM. Statistically significant differences were assessed by one-way ANOVA, and p values less than 0.05 were considered significant.

4.3 Results

Olig1 enhances NPC oligodendroglial commitment

To investigate the importance of Olig1 in oligodendrocyte lineage commitment, NPCs were cultured from the brains of EGFP-Olig1+/+ (WT) and EGFP-Olig1-deficient mice (Olig1-/- mice). NPCs were cultured on matrigel-coated slides for 5 days to induce differentiation under defined conditions at which point defined cellular antigens were used to identify lineage commitment by immunocytochemical staining. By 5 days post-differentiation, the majority of cells cultured from WT mice expressed antigens NG2 and/or GalC that are markers associated with cells of the oligodendrocyte lineage (**Figures 4.1A and C**). Approximately 5% of cultured WT cells expressed the astrocyte-associated marker GFAP (**Figures 4.1D and F**). In contrast, genetic ablation of Olig1 in NPCs resulted in reduced expression of both NG2 and GalC (**Figures 4.1B and C**) while there was an ~ 2-fold increase in GFAP expression when compared to WT cells (**Figures 4.1E and F**). Immunocytochemical staining of differentiated WT and Olig1-/- NPCs revealed GalC-positive cells displaying an arborized morphology consistent with oligodendroglia (**Figures 4.1A and B**). GFAP-positive cells derived from either WT or Olig1-/- NPCs exhibited a more flat or stellate morphology (**Figures 4.1D and E**).

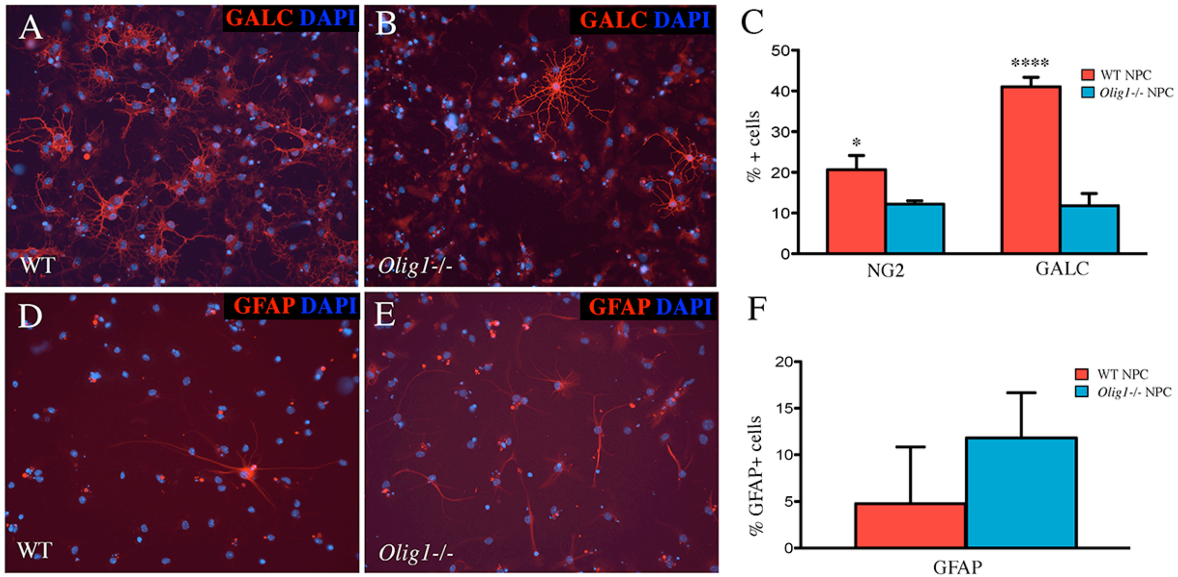


Figure 4.1. Differentiation of neural progenitor cells derived from wild type and *Olig1* deficient mice yields oligodendrocyte and astrocyte enriched cell populations *in vitro* respectively

Neural progenitor cells were cultured from the brains of wild type and *Olig1* deficient mice, 1-day postnatal pups, in the presence of EGF using the neurosphere culture method. **(A)** Immunofluorescence staining of Galc-positive oligodendrocyte following 5 days *in vitro* differentiation of wild type NPC and **(B)** *Olig1-/-* NPC. **(C)** Quantification of immunofluorescence staining for Galc and NG2 was performed 4-5 days following the simultaneous removal of growth factors and transfer of neurospheres to matrigel coated slides. **(D)** Immunofluorescence staining of Gfap-positive astrocytes following 5 days *in vitro* differentiation of wild type NPC and **(E)** *Olig1-/-* NPC. **(F)** Quantification of immunofluorescence staining for GFAP. Data are presented as average±SEM and are representative of results of three independent cultures.

GFP-NPC transplantation does not modulate the severity of demyelination

To avoid toxicity and induction of senescence associated with BrdU labeling of NPC [13], we generated GFP-Olig1-/- mice and used NPC cultured from these mice for subsequent transplantation into mice. NPC cultured from GFP-C57BL/6 mice were used as WT controls. Two weeks prior to transplantation, recipient C57BL/6 mice were infected by i.c. injection of 200 PFU of JHMV, which is a sufficient viral dose to induce immune-mediated demyelination. Transplantation consisted of a single intraspinal injection of $2.5x10^5$ cells (or vehicle control, HBSS) at thoracic vertebrae 9 and recipient animals were sacrificed one week and three weeks post-transplant to evaluate the extent of exogenous cell migration and engraftment. Luxol fast blue (LFB) staining of a representative JHMV-infected mouse 14 days p.i. indicated that at the time of transplantation, white matter demyelination was localized in the ventral white matter in the vicinity of the central canal (**Figure 4.2A**). Three weeks post-transplant of either WT or Olig1-/- NPCs, demyelinated lesions were found to extend from the ventral to the lateral white matter (**Figure 4.2A**). Quantification of demyelination in experimental mice indicated a similar level of myelin damage in recipients of either WT or Olig1-/- NPCs compared to vehicle control (**Figure 4.2B**). Importantly, these findings are consistent with earlier results and indicate that transplantation of NPCs neither exacerbate nor ameliorate JHMV-induced immunopathology and this was associated with no attenuation in neuroinflammation [7, 8].

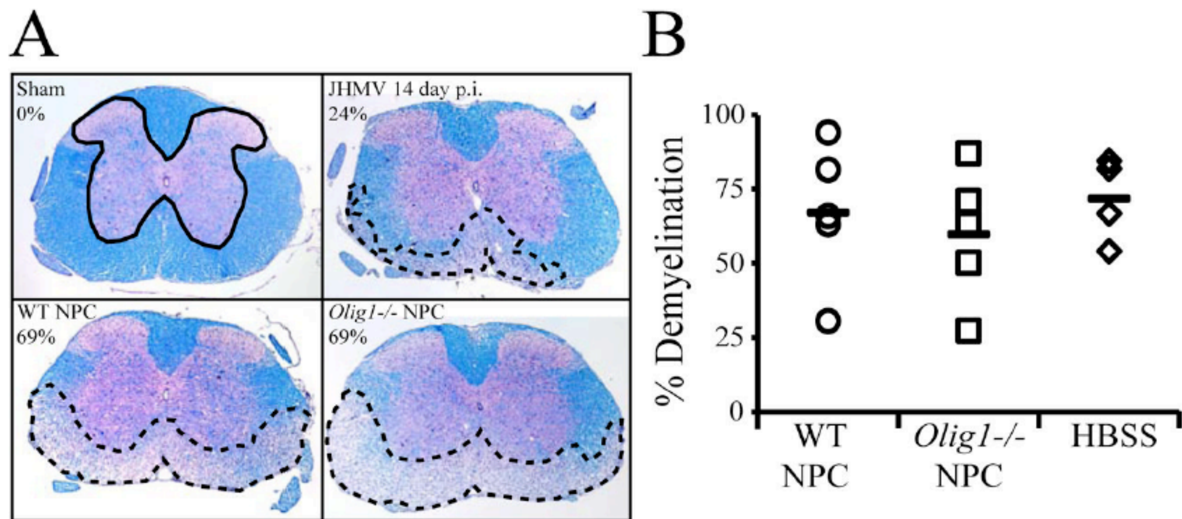


Figure 4.2. Transplantation of wild type and Olig1 deficient NPC does not modulate JHMV-induced demyelination

(A) Representative OCT embedded spinal cords stained with luxol fast blue (intact myelin is bright blue) in combination with H&E staining. The intact myelinated white matter of sham-infected mouse (upper left) stains bright blue while demyelinated lesions (dotted outline) are present in the ventral white matter 14 day p.i. JHMV infection (upper right). Representative tissue from mice that received wild type GFP+ NPC transplant (lower left) or GFP+*Olig1-/-* NPC transplant (lower right) indicate that demyelination spread evenly through the ventral white matter during the three-week survival period. **(B)** Quantification of the overall percent demyelination following transplantation of wild type NPC, *Olig1-/-* NPC or vehicle control indicates that all three groups had equivalent average. A black bar indicates the average of each group, while shapes represent individual animals. The percent demyelination for each individual animal is the average demyelination from 12 spinal cord sections spaced over 16mm, 8mm anterior and 8mm posterior from the transplant injection site. Percent demyelination = (area of demyelination / total area white matter) X 100. Results are representative of two independent experiments.

Similar migration of WT and Olig1-/- NPCs

Surgically engrafted NPCs preferentially migrate and accumulate within areas of white matter damage in JHMV-infected mice [7, 8]. Moreover, we have determined that migration is mediated through CXCR4 expressed on transplanted NPCs responding to CXCL12 that is expressed within demyelinating lesions [9]. Transplanted WT and Olig1-/- NPCs exhibited similar migration both rostral and caudal to the site of implantation at 1 and 3 weeks post-transplant indicating that Olig1 function does not regulate migration (**Figure 4.3A**). In addition, similar numbers of WT and Olig1-/- NPCs were detected in transplanted mice indicating that replication is not negatively affected in the absence of Olig1 (**Figure 4.3B**). Importantly, positional migration of transplanted NPCs was not affected in the absence of Olig1 as both populations of NPCs preferentially migrated into ventral and lateral white matter columns of JHMV-infected mice (Figure 3C). Collectively, these findings provide compelling evidence that Olig1 function is not required for NPC migration, proliferation, or preferential accumulation within areas of white matter damage.

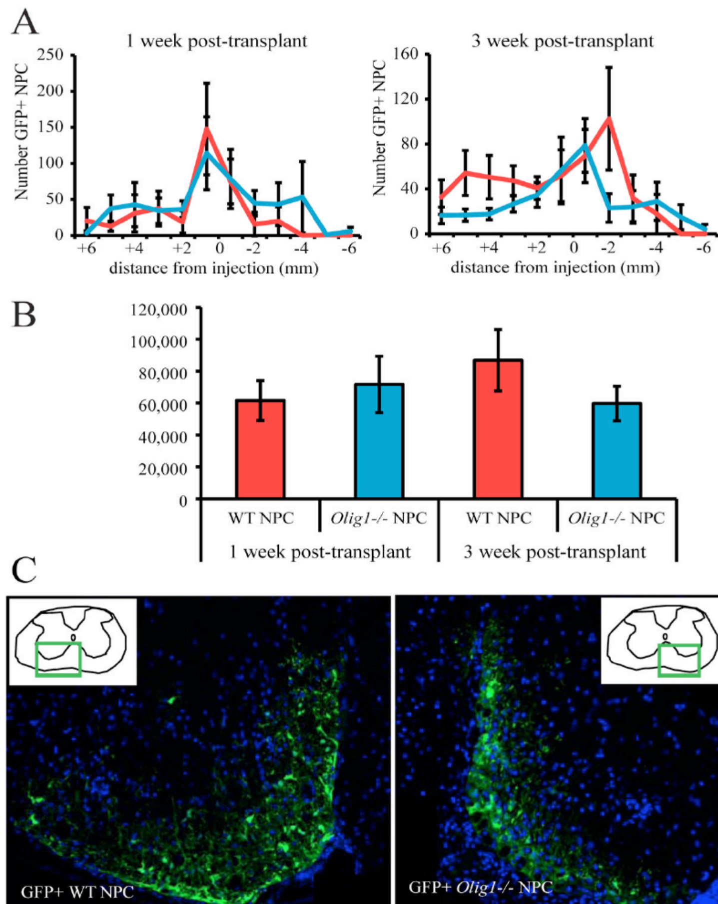


Figure 4.3. Wild type and *Olig1* deficient neural progenitor cells migrate to demyelinated white matter after intra-spinal transplant

JHMV-infected mice with established demyelination were transplanted with wild type GFP+ NPC, GFP+*Olig1-/-* NPC, or vehicle control (HBSS) by intra-spinal injection. Animals were sacrificed 1 week and 3 weeks following transplant and the 16mm region of spinal cord surrounding the transplant site was removed, cut into 12 even pieces, and embedded in OCT for analysis. **(A)** Quantification of the number of wild type GFP+ cells and GFP+*Olig1-/-* cells per tissue section at one-week and three-weeks post-transplant show anterior and posterior migration of NPC away from the site of injection. The distribution and extent of migration of wild type and *Olig1-/-* GFP+ NPC overlap at both time points. **(B)** An approximation of total numbers of transplanted cells one-week and three-weeks after transplanted was calculated using area under the curve analysis of cell number/section data shown in **(A)**. This analysis indicated that wild type and *Olig1-/-* NPC have similar survival and proliferation *in vivo*. Results are representative of two independent experiments. **(C)** Representative images of GFP+ wild type (left) and *Olig1-/-* (right) NPC indicate that the transplanted cells migrate to ventral white matter. The anatomical distribution of GFP+ NPC mirrors the distribution of demyelination at the time of transplant (14 days post-JHMV infection), Figure 2A. Images are from animals sacrificed 21 days post-transplant. n = 4 to 5. Results are representative of two independent experiments.

Olig1-/- NPCs do not promote remyelination

We next evaluated the extent of remyelination in JHMV-infected recipients of either WT or Olig1-/- NPCs at 3 weeks post-transplant. Spinal cord sections were used for either tracking migration of GFP-labeled NPCs or measuring remyelination to ensure that assessment of histopathology was performed within areas in which transplanted cells were present (**Figure 4.4A**). A representative spinal cord from a non-infected, non-transplanted mouse is shown to demonstrate normal myelin thickness (**Figure 4.4B**). Numerous demyelinated axons were present among vacuoles, myelin debris, and activated macrophages in JHMV-infected mice receiving either vehicle control (**Figure 4.4C**) or Olig1-/- NPCs (**Figure 4.4D**). In contrast, infected mice transplanted with WT NPCs exhibited numerous axons with thin myelin sheaths consistent with remyelination (**Figure 4.4E**). Quantification of remyelination in experimental groups of mice revealed an overall increase in the frequency of remyelinated axons in mice transplanted with WT NPCs when compared to either Olig1-/- NPCs or vehicle control (**Figure 4.4F**) [8]. Furthermore, immunofluorescent staining in combination with confocal microscopy revealed GFP signal overlying with MBP staining (**Figure 4.5A**) as well as GFP-positive wraps surrounding numerous axons (defined by neurofilament staining) (**Figure 4.5B**) in mice receiving WT NPCs yet this was absent in recipients of Olig1-/- NPCs. These findings support and extend previous work from our laboratory indicating that transplantation of NPCs into JHMV-infected mice results in extensive remyelination [8, 9]. The overall paucity in remyelinated axons in Olig1-/- NPCs suggests that although these cells are able to migrate and accumulate within areas of pathology they are not able to remyelinate demyelinated axons. Importantly, the demonstration of transplanted NPC-derived myelin wraps surrounding

axons in WT NPC recipients provides compelling evidence that transplanted cells directly remyelinate axons.

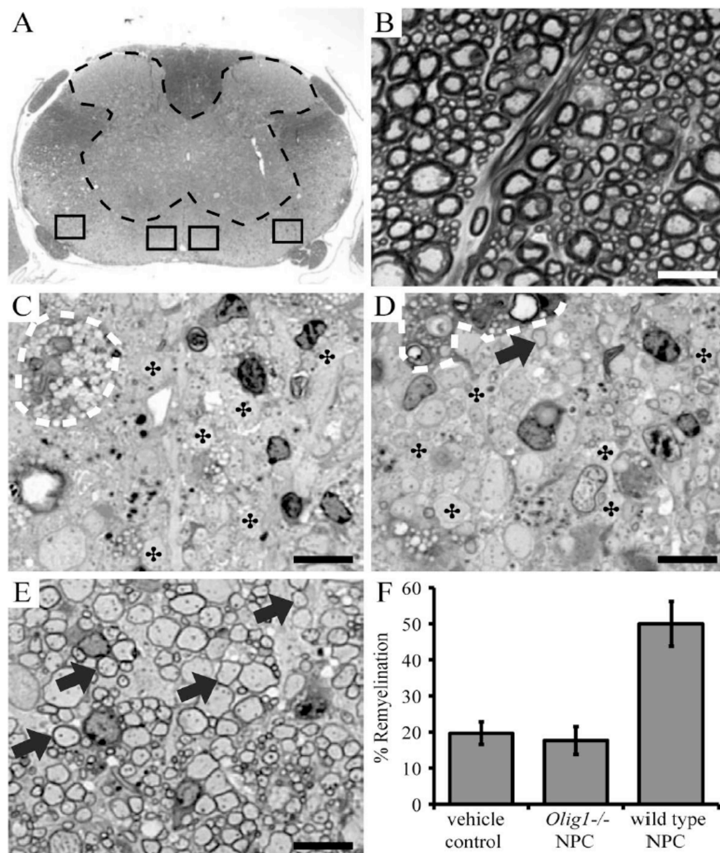


Figure 4.4. Transplantation of Olig1-/- NPC does not promote remyelination
JHMV-infected mice with established demyelination were transplanted with wild type NPC, *Olig1-/-* NPC, or vehicle control (HBSS) by intra-spinal injection. Animals were sacrificed 3 weeks following transplant and the 16mm region of spinal cord surrounding the transplant site was removed, cut into 12 even pieces, and the even pieces were embedded spur resin to evaluate remyelination following NPC transplant. **(A)** Toluidine blue-stained transverse spinal cord section 4 boxed areas indicating the 4 regions of remyelination analysis. For each even section, the number of normal, demyelinated and remyelinated axons were counted and summed. **(B)** Resin embedded toluidine-blue stained spinal cord from an uninfected animal showing normal axons with thick myelin sheaths. Resin embedded toluidine-blue stained spinal cord from MHV-infected mice transplanted with **(C)** vehicle control, **(D)** *Olig1-/-* NPC, or **(E)** wild type NPC. Note the predominance of demyelinated axons (asterisk), infiltrating cells and tissue damage in the vehicle control and *Olig1-/-* NPC recipient mice. In contrast, the majority of the axons in the mouse transplanted with wild type NPC have thin myelin sheaths (black arrow) indicative of remyelination. Demyelinated axons, asterisk; remyelinated axons, black arrow. **(F)** Quantification of the percent remyelination by group shows that transplantation of wild type NPC results in 50% remyelinated axons compared to 19% and 18% remyelinated axons in the vehicle control and *Olig1-/-* NPC groups respectively. **(B-E)** scale bar represents 10μm.

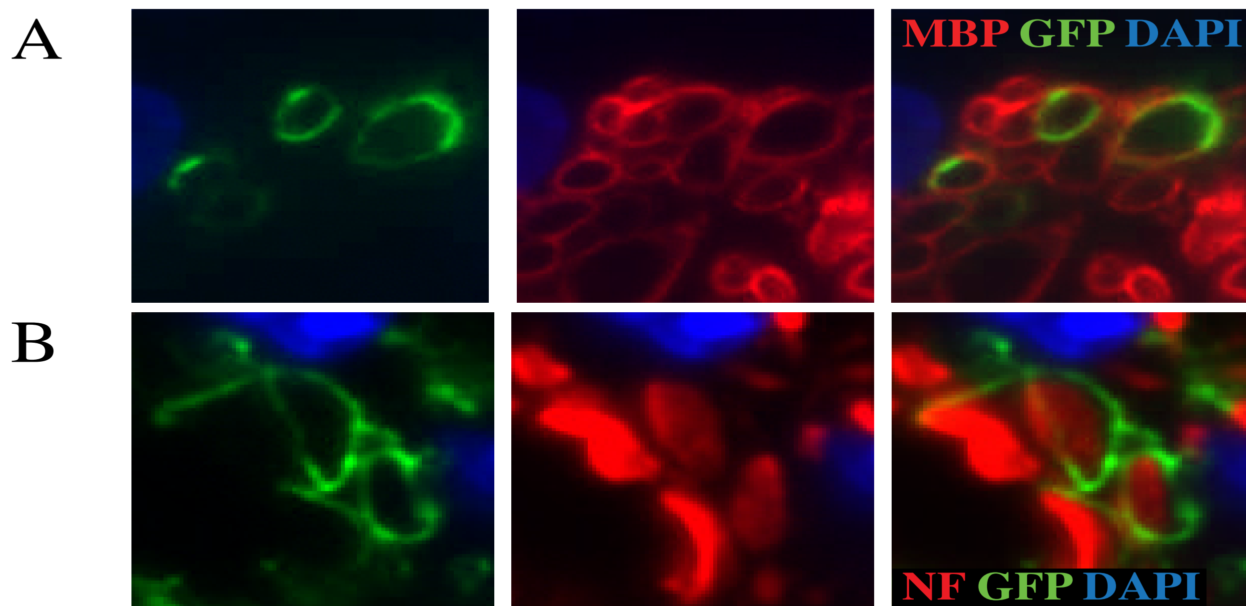
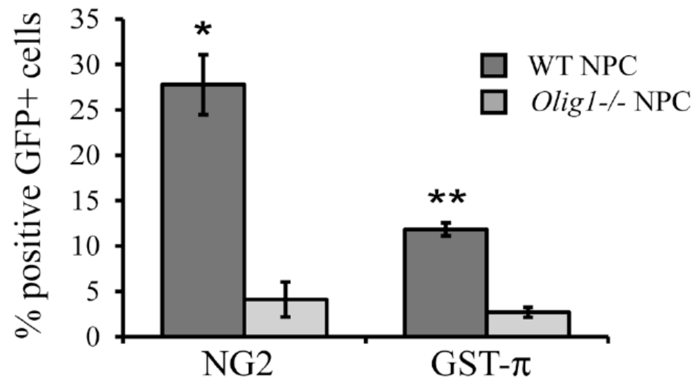


Figure 4.5. Wild type NPC derived cells form processes that encircle axons *in vivo*
Wild type GFP+ NPCs form thin circular GFP+ processes in vivo. **(A)** MBP staining of tissue from representative recipient of GFP+ wild type NPC showing overlap between MBP and GFP+ process. **(B)** Neurofilament staining of tissue from representative recipient of GFP+ wild type NPC showing GFP+ process encircling neurofilament labeled axons.

In vivo differentiation of transplanted WT and Olig1-/- NPCs

Evaluation of differentiated WT and Olig1-/- NPCs at 3-weeks post-transplant in JHMV-infected mice was performed by immunofluorescence staining. Consistent with previous findings [9], approximately 40% of the engrafted WT NPCs differentiated to an oligodendrocyte lineage as determined by NG2 and GST-p staining (**Figure 4.6A**). The majority of WT NPCs had differentiated into NG2-positive OPCs (27.78% ± 3.31) rather than mature oligodendrocytes as evidenced by GST-p staining (11.83% ± 0.72). In marked contrast, very few (<5%) of Olig1-/- NPCs expressed either NG2 (4.11% ± 1.92) or GST-p (2.7%± 0.55) indicating these cells did not preferentially differentiate into a myelin-competent cell (**Figure 4.6A**). Indeed, staining for the astrocyte marker GFAP revealed that the majority (>70%) of transplanted Olig1-/- NPCs were GFAP-positive (73.04% ± 3.1) and displayed morphology consistent with an activated astrocyte (**Figure 4.6B**). However, fewer than 20% of transplanted WT NPCs differentiated in GFAP-positive cells (17.05% ± 3.57) (**Figure 4.6B**).

A



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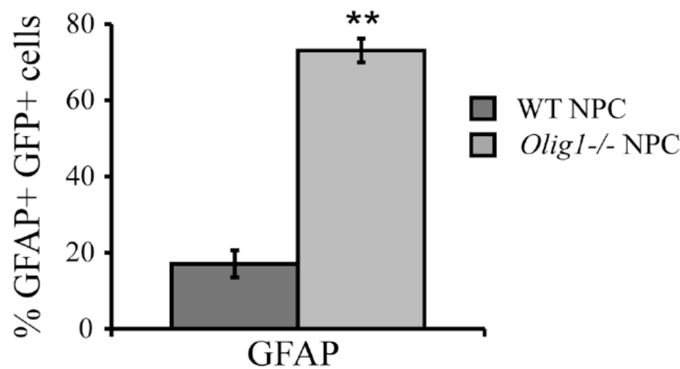

Figure 4.6. Wild type GFP+ NPCs differentiate into oligodendrocytes and *Olig1* deficient NPCs differentiate into astrocytes *in vivo*
Analysis of *in vivo* differentiation of transplanted cells into NG2+ oligodendrocyte progenitor cells, GST-p+ oligodendrocytes and GFAP+ astrocytes in frozen OCT embedded spinal cord, 3 weeks post-transplant. **(A)** Immunofluorescence staining for NG2 of spinal cord from a recipient that received wild type GFP+ NPC shows that 27.5% of the transplanted cells express the NG2 protein. In contrast, in a recipient that received GFP+*Olig1-/-* NPC, only 10% of the transplanted cells express NG2. The overall percent of NG2 positive and GST-p positive transplanted cells from each group was determined from 6 sections distributed evenly in the 16mm region surrounding the injection site. This analysis showed that 3 weeks after transplant, about 40% of the wild type NPCs differentiated into cells of the oligodendrocyte lineage, compared to less than 10% of Olig1-/- NPCs. n = 4. *, $p < 0.01$; **, $p < 0.001$. **(B)** The overall percent of GFAP positive transplanted cells from each group was determined from 6 sections distributed evenly in the 16mm region surrounding the injection site. This analysis showed that 3 weeks after transplant, the majority of *Olig1-/-* NPC (73 ± 3) differentiates in GFAP expressing astrocytes while less than 20% of transplanted wild type NPCs express GFAP. n = 4. **, $p < 0.001$.

4.4 Discussion

There are no effective therapies available for patients with progressive forms of MS. For this and other devastating human white matter disorders, exogenous NPC and OPC transplantation are of great interest as having potential therapeutic roles [2, 4, 14-19]. The use of a viral model of demyelination to evaluate the remyelination potential of NPCs has unique features that make this a relevant experimental model system. First, the etiology of the human demyelinating disease is enigmatic with both genetic factors and environmental influences considered important in initiation and maintenance of disease. Viral infection has long been viewed as 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 CNS and damage the myelin sheath [20-22]. Therefore, viral models of demyelination are clearly relevant and have provided important insight into mechanisms associated with disease initiation, neuroinflammation and demyelination. Given the possibility of viral infection in initiating demyelination as well as the fact that numerous neurotropic viruses exist that are capable of persisting within the CNS, it is imperative to evaluate the remyelination potential of stem cells in the presence of a persistent viral infection that is correlative with chronic neuroinflammation and demyelination

The present study sought to determine the importance of Olig1 in NPC-mediated remyelination in JHMV-infected mice with established demyelination. To address this, we used NPCs derived from mice deficient in the transcription factor Olig1. The genes Olig1 and Olig2 encode basic helix-loop helix transcription factors that are expressed in neural progenitor cells [10] and are required for fundamental processes of CNS development

including oligodendrocyte formation [11]. The Olig1-deficient mice we employed are viable and have mildly delayed myelination during development [11]. However, they showed a profound deficiency in remyelination, indicating a nonredundant role for Olig1 in remyelination due to a defect in OPC differentiation to oligodendrocytes after white matter injury [12]. We note that this contrasts findings with a different Olig1-null line that shows a lethal developmental hypomyelination phenotype [23]. Thus, Olig1 is critical for normal myelination and remyelination.

We took advantage of this genetic requirement of Olig1 in repair to determine if intraspinal transplantation of Olig1-/- NPCs resulted in remyelination. Our findings reveal that engrafted Olig1-/- NPCs were well tolerated, replicated, and migrated to a similar extent along white matter spinal cord tracts compared to WT NPCs. However, there was a marked reduction in the number of remyelinated axons in comparison to recipients of control cells. Paucity of remyelination from Olig1-/- donor cells was associated with an apparent change in lineage fate commitment, as Olig1-null mice preferentially differentiated into GFAP-positive cells whereas WT cells exhibited commitment to an oligodendrocyte lineage. This was confirmed by our in vitro studies showing the importance of Olig1-/- to enhance oligodendroglial lineage commitment, as the absence of this transcription factor seemed to favor the differentiation into astrocytes by NPCs.

In all recipients, WT NPCs migrated to demyelinated white matter and formed MBP-containing processes that encircle neurofilament positive axons. These findings are consistent with several possibilities. First, Olig1 may be required to regulate cell fate differentiation of NPCs into OPCs; alternatively, Olig1 may be required to maintain OPC fate and in the absence of Olig1 function OPCs will “trans-differentiate” to astrocytes. We favor

the former argument as in Olig1-/- mice OPC fate and myelination proceeds to a level comparable to wildtype mice demonstrating Olig1 is not strictly required to maintain OPC fate. Therefore, our findings indicate that Olig1 is not required for migration into lesions but is important in selecting an oligodendrocyte lineage fate. These findings differ from, but are not in conflict, with Arnett et al [12] where OPCs were recruited into demyelinating lesions induced by lysolethicin treatment but failed to mature to participate in remyelination. Of note, in this study no increased astrogliosis in lesions was observed. Similarly, transplanted Olig1-/- NPCs migrated yet did exhibit differentiation primarily into astrocytes. An important difference in these results may reflect differences in model systems utilized. While lysolethicin induces focal demyelinating lesions in the absence of infiltration of activated T lymphocytes and monocyte/macrophages, JHMV-induced demyelination is characterized by the presence of activated T lymphocytes as well as other inflammatory cells that results in the secretion of numerous proinflammatory cytokines/chemokines [5, 24, 25]. Therefore, one intriguing possibility is that the inflammatory microenvironment may tailor NPCs fate decision through an Olig1-regulated mechanism. In addition, these findings are consistent with other studies highlighting the importance of Olig1 in contributing to myelin repair following experimental demyelination [16, 26-28].

Our findings suggest that exogenous NPCs actively participate in remyelination following engraftment. This is supported by counting remyelinated axons in experimental animals as well as the presence of GFP-positive wraps encircling axons following transplantation of wild type NPCs. Our findings are consistent with earlier studies by Cummings et al. [29] that demonstrated engraftment of human NPCs promoted locomotor

recovery in a rodent model of spinal cord injury. Importantly, recovery was abolished by selective ablation of engrafted cells suggesting that the therapeutic benefit was mediated primarily by engrafted cells. However, a recent report indicated that transplanted neural progenitors were shown to enhance proliferation of host OPCs [30]. This was associated with increased remyelination in a model of cuprizone-mediated demyelination indicating that transplanted neural progenitors stimulated endogenous cells to participate in repair. Differences between experimental outcomes most likely reflect differences within the model systems employed as cuprizone represents an acute and focal model of demyelination with a limited role for activated lymphocytes in participating in myelin destruction and this is dramatically different compared to JHMV-induced demyelination. Therefore, the environmental signals encountered by engrafted cells will modulate the ability of the engrafted cell to home, differentiate, and participate in repair. With this in mind, our reveal insight into the importance of Olig1 within the context of engrafted NPCs into an ongoing immune-mediated demyelinating disease initiated by viral infection: i) Olig1 does not influence NPC positional migration, ii) Olig1 is required for differentiation into OPCs/oligodendrocytes, and iii) preferential differentiation into oligolineage cells is associated with increased remyelination.

4.5 References

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CHAPTER FIVE

Summary and Significance

5.1 Summary and Significance

FTY720 is the first oral drug for treating relapsing remitting forms of MS [1]. Patients show a significant reduction in relapses that correlates with reduced numbers of new lesions [2, 3]. However, due to its mode of action whereby FTY720 prevents egress of T cells from lymph nodes, its effect on immune responses remains undefined [4-7]. Indeed, during clinical trials, patients treated with FTY720 showed herpes zoster recrudescence [8, 9]. We have thus analyzed the effect of FTY720 during acute and chronic immune responses to JHMV infection of the CNS (**Chapter 2**).

During the acute phase of MHV disease, virus specific T cells infiltrate the CNS following BBB permeabilization by innate immune cells such as neutrophils [10]. While T cells are important for viral clearance, they also induce demyelination [11]. FTY720 treatment starting at day 5 post-infection in mice infected with MHV resulted in increased morbidity and mortality associated with increased viral load in the brain and spinal cord. This was correlated with a decrease in virus specific IFNγ-producing CD4 and CD8+ T cell infiltration into the CNS. Moreover, increased retention of CD4 + and CD8+ T cells in the draining cervical lymph nodes was highlighted during the acute disease. Investigation of T cell function revealed no negative effect via FTY720 indicating that inability to clear viral load and increase mortality is due to T cell trafficking impairment and not direct effect on T cell effector functions. Indeed, IFNγ and TNFα cytokine secretion, proliferation by virus-specific T cells was not affected by FTY720. Moreover, CD8+ T cell effector function was not impacted, as virus specific CD8+ T cells treated with FTY720-phosphate were able to induce target cell lysis. Neutralizing antibody levels were not changed in mice treated with

FTY720 suggesting that viral recrudescence would not be impacted during treatment. Interestingly, neuroinflammation in the surviving mice in the chronic phase of disease was significantly diminished in the spinal cords and the severity of demyelination was reduced. This is due to T cell trafficking impairment but could arguably be due to a direct effect of FTY720 on resident cells as this compound penetrates the CNS.

There are no available treatments for secondary progressive MS and the mechanisms involved in myelin destruction are not well understood. Importantly, successful therapies should not only limit inflammation but also promote repair and remyelination. Stem cell therapy to promote repair of damaged lesions of the CNS seems very promising [12] and could be given in conjunction with FTY720 thereby limiting inflammation in patients with MS. Indeed stem cells transplants have shown to promote remyelination in various models of MS and in the viral MHV model; NPCs migrate to sites of damage, differentiate and induce remyelination [13].

We thus investigated the potential effect of FTY720 on engrafted neural progenitor cells into the spinal cord of mice with established immune-mediated demyelination (**Chapter 3**). We have previously demonstrated in our laboratory the importance of CXCR4 expression for NPC migration upon engraftment in the spinal cord [14]. Our data highlights the complexity of environmental cues important for NPC migration. We investigated the migration towards CXCL12 of treated NPCs with FTY720 *in vitro* and demonstrated that FTY720 enhances NPC migration. We confirmed that FTY720 enhances migration of GFP labeled NPC engrafted into the spinal cord of MHV infected mice with established

demyelination and thus further highlight S1P as another environment cue for NPC migration. Interestingly, FTY720 did not increase CXCR4 expression by NPCs suggesting that FTY720 affects NPC migration through a different mechanism or through modulation of CXCR4 function but not expression. In addition, FTY720 promoted NPCs proliferation but not differentiation.

Importantly, these studies were investigated in a viral model of demyelination and viral triggers have been suspected to initiate MS [17, 18]. As FTY720 is currently used in patients with MS, an important question is whether the modulation of immune responses can affect viral clearance and we have shown that FTY720 can indeed delay viral clearance during MHV disease. However, our studies also demonstrate that FTY720 can have a direct effect on the CNS and serve as an environmental cue for NPC migration throughout the spinal cord. As FTY720 is being used therapeutically in patients with MS to reduce inflammation and modulate circulating autoreactive T cells, it remains to be determined if FTY720 can promote repair, remyelination and have a neuroprotective effect in humans.

Previous studies have demonstrated the importance of *Olig1* for remyelination in the adult CNS [15]. We demonstrated the importance of *Olig1* for remyelination under inflammatory conditions such as MHV infection (**Chapter 4**) [16]. Engrafted *Olig1-/-* NPC isolated from the SVZ of neonatal pups showed similar migration properties as *Olig1+/+* NPC. However, *Olig1-/-* NPC preferentially differentiated into astrocytes whereas *Olig1+/+* NPC differentiated into oligodendrocytes. Importantly, *Olig1-/-* NPC did not induce a robust remyelination when compared to *Olig1+/+* NPC [16].

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