CXCL1/CXCR2 SIGNALING AXIS AND DISEASE PROGRESSION IN PRE-CLINICAL ANIMAL MODELS OF MULTIPLE SCLEROSIS

by

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ABSTRACT

The Glu-Leu-Arg (ELR)(+) chemokine C-X-C ligand 1 (CXCL1) is widely known to be involved in the recruitment of neutrophils to sites of inflammation by binding to the receptor C-X-C receptor 2 (CXCR2) expressed on the surface of neutrophils. The role of neutrophils in central nervous system (CNS) diseases has not been well characterized. Thus, we wanted to better understand the role of the CXCL1/CXCR2 signaling axis in the context of mouse models of the human demyelinating disease multiple sclerosis (MS), including infection with the JHM strain of mouse hepatitis virus (JHMV) and the myelin oligodendrocyte glycoprotein (MOG) peptide from amino acid 35 to 55 ($MOG_{35\text{-}55}$)-induced Experimental Autoimmune Encephalomyelitis (EAE) model. In both models, the doxycycline (Dox)-inducible expression of CXCL1 from astrocytes selectively increased the recruitment of neutrophils into the spinal cord, correlating with an increase in both morbidity and demyelination. Antibody-mediated depletion of neutrophils during the overexpression of CXCL1 in the CNS correlated with a decrease in the severity of demyelination, indicating a role for neutrophils in white matter disease in JHMV-infected mice and $MOG_{35\text{-}55}$-induced EAE.

During CNS development, proliferation and migration of oligodendrocyte progenitor cells (OPCs) was dependent upon surface expression of the chemokine receptor CXCR2; genetic ablation of *Cxcr2* resulted in misalignments in oligodendrocyte lineage cells (OLCs), decreased white matter in the spinal cord, and decreased myelin

sheath thickness. Subsequent studies have determined that CXCR2 signaling has important roles on both hematopoietic and non-hematopoietic cells in remyelination in different models of demyelination. To better determine the role the CXCR2 signaling on OLCs during the pathogenesis of JHMV and $MOG_{35\text{-}55}$ EAE, mice were engineered in which CXCR2 expression was inducibly ablated on oligodendroglia upon treatment with tamoxifen. Using both the JHMV infection and EAE models, we observed no significant changes in clinical disease, demyelination, or leukocyte infiltration into the CNS following tamoxifen-induced ablation in oligodendrocytes, indicating that CXCR2 in OLCs does not appear to be important in the pathogenesis of JHMV and $MOG_{35\text{-}55}$ EAE.

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LIST OF ABBREVIATIONS

4-OHT 4-Hydroxytamoxifen

AD .... Alzheimer's disease

ADCC .... antibody-dependent cell-mediated cytotoxicity

APCs .... antigen presenting cells

ASCs .... antibody-secreting cells

BBB .... blood brain barrier

Bcl .... B-cell lymphoma

CCL .... C-C ligand

CCR .... C-C receptor

c*Cxcr2*$^{fl/fl}$ .... *Plp-Cre-ER(T) :: Cxcr2*$^{fl/fl}$ *:: R26-stop-Td*$^{+/-}$

CEACAM1a .... carcinoembryonic antigen cell adhesion molecule 1

CFA .... Complete Freund's Adjuvant

CNPase .... cyclic nucleotide phosphodiesterase

CNS .... central nervous system

CSF .... cerebrospinal fluid

CXCL .... C-X-C ligand

CXCR .... C-X-C receptor

DCs .... dendritic cells

DMTs .... disease modifying therapies

Dox .... Doxycycline

d.p.i. .... days post-infection

EAE..Experimental Autoimmune Encephalomyelitis

EBV..Epstein-Barr virus

ELR .... Glu-Leu-Arg

FDA ....Food and Drug Administration

G-CSF ....granulocyte-colony stimulating factor

GFAP .... glial fibrillary acidic protein

GM-CSF..granulocyte-macrophage colony-stimulating factor

GST-π .... glutathione S-transferase

H&E ....hematoxylin and eosin

HERV... human endogenous retroviruses

HHV-6..human herpesvirus 6

HIV-1 .... human immunodeficiency virus 1

i.c.. intracranial

IFN ....interferon

IL.. interleukin

Iono .... Ionomycin

i.p. .... intraperitoneal

JHMV..........J2.2v-1 variant of the John Howard Mueller strain of Mouse Hepatitis Virus

LCMV .... lymphocytic choriomeningitis virus

LFB .... luxol fast blue

mAb... monoclonal antibody

MBP .... myelin basic protein

MHC .... major histocompatibility complex

MHV .... Mouse Hepatitis Virus

MMPs.. matrix metalloproteinases

MOG_{35-55} .... myelin oligodendrocyte glycoprotein peptide from amino acid 35 to 55

mRNA .... messenger ribonucleic acid

MS .... Multiple Sclerosis

NETs .... neutrophil extracellular traps

NK .... natural killer

NMO .... neuromyelitis optica

NPCs .... neural progenitor cells

OLCs .... oligodendrocyte lineage cells

OPCs .... oligodendrocyte progenitor cells

P1 .... post-natal day 1

p.i. .... post-infection

$PLP_{139-151}$ .... proteolipid peptide from amino acids 139 to 151

PMA .... phorbol myristate acetate

PP-MS .... primary progressive Multiple Sclerosis

RNA .... ribonucleic acid

RR-MS .... relapsing remitting Multiple Sclerosis

rtTA .... reverse tetracycline-controlled trans activator

SOCS3 .... suppressor of cytokine signaling 3

SP-MS .... secondary progressive Multiple Sclerosis

tg .... transgenic

TGF .... transforming growth factor

Th .... T helper

TNF .... tumor necrosis factor

TRE .... tetracycline-responsive promoter element

Tregs .... regulatory T cells

UTR..untranslated region

WNV .... West Nile virus

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CHAPTER 1

INTRODUCTION

1.1 Multiple sclerosis

Multiple sclerosis (MS) is a chronic inflammatory and demyelinating autoimmune disease that affects the central nervous system (CNS) (1, 2). Diagnosis of the disease typically ranges between 20 and 40 years of age and similar to most other autoimmune diseases, affects more women than men at a 2-3:1 ratio (3, 4). To date, over 2.5 million people have MS and this number continues to rise (3, 5). MS is considered to be a misguided T cell attack to proteins embedded within the myelin sheath in the CNS. Nonetheless, both the innate and adaptive immune response have been shown to be involved in the pathogenesis of MS, including infiltrating macrophages, activated microglia, natural killer (NK) cells, dendritic cells (DCs), T helper 1 (Th1) and T helper 17 (Th17) CD4+ T cells, CD8+ T cells, B cells, and plasma cells (3). In addition, emerging evidence has begun to show that neutrophils may also be involved in the pathogenesis of MS, by both studies in preclinical animal models of MS e.g. Experimental Autoimmune Encephalomyelitis (EAE) and indirect evidence in MS patients (4, 6-11). While there is no cure for MS, there are many approved therapies that can dampen disease and decrease relapses (12). Current disease modifying therapies (DMTs) that have been effective for MS have mainly been developed to dampen the adaptive immune response, indicating that B and T cells play a large role in the pathogenesis of the disease by dampening new lesion formation and rate of relapse in relapsing remitting MS (RR-MS) patients (5, 13, 14). Symptoms of MS can still occur in patients on these DMTs, arguing that both B and T cells are not the only cell types involved in MS pathogenesis, thus new DMTs for the innate immune response need to be studied and developed to dampen MS symptoms (5, 13, 14).

Environmental factors that may contribute to development of MS include prepubescent development in latitudes higher than 40°, vitamin D deficiency, Epstein-Barr virus (EBV) infection as a child, and cigarette smoke (15-17). In addition to EBV, other pathogens have been identified to be linked to the development and/or exacerbation of MS, including human herpesvirus 6 (HHV-6), and human endogenous retroviruses (HERV) that may lead to induction of MS through molecular mimicry (18-20). Bacterial infections by *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, *Streptococcus pneumoniae*, and the production of superantigens from *Staphylococcus aureus*, as well as infection by the protozoa *Acanthamoeba castellanii* have also been suggested to be risk factors associated with development of MS (18). In contrast, many species of helminthes infections have been associated with a decrease in risk for the development of MS and this has been suggested to be associated with elevated T helper 2 (Th2) immune responses (18). The fact that autoimmune diseases, such as MS, have risen in the past 3 decades in developing countries has given evidence to the hygiene hypothesis, which states that the decrease in the incidence of infectious diseases due to clean water, improved hygiene, antibiotics, and vaccinations leads to an increase in autoimmune diseases (21, 22).

During the early stage of MS pathology, there are acute regions of inflammation within the white matter of the CNS resulting in demyelination (23). Oligodendrocytes will then become destroyed and/or damaged; in addition, axonal conduction will become impeded due to the loss of myelin. This is why many patients will have the following common symptoms that can impair their quality of life, including walking difficulties, sensory disturbances, vision problems, cognitive issues, depression, and intestinal and

sexual dysfunctions (2, 4, 24). In RR-MS patients, episodic focal attacks correlating with many of these common symptoms typically occur, varying in duration from 24 hours to many weeks, which typically completely resolve (25). The remission in these patients is related to decreases in inflammation and partial remyelination to restore some of the lost axonal conduction (26, 27). While oligodendrocyte progenitor cells (OPCs) are typically dispersed throughout a healthy CNS, in subacute lesions within the CNS in MS patients, they can be enriched (28). In addition, perivascular infiltrates into the CNS that migrate and accumulate within subacute lesions of demyelination include activated CD4+ T cells, CD8+ T cells, and macrophages interacting with local reactive microglia to release various proinflammatory cytokines dysregulating oligodendrocytes (23, 29). In addition, CD8+ T cells that are clonally expanded and major histocompatibility complex (MHC) class I restricted appear close to demyelinated axons during MS, thought to interact with myelin protein epitopes (30). Patients will form what are known as shadow plaques in previously demyelinated areas; these are remyelinated white matter areas due to the maturation of OPCs and produce very thin myelin sheath surrounding axons (28, 31-34).

There are two main forms of MS with which patients can be diagnosed; 85% of diagnosed patients have the RR-MS form, while 15% of patients are diagnosed with the primary progressive form (PP-MS) (4). After 30 years with RR-MS, approximately 66% of patients will develop a more progressive form of the disease termed secondary progressive MS (SP-MS) (4). Progressive forms of MS do not have stages of remission and instead, symptoms will progressively get worse. The progressive form of disease does not consist much of the adaptive immune system, although axonal degeneration and grey matter neuropathy persist (35-38). Microglial activation within the CNS, plus

macrophages, T cells, and B cells, are observed near the border of preformed MS lesions (39, 40). While OPCs are known to aid in the remyelination process, OPCs ultimately fail at remyelinating damaged areas of the CNS, resulting in an inability to recover (26, 41).

1.2 Introduction to mouse hepatitis virus

Mouse hepatitis virus (MHV) is a member of the family *Coronaviridae*, a virus family of enveloped positive-sense single-stranded ribonucleic acid (RNA) (26-32kb) viruses (42). MHV has many different strains that can cause various pathologies in mice depending on both strain and age. These include the enterotropic strain MHV-Y, which is highly contagious and can lead to outbreaks in housed lab mice colonies causing enteritis; the hepatotropic strain MHV-2, causing hepatitis by intraperitoneal (i.p.) injection or neurotropic after intracranial (i.c.) injection leading to minor meningitis; and the MHV A59 strain, which is both neurotropic and hepatotropic following i.c. injection that infects neurons and glia in the CNS giving the mice mild encephalomyelitis and some demyelination, while only i.p. injection gives rise to hepatitis (43-46). In addition, the well-studied neurotropic strain of MHV is the neuroattenuated J2.2v-1 variant of the John Howard Mueller strain, termed JHMV, causes acute encephalitis that leads to chronic demyelination in susceptible adult mouse strains (47-50). Demyelination and chronic neuroinflammation in JHMV-infected mice occurs due to the chronic persistence of viral RNA in the white matter tracts (51). Lab strains of mice susceptible to JHMV infection within the CNS are used as a model of viral-induced demyelination and encephalomyelitis to study the human demyelinating disease MS (52). There are many preclinical models that recapitulate clinical and histological symptoms of MS. An

environmental trigger that is proposed to contribute to inducing MS clinical symptoms in genetically susceptible people is viral infection; thus, studying JHMV will give insights into viral-induced demyelination/remyelination during persistent neurotropic viral infection.

JHMV was derived from the highly lethal JHM-DL laboratory strain using a monoclonal antibody (mAb) (49, 50). JHMV is a model to study aspects of viral infection within the CNS for both viral-induced encephalomyelitis and mechanisms regarding neuroinflammation. It is also used as a model for the human demyelinating disease MS to study immunological mechanisms contributing to viral-induced demyelination as well as to study remyelination during in the chronic stage of disease.

The route of infection of JHMV is by direct i.c. infection that first leads to infection and replication of ependymal cells of the lateral ventricles and subsequently infects astrocytes, oligodendrocytes, and microglia by binding to the surface-expressed receptor carcinoembryonic antigen cell adhesion molecule 1 (CEACAM1a) (47-49, 53). Viral titers analyzed by plaque assay within the CNS peak around day 5-7 post-infection (p.i.) and by 14 days post-infection (d.p.i.), no viral replication is detectable, although viral RNA persists within the CNS and leads to a continued neuroinflammation, immune cell infiltration, and demyelination that does not completely resolve (42, 48, 54, 55).

Following i.c. infection with JHMV, the innate antiviral response is rapidly initiated within 48 hours with localized expression of proinflammatory cytokines and chemokines including interleukin-1α (IL-1α), IL-1β, IL-6, IL-12, and tumor necrosis factor-α (TNF-α) and the production of matrix metalloproteinases (MMPs) (47, 56-58). Similar to almost all viral infections, the type I interferon response is an important

immune response for host defense to JHMV infection through the production of interferon-α (IFN-α) early during disease (57, 58). The first cell types infiltrating into the CNS are part of the innate immune response and these include neutrophils, NK cells, and infiltrating monocytes/macrophages (42, 48). During JHMV infection, monocytes/macrophages expressing the chemokine C-C receptor 2 (CCR2) are recruited to the CNS by responding to its C-C ligand 2 (CCL2) (59-61). Extensive research into aspects of the innate immune response have shown that both neutrophils and monocytes/macrophages are important for breaking down the blood brain barrier (BBB) by the production of MMPs and this facilitates CNS entry of virus-specific T cells that aid in controlling viral replication (56, 61-64). NK cells have been shown to not be important for viral clearance (65).

Ultimately, the adaptive immune response is required for control of JHMV within the CNS. This is done primarily by virus-specific CD4+ and CD8+ T cells, which first expand in the draining cervical lymph nodes, and then traffic through the BBB and into the CNS by responding to the chemokines CCL5, CXCL9, and CXCL10 (66-71). CCR2 ablation, but not its ligand CCL2, impeded recruitment of virus-specific T cells to the CNS, resulting in an increase in mortality following JHMV infection of the CNS and it is believed to be due to CCR2 expression on endothelial cells to aid in the breakdown of the BBB (59, 72). In addition, CCL2 signaling is involved in the recruitment of monocytes/macrophages to the CNS during JHMV infection by binding to CCR2 expressed on the surface of these cells (59-61). To help control viral replication during JHMV infection, antigen presenting cells (APCs) will increase MHC class I and II molecules and by the recruitment of virus-specific CD4+ and CD8+ T cells into the CNS

limiting replication within astrocytes, oligodendrocytes, and microglia via production of IFN-γ; in contrast to perforin secretion by CD8+ viral-specific T cells for the control of replicating virus from astrocytes and microglia (42, 54, 73-76).

Although virus-specific T cells help control JHMV replication within infected cells, sterile immunity is not achieved and virus persists primarily within white matter tracts. During chronic JHMV infection, viral recrudescence is controlled by antibody-secreting cells (ASCs) and ablation of these cells results in the reappearance of replicating virus (54, 55, 77-80). While infiltrating T cells and macrophages are important in controlling viral replication during acute disease, these cells are also responsible for the demyelination observed in the spinal cord of JHMV-infected mice (48). In addition, during both the acute and chronic stages of disease, chemokine and chemokine receptor production within the CNS leads to chronic neuroinflammation and demyelination (42, 48).

1.3 Neutrophils, BBB breakdown, and CXCR2 signaling during acute JHMV-induced disease

Neutrophils are part of the innate immune system and are well studied in the context of killing of invading pathogens by releasing toxic factors, including the release of microbicidal granules, neutrophil extracellular traps (NETs), reactive oxygen and nitrogen species, and the ability to phagocytize invading pathogens to digest them (81-84). However, how neutrophils aid in host defense in response to viral infection of the CNS has been less well characterized. Some examples of how neutrophils are involved in CNS viral infection include promoting vascular leakage and lethality in the acute phase

of disease during lymphocytic choriomeningitis virus (LCMV) and enhancement of the expression of the neutrophil attracting chemokine, CXCL5, during human immunodeficiency virus 1 (HIV-1) that contributes to neuronal death (85, 86). Furthermore, neutrophil depletion during West Nile virus (WNV)-induced neurologic disease in mice has shown to play either a beneficial or detrimental role, depending on when neutrophils were depleted (87).

In other preclinical CNS models of neurodegenerative disease, the depletion of neutrophils was beneficial. Decreased recruitment of neutrophils into the CNS by CXCR2 antagonism during middle cerebral artery occlusion resulted in a reduction in neurological deficits and infarct volume (88). In addition, when neutrophils were depleted in a model of aneurysmal subarachnoid hemorrhage, the mice showed memory improvements (89, 90). Lastly, in a model for Alzheimer's disease (AD), targeting neutrophil recruitment to the CNS reduced gliosis and cognitive decline (91). Neutrophils have been shown to aid in breakdown of the BBB early during disease/injury, leading to the enhancement of CNS inflammation in animal models of spinal cord injury, autoimmune demyelination, and viral-induced demyelination (56, 92-95). Together, these data show that neutrophils within the CNS can act as a double-edged sword and that early during infection, they can help open the BBB to allow for clearance of infectious virus, although it is possible that neutrophils can amplify disease due to the secretion of toxic factors and new studies will be needed to better understand the role of prolonged neutrophil infiltration into the CNS during JHMV infection.

Chemokines and chemokine receptors are important for the recruitment of leukocytes to sites of inflammation (96-99). Chemokines have been shown to be

important during different biological processes, including organogenesis, maturation and migration of stem cells, formation of secondary lymphoid tissues, and angiogenesis (98-105). Of particular interest is the chemokine receptor CXCR2, which is present mainly on neutrophils and is important for neutrophil chemotaxis out of the bone marrow and to sites of inflammation (84). The chemotaxis of the CXCR2+ neutrophils occurs when ELR(+) CXC chemokines e.g. CXCL1, 2, 5, and 7 are expressed in inflamed tissue (84, 96). In addition to neutrophil chemotaxis, signaling through CXCR2 is important in oligodendrocyte precursor positioning during CNS development, can be anti-apoptoic, enhance the production of growth factors, and can modulate synaptic transmission (96). In humans, these ELR(+) CXC chemokines will bind the receptors CXCR1 and CXCR2, (96, 106).

In the cerebrospinal fluid (CSF) of MS patients, CXCL8 was elevated and neutrophils were observed in the CSF correlating with clinical relapse (8, 96, 107). In addition, Segal and colleagues have recently shown that in the plasma of RR-MS patients, there is an increase in CXCL1, CXCL5, and neutrophil elastase in patients with new lesion formation and an increase in plasma levels of CXCL1, neutrophil elastase, CCL11, and granulocyte-colony stimulating factor (G-CSF) in SP-MS patients correlated with overall CNS tissue damage by T2 lesion volume analysis (6, 11). Transcriptional profiling studies of lesions from MS patients showed an increase of IL-6, IFN-γ, and IL-17 (10). Thus, the elevated levels of ELR(+) CXC chemokines in MS patients may be due to the production of IL-17 from Th17 cells, which are known to be recruited to the CNS and can signal through the IL-17 receptor, potentially enhancing expression of these chemokines (108-114).

In an early study of transgenic mice in which CXCL1 was constitutively expressed from oligodendrocytes, adult mice showed an increase in neutrophil infiltration into the CNS, causing an increase in mortality (115). We wanted to expand on the understanding of how ELR(+) CXC chemokines played a role in preclinical disease models of MS when overproduced from a natural source within the CNS, specifically CXCL1 from astrocytes. We designed transgenic mice utilizing the reverse tetracycline-controlled transcriptional activation system to allow for controlled overexpression of CXCL1 from astrocytes in response to doxycycline (Dox) treatment (116). Utilizing this "tet-on" system, we used a doxycycline-inducible reverse tetracycline-controlled trans activator (rtTA) protein termed rtTA2s-M2, which will bind more efficiently to the tetracycline-responsive promoter element (TRE) in the presence of Dox, allowing for overexpression CXCL1 (117-119). How inducible expression of CXCL1 affects host defense and disease following JHMV infection of the CNS will be covered in Chapter 3.

1.4 Experimental autoimmune encephalomyelitis: A model of autoimmune inflammatory demyelination

In addition to the JHMV model of neurologic disease and demyelination, EAE is another animal model used to study immune-mediated demyelination and serves as a well-accepted preclinical model of MS. There are many different variations of EAE that can be induced in animal models that can vary depending on the antigen used in immunization, age, sex, and genetic strain of animal (120). EAE has been used for decades as a model for MS and has helped in the understanding of neuroinflammation, immune-mediated tissue damage, and immune surveillance of the CNS (12). A few

commonly studied models of EAE include the use of Lewis rats, DA rats, SJL mice, and C57BL/6 mice (1). The underlying protocol for inducing EAE involves an administration of an encephalitogenic peptide from a myelin-specific protein emulsified in Complete Freund's Adjuvant (CFA) and subsequently injected with pertussis toxin (121-123). For example, SJL mice can be immunized with the proteolipid peptide from amino acids 139 to 151 ($PLP_{139\text{-}151}$), causing a relapsing remitting form of disease (120-123). Another example utilizes the $MOG_{35\text{-}55}$ injected into the C57BL/6 mouse strain, causing an acute neuroinflammation and demyelination that does not completely resolve (121-123). Mechanistic studies of EAE pathogenesis and drug development have employed both EAE models due to the large array of transgenic mice available on these backgrounds (1, 12). Thus, EAE has been used as a proof of concept model for MS, aiding in the development of Food and Drug Administration (FDA)-approved therapies, including glatiramer acetate, mitoxantrone, natalizumab, fingolimod, fumarate, and teriflunomide, as well as drugs that are under clinical trials for MS patients, including rituximab and laquinimod (12, 124, 125).

In EAE, autoreactive T cells in the CNS will interact with CNS resident antigen presenting cells (APCs), including resident microglia, macrophages, DCs, and B cells, that cause an inflammatory cascade leading to white matter damage (124, 126). Infiltrating monocytes/macrophages can migrate into the CNS by an inflammatory cascade through production of cytokines and chemokines produced from these autoreactive T cells (124, 126). These monocytes/macrophages, in combination with autoreactive antibodies produced from plasma cells, lead to myelin destruction and axonal damage through mechanisms, including antibody-dependent cell-mediated

cytotoxicity (ADCC), phagocytosis, and the production of toxic factors (124, 126). It has also been shown that myelin-loaded macrophages can drain back into cervical and lumbar lymph nodes and the spleen where new autoreactive T cells specific for additional myelin-specific proteins can arise (126). Lastly, MOG-specific CD8+ T cells, B cells, microglia, astrocytes, and even neutrophils have been shown to play a role in EAE pathogenesis (4, 124, 127-129). For instance, Th17+ T cells produce many proinflammatory cytokines, including granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-2, IL-9, IL-17, and IL-21 that can all disturb glial cell survival (130, 131).

1.5 Neutrophils and EAE pathogenesis

During EAE, ELR(+) CXC chemokines are elevated in the CNS during the acute stage of disease (132, 133). Neutrophils have been implemented in the pathogenesis of EAE and depletion of neutrophils by anti-Ly6G and/or anti-CXCR2 has shown to delay onset or decrease exacerbation of clinical disease as well as decreased demyelination (132-135). Both G-CSF and CXCL1 drive the migration/recruitment of neutrophils out of the bone marrow and into the blood within 48 hours after MOG_{35-55} peptide immunization (11). There is a significant increase from the preclinical to the onset of symptoms of the ELR(+) CXC chemokines CXCL1, CXCL2, and CXCL5, in the brain and spinal cord of mice immunized with MOG_{35-55} EAE (133). When neutrophils from wild type mice are adoptively transferred into *Cxcr2 -/-* mice, it results in the re-establishment of clinical symptoms, although the clinical scores do not return to wildtype levels, indicating a role for CXCR2+ neutrophils during EAE pathogenesis (132). When MOG_{35-55} EAE mice were treated with anti-CXCL1, onset of clinical disease was delayed

by approximately 3 days, correlating with a decrease in Ly6G+ neutrophils being recruited to the cerebral cortex (133). During MOG_{35-55}, previous studies by Friese and colleagues showed by anti-Ly6G studies that neutrophils are important contributors early during pathogenesis likely by aiding in BBB breakdown and maturing APCs within the perivascular regions within the spinal cord (134). *Cxcr2 -/-* mice were resistant to demyelination in the cuprizone model, demonstrating that CXCR2+ neutrophils were important in inducing demyelination in this model (136).

In atypical EAE, it has been shown that the neutrophil-chemoattractant chemokine CXCL2 is increased in the brainstem, resulting in increased neutrophil accumulation within the CNS and exacerbation of disease (137-139). When the suppressor of cytokine signaling 3 (SOCS3) gene is knocked out in neutrophils, mice are more susceptible to atypcial signs of EAE by allowing for increased recruitment of neutrophils to the cerebellum and brainstem (140). Adoptive transfer studies in EAE have shown that both IFN-γ-producing Th1 cells, and IL-17-producing Th17 cells can independently produce disease and potentially have independent mechanisms (141, 142). Specifically IL-17 has been shown to enhance the expression of ELR(+) CXC chemokines through the IL-17R and knockout studies of *Il-17rc* has shown a dampening of EAE disease correlating with a decrease in ELR(+) CXC chemokines (90). In addition, when *Act1*, part of the IL-17 receptor complex, is knocked out in the CNS - specifically in NG2+ OPCs and to a lesser extent astrocytes (but not in mature oligodendrocytes or neurons), there is a reduction in EAE pathogenesis correlating with a decrease in neutrophil recruitment and ELR(+) CXC chemokine production, including messenger ribonucleic acid (mRNA) of *Cxcl1* and *Cxcl2* in the spinal cord (130). This illustrates that

signaling through OPCs and astrocytes of the Th17 pathway increases ELR(+) CXC chemokines and overall neuroinflammation (130). Together these data indicate a role for ELR(+) CXC chemokines and their receptor, CXCR2, in neutrophils to aid in the pathogenesis of demyelinating mouse models, EAE and cuprizone. Thus, further studies will require determining the pathogenic role of neutrophils during MOG_{35-55} EAE pathogenesis following the breakdown of the BBB; to this end, we utilized the mice described previously that overexpress CXCL1 from glial fibrillary acidic protein (GFAP)+ astrocytes in a Dox-dependent manner and will be discussed in Chapter 4.

1.6 ELR(+) CXC chemokine signaling during chronic JHMV-induced disease and EAE

During chronic JHMV infection, oligodendrocytes serve as a reservoir for JHMV and these animals experience chronic neuroinflammation and demyelination (90). Direct viral-induced lysis does not appear to be a cause of demyelination as immunodeficient mice showed replication in oligodendrocytes with minimal demyelination (49, 53, 143). Adoptive transfer of splenocytes isolated from JHMV infected mouse into JHMV-infected immunodeficient mice results in widespread demyelination, indicating that T cells are important in white matter damage (143-145). Similarly, JHMV infection of CD4 or CD8 knockout mice resulted in demyelination, although the data indicated a more important role for CD4+ T cells in contributing to white matter damage (70). CD4+ T cells have been shown to secrete CCL5, a chemokine that attracts inflammatory macrophages enhancing demyelination in the CNS during JHMV, arguing this is one potential mechanism by which CD4+ T cells contribute to disease (70, 143, 146-148).

Nonetheless, the mechanisms of action of both CD4+ T cells and CD8+ T cells is somewhat controversial during JHMV infection in that, IFN-γ secretion by CD8+ T cells has been shown to enhance demyelination by recruiting macrophages/microglia to white matter tracts, while a different study showed CD4+ T cell secretion of IFN-γ was protective and aided in controlling of viral replication in the CNS (149-151). Activated bystander CD4+ T cells that were not viral antigen specific within the CNS did not appear to contribute to demyelination, arguing that white matter damage is mediated by virus-specific T cells (152). Adoptive transfer of CD4+CD25+FoxP3+ regulatory T cells (Tregs) into JHMV-infected mice resulted in muted clinical disease severity accompanied by reduced neuroinflammation and demyelination (153). Together this shows insights into the mechanisms of how T cells are required for clearance of JHMV from the CNS, although they also contribute to enhanced neuroinflammation and demyelination and into the chronic stage of disease.

T cell production of IFN-γ has been observed out to 4 weeks following JHMV infection and this potentially contributes to dysregulation of oligodendrocytes and demyelination (58). IFN-γ production from CD8+ T cells contributes to demyelination and oligodendrocyte damage and destruction during an adoptive transfer study (149). OPCs have displayed more sensitivity to IFN-γ induced apoptosis compared to mature oligodendrocytes, although apoptosis still occurs in IFN-γ receptor knockout oligodendrocyte lineage cells (OLCs), suggesting the mechanism of demyelination is not only due to IFN-γ; release of chemokines such as CXCL10 by activated glia may cause dysregulation and/or death of oligodendrocytes whereas CXCL1 may be beneficial (74, 154-160). Activated microglia and macrophages are involved in demyelination by

directly stripping myelin from axons (146, 161, 162). While defining the specific roles of microglia and macrophages in demyelination in JHMV-infected mice, recent studies in EAE argue that infiltrating macrophages are more pathogenic than microglia, suggesting this may be true in JHMV infection as well (163). The process of demyelination is multifaceted and many factors contribute to its mechanism, thus utilizing the mouse model of chronic JHMV infection will continue to give us insights into this process and how to combat it.

Growing evidence has suggested that CXCR2 in neutrophils, as well as CXCR2 on resident CNS cells, may play a role in the pathogenesis of MS (4). When MS and neuromyelitis optica (NMO) patients were administered the neutrophil-activating molecule G-CSF, symptoms of disease were exacerbated, arguing that neutrophils may play a role in the pathogenesis of MS and NMO (164, 165). In addition, neutrophils have been shown to be higher in MS patients and in a more primed state than healthy patients (34). In the CNS of MS patients, CXCL1 was upregulated in astrocytes within active lesions adjacent to proliferating CXCR2+ oligodendrocytes (166). Cultured mouse OPCs began to proliferate when given spinal cord astrocytes secreting CXCL1 and human fetal oligodendrocytes from early to mature showed expression of CXCR2, while fetal human astrocytes showed production of CXCL1 when induced by IL-1β, but not IFN-γ or transforming growth factor β (TGFβ) (166-168). It is also known that the CXCL1/CXCR2 signaling axis is important for spinal cord OPC proliferation and positioning during development (169). Adult *Cxcr2 -/-* mice have misaligned glial cells within the CNS including OPC numbers and positioning, as well as decreased white matter area and myelin sheath thickness in the spinal cord and OPCs derived from these

Cxcr2 -/- mice have a decreased capacity to differentiate into mature oligodendrocytes (170). Adoptive transfer of bone marrow transfer of wildtype mouse cells into *Cxcr2 -/-* mice revealed an increase in recovery that was attributed to OPCs increased replication and oligodendrocyte differentiation in demyelinated regions in both EAE and cuprizone models of demyelination (171). This indicated that resident glial cell expression of CXCR2 inhibits myelin repair after demyelination occurred. During both EAE and spinal cord injury, CXCR2 antagonism increased oligodendrocyte differentiation and aided in recovery, suggesting that CXCR2 increases clinical severity, although antagonistic studies are difficult to differentiate between decreased immune cell infiltration and on glial cells (172, 173). All together, this points to a clear role of CXCR2 in OPCs during development and potentially OPC differentiation in general.

During the chronic stage of JHMV infection, our lab has previously determined that anti-CXCR2 treatment increased clinical disease severity that was associated with increased apoptosis of oligodendrocytes and demyelination (160). These data correlated with *in vitro* data from cultured OPCs derived from mouse neural progenitor cells (NPCs) in which CXCL1 administration blocked OPC apoptosis (160). Not only is OPC apoptosis decreased in the presence of CXCL1 during JHMV infection, but also when treated with IFN-γ, possibly by inducing CXCL10, which signals through the specific receptor CXCR3 (158). When cultured OPCs from *Cxcr3 -/-* mice were treated with IFN-γ, there was also a reduction in apoptosis, further arguing that CXCL10 may augment demyelination by directly killing oligodendrocytes (158). Mechanisms of how CXCL1 aids in CXCR2+ OPC protection from apoptosis include decreasing caspase 3, a pro-apoptotic protein, and increasing the anti-apoptotic protein B-cell lymphoma 2 (Bcl2)

(158).

Collectively, findings derived from *in vitro* and *in vivo* models advocate that CXCR2 signaling is involved in oligodendrocyte biology. However, all of these experiments have employed either *Cxcr2* whole-body knockout mice or treatment with blocking antibodies or small molecule antagonists to assess how CXCR2 signaling controls these events. Therefore, accurate interpretation of how CXCR2 signaling modulates oligodendroglia biology is confounded by the fact that microglia, astrocytes, and neurons may express CXCR2, leading to potential off-target effects of CXCR2-targeted therapies. To circumvent this issue, we have generated mice in which CXCR2 is ablated in a tamoxifen-inducible manner in oligodendroglia in adult mice in order to evaluate how signaling through this receptor affects disease progression in EAE and JHMV-induced neurologic disease. These findings are detailed in Chapter 5.

1.7 Summary

Chapters 3 and 4 examine how CXCL1 influences the pathogenesis of JHMV and MOG_{35-55}-induced EAE. Transgenic mice were developed on a C57BL/6 background to overexpress the chemokine CXCL1, driven by a GFAP promoter when induced by the tetracycline derivative, doxycycline, to allow for controlled overexpression of CXCL1 in astrocytes (174). Increased expression of CXCL1 from GFAP+ astrocytes correlated with an enhancement recruitment of neutrophils, but not other immune cells, into the CNS, mainly into the spinal cord, enhancing clinical disease and demyelination in both JHMV and MOG_{35-55} EAE. When neutrophils were depleted by antibody treatment, there was a decrease in CNS pathology, suggesting that neutrophils were directly leading to

demyelination.

Chapter 5 evaluates the role of CXCR2 signaling on OLCs within the JHMV and MOG_{33-55} EAE mouse models of neuroinflammation/demyelination. To accomplish this, we designed transgenic mice in which *Cxcr2* is selectively ablated within oligodendrocytes upon treatment with tamoxifen (175). Neuroinflammation and demyelination were not affected during both the JHMV and MOG_{35-55} EAE pathogenesis, suggesting that CXCR2 signaling on oligodendrocytes is not important in controlling disease progression. Future studies understanding the roles of chemokine and chemokine receptor signaling in glial cells will help us determine novel therapeutic targets for MS patients. Taken together, this indicates that blocking of neutrophil attracting molecules by the use of therapeutic agents may aid in decreasing neuroinflammation and demyelination in MS patients.

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CHAPTER 2

NEUTROPHILS AND VIRAL-INDUCED NEUROLOGIC DISEASE

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Neutrophils and viral-induced neurologic disease

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ABSTRACT

Infection of the central nervous system (CNS) by neurotropic viruses represents an increasing worldwide problem in terms of morbidity and mortality for people of all ages. Although unique structural features of the blood-brain-barrier (BBB) provide a physical and physiological barrier, a number of neurotropic viruses are able to enter the CNS resulting in a variety of pathological outcomes. Nonetheless, antigen-specific lymphocytes are ultimately able to accumulate within the CNS and contribute to defense by reducing or eliminating the invading viral pathogen. Alternatively, infiltration of activated cells of the immune system may be detrimental, as these cells can contribute to neuropathology that may result in long-term cellular damage or death. More recently, myeloid cells e.g. neutrophils have been implicated in contributing to both host defense and disease in response to viral infection of the CNS. This review highlights recent studies using coronavirus-induced neurologic disease as a model to determine how neutrophils affect effective control of viral replication as well as demyelination.
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1. Introduction

Intracranial infection of susceptible mice with the JHM strain of mouse hepatitis virus (JHMV) causes an acute encephalomyelitis followed by a chronic demyelinating disease similar to the human demyelinating disease multiple sclerosis (MS). Early following JHMV infection of the CNS, the virus targets ependymal cells lining the ventricles, replicates, and rapidly disseminates into the brain parenchyma at which point the virus infects and replicates within astrocytes, oligodendroglia, and microglia throughout the brain and spinal cord [45]. In response to viral infection of glial cells, a rapid and orchestrated expression of chemokines occurs that contribute to attracting inflammatory cells into the CNS. In terms of host defense, secretion of chemokines derived from the CNS, including CXCL10 and CCL5, promote the migration and accumulation of virus-specific CD4+ and CD8+ T cells that control viral replication via secretion of IFN-γ and cytolytic activity. While inflammatory T cells are effective in eliminating virus, sterile immunity is not achieved; viral protein and/or RNA persist within astrocytes and oligodendroglia resulting in chronic expression of chemokine genes leading to chronic neuroinflammation and demyelination. Histological features associated with viral persistence include the development of an immune-mediated demyelinating disease similar to the human demyelinating disease MS in that both T cells and macrophages are critical mediators of disease severity and contribute to myelin damage [5,32].

Through the course of both acute and chronic JHMV-induced neurologic infection, there is a coordinated expression of chemokines and chemokine receptors that regulate inflammation contributing to both host defense and disease exacerbation. Among the chemokines expressed during infection are members of the ELR(+) chemokine family CXCL1, CXCL2, and CXCL5. CXCL1 and CXCL2 are potent chemoattractants for neutrophils via binding and signaling through the receptor CXCR2 [28,39,48]. Moreover, PMNs have been shown to enhance CNS inflammation by disrupting blood brain barrier (BBB) integrity in various animal models of chronic neuroinflammation including spinal cord injury (SCI) [9,44] and autoimmune demyelination [4] while blocking or silencing of CXCR2 signaling mutes inflammation and tissue damage in mouse models in which PMN infiltration is critical to disease initiation [2,4,9,18,24,25,43,47].

2. Neutrophils and acute viral-induced encephalomyelitis

Neutrophils represent a component of the innate immune response and provide an essential role in killing invading pathogens through an arsenal of defense mechanisms including release of microbicidal granules and release of reactive oxygen/nitrogen species [3,30]. While a clear role for neutrophils in combating bacterial pathogens is documented [3,30], how these cells contribute to host defense and disease in response to CNS viral infection is less well characterized. McGavern et al. [19] employed two-photon microscopy to elegantly demonstrate that neutrophils, along with monocytes, were responsible for vascular leakage and acute lethality following lymphocytic choriomeningitis virus (LCMV) infection of the CNS. Human immunodeficiency virus-1 (HIV-1) infection of monocyte-derived macrophages increases expression of CXCL5 that serves to attract neutrophils that may augment neuropathology by contributing to neuron death [10]. Experimental

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infection of mice with West Nile virus (WNV) in which neutrophil trafficking to the CNS is impaired results in increased protection from WNV encephalitis by limiting immune cell access to the CNS thus diminishing neuropathology [46]. With regards to JHMV-induced encephalomyelitis, early work by Stohlman et al. [50] highlighted a previously unrecognized role for neutrophils in effectively controlling viral replication within the CNS. The underlying mechanisms by which neutrophils contribute to an effective host defense are related to neutrophil-mediated permeabilization of the blood-brain-barrier (BBB) through release of matrix metalloproteinase 9 (MMP-9) [50] although other factors independent of MMP-9 may also be involved [37]. In addition, monocytes can also enhance T cell accumulation within the CNS of JHMV-infected mice through the glia limitans [37]. Neutrophils are rapidly mobilized from the bone-marrow and into the blood in response to CNS infection by JHMV and this most likely reflects the precipitous increase in expression of the neutrophil chemoattractants CXCL1, CXCL2, and CXCL5 that all bind to their cognate receptor CXCR2 with high binding [12]. Indeed, treatment of JHMV-infected mice with a blocking antibody specific for CXCR2 resulted impaired migration of CXCR2-bearing neutrophils to the CNS and this resulted in increased mortality that was associated with impaired ability to control viral replication within the CNS [12]. Blocking neutrophil accumulation within the CNS resulted in reduced expression of MMP-9, limited permeabilization of the BBB, and diminished infiltration of virus-specific T cells [12]. Collectively, these findings illustrate that neutrophils are an important component of an effective host defense following CNS infection with a neurotropic virus.

3. Neutrophils and viral-induced demyelination

Neutrophil infiltration into the CNS has been associated with neurologic disease in pre-clinical animal models [6,14,34,38,40]. Herz et al. [11] have recently demonstrated that CXCR2 antagonization reduced neurological deficits and infarct volumes following middle cerebral artery occlusion and this was associated with reduced neutrophil infiltration into the CNS. Similarly, depletion of neutrophils following subarachnoid hemorrhage was found to improve memory in a model of aneurysmal subarachnoid hemorrhage (SAH) [33]. Additionally, Zenaro et al. [49] have demonstrated a role for the adhesion molecule lymphocyte function-associated antigen 1 (LFA-1) in promoting neutrophil accumulation within the CNS and amplifying AD-like pathology in transgenic models of Alzheimer's disease (AD). Depletion of neutrophils and/or a deficiency in LFA-1 resulted in protection from cognitive decline and reduced gliosis arguing that blocking neutrophil trafficking may be beneficial in AD [49]. Within models of spinal cord injury/trauma, neutrophils are among the first cells to accumulate within the site of injury and a number of studies argue for a pathogenic role for these cells through limiting tissue sparing and motor recovery while increasing expression of pro-inflammatory cytokines [1,36]. Collectively, these studies demonstrate that in animal models of chronic neuroinflammation/neurodegeneration neutrophils can amplify the severity histologic disease and argue that blocking entry into the CNS may limit the severity of neurologic disease.

A role for neutrophils in immune-mediated demyelination remains to be well characterized. Ransohoff et al. [22], have shown that CXCR2-positive neutrophils are essential for cuprizone-induced demyelination and potentially contribute to oligodendrocyte cell loss. Questions remain regarding the importance of neutrophils in the pathogenesis of MS given the paucity of these cells in active lesions; however, elevated neutrophil numbers within the cerebrospinal fluid (CSF) of MS patients have been correlated with clinical relapse [20]. Administration of granulocyte-colony-stimulating factor (G-CSF), a neutrophil activating molecule, to MS and neuromyelitis optica (NMO) patients resulted in disease exacerbation arguing for a role for these cells in amplifying disease severity [16,31]. Additionally, neutrophils have been reported to be more numerous and exhibit a more primed state in MS patients [29]. Recent studies [15,35] highlight the importance of CXCL1 as well as other myeloid-chemoattractant molecules as having a possible role in potentiating disease in patients with either relapsing-remitting or progressive forms of MS, suggesting that soluble factors that attract neutrophils and/or neutrophil-related molecules may be important therapeutic targets for MS patients. Support for this notion is derived from studies employing experimental autoimmune encephalomyelitis (EAE) as a model for MS in which disease onset is mute when neutrophil trafficking to the CNS is disrupted [4,27]. More recently, Stoolman et al. [42] have expanded on these findings to show that enriched expression of CXCL2 within the brainstem attracts neutrophils that substantially contribute to atypical EAE. Similarly, mice in which neutrophils lack suppressor of cytokine signaling 3 (SOCS3) exhibit an increase in susceptibility to the atypical EAE and this correlates with preferential recruitment of neutrophils into the cerebellum and brainstem [23]. The site of neutrophil recruitment may be critical in terms of amplifying histopathology as neutrophil accumulation within the brain, but to a limited extent in the spinal cord, contribute to tissue injury [41]. Collectively, these findings indicate that neutrophils can affect the severity of clinical disease and neuroinflammation in EAE.

4. A transgenic model to study viral-induced neutrophil-mediated neuropathology

In attempt to better understand how neutrophils influence both host defense and disease following CNS viral infection, we have recently engineered transgenic mice to utilize the tetracycline-controlled transcriptional activation system in which the human glial fibrillary acidic protein (hGFAP) promoter drives expression of a modified version of the reverse tetracycline transactivator protein (rtTA*M2) [26] (Fig. 1A). Astrocytes were chosen for targeted expression of CXCL1 as previous studies [7,8,17] have shown that JHMV-infected astrocytes express CXCL1 [13,21]. In the presence of doxycycline (Dox), transcription initiates at a tet-operon and leads to production of recombinant CXCL1 mRNA transcripts. Double transgenic (tg) mice (pBI-CXCL1-rtTA) and single tg mice (pBI-CXCL1) were generated; characterization of double tg mice revealed Dox-dependent expression of CXCL1 from cultured astrocytes as determined by ELISA (Fig. 1B) [26]. I.c. infection of Dox-treated double tg mice with JHMV resulted in a selective increased expression of CXCL1 mRNA transcripts and protein within the brain and spinal cords when compared to Dox-treated single tg mice infected with JHMV (Fig. 1C) [26]. Dox-induced overexpression of CXCL1 did not enhance control of viral replication within the CNS as both infected double and single tg mice exhibited similar viral titers at defined times post-infection (p.i.) nor were there differences in either frequency or numbers of virus-specific CD4 + and CD8 + T cells within the CNS of double tg mice compared to single tg mice [26]. However, Dox-treatment of JHMV-infected double tg mice resulted in increased clinical disease and mortality when compared to infected single tg mice [26].

In conjunction with increased expression of CXCL1 initiated within the CNS of Dox-treated double tg mice infected with JHMV, there was a rapid increase in CXCL1 protein levels in serum [26]. Correspondingly, there is a rapid increase in neutrophils within the blood at days 4 ($p < 0.05$) and 7 ($p < 0.001$) in double tg mice compared to infected single tg controls [26]. Dox-induced CXCL1 production in JHMV-infected double tg mice also resulted in an increase in neutrophil frequency within the brain at days 4 and 7 p.i. [26]. Similarly, there was an increase in neutrophil frequency within spinal cords of double tg mice at days 4 ($p < 0.01$) and 7 ($p < 0.05$) p.i. compared to single tg mice [26]. Immunofluorescence staining for neutrophils (Ly-6B.2) supported the flow cytometric data and revealed increased numbers of neutrophils accumulating within the meninges of double tg mice at day 7 p.i. [26]. The increased presence of neutrophils within the CNS of double tg mice suggested that there would also be a corresponding increase in blood-brain-barrier (BBB) permeability. Surprisingly, no differences were observed in BBB permeability within the brain or spinal cord at

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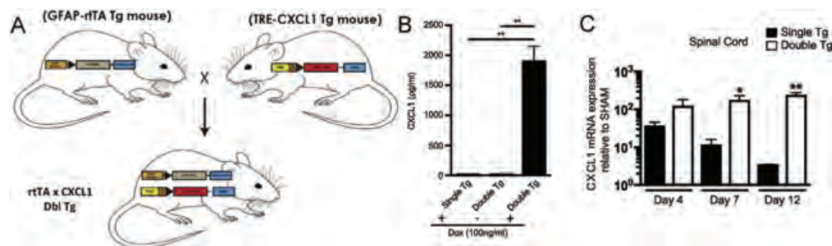

Fig. 1. Derivation and characterization of a mouse model in which CXCL1 expression within the CNS is under control of a Doxycycline promoter. (A) Cartoon depiction of experimental strategy to generate double (dbl) transgenic (tg) mice in which expression of mouse CXCL1 is under control of the GFAP promoter upon doxycycline treatment. (B) Cortex tissue from double tg and single tg post-natal day 1 (P1) mice was dissociated and enriched for astrocytes. Following 24-h of Dox (100 ng/ml) treated double tg astrocyte cultures, immunofluorescence confirmed CXCL1 expression within GFAP-positive astrocytes while vehicle treatment yielded no CXCL1 fluorescence. (C) Within the SC, dox-treated double tg mice had statistically significant increases in CXCL1 mRNA expression over Dox-treated single tg mice at days 7 and 12 p.i. Images adapted from [26] with permission.

day 4 p.i. as measured by sodium fluorescein (NaF) uptake [26]. Examination of spinal cords from JHMV-infected Dox-treated double tg mice revealed an overall increase ($p < 0.05$) in the severity of demyelination when compared to infected single tg animals (Fig. 2A) [26]. The increase in demyelination in double tg mice was associated with a significant ($p < 0.05$) loss of mature oligodendrocytes (as determined by expression of GST-π) within the spinal cords and increased numbers of microglia in Dox-treated JHMV-infected double tg mice compared to

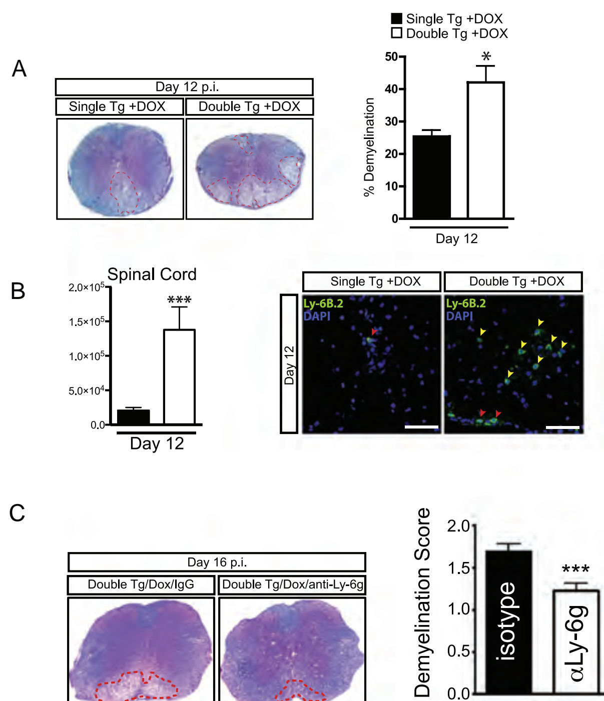

Fig. 2. Elevated CXCL1 expression is associated with increased demyelination. Histopathological analysis of spinal cords of double tg mice reveals an increase in demyelination. (A) Representative luxol fast blue (LFB)-stained spinal cords reveals increased ($p < 0.05$) demyelination in JHMV-infected Dox-treated double tg mice compared to single tg controls. (B) Flow cytometric analysis revealed a significant increase in the frequency and total number of neutrophils within the spinal cord of JHMV-infected Dox-treated double tg mice compared to single tg mice. Representative immunofluorescence staining further demonstrated a significant increase in the number of Ly6B.2-positive neutrophils (yellow arrowheads) within the spinal cord parenchyma of JHMV-infected double tg compared to single tg mice; red arrowheads indicate neutrophils located within the spinal cord meninges. Quantification of neutrophils within the spinal cords indicated an overall increase ($p < 0.05$) in Dox-treated double tg mice compared to Dox-treated single tg mice. (C) Representative LFB-stained spinal cord sections from JHMV-infected double tg mice treated with either control IgG2a or anti-Ly6G antibody between days 3–15 p.i. Quantification of the severity of demyelination revealed reduced white matter damage in mice treated with anti-Ly6G antibody compared to mice treated with isogenic IgG2a control antibody. Images adapted from [26] with permission.

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infected single tg mice [26]. Flow cytometric data indicated that neutrophil frequencies within the spinal cords of infected double tg were significantly increased ($p < 0.01$) as well as their total numbers ($p < 0.001$) at day 12 p.i. compared to single tg mice (Fig. 2B) [26]. Additionally, neutrophils were detected within the spinal cord parenchyma of double tg mice compared to single tg mice (Fig. 2B) [26]. Elimination of neutrophils via administration of anti-Ly6g monoclonal antibody injection into JHMV-infected double tg mice treated with Dox resulted in a reduction in the severity of demyelination when compared to mice treated with isotype control antibody (Fig. 2C) thus demonstrating that neutrophils are capable of augmenting the severity of white matter damage [26].

5. Perspectives

Although a role for neutrophils in host defense following infection with bacterial pathogens has been appreciated for a number of years, how neutrophils affect host defense in response to viral infection of the CNS has not been as well studied. However, it is now clear that neutrophils are capable of enhancing control of viral replication within the CNS through increasing the permeabilization of the BBB thereby allowing antigen-specific lymphocytes access to sites of infection. Equally interesting is how neutrophil infiltration into the CNS contributes to neuropathology e.g. demyelination. Compelling new information derived from clinical studies from MS patients as well as preclinical animal models of MS have emphasized a potential role for these cells in amplifying white matter damage opening the possibility of targeting neutrophil migration into the CNS as a therapeutic strategy to limit CNS damage.

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CHAPTER 3

INDUCIBLE EXPRESSION OF CXCL1 WITHIN THE CENTRAL NERVOUS SYSTEM AMPLIFIES VIRAL-INDUCED DEMYELINATION

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The Journal of Immunology

Inducible Expression of CXCL1 within the Central Nervous System Amplifies Viral-Induced Demyelination

Brett S. Marro,* Jonathan J. Grist,† and Thomas E. Lane†

The functional role of the ELR$^+$ chemokine CXCL1 in host defense and disease following infection of the CNS with the neurotropic JHM strain of mouse hepatitis virus (JHMV) was examined. Mice in which expression of CXCL1 is under the control of a tetracycline-inducible promoter active within glial fibrillary acidic protein–positive cells were generated and this allowed for selectively increasing CNS expression of CXCL1 in response to JHMV infection and evaluating the effects on neuroinflammation, control of viral replication, and demyelination. Inducible expression of CNS-derived CXCL1 resulted in increased levels of CXCL1 protein within the serum, brain, and spinal cord that correlated with increased frequency of Ly6G$^+$CD11b$^+$ neutrophils present within the CNS. Elevated levels of CXCL1 did not influence the generation of virus-specific T cells, and there was no difference in control of JHMV replication compared with control mice, indicating that T cell infiltration into the CNS is CXCL1-independent. Sustained CXCL1 expression within the CNS resulted in increased mortality that correlated with elevated neutrophil infiltration, diminished numbers of mature oligodendrocytes, and an increase in the severity of demyelination. Neutrophil ablation in CXCL1-transgenic mice reduced the severity of demyelination in mice, arguing for a role for these cells in white matter damage. Collectively, these findings illustrate that sustained CXCL1 expression amplifies the severity of white matter damage and that neutrophils can contribute to this process in a model of viral-induced neurologic disease. ***The Journal of Immunology*****, 2016, 196: 000–000.**

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Inoculation of susceptible mice with the neurotropic JHM strain of mouse hepatitis virus (JHMV) into the CNS results in widespread dissemination of virus, leading to infection and replication within glial cells. This is followed by infiltration of virus-specific T lymphocytes that control viral replication through cytokine secretion and cytolytic activity (1). Sterilizing immunity is not achieved, as viral Ag and viral RNA are detected within white matter tracts of surviving mice, resulting in demyelination that is amplified by inflammatory T cells and macrophages. In response to infection, numerous cytokines/chemokines are secreted by inflammatory leukocytes and resident cells of the CNS, generating an inflammatory milieu that contributes to host defense while also promoting disease. Our previous work has evaluated the functional roles for chemokines and chemokine receptors in orchestrating leukocyte recruitment into the CNS in response to JHMV infection that aid in host defense as well as their contribution to demyelination (2–5).

Among the chemokines expressed during acute and chronic JHMV-induced neurologic disease are the ELR$^+$ CXC chemokines CXCL1, CXCL2, and CXCL5 and its signaling receptor CXCR2 (4, 6). Following JHMV infection of the CNS, expression of CNS-derived ELR$^+$ ligands promote the migration of CXCR2-expressing neutrophils (Ly6G/C^{hi}CD11b$^+$) to the blood–brain barrier (BBB), where they can assist in breaking down structural components of the glial limitans through release of matrix metalloproteinases (MMPs) from preformed granules. Indeed, Ab-mediated neutralization of CXCR2 within JHMV-infected mice prevented neutrophil migration to the CNS, and this was associated with a significant increase in mortality and inefficient control of virus replication as a result of impaired parenchymal access of virus-specific T cells (4). Anti-CXCR2 treatment was also found to diminish macrophage recruitment to the CNS as well, making it challenging to define the precise contributions of both immune cell subsets in establishing host defense while also promoting disease when CXCR2 signaling is neutralized (4). Other studies have demonstrated that neutrophil depletion in JHMV-infected mice in which monocyte trafficking to the CNS is compromised does not alter early CNS inflammation, clinical disease severity, or death, but rather it limits parenchymal access of leukocytes at later times following infection, suggesting that neutrophils and monocytes may act separately to shape adaptive immunity following JHMV infection of the CNS (7, 8).

The present study was undertaken to expand our understanding of how ELR$^+$ chemokines influence host defense and disease progression following JHMV infection of the CNS. To this end, we have generated mice in which expression of CXCL1 is under the control of a tetracycline-inducible promoter that is active within glial fibrillary acidic protein (GFAP)$^+$ cells that express an enhanced version of the reverse tetracycline transactivator (rtTA*M2) protein. The successful generation of these pBI-CXCL1-rtTA mice has allowed us to selectively increase CNS expression of CXCL1 in adult mice following JHMV-induced disease and evaluate the effects on neuroinflammation, control of viral replication, and demyelination.

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The online version of this article contains supplemental material.

Abbreviations used in this article: BBB, blood–brain barrier; DOX, doxycycline; GFAP, glial fibrillary acidic protein; i.c., intracerebral(ly); JHMV, JHM strain of mouse hepatitis virus; MMP, matrix metalloproteinase; NaFl, sodium fluorescein; p.i., postinfection; rtTA, reverse tetracycline transactivator; tg, transgenic.

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Materials and Methods

pBI-CXCL1 vector construction

CXCL1 cDNA incorporating XhoI and NheI restriction site ends was generated from the brains of C57BL/6 mice infected with 250 PFU JHMV. CXCL1 cDNA was cloned into a pBI-MCS-EGFP plasmid (9) downstream of a bidirectional tetracycline responsive element. The purified pBI-CXCL1 plasmid was linearized following an overnight incubation with AatII and AseI restriction enzymes to generate a 3350-bp fragment containing the pBI-CXCL1 construct.

Virus and mice

pBI-CXCL1 transgenic (tg) mice were generated by the University of California, Irvine tg mouse facility through DNA microinjection of fertilized C56BL/6 eggs using the linearized pBI-CXCL1 construct (9). The five resulting founder tg mice where mated to wild-type C57BL/6 mice to indentify F_1 offspring containing the transgene. To generate double-tg mice, hemizygous pBI-CXCL1 tgs were crossed to hemizygous GFAP-rtTA*M2 mice (The Jackson Laboratory), resulting in double-tg mice (pBI-CXCL1-rtTA), single-tg mice (rtTA-GFAP or pBI-CXCL1), or wild-type mice. For evaluation of CXCL1 induction in experimental mice, age-matched 5- to 6-wk-old single-tg or double-tg mice were infected intracerebrally (i.c.) with 250 PFU JHMV strain J2.2v-1 in 30 μl sterile HBSS; subsequently, animals were injected with doxycycline (Dox) (50 mg/kg via i.p. injection) starting at day 2 postinfection (p.i.) and continuing through day 12 p.i. SHAM-infected animals received 30 μl HBSS via i.c. injection. For viral titer analysis, one half of each brain or whole spinal cord was homogenized and used in a plaque assay as previously described (10).

Primary astrocyte cultures

Cortices from postnatal day 1 pBI-CXCL1-rtTA (double-tg) and rtTA-GFAP or pBI-CXCL1 (single-tg) mice were dissected and triturated according to previously published protocols (11). In brief, following removal of the meninges, cortical tissue was minced with a razor and placed in prewarmed DMEM containing papain to completely dissociate the tissue. Following further dissociation with a Pasteur pipette, single-cell suspensions were added to poly-D-lysine–coated culture flasks and grown for 9 d in DMEM supplemented with 10% FBS. Flasks were then transferred to an orbital shaker in a 5% CO_2 tissue culture incubator and shaken for ~16 h at 220 rpm to remove loosely adherent contaminating cells. Cells were passaged in 0.05% trypsin and replated. When cells regained confluency, 10 μM Ara-C (Sigma-Aldrich, St. Louis, MO) was added for 3 d to kill dividing cells. Cells were passaged again and added to 24-well culture dishes or Nunc Lab-Tek II chamber slides (Thermo Fisher Scientific, Waltham, MA).

Gene expression analysis

Total cDNA from brains and spinal cords of sham- and JHMV-infected mice at days 4, 7, and 12 p.i. was generated via SuperScript III (Life Technologies, Carlsbad, CA) following homogenization in TRIzol (Life Technologies). To determine relative CXCL1 mRNA expression, real-time SYBR Green analysis was performed using mouse β-actin primers (forward, 5′-GGCCCAGAGCAAGAGAGGTAT-3′; reverse, 5′-ACGCACGATTTCCCTCTCAGC-3′) and mouse CXCL1 primers (forward, 5′-GGCGCCTATCGCCAATG-3′; reverse, 5′-CTGGATGTTCTTGAGGTGAATCC-3′) using a Roche LightCycler 480 (12). CXCL1 expression was determined by normalizing the expression of each sample to β-actin and then quantifying fold change relative to sham-infected double-tg mice. Expression of defined mouse chemokine and cytokine genes within the CNS of experimental mice was determined using a mouse cytokine and chemokine RT^2 Profiler PCR array (Qiagen, Valencia, CA).

ELISA

Assessment of CXCL1 protein within the supernatants of Dox-treated astrocyte cultures was determined using a CXCL1 (KC) DuoSet sandwhich ELISA kit (R&D Systems, Minneapolis, MN) according to manufacturer specifications. To determine CXCL1 protein levels within the spinal cords of double- and single-tg mice, spinal cords were homogenized in RIPA buffer and clarified by high-speed centrifugation. Following quantification of total protein, samples were diluted in RIPA buffer to a standard protein concentration before performing the ELISA assay.

Flow cytometry

Flow cytometry was performed to immunophenotype inflammatory cells entering the CNS using established protocols (3, 13). In brief, single-cell suspensions were generated from tissue samples by grinding with frosted microscope slides. Immune cells were enriched by a two-step Percoll cushion (90 and 63%) and cells were collected at the interface of the two Percoll layers. Before staining with the fluorescent Abs, isolated cells were incubated with anti-CD16/32 Fc Block (BD Biosciences, San Jose, CA) at a 1:200 dilution. Immunophenotyping was performed using either rat anti-mouse IgG or Armenian hamster anti-mouse IgG Abs for the following cell surface markers: F4/80 (Serotec, Raleigh, NC), MHV S510-tetramer (National Institutes of Health), MHV M133-tetramer (National Institutes of Health), CD4, CD8, Ly6g, CD11b, IFN-γ (BD Biosciences), and Ly6c (eBioscience, San Diego, CA). The following flow cytometric gating strategies were employed for immunophenotyping inflammatory infiltrates in the brain: neutrophils ($CD45^{hi}CD11b^{+}Ly6G^{+}$), monocytes ($CD45^{hi}CD11b^{+}Ly6C^{+}Ly6G^{-}$), macrophages ($CD45^{hi}CD11b^{+}F4/80^{+}$), microglia ($CD45^{lo}$, $CD11b^{+}$, $F4/80^{lo}$), M133-specific $CD4^{+}$ T cells ($CD45^{hi}$, $CD4^{+}$, M133-tetramer^{+}), and S510-specific $CD8^{+}$ T cells ($CD45^{hi}$, $CD8^{+}$, S510-tetramer^{+}).

Sodium fluorescein BBB permeability assay

JHMV-infected double- or single-tg mice received 100 μl 10% sodium fluorescein (NaFl) in PBS via i.p. injection. After 30 min, mice were euthanized and perfused with 30 ml PBS to remove NaFl from the vasculature. Brains and spinal cords were dissected, weighed, and homogenized in PBS. Homogenates were clarified by centrifugation at 500 × *g* for 10 min at 4°C. A 1:1 ratio of supernatant was combined with 15% trichloroacetic acid and incubated for 24 h at 4°C. Samples were centrifuged and supernatants were neutralized with 5 N NaOH. Fluorescence intensity was assessed with a Synergy H1 (BioTek, Winooski, VT) set at a 485 nm excitation, and NaFl concentrations were determined through extrapolation from a standard curve generated from defined NaFl concentrations.

Clinical severity and histopathology

Clinical disease severity was assessed using a four-point scoring scale as previously described (3). Mice were euthanized according to Institutional Animal Care and Use Committee guidelines and perfused with 30 ml 4% paraformaldehyde. Spinal cords were removed, fixed overnight in 4% paraformaldehyde at 4°C, and separated into eight 1.5-mm sections. Each section was cryoprotected in 20% sucrose for 5 d before embedding in OCT; 8-μm-thick coronal sections were cut and stained with Luxol fast blue and H&E. The severity of demyelination for each mouse was determined using 6–12 spinal cord coronal sections per mouse using either a previously described four-point scoring scale (14) and calculated as average ± SEM or determining the percentage demyelination using ImageJ software (National Institutes of Health).

Immunofluorescence

Spinal cord sections were processed as above. For immunofluorescence, slides were first desiccated for at least 2 h and blocked with 5% normal donkey serum with or without 0.3% Triton X-100. Primary Abs were incubated overnight at 4°C, including goat anti-CXCL1 1:50 (R&D Systems), rabbit anti-GFAP 1:500 (Life Technologies), rat anti-Ly6B.2 1:100 (Serotec, Raleigh, NC), rabbit anti-Iba11:500 (Wako Chemicals, Richmond, VA), and rabbit anti–GST-π 1:1000 (MBL International, Woburn, MA).

Neutrophil depletion

Mice received either 250 μg anti-Ly6g clone 1A8 or isotype control (Bio X Cell, West Lebanon, NH) via i.p. injection every other day beginning between days 3 and 15 p.i. Targeted depletion of neutrophils was confirmed by flow staining of circulating neutrophils at defined times after treatment with Ly6g-specific Ab.

Statistical analysis

Flow cytometric data were analyzed with FlowJo (Tree Star), and statistical analyses were performed using Prism (GraphPad Software)

Results

Dox treatment of pBI-CXCL1-rtTA double-tg mice elevates CXCL1 mRNA and protein within the CNS

In response to CNS infection of C57BL/6 mice with JHMV, the ELR^{+} chemokine CXCL1 is expressed at early times after infection and serves to attract CXCR2-expressing neutrophils to the CNS that aid in host defense by contributing to the

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permeabilization of the BBB (4). To better understand how CXCL1 signaling affects host defense and disease progression following viral infection of the CNS, tg mice were engineered to use the tetracycline-controlled transcriptional activation system in which the human GFAP promoter drove expression of a modified version of the rtTA protein (rtTA*M2). Astrocytes were chosen for targeted expression of CXCL1, as previous studies (15–17) have shown these cells to be the primary source of CXCL1 in models of neuroinflammation as well as following infection of astrocytes with JHMV (5, 6). In the presence of Dox, transcription initiates at a tetracycline operon and leads to production of rCXCL1 mRNA transcripts. Double-tg mice (pBI-CXCL1-rtTA) were generated and genotyped to confirm that they incorporated both constructs (Fig. 1A). Characterization of double-tg mice revealed Dox-dependent expression of CXCL1 from cultured astrocytes as determined by immunofluorescence staining and ELISA (Fig. 1B, 1C).

We tested whether treatment of tg mice with Dox resulted in overproduction of CXCL1 within the brain and spinal cord of the CNS of JHMV-infected mice. Double-tg or single-tg mice were infected i.c. with JHMV (250 PFU) and subsequently treated daily with Dox (50 mg/kg) via i.p. injection starting at day 2 p.i. and continuing through day 12 p.i. (Fig. 2A). Dox treatment resulted in increased mRNA transcripts specific for CXCL1 in the brains of infected double-tg mice at days 4, 7, and 12 p.i. compared with single-tg mice (Fig. 2B). Similarly, CXCL1 transcripts were elevated in the spinal cords at days 4, 7 ($p < 0.05$), and 12 ($p < 0.01$) p.i. when compared with single-tg mice (Fig. 2C). To confirm that that CXCL1 mRNA was derived from the transgene and not from germline-encoded CXCL1, we employed quantitative PCR primers that specifically anneal to an untranslated region region specific for endogenous CXCL1 mRNA and not tg CXCL1. As shown in Fig. 2D, there was only a marginal increase in endogenously produced CXCL1 mRNA transcripts when compared with transgene-encoded CXCL1. These results confirm that increased CXCL1 mRNA transcripts are derived from the CXCL1 transgene. CNS-derived CXCL1 in Dox-treated double-tg mice resulted in increased CXCL1 protein in both the spinal cord (Fig. 2E) and serum (Fig. 2F) compared with single-tg mice at day 7 p.i. Immunofluorescence staining confirmed increased CXCL1 protein present within the CNS and astrocytes as the primary cellular source in Dox-treated double-tg mice (Fig. 2G). Finally, overproduction of rCXCL1 within the CNS of JHMV-infected mice did not result in increased expression of other proinflammatory cytokines and chemokines within the spinal cord (Fig. 2H) or brain (data not shown). These data demonstrate that double-tg mice are responsive to Dox treatment, resulting in increased levels of CXCL1 derived within the CNS, and this corresponds to elevated levels of circulating CXCL1 within the serum. However, CXCL1 overproduction does not impact the expression of other proinflammatory factors involved in JHMV host defense and disease.

CXCL1 overexpression increases morbidity/mortality in double-tg mice

Dox-induced overexpression of CXCL1 within the CNS resulted in a statistically significant increase in clinical disease severity (Fig. 3A) and this correlated with an increase in mortality (Fig. 3B). This was not associated with impaired ability to control viral infection in Dox-treated double-tg mice, as there were comparable titers in the brains at days 4 and 7 p.i. whereas lower viral titers ($p < 0.05$) were found in spinal cords at days 4 and 7 p.i. (Fig. 3C, 3D). Both double-tg and single-tg animals reduced CNS viral titers to below detectable titers as measured by plaque assay within the brain by day 12 p.i. (Fig. 3C) whereas viral titers within the spinal cord at day 12 p.i. were near the limit of detection (Fig. 3D). Moreover, no differences were observed with viral RNA load between double-tg and single-tg mice at day 12 p.i. (data not shown). Treatment of JHMV-infected astrocytes with rCXCL1 did not decrease viral titers, ruling out that CXCL1 did

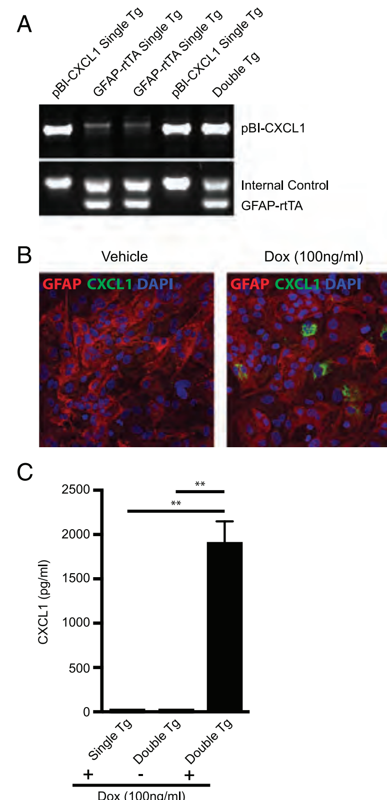

FIGURE 1. Generation and in vitro characterization of a Dox-inducible, astrocyte-specific CXCL1 overexpressing mouse line. (**A**) Double-tg mice utilizing the tetracycline operon–controlled Dox-inducible system were generated by crossing hemizygous human GFAP-rtTA*M2 mice to a tg mouse line incorporating a tetracycline responsive element driving expression of CXCL1 (pBI-CXCL1). Shown are PCR results with genomic DNA from mice revealing amplicons specific for either pBI-CXCL1 or GFAP-rtTa. The internal control was performed when detecting GFAP-rtTa amplicons according to defined Jackson Laboratory protocols. (**B**) Cortex tissue from double-tg and single-tg postnatal day 1 mice was dissociated and enriched for astrocytes. Following 24 h of Dox (100 ng/ml)-treated double-tg astrocyte cultures, immunofluorescence (original magnification ×20) confirmed CXCL1 expression within $GFAP^{+}$ astrocytes whereas vehicle treatment yielded no CXCL1 fluorescence. (**C**) Supernatants from Dox-treated double-tg and single-tg astrocytes cultures were collected and levels of CXCL1 were measured by ELISA. In the presence of Dox, double-tg cultures yielded significantly elevated CXCL1 protein levels, whereas single-tg cultures did not. Data from (C) represent supernatants pooled from triplicate wells derived from three separate double-tg and single-tg mouse cultures. Data are presented as average ± SEM; statistical significant was measured with an unpaired two-tailed Student *t* test. **$p < 0.01$.

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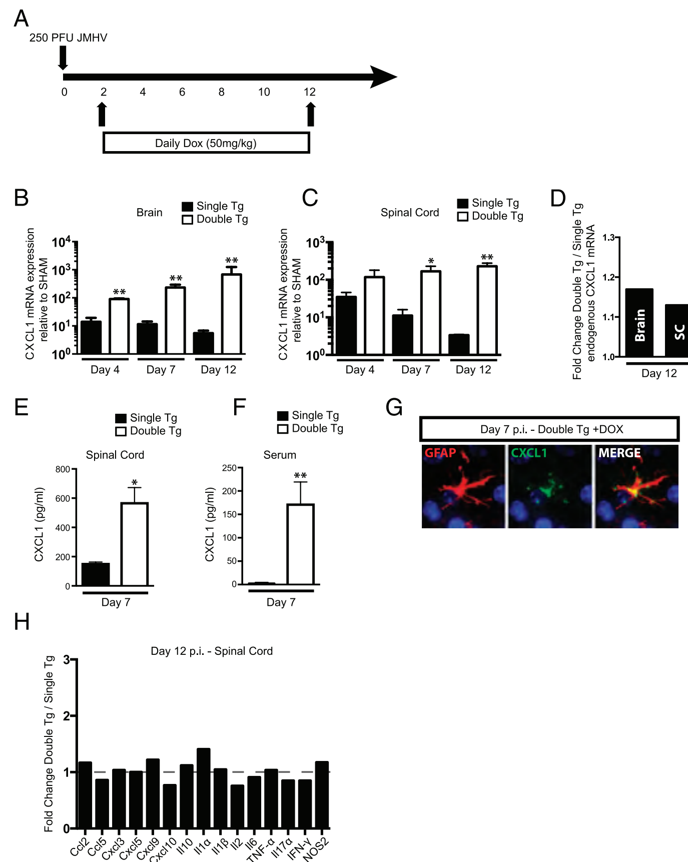

FIGURE 2. CXCL1 is induced in vivo following administration of Dox into double-tg mice during acute JHMV infection. (**A**) To test the ability of Dox to induce CXCL1 overproduction in vivo, double-tg and single-tg mice were infected with 250 PFU JHMV and treated daily with 50 mg/kg Dox between days 2 and 12 p.i. (**B**) Administration of Dox to double-tg mice resulted in a significant increase in the expression of CXCL1 mRNA compared with Dox-treated single-tg mice within the brain at days 4, 7, and 12 p.i. as measured by quantitative real-time PCR. (**C**) Within the spinal cord, dox-treated double-tg mice had statistically significant increases in CXCL1 mRNA expression over Dox-treated single-tg mice at days 7 and 12 p.i. (**D**) CXCL1 transgene expression within the brain and spinal cord did not impact endogenous CXCL1 production within Dox-treated double-tg mice compared with Dox-treated single-tg mice. Overproduction of CXCL1 protein was observed within the spinal cord at day 7 p.i. within double-tg mice (**E**), and this correlated with an increase in CXCL1 protein within the blood serum (**F**) at day 7 p.i. as measured by ELISA. (**G**) Immunofluorscence analysis (original magnification ×40) of spinal cord tissue from Dox-treated double-tg mice confirmed that astrocytes were the source of CXCL1 production at day 7 p.i. (**H**) No significant changes in proinflammatory gene expression between double-tg and single-tg mice were observed within the spinal cord at day 12 p.i. using an RNA superarray. Data from (B) represent two experiments with a minimum of four mice per group. All quantitative real-time PCR samples were run in triplicate. (D) and (E) represent a minimum of three mice per group. Superarray data were compiled used the average value of two mice per group run in duplicate. Data are presented as average ± SEM; statistical significance was measured using an unpaired two-tailed Student *t* test. $*p < 0.05$, $**p < 0.01$.

not exert a direct antiviral effect on infected glial cells (data not shown). Collectively, these data indicate that the augmented clinical disease severity in Dox-treated double-tg mice is not a result of an inability to control viral replication within the CNS.

Neutrophil migration to the CNS is increased in Dox-treated double-tg mice

We have previously shown that neutrophils migrate into the CNS of JHMV-infected mice through a CXCR2-dependent mechanism (4). Therefore, we determined whether Dox-induced CXCL1 overexpression within the CNS resulted in enhanced mobilization of neutrophils to the CNS in response to JHMV infection. JHMV-infected double-tg and single-tg mice were i.c. infected with virus, treated with Dox, and mice were sacrificed at days 4 and 7 p.i. and neutrophil (Ly6G^{hi}CD11b^{+}) levels in blood were measured by flow cytometry. CXCL1 overexpression from the CNS resulted in significantly increased frequency of neutrophils within the blood at days 4 ($p < 0.05$) and 7 ($p < 0.001$) p.i. in double-tg mice compared with infected single-tg controls (Fig. 4A). Dox-induced CXCL1 production in JHMV-infected

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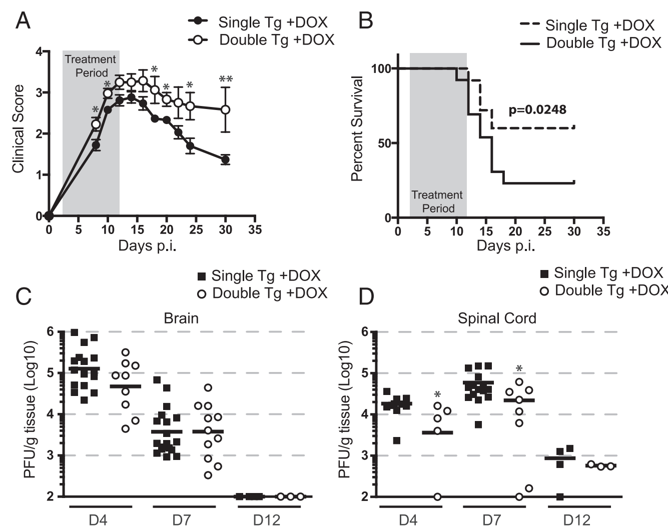

FIGURE 3. CXCL1 overproduction amplifies JHMV-induced clinical disease and mortality but is not a result of delayed viral clearance. Double-tg and single-tg mice were infected with 250 PFU JHMV and treated with 50 mg/kg Dox daily starting at day 2 p.i. through day 12 p.i. (**A**) Clinical severity was assessed until day 30 p.i. using a four-point scale. (**B**) Dox treatment of double-tg mice led to significantly increased mortality compared with Dox-treated single-tg controls. Viral titers of Dox-treated double-tg and single-tg mice were measured within both the brain (**C**) and spinal cord (**D**) by plaque assay. Each symbol represents one individual mouse; solid black lines represent geometric means. Statistical significance was measured with the Mann–Whitney *U* test. Clinical disease and mortality scoring represents an average of double-tg and single-tg mice. For (A) and (B), $n = 23$ single-tg mice and $n = 12$ double-tg mice. Statistical significance for the clinical scoring data was determined by a Student *t* test for each time point assessed. Statistical comparison of the survival curves was measured using the Mantel–Cox test. $*p < 0.05$, $**p < 0.01$.

double-tg mice also resulted in a significant increase in neutrophil frequency within the brain at days 4 ($p < 0.0001$) and 7 ($p < 0.01$) p.i. (Fig. 4B). Similarly, there was an increase in neutrophil frequency within spinal cords of double-tg mice at days 4 ($p < 0.01$) and 7 ($p < 0.05$) p.i. compared with single-tg mice (Fig. 4C). Immunofluorescence staining for neutrophils (Ly6B.2) supported the flow cytometric data and revealed increased numbers of neutrophils accumulating within the meninges of double-tg mice at day 7 p.i. (Fig. 4D). The increased presence of neutrophils within the CNS of double-tg mice suggested that there would also be a corresponding increase in BBB permeability. Rather, no differences were observed in BBB permeability within the brain or spinal cord at day 4 p.i. as measured by NaFl uptake (Fig. 4E) and at day 7 p.i. (data not shown). We also determined whether Dox-induced expression of CXCL1 affected mobilization and migration of $Ly6C^+$ monocytes to the CNS, as previous studies revealed an important role for these cells in amplifying the severity of autoimmune-mediated demyelination (18). There were no significant differences in the frequency $CD11b^+Ly6C^+Ly6G^-$ cells within the blood or brains of Dox-treated double-tg mice as compared with single-tg mice at defined times after infection with JHMV as determined by flow cytometric analysis (Fig. 4F, 4G). However, there was a slight but significant ($p < 0.05$) decrease in numbers of $CD11b^+Ly6C^+Ly6G^-$ cells within the spinal cords of Dox-treated double-tg mice at day 12 p.i. compared with single-tg mice (Fig. 4G) There were no differences in numbers of $CD11b^+$ $Ly6C^+Ly6G^-$ cells within the blood or CNS of Dox-treated double-tg mice compared with single-tg mice (data not shown).

Demyelination is increased in response to elevated CNS expression of CXCL1

Examination of spinal cords from JHMV-infected, Dox-treated double-tg mice revealed an overall increase ($p < 0.05$) in the severity of demyelination when compared with infected single-tg animals (Fig. 5A). Analysis of defined spinal cord sections revealed increased demyelination along the length of the spinal cords of infected double-tg mice compared with single-tg mice, although pathology was enhanced within the upper thoracic region and to a lesser extent within the lower thoracic/lumbar regions (Fig. 5A). The increase in demyelination in double-tg mice was associated with a significant ($p < 0.05$) loss of mature oligodendrocytes (as determined by expression of GST-π) within the spinal cords (Fig. 5B). Additionally, there were increased numbers of microglia ($CD45^{lo}F4/80^+$) cells in Dox-treated, JHMV-infected double-tg mice compared with infected single-tg mice within the spinal cord as determined by flow cytometry as well as immunohistochemical staining for Iba1 (Fig. 5C). Immunophenotyping cells infiltrating into the brain via flow cytometry revealed no differences in the frequencies or numbers of $CD45^{hi}$ cells at defined times after infection (Supplemental Fig. 1). Within the spinal cord, there was a significant increase in the frequency of $CD45^{hi}$ infiltrates at day 4 p.i. and reflects the early enhancement of neutrophils in double-tg mice treated with Dox (Supplemental Fig. 1). However, there were no differences in $CD4^+$ T cells (Supplemental Fig. 1), $CD8^+$ T cells (Supplemental Fig. 1), or virus-specific $CD4^+$ (Supplemental Fig. 1) or $CD8^+$ T cells (Supplemental Fig. 1) infiltration into the brain or spinal cords of experimental mice. Overexpression of CXCL1 in Dox-treated, JHMV-infected double-tg mice did not increase numbers of infiltrating macrophages

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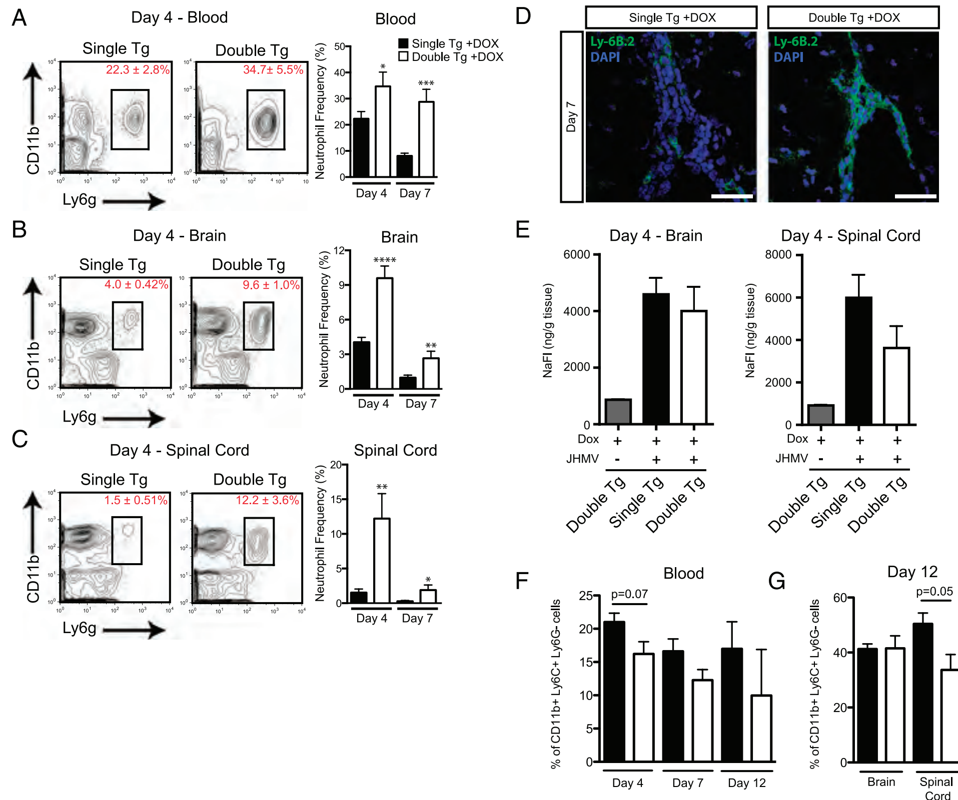

FIGURE 4. CXCL1 overproduction from astrocytes mobilizes neutrophils and directs them to the CNS. Blood from Dox-treated, JHMV-infected double-tg and single-tg mice was isolated at day 4 and day 7 p.i. and used for flow cytometric analysis. (**A**) The frequency of $CD11b^+Ly6G^+$ neutrophils was significantly higher within the blood at days 4 and 7 p.i. Neutrophil migration to the brains (**B**) and spinal cords (**C**) of both groups was assessed by flow cytometry at days 4 and 7 p.i. A significant increase in neutrophil frequency within the brain and spinal cord was observed in Dox-treated double-tg mice at both time points. (**D**) Immunofluorescence analysis indicated that $Ly6B.2^+$ neutrophils were primarily located at the ependymal lining and perivascular space at the spinal cord, with minimal neutrophil presence within the parenchyma. Scale bars, 50 μM. (**E**) At day 4 p.i., brains and spinal cords from Dox-treated single-tg and double-tg mice treated with NaFl were removed and total NaFl uptake was measured. No differences in the frequency of $CD11b^+Ly6C^+Ly6G^-$ cells were detected within either the blood (**F**) or brain and spinal cords (**G**) between Dox-treated double-tg or single-tg mice at defined times after infection with JHMV. (A)–(C) represent three independent experiments with a minimum of three mice per group per experiment at each time point analyzed; (F) and (G) represent two independent experiments with a minimum of three mice per group per experiment at each time point analyzed. Data are presented as average ± SEM; statistical significance was measured using an unpaired two-tailed Student t test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

($CD45^{hi}F4/80^+$) in either the brains (Supplemental Fig. 2) or spinal cords (Supplemental Fig. 2) as compared with infected single-tg mice (Supplemental Fig. 2). Additionally, no differences were detected in expression of activation markers MHC class II (Supplemental Fig. 2) or CD80 (Supplemental Fig. 2), arguing that overexpression of CXCL1 in response to Dox treatment was not enhancing the activation state of inflammatory macrophages that contributed to increased myelin destruction.

Neutrophil accumulation within the spinal cord correlates with increased demyelination

We next determined whether neutrophil infiltration into the CNS was associated with the increase in both clinical and histologic disease in double-tg mice. Flow cytometric data indicated that neutrophil frequencies within the spinal cords of infected double-tg mice were significantly increased ($p < 0.01$) as well as their total numbers ($p < 0.001$) at day 12 p.i. compared with single-tg mice (Fig. 6A). CXCL1 expression, as determined by immunohistochemical staining, was elevated within the spinal cords of Dox-treated double-tg mice as compared with treated single-tg mice (Fig. 6B). Correspondingly, in double-tg mice, a significant increase in neutrophils ($p < 0.05$) was detected within the spinal cord parenchyma of double-tg mice compared with single-tg mice (Fig. 6C, 6D). Notably, neutrophils were enriched within the upper thoracic region of the spinal cord undergoing demyelination whereas these cells were relatively absent in demyelinating lesions in single-tg mice (Fig. 6E). To determine whether the robust parenchymal presence of neutrophils within the spinal cords of double-

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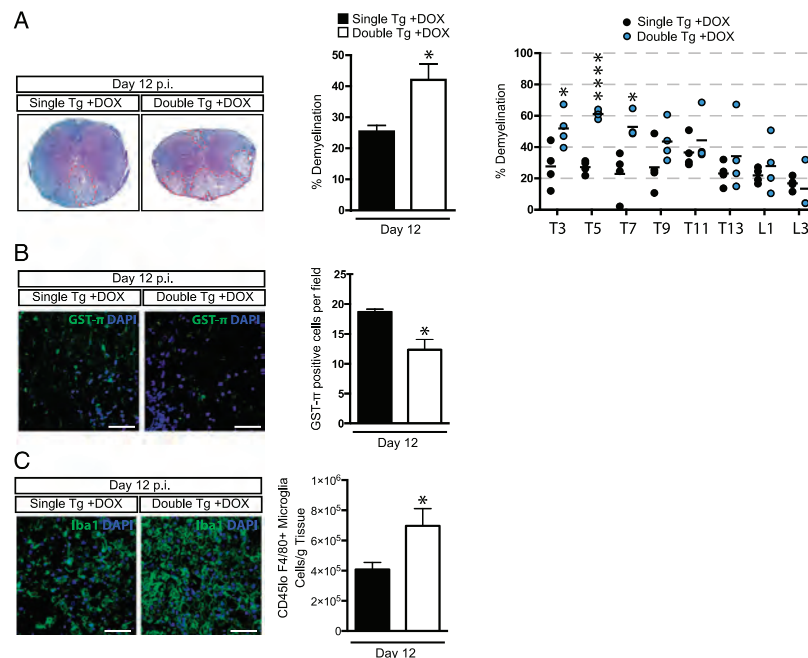

FIGURE 5. Elevated CXCL1 expression is associated with increased demyelination. Histopathological analysis of spinal cords of double-tg mice reveals an increase in demyelination. (**A**) Representative Luxol fast blue–stained spinal cords (original magnification ×4) reveal increased ($p < 0.05$) demyelination in Dox-treated double-tg mice compared with single-tg controls. Assessment of demyelination in defined spinal cord sections indicates white matter damage is more concentrated within the upper thoracic (T) regions (T3–T7) compared with lower T9–T13 as well as lumbar (L) regions examined. (**B**) Increased demyelination was associated with a reduction in the number total GST-π^+ oligodendrocytes within the white matter. The dot plot presented in (A) represents the percentage demyelination at specific anatomical regions of the spinal cord. (**C**) Immunofluorescence staining for Iba1 revealed increased numbers of positive cells within areas of demyelination in spinal cords of double-tg compared with single-tg mice at day 12 p.i., and flow analysis indicated increased ($p < 0.05$) numbers of microglia ($CD45^{lo}F4/80^+$). Scale bars, 50 μM. (A)–(C) represent a minimum of four mice per group. Data are presented as average ± SEM; statistical significance was measured using an unpaired two-tailed Student *t* test. *$p < 0.05$.

tg mice was associated with the enhanced lesion load, neutrophils were eliminated from the periphery using anti-Ly6g mAb injection from days 3 to 15 p.i. (Fig. 7A). Flow analysis of immune cell infiltrates within the spinal cord confirmed neutrophil depletion following anti-Ly6g treatment (Fig. 7A). Supporting our previous findings that double-tg mice display an increase in white matter demyelination compared with single-tg mice was the observation that isotype-treated double-tg mice displayed a significant increase ($p < 0.001$) in the percentage of total white matter demyelination compared with anti-Ly6g–treated single-tg mice (Fig. 7B).

Discussion

The ELR^+ CXC chemokines (CXCL1–3 and CXCL5–8) are high-affinity ligands for their cognate receptor CXCR2. This signaling axis serves critical functions in host defense and disease, as it is the dominant chemotactic pathway for neutrophil homing to sites of inflammation. CXCL1, CXCL2, and CXCL5 are upregulated within the CNS shortly following JHMV infection and control neutrophil migration to the BBB, resulting in peak accumulation by 3 d p.i (4). The importance of neutrophils in host defense to JHMV infection has been investigated with the use of Abs that either eliminate neutrophils from the periphery or prevent their chemotaxis to the CNS. Many of these studies (4, 19, 20) have implicated the short-lived neutrophils as a primarily cellular source for the protease MMP9 during acute JHMV infection. However, no studies have investigated how chronic ELR chemokine overproduction from the CNS impacts neutrophil function as well as host defense and disease following JHMV infection.

The successful generation of a Dox-dependent CXCL1-expressing tg mouse has allowed for selective CXCL1 production from astrocytes within mice infected with JHMV. Dox administration resulted in elevated CXCL1 transgene expression within the brains and spinal cords of double-tg mice, correlating with rapid and selective neutrophil migration to the CNS compared with Dox-treated controls. As a result of CXCL1 transgene expression, double-tg mice displayed a sustained increase in clinical disease severity congruent with an increase in mortality. This observation shares similarities to mice infected with the lethal DM-JHMV variant that is characterized by a severe disease whereby neutrophils dominate the immune cell composition early following infection (20). Overaccumulation of neutrophils in the CNS did not result in an increase in BBB permeability or affect the expression of proinflammatory factors that could potentially attract inflammatory leukocytes. Indeed, we observed

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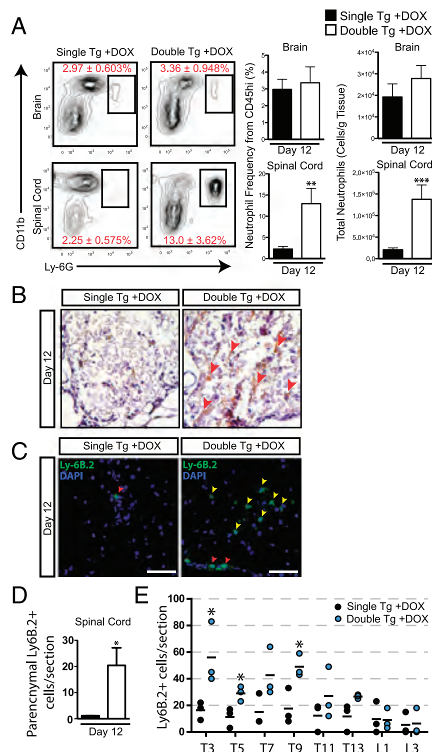

FIGURE 6. Neutrophils are found within parenchymal regions of the spinal cord in double-tg mice. (**A**) At day 12 p.i. brains and spinal cords were isolated and single-cell suspensions were prepared from double-tg and single-tg mice infected with JHMV. Flow cytometric analysis revealed a significant increase in the frequency and total number of neutrophils within the spinal cord of double-tg mice. (**B**) CXCL1 protein was detected by immunohistochemical staining (red arrowheads) within spinal cords of Dox-treated double-tg mice, and (**C**) immunofluorescence staining further demonstrated a significant increase in the number of Ly6B.2^{+} neutrophils (yellow arrowheads). Scale bars, 50 μM. (**D**) Quantification of neutrophils within the spinal cords indicated an overall increase ($p < 0.05$) in Dox-treated double-tg mice compared with Dox-treated single-tg mice. (**E**) Quantification of neutrophils in defined anatomic regions of spinal cords showed increased numbers of neutrophils ($p < 0.05$) within upper thoracic (T) regions (T3–T9) and lower numbers at T11–T13 and lumbar (L) regions in Dox-treated double-tg mice compared with Dox-treated single-tg mice. (A) represents three independent experiments with a minimum of three mice per group per experiment. (D) and (E) represent a minimum of three mice per group. Data are presented as average ± SEM; statistical significance was measured using an unpaired two-tailed Student *t* test. *$p < 0.05$.

no statistical difference in the frequencies of macrophages or T cells between double-tg and single-tg mice at day 12 p.i. as measured by flow cytometry. These results are partially supported by Savarin et al. (7, 8) who have provided evidence that neutrophils are not essential for promoting access of activated immune cells into the CNS, as neutropenic mice or MMP9-deficient mice infected with sublethal strains of JHMV do not show appreciable differences in the early recruitment of activated leukocytes into the parenchyma. Overall, these data indicate that the increased presence of neutrophils within the CNS following JHMV infection does not influence BBB integrity and has no impact on the recruitment of other inflammatory cells from the periphery. Importantly, Dox-induced CXCL1 within the CNS did not affect mobilization of CD11b^{+}Ly6C^{+}Ly6G^{-} monocytes within the blood or migration into the CNS. Rather, there were decreased numbers of these cells within the spinal cords of Dox-treated double-tg mice compared with single-tg mice by day 12 p.i. Whether CXCL1 modulates trafficking of CD11b^{+}Ly6C^{+}Ly6G^{-} monocytes in chronic stages of CNS inflammatory disease is not known at this time.

An increase in the severity of white matter demyelination was observed at day 12 p.i. in Dox-treated double-tg mice, and this correlated with an overall decrease in the total number of GST-π^{+} mature oligodendrocytes. We do not think that CXCL1 had any direct cytotoxic activity on oligodendroglia, as we have previously

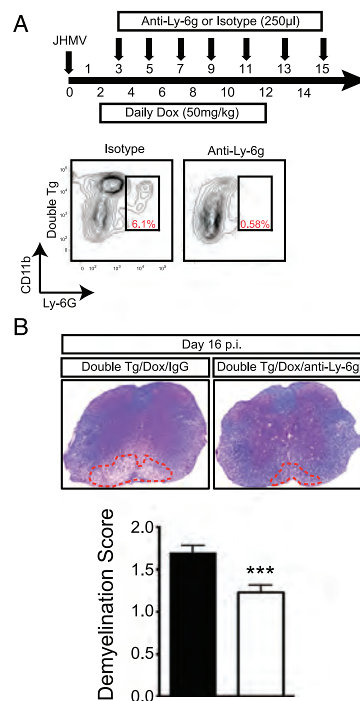

FIGURE 7. Neutrophils amplify the severity of demyelination. (**A**) Dox-treated JHMV-infected double-tg mice were treated with either anti-Ly6g Ab or isotype-matched control starting between days 3 and 4 p.i. and continuing every other day until day 15 p.i. Representative flow analysis of spinal cords confirmed anti-Ly6G treatment successfully depleted neutrophils. (**B**) Representative Luxol fast blue–stained spinal cord sections from JHMV-infected double-tg mice treated with either control IgG2a or anti-Ly6G Ab between days 3 and 15 p.i. Quantification of the severity of demyelination revealed reduced white matter damage in mice treated with anti-Ly6G Ab compared with mice treated with isogenic IgG2a control Ab; data are derived from two independent experiments with a minimum of three mice per group per experiment. Data are presented as average ± SEM; statistical significance was measured using an unpaired two-tailed Student *t* test. *$p < 0.001$.

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shown that treatment of differentiating mouse oligodendrocyte progenitor cells with recombinant CXCL1 does not kill these cells (21). However, our findings revealed a significant parenchymal presence of neutrophils associated with regions of severe demyelination in JHMV-infected double-tg mice, and ablation of these cells limited the severity of white matter damage, arguing for an important role for neutrophils in amplifying disease. Although recent studies suggest that Ly6C+ myeloid precursor cells play a pathogenic role during autoimmune demyelination (17), our findings reveal that the increase in tissue damage was not the result of enhanced migration of these cells to the CNS of Dox-treated double-tg mice when compared with single-tg mice. Neutrophils are known to possess an arsenal of proteases and toxic factors that can impact cell survival, including neutrophil elastase, cathepsin G, and MMPs (22). Moreover, respiratory burst from the neutrophil phagosome and the generation NO as well as reactive oxygen species through the NADPH oxidase complex can contribute to both vascular and parenchymal damage, resulting in bystander destruction of axons and oligodendrocytes within white matter tissue (23, 24). This has been demonstrated in mouse models of ischemic injury where inhibition of the NADPH complex results in less injury to vascular endothelium and reduced cellular damage within brain parenchyma (25). Furthermore, neutrophils are implicated in exacerbating lesion development within spinal cords on patients with neuromyelitis optica (26), and inhibition of neutrophil elastase, a serine protease released from the primary granules of neutrophils, within mouse models of neuromyelitis optica resulted in reduced neuroinflammation and myelin loss (27, 28). The potential destructive force of neutrophils has also been demonstrated using neurovirulent JHM recombinant variants, as neutrophil depletion or inhibition of NO synthase correlated with reduced apoptosis of glial cells within the brain (29). Another scenario is that neutrophils could be promoting pathology by drawing in macrophages within more cervical and upper thoracic regions of the spinal cord. Within the EAE model of chronic neurologic disease, neutrophils have recently been reported to have a role in maturating local APCs within the CNS, thus potentially contributing to enhanced disease severity by increasing numbers of autoreactive T cells (30). We observed dense clusters of macrophages in heavily demyelinated white matter regions that also contained significant neutrophil populations in double-tg mice. However, we think it unlikely that the increase in disease severity is the result of macrophages presenting viral Ag, as we did not detect elevated numbers of virus-specific T cells within either the brain or spinal cord.

Tani et al. (31) have shown that chronic overexpression of CXCL1 from oligodendrocytes within naive mice results in a neurologic disease associated with microglia and astrocytic reactivity as a result of pronounced neutrophil accumulation within the brain. Indeed, we observed increased numbers of microglia within infected double-tg mice, and astrocytes displayed distinct phenotypic changes within heavily demyelinated regions, including truncated morphologies and hypertrophy (unpublished observations). We are currently evaluating how microglia may augment demyelination and whether this is dependent on the presence of neutrophils. More importantly, these findings argue that sustained neutrophil infiltration into the CNS enhances clinical disease severity associated with an increase in white matter destruction. Additionally, our observations are consistent with recent studies from Segal and colleagues (32) and argue that therapies targeting neutrophil accumulation within the CNS may offer novel alternative therapies for treating neuroinflammatory diseases.

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Disclosures

The authors have no conflicts of interest.

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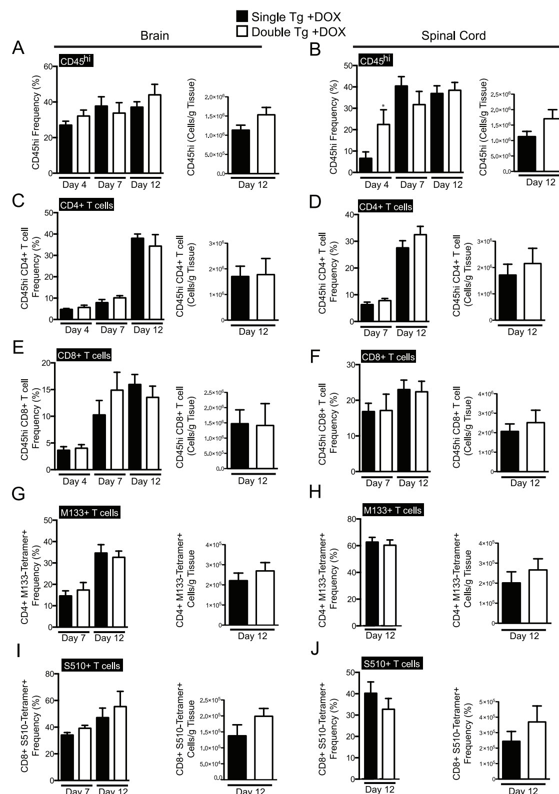
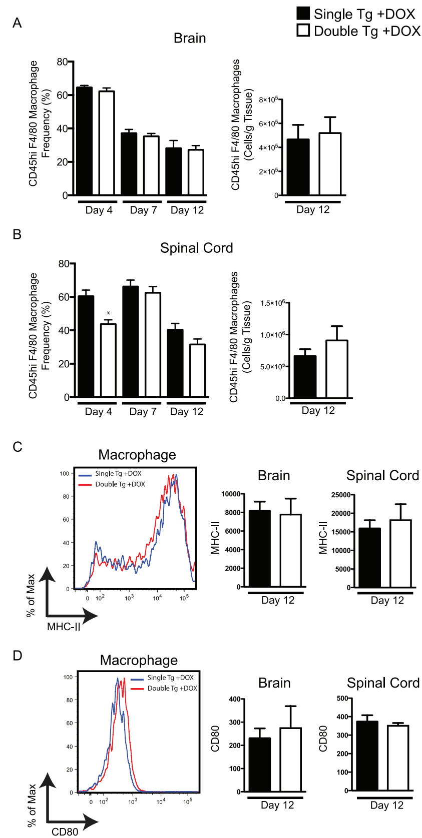


SUPPLEMENTAL FIGURE 1. CXCL1 overproduction from the CNS does not affect T cell infiltration into the CNS. Single cell suspensions from the brains and spinal cords from Dox-treated JHMV-infected double tg and single tg mice were isolated and immunophenotyped by flow cytometry. **(A)** No statistical difference was observed in the frequency of CD45^{hi} inflammatory infiltrates within the brain at day 4, 7 or 12 post-infection (p.i.). **(B)** Within the spinal cord, CD45^{hi} cells were elevated ($p<0.05$) at day 4 p.i. in dox-treated double tg mice; however, this increase was no longer observed at day 7 or 12 p.i. Furthermore, no changes were observed in the frequency of CD4+ **(C,D)** and CD8+ **(E,F)** T cells within the brain and spinal cord at day 4, 7 or 12 p.i.. The frequency and number of virus-specific CD4+ **(G,H)** and CD8+ **(I,J)** T cell also remained unchanged at all examined time-points. Flow cytometric data represents 3 independent experiments with a minimum of 3 mice per group per experiment at each time-point. Data is presented as average$\pm$SEM. Statistical significance was measured using an unpaired 2-tailed Student’s t test; *$p<0.05$.



SUPPLEMENTAL FIGURE 2. Macrophage infiltration into the CNS is not affected by overproduction of CXCL1. Macrophage ($CD45^{hi}$F480+) infiltration within the brain **(A)** and spinal cord **(B)** remained relatively unchanged in infected Dox-treated double tg mice compared to Dox-treated single tg mice. No differences were observed in the median fluorescence intensity (MFI) of MHC-II **(C)** and CD80 **(D)** within the brain or spinal cord of double tg and single tg mice. Flow cytometric data represents 3 independent experiments with a minimum of 3 mice per group per experiment at each time-point. Statistical significance was measured using an unpaired 2-tailed Student's t test; *p<0.05.

CHAPTER 4

INDUCED CENTRAL NERVOUS SYSTEM EXPRESSION OF CXCL1 AUGMENTS NEUROLOGIC DISEASE IN AN AUTOIMMUNE MODEL OF MULTIPLE SCLEROSIS VIA ENHANCED NEUTROPHIL RECRUITMENT

4.1 Abstract

The functional role of the chemokine CXCL1 in contributing to neuroinflammation and demyelination in EAE was examined. Through use of transgenic (tg) mice in which expression of CXCL1 is under the control of a tetracycline-inducible promoter active within GFAP+ cells, we have shown that sustained CXCL1 expression within the CNS increases clinical and histologic disease that is independent of an increase in either the frequency of encephalitogenic myelin-reactive Th1 or Th17 cells. Rather, increased disease was associated with enhanced recruitment of CD11b(+)Ly6G(+) neutrophils into the spinal cord. Neutrophil ablation resulted in a reduction in demyelination, arguing for a role for these cells in myelin damage. Collectively, these findings emphasize that CXCL1-mediated attraction of neutrophils into the CNS augments demyelination, arguing that this signaling pathway may offer new targets for therapeutic intervention.

4.2 Introduction

EAE is a T cell-driven autoimmune disease sharing many clinical and pathologic features with the human demyelinating disease MS (1-3). In EAE, CD4+ and CD8+ T cells specific for self-antigens expressed in CNS myelin initiate a localized inflammatory process that results in demyelination, axonopathy, and clinical deficits (4-6). The concept that the adaptive immune response is critical for new lesion development and disease progression in MS is emphasized in that FDA-approved disease modifying therapies are designed to limit infiltration of encephalitogenic lymphocytes into the CNS (7). Among the mechanisms by which myelin-reactive CD4+ T cells contribute to disease is through

localized secretion of cytokines e.g. IL-17 that increases expression of chemokines that attract myeloid cells into the CNS (8). Monocytes and macrophages amplify white matter damage through active stripping of the myelin sheath, leading to axonal damage, presentation of novel myelin epitopes to T cells, and amplifying neuroinflammation through secretion of proinflammatory molecules. In addition, IL-17 secretion increases expression of neutrophil chemoattractant chemokines CXCL1 and CXCL2, resulting in increased recruitment and accumulation of these cells within the CNS (9).

Increasing evidence in both preclinical models of MS as well as from MS patients have highlighted a potentially important role for neutrophils in demyelination (10-20). Neutrophil depletion delays the onset of clinical symptoms in EAE mice, arguing for a role for these cells in disease initiation and/or lesion formation (9, 13, 17, 19). Studies designed to decipher the mechanisms by which neutrophils augment disease progression point to increases in vascular permeability as well as secretion of reactive nitrogen and oxygen species (14, 18). In patients with relapsing-remitting MS, there are elevated systemic levels of neutrophil-activating chemokines, including CXCL1, CXCL5, and neutrophil elastase accompanied by increased numbers of neutrophils having a primed phenotype (10, 12). Collectively, these findings from preclinical models as well as MS patients strongly argue for a role for neutrophils in contributing to disease.

Given the transient nature of neutrophils in terms of having a short half-life, limited presence within lesions, and tightly regulated expression of chemotactic signals underscores the challenging aspects involved in understanding the role of these cells in autoimmune demyelination. To overcome some of these limitations, we have derived a transgenic mouse whereby expression of CXCL1 is under the control of a tetracycline-

inducible promoter that is active within GFAP+ cells (21). Upon Dox treatment, these animals exhibit increased CXCL1 expression within the CNS, resulting in increased and sustained neutrophil infiltration into the spinal cord of mice infected with the neurotropic JHMV (21). Using these transgenic mice, we have recently shown that increased neutrophil infiltration into the CNS of mice infected with JHMV increased neuroinflammation and demyelination, emphasizing an important role for neutrophils in participating in white matter damage (21). Our present study was undertaken to assess how increased and sustained neutrophil recruitment into the CNS impacts EAE disease progression. Our findings indicate that increased expression of CXCL1 within the CNS results in more severe EAE as measured by both clinical disease and demyelination. The increase in disease was not dependent on an increase in myelin-reactive Th1 or Th17 cells but correlated with increased numbers of neutrophil infiltration into the spinal cord. Indeed, depletion of neutrophils resulted in a reduction in the severity of white matter damage, highlighting a role for these cells in demyelination.

4.3 Materials and methods

4.3.1 Mice

pBI-CXCL1-rtTA double transgenic mice (developed on the C57BL/6 background) were developed as previously described (21). In brief, pBI-CXCL1 transgenic mice were generated by the University of California, Irvine transgenic mouse facility through DNA microinjection of fertilized C56BL/6 eggs using the linearized pBI-CXCL1 construct (22). The five resulting founder tg mice were mated to wildtype C57BL/6 mice to identify F1 offspring containing the transgene. To generate double tg

mice, hemizygous pBI-CXCL1 transgenics were crossed to hemizygous GFAP-rtTA*M2 mice (JAX), resulting in double transgenic mice (pBI-CXCL1-rtTA), single tg (rtTA-GFAP or pBI-CXCL1), or wildtype. Dox administration to double tg mice results in elevated CXCL1 transgene expression within astrocytes corresponding with increased CXCL1 protein and neutrophil accumulation whereas Dox treatment of single tg mice had no effect on CXCL1 expression nor neutrophil accumulation within the CNS (21).

4.3.2 $MOG_{35\text{-}55}$ EAE immunization

For induction of EAE, experimental mice (aged 6-8 weeks old) were injected subcutaneously in the flanks with 200 μL of 1 mM $MOG_{35\text{-}55}$ peptide (DNA/Peptide Synthesis Core Facility, University of Utah) emulsified with reconstituted complete Freund's adjuvant (Pierce Biotechnology, Waltham, MA, USA) containing *Mycobacterium tuberculosis* H37 Ra (2 mg/ml) (Difco Laboratories, Franklin Lakes, NJ, USA). Mice were injected intravenously with 100 μL *Bordetella pertussis* toxin (Ptx) (List Biological Laboratories Inc., Campbell, CA, USA) at 0.2 μg/mouse on days 0 and 2 following sensitization. Double and single transgenic animals were injected with Dox (50mg/kg via i.p. injection) starting at day 9 following EAE induction and continuing through day 19 post-immunization. Experimental mice injected with $MOG_{35\text{-}55}$ were scored daily for clinical signs through day 21 following immunization using previously described methods (23).

4.3.3 Flow cytometry

Inflammatory leukocytes infiltrating into the CNS were isolated using an established protocol (3,4). In short, CNS tissue was minced and leukocytes were isolated using a two-step Percoll gradient (90% and 63%). The isolated cells were collected and then washed prior to staining. Cells were incubated in an anti-CD16/32 Fc Block (BD Biosciences, San Jose, CA) at a dilution of 1:200. Cells were stained with fluorescently tagged rat anti-mouse IgG for the following cell surface antibodies, Ly6G, CD11b, CD45, CD8, (BD Biosciences), Ly6C, CD4, F4/80, I-A/I-E, CD11c (Biolegend, San Diego, CA), and CD4, B220 (eBiosciences, San Diego, CA), or Armenian hamster anti-mouse IgG for CD80 (BD Biosciences).

4.3.4 *Ex vivo* peptide restimulation

Spinal cord leukocytes that were isolated using a Percoll gradient as above were stimulated with MOG_{35-55} peptide or 50ng/mL phorbol myristate acetate (PMA) (Sigma, St. Louis, MO) with 2ug/mL Ionomycin (Iono) (Sigma, St. Louis, MO) and BD Cytofix/Cytoperm Plus Fixation/Permeabilization Kit (with BD GolgiPlug protein transport inhibitor containing brefeldin A) (BD Biosciences, San Jose, CA) and incubated at 37°C for 6 hours or 5 hours, respectively. Cells were stained with the surface marker CD4 (eBiosciences, San Diego, CA) and for intracellular cytokines including Rat anti-mouse IgG, IL-17A (eBiosciences, San Diego, CA), and IFN-γ (BD Biosciences).

4.3.5 Quantitative real-time PCR

Total cDNA from brains and spinal cords of sham and EAE immunized mice at day 12 following disease induction was generated via Superscript III (Life Tech., Carlsbad, CA) following homogenization in Trizol (Life Tech., Carlsbad, CA). Expression of mRNA from defined mouse chemokine and cytokine genes including *Cxcl1, Cxcl2, Cxcl10, Ccl2, Ccl5, IFN-γ*, and *IL-17A* within the brain of experimental mice was determined by qPCR using SYBR Green I Master (Roche) on a LightCycler 480 (Roche) and normalized to β-actin. (24)

4.3.6 Histology

Mice were euthanized according to IACUC guidelines and perfused with 20ml of 4% paraformaldehyde (PFA). Spinal cords were removed, fixed overnight in 4% PFA at 4°C, and separated into eight 1.5mm sections. Each section was cryoprotected in 30% sucrose for 5 days before embedding in OCT; 8 µM thick coronal sections were cut and stained with luxol fast blue (LFB) combined with hematoxylin and eosin (H&E). Pathology scoring of spinal cord sections was performed using previously described methods using 6-8 sections per cord (25).

4.3.7 Immunofluorescence

Spinal cord sections were processed as described above. For immunofluorescence, slides were first desiccated for 2 hours and blocked with 5% Normal Donkey Serum with or without 0.3% Triton-X 100. Primary antibodies were incubated overnight at 4°C: goat anti-CXCL1 1:50 (R&D Systems, Minneapolis, MN),

rabbit anti-GFAP 1:500 (Life Technologies, Carlsbad, CA), and rat anti-Ly-6B.2 1:100 (Serotec, Raleigh, NC). Images were analyzed using the Image J software (NIH) according to previously described methods (26, 27).

4.3.8 Antibody administration

Rabbit polyclonal antiserum was generated to a 17-amino acid portion of the amino-terminus ligand binding domain of CXCR2 (MGEFKVDKFNIEDFFSG) (Cocalico Biologicals, Inc., Stevens, PA) and used to target neutrophils as previously described (9, 28, 29). Normal rabbit serum (NRS) from pre-immunized rabbits was used as controls. Experimental mice were injected i.p. with 0.5 mL with either anti-CXCR2 or NRS every 2-3 days starting on day 9 following EAE induction and ending on or before day 19.

4.3.9 Statistical analysis

Flow cytometry data were analyzed using FlowJo, while all graphs were analyzed using Prism. Significance was given if the p value was below 0.05 and averages were given in ±SEM.

4.4 Results

4.4.1 Increased clinical disease following Dox-induced expression of CXCL1

Following induction of EAE, CD4+ T cell-derived IL-17 expression in the CNS potentiates disease by enhancing expression of the neutrophil chemoattractants CXCL1

and CXCL2 (9). The accumulation of neutrophils in the CNS is rapid yet transient and reflects the expression kinetics of CXCL1 and CXCL2. To better understand how neutrophil infiltration impacts demyelination and disease progression in EAE, tg mice were engineered to express CXCL1 within astrocytes upon doxycycline administration, as previous studies have shown these cells to be the primary source of CXCL1 in models of neuroinflammation (30, 31). Dox responsive double tg mice (pBI-CXCL1-rtTA) or single tg controls lacking the rtTA were immunized with MOG_{35-55} peptide and treated daily with Dox (50 mg/kg) i.p. between days 9 through 19 post-immunization (**Fig. 4.1A**). Onset of clinical disease was similar between Dox-treated double tg and single tg mice (day 9 p.i.), but double tg mice exhibited a significant increase in clinical disease severity compared to single tg mice (**Fig. 4.1B**). Examination of mRNA transcripts encoding proinflammatory cytokines indicated a selective >2-fold increase in CXCL1 in Dox-treated double tg mice versus single tg mice at day 12 post-immunization, which represents a time in which there is a separation in clinical disease between double and single tg mice following MOG_{35-55} immunization. No differences in expression of transcripts encoding Cxcl2, Cxcl10, Ccl2, Ccl5, IFN-γ, and Il-17A at day 12 between Dox-treated double tg and single tg mice were observed (**Fig. 4.1C**). A significant increase in CXCL1 protein was detected only in the spinal cord (**Fig. 4.1E**) and not in the serum (**Fig. 4.1D**) of double tg mice 12 days post-immunization compared to single tg controls . In contrast, we did not detect differences in proteins levels of G-CSF, GM-CSF, or IL-17 (**data not shown**) either prior to or following Dox treatment of double tg or single tg EAE mice. Immunofluorescence staining confirmed astrocytes (GFAP+) as the primary cellular source of CXCL1 in Dox-treated double tg EAE mice (**Fig. 4.1F**).

Together, these findings indicate that CXCL1 expression was specifically elevated in the CNS of Dox-treated double tg mice that correlated with an increase in clinical disease.

4.4.2 Induced expression of CXCL1 does not increase either T cell or macrophage infiltration into the CNS

MOG_{35-55}-induced EAE disease correlates with CNS infiltration of Th1 and Th17 CD4+ T cells reactive against the encephalitogenic MOG peptide (32). In addition, other immune subsets including CD8+ T cells, B cells, and macrophages are also considered important in amplifying clinical disease and pathology (32). We determined if overexpression of CXCL1 in the CNS of Dox-treated double tg mice immunized with MOG_{35-55} peptide altered the infiltration of inflammatory leukocytes into the CNS. We did not detect differences in the frequency or numbers of $CD45^{hi}$ leukocytes (**data not shown**), CD4+ T cells (**Fig. 4.2A and B**), or CD8+ T cells (**Fig. 4.2C and D**) in spinal cords of Dox-treated double tg mice compared to single tg mice. Moreover, there was no difference in intracellular CD4+ T cell cytokine expression of IFN-γ, IL-17A, or co-expressing IFN-γ and IL-17A within the spinal cords at day 12 p.i. following PMA/ionomycin stimulation (**Fig. 4.2E and F**). Moreover, we did not observe changes in macrophages ($CD45^{hi}F4/80^{+}$) (**Fig. 4.3A**), microglia ($CD45^{lo}F4/80^{+}$) (**Fig. 4.3B**), or macrophages or microglia co-expressing MHC class II and CD80 (**Fig. 4.3C and D**) of Dox-treated double and single tg EAE mice. Collectively, these findings argue that the increase in clinical disease following Dox-induced CXCL1 expression within the CNS is not due to increased infiltration of myelin-reactive CD4+ T cells nor other immune cells that are known to contribute to EAE disease severity.

4.4.3 Increased neutrophil accumulation within the spinal cord in response to Dox-induced expression of CXCL1

CXCL1 is a potent chemoattractant of neutrophils expressing the chemokine receptor CXCR2 and its overexpression from astrocytes enhances the migration and accumulation of neutrophils in the white matter tracts of the spinal cords in a model of viral-induced encephalomyelitis (21, 28). We next investigated whether Dox-induced overexpression of CXCL1 promotes neutrophil accumulation in double tg mice induced with EAE. Histopathologic examination of spinal cord sections revealed increased localization of inflammatory cells in the anterior median fissure and meninges of the spinal cords of Dox-treated double tg mice compared to single tg EAE mice (**Fig. 4.4A**). The majority of inflammatory cells within spinal cords of double tg mice had a multi-lobed nucleus characteristic of neutrophils (**Fig. 4.4B**). Immunofluorescence staining revealed increased numbers of cells positive for the neutrophil-associated surface antigen Ly6B.2 within the spinal cords of Dox-treated double tg EAE mice compared to single tg mice treated with Dox (**Fig. 4.4C**). In addition, flow analysis of spinal cords isolated from experimental mice indicated the frequency and numbers of neutrophils ($CD45^{hi}CD11b^{+}Ly6G^{+}$) within the spinal cords of Dox-treated double tg mice were significantly ($p<0.01$) increased compared to single tg mice (**Fig. 4.4D and E**). Importantly, we did not observe an increase in Ly6C+ monocytes in either brain or spinal cords of double tg mice treated with Dox (**data not shown**). Together, these findings argue that the Dox-induced increase in CXCL1 expression within the CNS enhances the recruitment and accumulation of neutrophils within the spinal cords is associated increased with clinical disease severity.

4.4.4 Neutrophils increase the severity of demyelination in Dox-treated double tg EAE mice

Using a model of viral-induced demyelination, we have previously shown that Dox-induced CXCL1 expression within the CNS increases the severity of white matter damage and this was correlated with enhanced neutrophil recruitment into the spinal cord (21). Assessment of spinal cord pathology between Dox-treated single tg and double tg mice was assessed using H&E/LFB staining at day 21 p.i. (**Fig. 4.5A**). Pathology scoring was evaluated by examining meningeal infiltration, perivascular cuffing, and demyelination. An increase in both meningeal infiltration ($p<0.05$) and demyelination ($p<0.05$) of double tg compared to single tg was observed (**Fig. 4.5B**). Furthermore, to determine whether neutrophils contributed to the enhancement of demyelination, MOG_{35-55}-immunized double tg mice treated with Dox were administered anti-CXCR2 antisera which we have previously shown to effectively target neutrophils (28, 31). Anti-CXCR2 or NRS was administered to Dox-treated double tg mice at defined times following EAE induction (**Fig. 4.5C**). Administration of anti-CXCR2 antisera to Dox-treated double tg mice resulted in a dramatic reduction in clinical disease severity compared to animals treated with NRS alone (**Fig. 4.5D**) and this was correlated with reduced numbers of circulating $CD45^{hi}CD11b^{+}Ly6C^{+}Ly6G^{+}$ neutrophils (**Fig. 4.5E**). The reduction in clinical disease in Dox-treated double tg mice treated with anti-CXCR2 correlated with a significant ($p<0.01$) reduction in the severity of demyelination compared to NRS treated mice (**Fig. 4.5F and G**) and this was associated with reduced numbers of neutrophils present within the spinal cords (**Fig. 4.5H and I**). Together, these findings indicate that neutrophils can enhance the severity of demyelination in MOG_{35-55}-induced EAE.

4.5 Discussion

The pathogenesis of MS is characterized by CNS inflammatory lesions consisting of activated lymphocytes and monocyte/macrophages (33, 34). The importance of these cells to disease is highlighted in that FDA-approved DMTs used in treatment of relapsing-remitting MS limit immune cell infiltration and lesion development (35, 36). Nonetheless, there is increasing interest in the potential role of other cell types including neutrophils in participating in demyelination. The diverse functions of neutrophils include phagocytosis, release of toxic granules, and secretion of reactive oxygen species (ROS), nitrogen species, and NETs that can lead to bacterial clearance and tissue damage (37-40). In addition, neutrophils can produce many different cytokines and chemokines that participate in tailoring the immune response (37-40). Although there is direct evidence that neutrophils are important in both disease initiation and progression in EAE models, only indirect evidence exists showing these cells also play a role in MS pathogenesis (41). Since neutrophils are typically short-lived and have a half-life in circulation between 6 and 8 hours in humans (42), direct evidence of neutrophils in MS lesions from available autopsy tissue samples has been limited. Nonetheless, recent studies from Segal and colleagues (12) have demonstrated increased systemic levels of the chemokines CXCL1 and CXCL5 as well as neutrophil elastase that correlated with lesion burden and clinical disability in MS patients, arguing for a role for neutrophils in augmenting disease.

The current study was performed to better understand the functional role of CXCL1 within the central nervous system following induction of MOG_{35-55}-immunized experimental autoimmune encephalomyelitis (EAE) in mice. CXCL1 is a member of the

ELR(+) family of CXC chemokines that bind the chemokine receptor CXCR2 expressed on neutrophils that are important in allowing egress of neutrophils out of the bone marrow and to sites of inflammation (37). In the context of EAE, the highest expressing ELR(+) chemokines within the CNS are CXCL1 and CXCL5 during early stages of disease onset (34) and CXCL1 protein levels in the spinal cord were increased prior to and following disease onset (12). Mice treated with anti-CXCL1 showed a delayed onset of disease and decreased clinical disease, correlating with diminished numbers of $Ly6G^+$ neutrophils, yet there was no change in infiltration of $CD3^+$ T cells into the CNS, arguing for a role for neutrophils in disease (34). More recently, we have shown that over-expression of CXCL1 within the CNS following infection with JHMV elevates clinical disease severity that correlates with an increase in demyelination; neutrophil ablation resulted in diminished white matter disease, highlighting that neutrophils can augment demyelination.

The successful generation of a Dox-dependent CXCL1 expressing transgenic mouse (21) has allowed for selective CXCL1 production from astrocytes and subsequently a better understanding of the functional role of this chemokine in MOG_{35-55}-induced EAE. Previously, Lira and colleagues (43) have shown that chronic overexpression of CXCL1 from oligodendrocytes within naïve mice results in a neurologic disease associated with microglia and astrocytic reactivity as a result of pronounced neutrophil accumulation within the brain. Using the CXCL1 double tg animals described in this study, we recently showed that astrocyte-derived overexpression of CXCL1 within the CNS was associated with increased clinical disease and demyelination in animals infected with the neurotropic JHMV and this was

dependent upon neutrophil infiltration into the spinal cords (21). In contrast, Raine and colleagues (44) demonstrated that overexpression of CXCL1 by astrocytes protected animals from EAE by limiting neuroinflammation as well as increasing numbers of oligodendrocytes associated with enhanced remyelination. These differences in clinical and histologic most likely reflect that, unlike Raine and colleagues, the *Cxcl1* transgene employed in our study was lacking the 3' untranslated region (UTR), resulting in an increase in the half-life of CXCL1 mRNA and therefore sustained CXCL1 production and subsequently neutrophil accumulation (44). The importance of neutrophils in enhancing white matter damage in Dox-treated double tg mice was emphasized by demonstrating that blocking neutrophil accumulation within the CNS via anti-CXCR2 treatment dramatically reduced demyelination.

Along with our data reported here, increasing evidence has shown that neutrophils play a key role in EAE and potentially MS. Ongoing studies are concentrating on defining mechanisms by which neutrophils increase demyelination in white matter tracts. Our findings demonstrating that the overproduction of CXCL1 in the CNS enhances white matter damage by the increase of neutrophil infiltration into the spinal cord, in conjunction with others in the EAE model and recent data from MS patients, suggest that impeding the production of CXCL1 would be an important target for a prospective therapy for neuroinflammatory diseases.

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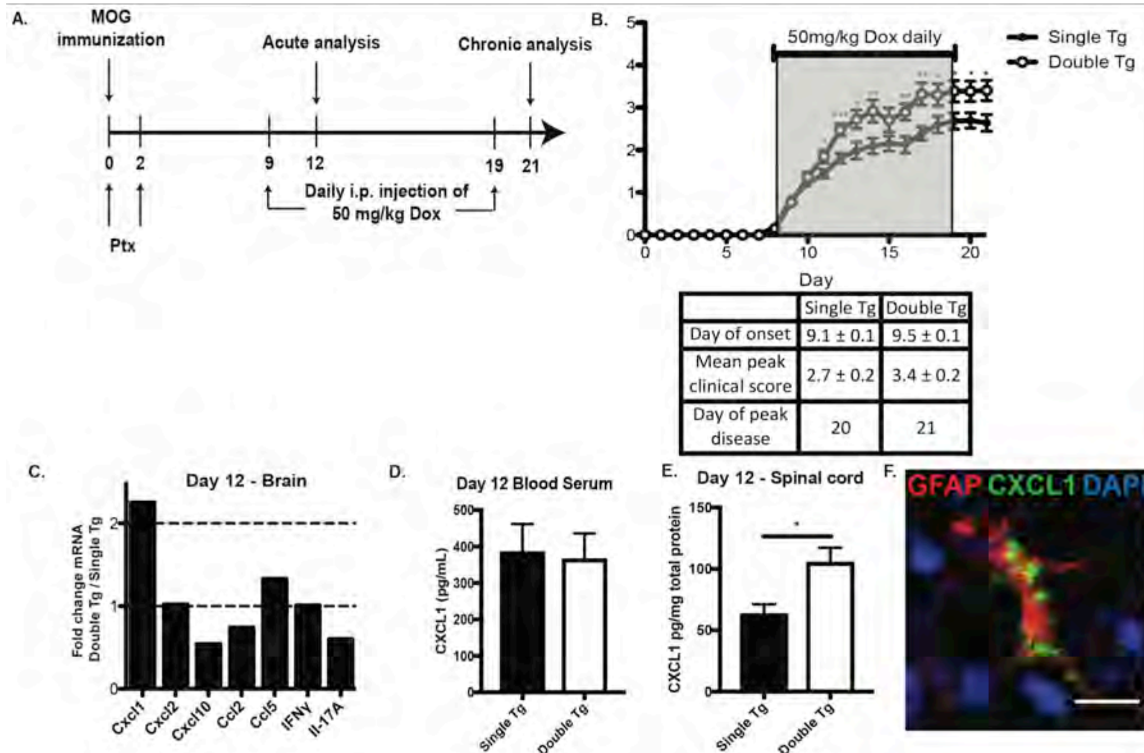


	Single Tg	Double Tg
Day of onset	9.1 ± 0.1	9.5 ± 0.1
Mean peak clinical score	2.7 ± 0.2	3.4 ± 0.2
Day of peak disease	20	21

Figure 4.1. Dox-induced CXCL1 expression increases clinical disease severity following MOG$_{35\text{-}55}$-induced EAE. (**A**) Schematic outline of experimental approach for disease induction and Dox administration. (**B**) Clinical disease is increased in MOG$_{35\text{-}55}$-immunized double tg mice (n=26) compared to single tg (n=29) following Dox treatment. Dox-treated double tg mice had an overall increase in peak clinical disease compared to single tg mice. Data represent four independent experiments. (**C**) Dox-treatment of double tg mice (n=2) resulted in an selective 2-fold increase in *Cxcl1* mRNA transcripts compared to single tg mice (n=2) at day 12 p.i whereas there was no difference in expression of transcripts encoding other pro-inflammatory cytokines/chemokines between experimental animals. On day 12, ELISA indicates Dox-treatment of either double tg or single tg mice did not change CXCL1 levels in serum, (**D**) yet there was a significant ($p<0.05$) increase in the spinal cords of double tg mice compared to single tg mice (**E**). Data represents a minimum of three independent experiments with between 2-4 mice for each experiment. Representative immunofluorescence staining (60X) showing co-localization of CXCL1 protein (green) with GFAP-positive (red) astrocytes in double tg mice at day 12 following Dox treatment (scale bar = 5μm). Statistical analysis employed unpaired two-tailed Student's *t* test; data presented as average $\pm$SEM. *p<0.05; **p<0.01; ***p<0.001.

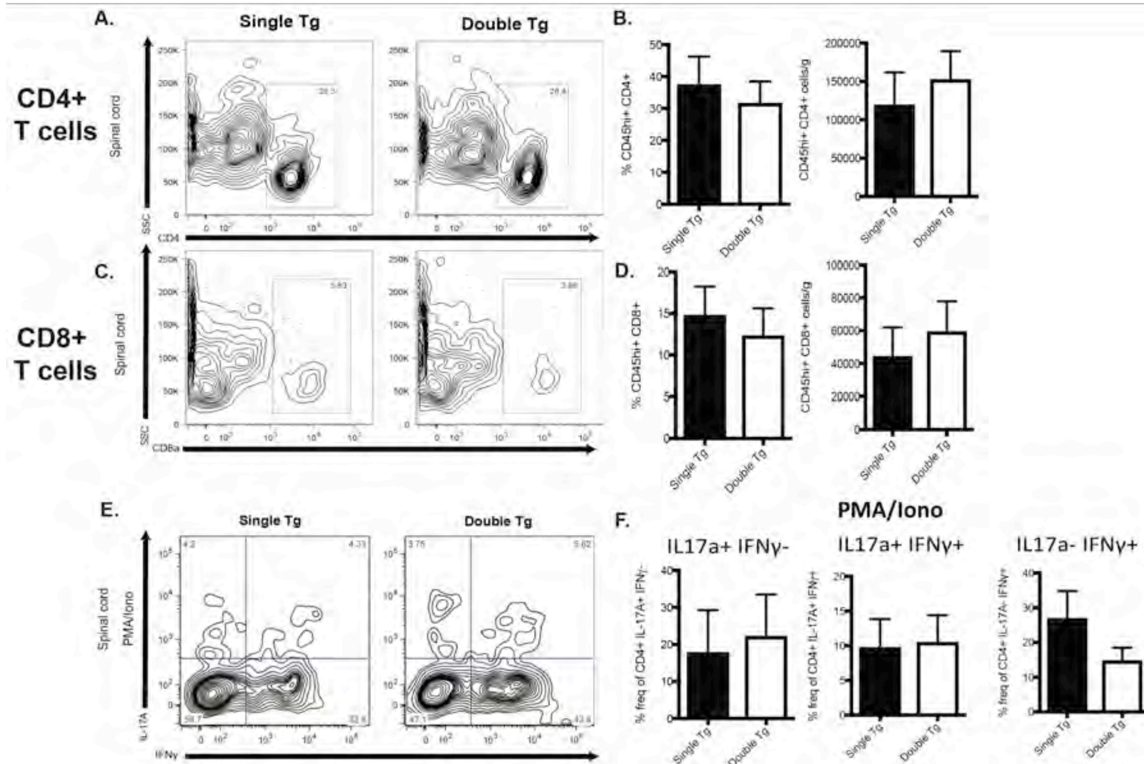


Figure 4.2. T cell infiltration into the CNS is not altered following Dox-induced expression of CXCL1. Spinal cords were removed at day 12 following MOG_{35-55} immunization of Dox-treated single and double tg mice and T cell infiltration determined by flow cytometry. Increased CXCL1 expression and disease severity in Dox-treated double tg (n=10) was not associated with an increase in either CD45+CD4+ T cells (**A, B**) or CD45+CD8a+ T cells (**C, D**) when compared to single tg mice (n=10). Representative contour blots from experimental animals are shown in panels *A* and *C*. Data in panels *B* and *D* are representative of a minimum of two independent experiments and data are presented as average$\pm$SEM. (**E, F**) No differences in expression of cytokines IL-17A and/or IFN-γ between Dox-treated double tg (n=3) or single tg (n=3) following PMA/Iono treatment of CD4+ T cells isolated from spinal cords at day 12 following MOG_{35-55} immunization. Data in panel *E* are representative contour plots showing the results of intracellular staining for IL-17A and IFN-γ following PMA/Iono treatment of CD4+ T cells; data in panel *F* represent quantification of intracellular cytokine staining. Data are presented as average$\pm$SEM and statistical analysis employed unpaired two-tailed Student's *t* test.

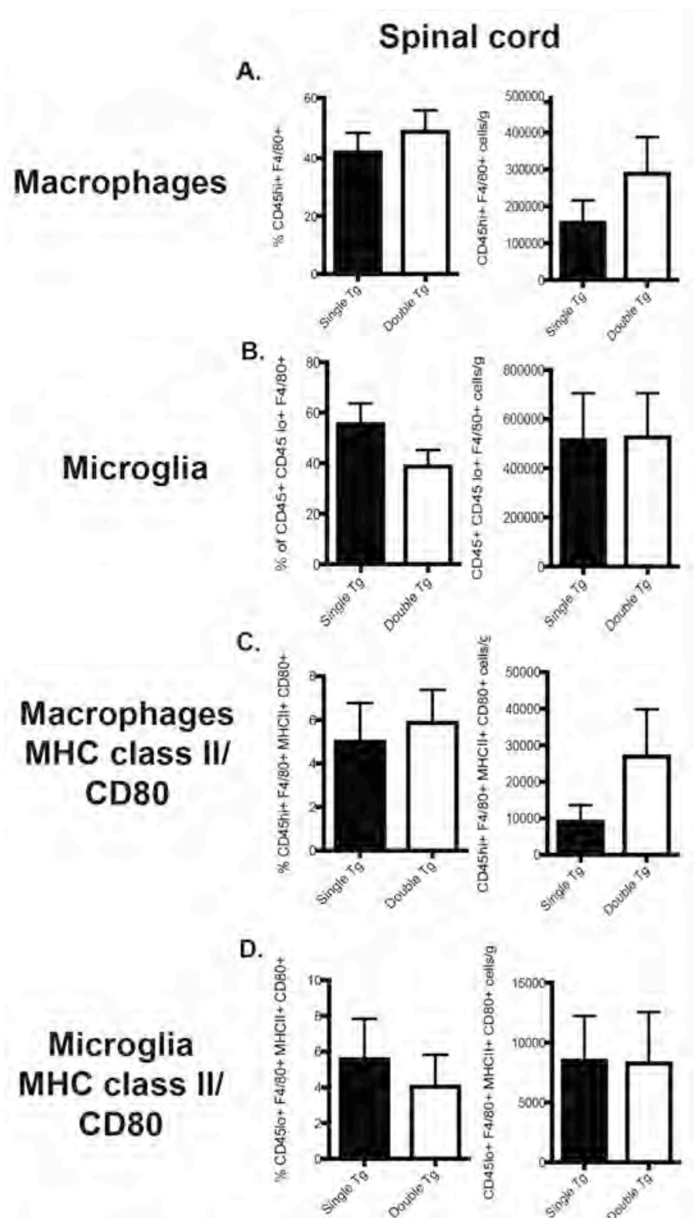


Figure 4.3. Dox-induced CXCL1 within the CNS does not increase microglia/macrophage activation. Dox-treated double and single tg mice were sacrificed at day 12 following MOG_{35-55} immunization and spinal cords removed to assess macrophage ($CD45^{hi}$ $F4/80^{+}$) and microglia ($CD45^{lo}F4/80^{+}$) activation via flow cytometric staining. Such analysis revealed no differences in either the frequency or numbers of either macrophages (**A**) or microglia (**B**) between double tg (n=10) or single tg (n=10) mice. Surface expression of activation markers MHC class II and CD80 was also examined and there were no differences in frequency or numbers of dual-positive macrophages (**C**) or microglia (**D**) between double tg (n=7) and single tg (n=7) mice. Data in panels *A* and *B* were derived from three independent experiments; data in panels *C* and *D* from two independent experiments. Data are presented as average±SEM and statistical analysis employed unpaired two-tailed Student's *t* test.

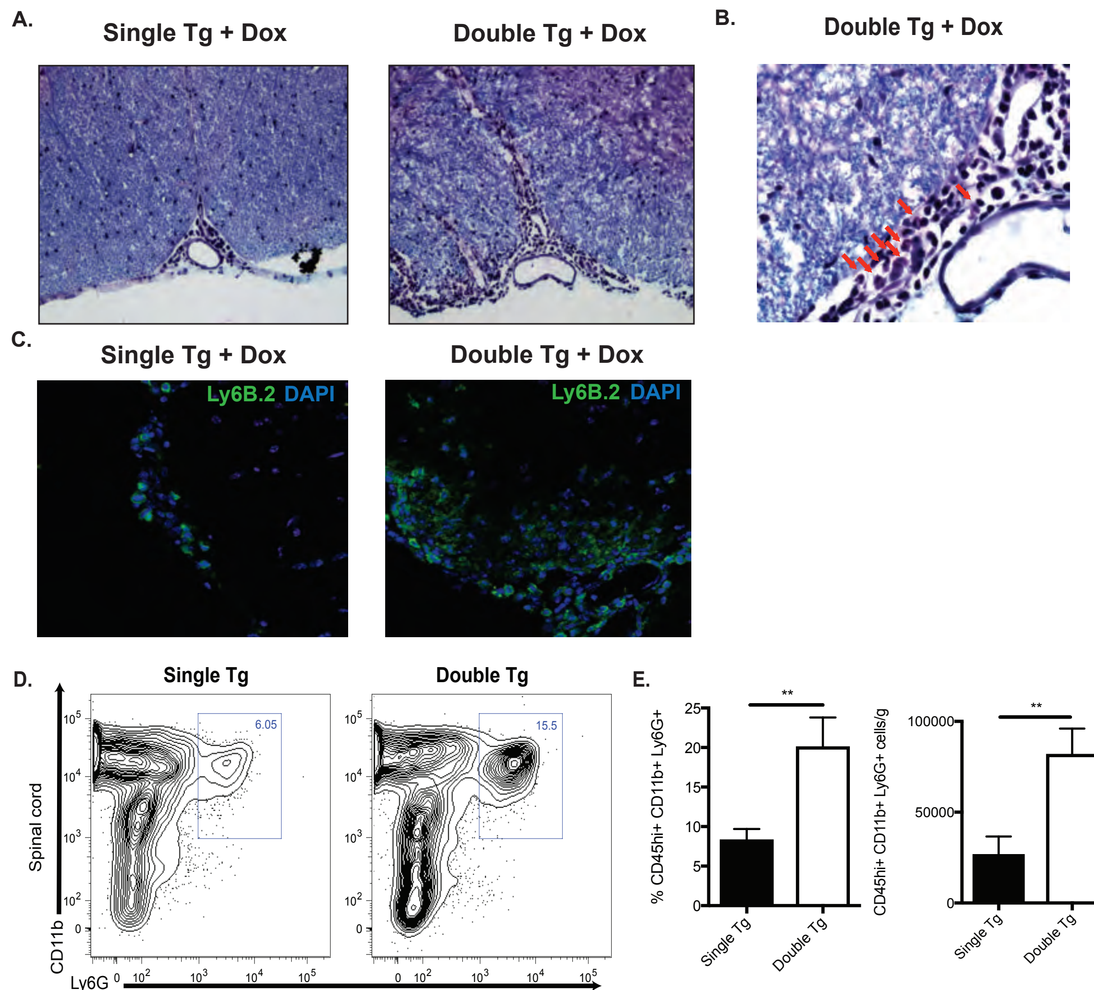
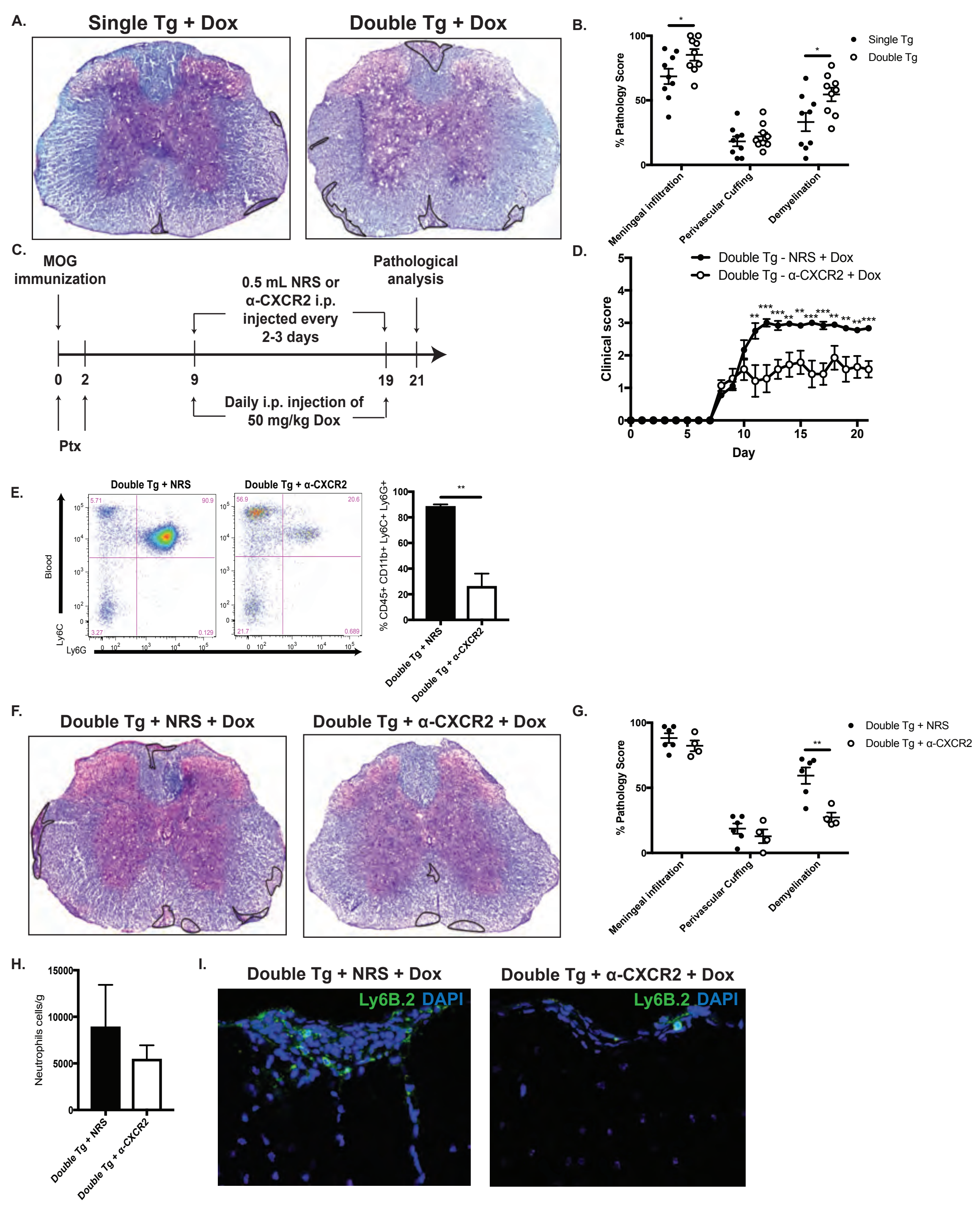


Figure 4.4. Neutrophil infiltration into the CNS is increased in response to Dox-induced expression of CXCL1. $MOG_{35\text{-}55}$-immunized double and single tg mice treated with Dox were sacrificed at day 12 post-immunization and spinal cords removed to assess histopathology and myeloid cell infiltration. (**A**) Representative H&E/Luxol fast blue staining of the spinal cords of experimental mice revealed increased inflammation within the anterior median fissure of double tg mice compared to single tg mice, 20X magnification. (**B**) Representative image of a spinal cord of Dox-treated double tg mouse showing cells with multilobed nuclei (red arrows) characteristic of neutrophils, 60X magnification. (**C**) Representative immunofluorescent staining for the neutrophil-specific surface antigen Ly6B.2 indicates increased expression within the spinal cords of double tg mice compared to single tg mice, 20X magnification. (**D**) Representative contour flow graphs showing increase in neutrophils ($CD45^{hi}$ $CD11b^{+}$ $Ly6G^{+}$) within the spinal cords of double tg mice compared to single tg mice. (**E**) Quantification of neutrophil flow staining revealed a significant increase in both the frequency and numbers of neutrophils in the spinal cords of double tg mice (n=10) when compared to single tg mice (n=10) that is significant in the percentages and cells/g spinal cord tissue. Data in panel *E* were derived from three independent experiments and data presented as average±SEM. Statistical analysis employed unpaired two-tailed Student's *t* test, **p<0.01.

Figure 4.5. Increased neutrophil infiltration augments spinal cord demyelination. MOG_{35-55}-immunized double and single tg mice treated with Dox were sacrificed at day 21 post-immunization and spinal cords removed to assess histopathology and myeloid cell infiltration. (**A**) Representative H&E/LFB stained spinal cord images (4X) (areas of demyelination outlined in black) obtained from double tg and single tg mice. (**B**) Quantification of % pathology score at day 21 observing meningeal infiltration, perivascular cuffing, and demyelination revealing a significant increase in meningeal infiltration and demyelination in double tg mice (n=9) compared to single tg mice (n=9) including 3 independent experiments. (**C**) Schematic outline for experimental design to deplete neutrophils in double tg mice via injection of either anti-CXCR2 or NRS control antibody. (**D**) Clinical score analysis of double tg mice given anti-CXCR2 showed a significant decrease compared to NRS control double tg mice during MOG_{35-55} EAE. (**E**) *In vivo* representation of double tg mice treated with anti-CXCR2 (n=3) showing a significant ($p<0.01$) decrease in neutrophils ($CD45^{hi}$ $CD11b^{+}$ $Ly6C^{+}$ $Ly6G^{+}$) compared to double tg mice treated with NRS 3 hours following treatment in the blood at 18 days post-immunization in one independent experiment. (**F**) Representative H&E/LFB staining reveals a reduction in the severity of demyelination (outlined in black) in double tg mice treated with the neutrophil-depleting anti-CXCR2 antibody compared to the NRS control antibody. (**G**) Quantification of pathology scoring of meningeal infiltration, perivascular cuffing, and demyelination in anti-CXCR2 treated double tg mice (n=4) compared to NRS treated mice (n=6) including 2 independent experiments at day 21 indicates that neutrophil depletion significantly decreases demyelination ($p<0.01$). (**H**) No significant difference in neutrophils ($CD45^{hi}$ $CD11b^{+}$ $Ly6C^{+}$ $Ly6G^{+}$) in the spinal cord at day 21, n=3 per group and one experiment. (**I**) Representative immunofluorescence (60X) of the dorsal funiculus in the spinal cord indicating the presence of Ly6B.2+ neutrophils within the parenchyma in double tg treated with anti-CXCR2 that is not present in the double tg NRS treated mice at 21 days post-immunization. Data presented as average$\pm$SEM. Statistical analysis employed unpaired two-tailed Student's *t* test. *$p<0.05$; **$p<0.01$; ***$p<0.001$.



A.
Single Tg + Dox
Double Tg + Dox
B.
% Pathology Score
Single Tg
Double Tg
Meningeal infiltration
Perivascular Cuffing
Demyelination
C.
MOG immunization
0.5 mL NRS or α-CXCR2 i.p. injected every 2-3 days
Pathological analysis
0 2 9 19 21
Daily i.p. injection of 50 mg/kg Dox
Ptx
D.
Double Tg - NRS + Dox
Double Tg - α-CXCR2 + Dox
Clinical score
Day
E.
Double Tg + NRS
Double Tg + α-CXCR2
Blood
Ly6C
Ly6G
% CD45+ CD11b+ Ly6C+ Ly6G+
Double Tg + NRS
Double Tg + α-CXCR2
F.
Double Tg + NRS + Dox
Double Tg + α-CXCR2 + Dox
G.
% Pathology Score
Double Tg + NRS
Double Tg + α-CXCR2
Meningeal infiltration
Perivascular Cuffing
Demyelination
H.
Neutrophils cells/g
Double Tg + NRS
Double Tg + α-CXCR2
I.
Double Tg + NRS + Dox
Double Tg + α-CXCR2 + Dox
Ly6B.2 DAPI
Ly6B.2 DAPI

CHAPTER 5

DISRUPTED CXCR2 SIGNALING IN OLIGODENDROGLIA LINEAGE CELLS DOES NOT AFFECT SUSCEPTIBILITY TO NEUROINFLAMMATORY DEMYELINATION

5.1 Introduction

MS is a chronic neurodegenerative disease characterized by multifocal regions of neuroinflammation, demyelination, and axonal loss that ultimately results in extensive neurologic disability (1). Animal models of MS indicate that CNS infiltration of neutrophils, monocyte/macrophages, and inflammatory T cells, including those that are autoreactive to specific proteins embedded in the myelin sheath, are important in disease initiation and maintaining demyelination (2, 3). In support of this is the demonstration that drugs designed to limit immune cell infiltration into the CNS impede new lesion formation in MS patients (4, 5) and improve clinical outcome, as well as dampen demyelination in animal models of MS (6-9). Therefore, understanding the signaling events that shape immune cell trafficking into and within the CNS offers new insights for identification of novel targets for therapeutic intervention.

Through use of preclinical mouse models of MS, chemokines and chemokine receptors have been implicated as important in attracting activated immune cells into the CNS and have been considered relevant targets for clinical intervention for MS patients (10-19). CXCR2 is a receptor for ELR(+) CXC chemokines e.g. CXCL1 and CXCL2 and is expressed on polymorphonuclear neutrophils (PMN) as well as glia and neurons (20-24). CXCR2 signaling on neutrophils promotes demyelination in EAE and cuprizone-induced demyelination (16, 25). CXCR2 has additional roles that extend beyond influencing neutrophil activity as it is expressed on immature OPCs as well as mature myelinating oligodendrocytes (26). Signaling through CXCR2 influences OPC proliferation and differentiation (27), controls the positional migration of OPCs during the development of the mouse spinal cord (28), and regulates the numbers of OPCs to

ensure the structural integrity of the white matter during CNS development (28). Indeed, mice devoid of CXCR2 exhibit a paucity of OPCs and structural misalignments that persist into adulthood of the mouse, resulting in reduced numbers of mature oligodendrocytes and total myelin within the white matter (29). Moreover, either genetic deletion or antibody-mediated targeting of CXCR2 increases myelin synthesis in demyelinated cerebellum slice cultures (30). It remains uncertain whether CXCR2 signaling on oligodendrocyte lineage cells is implicated in the early inflammatory events that occur in MS and its models. The answer to this question is consequential, as interpretations of results of CXCR2-directed remyelination or oligodendrocyte-injury studies are markedly sharpened if a primary effect of oligodendrocyte lineage CXCR2 can be excluded as a mediator of the initiating inflammatory events.

The present study was undertaken to better understand how selective deletion of *Cxcr2* within oligodendroglial lineage cells in adult mice influences CNS disease using models of viral-induced and autoimmune-mediated inflammatory demyelinating disease. We demonstrate that conditional ablation of *Cxcr2* signaling in oligodendroglia did not alter the extent of clinical disease severity nor affect neuroinflammation or demyelination in either of these preclinical MS models. Therefore, our findings suggest that CXCR2 signaling on oligodendrocytes does not modulate initial disease in these models of neuroinflammation and demyelination. The data indicate that either model can be used with confidence, to examine the downstream effects of oligodendrocyte lineage CXCR2 on long-term disease outcomes including tissue repair and oligodendrocyte cell death.

5.2 Methods

5.2.1 Mice and tamoxifen treatment

Plp-Cre-ER(T) :: Cxcr2 $^{fl/fl}$ mice were kindly donated by Dr. Richard Ransohoff (Cleveland Clinic). Mice were crossed to the reporter strain B6.Cg-*Gt(ROSA)26Sor*$^{tm9(CAG\text{-}tdTomato)Hze}$/J (The Jackson Laboratories, stock 007909) to generate *Plp-Cre-ER(T) :: Cxcr2* $^{fl/fl}$ *:: R26-stop-Td* $^{+/-}$ mice (c*Cxcr2* $^{fl/fl}$). Tamoxifen was prepared by resuspending at 10mg/ml in prewarmed sesame seed oil. The mixture was placed on an orbital shaker at 37°C and shaken overnight to completely dissolve solution. Four-week-old c*Cxcr2* $^{fl/fl}$ mice received 1mg/ml tamoxifen twice daily for 5 days via i.p. injection, rested for 5 weeks and subsequently immunized with MOG_{35-55} peptide or infected intracranially with JHMV (31).

5.2.2 Viral infection

Age-matched 5-6-week-old c*Cxcr2* $^{fl/fl}$ mice were infected i.c. with 250 plaque forming units (PFU) of JHMV in 30μl of sterile HBSS. SHAM infected animals received 30ul HBSS via i.c. injection (32, 33). For viral titer analysis, one half of each brain or whole spinal cord was homogenized and used in a plaque assay as previously described (34, 35). Clinical disease severity was assessed using a previously described scoring system (34, 35).

5.2.3 EAE induction

CFA was prepared by mixing 10mL incomplete Freund's adjuvant (Thermo Scientific, Rockford, IL) with 40mg *M tuberculosis* H37 (BD Biosciences, San Jose, CA)

to make a 4mg/ml solution of CFA. MOG_{35-55} peptide was prepared by resuspending in PBS at 1 μmol/mL. A 1:1 CFA-MOG emulsification was generating an emulsified by homogenization. To induce EAE, mice received two subcutaneous 100 mL injections of the CFA-MOG mixture and 100 mL of pertussis toxin (Ptx) (LIST Biological, Campbell, CA) via retroorbital injection. Mice received a second injection of Ptx at day 2 post-immunization (36). Clinical disease was assessed using a previously described scoring system (37).

5.2.4 Flow cytometry

Flow cytometric analysis was performed to assess the composition of inflammatory cells entering the CNS using established protocols (38, 39). In brief, single cell suspensions were generated from tissue samples by grinding with frosted microscope slides. Immune cells were enriched by a 2-step Percoll cushion (90% and 63%) and cells were collected at the interface of the two Percoll layers. Before staining with the fluorescent antibodies, isolated cells were incubated with anti-CD16/32 Fc block (BD Biosciences, CA) at a 1:200 dilution. Immunophenotyping was performed using either rat anti-mouse IgG or Armenian hamster anti-mouse IgG antibodies for the following cell surface markers: F4/80 (Serotec, Raleigh, NC), MHV S510-tetramer (NIH), MHV M133-Tetramer (NIH) and CD4, CD8, Ly6g, CD11b, IFN-γ, IL-17A, and CD45 (BD Biosciences, San Jose, CA).

5.2.5 Histopathology

Mice were euthanized according to IACUC guidelines and perfused with 20ml of 4% paraformaldehyde (PFA). Spinal cords were removed, fixed overnight in 4% PFA at 4°C, and separated into eight 1.5mm sections. Each section was cryoprotected in 30% sucrose for 5 days before embedding in OCT. Eight micron thick coronal spinal cord sections were cut and stained with LFB as well as H&E from JHMV infected spinal cords, while MOG_{35-55} EAE immunized spinal cords were stained with FluroMyelin (Thermo Fisher Scientific, Waltham, MA). The percent demyelination was calculated by taking the total average demyelination within the white matter of eight spinal cord coronal sections using Image J software (NIH).

5.2.6 Immunofluorescence/Immunocytochemistry

Spinal cord sections were desiccated at room temperature (RT) for 2 hours before beginning the staining process. Slides were then washed in PBS and blocked with 5% normal donkey serum (NDS) for 1 hour at RT. Rabbit anti-glutathione S-transferase (GST-π) (MBL Life Science, Woburn, MA) was diluted to 1:1000 in 5% NDS and stained overnight at 4c. Slides were washed in PBS and Alexa 594-conjugated donkey anti-rabbit was added at a 1:500 dilution (Invitrogen, Carlsbad, CA). For cultured oligodendrocytes, cells were fixed for 20 mins in 4% PFA before being blocked with 5% normal goat serum (NGS). Cells were then stained with mouse anti-mouse O1 at a dilution of 1:50 (eBiosciences, San Diego, CA) followed by a secondary stain with Alexa 488-conjugated goat anti-mouse 1:500 (Invitrogen, Carlsbad, CA).

5.2.7 Primary oligodendrocyte cultures

Cortices from postnatal day 1 c*Cxcr2* $^{fl/fl}$ mice were dissected and processed according to previously published protocols (40). In brief, following removal of the meninges, cortical tissue was minced with a razor and placed in pre-warmed DMEM containing papain in order to completely dissociate the tissue. Following further aspiration through a Pasteur pipette, single cell suspensions were added to Poly-D-Lysine coated culture flasks and grown for 9 days in DMEM supplemented with 10% FBS. Flasks were then transferred to an orbital shaker in a 5% CO_2 tissue culture incubator and shaken for approximately 16 hours at 220 rpm in order to remove loosely adherent OPCs. Media containing OPCs was transferred to 10 cm dishes for 30 minutes to remove strongly adherent astroglial contaminants. OPCs were transferred to a 15ml conical and centrifuged at 300x*g* for 5 minutes. Cells were counted and plated onto matrigel-coated Nunc™ Lab-Tek II Chamber slides (ThermoFisher Scientific, Waltham, MA) at 50,000 OPCs per chamber in N2 media supplemented with 3, 3', 5-Triiodo-L-thyronine sodium salt hydrate (T3, Sigma, St. Louis, MO). After 2 days, fresh media was used supplemented with (Z)-4-Hydroxytamoxifen (4-OHT, Sigma, St. Louis, MO) at 100nM to induce Cre-mediated recombination. Cells were cultured for an additional 6 days.

5.2.8 Western blotting

Samples were isolated from OPC cultures treated with either 4-OHT or vehicle control and separated by SDS-PAGE on a 10% gel (BioRad Mini PROTEAN TGX), transferred to a PVDF membrane in a Tris-Glycine buffer containing 20% methanol at 30V for 3.5 hours, and blocked for 1 hour at room temperature (5% milk in PBS-T).

Membranes were then incubated overnight at $4^{o}C$ in primary antibody diluted in 5% BSA in PBS-T. Primary antibodies used were myelin basic protein (MBP) (Rabbit anti-mouse, Abcam ab40390 @ 1:1000), cyclic nucleotide phosphodiesterase (CNPase) (Mouse anti-mouse, Abcam ab6319 @ 1:1000), and β-actin (Rabbit anti-mouse, Abcam ab8227 @ 1:1000). Primary antibodies were detected using horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit, Abcam ab97051 or goat anti-mouse, Abcam ab97023) diluted 1:5000 in 5% milk in PBS-T for 1 hour at room temperature. Membranes were developed with Western Lightning Plus-ECL Chemiluminescence Substrate (Perkin Elmer, Waltham, MA).

5.2.9 PMA/Ionomycin stimulation assay

Brains were isolated from experimental mice and processed to generate single cell suspensions. A total of $1x10^6$ cells mononuclear cells were added to each well of a 96-well plate in the presence of 50ng/ml PMA (Sigma, St Louis, MO) + 2ug/ml Iono (Sigma, St Louis, MO) for 5 hours. Following the incubation period, cells were stained for surface and intracellular antigens.

5.3 Results

5.3.1 Generation and characterization of *Plp-Cre-ER(T); Cxcr2* $^{fl/fl}$ (c*Cxcr2* $^{fl/fl}$) mice

We sought to ablate *Cxcr2* signaling within oligodendrocytes and their progenitors and assess the impact on two well-accepted preclinical mouse models of MS. A *Plp-Cre-ER(T) :: Cxcr2* $^{fl/fl}$ mouse line (referred as c*Cxcr2* $^{fl/fl}$) was employed that

utilizes Cre recombinase to selectively ablate *Cxcr2* (41-46). The PLP regulatory element promotes expression of Cre-ER(T) (47) and has previously been shown to accurately reflect endogenous PLP expression spatially and temporally in oligodendroglia (47). To aid in visualizing cells actively expressing Cre recombinase, c*Cxcr2* $^{fl/fl}$ tg mice were crossed to a Cre-inducible *Rosa26*-Tdtomato red reporter line on the C57BL/6 background (**Figure 5.1A**).

Ablation of *Cxcr2* was confirmed within oligodendrocyte-lineage cells following *ex vivo* culture of OPCs generated from the brains of post-natal day 1 (P1) c*Cxcr2* $^{fl/fl}$ mice. Addition of 4-OHT (100nM) induced Cre-mediated recombination at the *Cxcr2* locus as detected by PCR (**Figure 5.1B**). Further, 4-OHT induced expression of Tdtomato red in greater than 90% of OPCs expressing the late stage O1 surface marker (**Figure 5.1C**). Ablation of CXCR2 signaling within cultured cells treated with 4-OHT did not alter synthesis of MBP nor CNPase, which are proteins associated with oligodendrocyte maturation and myelin synthesis (**Figure 5.1D-F**).

To determine the cellular specificity of Cre *in vivo,* 4-week-old c*Cxcr2* $^{fl/fl}$ mice were treated with 1mg tamoxifen by i.p. administration twice daily for 5 days. Recombination at the *Cxcr2* locus was detected by PCR in the brain and spinal cord of tg mice but not in the spleen, liver, or kidney (**Figure 5.2A**). Selective expression of Tdtomato red was observed in over 70% of GST-π –positive oligodendrocytes within the spinal cord (**Figure 5.2B**). Previous studies (16) have demonstrated that neutrophils require CXCR2 to traffic and accumulate within the CNS and promote EAE (16). We confirmed that tamoxifen treatment did not induce aberrant Cre activity in neutrophils, as no differences were observed in expression of CXCR2 on neutrophils in the spleen at 14

days post-tamoxifen treatment (**Figure 5.2C**). Collectively, these results demonstrate that deletion of *Cxcr2* is specific to oligodendrocyte-lineage cells.

5.3.2 Disrupted CXCR2 signaling in oligodendroglia does not affect susceptibility to EAE

Tamoxifen has been reported to mute the severity of EAE by dampening immune responses to myelin-specific antigens (31). To maximize tamoxifen clearance prior to MOG_{35-55} peptide immunization, c*Cxcr2* $^{fl/fl}$ mice were rested for 30 days following tamoxifen treatment (48) (**Figure 5.3A**). Enriched expression of Tdtomato red was observed within the white matter tracts of the spinal cords of tamoxifen-treated tg mice compared to vehicle-treated control mice (**Figure 5.3B**) and was specific to GST-π oligodendrocytes (**Figure 5.3C**). Tamoxifen-treated tg mice were immunized with MOG_{35-55} peptide with adjuvants (see methods) and the severity of clinical disease was monitored. Ablation of *Cxcr2* had no effect on clinical disease to at least 35 days post-immunization (**Figures 5.3D**). Fluoromyelin staining revealed similar levels of demyelination between vehicle and tamoxifen-treated EAE mice (**Figure 5.3E**) and quantification of demyelination by LFB analysis confirmed no difference in white matter pathology between groups (**Figure 5.3F**). These results argue that CXCR2 signaling on oligodendroglia is dispensable for induction of EAE.

5.3.3 Silencing of CXCR2 signaling on oligodendroglia does not impair immune cell infiltration into the CNS upon EAE induction

We investigated whether deletion of *Cxcr2* in oligodendroglia influenced leukocyte infiltration into the CNS, as engagement of CXCR2 on oligodendrocytes in culture promotes oligodendroglia-specific autoamplification of ELR-chemokines (49). No differences in frequency or numbers of total CD4+ or CD8+ T cells were detected in the brain or spinal cord of tamoxifen-treated EAE mice compared to vehicle-treated EAE mice at day 20 post-MOG_{35-55} immunization (**Figures 5.4A and B**). *Ex vivo* PMA/Iono stimulation of CNS-derived CD4+ T cells isolated from brains of tamoxifen-treated tg mice displayed similar expression of IFN-γ^{+}IL17A^{-}, IFN-γ^{-}IL-17A^{+}, and dual-positive IFN-γ^{+}IL-17A^{+} expression compared to vehicle controls (**Figures 5.4C and D**). No differences were detected in CNS infiltration of neutrophils ($CD45^{hi}CD11b^{+}Ly6G^{+}$) or macrophages ($CD45^{hi}F480^{+}$), and the total numbers of microglia were the same ($CD45^{lo}F480^{+}$) between groups (**Figures 5.5A and B**).

5.3.4 *Cxcr2* ablation within oligodendrocytes does not affect viral-induced demyelination

Intracranial instillation of the neuroadapted JHMV results in an acute encephalomyelitis followed by a chronic demyelinating disease characterized by viral persistence in white matter tracts accompanied by immune-mediated damage (50). The JHMV model differs from MOG_{35-55}-induced EAE as it is characterized by transient neutrophil accumulation within the CNS and a Th1-mediated pathology whereby virus-specific IFN-γ-expressing CD4+ T cells and inflammatory macrophages contribute to

white matter pathology (3, 51). During chronic JHMV disease, ELR-chemokine signaling and the appearance of IL-17A and Th17+ T cells remains minimal and are not considered important in contributing to either clinical disease or white matter pathology (52, 53). Tamoxifen or vehicle treated c*Cxcr2* $^{fl/fl}$ mice were infected with 250PFU JHMV and clinical disease was monitored. No differences were observed in morbidity or mortality in tg mice (**Figure 5.6A**). Similar frequencies of neutrophils and macrophages were observed day 3 p.i. (**Figure 5.6B**) and the frequency and total numbers of macrophages, neutrophils, and T cells were similar between groups at day 6 p.i. (**Figure 5.6C**). No differences were observed in the infiltration of virus-specific CD4+ and CD8+ T cells into the CNS of tamoxifen treated tg mice (**Figure 5.7A**), resulting in similar viral titers compared to vehicle treated tg controls (**Figure 5.7B**). Finally, assessment of the extent of demyelination in JHMV-infected mice either treated with tamoxifen or vehicle revealed no differences in white matter damage (**Figure 5.7C**).

5.4 Discussion

Chemokine signaling networks that are associated with chronic CNS diseases such as MS or persistent viral infections are thought to amplify disease severity by attracting targeted populations of leukocytes into the CNS (54). One chemokine signaling pathway that is emerging as important in chronic CNS disease involves the CXCR2 receptor and its cognate ELR(+) chemokine ligands CXCL1 -2 -3 and CXCL5 -6 -7 -8. CXCR2 is highly expressed on circulating neutrophils, enabling these cells to rapidly migrate to the BBB in response to CNS-derived ELR-ligand expression whereby these cells participate in degrading components of the BBB. Evidence for a proinflammatory

role of neutrophils in chronic neurologic disease is supported by Segal and colleagues (16) who have shown that genetic silencing or antibody-mediated blockade of CXCR2 in mice following PLP-induced EAE resulted in reduced clinical disease and relapses as a result of limiting BBB permeability. Within the context of acute viral infection of the CNS, we have previously reported that antibody-mediated neutralization of CXCR2 during JHMV-infection enhanced disease (55). This outcome was a result of reduced neutrophil and monocyte trafficking, as these cells are critical in permeabilizing the BBB that subsequently allows penetration of virus-specific T cells in the parenchyma to combat viral replication.

In addition to being expressed on circulating myeloid cells, CXCR2 is also expressed on neurons as well as resident glial cells including oligodendroglia. With regards to a putative functional role for CXCR2 signaling during chronic JHMV-induced neuroinflammation and demyelination, administration of a CXCR2-specific blocking antibody to infected mice increased clinical disease and correlated with a significant increase in oligodendrocyte apoptosis and demyelination (56). Further, addition of IFN-γ to cultured OPCs derived from mouse neural progenitor cells results in apoptosis, while inclusion of CXCL1 protein blocks OPC apoptotic death (22).

CXCR2 is believed to influence OPC proliferation and differentiation (27) as well as controlling migration of spinal cord OPCs during development (28). Ablation of *Cxcr2* results in a paucity of OPC numbers and structural misalignments that persist into adulthood of the mouse, and manifest as reduced numbers of mature oligodendrocytes and total myelin within the white matter (29). The functional role of CXCR2 signaling in mouse models of demyelination within the CNS is somewhat enigmatic. Some findings

suggest that the CXCR2 signaling axis downregulates myelin production by oligodendrocytes (57), while others have reported that CXCR2 signaling is a survival mechanism for OPCs needed to halt apoptosis induced by cytotoxic factors secreted during an inflammatory response (22, 56, 58, 59). Liu and colleagues (30) used bone marrow chimeric mice to partition the contribution of CXCR2 expression on either hematopoietic or CNS derived cells. Adoptive transfer of hematopoietic cells derived from the bone-marrow of *Cxcr2* $^{+/+}$ mice into *Cxcr2* $^{-/-}$ mice led to increased oligodendrocyte differentiation in both EAE and cuprizone models of demyelination suggesting that CXCR2 may be an inhibitory signaling cue for myelin repair (30). Using CXCR2 antagonism via a neutralizing antibody therapy resulted in enhanced oligodendrocyte differentiation and clinical recovery during EAE, further supporting a detrimental role for CXCR2 signaling within the CNS in mouse models of chronic inflammatory demyelinating disease (60). Conversely, inducible overproduction of CXCL1 by astrocytes reduced EAE clinical disease; although it is unclear whether increased levels of CXCL1 directly impacted oligodendrocyte biology or modulated immune cell recruitment into the CNS (58). Previous work from our laboratory employing antibody-mediated targeting of CXCR2 shows that blocking signaling in JHMV-infected mice increases the severity of demyelination arguing for a protective role for CXCR2 in a model of viral-induced demyelination as previously discussed (49). However, whether this is a direct effect of blocking CXCR2 signaling on oligodendroglia and/or other CNS resident cells is unknown.

Our present study built upon these studies to further our understanding of how CXCR2 signaling on oligodendrocytes affects experimental models of

neuroinflammatory demyelination. Using both the MOG_{35-55}-induced EAE and the JHMV model of viral-induced demyelination, we provide evidence that tamoxifen-induced silencing of CXCR2 on oligodendroglia did not affect clinical disease. More importantly, we did not observe any changes in the extent of neuroinflammation in either model, suggesting that muting CXCR2 on oligodendroglia alone did not influence proinflammatory gene expression within the CNS. This notion is supported by evidence indicating that neither generation of antigen-specific T lymphocytes was impacted nor was recruitment of T cells, neutrophils, or macrophages affected following tamoxifen treatment of experimental c*Cxcr2* $^{fl/fl}$ mice compared to mice treated with vehicle control. These findings argue that CXCR2 signaling on oligodendroglia has no effect on proinflammatory gene signatures responsible for recruiting either EAE-associated Th1/Th17 cells nor JHMV-associated Th1 cells. This is interesting in light of a recent report demonstrating that NG2-positive glia, precursors to glial cells including oligodendroglia, respond to the cytokine IL-17, and mediate the effector phase of Th17-mediated EAE (61). In addition, IL-17 impairs maturation of oligodendroglia and reduces their survival (61). Therefore, oligodendroglia are responsive to the cytokine/chemokine milieu within the CNS microenvironment during neuroinflammatory disease. Nonetheless, our findings clearly indicate typical neuroinflammation occurs in the absence of CXCR2 signaling on oligodendroglia.

In vitro treatment of oligodendroglia isolated from c*Cxcr2* $^{fl/fl}$ mice with 4-OHT did not increase or decrease synthesis of myelin-associated proteins MBP and CNPase. These findings contrast with reports suggesting that blocking CXCR2 increases myelin formation (30, 62, 63). These studies were performed using either small molecule

antagonists for CXCR2 or *Cxcr2* germline knock-out mice that would lack specificity for selectively targeting CXCR2 signaling to oligodendroglia lineage cells. More recently, Ransohoff and colleagues generated mice in which *Cxcr2* was selectively ablated in oligodendrocytes (64). Similar to the animals we have generated, *Cxcr2* is silenced within oligodendroglia in adult mice upon tamoxifen treatment (64). Employing toxin models of demyelination, the authors clearly show enhanced remyelination in animals in which CXCR2 signaling in oligodendrocytes is inhibited arguing for an important role for this chemokine receptor in influencing oligodendrocyte biology. Our findings showing that silencing of CXCR2 did not improve clinical disease in either autoimmune or viral-induced neuroinflammatory demyelination argues that remyelination was not occurring. These results highly suggest that the effect of CXCR2 on remyelination may be model dependent. In the non-inflammatory toxin models of demyelination, blocking CXCR2 enhances OPC maturation and increases remyelination whereas in neuroinflammatory models, selective ablation of CXCR2 does not have any appreciable effects on remyelination. Whether this is the result of the inflammatory microenvironment on masking the potentially protective effect of targeting CXCR2 on oligodendroglia is unknown at this time and is the focus of ongoing studies.

5.5 Conclusions

This study demonstrates that targeted silencing of *Cxcr2* expression within oligodendroglia did not impact neuroinflammation nor demyelination using either a viral or autoimmune model of MS. These findings argue that signaling through CXCR2 on oligodendroglia is not a key factor in amplifying proinflammatory gene expression and

immune cell infiltration into the CNS. Nonetheless, compelling evidence indicate that CXCR2 plays an important role in OPC maturation and subsequent remyelination. Therefore, within the context of oligodendroglia, CXCR2 is not important in disease initiation yet has an important role in repair.

5.6 References

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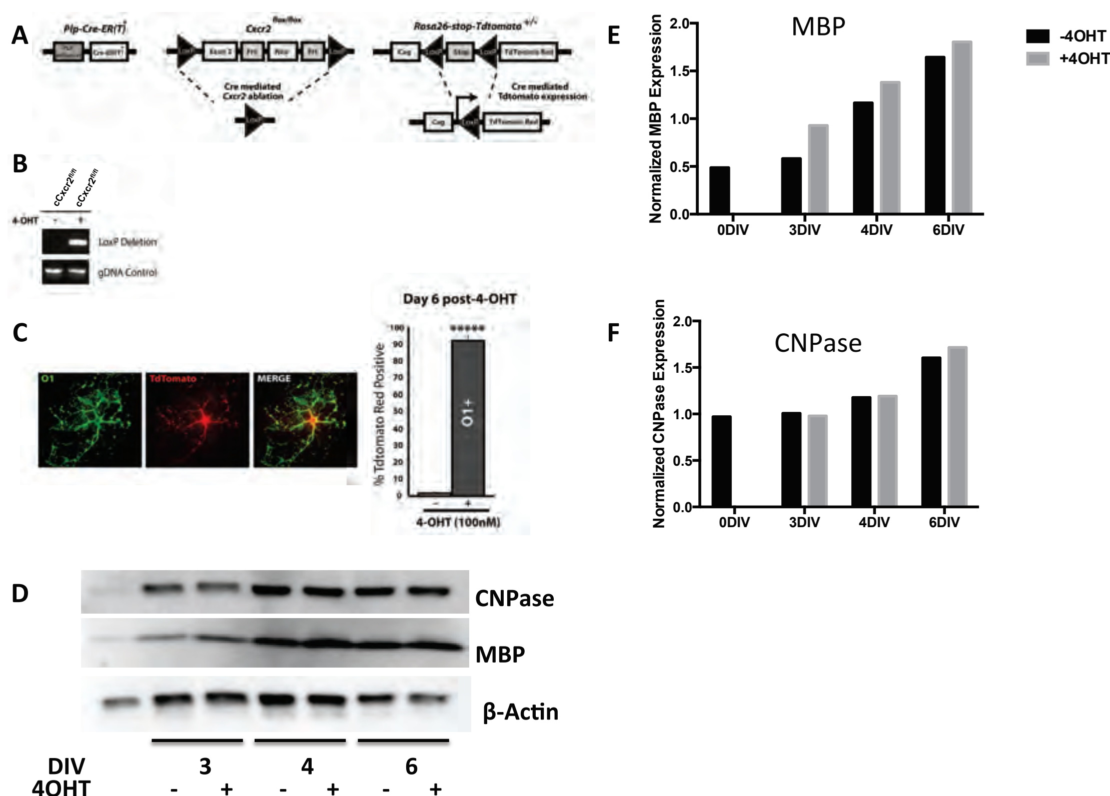

Figure 5.1: Cre-mediated recombination is detected *in vitro*. (**A**) Schematic diagram showing genetic strategy to generate tamoxifen-inducible knock-out of *Cxcr2* within oligodendroglia. (**B**) Cre-mediated recombination at *Cxcr2* locus was detected by PCR from oligodendrocyte-enriched cultures derived from P1 c*Cxcr2* $^{fl/fl}$ mice following addition of 4-OHT. (**C**) Treatment with 4-OHT induced expression of Tdtomato red in >90% O1-positive oligodentrocytes derived from P1 c*Cxcr2* $^{fl/fl}$ mice. (D) Western blot of CNPase and MBP from 4-OHT treated oligodendrocyte cultures. (**E**) Normalized expression of MBP or (**F**) CNPase following 4-OHT treatment. Quantification of Tdtomato red-positive O1-positive oligodendrocytes in panel ***C*** represents 4 independent OPC cultures derived from c*Cxcr2* $^{fl/fl}$ mice; data presented as average±SEM. DIV, days *in vitro*; *****$p<0.00001$.

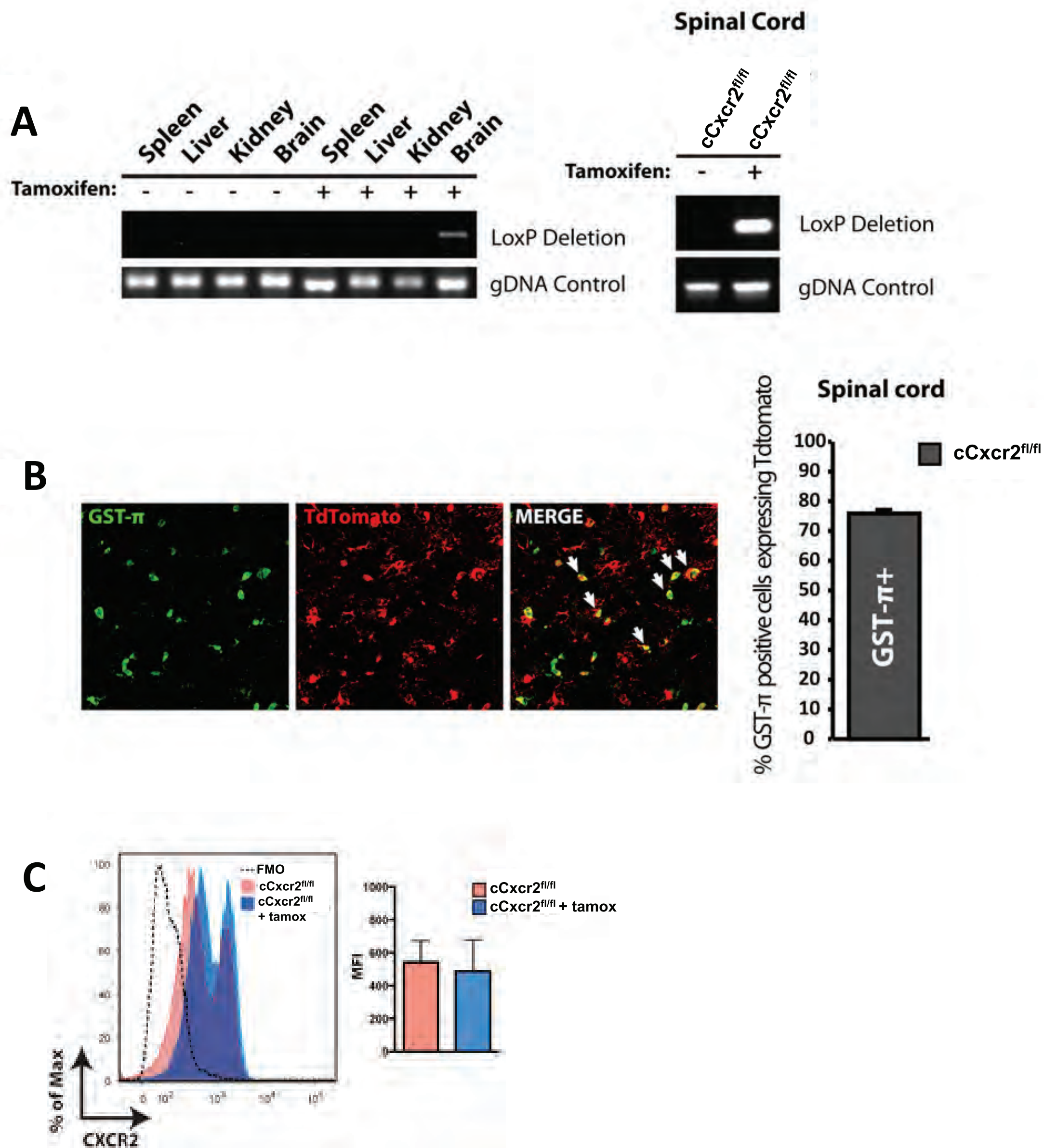

Figure 5.2: Cxcr2 is ablated *in vivo* following tamoxifen treatment. (**A**) Detection of Cre-mediated recombination at the *Cxcr2* locus in the brain and spinal cord of c*Cxcr2* $^{fl/fl}$ mice 2 weeks following tamoxifen treatment. (**B**) Quantification revealed ~75% of GST-π-positive oligodendrocytes expressed Tdtomato following tamoxifen treatment. (**C**) CXCR2 expression on splenic neutrophils following tamoxifen-treated c*Cxcr2* $^{fl/fl}$ mice. Panel *B* is representative of 8 separate coronal spinal cord sections from 4 mice and presented as average±SEM. Panel *C* represents 2 independent experiments with a minimum of 4 mice per experiment; bar graphs represent average±SEM.

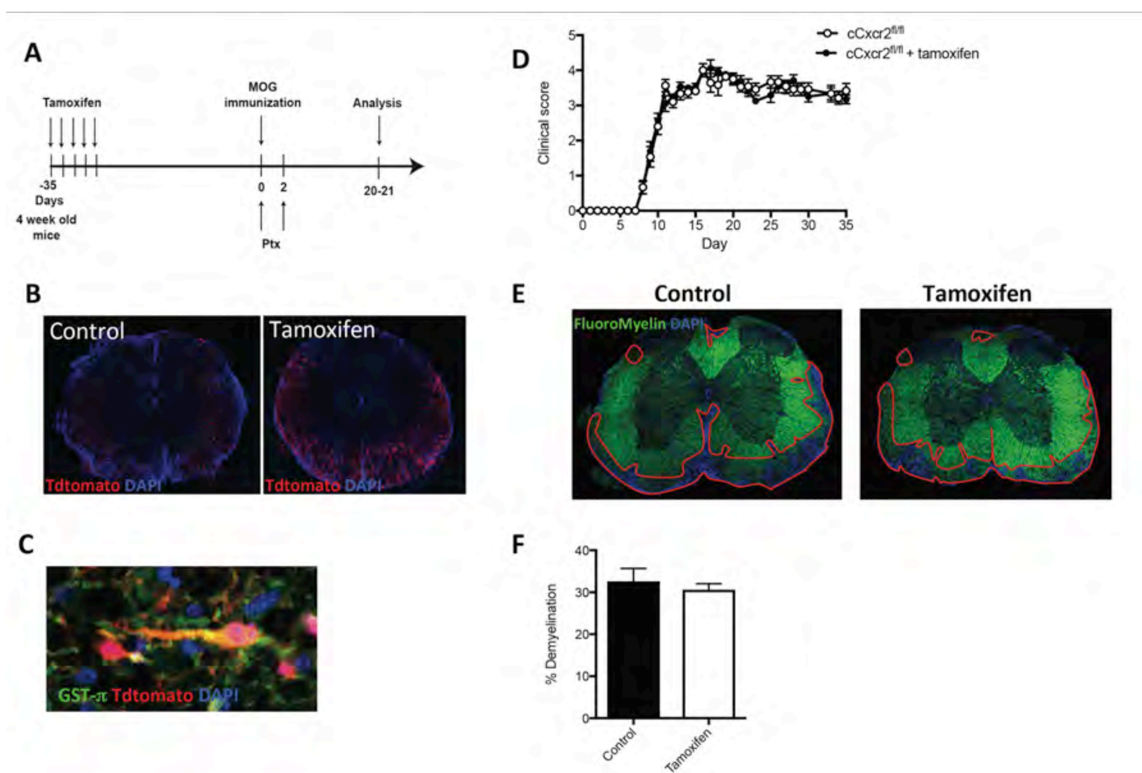

Figure 5.3: Clinical and histologic disease is not affected following $MOG_{35\text{-}55}$-induced EAE in c*Cxcr2* $^{fl/fl}$ mice. (**A**) Schematic depicting tamoxifen injections and $MOG_{35\text{-}55}$ immunization in c*Cxcr2* $^{fl/fl}$ mice. (**B**) Representative spinal cord images of $MOG_{35\text{-}55}$-immunized treated with vehicle (control) or tamoxifen at day 21 following $MOG_{35\text{-}55}$ immunization. (**C**) Representative immunofluorescent staining for GST-π confirmed that Tdtomato red expression is restricted to oligodendrocytes. (**D**) Clinical disease scoring of tamoxifen-treated c*Cxcr2* $^{fl/fl}$ mice (n=15) compared with control-treated mice (n=16) immunized with $MOG_{35\text{-}55}$ peptide. (**E**) Representative fluoromyelin staining of spinal cords from control (n=5) and tamoxifen-treated c*Cxcr2* $^{fl/fl}$ mice (n=7); areas of demyelination are outlined by red lines and (**F**) quantification on total demyelination in spinal cord sections. Clinical scoring data are derived from two independent experiments. Fluoromyelin staining represents 2 independent experiments including 8 spinal cord sections averaged for each mouse; bar graphs presented as average±SEM.

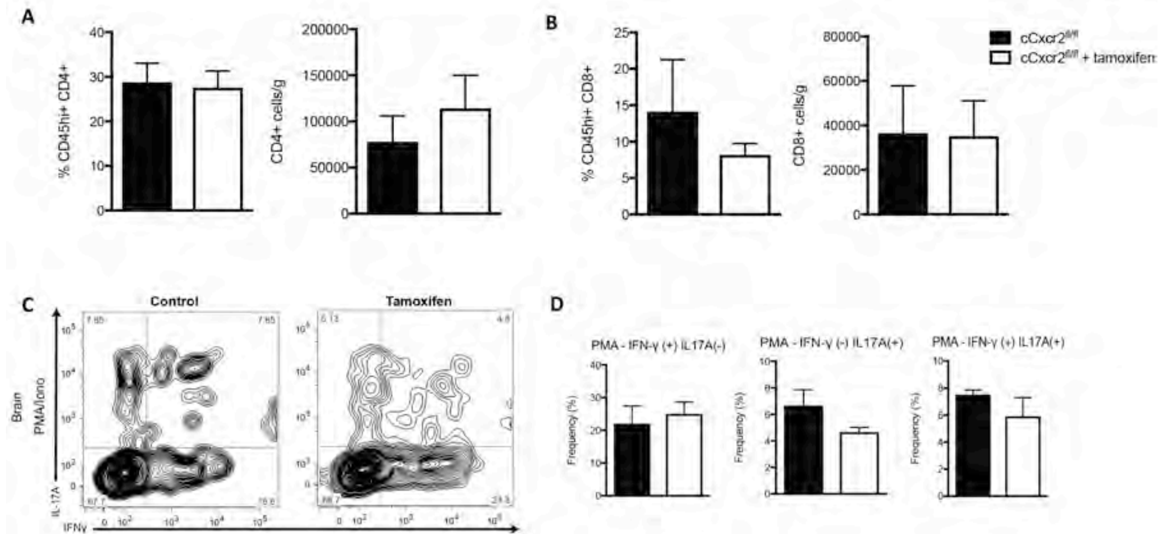

Figure 5.4: MOG$_{35-55}$-specific CD4+ T cell responses are intact following tamoxifen treatment. MOG$_{35-55}$-immunized c*Cxcr2* $^{fl/fl}$ mice were treated with either tamoxifen (n=8) or vehicle control (n=6) including 2 independent experiments and spinal cords removed at day 20-21 post-immunization to measure the frequency and number of (**A**) Percent and numbers of CD4+ T cells and (**B**) CD8+ T cells in spinal cords. (**C-D**) On day 20 post-immunization, isolated brain leukocytes were stimulated PMA/Iono for 5 hours and intracellular cytokine production of IFN-γ and/or IL-17A was measured in CD4+ T cells. (**D**) Data are derived from one independent experiment n=3 per group and presented as average±SEM.

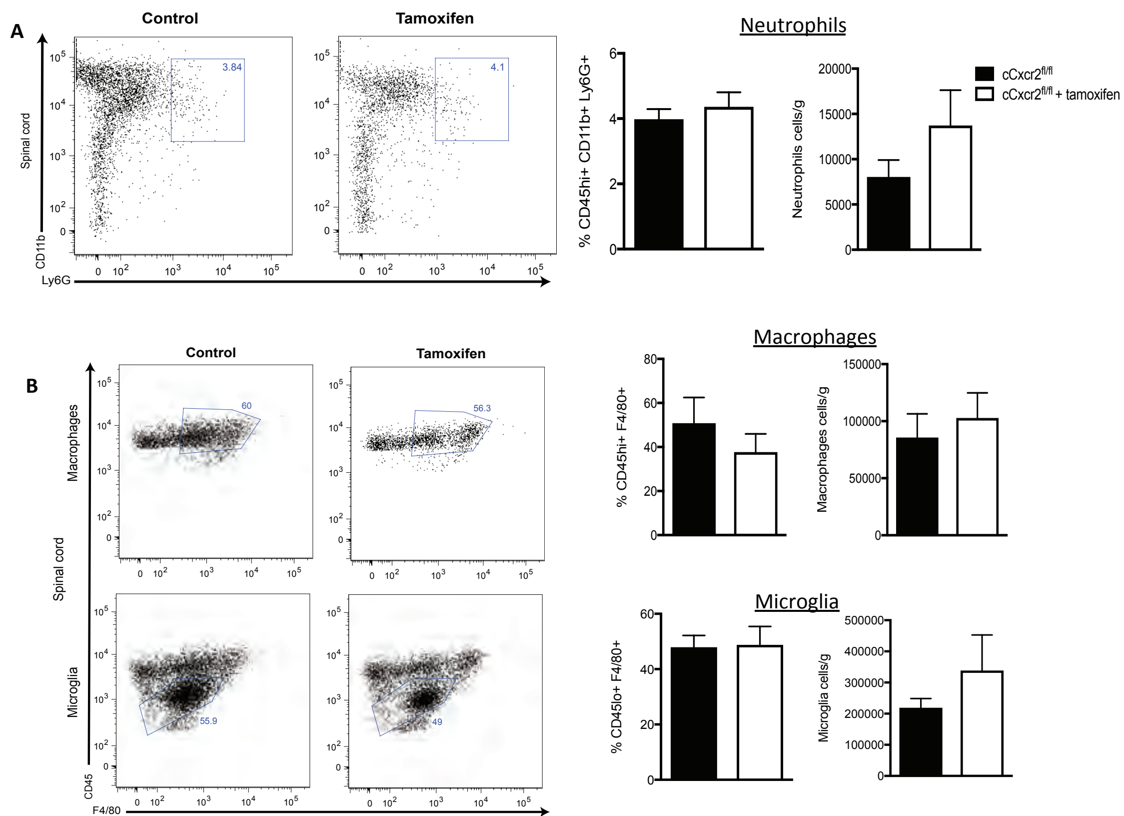

Figure 5.5: EAE neuroinflammation is not affected in response to tamoxifen treatment. MOG_{35-55}-immunized c*Cxcr2* $^{fl/fl}$ mice were treated with either tamoxifen (n=8) or vehicle control (n=6) and spinal cords removed at day 20-21 following peptide injection to measure the frequency and numbers of (**A**) neutrophils ($CD45^{hi}CD11b^{+}Ly6G^{+}$), (**B**) macrophages ($CD45^{hi}F4/80^{+}$) and microglia ($CD45^{hi}F4/80^{-}$). Data represent 2 independent experiments with at least 3 mice per group and presented as average±SEM.

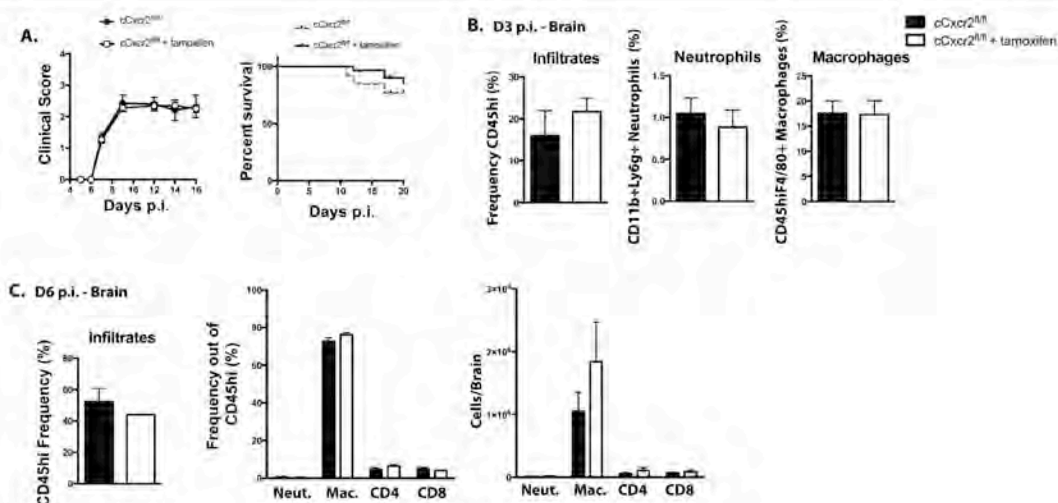

Figure 5.6: Tamoxifen-mediated ablation of Cxcr2 does not affect neuroinflammation in response to JHMV infection of the CNS. c*Cxcr2* $^{fl/fl}$ mice were treated with tamoxifen or vehicle control and infected with 250 PFU JHMV. (**A**) Clinical disease severity and mortality was recorded following JHMV infection. (**B**) Flow cytometric analysis of the frequency leukocytes ($CD45^{hi}$), neutrophils ($CD45^{hi}CD11b^{+}Ly6G^{+}$), and macrophages ($CD45^{hi}F4/80^{+}$) in the brains at day 3 p.i. or (**C**) day 6 p.i.. Also included are the numbers of infiltrating leukocytes, neutrophils, macrophages, CD4+ T cells, and CD8+ T cells in the brain at day 6 p.i.. Clinical disease and mortality is representative of 10 vehicle treated and 22 tamoxifen treated c*Cxcr2* $^{fl/fl}$ mice. Flow cytometric data at day 3 and 6 p.i. are derived from at least 5 mice per group and average$\pm$SEM.

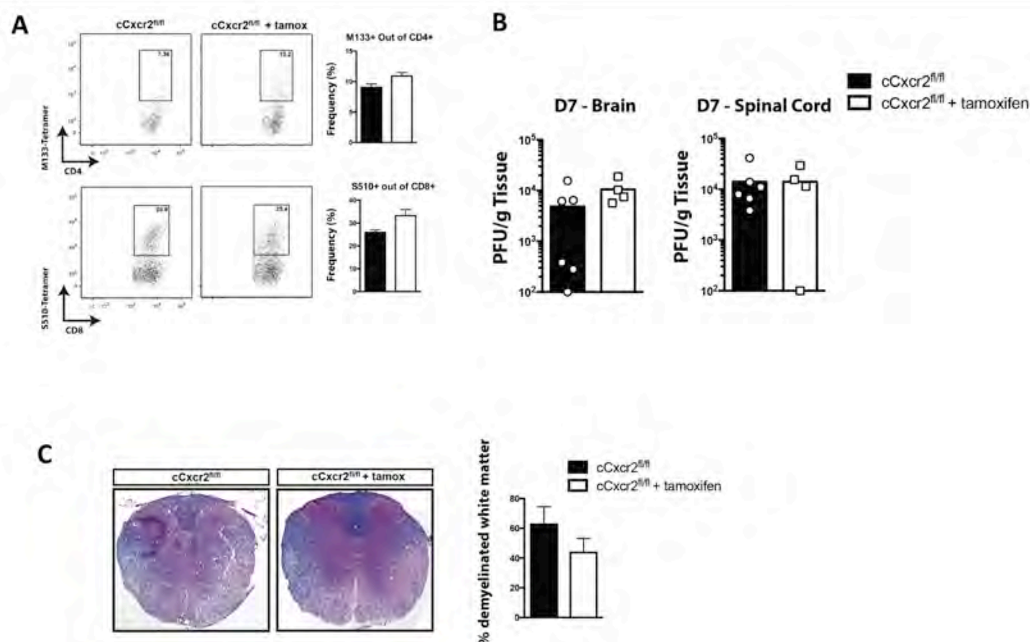

Figure 5.7: *Cxcr2* ablation does not alter antiviral immune responses nor demyelination. JHMV-infected c*Cxcr2* $^{fl/fl}$ mice treated with either tamoxifen or vehicle control and infected with 250 PFU JHMV. (**A**) M133-specific CD4+ T cells or S510-specific CD8+ cell frequencies were determined in by tetramer staining from cells derived from the brains of mice at day 6 p.i. **(B**) Viral titers in brain and spinal cord of mice were measured at day 7 p.i. (**C**) Representative (left) spinal cord sections showing demyelination within the white matter tracts of mice was measured by H&E in conjunction with LFB staining. Percent demyelination was quantified (right). Data in panel **A**, **B**, and **C** represent at least 4 mice per group and 6-8 spinal cord sections per spinal cord averaged for percent demyelination for each mouse. Data represented with averages±SEM.

CHAPTER 6

CONCLUSION

6.1 Overexpression of CXCL1 in models of MS

The ELR(+) CXC chemokines bind to the receptor CXCR2 that is expressed on neutrophils and is important in their ability to traffic to sites of inflammation (1). In the context of a model of viral-induced demyelination e.g. JHMV-infection of the CNS of susceptible mice, the ELR(+) CXC chemokine CXCL1 as well as others are increased early following infection. In EAE, the autoimmune model of MS, expression of ELR(+) chemokines are also increased near onset of disease (2-4). During acute JHMV, treatment with anti-CXCR2 depleted neutrophils in the brain, showing that the release of MMP-9 by neutrophils early during disease is important in aiding the breakdown of the BBB resulting in decreased immune cell recruitment to the CNS, unimpaired viral replication, and increased mortality in the mice (2). Blocked neutrophil trafficking in EAE models via anti-Ly6G, anti-CXCL1, or anti-CXCR2 treatment resulted in a delay in clinical disease onset (4-6). Conversely, in transgenic mice in which CXCL1 is constitutively expressed from oligodendroglia resulted in increased neutrophil accumulation within the CNS and enhanced microglial and astrocyte activation that correlated with pronounced ataxia and decreased lifespan (7). Astrocytes express the chemokine CXCL1 and we wanted to better understand how overexpression of CXCL1 influenced disease in two preclinical mouse models of MS and if neutrophils participated in demyelination (4, 8, 9).

Dox-dependent overexpression of CXCL1 from astrocytes in JHMV-infected mice and $MOG_{35\text{-}55}$-induced EAE resulted in a significant increase in clinical disease correlating with an increase in spinal cord neutrophil infiltration. In both models, there is an increase in demyelination when CXCL1 is overproduced; either depletion of neutrophils via anti-Ly6G treatment or blocking neutrophil trafficking via anti-CXCR2

decreased the severity of spinal cord demyelination. In the JHMV model, increased neutrophil accumulation in the CNS correlated with an increase in mortality independent of changes in BBB permeability, alterations in other proinflammatory cytokine levels, or infiltration of virus-specific T cells or monocyte/macrophages. Similar results appeared in the MOG_{35-55} EAE model in which the increase in disease was associated with increased neutrophil infiltration, while myelin-reactive T cells as well as other leukocyte were not affected. Together, these findings suggest that the increase in CXCL1 in the CNS during mouse models of MS contributes to increased disease severity mediated by neutrophils. Overexpression of CXCL1 within astrocytes showed an increase in macrophages/microglia within the spinal cords of experimental mice, but we do not think that these cells are contributing to the increase in morbidity since there was no increase in the activation state as determined by measuring MHC class II and CD80/86 expression. In addition, previous studies have suggested that Ly6C+ myeloid precursor cells can also contribute to disease severity in EAE (10).

6.2 Oligodendrocyte expression of CXCR2 role in MS models

The role of CXCR2 on neutrophils has been mainly studied in mouse models of MS, either by antibody blockage, antagonist administration, or use of whole-body *Cxcr2 -/-* mice (2, 5, 11, 12). During acute JHMV infection, antibody neutralization of CXCR2 increased morbidity and mortality in mice by an inability to breakdown the BBB due to the decrease in neutrophil recruitment and mice succumb to viral infection (2). In a toxin model of demyelination, CXCR2+ neutrophils contribute to white matter damage (11). In both the lysolecithin (LPC) and MOG_{35-55} EAE model, CXCR2 antagonist administration

resulted in a decrease in demyelination and increase in remyelination, although the specific cell type/types involved were not deduced (12). In the $PLP_{139-151}$ EAE and cuprizone model of demyelination, CXCR2 expression in nonhematopoietic cells impeded the ability for myelin repair, indicating that CXCR2 in resident cells of the CNS are involved in myelin repair (11). During the chronic stage of JHMV infection, anti-CXCR2 treatment increased clinical disease and this was associated with oligodendrocyte apoptosis and demyelination, suggesting a protective role of CXCR2 on oligodendrocytes in the late stages of this model (3). In addition, OPCs administered CXCL1 impedes apoptosis in response to viral infection by inhibiting cleavage of caspase 3 (13).

While the role of CXCR2 in neutrophil recruitment to sites of inflammation has been studied more extensively, CXCR2 expressed in resident cells within the CNS including neurons, OPCs, oligodendrocytes, activated astrocytes, and microglia has not been analyzed (8, 14-19). Previous studies have indicated that CXCR2 is important in OPC proliferation and migration during development and *Cxcr2 -/-* mice show spatial alterations in OPC levels in the CNS and a decrease in white matter and myelin thickness in the spinal cord of adult mice (20, 21). Since CXCR2 is important in OLCs during development, we wanted to better understand its role in PLP+ OLCs during demyelination/remyelination in MS mouse models. As such, we developed mice in which we could selectively ablate *Cxcr2* within oligodendroglia upon tamoxifen treatment and this allowed us to better understand the functional role of this receptor in disease and repair in JHMV-infected mice as well as animals with EAE.

Our findings argue that genetic ablation of CXCR2 in oligodendroglia was not important in the clinical course of both MOG_{35-55} EAE and JHMV-infected mice. No

differences in the recruitment of T cells, neutrophils, or macrophages into the CNS during either JHMV or MOG_{35-55} EAE were observed. Nor was there a difference in Th1, Th17, or Th1/Th17 cells in EAE or viral specific T cells during JHMV infection. *In vitro* deletion of CXCR2 from oligodendroglia did not alter myelin protein alterations, MBP and CNPase (12, 21, 22). Collectively, these findings would argue that CXCR2 signaling in oligodendroglia does not influence either demyelination or remyelination in neuroinflammatory models of demyelination yet is important in positional migration of OPCs and myelination during development.

6.3 Future directions

The FDA-approved drugs for MS patients have focused on the adaptive immune response, although none completely subside symptoms or relapse, thus designing drugs that modulate the innate immune system need to be tested. There has been limited direct evidence for neutrophils in MS patients due to limited autopsy tissue samples and due to the short-lived nature (6-8 hours in humans) of these cells (23, 24). Neutrophils can release toxic factors including microbicidal granules such as cathepsin G, reactive oxygen and nitrogen species, neutrophil extracellular traps (NETs), MMPs, and many cytokines and chemokines, as well as their ability to phagocytize pathogens (1, 25-28). Although neutrophils are short-lived, bystander destruction of host cells and tissue including axons and oligodendrocytes can occur through NADPH oxidase producing both nitric oxide (NO) and reactive oxygen species (ROS), which has been shown to increase oxidative stress, inflammation, and ischemia-reperfusion damage in the brain in a mouse model of ischemic injury has been observed (29-32). Neutrophils have been shown to enhance

spinal cord lesions in patients with NMO and blocking neutrophil elastase in a mouse model of NMO decreased neuroinflammation and demyelination (33-35). Neutrophils and nitric oxide synthase production enhanced glial cell death in the brains of virally infected mice (36). It has been proposed that neutrophils may augment macrophage recruitment as well as antigen presentation (6). In the MOG_{35-55} EAE model, it was observed that neutrophils activate macrophages and microglia express MHC class II and subsequently antigen presentation (6). CNS infiltrating neutrophils also contribute to disease by the production of many proinflammatory cytokines (6). During EAE, there is a distinct difference in cytokine expression between neutrophils in the CNS compared to the bone marrow and during JHMV infection neutrophils have been shown to produce MMP9 to aid in the breakdown of the BBB early during infection (2, 6)

In MOG_{35-55} EAE, it has been shown that neutrophils within the CNS produce many proinflammatory cytokines including TNF-α, IL-6, IL-12/23p40, and IFN-γ, but not IL-10 or IL-17 (6). *In vitro* studies showed that CNS neutrophils are important in enhancing MHC class II, CD80, and CD86 on bone marrow dendritic cells, but could not pinpoint the cytokine or other mechanism of how neutrophils accomplished this feat (6). In addition, neutrophils do not present MHC class II, although they reportedly increase the APC capacity of microglia/macrophages, but without altering levels of antigen-specific CD4+ T cells (6). This article was the first to show a specific role for neutrophils in MOG_{35-55} EAE, although it did not determine a mechanism. It was found that myeloperoxidase and neutrophil elastase did not alter overall clinical score in MOG_{35-55} EAE mice, suggesting is not important in pathogenesis of disease (6). In JHMV infection, we now know that neutrophils express MMP-9 and aid in breakdown of the BBB (2). The

observation that neutrophils increasing morbidity viral and autoimmune models of MS combined with from Segal and colleagues reporting an increase in CXCL1, CXCL5, and neutrophil elastase in MS patient's serum correlating with MS lesion burden suggests that targeting ELR(+) CXC chemokines or its receptor may be used as novel therapies to treat MS (37). Therefore, future studies will require understanding specifically how neutrophils contribute to neurologic disease and specifically demyelination.

We know that expression of CXCR2 on OPCs are important in proliferation and migration during development and adult mice that are *Cxcr2 -/-* show an altered spatial formation of OPCs, decreased myelin thickness in the spinal cord, and impaired remyelination (20-22). To understand the mechanism of impaired remyelination by CXCR2 within the CNS, the first CNS resident cell types to look at were OLCs. The recent article by Ransohoff and colleagues demonstrated that genetic ablation of CXCR2 in oligodendroglia enhanced remyelination arguing that CXCR2 signaling may impede OPC maturation and/or myelin synthesis in non-inflammatory models of demyelination (38). We are currently analyzing remyelination in both of these mouse models to see if CXCR2 on OLCs impedes myelin repair. To better understand the mechanism of CXCR2 in myelin repair, future studies will require the ablation of CXCR2 from other resident glial cell populations that have been shown to express CXCR2 including astrocytes, microglia, and neurons followed by observations of alterations in the pathogenesis/myelin repair in mouse models of demyelination. Overall, these studies all suggest that the development of drugs to block CXCR2 may aid in decreasing clinical symptoms in MS patients either by impeding neutrophil recruitment to the CNS or blocking the role on CNS resident cells to enhance myelin repair.

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APPENDIX

SPHINGOSINE-1-PHOSPHATE RECEPTOR ANTAGONISM ENHANCES PROLIFERATION AND MIGRATION OF ENGRAFTED NEURAL PROGENITOR CELLS IN A MODEL OF VIRAL-INDUCED DEMYELINATION

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IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

Sphingosine-1-Phosphate Receptor Antagonism Enhances Proliferation and Migration of Engrafted Neural Progenitor Cells in a Model of Viral-Induced Demyelination

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The oral drug FTY720 affects sphingosine-1-phosphate (S1P) signaling on targeted cells that bear the S1P receptors S1P1, S1P3, S1P4, and S1P5. We examined the effect of FTY720 treatment on the biology of mouse neural progenitor cells (NPCs) after transplantation in a viral model of demyelination. Intracerebral infection with the neurotropic JHM strain of mouse hepatitis virus (JHMV) resulted in an acute encephalomyelitis, followed by demyelination similar in pathology to the human demyelinating disease, multiple sclerosis. 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 expressed transcripts for S1P receptors S1P1, S1P2, S1P3, S1P4, and S1P5. FTY720 treatment of cultured NPCs resulted in increased mitogen-activated protein kinase phosphorylation and migration after exposure to the chemokine CXCL12. Administration of FTY720 to JHMV-infected mice resulted in enhanced migration and increased proliferation of transplanted NPCs after spinal cord engraftment. FTY720 treatment did not improve clinical disease, diminish neuroinflammation or the severity of demyelination, nor increase remyelination. These findings argue that FTY720 treatment selectively increases NPC proliferation and migration but does not either improve clinical outcome or enhance remyelination after transplantation into animals in which immune-mediated demyelination is initiated by the viral infection of the central nervous system. *(Am J Pathol 2015, 185: 2819–2832; http://dx.doi.org/10.1016/j.ajpath.2015.06.009)*

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 central nervous system (CNS), axonal damage, and demyelination.[1–7] Previous studies from our laboratory have used the JHMV model of neuroinflammation-mediated demyelination to evaluate the therapeutic benefit of mouse neural progenitor cell (NPC) engraftment on remyelination.[8–10] 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.[8–10] Engrafted NPCs physically engage damaged axons, and this ultimately leads to increased axonal integrity that correlates with remyelination.[8,11] These findings, along with others,[12–14] argue that engraftment of NPCs may provide an important unmet clinical need for treatment of human demyelinating

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diseases, including multiple sclerosis (MS), by facilitating sustained remyelination that can restore motor function and ameliorate clinical symptoms.

After engraftment of NPCs into the spinal cords of JHMV-infected mice, transplanted cells migrate both rostral and caudal from the implantation site.[8,9] 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 on 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.[9] However, the molecular mechanisms governing positional migration of NPCs are likely complex and consist of additional soluble factors that affect 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.[15−18] Although the importance of S1P signaling in controlling lymphocyte homing and egress from lymphatic tissues is well documented,[19−21] 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.[22,23] Activation of these receptors yields different effects on migration and survival of astrocytes, microglia, and oligodendrocytes.[24−26] In addition, NPCs express S1P receptors, and signaling has previously been reported to influence *in vitro* differentiation.[27] Moreover, Kimura et al[28] demonstrated an important role for S1P signaling in controlling migration of transplanted NPCs to an injury site in a model of spinal cord injury.

We examined the functional role of S1P signaling after NPC transplantation into the spinal cords of JHMV-infected mice. FTY720 is a U.S. Food and Drug Administration−approved oral drug for treatment of patients with relapsing MS.[22,23,29−31] FTY720 exerts immunomodulatory effects that reduce acute relapses, new lesion formation, and disability progression and brain volume loss in MS patients.[32] The mechanism(s) behind 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. FTY720 is a functional antagonist of S1P1 on lymphocytes,[20] yet also can act as a nonselective agonist of S1P1, S1P3, S1P4, and S1P5.[33] Therefore, the available evidence suggests that cellular source and receptor expression profile are critical in terms of how FTY720 affects S1P signaling, and likely lead to a dampening of autoreactive T cells specific for myelin antigens infiltrating into the CNS. More important, FTY720, because of its lipophilic nature, penetrates the blood-brain barrier 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. Our findings reveal that treatment of cultured NPCs with FTY720P led to an active signaling response, as determined by phosphorylation of mitogen-activated protein (MAP) kinase, yet did not influence lineage fate commitment. FTY720 treatment of JHMV-infected mice, transplanted with NPCs, demonstrated enhanced migration associated with increased numbers of NPCs compared with vehicle-treated control animals. FTY720 treatment did not affect the accumulation of T cells or macrophages within the CNS. Finally, after treatment in animals in which demyelination is established, FTY720 did not augment the effects of NPCs on influencing remyelination, indicating a selective effect on migration/proliferation on spinal cord engraftment into JHMV-infected mice.

Materials and Methods

Mice and Virus

Age-matched (5 to 7 weeks) S1P1 enhanced green fluorescent protein (eGFP) knock-in mice (C57BL/6 background)[34] and C57BL/6 mice were anesthetized with an i.p. injection of 150 μL of a mixture of ketamine (Western Medical Supply, Arcadia, CA) and xylazine (Phoenix Pharmaceutical, Saint Joseph, MO) in Hanks' balanced salt solution. Mice were injected intracranially with 150 plaque-forming units of JHMV (strain V2.2-1) suspended in 30 μL saline.[9] Clinical severity was assessed by blinded investigators (T.E.L. and C.A.B.) using a previously described four-point scoring scale.[35] 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). FTY720 or the vehicle was administered by daily i.p. injections of 100 μL, starting at day 13 postinfection (p.i.). Experiments for all animal studies were reviewed and approved by the University of Utah (Salt Lake City) and the University of California (Irvine) Institutional Animal Care and Use Committees.

NPC Isolation and Culture

Neurosphere cultures were prepared from brains of perinatal *S1P1* eGFP knock-in mice, as previously described.[8,36] Briefly, dissected striata were razor minced and triturated in 0.05% trypsin for 10 minutes, followed by anti-trypsin to inactivate the digestion. Single cells were resuspended in Dulbecco's modified Eagle's medium:F12 (Invitrogen, Carlsbad, CA) supplemented with B27 (Invitrogen), insulin-transferrin-selenium-X (Invitrogen), penicillin-streptomycin (Invitrogen), 40 ng/mL T3 (T67407; Sigma, St. Louis, MO), and 20 ng/mL human recombinant epidermal growth factor (E9644; Sigma). Cells were cultured for 6 days with replacement of media every other day, at which point

mature neurospheres were isolated and GFP expression was visualized by fluorescence microscopy.

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: S1P1, 5′-TTTCCATCGCCATCCTCTAC-3′ (forward) and 5′-GCAGGCAATGAAGACACTCA-3′ (reverse); S1P2, 5′-TCTCAGGGCATGTCACTCTG-3′ (forward) and 5′-CAGCTTTTGTCACTGCCGTA-3′ (reverse); S1P3, 5′-GTGTGTTCATTGCCTGTTGG-3′ (forward) and 5′-TTGACTAGACAGCCGCACAC-3′ (reverse); S1P4, 5′-GGCTACTGGCAGCTATCCTG-3′ (forward) and 5′-AAGGCCACCAAGATCATCAG-3′ (reverse); and S1P5, 5′-GATCCCTTCCTGGGTCTAGC-3′ (forward) and 5′-TAGAGCTGCGATCCAAGGTT-3′ (reverse). Primers were purchased from Invitrogen. Sequencing of PCR amplicons confirmed primer specificity.

Western Blot Analysis

NPCs were plated on Matrigel-coated 6-well plates and treated with either 100 nmol/L FTY720 phosphate or vehicle for 5 minutes, 30 minutes, 1 hour, 2 hours, and 4 hours. The cells were then lysed using radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCL (pH 7.4), 175 mmol/L NaCl, 5 mmol/L EDTA, 1% NP-40, 0.1% SDS, and 0.5% deoxycholic acid] supplemented in protease and phosphatase inhibitors (Roche). P44/42 MAP kinase (1:2000; Cell Signaling, Danvers, MA) and phosphorylated p44/42 MAP kinase (1:15,000; Cell Signaling) were detected by Western blot analysis using horseradish peroxidase−conjugated secondary antibodies (1:25,000; Jackson ImmunoResearch Laboratory, West Grove, PA) and exposed to Supersignal West-Femto chemiluminescent reagent (Pierce, Rockford, IL).

NPC Differentiation

To assess differentiation potential, NPCs expressing GFP[8,9,11,37] were grown on Matrigel-coated chamber slides with epidermal growth factor for 24 hours, at which point growth factor was removed and NPCs were allowed to differentiate for up to 7 days.[8] Cells were treated daily with 100 nmol/L FTY720P or vehicle subsequently fixed in 4% paraformaldehyde for 20 minutes at room temperature. Immunofluorescence was performed using established protocols: rabbit−anti-mouse Olig 2 (1:200; Millipore, Darmstadt, Germany), rabbit−anti-mouse glial fibrillary acidic protein (1:1000; Invitrogen), mouse−anti-mouse Map2 (1:750; Sigma), Alexa-594 anti-rabbit (1:500; Invitrogen), and anti-mouse (1:1000; Invitrogen) were used as secondary antibodies. Samples were then washed in phosphate-buffered saline (PBS), and coverslip was mounted with DAPI Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA). The percentage 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, at which point infected mice have established demyelination.[8,9,11] Mice were anesthetized with an i.p. injection of 150 μL of a mixture of ketamine (Western Medical Supply, Arcadia, CA) and xylazine (Phoenix Pharmaceutical, Saint Joseph, MO) in Hanks' balanced salt solution, a laminectomy was performed at T9, and 2.5×10^5 NPCs resuspended in 2.5 μL solution were injected into the spinal cord using a 10-μL Hamilton syringe, as previously described.[8,38−40] Recipient mice also received daily i.p. injections of 3 mg/kg FTY720 starting at day 13 p.i., whereas a control group received vehicle starting at day 13 p.i.

NPC Chemotaxis

In vitro chemotaxis assays were performed using a NeuroProbe ChemoTx system, according to the manufacturer's protocol. Briefly, GFP-NPCs treated with 100 nmol/L, 10 nmol/L, and 1 μmol/L FTY720P or vehicle control were allowed to migrate in response to recombinant mouse CXCL12 (Peprotech, Rocky Hill, NJ). Migration after 16 hours of culture was assessed by a fluorescent microplate reader (Synergy H1; BioTek, Winooski, VT), according to manufacturer's specifications.

Histology

Spinal cords were isolated at defined time points and fixed overnight with 4% paraformaldehyde at 4°C. Individual spinal cords were divided into sections, and twelve coronal sections (1 mm thick) were cryoprotected in 20% sucrose and embedded in OCT (VWR, Radnor, PA). Coronal sections (8 μm thick) were cut, and sections were stained with luxol fast blue. Areas of total white matter and demyelinated white matter were determined with ImageJ software version 1.48 (NIH, Bethesda, MD; *http://imagej.nih.gov/ij*).[41] Demyelination was scored as a percentage of total demyelination along the entire length of the spinal cord. The total numbers of GFP-positive cells were determined in each of the twelve spinal cord sections surrounding the transplant site by counting GFP-positive cells colocalized with DAPI-positive nuclei. Determination of both the severity of demyelination and numbers of GFP-positive cells was performed blinded (T.E.L. and C.A.B.).

Electron Microscopy

Mice were perfused with 0.1 mol/L cacodylate buffer containing 2% paraformaldehyde/2% glutaraldehyde, and spinal

cords were isolated, and then embedded in Epon (Danbury, CT) epoxy resin. Serial ultrathin sections were stained with uranyl acetate−lead citrate and analyzed as previously described. Images at ×1200 magnification were analyzed for *g*-ratio using ImageJ software.[42] A minimum of 150 axons were analyzed per mouse, and the *g*-ratio was calculated by dividing the axon diameter by the total fiber diameter.

Immunofluorescence

For immunophenotyping of glial cells, fixed spinal cord sections were incubated in 10% normal goat serum (Jackson ImmunoResearch, West Grove, PA) and 1% bovine serum albumin for 1 hour at room temperature. This was followed by an overnight incubation at 4°C with the following primary antibodies in 1% bovine serum albumin: rabbit anti-mouse glutathione *S*-transferase (GST)-π (1:1000; MBL, International Corporation, Woburn MA), rabbit−anti-mouse glial fibrillary acidic protein (GFAP; 1:1000; Invitrogen), and rabbit−anti-mouse Ki-67 (1:300; Abcam, Cambridge, MA). Sections were then washed in PBS and incubated for 1 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). Next, sections were washed in PBS and coverslip mounted using DAPI Vectashield Mounting Medium (Vector Laboratories). The percentage 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, spinal cords, and blood were isolated at day 21 or 28 p.i. from infected mice treated with 3 mg/kg FTY720 or vehicle, starting at day 13 p.i. and transplanted with GFP-labeled NPCs. By using previously described protocols,[41] tissues were then homogenized and immunophenotyped by flow cytometry using the following antibodies: rat−anti-mouse CD4-allophycocyanin (1:50; Biolegend, San Diego, CA), rat−anti-mouse CD8-allophycocyanin (1:50; Biolegend), S510 to S518 tetramer-phosphatidylethanolamine (1:300; NIH), and M133 to M147 tetramer-phosphatidylethanolamine (1:150; NIH). Blood was collected by cardiac heart puncture, and cells were stained with rat−anti-mouse CD4-allophycocyanin and CD8-phosphatidylethanolamine after red blood cell lysis. Samples were analyzed using a BD-Fortessa X-20 Flow Cytometer (BD Biosciences, Franklin Lakes, NJ).

Results

FTY720 Treatment Activates Cultured NPCs

FTY720 functions as both an antagonist and agonist for members of the S1P receptor family whose natural ligand

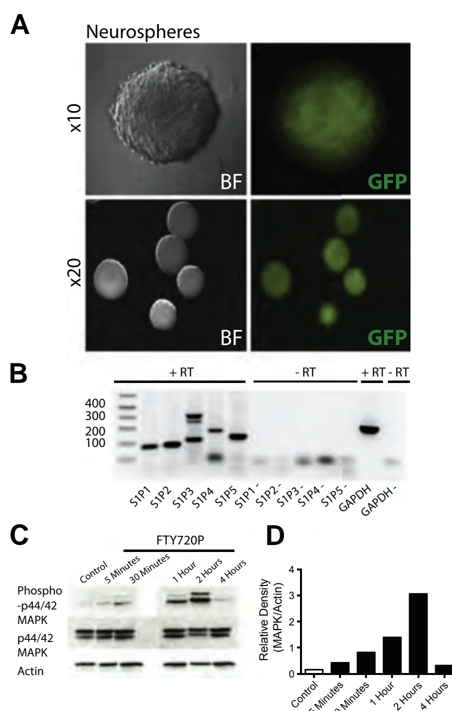

Figure 1 FTY720 treatment activates cultured neural progenitor cells (NPCs). Neurospheres were isolated from the subventricular zone of sphingosine-1-phosphate receptor 1 (S1P1) enhanced green fluorescent protein (eGFP) neonatal pups. **A:** Representative immunofluorescence images confirm that neurospheres express S1P1, as evidenced by GFP expression. **B:** Analysis of S1P receptor expression by NPCs at the mRNA level demonstrates expression of transcripts specific for S1P1 to S1P5; the sequence of amplicons confirmed primer specificity. **C:** Western blot analysis of cultured NPCs treated with either vehicle or 100 nmol/L phosphorylated active form of FTY720 (FTY720P) reveals increased phosphorylation over time. **D:** Quantitative analysis of Western blot data confirms increased phosphorylation of mitogen-activated protein kinase (MAPK). Analyses of band intensity on films are presented as the relative ratio of phosphorylated MAPK/actin. BF, brightfield microscopy; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

is S1P. Previous studies have demonstrated that FTY720 preferentially binds S1P1, S1P3, S1P4, and S1P5 receptors, including lower affinity for S1P4, but does not bind to S1P2.[19] We tested whether mouse NPCs expressed S1P receptors and if FTY720 treatment affected defined responses. Neurospheres were isolated from the subventricular zone of day 1 old eGFP-S1P1 knock-in mice,[8,36] and immunofluorescence confirmed that NPCs express S1P1, as evidenced by GFP expression (Figure 1A). Subsequent analysis of additional S1P receptor expression by RT-PCR demonstrated expression of transcripts for all five defined

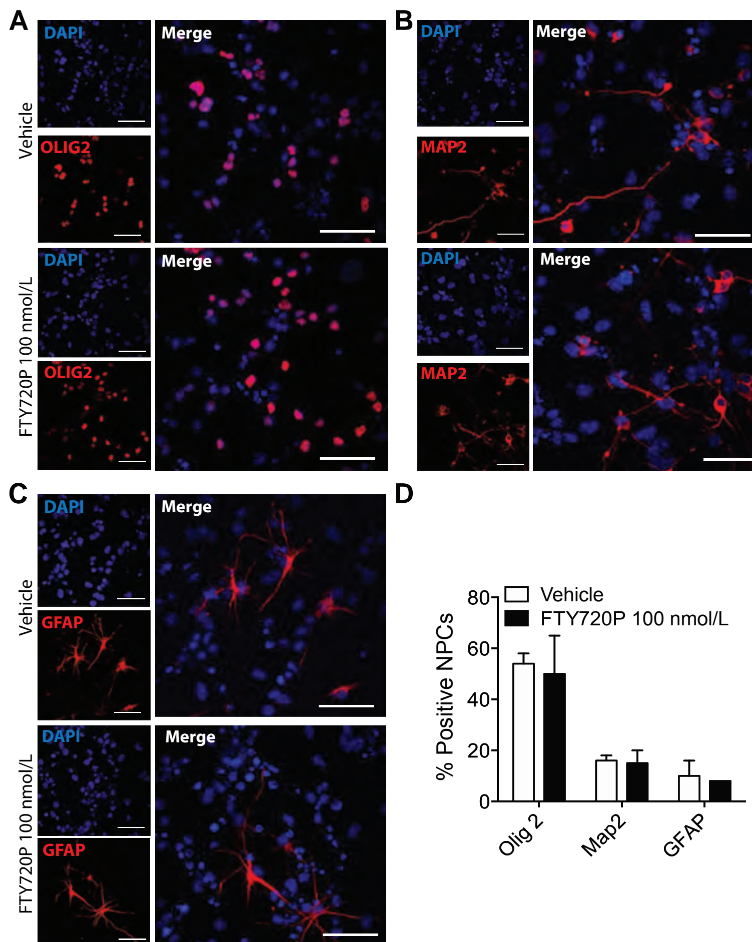

Figure 2 FTY720 does not affect neural progenitor cell (NPC) differentiation. Exposure of cultured NPCs to daily 100 nmol/L phosphorylated active form of FTY720 (FTY720P) for 5 days does not influence lineage fate commitment to either oligodendroglia (Olig2; **A**), neurons [mitogen-activated protein (Map) 2; **B**], and astrocytes [glial fibrillary acidic protein (GFAP); **C**] compared with vehicle-control–treated cultures. **D:** Quantification of immunocytochemical staining for defined cell lineages indicates similar frequencies of Olig2-, MAP2-, and GFAP-positive cells after treatment of cultured NPCs with either vehicle or FTY720P. Data were presented as means ± SEM (**D**). $n = 3$ independent experiments (**D**).

S1P receptors (Figure 1B). Previous studies have demonstrated that FTY720 treatment activates several intracellular signaling cascades, including phosphorylation of MAP kinase.[43,44] Treatment of cultured NPCs with the activated FTY720P (100 nmol/L) resulted in phosphorylation of MAP kinase in a time-dependent manner, indicating receptor binding and activation (Figure 1, C and D). These findings support earlier studies[15] demonstrating that NPCs express S1P receptors and FTY720 treatment initiates activation of intracellular signaling pathways.

FTY720 Does Not Affect NPC Differentiation

We next tested whether exposure of cultured NPCs to FTY720 influenced lineage fate commitment. Under defined conditions, cultured NPCs will preferentially differentiate into oligodendroglia, although astrocytes and neurons are

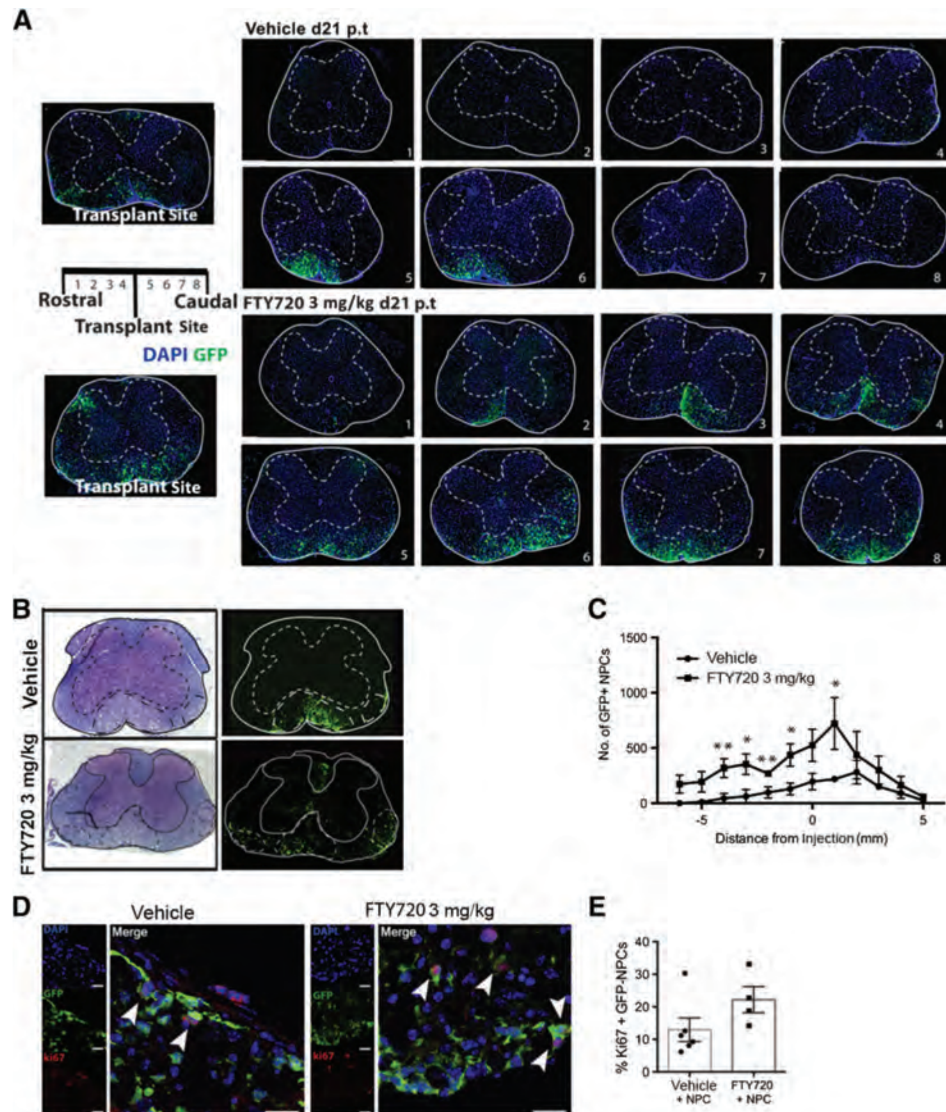

Figure 3 FTY720 treatment enhances migration of engrafted green fluorescent protein (GFP)—neural progenitor cells (NPCs). JHMV-infected mice were treated with FTY720 (3 mg/kg daily via i.p. injection) or vehicle control beginning at day 13 postinfection (p.i.). GFP-expressing NPCs were transplanted into the spinal cords at day 14 p.i., and migration of transplanted cells rostral and caudal to the implantation site was assessed 3 weeks posttransplant (p.t.). **A:** Transplanted GFP-NPCs migrate both rostral and caudal from the implantation site in both control and FTY720-treated mice. Images represent spinal cord sections rostral (1 to 4) and caudal (5 to 8) from the transplantation site. **B:** Transplanted GFP-NPCs congregate within areas of demyelination located in the anterior and lateral funiculus in both FTY720-treated mice and vehicle control. **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 Ki-67 staining by transplanted GFP-NPCs in vehicle control and FTY720-treated mice. **Arrowheads** represent Ki-67$^+$ transplanted GFP-NPCs. **E:** Quantification of GFP-NPCs expressing Ki-67. Data are presented as means ± SEM (**C** and **E**). n = 2 or more independent experiments with n = 4 or more mice per group (**C** and **E**). *$P < 0.05$, **$P < 0.01$.

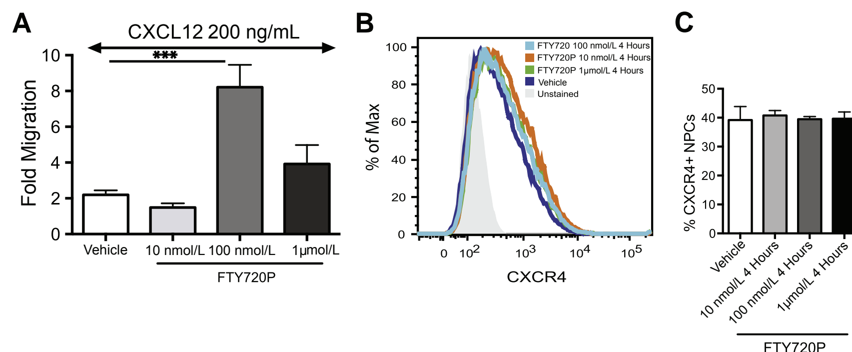

Figure 4 FTY720 does not affect CXCR4 expression by neural progenitor cells (NPCs). **A:** Cultured NPCs were treated with increasing concentrations of phosphorylated active form of FTY720 (FTY720P; 10 nmol/L, 100 nmol/L, and 1 μmol/L), and migration in response to 200 ng/mL recombinant mouse CXCL12 was determined after 16 hours. Exposure to FTY720P results in a dose-dependent migration response. **B:** Representative flow analysis reveals that FTY720 treatment does not affect surface expression of CXCR4 by cultured NPCs at a defined concentration. **C:** This is confirmed by quantification of mean fluorescence intensity of CXCR4 expression of cultured cells under experimental conditions. Data are given as means ± SEM (**A** and **C**). $n = 2$ independent experiments (**A**); $n = 3$ independent experiments (**C**). $***P < 0.001$. Max, maximum.

also present.[8,9] Exposure of NPCs to 100 nmol/L FTY720P did not influence differentiation because we detected similar frequencies of oligodendroglia (Olig2 positive), astrocytes (GFAP), and neurons (Map2) when compared with vehicle-control−treated cultures (Figure 2, A−D). Therefore, S1P receptor antagonism does not affect NPC differentiation.

FTY720 Treatment Enhances Migration of Engrafted GFP-NPCs

To test if FTY720 affects migration of engrafted NPCs, JHMV and GFP-expressing NPCs (GFP-NPCs) were transplanted into the spinal cord at day 14 p.i.,[8,11,37,39] and FTY720 treatment (3 mg/kg, daily, via i.p. injection) was initiated at day 13 p.i. Control groups consisted of JHMV-infected mice transplanted with GFP-NPCs treated with vehicle only. Mice were sacrificed at 3 weeks posttransplant, and GFP-NPC distribution was assessed histologically in spinal cord cross sections. In both FTY720 and vehicle-treated control mice, GFP-NPCs were distributed rostral and caudal from the implantation site and colonized areas of demyelination within the ventral funiculus and lateral white matter columns (Figure 3, A 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 with vehicle-treated mice (Figure 3C). Immunostaining for Ki-67 revealed increased numbers of Ki-67−positive GFP-NPCs throughout the spinal cord in FTY720-treated mice, consistent with increased proliferation (Figure 3, D and E).

These findings suggest a role in S1P receptor antagonism in controlling proliferation and/or migration of NPCs engrafted into the spinal cord. We have previously shown that CXCL12 is critical in controlling the positional migration of engrafted NPCs by signaling through CXCR4 expressed on the surface of NPCs.[9] Furthermore, Kimura et al[45] have reported that FTY720 treatment promotes migration of human $CD34^+$ hematopoietic progenitor cells by enhancing CXCR4 function. Cultured NPCs were treated with increasing concentrations of FTY720P (10 nmol/L, 100 nmol/L, and 1 μmol/L), and the *in vitro* migration in response to 200 ng/mL recombinant mouse CXCL12 resulted in a dose-dependent migration response (Figure 4A). Flow analysis revealed that FTY720 treatment did not affect surface expression of CXCR4 at any concentration tested (Figure 4, B and C). Therefore, administration of FTY720 enhances migration of NPCs potentially by enhancing CXCR4 function, consistent with earlier studies.[45]

FTY720 Treatment of NPC-Transplanted Mice Does Not Affect NPC Differentiation or Demyelination

FTY720 did not affect clinical disease in JHMV-infected mice, regardless if transplanted with GFP-NPCs or treated with vehicle (data not shown). We next examined if FTY720 treatment influenced the ability of engrafted NPCs to differentiate into oligodendroglia, because our previous studies have shown that most transplanted cells preferentially differentiate into these cells.[8,9] By 14 days posttransplant, FTY720 did not affect lineage fate commitment of NPCs because similar frequencies of GST-π−positive cells (a marker for mature myelin-producing oligodendrocytes) were observed in FTY720 versus vehicle-treated mice (Figure 5, A and B). The severity of spinal cord demyelination in transplanted mice treated with FTY720 was examined by staining serial coronal sections rostral and caudal to the implantation site with luxol fast blue and quantifying the percentage of white matter damage.[41,42] By day 14 posttransplant, the severity of demyelination was similar in transplanted mice treated with

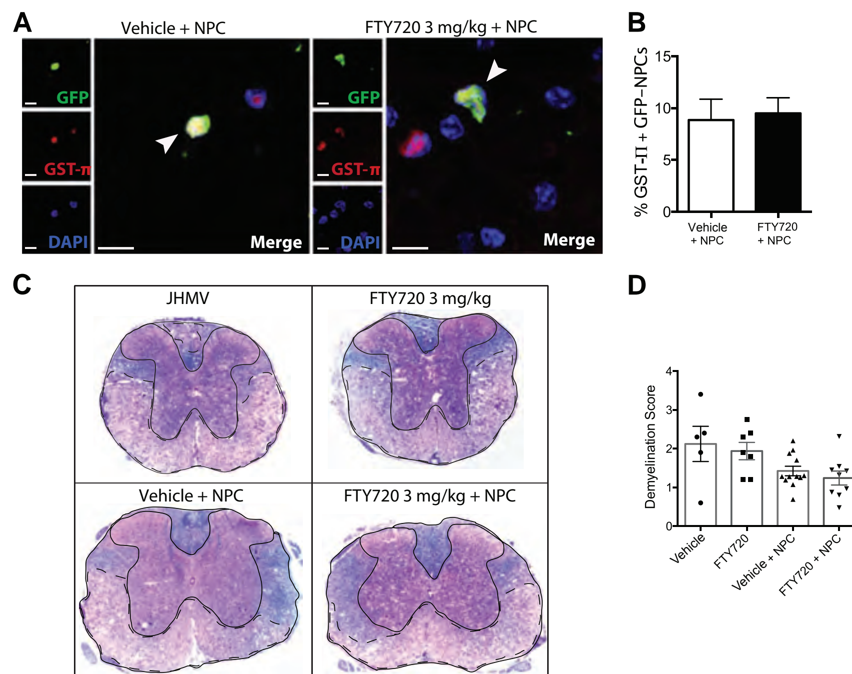

Figure 5 FTY720 does not decrease the severity of demyelination. Mice were infected with 150 plaque-forming units of JHMV and 3 mg/kg FTY720 or control vehicle treatment initiated at day 13 postinfection (p.i.) and transplanted with green fluorescent protein (GFP)–neural progenitor cells (NPCs) at day 14 p.i. In addition, JHMV-infected mice treated with FTY720 or vehicle alone served as an additional control. **A:** Representative glutathione *S*-transferase (GST)-π immunofluorescence staining of spinal cords isolated at day 14 posttransplant (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. **Arrowheads** represent GST-π–positive transplanted GFP-NPCs. **B:** Similar frequencies of GFP-positive mature oligodendrocytes in GFP-NPC–transplanted mice treated with either FTY720 or vehicle. Twelve spinal cord sections per mouse were counted to determine the frequency of transplanted GFP-NPCs that differentiated into GST-π–positive cells. **C:** Representative luxol fast blue–stained spinal cord sections from NPC-transplanted mice treated with either FTY720 or control vehicle, or nontransplanted mice treated with FTY720 or control vehicle at day 14 p.t. **D:** Quantification of demyelination indicates no differences in the severity of white matter damage in experimental groups of mice at day 14 p.t. Data are presented as means ± SEM (**B** and **D**). $n = 2$ independent experiments with $n = 4$ or more mice per experimental group (**B**); $n = 2$ independent experiments with $n = 5$ or more mice per experimental group (**D**). Scale bar = 50 μm (**A**).

FTY720 when compared with control animals (Figure 5, C and D). Electron microscopic analysis of spinal cords from experimental mice was performed to better assess whether FTY720 treatment of mice promoted remyelination. Determination of the *g*-ratio, the ratio of the inner axonal diameter/the total outer fiber diameter, is an established structural index of remyelination, with lower ratios indicating more extensive remyelination.[42] Regions of spinal cord ventral and lateral white matter tracts of JHMV-infected mice, transplanted with GFP-NPCs and treated with FTY720 or vehicle, and JHMV-infected mice treated with vehicle or FTY720 alone were analyzed (Figure 6A).

We first determined whether FTY720 treatment alone increased remyelination in JHMV-infected mice. Our findings indicate that, although there was an overall trend toward remyelination after FTY720 treatment compared with control mice, this did not reach significance (Figure 6, B, C, and F). Increased remyelination was observed in JHMV-infected mice that were transplanted with GFP-NPCs compared with vehicle treatment alone (Figure 6, B, D, and F). However, FTY720 did not result in a significant increase in remyelination in GFP-NPC–treated animals compared with transplanted animals treated with vehicle alone (Figure 6, B, E, and F). Therefore, these findings argue that FTY720 treatment does not enhance remyelination in JHMV-infected mice regardless if transplanted with GFP-NPCs.

Treatment with FTY720 Does Not Affect Neuroinflammation in JHMV-Infected Mice

We have previously determined that FTY720 treatment of JHMV-infected mice during acute disease results in

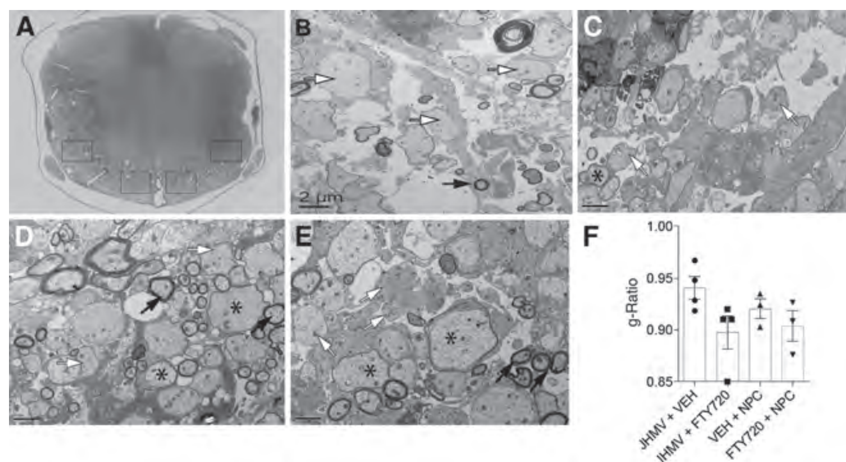

Figure 6 FTY720 treatment does not promote remyelination. **A:** Representative transverse spinal cord section; **boxed areas** indicate the regions in which demyelinated and remyelinated axons were determined. Representative electron microscopic (EM) images of spinal cords of JHMV—infected mice treated with vehicle (VEH) alone (**B**), FTY720 (**C**), green fluorescent protein (GFP)—neural progenitor cells (NPCs) and vehicle (**D**), and GFP-NPCs and FTY720 (**E**). **Black arrows** indicate myelinated axons; **white arrows**, demyelinated axons; **asterisks**, remyelinated axons. **F:** Calculation of *g*-ratio, as a measurement of axonal remyelination, shows no significant differences between experimental mice. Data are presented as means ± SEM (**F**). $n = 3$ per group with $n = 150$ or more axons per mouse analyzed (**F**). Original magnification, ×1200 (**B**—**E**).

increased mortality and limited infiltration of T cells into the CNS, which correlated with impaired ability to control viral replication within the CNS.[41] We next examined whether FTY720 treatment affected T-cell infiltration into the CNS of mice either infected with JHMV or infected and transplanted with GFP-NPCs. Mice were infected intracranially with JHMV, and mice received daily i.p. injections of FTY720 beginning at day 13 p.i. Flow analysis of T-cell infiltration into the spinal cords of infected mice isolated at day 28 p.i. indicated no differences in $CD4^+$ or $CD8^+$ T cells within the spinal cords of mice treated with either FTY720 or vehicle alone (Figure 7, A and B). Moreover, infiltration of virus-specific $CD4^+$ and $CD8^+$ T cells was not affected after FTY720 treatment (Figure 7, A and B).

Transplantation of GFP-NPCs into JHMV-infected mice did not affect infiltration of total $CD4^+$ and $CD8^+$ T cells nor virus-specific T cells into the spinal cord (Figure 7, C and D), and this is consistent with our previously published studies.[46] Similarly, administration of FTY720 to infected mice transplanted with GFP-NPCs did not prevent total T-cell or virus-specific T-cell entry into the CNS (Figure 7, C and D). In addition, FTY720 did not affect T-cell infiltration into the brains of mice infected with JHMV alone or transplanted with GFP-NPCs (data not shown). We confirmed the biological 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 $CD4^+$ (Figure 8A) and $CD8^+$ (Figure 8C) T cells within the blood compared with control mice. In addition, by 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 $CD4^+$ (Figure 8B) and $CD8^+$ T cells (Figure 8D) in FTY720-treated mice when compared with control animals. Therefore, FTY720 treatment of JHMV-infected mice results in diminished levels of circulating lymphocytes that correlates with decreased S1P1 expression, and this is consistent with our earlier findings.[41] These results indicate that FTY720 does not affect T-cell migration into the CNS during chronic disease in animals, regardless of whether transplanted with GFP-NPCs or vehicle alone.

Discussion

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.[11,42,47–50] After 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.[9] However, it is likely that other signaling cues are present within this inflammatory demyelinating environment that influence NPC migration. The S1P/S1P1 axis has been shown to be involved in NPC migration to sites of damage in a model of spinal cord injury highlighting the importance of S1P receptors in mediating positional migration of NPCs.[28] Treatment of mice with FTY720

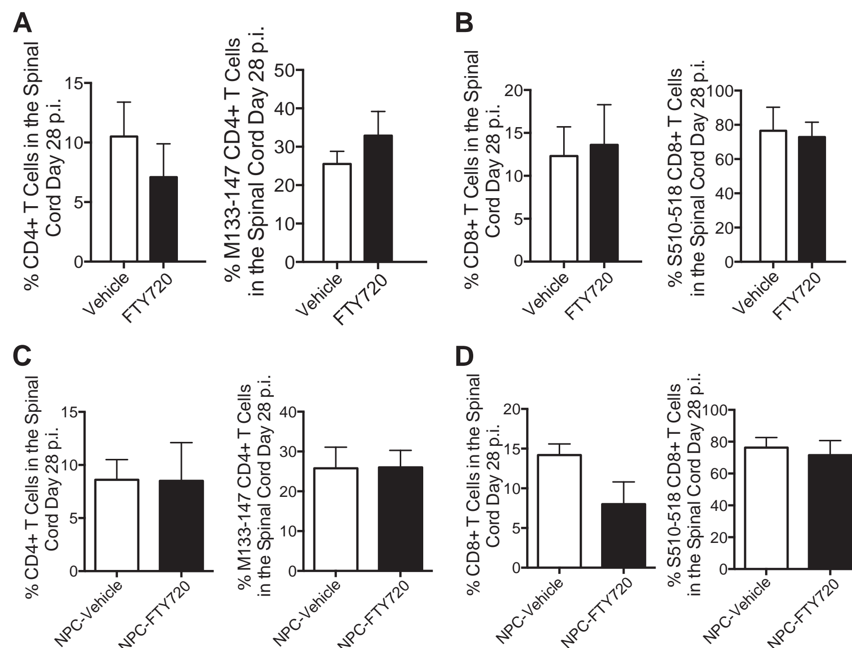

Figure 7 Treatment with FTY720 does not affect neuroinflammation in JHMV infected mice. The effect of FTY720 treatment on T-cell infiltration into the spinal cord after either JHMV infection alone or GFP-NPCs transplantation was examined. Spinal cords were removed at day 28 postinfection [p.i.; day 14 posttransplant (p.t.) of GFP-NPCs], and the frequency of total T-cell subsets and virus-specific T cells was determined. There is no difference in frequencies of $CD4^+$ (**A**) or $CD8^+$ (**B**) T cells and virus-specific $CD4^+$ (**A**) or $CD8^+$ (**B**) T cells in JHMV-infected mice treated with either vehicle control or FTY720. FTY720 does not reduce overall frequencies of infiltrating $CD4^+$ (**C**) or $CD8^+$ (**D**) T cells compared with vehicle-treated control mice nor are there differences in the frequencies of virus-specific $CD4^+$ (**C**) and $CD8^+$ (**D**) T cells following spinal cord transplantation of GFP-NPCs. Data represent means ± SEM (**A**–**D**). $n = 4$ mice or more per group (**A**–**D**).

augments CXCR4 signaling and potentiates migration of hematopoietic stem cells.[45] Moreover, FTY720 readily penetrates the CNS,[19,24,33,51,52] arguing that it can modulate the biology of transplanted NPCs by binding to S1P receptors. Indeed, Gonzalez-Cabrera et al[53] have shown that chronic FTY720 degrades and down modulates the receptor in the CNS. FTY720 has a 17:1 brain/plasma ratio and is accumulated in the brain, providing long-term steady-state levels that drive complete receptor occupancy and degradation. Therefore, we investigated the effects of FTY720 treatment in conjunction with NPC therapy in a viral model of MS.

Herein, we show that FTY720 treatment of JHMV-infected mice transplanted with GFP-NPCs results in enhanced migration of transplanted cells when compared with transplanted animals treated with vehicle control (Figure 3, A and C). FTY720 treatment did not alter positional migration of transplanted NPCs because these cells efficiently congregated within areas of demyelination (Figure 3, A and B). The *in vivo* migration data support our *in vitro* experimental results showing that FTY720 treatment of cultured NPCs increases migration after exposure to recombinant mouse CXCL12, and this was independent of elevated surface expression of CXCR4 on NPCs (Figure 4, A and B). These findings argue that a mechanism underlying enhanced NPC migration could involve 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 Janus-activating kinase 2 in progenitor cells,[54] and affects CXCR4-mediated migration in hematopoietic stem cells after exposure to CXCL12.[45]

Future work focusing on defining the specific S1P receptor(s) involved in elevated CXCR4 function will be critical to better understand the molecular mechanisms governing how receptor agonists/antagonists influence NPC migration mediated by CXCR4.

Our findings also indicated increased numbers of GFP-NPCs within demyelinated white matter tracts of GFP-NPCs

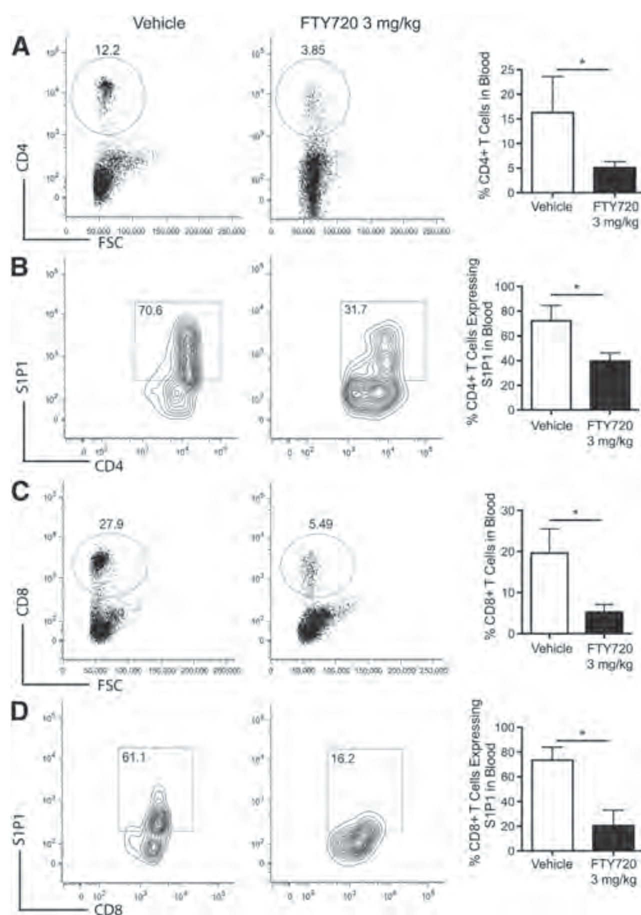

Figure 8 FTY720 induces lymphopenia and down-regulates sphingosine-1-phosphate receptor 1 (S1P1) on T cells. Frequencies of $CD4^+$ and $CD8^+$ T cells in the blood day 7 posttransplant with GFP-NPCs into JHMV-infected S1P1 eGFP mice treated daily with FTY720 or control starting at day 13 postinfection. FTY720 significantly diminishes the frequency of both $CD4^+$ (**A**) and $CD8^+$ (**C**) T cells and S1P1 expression measured by GFP expression on $CD4^+$ (**B**) and $CD8^+$ (**D**) T cells. Data are presented as means ± SEM (**A**–**D**). $n = 2$ or more experiments with a minimum $n = 4$ per group (**A**–**D**). $^*P < 0.05$. FSC, forward scatter.

in JHMV-infected mice treated with FTY720 when compared with transplanted mice treated with vehicle control, suggesting proliferation is increased *in vivo*. Collectively, these results argue that FTY720 treatment increased NPC migration and proliferation following engraftment.

We have recently shown that FTY720 treatment of JHMV-infected mice during acute disease 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.[41] 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.[19,20,51,52] More important, administration of FTY720 to JHMV-infected mice during acute disease was associated with diminished severity of demyelination. These findings highlight an important role for S1P signaling in host defense during acute viral-induced neurological disease, most likely by enhancing T cells to efficiently migrate from lymphatic tissue into the CNS. In marked contrast, the present study indicates that when FTY720 treatment is initiated at day 13 p.i. with JHMV, there is no effect on T-cell accumulation within the CNS, and the severity of demyelination is not affected. We believe these differences in outcomes with regard to neuroinflammation reflect the stage of disease at which point FTY720 is administered. As indicated above, FTY720 treatment during acute disease limited egress of virus-specific T cells from draining cervical lymph nodes, thereby reducing the severity of neuroinflammation and demyelination. By day 13 p.i., surviving

mice have reduced viral titers below the level of detection as a result of infiltration of virus-specific T cells into the CNS that can effectively control viral replication. Correspondingly, treatment with FTY720 would have limited-to-no effect on disease progression at this stage of disease because the bulk of virus-specific lymphocytes have already expanded and exited the draining cervical lymph nodes and subsequently infiltrated the CNS. In addition, these data also indicate that T cells do not rely on S1P signaling for T-cell migration to the CNS during chronic disease but rather use other inflammatory signaling cues (eg, chemokines) to gain access to the CNS of JHMV-infected mice.[2,55]

Although FTY720 treatment of cultured NPCs resulted in activation, increased proliferation, and enhanced migration in response to CXCL12, there was no effect on lineage fate commitment because similar frequencies of oligodendroglia, astrocytes, and neurons were observed compared with NPCs treated with control vehicle. Similar frequencies of GFP-positive oligodendroglia expressing GST-π were detected within spinal cords of GFP-NPC−transplanted mice treated with either FTY720 or vehicle control. Although there were increased numbers of GFP-NPCs within areas of demyelination in FTY720-treated mice, we did not observe any discernable increase in remyelination. Whether this is because of functional deficits in these cells is currently unknown and is the focus of ongoing studies.

We have previously shown that engrafted NPCs can remyelinate demyelinated axons,[11] and these findings would argue that FTY720 does not increase the remyelination potential of engrafted NPCs in our model of viral-induced demyelination. This is similar to previous studies that determined that FTY720 does not induce remyelination in either the cuprizone or lysophosphatidyl choline models of demyelination.[56] However, Miron et al[57] have demonstrated that FTY720 treatment resulted in increased remyelination in organotypic cerebral slices where demyelination was induced by lysolecithin. These findings support other studies demonstrating FTY720 treatment in augmenting neurogenesis and repair in models of CNS injury, possibly by activating endogenous NPCs and/or oligodendrocyte progenitor cells.[58−62] We are currently examining whether FTY720 activates endogenous progenitor cells within the CNS of JHMV-infected mice. The conflicting reports on the effects of FTY720 on remyelination highlight differences in model systems used, emphasizing the potential importance of targeting specific receptors for promoting OPC maturation and myelin synthesis. For example, by using a lysophosphatidyl choline−induced model of demyelination, administration of S1P5 agonist has been reported to have a greater effect on remyelination compared with S1P1 agonists.[63] FTY720 treatment of MS patients with the relapsing-remitting form of disease reduced the risk of disability progression; yet, it is not clear if this is because of an increase in remyelination.[64] The fact that we did not observe any increase in remyelination in JHMV-infected mice treated with FTY720 alone would argue additional studies in preclinical models of MS with more selective S1P receptor agonists or antagonists to better understand the effects on both endogenous glial cells and transplanted NPCs with regard to promoting remyelination.

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