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## Neutrophils and viral-induced neurologic disease

Jonathan J. Grist<sup>1</sup>, Brett Marro<sup>2</sup>, and Thomas E. Lane<sup>1</sup>

<sup>1</sup>Department of Pathology, University of Utah School of Medicine, Salt Lake City, UT 84112

<sup>2</sup>Department of Immunology & Microbial Science, The Scripps Research Institute, La Jolla CA 92037

### 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.

### Keywords

neutrophils; mouse hepatitis virus; chemokines; chemokine receptors

## 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 (Wang et al., 1992). In response to viral infection of glial cells, a rapid and orchestrated expression of chemokines occurs that contribute to attracting inflammatory cells into the

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Address Correspondence: Thomas E. Lane, Ph.D., Department of Pathology, University of Utah, School of Medicine, Salt Lake City, UT 84112, tom.lane@path.utah.edu, Telephone: 801-585-5554.

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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- $\gamma$  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 (Cheever et al., 1949; Perlman et al., 1999).

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 (Moser et al., 1990; Schumacher et al., 1992; Wolpe et al., 1989). 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) (Gorio et al., 2007; Tonai et al., 2001) and autoimmune demyelination (Carlson et al., 2008) while blocking or silencing of CXCR2 signaling mutes inflammation and tissue damage in mouse models in which PMN infiltration is critical to disease initiation (Belperio et al., 2005; Carlson et al., 2008; Gorio et al., 2007; Kielian et al., 2001; Londhe et al., 2005a, b; Strieter et al., 2005; Wareing et al., 2007).

## 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 (Borregaard, 2010; Nathan, 2006). While a clear role for neutrophils in combating bacterial pathogens is documented (Borregaard, 2010; Nathan, 2006), how these cells contribute to host defense and disease in response to CNS viral infection is less well characterized. McGavern and colleagues (Kim et al., 2009) 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 (Guha et al., 2015). Experimental 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 (Wang et al., 2012). With regards to JHMV-induced encephalomyelitis, early work by Stohlman and colleagues (Zhou et al., 2003) 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) (Zhou et al., 2003) although other factors independent of MMP-9 may also be involved (Savarin et al., 2010). In addition, monocytes can also enhance T cell accumulation within the CNS of JHMV-infected mice through the glia limitans (Savarin et al., 2010). 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 (Hosking et al., 2009). Indeed, treatment of JHMV-infected mice with a blocking antibody specific for CXCR2 resulted in 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 (Hosking et al., 2009). 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 (Hosking et al., 2009). 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 (Christy et al., 2013; Howe et al., 2012; Ransohoff and Brown, 2012; Sayed et al., 2010; Sewell et al., 2004). Herz and colleagues (Herz et al., 2015) 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) (Provencio et al., 2016). Additionally, Zenaro et al. (Zenaro et al., 2015) 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 (Zenaro et al., 2015). 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 (Bao et al., 2012; Saiwai et al., 2010). 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 and colleagues (Liu et al., 2010), 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 (Kostic et al., 2014). 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 (Jacob et al., 2012; Openshaw et al., 2000). Additionally, neutrophils have been reported to be more numerous and exhibit a more primed state in MS patients (Naegele et al., 2012). Recent studies (Huber et al., 2014; Rumble et al., 2015) 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 muted when neutrophil trafficking to the CNS is disrupted (Carlson et al., 2008; McColl et al., 1998). More recently, Stoolman et al. (Stoolman et al., 2014) 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 (Liu et al., 2015). 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 (Simmons et al., 2014). 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*) (Marro et al., 2016) (Figure 1A). Astrocytes were chosen for targeted expression of CXCL1 as previous studies (Glabinski et al., 1999; Glabinski et al., 1998; Kang et al., 2013) have shown that JHMV-infected astrocytes express CXCL1 (Hosking et al., 2010; Lane et al., 1998). 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 (Figure 1B) (Marro et al., 2016). 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 (Figure 1C) (Marro et al., 2016). 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<sup>+</sup> and CD8<sup>+</sup> T cells within the CNS of double tg mice compared to single tg mice (Marro et al., 2016). However, Dox-treatment of JHMV-infected double tg mice resulted in increased clinical disease and mortality when compared to infected single tg mice (Marro et al., 2016).

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 (Marro et al., 2016). 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 (Marro et al., 2016). 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. (Marro et al., 2016). 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 (Marro et al., 2016). 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. (Marro et al., 2016). 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 day 4 p.i. as measured by sodium fluorescein (NaF) uptake (Marro et al., 2016). 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) (Marro et al., 2016). 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- $\pi$ ) within the spinal cords and increased numbers of microglia in Dox-treated JHMV-infected double tg mice compared to infected single tg mice (Marro et al., 2016). 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) (Marro et al., 2016). Additionally, neutrophils were detected within the spinal cord parenchyma of double tg mice compared to single tg mice (Fig. 2B) (Marro et al., 2016). 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 (Marro et al., 2016)

## 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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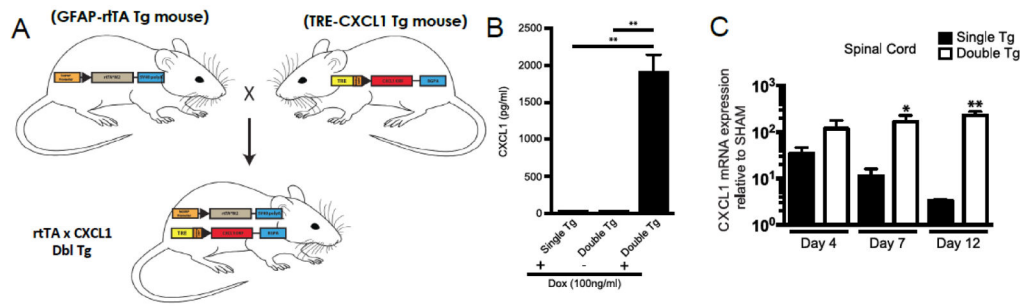
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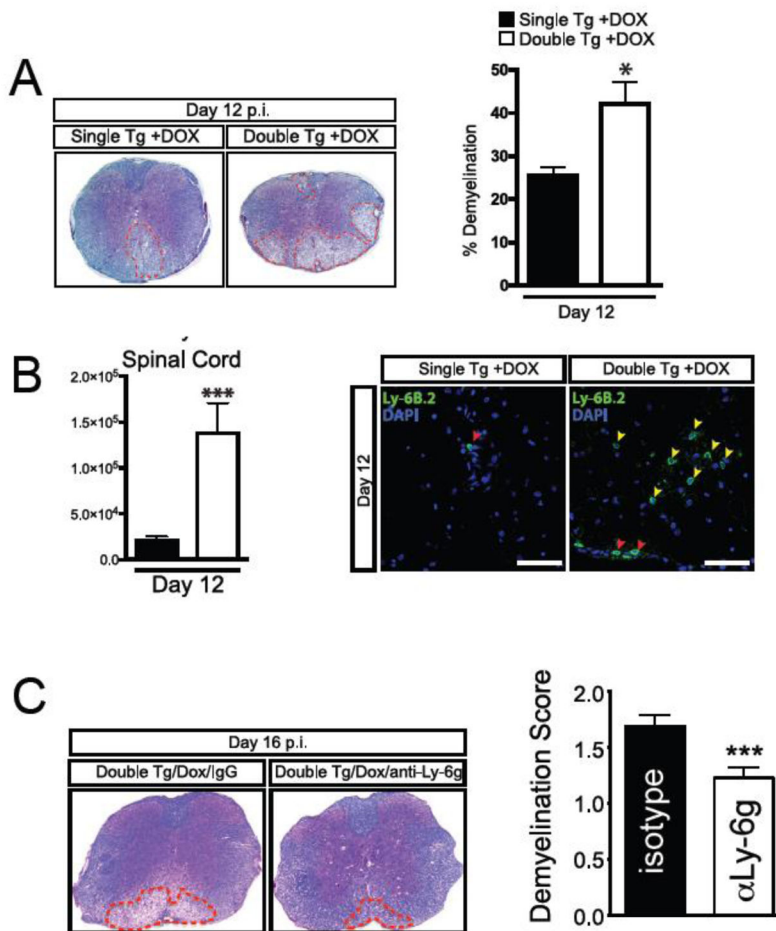
### Highlights

- Neutrophils are rapidly mobilized from the bone-marrow to the central nervous system following infection with neurotropic viruses.
- Neutrophils respond to chemokine ligands including CXCL1 and CXCL2 by binding to the receptor CXCR2 expressed upon the neutrophil cell surface.
- Contributions to host defense by neutrophils include increasing the permeability of the blood-brain-barrier to allow access by virus-specific lymphocytes.
- Neutrophils can also increase the severity of neuropathology e.g. demyelination as evidenced through use of transgenic mouse model systems.



**Figure 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-hours of Dox (100ng/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 Marro et al., 2016 with permission.



**Figure 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 Marro et al., 2016 with permission.