

Immune Modulation of Adult Neurogenesis during Experimental Herpes Simplex  
Encephalitis

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## Abstract

Herpes Simplex Virus-1 (HSV-1) is the primary cause of sporadic viral encephalitis in the United States. While prompt treatment results in high survival rates, greater than fifty percent of patients develop severe, life-altering neurological deficits subsequent to Herpes Simplex encephalitis (HSE). Inflammatory cues generated during non-HSE viral encephalitis have been shown to alter reparative neurogenesis in humans and mice. However, interactions between induced immune responses and the normal processes of adult neurogenesis remain unexplored during HSE. The present thesis hypothesized that **inflammation induced by experimental HSV-1 infection of laboratory mice alters neural stem/progenitor cell (NSC) proliferation.** The response of NSCs was dynamic throughout HSV-1 infection of adult BALB/c mice, with a significant increase in the NSC population during acute HSE at 6 d p.i. and a significant decrease by 15 d p.i.. The 6 d p.i. and 15 d p.i. time points coincided with macrophage infiltration and CD8(+) T cell infiltration respectively.

At 6 d p.i., infiltrating macrophages were observed to be alternatively activated (M2) whereas macrophages at 15 d p.i. were classically activated (M1 macrophages). Treatment with M2 macrophages resulted in significant increases in the NSC population size both *in vivo* and *in vitro*. *In vitro*, the increase in NSC proliferation was found to be mediated via Wnt5a. *In vivo*, transplanted M2 macrophages increased the number of NSCs and Wnt5a(+)CD11b(+) macrophages were observed in the brains of 5 d p.i. mice. On the other hand, M1 macrophages activated with interferon- $\gamma$  did not support NSC

proliferation, which suggested that macrophage effects may be modulated by other immune factors present in the infected brain.

CD8(+) T cells are the largest population of infiltrating immune cells during chronic HSE and produce robust amounts of IFN- $\gamma$ . The interactions of CD8(+) T cells and NSCs were addressed *in vitro* and *in vivo* experiments. Co-culture of activated CD8(+) T cells and NSCs abrogated NSC proliferation *in vitro* while *in vivo* depletion of CD8(+) T cells restored the NSC population in infected mice. Further evaluation of the effect of CD8(+) T cells on NSC proliferation was dependent on IFN- $\gamma$  both *in vivo* and *in vitro*, as IFN- $\gamma$  receptor 1 (IFN- $\gamma$ R1) knockout mice were unresponsive to CD8(+) T cells in culture and infected IFN- $\gamma$ R1 knockout mice had no reduction in the size of the NSC population.

To continue evaluation of interactions of NSC proliferation and IFN- $\gamma$ , alternative models of HSE were assessed in C57BL/6 mice. The kinetics of immune cell and NSC proliferation were similar between BALB/c and C57BL/6 mice, but macrophage and T cell phenotypes were different between the strains. Macrophages infiltrating the brain, at any time point examined, were persistently classically activated with an M1 phenotype, and T cell infiltration was dominated by CD4(+) T cells in C57BL/6 mice. On the other hand, NSC proliferation was significantly decreased when IFN- $\gamma$  was expressed suggesting that the interactions of IFN- $\gamma$  and NSCs were similar in both strains of mice tested.

The studies presented in this dissertation are the first to suggest a connection between IFN- $\gamma$  produced by infiltrating T cells and impaired NSC proliferation. The

mechanism by which T cells mediate this effect may be direct or through interaction with other cell types in the brain. However, there are implications that NSC proliferation and associated reparative neurogenesis may be impacted in any model of brain damage that exhibits prominent IFN- $\gamma$  expression.

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## **Chapter 1: Thesis Introduction**

## **Introduction**

Herpes simplex virus type 1 infection (HSV-1) is the most common cause of sporadic viral encephalitis (HSE) of immunocompetent people in the United States. Acyclovir treatment is effective at preventing mortality, however approximately 70% of surviving patients develop life altering neurological deficits (Kapur N 1994; McGrath, Anderson et al. 1997; Whitley 2006; Baringer 2008). The predominant clinical conditions observed following HSE include anterograde memory loss, anosmia, and dysphasia (McGrath, Anderson et al. 1997). Classically, adult HSE manifests as bilateral lesions of the temporal lobes, with or without parietal and frontal lobe involvement (Margaret 1982). Damage produced by HSV-1 is confined functionally and anatomically to the limbic system, particularly the amygdaloid nucleus, hippocampus, and insula.

HSE is also associated with persistent immune activation in human patients. Studies evaluating the cerebrospinal fluid (CSF) have found circulating CD8(+) T cells two months after the presentation of disease (Lellouch-Tubiana, Fohlen et al. 2000; Kimberlin 2007; Studahl, Lindquist et al. 2013). Abnormally elevated levels of neopterin, a biomarker of immune cell activation, were detected in the CSF of HSE patients more than one year after the resolution of disease, whereas serum levels of neopterin in the same patients returned to normal limits within three weeks after viral activity ceased to be detectable (Aurelius, Forsgren et al. 1993). As expected, robust increases in CSF concentration of interleukin-6, interferon- $\gamma$ , and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) were observed in HSE patients during the symptomatic phase. However, CSF concentrations of these three cytokines remained abnormally elevated for several weeks after disease symptoms were resolved (Aurelius, Andersson et al. 1994; Studahl, Lindquist et al.

2013), suggesting that a robust and long-lasting immune response had been induced in the context of HSE in human patients.

HSV-1 brain infection in the BALB/c mouse model used in this dissertation, induces a Th1-type immune response that persists long after virus ceases to be detectable which similar to the human condition. Robust secretion of TNF- $\alpha$  and IFN- $\gamma$  coincides with peak macrophage and T cell infiltration during the acute phase of infection in the mouse model (Marques, Cheeran et al. 2008) and human patients (Lellouch-Tubiana, Fohlenet al. 2000) Moreover, the experimental infection model in BALB/c mice enables evaluation of both immune cells as well as the soluble components composing the inflammatory response to HSV-1 brain infection. Previous studies using this mouse model have shown that the cellular immune response is complex and highly dynamic. Peak macrophage infiltration occurs around 6 d p.i. and is followed by peak T cell infiltration at 15 d p.i. (Marques, Cheeran et al. 2008). In contrast to other models of encephalitis, HSE in BALB/c mice is marked by persistent infiltration (or possibly retention) of CD8(+) T cells, which is observed in the brain through 60 d p.i., long after apparent viral clearance. The prolonged retention of CD8(+) T cells in the HSV-1 infected brain suggests that these cells may influence recovery from virus induced damage. Observations in the mouse models of West Nile encephalitis and HSV-1infected trigeminal ganglion have demonstrated that CD8(+) T cells are important for viral clearance from CNS tissue (Wang, Lobigs et al. 2003; Mackay, Wakim et al. 2012). However, CD8(+) T cells have also been shown to facilitate long-term immunopathology resulting from viral encephalitis or non-infectious brain damage (Fontana A 1987; Steinman 2001; Wang, Lobigs et al. 2003).

Neural stem/progenitor cells (NSCs) are remnants of the neuroectoderm layer from embryonic development that persist in the adult brain. There are two major germinal niches in the adult where neurogenesis occurs, the subventricular zone of the lateral ventricles (SVZ) and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus (Doetsch and Alvarez-Buylla 1996; Kornblum 2007). The SVZ is composed of multiple cell types including, a population of slowly dividing, radial astroglial stem cells called B cells, the transit amplifying intermediate C cells, and a population of immature, migratory neuroblasts called A cells (Doetsch, García-Verdugo et al. 1997; Doetsch, Caillé et al. 1999; Danilov, Gomes-Leal et al. 2009). All three cell types are capable of proliferation while in close proximity to the neurogenic niche and are referred to collectively as neural stem/progenitor cells (NSCs) throughout this dissertation. In the healthy adult murine brain, the primary function of NSCs is to facilitate turnover of olfactory interneurons and provide new pyramidal neurons in the hippocampus during novel memory formation. Numerous murine model systems have suggested that this physiological process of adult neurogenesis may have the capacity to ameliorate brain damage by replacing damaged neuronal circuitry (Jordan, Ojeda et al. 2009).

Brain damage, regardless of the etiology, has been shown to stimulate reparative adult neurogenesis in humans and mice. Following a brain insult, the neurogenic process begins with proliferation of endogenous NSCs (Lichtenwalner and Parent 2005; Parent, Elliott et al. 2006). NSCs migrate towards areas of damage via a chemokine gradient (Imitola, Raddassi et al. 2004; Belmadani, Tran et al. 2006). At sites of damage NSCs are thought to have two primary functions: 1) differentiating into neurons to integrate into the

existing neuronal circuitry, and 2) trophic functions that promote an environment permissive of repair in the brain. Particularly, NSCs have been shown to increase production of anti-inflammatory cytokines and thereby suppress T cell activation (Fainstein, Einstein et al. 2013; Zhang, Liu et al. 2013). However, the pro-inflammatory milieu around lesion sites can decrease NSC survival and impair their ameliorative functions. This phenomenon was elegantly demonstrated in a rodent model of ischemic stroke where only 20% of NSCs generated survive this pro-inflammatory environment and become neurons (Arvidsson, Collin et al. 2002).

To evaluate interactions between the immune system and NSCs during HSE, this research project makes use of a murine model, where the virus replication at the initial site of infection and subsequent spread into the brain via retrograde transport in discrete neural tracts is similar to that seen in humans. Intranasal inoculation of BALB/c mice with Herpes Simplex Virus (HSV)-1 results in initial infection of the olfactory nerve terminals and subsequent spread of the virus through olfactory nerves into the entorhinal cortex and fimbria of the hippocampus. In both humans and mice, the virus also traffics through the trigeminal nerve tract to associated brain stem regions (Webb SJ 1989; Esiri, Drummond et al. 1995; Armien, Hu et al. 2010). Importantly, and similar to the human condition, while the virus stays localized to the specific neural tracts used for trafficking within the infected mouse brain, the resultant inflammation is wide-spread throughout the brain.

Interactions between inflammation and adult neurogenesis have been observed in other animal models of viral encephalitis, yet it remains unclear if such an association exists during in HSE. In addition, the immune components involved in bilateral

interactions with NSCs, during recovery from brain damage, are not well understood. The development of neurological sequelae in the presence of activated T cells suggests that such interactions may indeed occur during HSE. However, the mechanisms altering adult neurogenesis during HSE, if any, are unknown. Given the chronic presence of activated immune cells in the brain during HSE, the hypothesis tested in this dissertation is that **inflammation induced by HSV-1 brain infection alters NSC proliferation**. The first objective was to evaluate the response of the neurogenic niche during HSE (see Chapter 3). As immune responses have been shown to promote anti-proliferative effects on NSCs in mouse models of Japanese encephalitis (JEV) and West Nile (WNV) encephalitis (Wang, Lobigs et al. 2003; Dutta, Mishra et al. 2010), the first hypothesis tested was **that NSC proliferation is impaired during HSE with potential contributions to the development of neurological deficits**. These experiments established strong associations between inflammatory cell infiltrates and NSC proliferation. During peak macrophage infiltration, at 6 d p.i., proliferation of nestin/Sox2(+) NSCs was significantly increased, relative to uninfected age-matched BALB/c mice. Conversely, nestin/Sox2(+) NSC proliferation was significantly decreased relative to uninfected mice at 15 d p.i., at a time corresponding to peak T cell infiltration.

Specific macrophage activation profiles have been implicated in promoting tissue regeneration in a variety of damage models. Increased levels of tissue restoration and anti-inflammatory products have been shown to be associated with M2 macrophage function, particularly in myocardial infarction (Aurora, Porrello et al. 2014; Weirather, Hofmann et al. 2014), DSS-induced colitis (Takada, Hisamatsu et al. 2010), and ischemic stroke in rodent models (Hu, Li et al. 2012). To investigate the impact of macrophages on



NSCs during HSE, the hypothesis **that alternatively activated monocytes/macrophages stimulate NSC proliferation between 3 and 6 d p.i.** was tested. Data from these studies, as described in Chapter 4, demonstrated that macrophages infiltrating the brain at 5 d p.i. were alternatively activated (M2). While at 15 d p.i., macrophages present in the brain exhibited a classically activated (M1) phenotype. Furthermore, treatment of NSCs with M2 conditioned macrophage supernatants significantly increased cell proliferation both *in vitro* and *in vivo*. Additional evaluation demonstrated that the M2 macrophage effect on NSC proliferation *in vitro* were dependent on the production of WNT5a. In contrast, M1 macrophage supernatants abrogated NSC proliferation, suggesting that M1 macrophage polarization may decrease NSC proliferation *in vivo*.

IFN- $\gamma$  is a known inducer of the M1 macrophage phenotype.. Significant amounts of IFN- $\gamma$  are expressed during HSE (Marques, Cheeran et al. 2008), suggesting that IFN- $\gamma$  may mediate the M1 macrophage polarization observed in the brain at 15 d p.i... The next objective of this research was to determine whether IFN- $\gamma$  would alter NSC proliferation (see Chapter 5). The hypothesis underlying these studies was **that CD8(+) T cells inhibit NSC proliferation in an IFN- $\gamma$  dependent manner.** Activated CD8(+) T cells, but not CD4(+) T cells, inhibited NSC proliferation *in vitro* and promoted NSC differentiation into GFAP(+) astrocyte-like cells. The inhibitory effects of activated CD8(+) T cells on NSC proliferation were mediated by IFN- $\gamma$ , given that activated CD8(+) T cells did not alter NSC proliferation in IFN- $\gamma$  receptor knockout NSCs *in vitro*.

To further investigate the *in vivo* relevance of IFN- $\gamma$  during HSE, the next series of experiments investigated the impact of CD8(+) T cells on adult neurogenesis in HSV-1

infected mouse brains at 15 d p.i. (see Chapter 6). Specifically the hypothesis tested was **that CD8(+) T cells inhibit NSC proliferation in an IFN- $\gamma$  dependent manner during chronic HSE.** Removal of CD8(+) T cells using a depleting antibody resulted in a significant increase in the NSC population in infected BALB/c mice, without changing the numbers or phenotypes of the CD4(+) T cells present in CD8(+) T cell depleted mice. There was also a significant reduction in expression of IFN- $\gamma$  compared to isotype antibody-treated controls. The specific role of IFN- $\gamma$  was evaluated using HSV-1 infected IFN- $\gamma$  receptor 1 knock out mice (IFN- $\gamma$ R1 KO). NSC proliferation in infected IFN- $\gamma$ R1KO mice was assessed at 15 d p.i.. Total numbers of NSCs in the brains of infected mice were significantly increased compared to infected wild-type mice. Furthermore, the total numbers of NSCs in infected IFN- $\gamma$ R1 KO animals were consistent with numbers seen in uninfected IFN- $\gamma$ R1KO and wild-type BALB/c mice.

The final objective of this dissertation was to develop a C57BL/6 mouse model of HSE as a possible alternative model of corroborating and extending the findings described above. The BALB/c mouse model of HSE is limited by the lack of genetically modified mice available on this background and thus the degree to which disease processes can be manipulated to test the role of IFN- $\gamma$  and CD8(+) T cells on adult neurogenesis. The objective of this section of the dissertation was **to validate the reproducibility of disease, assess HSV-1 induced neuroinflammation, and investigate its effect on NSC proliferation in a C57BL/6 mouse model.** Mouse mortality and clinical disease scores were not significantly different in HSV-1 infected BALB/c vs C57BL/6 mice. While NSC proliferation followed roughly the same kinetics in C57BL/6 mice as in BALB/c mice, the profile of activated immune cells infiltrating

the brain was different. Infiltrating macrophages were consistently polarized to the M1 phenotype and CD4(+) T cells comprised the majority of infiltrating T cell population from 10 d p.i. to 30 d p.i. in HSV-1 infected C57BL-6 mice.

In summary, there are four major findings from these studies. First, NSCs respond dynamically to immune cues during HSE. Second, M2 macrophages promote NSC proliferation via a Wnt5a-dependent mechanism. Third, activated CD8(+) T cells inhibit NSC proliferation and promote an M1 phenotype in macrophages through the production of IFN- $\gamma$ . Lastly, a C57BL/6 mouse model may prove valuable to examine the neuroimmune response in a more detailed fashion than is currently possible in the BALB/c mouse model as the C57BL/6 mouse macrophages are prone to classical activation during acute HSE. Importantly, these findings provide the first steps in understanding the reparative processes occurring in response to viral encephalitis. Comprehensive insight into these processes may facilitate development of novel therapeutic approaches for patients recovering from viral encephalitis.

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## **Chapter 2: Review of Literature**

## **Introduction:**

Viral encephalitis results in severe neurological damage for surviving patients. Herpes Simplex virus type 1 (HSV-1) is the most common cause of sporadic viral encephalitis in the United States, leading to Herpes Simplex encephalitis (HSE) (Kimberlin 2007). Greater than 50% of HSE survivors develop life-altering neurological deficits following infection. HSE induces robust inflammatory responses during infection that contribute to the development of brain lesions in both humans (Esiri, Drummond et al. 1995) and in mouse models (Armien, Hu et al. 2010). Emerging evidence suggests that a small population of neural stem/progenitor cells in the brain respond to damage in an attempt to restore disrupted neuronal circuitry in murine ischemic stroke (Imitola, Raddassi et al. 2004). On the other hand, understanding of how the brain responds to intraorgan damage is poorly understood. This literature review examines the development and persistence of brain lesions during HSE and to address the potential of adult neurogenesis to improve associated tissue damage.

## **Herpes Simplex encephalitis**

While survival rates with timely HSE treatment are approximately 97%, greater than 50% of HSE patients will develop life altering neurological deficits (Kimberlin 2007). The nature of reported deficits range from anosmia to significant reductions in verbal memory tasks and are consistent with damage lesions in the sensory motor cortex regions of the frontal cortex (Hokkanen, Poutiainen et al. 1996). Similar to the human condition, significant impairment during spatial memory tasks has been observed in the BALB/c mouse model of HSE, as animals are unable to find a platform during the Morris water maze task and have lesions in their sensory motor cortex (Armien, Hu et al. 2010).

While the physical damage resulting from HSE has been well characterized in both humans and mice, understanding of the pathophysiology of HSE is incomplete.

Greater than 70% of the adult population in the United States is seropositive for Herpes Simplex Virus-1 (HSV-1) (Xu, Sternberg et al. 2006) and yet the incidence of HSE is very low (James, Kimberlin et al. 2009). It remains unknown if HSE results from primary infection of a previously unexposed individual with HSV-1 or if the encephalitis is the result of reactivation of HSV-1 latent in one or more nerve tracts (Aurelius, Forsgren et al. 1993; Kimberlin 2007; Baringer 2008). While the specific insult resulting in HSV-1 migration into the brain is unknown, the progression of the virus through the human brain has been suggested from damage patterns and sites of viral latency in HSE survivors. Progression of the virus through the human brain would seem to begin with two possible origins of the virus in the brain, the olfactory bulb and the trigeminal ganglia (Margaret 1982; Kimberlin 2007; James, Kimberlin et al. 2009). From those two sites, the virus would seem to progress through the thalamus to the temporal lobe, creating characteristic lesions (Margaret 1982; McGrath, Anderson et al. 1997; James, Kimberlin et al. 2009).

Based on this understanding of the progression of HSV-1 through the human brain, intranasal induction of HSV-1 infection in BALB/c mice can be regarded as a highly relevant experimental model of HSE. HSV-1 in BALB/c mice travels up neural tracts to infect the olfactory bulb and also travels up the trigeminal ganglia into the brainstem resulting in localization of the virus to specific neural tracts and virus lesion formation in regions that match the affected areas in human HSE patients (Margaret 1982; Whitley 2006; Kimberlin 2007; Marques, Cheeran et al. 2008; Armien, Hu et al.

2010). Behaviorally, mice infected with HSV-1 via intranasal inoculation experience major deficits in their spatial memory (Armien, Hu et al. 2010), a type of memory that is also disrupted in human HSE patients (Whitley 2006), suggesting that the survivor phenotypes found in experimentally infected mice are consistent with humans surviving HSE.

### **Macroscopic brain inflammation during Herpes Simplex Encephalitis**

Unlike Japanese encephalitis or West Nile encephalitis, HSE has two distinct phases to the inflammatory response corresponding to an acute phase of lytic viral infection where the immune response to the virus is markedly similar to JEV, and a chronic phase consisting of latent viral infection of brain neurons and persistent CD8(+) T cell infiltration in the brain despite a lack of detectable viral antigen (Marques, Cheeran et al. 2008; Armien, Hu et al. 2010). During the chronic phase, significant brain remodeling occurs as disease lesions are replaced with astrocytes (Armien, Hu et al. 2010). HSE surviving animals had severe deficits in their spatial memory one month after the initial infection with the virus which were unresolved at the last time point tested, 60 d p.i. (Armien, Hu et al. 2010). The formation of astroglial scars coincides with a robust CD8(+) T cell infiltration that has been shown to inhibit reparative brain mechanisms, such as adult neurogenesis in other mouse models of viral encephalitis such as Japanese encephalitis (Das, Dutta et al. 2011) and West Nile encephalitis (Wang, Lobigs et al. 2003).

Interactions of the immune system and the brain are dramatically different from interactions of the immune system and peripheral tissues due to physical and molecular constraints imposed by the blood-brain barrier (BBB) and the active promotion of an

immunosuppressive milieu in the brain parenchyma. The extreme regulation of permeability in the BBB minimizes immune surveillance in the brain parenchyma in the absence of infection, while many brain cell types, including astrocytes and microglia, constitutively produce low levels of anti-inflammatory cytokines (Aurelius, Andersson et al. 1994; Ekdahl, Kokaia et al. 2009; Obermeier, Daneman et al. 2013). Damage inducing stimuli, such as viruses, result in disruption of the BBB and an alteration in the immune activation state of the brain. However, the duration and scope of disruption to the BBB is highly variable and dependent both on the quantity and duration of damage processes (Schoknecht and Shalev 2012). Persistent disruptions, as observed with HSE, tend to be associated with more substantive brain pathology resulting in worse neurological deficits (Lellouch-Tubiana, Fohlen et al. 2000; Schoknecht and Shalev 2012). Importantly, with immune cell infiltration, the immunosuppressive microenvironment of the brain is shifted to allow development of robust and often pathological immune responses depending on the nature of the insult (Obermeier, Daneman et al. 2013).

#### **Cellular inflammation and brain damage:**

During times of brain damage immune cell infiltration follows similar kinetics and mechanisms as the in the periphery. Cellular damage results in secretion of danger signaling molecules which are then detected by other local cells and the signal is perpetuated to induce opening of the BBB to allow infiltration of immune cells (Murphy, Lalor et al. 2010; Nishimura, Yasuda et al. 2013). In HSE, immune cell infiltration is temporally regulated with distinct peaks of immune cell infiltration. Initial inflammation is mediated by neutrophils, however by three days post-infection macrophage infiltration is noticeable. Macrophage infiltration peaks soon after that, between five and six days

post-infection before subsiding. By eight days post-infection, T cell infiltration comprises a prominent part of the inflammatory compartment (Marques, Cheeran et al. 2008). In contrast to the neutrophil and macrophage infiltration patterns, T cell infiltration remains persistent with T cells being detected in the brains of surviving humans for months (Aurelius, Forsgren et al. 1993) and mice past 60 days (Armien, Hu et al. 2010). Surprisingly, the phenotype of these T cells is altered over time going from a one to one ratio of CD4(+) to CD8(+) T cells to a two to one ratio of CD8(+) to CD4(+) T cells by 15 d p.i.. Importantly, these CD8(+) T cells persist after viral antigen ceases to be detectable (Marques, Cheeran et al. 2008).

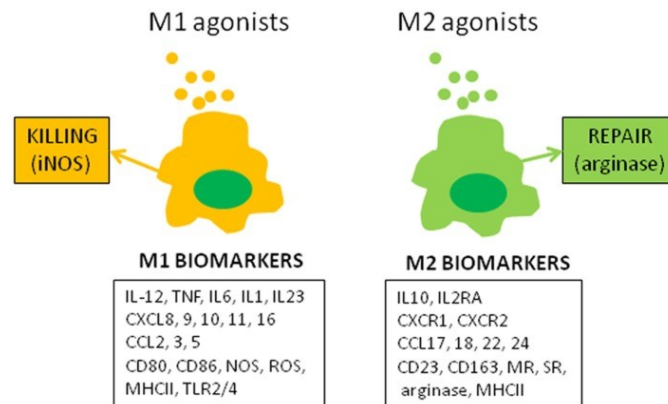
### **Macrophage responses to brain damage:**

Macrophage response to brain damage has been shown to be largely pro-inflammatory, and damaging to the brain parenchyma. However, recent evidence in mouse models of stroke suggest that early after ischemia macrophage functions are anti-inflammatory (Hu, Li et al. 2012). These dichotomous functions can be grouped into two large activation states: the classical (M1) phenotype or the alternative (M2) phenotype (Fig. 2.1; Ka, Dumas et al. 2014) Classically activated macrophages predominantly produce pro-inflammatory cytokines and promote cell death in murine models of septic shock (Li, Ma et al. 2014; Lin, Yang et al. 2014). They also have been shown to worsen allergenic asthma (Hong, Chung et al. 2014). Predictably, macrophages responding to viral infections such as Japanese encephalitis (Dutta, Mishra et al. 2010), West Nile virus (Getts, Terry et al. 2012), Herpes Simplex virus (Aravalli, Hu et al. 2005; Marques, Hu et al. 2006), coronaviruses (Chen, Kuziel et al. 2001) and rhinoviruses (Hong, Chung et al.

2014) also are classically M1 activated and express high levels of CD32 and iNOS as well as tumor necrosis factor- $\alpha$  (Kigerl, Gensel et al. 2009).

In contrast, alternatively activated M2 macrophages have been associated with production of anti-inflammatory cytokines and increased production of growth factors. While this type of macrophage has been associated with some disease states, most prominently asthma (Müller, Jaunin et al. 1993), M2 macrophages are more frequently associated with brain and intestinal tissue growth during development and tissue remodeling post injury (Kigerl, Gensel et al. 2009). Specifically, M2 macrophages have been shown to have important roles in the development of mouse cortices *in utero* (Cunningham, Martínez-Cerdeño et al. 2013) as well as being essential for spinal cord repair in transection models (Kigerl, Gensel et al. 2009; Schwartz 2010; Nishimura, Yasuda et al. 2013). Furthermore, despite the dramatic differences in function, these macrophage polarization states are not terminal as crossover between macrophage phenotypes has been shown, *ex vivo* in sheep macrophages (Crespo, Bertolotti et al. 2013).

In the context of brain damage, *in vivo* macrophage activation in murine models of stroke has demonstrated that macrophages undergo changes in function from a beneficial regenerative phenotype to a more pro-inflammatory damaging phenotype following induction of ischemia (Hu, Li et al. 2012). Importantly, pharmacological induction of an M2 like phenotype following stroke was associated with better behavioral outcomes in ischemic animals (Jin, Cheng et al. 2014). Furthermore, in murine models of experimental autoimmune encephalomyelitis, the M2 phenotype was associated with marked reduction in both inflammation and disease during remitting phases of disease



**Figure 2.1: M1 and M2 macrophage polarization.** The figure represents canonical M1 and M2 agonists that induce the production of M1 and M2 markers by human macrophages *in vitro*. These markers, isolated or combined, have been used to describe the polarization of monocytes and macrophages in clinical investigations. Figure reprinted from Ka et al. *Frontiers in Immunology* 2014.



(Khoury, Hancock et al. 1992). These findings suggest that the M2 phenotype is beneficial during both acute and chronic disease states.

### **T cell responses to brain damage:**

Classically activated macrophages and Th1 T cells are often produced together in response to antigen while alternatively activated macrophages and Th2 T cells are produced together (Stout and Suttles 1993). West Nile virus (WNV) (Kitaura, Fujii et al. 2011), Japanese Encephalitis virus (JEV) (Das, Dutta et al. 2011), murine (Mutnal, Cheeran et al. 2011) and human (Cheeran, Jiang et al. 2008) Cytomegalovirus (mCMV), and Herpes Simplex virus (HSV) (Marques, Cheeran et al. 2008) all induce a Th1 type immune response. Interestingly, while both CD4(+) and CD8(+) T cells contribute to peripheral Th1 type immune responses in these mouse models, CD8(+) T cells predominate in the immune response to JEV (Das, Dutta et al. 2011), WNV (Kitaura, Fujii et al. 2011), and HSV-1 (Marques, Cheeran et al. 2008). In addition, CD8(+) T cells in the brains of mice infected with either WNV or JEV have been shown to mediate development of immunopathology and a worsened neurological outcome (Wang, Lobigs et al. 2003; Das, Dutta et al. 2011). On the other hand, removal of CD8(+) T cells from the brains of encephalitic WNV infected mouse brains also decreased survival indicating that while CD8(+) T cells may mediate immunopathology, they are essential for control of the virus (Wang, Lobigs et al. 2003).

Mechanistically, effects of CD8(+) T cells are thought to be dependent on two specific mechanisms. First, CD8(+) T cells are known to induce cell death in bystander cells during Th1 type immune responses (Lee, Chanamara et al. 2012; Too, Ball et al. 2014). Modulation of CD8(+) T cell activation using the antibiotic minocycline has been

shown to greatly improve NSC proliferation following Japanese encephalitis (Dutta, Mishra et al. 2010). Secondly, CD8(+) T cells produce interferon- $\gamma$  (a T cell cytokine), which while improving dendrite and synapse survival, has been shown to inhibit NSC proliferation following viral encephalitis (Kreutzfeldt, Bergthaler et al. 2013).

Furthermore IFN- $\gamma$  has been shown to stimulate an increase in major histocompatibility complex I expression on cultured NSCs (Cheeran, Jiang et al. 2008) thus increasing the visibility of NSCs to the immune system and increasing the possibility of NSC death via cytotoxic T cells. Induction of oligodendrocyte precursor proliferation during multiple sclerosis is also thought to be dependent on down regulating CD8(+) T cell activity as decreasing IFN- $\gamma$  secretion during remitting phases of the disease is associated with increases in the proliferation of oligodendrocyte precursor cells (Khoury, Hancock et al. 1992).

### **Immunopathology and Neurological Deficits:**

The net effect of inflammatory responses to brain damage is dependent on the nature of the initial insult being either infectious or sterile, as these two types of brain damage trigger both different immune activation states and immune cell infiltration kinetics. Mouse models of ischemia (Hu, Li et al. 2012) and traumatic brain injury (Yoles, Hauben et al. 2001) have shown that there is greater infiltration of CD206, interleukin-10 expressing macrophages (M2-like) and earlier infiltration of regulatory T (Tregs) cells compared to infectious stimuli at acute time points suggesting that the immune response is less damaging to surrounding tissues. Specific induction of alternatively activated macrophages and immunosuppressive Tregs are important as they control multiple divergent phenotypes in T cells (Mosmann, Cherwinski et al. 1986;

Fiorentino, Bond et al. 1989) and macrophages (Stout and Suttles 1993; Kigerl, Gensel et al. 2009) thus having the capacity to directly affect the pro or anti-neurogenic nature of the brain milieu. Activation of these anti-inflammatory or immunosuppressive immune cell subsets is associated with decreased tissue damage in the brain (Kalluri and Dempsey 2008; Hu, Li et al. 2012; Jin, Cheng et al. 2014) and the gut (Ito, Shin-Ya et al. 2006; Lin, Yang et al. 2014).

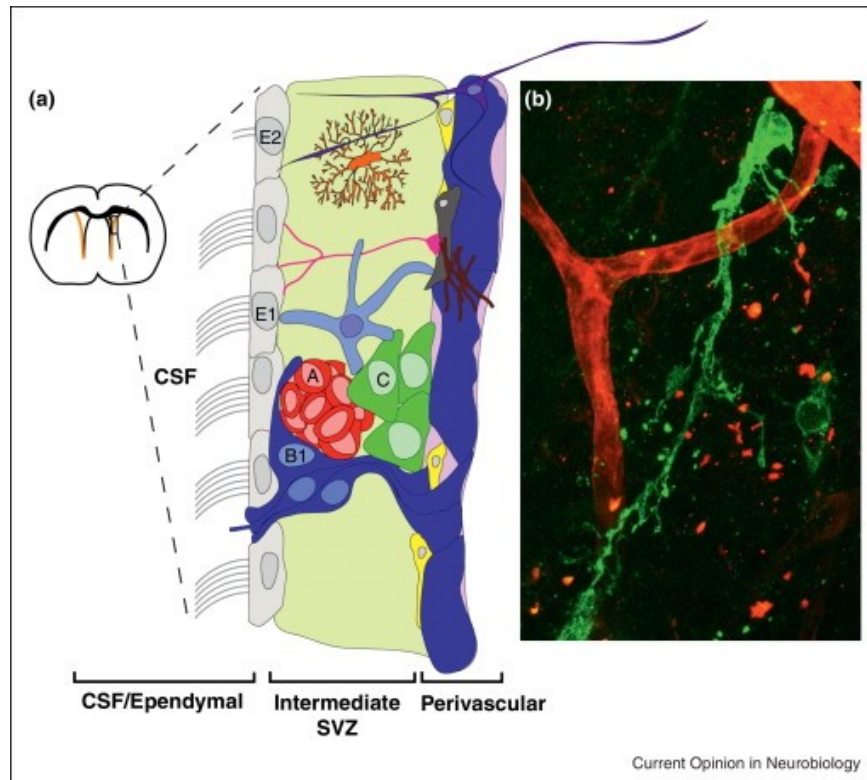
Development of neurological deficits following viral encephalitis is the result of damage to the neural tracts responsible for learning, memory, sensory perception, and other brain functions. Immune mediated brain damage during viral encephalitis has been shown to occur by two distinct mechanisms: destruction of infected cells and by-stander damage (Hokkanen, Poutiainen et al. 1996; Wang, Lobigs et al. 2003; Das and Basu 2008). While destruction of infected cells is an essential feature of virus control, by-stander damage is the destruction of uninfected cells in physical proximity to infected cells (Capone, Frigerio et al. 2007). Regeneration of neural tracts is dependent on replacement of neurons with neurons and not scar generating cells such as astroglia, furthermore these neurons must have the same functional connectivity as the neurons they replace (Kornblum 2007; Martino, Pluchino et al. 2011; L'Episcopo, Tirolo et al. 2012). Currently, the best possible therapy for achieving these goals is the use of neural stem cells, a primordial cell population left over from embryogenesis.

### **Neurogenesis and brain homeostasis in the healthy brain:**

Several regions experience high levels of neuron turnover in the healthy adult brain. In order to maintain the functions of these regions, new neurons are continually generated in the healthy brain. The process of neuron generation is termed adult

neurogenesis which is a multi-step process involving cellular changes mediated by a small population of cells identified as neural stem cells (NSCs). The process of adult neurogenesis is complex with five distinct phases: NSC proliferation, migration, differentiation, integration into the brain circuitry, and survival of newly integrated cells. Disruption of any or all of these stages can result in development or worsening of neurological sequelae. NSCs respond to brain injury by proliferating and migrating to distant sites of damage where they differentiate into neurons or glial cells and integrate into existing neural circuitry (Doetsch, Caillé et al. 1999). In the healthy adult brain, the purpose of adult neurogenesis in the subventricular zone of the lateral ventricles is to facilitate turnover of olfactory interneurons in the olfactory bulb and to supply the CA1 region of the hippocampus with neurons from the neurogenic niche in the subgranular zone of the hippocampus (Doetsch and Alvarez-Buylla 1996; Brown, Couillard-Després et al. 2003).

Neurogenesis is an elaborate process involving three distinct cell populations: true neural stem cells or B cells, transit amplifying cell or C cells, and migratory neuroblasts or A cells which are also known as neuroblasts (Doetsch, García-Verdugo et al. 1997). A schematic representation of the structure of the subventricular zone (SVZ) of the lateral ventricles is present as figure 2.2 (reprinted from (Silva-Vargas, Crouch et al. 2013). Importantly, B cells are located immediately adjacent to the ependymal cells lining the lateral ventricles. Of these cell populations only B cells possess true stem cell like



**Figure 2.2: Cell types in the V/SVZ niche. (A)** Coronal schema showing V/SVZ adjacent to the lateral ventricles. Box is expanded to right and shows compartments and cell types in the V/SVZ niche. The CSF/ependymal compartment comprises the CSF, ependymal cells (multi-ciliated E1 cells and bi-ciliated E2 cells) and B1 stem cells that contact the ventricle, as well as axons. The intermediate SVZ niche contains all SVZ cells (B1 stem cells, transit amplifying Type C cells, and neuroblasts (Type A cells). Other components of the intermediate SVZ niche include other astrocytes (light gray), microglia and long distance axon terminals. The perivascular niche has unique features. Blood vessels are made of endothelial cells, surrounded by pericytes and astrocyte endfeet. Stem cells and transit amplifying cells frequently contact the vasculature at specialized sites that lack pericytes and astrocyte endfoot coverage. Macrophages and fibroblasts (dark grey) are also present in the perivascular niche, as well as an enriched ECM and fractones that extend from blood vessels. **(B)** B1 stem cells have a polarized radial morphology and extend a long basal process (thin li) that contacts the vasculature (thick lines and puncta). Figure from Silva-Vargas et al. *Current Opinion in Neurobiology* 2013.

properties, as they maintain neurogenic niches in the brain via symmetrical cell division. Seminal work by Fiona Doetsch demonstrated that these cells are primordial cells with radial astrocyte phenotypes in the SVZ of the lateral ventricles (Doetsch, Caillé et al. 1999). Progression down the neurogenic pathway continues when B cells differentiate into C cells. These cells fill an essential neural progenitor role as they are capable of symmetrical cell division to maintain the population in homeostasis or asymmetrical cell division into A cell/neuroblasts, which have the migratory phenotype (Doetsch and Alvarez-Buylla 1996; Doetsch, García-Verdugo et al. 1997). Interestingly, the degree of differentiation of NSCs correlate to their location relative to the ependymal cells lining the SVZ, with B cells being the least differentiated and closest to the ependymal cells and the A cells being the most differentiated and farthest from the ependymal cells. In addition to the SVZ, proliferation of NSCs occurs in the subgranular zone of the hippocampus (Doetsch, García-Verdugo et al. 1997; Brown, Couillard-Després et al. 2003). In the undamaged brain, local sites of NSC proliferation provide continual cell renewal of discrete regions. In particular, the SVZ provides new olfactory interneurons in the olfactory bulb of humans and rodents (Altman and Das 1965; Eriksson, Perfilieva et al. 1998) as well as other species such as cats (Das and Altman 1971) and macaques (Rakic 1985). Numerous factors have been implicated in promotion of NSC proliferation including a variety of growth factors, such as FGF-2 (Bikfalvi, Klein et al. 1997; Matsuoka, Nozaki et al. 2003; Grote and Hannan 2007; Cheng, Wang et al. 2011), cytokines and hormones such as serotonin (Grote and Hannan 2007).

Control of neurogenesis beyond cell proliferation in healthy adult mammalian brains is largely dependent on expression of gamma-aminobutyric acid (GABA)

receptors expressed on C cells. In contrast to the usual neurotransmitter function of GABA, these GABA receptors are located extra-synaptically and do not play a role in the transmission of action potentials (Yokoyama, Mochimaru et al. 2011). Persistent stimulation of these GABA receptors is required to induce differentiation into A cells (neuroblasts) to direct A cell migration in the healthy brain (Peretto, Schellino et al. 2014). Successful completion of neurogenesis requires that neuroblasts mature into interneurons and develop both GABAergic and glutamatergic synapses on dendritic spines to complete neural circuitry (Lledo, Merkle et al. 2008; Khodosevich, Lazarini et al. 2013). In disease processes, GABA receptor mediated migration to the olfactory bulb or CA1 region of the hippocampus is effectively halted (Wang, Wang et al. 2014). However, in the diseased brain, NSCs have been shown to utilize chemokine signaling as a means of migrating to sites of damage (Belmadani, Tran et al. 2006; Hattermann, Ludwig et al. 2008).

Adult neurogenesis has been demonstrated in numerous animal systems, most notably mice, rats, cats, non-human primates, and humans (Eriksson, Perfilieva et al. 1998). Neurogenesis was first identified in the sub-granular zone of the hippocampus (SGZ) and the sub-ventricular zone of the lateral ventricles (SVZ) of rats by Robert Altman in mid-1960s. Proliferation of brain cells was observed by tritiated thymidine uptake after *intracerebroventricular* (*icv*) stereotactic injection into the lateral ventricles (Altman and Das 1965). Altman et al. identified these cells as astroglial in nature based on their morphology, an observation that was confirmed by Doetsch et al. in the late 1990s, who observed glial fibrillary acidic protein(+) (GFAP(+); a structural protein in astrocytes) BrdU(+) (a thymidine analog and proliferation marker) double positive cells

in the SVZ one cell layer below the ventricles (Doetsch, Caillé et al. 1999). Importantly, later studies identified comparable cell populations at numerous discrete brain regions of non-surgically manipulated mice (Brown, Couillard-Després et al. 2003); however the SVZ remains the largest neurogenic area identified. Importantly, this study demonstrated that the process of adult neurogenesis was able to produce functional neurons capable of conducting action potentials and thereby established the basis for the hypothesis that NSCs have the potential to replace damaged neurons (Brown, Couillard-Després et al. 2003).

Maintenance of the B and C cell populations in the neurogenic niche is dependent on physical proximity to the lateral ventricles or other blood/CSF sources. Blood products such as epidermal growth factor and fibroblast growth factor-2 are required for B cells to maintain their undifferentiated state (Frinchi, Bonomo et al. 2008; Danilov, Gomes-Leal et al. 2009). As B cells move away from the lateral ventricle wall they differentiate into transit amplifying cells (C cells) that are located within a few cell layers of the ventricle walls (reviewed in Doetsch et al. 1997). In fact, only the A cell population, or migratory neuroblasts, are observed distant from the lateral ventricles; however, expression of proliferation markers, such as proliferating cell nuclear antigen (PCNA) or Ki-67, in neuroblasts are only seen close to the ventricles (Brown, Couillard-Després et al. 2003; Kiyota, Ingraham et al. 2011). Removal of B or C cell populations from the lateral ventricles leads to terminal differentiation of these cells (Espinosa-Jeffrey, Becker-Catania et al. 2002) and a common problem in culturing embryonic NSCs is the spontaneous differentiation that occurs in prolonged passaging due to the absence of ventricle factors (Huh, Kataoka et al. 2009).



## **Neurogenesis in response to tissue damage:**

NSCs have long been suggested as a therapeutic option in response to damage of the central nervous system (CNS) as resident and transplanted NSCs have been shown to repair transected spinal cords in murine (Teng, Lavik et al. 2002; Boulland, Lambert et al. 2013; Kumamaru, Saiwai et al. 2013; Nishimura, Yasuda et al. 2013; Sabelström, Stenudd et al. 2013) and canine (Gericota, Anderson et al. 2014) spinal cord injury models. Studies of NSC reparative effects in the brain are more ambiguous with infrequent reports of complete functional repair. However, in sterile models of brain damage such as ischemic stroke (Chu, Kim et al. 2004; Imitola, Raddassi et al. 2004; Capone, Frigerio et al. 2007; Luheshi, Kovacs et al. 2011; Hu, Li et al. 2012; Sekeljic, Bataveljic et al. 2012) and traumatic brain injury (Faiz, Acarin et al. 2005; Crawford, Wood et al. 2009; Kernie and Parent 2010; Savage, Lopez-Castejon et al. 2012), supplementation with exogenous NSCs has been shown to improve functional outcomes. On the other hand, administration of exogenous NSCs can be challenging and typically requires injection of NSCs into the already damaged brain. Few studies have documented the effect transplanted NSCs have on inflammation associated with encephalitis as many pathogens responsible for encephalitis can infect and lyse NSCs (Arvidsson, Kirik et al. 2003; Das, Chakraborty et al. 2010; Kitaura, Fujii et al. 2011; Mutnal, Cheeran et al. 2011) thus eliminating any beneficial effects of the NSCs.

The alternative to using exogenous NSCs is to increase the activity of endogenous NSCs to improve behavioral outcomes. Complicating the use of endogenous NSCs is the intrinsic requirement that these cells survive the infection. Studies using a mouse model of Japanese encephalitis (JE) have suggested that NSC proliferation is abrogated early

during infection. Inhibition of NSC proliferation in JE is associated with increased neurological deficits in infected mice (Das, Dutta et al. 2011). Conversely, this group later demonstrated that expanding the NSC niche using the antibiotic minocycline to increase the proliferation of NSCs ameliorated the observed neurological deficits after the virus had been cleared (Dutta, Mishra et al. 2010). These findings suggest that increasing endogenous NSC proliferation following viral encephalitis may be a viable therapeutic option to improve behavioral outcomes.

The mechanisms to increase NSC action in the infected brain are somewhat ambiguous. Studies in the mouse model of multiple sclerosis, experimental autoimmune encephalitis (EAE), suggest that increases in NSC numbers are due to these cells migrating to sites of demyelination and differentiating into oligodendrocytes (Aharonowiz, Einstein et al. 2008). During demyelination, NSC proliferation is increased and the resulting neuroblasts differentiate into a subset of progenitor cells called oligodendrocyte precursor cells (OPCs) in both mice (Aharonowiz, Einstein et al. 2008) and primates (Pluchino, Gritti et al. 2009). OPCs synthesize myelin and induce remission of disease symptoms. However, this explanation fails to address the immunomodulatory effects that NSCs may be having on the brain milieu during times of damage. In the same EAE model mentioned above, OPCs have immunomodulatory functions such as inhibiting antigen presentation (Pluchino, Gritti et al. 2009) and limiting T cell activation (Einstein, Fainstein et al. 2007; Fainstein, Einstein et al. 2013) through secretion of anti-inflammatory cytokines (Aharonowiz, Einstein et al. 2008; Shahbazi, Kwang et al. 2013). OPC modulation of the inflammatory milieu reduces immune mediated damage and increases availability of trophic factors that promote NSC proliferation. Anti-

inflammatory effects have been observed in other models of brain damage not associated with the production of OPCs such as traumatic brain injury (Zhang, Liu et al. 2013) and ischemic stroke (Jin, Mao et al. 2010). In contrast, anti-inflammatory effects of NSCs have not been observed during acute viral Japanese encephalitis (Das and Basu 2008; Dutta, Mishra et al. 2010; Das, Dutta et al. 2011), Alzheimer's disease (Tatebayashi, Lee et al. 2003; Grote and Hannan 2007; Kiyota, Ingraham et al. 2011; Raj, Rothamel et al. 2014), and Parkinson's disease (Grote and Hannan 2007; L'Episcopo, Tirolo et al. 2012; Raj, Rothamel et al. 2014) although possible immunomodulatory effects of NSCs after resolution of virus in viral encephalitis remain unknown.

Many of the mechanisms of NSC response to brain damage utilize the same migration pathways as the immune response. NSC migration is mediated by chemokines, particularly CXCR4 (Imitola, Raddassi et al. 2004; Rasmussen, Imitola et al. 2011), and NSCs are capable of producing a variety of cytokines (Shahbazi, Kwang et al. 2013). Commonalities in cell signaling with the immune system may explain how adult neurogenesis responds to different types of brain damage. Particularly, changes in neural stem cell (NSC) proliferation, migration, and differentiation have been observed following diverse types of brain damage including, experimental autoimmune encephalitis (Aharoni, Arnon et al. 2005; Guo, Li et al. 2010; Huehnchen, Prozorovski et al. 2011; Tepavčević, Lazarini et al. 2011),

Depending on the etiologies of brain damage, neurogenesis is altered at multiple steps in the neurogenic pathway. Specifically, neurogenic changes resulting from sterile (non-infectious) brain damage are markedly different from neurogenesis resulting from infectious damage of the brain (Grote and Hannan 2007; Schoknecht and Shalev 2012).

Particularly, sterile models of brain damage tend to be associated with periods of active immune suppression immediately after damage (Teng, Lavik et al. 2002; Ekdahl, Kokaia et al. 2009; Kigerl, Gensel et al. 2009; Dietel, Cicha et al. 2012; Hu, Li et al. 2012) whereas with infectious causes of brain damage, the tissue microenvironment tends to remain in a pro-inflammatory state until the pathogen has been cleared (Das and Basu 2008; Marques, Cheeran et al. 2008; Armien, Hu et al. 2010; Getts, Terry et al. 2012). Importantly, sterile brain injuries are often associated with better neurological prognosis compared to brain infections (Ganguly and Poo 2013; Ma, Chan et al. 2014).

**Conclusion:**

Adult neurogenesis responds dynamically to damage in the brain. The success or failure of adult neurogenesis in repairing the brain seems to be dependent on the nature of the inflammatory milieu present in the brain during the repair process. Persistent activation of CD8(+) T cells and the activation phenotype of macrophages present in the brain have been shown to affect NSC proliferation during adult neurogenesis. However, the processes of adult neurogenesis and the influence of infiltrating immune cells during HSE have not been fully defined. The objective this thesis was to evaluate the effects of inflammation on NSC proliferation.

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**Chapter 3: Modulation of neural stem/progenitor cell proliferation during experimental Herpes Simplex encephalitis is mediated by differential FGF-2 expression in the adult brain.**

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## **Abstract**

Neural stem cells (NSCs) respond to inflammatory cues induced during brain injury and are thought to be involved in recovery from brain damage. Little is known about NSC response during brain infections. The present study evaluated NSC proliferation during Herpes Simplex Virus-1 brain infection. Total numbers of nestin(+) NSCs increased significantly in infected brains at 6 d post infection (p.i.). However, by 15 d p.i. the nestin(+) population decreased significantly below levels observed in uninfected brains and remained depressed through 30 d p.i.. This initial increase in NSC population occurred concurrently with increased brain cell proliferation, which peaked at 3 d p.i.. On closer examination, we found that while actively proliferating Sox2(+) NSCs increased in number at 6 d p.i., proliferating DCX(+) neuroblasts contributed to the increased response at 3 d p.i.. However, overall proliferation decreased steadily from 15 d p.i. to below control levels. To determine the mechanisms involved in altering NSC proliferation, neurotrophin and growth factor expression profiles were assessed. FGF-2 gene expression increased at 5 d p.i. and was robustly down-regulated at 15 d p.i. (>1000 fold), which was further confirmed by increased FGF-2 immunostaining around the lateral ventricles. Furthermore, supplementing infected animals with recombinant FGF-2, at 15 d p.i., significantly increased the number of proliferating brain cells. These findings demonstrate that the temporal changes in NSC proliferation are mediated through the regulation of FGF-2 and that the NSC niche may benefit from supplementation with FGF-2 during HSV-1 brain infection.

## **Introduction**

Herpes Simplex encephalitis (HSE) is the most common cause of sporadic viral encephalitis in the United States. While acyclovir treatment is a highly effective means to control HSV-1 brain infection (Whitley 2006); greater than fifty percent of human patients with HSE develop life altering neurological deficits (McGrath, Anderson et al. 1997; Kimberlin 2007). These neurological deficits are the result of severe brain damage manifesting as reactive astrogliosis and necrosis (Webb SJ 1989) which in human infections manifests with significant vascular changes with neuronal loss during early stages of disease and the development of astroglial scarring later in infection (Whitley 2006). The volume of glial scarring correlates to the level of neurological impairment, although the origins of HSV-1-mediated neurological damage and scar formation remain unclear.

In the murine model of experimental HSE, which closely resembles the human disease, immune responses to HSV-1 brain infection begins with infiltration of neutrophils and macrophages between 3 and 5 d p.i.. T cells infiltrate between 6 and 8 d p.i.. and peak between 14 and 16 d p.i. with significant persistence beyond 30 d p.i. (Marques, Cheeran et al. 2008; Armien, Hu et al. 2010). However, viral antigen is undetectable at 15 d p.i.. The role of persistent T cells at this stage of disease is unknown; however the presence of activated immune cells is indicative of on-going inflammation that facilitates development of damage and resultant neurological deficits (Armien, Hu et al. 2010). Similar studies in the murine model of Japanese encephalitis and experimental autoimmune encephalitis (EAE) have found that CD8(+) T cell mediated inflammation

may be responsible for brain damage and development of long term sequelae (Steinman 2001; Wang, Lobigs et al. 2003).

A small population of endogenous brain cells called neural stem/progenitor cells (NSCs) play a significant role in adult neurogenesis (Lichtenwalner and Parent 2005; Parent, Elliott et al. 2006). NSCs are maintained within distinct germinal niches, which support their multipotent phenotype and self-renewal properties. Adult neurogenesis is composed of highly complex and temporally non-linear progression of cell differentiation that begins with proliferation and ends with integration into existing brain circuitry. Three NSC sub-populations have been shown to proliferate during physiological adult neurogenesis: neural stem cells (B cells), transit-amplifying cells (C cells), and migratory neuroblasts (A cells) (Doetsch, García-Verdugo et al. 1997; Brown, Couillard-Després et al. 2003) Previous studies have shown that B/C cells (neural stem/progenitor cells) are immunoreactive for nestin, Sox2, mushashi, and oct4 although levels of expression of these markers may depend on cell progression through differentiation (Kempermann 2011). Doublecortin (DCX) is the primary cell marker expressed in A cells (Doetsch, García-Verdugo et al. 1997; Brown, Couillard-Després et al. 2003; Kempermann 2011). Brain injury and its resultant inflammation initiate neurogenesis by altering the niche microenvironment to stimulate proliferation and NSC migration (Merkle, Tramontin et al. 2004). NSCs express numerous receptors that respond to immune signals which influence proliferation, direct migration to sites of damage and determine the fate of these pluripotent cells in the injured brain (Matsuoka, Nozaki et al. 2003; Chu, Kim et al. 2004).

Inflammation plays a complex role in the post-injury recovery of the CNS. It has been shown that T cells specific to CNS antigens promote recovery from brain injury, provided their activity is controlled (Hauben, Nevo et al. 2000; Yoles, Hauben et al. 2001; Hofstetter, Sewell et al. 2003). However, during EAE, persistent neuroinflammation has been shown to abrogate proliferation of endogenous NSCs and neuroblast migration (Pluchino, Muzio et al. 2008). In fact, neurogenesis in several brain damage models, including acute ischemic injury (Arvidsson, Collin et al. 2002), neurotoxins (Tattersfield, Croon et al. 2004; Faiz, Acarin et al. 2005), and degenerative diseases like Alzheimer's and Parkinson's disease, have varying temporal neurogenic profiles (Capone, Frigerio et al. 2007), potentially directed by relatively unique inflammatory environments. The mechanisms instructing NSCs to maintain an undifferentiated state or differentiate within an inflamed tissue are poorly understood, particularly following viral encephalitis.

The objective of the present study was to evaluate NSC proliferation in the context of HSV-1 brain infection and its associated inflammation. Given the chronic inflammatory response observed during HSE, we hypothesized that NSC proliferation may be impaired during HSE which ultimately may contribute to the development of neurological deficits.

## **Materials and Methods:**

### **Virus and Infection**

HSV-1 brain infection that manifests as acute encephalitis in mice was performed as previously described (Marques, Cheeran et al. 2008). Briefly, HSV-1 strain Syn17+, a neurovirulent strain of HSV, was used in all experiments. The virus was propagated in



rabbit skin fibroblasts (CCL68; American Type Culture Collection) and titrated using standard plaque assay. Eight to ten week-old female BALB/c mice (Charles River Laboratories, Wilmington, MA) were infected with  $2.5 \times 10^5$  plaque forming units (PFU) by intranasal instillation in anesthetized animals as previously described (Marques, Hu et al. 2006).

### **Transplantation of luciferase(+) NSC**

Embryonic NSCs were isolated from the cortices of E14.5 BALB/c mice expressing luciferase (kindly provided by Dr. James Lokensgard, University of Minnesota). The luciferase(+) BALB/c transgenic mice were generated by backcrossing FVB mice expressing luciferase under the control of a CAGGs-CMV promoter into the BALB/c background for ten generations. Luciferase positive (luc(+)) NSCs ( $\geq 90\%$  nestin(+) by flow cytometry) were expanded in culture and transplanted by stereotaxic implantation into the subventricular zone (SVZ), within the striatum, of MHC-matched, non-transgenic BALB/c mice using previously described methods (Ni, Hu et al. 2004). Briefly, Luc(+) NSCs ( $10^6$  cells in  $3 \mu\text{L}$  serum free medium) or  $3 \mu\text{L}$  serum-free medium control was transplanted into the right SVZ using specific stereotaxic coordinates: rostrocaudal vector [AP]=0mm, interaural vector [ML]=+1.2mm and the dorsoventral vector [DV]= -3.0mm; delivered over a period of 7-10 minutes. The number of luc(+) NSCs to be transplanted was determined by measuring the minimum number of luc(+) cells necessary to provide consistent measurable baseline bioluminescent signal 7 d post transplantation, when recipient mice were infected by intranasal instillation of  $2.5 \times 10^5$  PFU HSV-1 (Syn17+). By 7 d post-transplant, surgery-induced neuroinflammation had subsided and did not affect establishment of and mortality from HSV-1 brain infection.

Bioimaging for luciferase expression in transplanted cells was performed using an *in vivo* imaging system, IVIS50 (Xenogen/Caliper Life Sciences, Alameda, CA) equipped with a charge-coupled camera device, as previously described (Marques, 2008). Briefly, 150 $\mu$ g of D-luciferin (Gold Biotechnology) was administered to anesthetized mice by i.p. injection. Animals were imaged 5-10 min after D-luciferin administration and data were acquired using a 5-min exposure window. Signal intensity of luciferase expression, as measured by the total amount of transmitted light, was quantified as a photons/sec/cm<sup>2</sup> using LivingImage (Caliper Life Sciences, Alameda CA) and Igor (Wavemetrics, Portland, OR) image analysis software. Change in bioluminescence was used as a measure of the numbers of luc(+) NSCs at indicated time points.

#### **Flow Cytometric Quantification of Endogenous Neural Stem Cells**

Mouse brain regions from -1 to +3 mm Bregma, which includes the neurogenic regions in the brain, was isolated using a coronal brain matrix (Braintree, Braintree, MA), and a papain based neural tissue dissociation kit (Miltenyi Biotec, Auburn, CA) was used to generate a single cell suspension. Myelin was depleted using myelin depletion beads (Miltenyi, CA). Live cells were enumerated, and 5x10<sup>5</sup> cells were immunostained for CD45 and nestin (BD Biosciences, San Jose, CA), Ki-67 (Abcam, Cambridge, MA) or SRY-related HMG box-gene (Sox)-2 (eBioscience, San Diego, CA) expression. For absolute quantification of immunostained cells expressing these markers, 50 $\mu$ L blank AccuCount particles (Spherotech, Lake Forest, IL) were added to samples immediately before analysis on the flow cytometer (BD FACSCanto). Absolute numbers of each cell population was calculated per the manufacturer's instruction, as a ratio of CD45(-

)nestin(+),CD45(-)Ki-67(+), or CD45(-)Ki-67(+)Sox2(+) events to number of AccuCount particles counted.

### **Immunohistochemistry**

Mice were deeply anesthetized using a mixture of ketamine and xylazine and perfused intracardially with 4% paraformaldehyde. Brains were post fixed in 4% paraformaldehyde for 24 hours and equilibrated in 30% sucrose. Fixed equilibrated tissue was frozen in OCT under liquid nitrogen vapor and sectioned at 30 $\mu$ m thickness onto gelatin-coated slides. Coronal sections thus obtained were quenched in a 0.3% peroxide solution for ten minutes and blocked with goat serum (5%) in PBS with 0.5% Triton-X for one hour at 25°C. Primary antibodies (Abs) were incubated in the blocking solution overnight at 4°C. Primary antibodies used were rabbit anti-doublecortin Ab (1:1000; Pierce Biotechnologies, Rockford, IL, and PerkinElmer Tyramide Signal Amplification PLUS Fluorescein, Perkin Elmer, Waltham, MA), rabbit or mouse anti-PCNA Ab (1:50; Abcam, Cambridge, MA), mouse anti-Sox2 or rabbit anti-FGF-2 Abs (1:200, Abcam), rat anti-Ki-67 (1:50, eBioscience), and goat anti-HSV-1 (1:100, ViroStat, Portland, MA). This HSV-1 polyclonal antibody is reactive to both immediate early and late structural antigens. Immunostaining using the Tyramide Signal Amplification kit utilized anti-rabbit IgG conjugated to horseradish peroxidase as secondary antibody. Fluorescein was used in the tyramide-horseradish peroxidase reaction step of the kit. Double immunostaining was performed following the Tyramide Signal Amplification kit using the Mouse-on-Mouse Immunohistochemistry kit (Vector Laboratories, Burlingame, CA) for mouse anti-PCNA Ab (1:50; eBioscience, San Diego, CA), or mouse anti-Sox2 Ab (1 $\mu$ g/mL; R&D Systems, Minneapolis, MN). The primary antibody incubation was

followed with a pan-mouse IgG biotinylated Ab, and an AlexaFluor 555 streptavidin Ab was used as the detecting antibody in this kit. Coronal brain sections were imaged using a Nikon ACT fluorescent microscope/camera system.

PCNA was used as a marker for IHC analysis of NSC proliferation, as an alternative to Ki-67, to identify cells in the S phase of the cell cycle. Sox2 expression was used as a surrogate marker of NSCs to confirm the findings observed within nestin(+) cell populations. Expression of these markers around the lateral ventricles is considered to be specific to NSC (Kempermann 2011). Immunostaining for NSC phenotypes was quantified exclusively around the SVZ. PCNA or Sox2 immunostaining was not observed in other brain regions including around HSV-1 induced lesions.

### **Quantification of IHC**

To quantify immunostained brain cells, ten sections from each brain were collected 320 $\mu$ m apart from (-1 to +3mm bregma) and double immunostained as described in the previous section. Images were collected using identical microscope and camera settings within a specific marker (i.e. PCNA or DCX) at 100X magnification. These images were analyzed using FIJI software (NIH, Bethesda, MD) to create merged images showing cell markers against the DAPI nuclear counterstain. The total numbers of single positive and double positive cells were counted on all ten sections from each brain, located around the sub-ventricular zone of the lateral ventricles and are presented as the average total number of cells per brain of all animals within a group.

### **RNA isolation and RT-PCR**

The neurogenic niche was isolated from infected and sham-infected mice as described above. Single cell suspensions from individual brains were generated using a

papain-based neural tissue dissociation kit (Miltenyi Biotec, Auburn, CA). Myelin was depleted as described before and the CD45(-) cell population was isolated by negative selection using CD45 selection beads (Miltenyi Biotec, Auburn, CA). Purity of the CD45(-) cell population was verified by flow cytometry ( $\geq 90\%$  purity), and total RNA was isolated from them using the Qiagen RNeasy kit (Valencia, CA). For the initial screening, 2 $\mu$ g of total RNA (260/280 ratio between 1.95 and 2.05) from CD45(-) cells harvested from three uninfected and three 15 d p.i. animals. To assess average changes in gene expression from infected and uninfected controls brains, RNA was pooled within the groups and was analyzed by two PCR arrays to assess expression profiles of genes involved in Neurogenesis and Neural Stem Cell Pathway and Neurotrophin/Neurotrophin Receptor genes (SABiosciences; Valencia, CA). Individual gene expression profiles were confirmed by real time RT-PCR was performed on whole brains isolated from intact hemispheres of infected or sham-infected mouse brains at various times post infection (p.i.) by using the Aurum Fibrous and Fatty Tissue RNA isolation kit (Bio-Rad, Hercules, CA) in a SYBR green based PCR reaction. 2 $\mu$ g of total RNA was used to synthesize cDNA using the RT<sup>2</sup> First Strand kit (Qiagen, Valencia, CA). Primers used in the single RT-PCR reactions were: Lif forward 5'-acggcaacctcatgaaccaga-3', reverse 5'-ctagaaggcctggaccaccac-3'; noggin forward 5'-cagatgtggctgtggtcac-3', reverse 5'-gcaggaacacttacactcgg-3'; Transforming Growth Factor- $\beta$  forward 5'-gactctccacctgcaagacat-3', reverse 5'-gggactggcgagccttagtt-3'; Fibroblast Growth Factor-2 forward 5'-gcgaccacacgtcaaaacta-3', reverse 5'-tccttgatagacacaactctc-3'; and Neurotrophin-3 forward 5'-ggagtttgccggaagactctc-3', reverse 5'-gggtgctctgtaatttcctta-3'.

### **FGF-2 Administration**

Infected animals at 15 d p.i. or uninfected controls were given either 1  $\mu$ g recombinant carrier-free bovine FGF-2 (R&D Systems, Minneapolis, MN) in 1  $\mu$ L saline or 1  $\mu$ L vehicle only by *intracerebroventricular* (*icv*) injection into the lateral ventricles (-0.9mm anterior/posterior, +0.5mm midline, and -2.5mm dorsal/ventral relative to bregma). Brains from experimental animals were collected 3 days later, and the neurogenic region was isolated (as in the previous section describing the quantification of endogenous NSCs) for analysis by flow cytometry for CD45 and nestin or CD45 and Ki-67 to assess stem cell numbers and proliferation of brain cells.

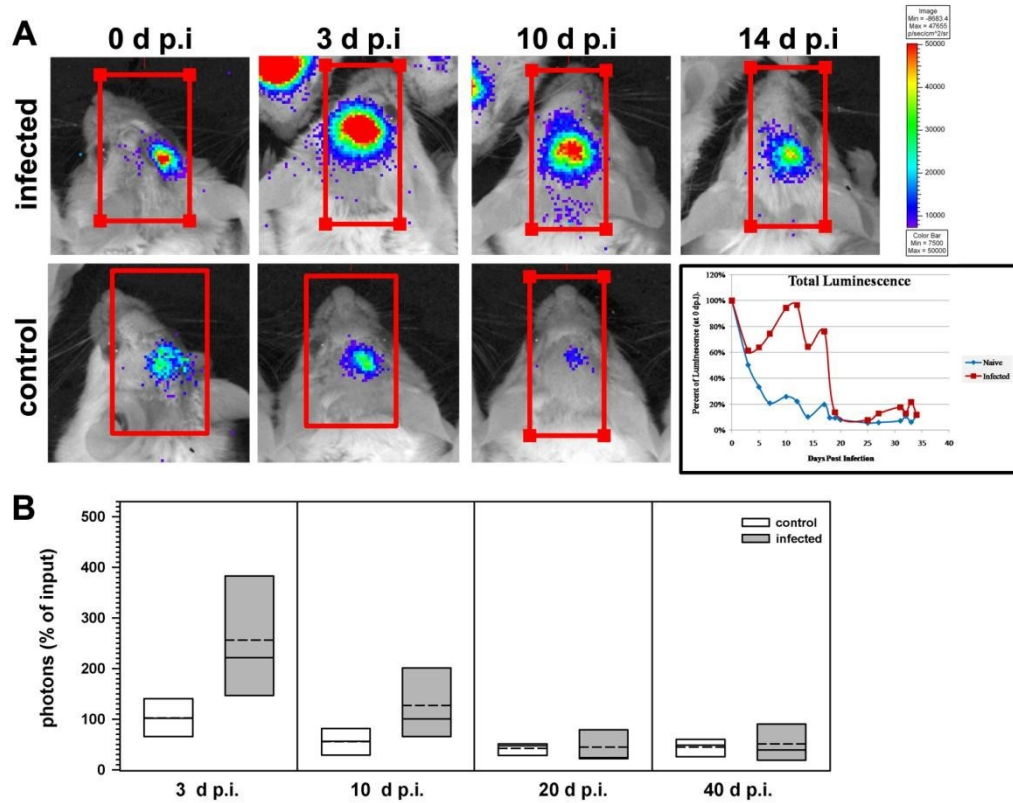
### **Statistics**

Student's T test was used to determine differences between groups. P values below 0.05 were considered to be significant. Analysis of Variance (ANOVA) was used for analysis of RT-PCR data.

### **Results**

#### **Transplanted NSCs in the adult brain respond to HSV-1 infection.**

To evaluate if HSV-1 infection altered neural stem/progenitor cell (NSC) proliferation in the adult brain, embryonic NSCs cultured from E14.5 luc(+) transgenic mice were transplanted into the striatal SVZ of MHC-matched recipient animals and evaluated only as a measure of proliferation. Infected animals exhibited temporally distinct changes in levels of bioluminescence from transplanted luc(+) NSCs at various times post infection (Fig. 3.1). A robust increase in bioluminescence was observed at 3 d p.i., which was greater than that observed in uninfected control animals (Fig. 3.1A). This increase was sustained until 15 d p.i., when bioluminescent signal decreased precipitously until it reached steady state at 20 d p.i. to levels observed in uninfected animals (Fig.



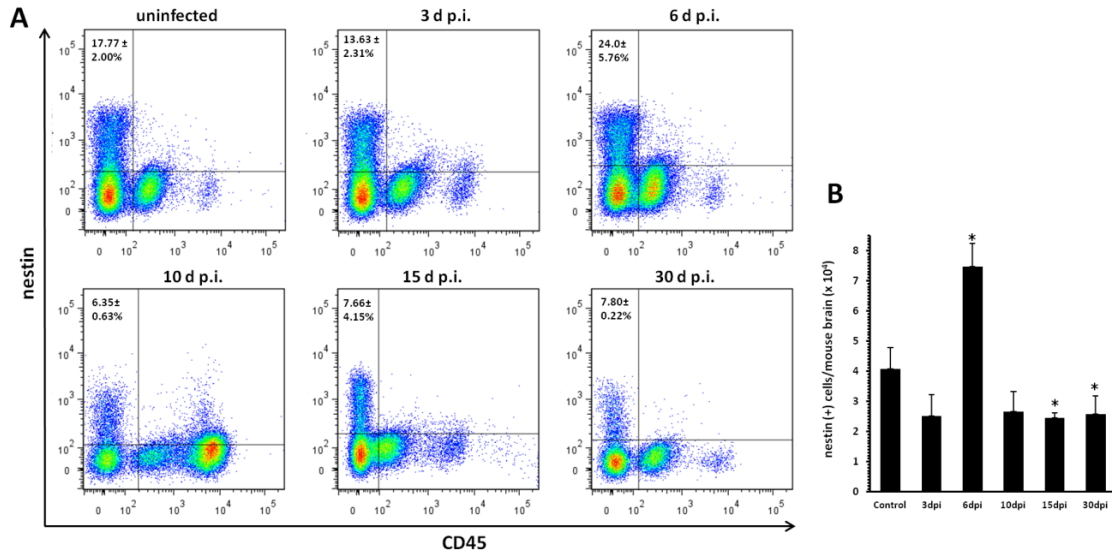
**Figure 3.1: NSCs proliferate during HSE.** Bioluminescence images obtained from animals receiving MHC-matched, luciferase-expressing ( $luc^+$ ) NSCs ( $1 \times 10^6$  cells/animal), transplanted into the SVZ by stereotactic injection (coordinates at bregma: 1.2 mm lateral, 0.5 mm caudal and 3.0 mm ventral from the skull surface). Animals were infected with HSV-1 17 syn<sup>+</sup>, 5 d post transplantation. **(A)** Levels of luciferase expression, indicative of the number of NSCs, were assessed by bioluminescence imaging longitudinally over the indicated time period. Bioluminescence images are presented on a pseudocolor scale showing photon flux values (photons/cm<sup>2</sup>/sec) at each time point in infected and uninfected animals. Representative images of the same animal (infected) at the indicated time points are shown. **(B)** Average percent change in total cranial luminescence normalized to the luminescence of each animal at time zero (0 d p.i.) is presented in HSV-1-infected (gray bars) and compared to uninfected animals (white bars). Mean values at each time point obtained from 4-6 animals followed longitudinally are presented as dashed lines in the plots and medians are solid lines. Luciferase expression levels were detected up to 40 d post-transplant, the latest time-point tested.

3.1A and B). *In vitro* analysis of luc(+) NSCs demonstrated that increased bioluminescence correlated with increased stem cell proliferation and total cell numbers (data not shown). These findings suggest that NSC exhibit an active proliferative response to HSV-1 brain infection during the acute phase of disease and that the increased cell numbers may not be sustained during the chronic phase.

### **Temporal divergence in endogenous NSC numbers following HSV-1 brain infection.**

To determine if endogenous NSCs proliferated in response to HSV-1 brain infection, changes in the nestin(+) NSC population were evaluated at 3, 6, 10, 15, and 30 d p.i. by flow cytometry. To exclude myeloid cells from the analysis, brain cells were concurrently immunostained with CD45 and the CD45(-)nestin(+) cell population was analyzed. Compared to uninfected controls, the proportion of nestin(+) NSCs among CD45(-) brain cells increased by 35% at 6 d p.i. from  $17.77 \pm 2.00\%$  to  $24.00 \pm 5.76\%$  ( $p \leq 0.05$ ). However, this increase was transient and by 10 d p.i. the percentage decreased to  $6.35 \pm 0.63\%$  ( $p \leq 0.01$ ) and remained at that level through 30 d p.i. (Fig. 3.2A). To further confirm the observed changes in the nestin(+) population during HSE, absolute numbers of CD45(-)nestin(+) cells were quantified against a fixed number of AccuCount particles added to each sample during flow cytometric analysis (Fig. 3.2B). The changes observed were preserved in the absolute quantification of total nestin(+) cell numbers. By 15 d p.i., the size of the nestin(+) NSC population decreased by approximately 33% of control, which was sustained through 30 d p.i.. These findings demonstrate that endogenous nestin(+) NSCs exhibit temporally divergent changes in population size, with



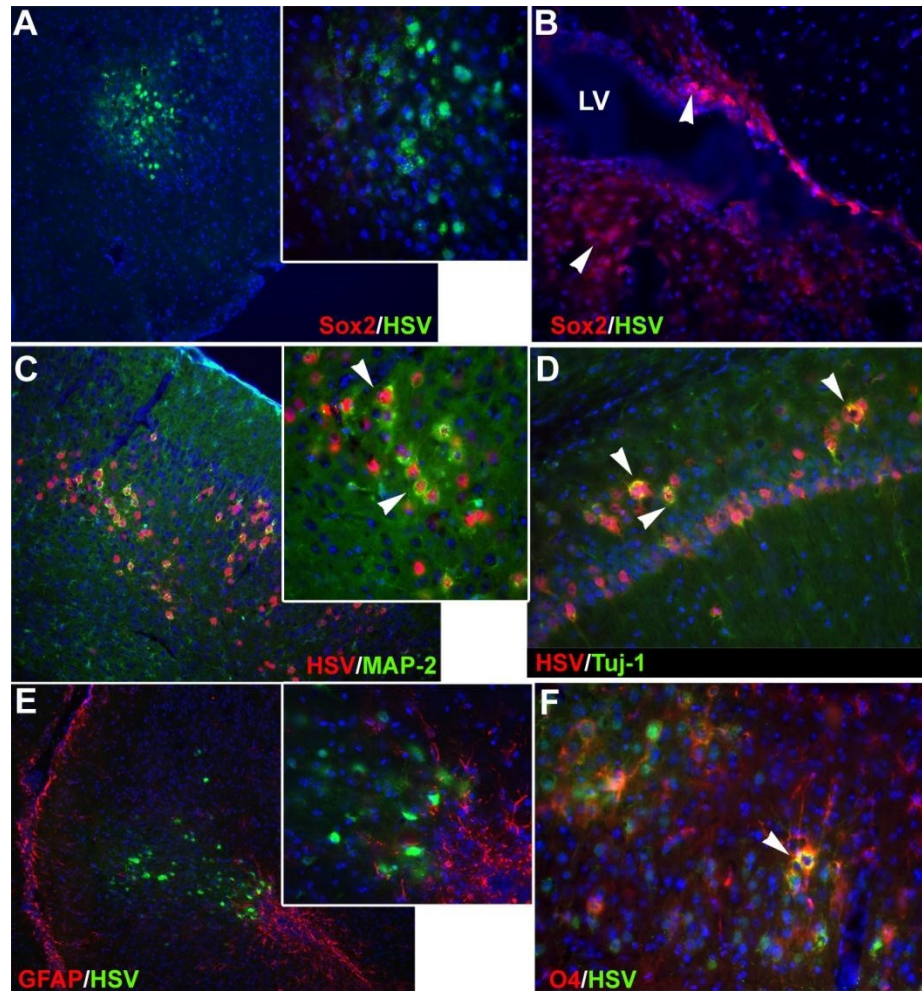


**Figure 3.2: Total number of nestin(+) NSCs increase in the HSV-1 infected brain.** Flow cytometry on single cell suspensions from infected or sham-infected BALB/c mouse brains trimmed to include neurogenic regions (-1.98 mm to 3.80 mm relative to bregma) were analyzed for nestin expression in the non-myeloid cell population (CD45(-)) and is shown at the indicated time points during HSV-1 infection. **(A)** Representative dot plots showing nestin and CD45 staining in brain cells demonstrate changes in a ratio of nestin(+) cells in the non-myeloid compartment (top left quadrant) over the course of HSV-1 infection. Quadrant gates were established based on isotype stained brain samples. Ratios of CD45(-)nestin(+) cells, presented as %±SEM, were obtained from 9-12 brains examined per time point. **(B)** The absolute number of nestin(+) cells was calculated from individual brain samples using a fixed number of AccuCount beads in each sample. Total number of live CD45(-)nestin(+) cells per brain was obtained from 3 replicates using 9 animals per replicate at each time point. The pooled average numbers in each group is presented as ±SEM. \* p≤0.05.

a robust increase in the nestin(+) cells during the acute phase of HSV-1 brain infection, and a persistent decrease in the size of this stem cell population late in the infection.

**Decrease in NSC numbers is not due to virus infection of stem cells during HSE.**

To ascertain if the decrease in NSCs could be explained by the HSV-1 infection of these cells, coronal brain sections from infected animals at 8 d p.i. were immunostained for both HSV-1 proteins and for representative brain cell phenotype markers. This specific time point was chosen based on previous studies in our laboratory demonstrating peak HSV-1 replication at 8 d p.i. in this murine model (Marques et al., 2008). HSV-1-infected cells in the brain shown by immunostaining for viral antigens were negative for Sox2 staining (Fig. 3.3A), while Sox2(+) cells in the subventricular zone were negative for viral antigens (Fig. 3.3B). Viral antigens were predominantly found in MAP-2(+) mature neurons (Fig. 3.3C) and in Tuj-1 (+) immature neurons (Fig. 3.3D). Tuj-1 is a neuronal specific class III  $\beta$ -tubulin expressed in immature neurons that precedes the expression of neurofilament protein and MAP-2 during differentiation (Memberg and Hall 1995; Ni, Hu et al. 2004; Mutnal, Cheeran et al. 2011). Tuj-1 and Map-2 (+) neurons were seen in cortical areas of the brain, distal from the SVZ and the NSC niche. Virus-infected GFAP positive astrocytes were not detected in brain sections obtained from the three animals tested (Fig. 3.3E) but occasional presence of O4<sup>+</sup> infected oligodendrocytes were observed (Fig. 3.3F). HSV antigens were undetectable by IHC in infected brains at 30 d p.i (Armien, Hu et al. 2010).

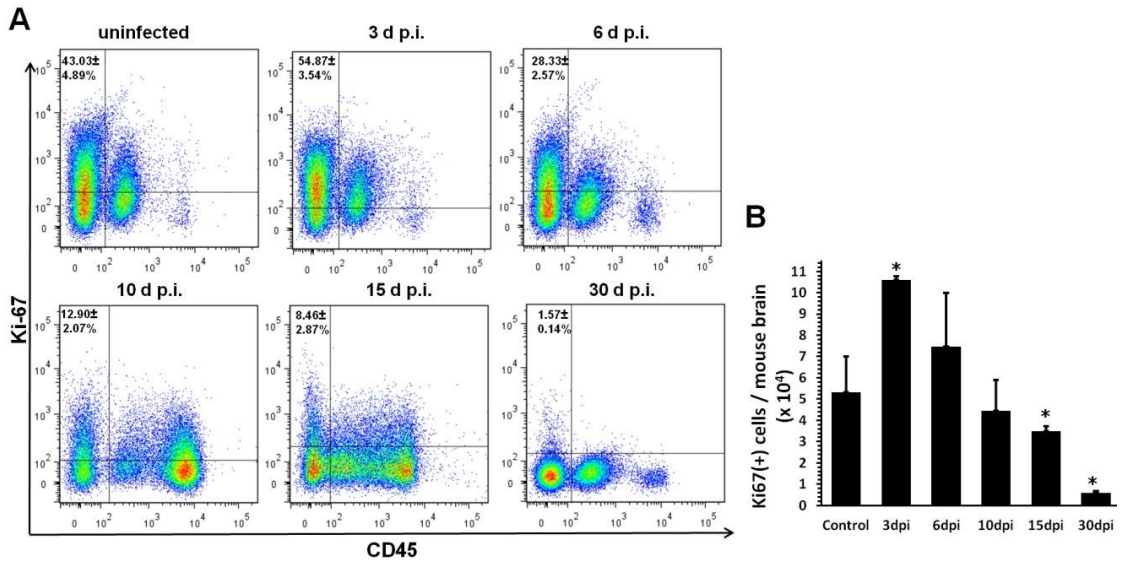


**Figure 3.3: NSCs are spared from HSV infection.** Coronal brain sections from HSV-1 infected animals were immunostained for viral antigen and brain cell specific markers at the peak of viral infection in the brain (8 d p.i.) to determine the brain cell types susceptible to viral infection. Photomicrographs demonstrating the presence of viral antigens in the different cell types are presented. **(A)** Cells expressing viral antigens in the cortex did not express Sox2 while **(B)** Sox2(+) cells observed in the subventricular zone of the same section were negative for viral antigens. **(C)** On the other hand, viral antigens were predominantly found in MAP-2(+) neurons **(D)** and in immature neurons, immunostained with Tuj-1. Nuclei were counterstained with DAPI and arrowheads denote viral positive neurons. **(E)** Viral infected GFAP(+) astrocytes were not detected in brain sections tested **(F)** but occasional O4(+) infected oligodendrocytes (arrow) were observed.

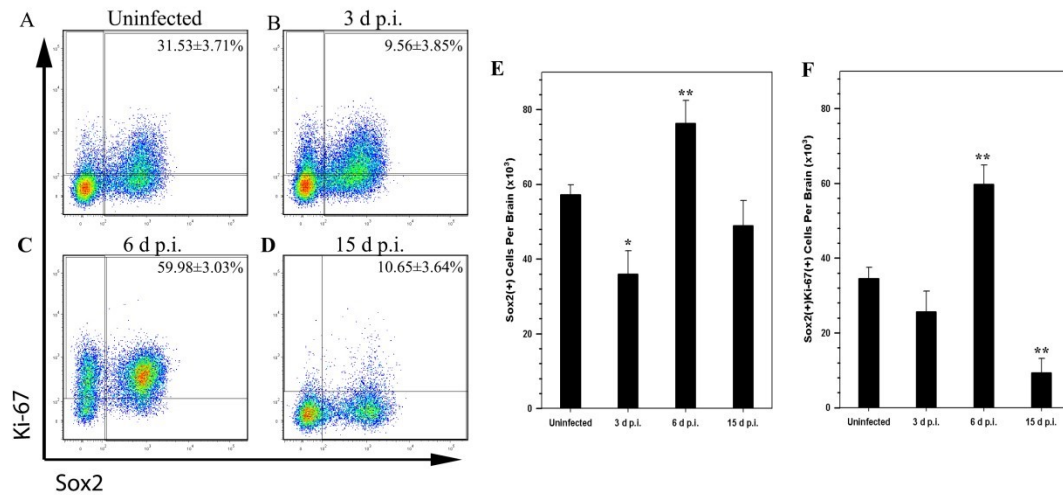
### **Temporal changes in endogenous brain cell proliferation during HSE.**

To determine if changes in the nestin(+) NSC population were associated with concomitant alteration in brain cell proliferation, Ki-67 expression in CD45(-) brain cells was evaluated. An increase in the ratio of Ki-67(+) cells was observed at 3 d p.i. ( $54.37 \pm 3.54\%$  vs.  $43.03 \pm 4.89\%$ , infected vs. uninfected respectively) which decreased steadily through 30 d p.i. to  $1.57 \pm 0.14\%$  ( $p \leq 0.01$ ; Fig. 3.4A). Most notably, the percentage of proliferating cells decreased significantly at 10, 15, and 30 d p.i. ( $12.90 \pm 2.07\%$ ,  $p \geq 0.05$ ;  $8.46 \pm 2.87\%$ ,  $p \leq 0.05$ ;  $1.57 \pm 0.14\%$ ,  $p \leq 0.01$  respectively) far below uninfected control levels ( $43.03 \pm 4.89\%$ ). The absolute numbers of CD45(-)Ki-67(+) per brain, quantified using AccuCount particles, decreased 20-fold from 3 to 30 d p.i. (from  $106,031 \pm 1628.1$  Ki-67(+) cells to  $5,745 \pm 1094$  cells per brain respectively,  $p \leq 0.01$ ; Fig. 3.4B). These findings indicated a robust proliferative response in the brain early after infection at 3 d p.i. (approximately a two-fold increase), but severely reduced proliferation later during the infection. Since the nestin(+) NSC population increased only at 6 d p.i., these findings also suggest that the increase in proliferation occurring at 3 d p.i. may be mediated by a nestin(-) cell population, such as transient amplifying migratory neuroblast cells (A cells), which are also involved in neurogenesis associated with injury (Doetsch et al., 1997).

To assess if NSC proliferation contributed to increased stem cell numbers, brain cells were co-immunostained with Sox2 and Ki-67 at 3, 6, and 15 d p.i., along with uninfected control brains, and analyzed by flow cytometry. These data revealed that trends observed by assessing nestin immunostaining were preserved, independent of



**Figure 3.4: Endogenous brain cells initiate proliferation at 3 p.i..** Single cell brain suspensions, immunostained for Ki-67 and CD45, were analyzed by flow cytometry. **(A)** Representative dot plots showing Ki-67 and CD45 staining in the brain cells of uninfected or infected animals at 3, 6, 10, 15, and 30 d p.i. demonstrated changes in a percentage of proliferating cells in the non-myeloid compartment (top left quadrant) over the course of HSV-1 infection. Quadrant gates were established based on isotype stained brain samples. Ratios of CD45(-)Ki-67(+) cells, presented in each quadrant as average values (%)  $\pm$  SEM, were obtained from 9-12 brains at each time point. **(B)** Absolute numbers of non-myeloid brain cells proliferating in response to HSV-1 infection was determined using counting beads in each brain sample analyzed for Ki-67 expression by flow cytometry. Data presented are representative plots obtained from 3 independent experiments with 9-12 animals per time point. Average values ( $\pm$ SEM) from pooled data at each time point are presented. \*  $p \leq 0.05$ .

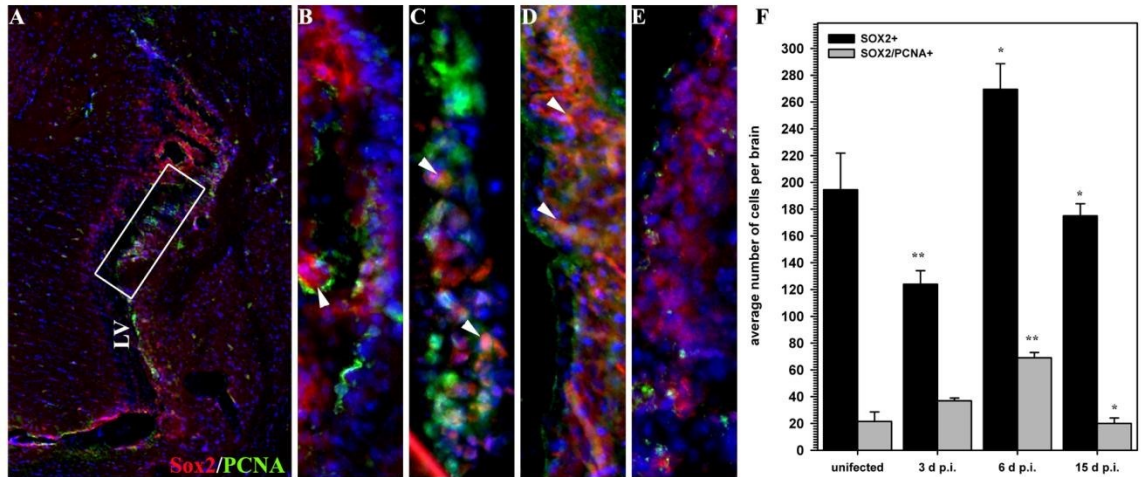


**Figure 3.5: Enhanced Sox2(+) NSC proliferation at 6 d p.i. is abrogated at 15 d p.i.** Single cell suspensions from mouse brains encompassing regions between -1.0 and +3.0mm Bregma of (A) uninfected and (B-D) infected animals at (B) 3 d p.i. (C) 6 d p.i. or (D) 15 d p.i. was analyzed for Sox2 and Ki-67 expression by flow cytometry. Data presented was based on analysis of CD45(-) brain cell population. Absolute numbers of (E) Sox2(+) and (F) proliferating Ki-67(+)Sox2(+) cells were quantified using counting beads in each brain sample analyzed, as described previously. Data are presented as averages  $\pm$  SEM of nine animals per time point. \*  $p \leq 0.05$  and \*\*  $p \leq 0.01$ .

analysis method (Fig. 3.5A-D). Notably, the total number of Sox2(+) cells was decreased at 3 d p.i. (Fig. 3.5E), but approximately 80% of the total Sox2(+) cells were proliferating (Ki-67(+)) at 6 d p.i. (Fig. 3.5F). However, both the total number of Sox2(+) and Ki-67(+)Sox2(+) cells decreased significantly at 15 d p.i. This suggests that while the brain initially responds to HSV-1 infection with robust NSC proliferation, by the time viral infection is resolved and no detectable viral antigen is seen in the brain, NSC proliferation is suppressed below physiological levels.

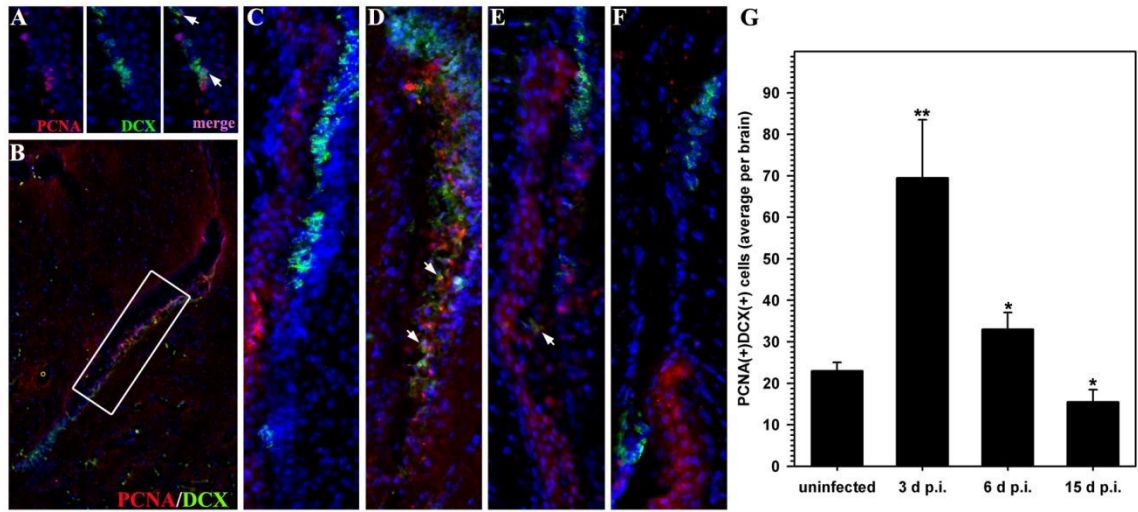
### **NSCs proliferative responses occur in the sub-ventricular zone of the lateral ventricles.**

We next proceeded to determine the neuroanatomical location of proliferative responses in HSV-1 infected brains by IHC and to determine the phenotype of NSCs located in the sub-ventricular zone, proliferating in response to viral infection. While the entire brain section was evaluated by IHC, PCNA(+)Sox2 populations were identified only within the SVZ. PCNA(+)Sox2(+) cell populations were quantified at 3, 6, and 15 d p.i. (Fig. 3.6B-E). Sox2 immunostaining confirmed that peak stem cell proliferation occurs at 6 d p.i., and that the initial increase in proliferation at 3 d p.i., measured by Ki-67 flow cytometry may result from expansion of other progenitor cell populations. Sox2(+) cells were located predominantly in the SVZ. Interestingly the total number of Sox2(+) cells decreased at 3 d p.i. ( $124.0 \pm 10.1$  total cells per infected brain vs  $194.5 \pm 27.3$  total cells per control brain,  $p \leq 0.05$ ; Fig. 3.6F), even though the number of PCNA(+) Sox2(+) stem/progenitor cells remained unchanged at this time point ( $37.0 \pm 2.0$  total cells per infected brain vs  $21.5 \pm 0.7$  total cells per control brain). However, both the total number of Sox2(+) and proliferating Sox2(+) cells were significantly increased at 6



**Figure 3.6: Sox2(+) NSC proliferation is increased in the SVZ at 6 d p.i.** Infected and sham treated brains were perfused with fixative and cryosectioned for immunohistochemistry. A total of 10 coronal sections were collected 320 $\mu$ m apart from the region between -1.0mm and +3.0mm relative to bregma. Photomicrographs of brain sections immunostained for Sox2, an NSC marker, and PCNA, proliferating cell nuclear antigen are presented. **(A)** The lateral ventricle (LV) from an uninfected mouse is shown to indicate the neuroanatomical location from which subsequent images **(B-E)** were obtained. Representative merged images showing Sox2 and PCNA immunostaining in the lateral ventricles from **(B)** uninfected animals, and infected animals at **(C)** 3 d p.i., **(D)** 6 d p.i., **(E)** 15 d p.i. are presented. Arrow heads show double positive cells. **(F)** Single- and double-positive cells were counted from all 10 coronal sections obtained from infected and sham-infected brains at the various time points indicated. The total numbers of Sox2 single-positive (black bars) and Sox2/PCNA double-positive (gray bars) cells are presented as the average ( $\pm$  SEM) number of cells observed in 10 sections obtained from 3 animals at each time point. \*  $p \leq 0.05$  and \*\*  $p \leq 0.01$ .





**Figure 3.7: Doublecortin(+) neuroblasts proliferate at 3 d p.i..** Brain sections processed and prepared as described, in the previous figure were immunostained for PCNA and doublecortin (DCX), a marker for proliferating cells and neuroblasts, respectively. **(A)** IHC analysis of control brains showing PCNA (left panel) or DCX (middle panel) staining on a representative coronal section and the merged image (right panel) showing the nuclear localization of PCNA antigen in the cell (arrows indicating double positive cells). **(B)** The lateral ventricle from a 3 d p.i. infected brain is shown to indicate the neuroanatomical location from which subsequent images **(C-F)** were obtained. Representative merged images showing patterns of PCNA and DCX immunostaining around the ventricles from **(C)** uninfected, **(D)** 3 d p.i., **(E)** 6 d p.i., **(F)** 15 d p.i. brains are presented. Arrows showing the presences of double positive cells in the infected brain at 3 and 6 d p.i. **(G)** Proliferating DCX(+)PCNA(+) neuroblasts were quantified from coronal sections obtained from infected and sham-infected brains at the various time points indicated (as described above). Increase in the total numbers of DCX(+)PCNA(+) cells in the brain was observed at 3 d p.i. but is significantly decreased at 15 d p.i. relative to uninfected controls. Data presented are averages ( $\pm$  SEM) cells counted from 3 animals at each time point. \*  $p \leq 0.05$  and \*\*  $p \leq 0.01$ .

d p.i. ( $269.5 \pm 19.11$  total cells per infected brain,  $p \leq 0.01$ , and  $69 \pm 4.02$  total cells per control brain,  $p \leq 0.01$ ). As expected both total Sox2(+) and proliferating Sox2(+) cell numbers, at 15 d p.i., decreased significantly compared to uninfected brains levels (total Sox2(+),  $175.00 \pm 9.02$  total cells per infected brain,  $p \geq 0.05$ ; double positive  $20 \pm 4.04$  total cells per infected brain,  $p \leq 0.01$ ). These findings show that NSC proliferation increased at early points during HSV-1 brain infection, but decreased significantly at later points.

### **The initial proliferative response is observed in doublecortin(+) migratory neuroblasts.**

Coronal brain sections were immunostained for doublecortin (DCX) and PCNA to determine if neuroblasts around the SVZ proliferated in response to HSV-1 infection (Fig. 3.7A-F). Proliferating DCX(+)PCNA(+) cells were significantly increased at 3 d p.i. ( $69.5 \pm 14.1$  total cells per infected brain vs  $23 \pm 2.1$  total cells per control brain,  $p \leq 0.01$ ). Although the number of proliferating DCX(+) neuroblasts around the SVZ contracted at 6 d p.i. their numbers were larger than seen in uninfected animals (Fig. 3.7G). However, during the chronic phase of infection (15 d p.i.), the number of proliferating DCX(+) cells decreased significantly relative to uninfected controls ( $15.5 \pm 2.97$  total cells per infected brain vs  $23 \pm 2.1$  total cells per control brain,  $p \leq 0.01$ ). These findings suggest that both Sox2(+) stem cells and neuroblasts respond to HSV-1 infection in a temporally distinct manner (Table 1), and viral infection results in a significant decrease in proliferation of both neural stem and progenitor cells at 15 d p.i..

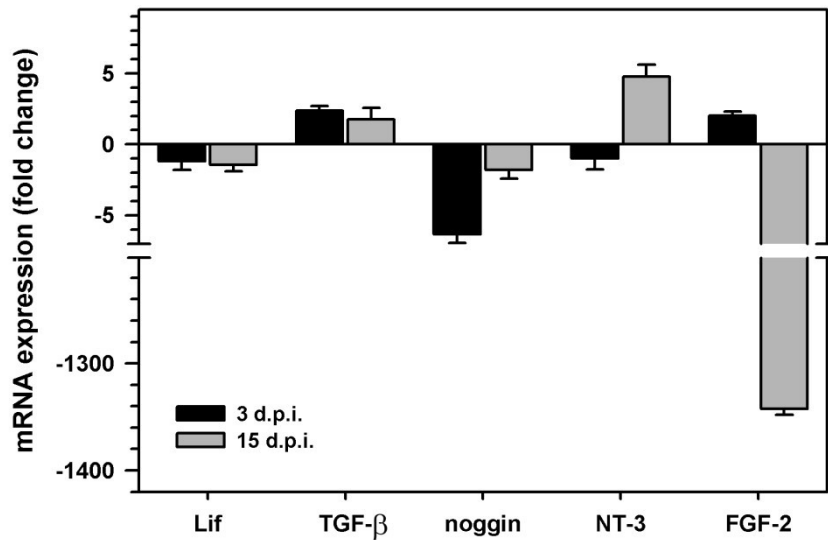
### **Differential expression of genes associated with neurogenesis at 3 and 15 d p.i..**

To further investigate the mechanisms that decrease NSC proliferation, a preliminary screen of 153 genes associated with neurogenesis was performed on mRNA

### Changes in NSC proliferation During HSE

Cell Markers	3 d p.i.	6 d p.i.	10 d p.i.	15 d p.i.	30 d p.i.
<i>Total Positive Cells</i>					
Sox2(+)	--	++		-	
nestin(+)	--	+++	--	--	--
Doublecortin(+)	+	-		++	
Ki-67(+)	+++	++	-	--	----
<i>Proliferating Cells</i>					
Sox2(+)	--	+++		----	
Doublecortin(+)	++++	++		--	
<b>Fold change value ranges: ----<math>\leq</math>0.3, ---=0.31 to 0.6, --=0.61 to 0.75,            -=0.76 to 1, +=1.01 to 1.25, ++=1.26 to 1.5, +++=1.51 to 2, ++++<math>\geq</math>2.01</b>					

**Table 3.1: Differential NSC proliferation during HSE.** The table presents observed trends of proliferation by cell marker and total endogenous brain cell proliferation as fold-change compared to the uninfected samples. The fold change values are presented as follows: (----)  $\leq$ 0.3; (---), 0.31 to 0.6; (--), 0.61 to 0.75; (-), 0.76 to 1; (+), 1.01 to 1.25; (++) , 1.26 to 1.5; (+++), 1.51 to 2, (++++),  $\geq$ 2.01

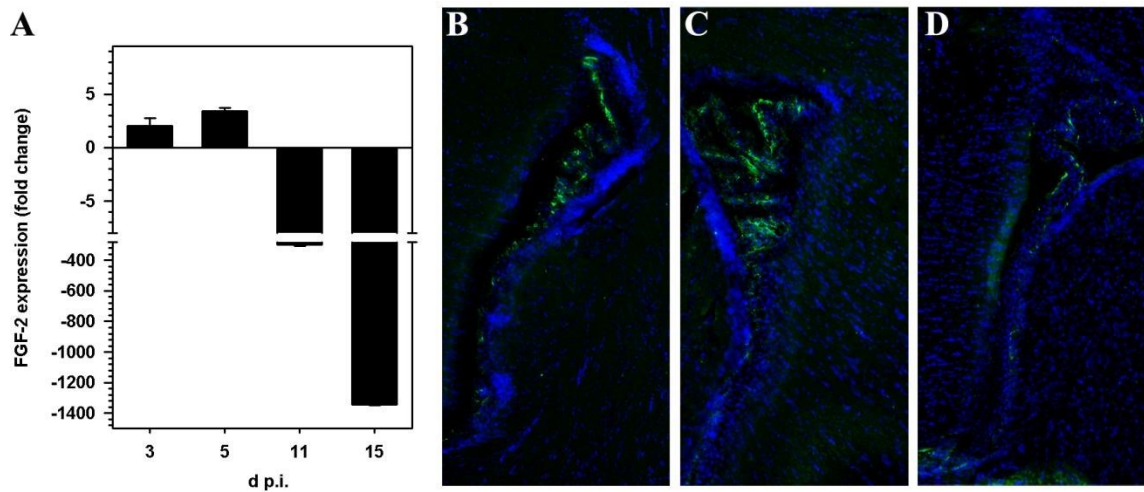


**Figure 3.8: NT-3 and FGF-2 are differentially regulated during acute and chronic HSE.** To identify mediators involved during the changes observed in NSC proliferation at 3 and 15 d p.i., genes known to affect neurogenesis were amplified by reverse transcriptase-polymerase chain reaction (RT-PCR). Five selected genes (Leukemia inhibitory factor (Lif), Transforming Growth Factor- $\beta$  (TGF- $\beta$ ), noggin, neurotrophic factor-3 (NT-3), and fibroblast growth factor-2 (FGF-2)) were assessed individually from total RNA obtained from whole brains of 3 infected animals at 3 d p.i. (black bars) and 15 d p.i. (gray bars). Data are presented as average fold change ( $\pm$  SD), in normalized mRNA expression, from uninfected controls.

isolated from CD45(-) brain cells from infected or uninfected animals at 15 d p.i.. Gene expression was analyzed on two PCR arrays (Neurogenesis/Neural Stem Cell Pathway and Neurotrophin/Neurotrophin Receptors arrays, SABiosciences; data not shown) from which, five genes selected based on change in expression ( $\geq 2$  fold) compared to uninfected controls. Expression of leukemia inhibitory factor (LIF), noggin, transforming growth factor- $\beta$  (TGF- $\beta$ ), neurotrophin-3 (NT-3), and fibroblast growth factor-2 (FGF-2) was further analyzed to determine if these selected genes exhibited differential expression at 3 and 15 d p.i. (Fig. 3.8). LIF and noggin were down regulated while TGF- $\beta$  was up regulated at both time points examined. However, NT-3 and FGF-2 were differentially regulated at 3 and 15 d p.i. NT-3 was down regulated by  $2.00 \pm 0.77$  fold at 3 d p.i. and up-regulated by  $4.76 \pm 0.84$  fold at 15 d p.i.. Conversely, FGF-2 expression increased by  $2.52 \pm 0.73$  fold at 3 d p.i., but decreased  $1342.84 \pm 33.33$  fold at 15 d p.i.. Since FGF-2 has been shown to induce stem cell proliferation (Dayer et al., 2007), the role of FGF-2 in altering NSC proliferation during HSE was further investigated.

### **Differential kinetics of FGF-2 gene and protein expression during acute and chronic HSE**

To evaluate if FGF-2 played a role in altering NSC proliferation during HSE, the kinetics of FGF-2 gene expression at 3, 5, 11, and 15 d p.i. was first analyzed in whole brain samples (Fig. 3.9A). Consistent with the data demonstrating increased brain cell proliferation at 3 and 6 d p.i., FGF-2 gene expression was up-regulated during the acute phase of infection ( $2.52 \pm 0.73$  and  $3.44 \pm 0.34$  fold respectively), and down-regulated



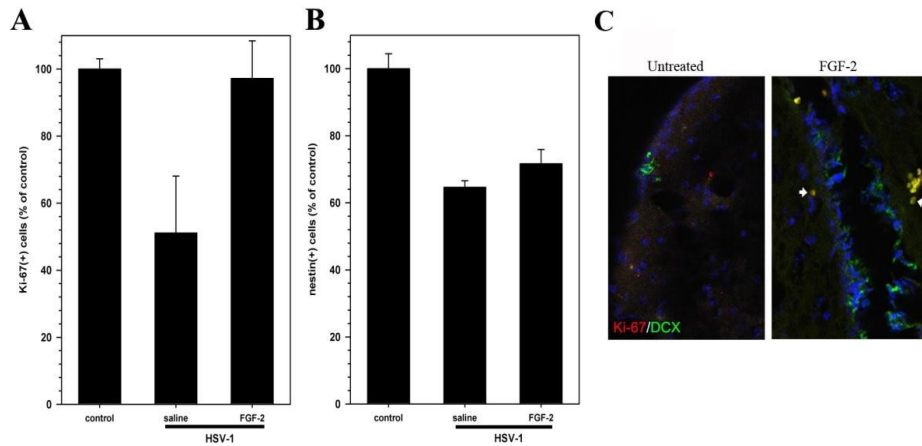
**Figure 3.9: Kinetics of FGF-2 expression during HSE.** FGF-2 expression was evaluated over the course of HSE. **(A)** Total RNA isolated from uninfected and HSV-1-infected brains was assayed for FGF-2 mRNA expression at the time points indicated. Change in FGF-2 expression over time was calculated as an average fold change ( $\pm$  SEM) from normalized gene expression in uninfected controls ( $n=3-5$  per time point). **(B-D)** To confirm FGF-2 mRNA changes were reflected in protein expression, coronal brain sections from uninfected and infected animals were evaluated by IHC. Representative photomicrographs of coronal brain sections showing the expression of FGF-2 protein around the lateral ventricles in **(B)** uninfected brains, compared to that observed in infected brains at **(C)** 3 d p.i., **(D)** 15 d p.i.. Images are representative of 3 brains assessed within each group.

during the chronic phase of infection at 11 and 15 d p.i. (288.35±13.33 and 1342.84±33.33 fold respectively).

To verify that reductions in mRNA expression were reflected by altered FGF-2 protein expression, brain sections from uninfected and infected animals at 3, 6 and 15 d p.i. were immunostained for FGF-2 and visualized by fluorescent microscopy (Fig. 3.9B). Consistent with the RT-PCR analysis, there was more FGF-2(+) immunofluorescence at 3 and 6 d p.i. around the subventricular zone, compared to the 15 d p.i. animals (coronal sections examined from -1 to +2mm bregma). Interestingly, there was FGF-2 immunostaining around the meninges, which also decreased at 15 d p.i. compared to uninfected controls (data not shown). These findings demonstrate that FGF-2 expression in infected brains correlates with observed changes in endogenous brain cell proliferation during HSE. These data also suggests that the decreased levels of FGF-2 may be responsible for decreased NSC proliferation at 15 d p.i..

### **FGF-2 treatment of chronically infected mice increases endogenous brain cell proliferation.**

To investigate if decreased FGF-2 levels in HSE animals influenced NSC proliferation, HSV-1 infected mice at 15 d p.i. were pulsed with 1µg FGF-2 or saline vehicle by *intracerebroventricular (icv)* injection. Brains were collected three days post-treatment and single cell suspensions from the neurogenic region were assessed for changes in CD45(-)Ki-67(+) proliferating brain cells (Fig. 10A) and nestin(+) neural stem/progenitor cells (Fig. 3.10B) by flow cytometry. In infected FGF-2 treated animals, CD45(-)Ki-67(+) proliferating endogenous brain cell population increased significantly



**Figure 3.10: FGF-2 treatment rescues inhibition of cell proliferation at 15 d p.i.** To determine if FGF-2 replacement impacts brain cell proliferation, infected mice at 15 d p.i. were given 1  $\mu$ g FGF-2 or saline by *icv* injection. At 3 d post treatment, brains were analyzed by flow cytometry for total number of **(A)** CD45(-)Ki-67(+) proliferating cells and **(B)** CD45(-) nestin(+). **(A)** Total numbers of proliferating Ki-67(+) endogenous brain cells reverted to uninfected (control) levels post FGF-2 treatment (FGF-2) compared to HSV-1-infected saline treated (saline) animals. Data are presented as % of control compared to uninfected (control) animals. **(B)** Similar analysis of nestin(+) NSC showed no difference in the total numbers whether infected animals were given vehicle (saline) or FGF-2. Data demonstrating differences among treatment groups are presented as average values ( $\pm$  SEM) obtained from 5 animals per group. \*  $p \leq 0.05$ . **(C)** FGF-2 treatment stimulates proliferation of DCX(+) cells in HSV-1 infected brains. Photomicrographs of coronal brains sections obtained at 15 d p.i from infected, untreated animals (left panel) or infected, FGF-2-treated animals (right panel) and immunostained for DCX and Ki67 are presented. Arrowheads indicate cells expressing DCX and Ki67. Representative images taken from four animals per treatment group are shown.



relative to the saline-treated infected animals and was not significantly different from uninfected controls (Fig. 3.10A). Although brain cell proliferation increased with FGF-2 treatment of infected animals, no increase was observed in the nestin(+) stem/progenitor cell population in these animals (Fig. 3.10B). We further characterized this increase in endogenous brain cell proliferation and observed that majority of proliferating cells with FGF-2 treatment in the SVZ were DCX(+) (Fig. 3.10C). These findings suggest that administration of exogenous FGF-2 in infected animals could stimulate brain cell proliferation and potentially be used to generate new stem/progenitor cells in virus-infected brains.

### **Discussion**

The present study demonstrates that adult murine neural stem/progenitor cells (NSCs) exhibit temporally distinct proliferative responses following Herpes Simplex Virus (HSV)-1 brain infection. In response to infection, luciferase(+) NSCs transplanted into the SVZ displayed robust expansion, indicated by an increase in total bioluminescence, which was sustained until 15 d p.i. This increase in bioluminescence was observed in infected animals, even as luminescence signals decreased in uninfected animals over time, indicative of NSC expansion or increased cell survival resulting from viral brain infection. Bioluminescence signals in infected animals subsequently dropped to levels observed in uninfected animals, during the chronic phase of infection (>15 d p.i). Bioluminescence imaging is a powerful non-invasive method to track the behavior of stem cells longitudinally *in vivo*, which when optimized can detect the presence of  $\leq 3000$  to 6000 transplanted cells in the brain (Aswendt, Adamczak et al. 2013). While this

method provides insight into acute responses of neural stem cells to HSV-1 brain infection, the fate of these implanted cells is not known.

To expand our understanding of the impact of HSV-1 brain infection on adult neurogenesis, endogenous NSCs, identified by either nestin or Sox2 expression, were analyzed in infected mouse brains. NSC numbers increased at 6 d p.i., while total brain cells proliferating in response to infection increased as early as 3 d p.i. These early proliferating cells at 3 d p.i. were identified as DCX(+) neuroblasts. This initial increase in NSC and neuroblast proliferation was abrogated during the chronic phase of infection and resulted in significantly lower numbers of nestin and Sox2 (+) NSCs in the infected brain. Expression of several growth factors was evaluated to determine the mechanisms involved in mediating these temporally divergent NSC responses. Most notably, the change in FGF-2 gene expression in HSV-1 infected brains mirrored the changes in NSC proliferation, suggesting that decreased FGF-2 consequent to infection may lead to suppressed NSC proliferation. Interestingly, a single dose of exogenous FGF-2 in chronically infected animals was sufficient to increase endogenous brain cell proliferation within 3 days post treatment, albeit without net increases in nestin(+) NSCs.

NSC proliferation in response to HSV-1 increased initially after infection. This initial increase in neurogenesis was followed by chronic suppression of proliferation in endogenous NSCs. This “window of neurogenesis” after the initiation of injury is not unique to HSE, but has been reported following non-infectious brain injury, like stroke and traumatic brain injury (Arvidsson, Collin et al. 2002; Kernie and Parent 2010; Zhou, Cheng et al. 2011). Interestingly, viral brain infections are reported to suppress

proliferative response (Das, Chakraborty et al. 2010), potentially due to virus infection and lysis of NSCs (Das and Basu 2008) or is mediated by inflammation resulting from viral infection (Das, Dutta et al. 2011). Our findings indicate that inhibition of NSC proliferation did not result directly from HSV-1 infection of NSCs, demonstrated by a distinct absence of viral antigen in the SVZ and in Sox2(+) NSCs. We and others have shown that experimental HSV-1 brain infection in mice typically occurs as a highly localized, focal neural tract infection without marked dissemination outside infected neurons in immunocompetent brains (Labetoulle, Maillet et al. 2003; Armien, Hu et al. 2010; Himmelein, St Leger et al. 2011). Furthermore, suppression of NSC proliferation, in the present study, was observed at the time when viral replication was not detected in the brain (Marques, Cheeran et al. 2008; Armien, Hu et al. 2010). This implies that the effect of HSV-1 on NSC proliferation is spatially and temporally separated, potentially mediated by indirect mechanisms resulting from the host response to infection.

Inflammatory mediators have been shown to influence NSC proliferation, migration and fate differentiation (Gonzalez-Perez 2010). The characteristics of inflammation in stroke and the early stages of HSV-1 brain infection, where NSC proliferation increases, are driven largely by macrophages and neutrophils (Arvidsson, Collin et al. 2002; Armien, Hu et al. 2010). These innate immune cells mediate both beneficial and harmful inhibitory effects on tissue regeneration (Dietel, Cicha et al. 2012; Hu, Li et al. 2012; Savage, Lopez-Castejon et al. 2012). On the other hand, acute inflammation following JEV infection, characterized by CD8(+) T cell infiltration, in addition to macrophages and neutrophils, results in inhibition of NSC proliferation (Das,

Dutta et al. 2011). Similarly, chronic T cell-mediated inflammation in EAE leads to decreased NSC proliferation and reduction of the NSC population (Ziv, Avidan et al. 2006) over extended periods following disease induction (Khoury, Hancock et al. 1992; Imitola, Raddassi et al. 2004). We demonstrate in the present study that the initial increase in proliferative response is followed by a stepwise decrease in both total NSC numbers and proliferation (Table 1) at a time when activated CD8(+) T cells dominate the inflammatory milieu (Marques, Cheeran et al. 2008; Armien, Hu et al. 2010). Activated CD8(+) T cells are essential for the maintenance of latency in HSV-1 infected neurons, effected through interactions between virus-infected cells and lymphocyte-derived lytic granule components viz. granzyme B (Knickelbein, Khanna et al. 2008; Jiang, Chentoufi et al. 2011). Release of granzyme B from activated CD8(+) T cells has been shown to impair NSC proliferation and differentiation via the voltage-dependent potassium channel, Kv1.3 (Wang, Lee et al. 2010). This suggests that persistent inflammation mediated by CD8(+) T cells following HSV-1 brain infection may similarly inhibit NSC proliferation thus impairing neurogenesis and brain recovery.

Closer examination of proliferating brain cell phenotypes revealed that multiple cell types compose the initial proliferative response in the brain. This endogenous brain cell response at 3 d p.i. was enabled by an increase in DCX(+) neuroblasts, while Sox2(+) proliferation peaked a few days later at 6 d p.i.. Proliferative response to brain injury has been shown to involve both cell types, which have different functions in the neurogenic process. This initial proliferative response has been shown to occur as the result of macrophage activation independent of the etiology of injury (Doetsch, García-

Verdugo et al. 1997; Kigerl, Gensel et al. 2009; Hu, Li et al. 2012). A number of studies have demonstrated unequivocally that proliferation of Sox2(+) cells occur early after injury, with few reporting the proliferation of DCX(+) neuroblasts. DCX(+) neuroblasts as the first identifiable proliferating cell type during neurogenesis in the adult brain (Brown, Couillard-Després et al. 2003), indicative of rapid expansion of these cells post injury. Involvement of DCX(+) neuroblasts have also been shown to be critical for the short-term outcomes associated with recovery from stroke (Jin, Mao et al. 2010). Similarly, progressive decrease in the proliferation and numbers of neuroblasts in EAE potentially influences clinical outcomes (Pluchino, Muzio et al. 2008; Huehnchen, Prozorovski et al. 2011; Tepavčević, Lazarini et al. 2011), indicative of the critical role neuroblasts play in recovery from brain injury. In the present study, the depressed proliferative response during the chronic phase of infection, where proliferation of both neural stem and progenitor cells was reduced, suggests clinical intervention at this point may be beneficial similar to that observed in ischemic injury following enhancement of NSC proliferation (Zhao, Singhal et al. 2007). Additional analysis of restorative neurogenesis in virus-infected brains is essential to demonstrate the contribution the different cell types make in the recovery from HSE-mediated damage.

Chronic inflammation alters the molecular constituents of the tissue microenvironment leading to suppression of NSC proliferation. Studies in EAE (Clemente, Ortega et al. 2011), stroke (Arvidsson, Kirik et al. 2003), and neuropathy (DeLeo, Colburn et al. 1997) have demonstrated that neurotrophic factors, such as FGF-2, glial-derived neurotrophic factor (GDNF), and anosmin-1, are down-regulated during

the chronic inflammation that develops subsequent to injury. This contrasts with findings during the acute injury where neurotrophic factors such as brain-derived neurotrophic factor (BDNF), stromal cell-derived factor (SDF)-1 $\alpha$ , and FGF-2 are up-regulated (Imitola, Raddassi et al. 2004; Kiprianova, Schindowski et al. 2004; Shruster, Ben-Zur et al. 2012). Our study found that during the course of HSV-1 infection and associated injury, NT-3 and FGF-2 gene expression is differentially regulated, increasing during the acute phase and decreasing in the chronic phase of infection.

Decreased expression of FGF-2 in HSV-1 infected mice was associated with concomitant inhibition of NSC proliferation. Although virus infection is spatially separated from the NSC niche in the SVZ, it is known that immune mediated effects following viral infections induce global changes to the tissue microenvironment (Marques, Cheeran et al. 2008; Mutnal, Cheeran et al. 2011). In uninjured brains, FGF-2 production is seen in GFAP(+) cells in the SVZ, which are unique from nestin(+) NSC (Frinchi, Bonomo et al. 2008), but the effects of virus induced inflammation on production of FGF-2 in the SVZ is poorly studied (Russo, Barlati et al. 2011). Changes in production of immune and brain produced growth factors, particularly FGF-2, have been documented consequent to inflammation resulting from ischemic injury (Dayer, Jenny et al. 2007; Cheng, Wang et al. 2011), multiple sclerosis (Clemente, Ortega et al. 2011), and Alzheimer's Disease (Kiyota, Ingraham et al. 2011). The types of growth factors and cytokines present in the injured tissue alter the outcomes of neurogenic processes initiated by injury (Haughey, Nath et al. 2002; Kalluri and Dempsey 2008). IL-1 $\beta$  treatment of brain cell cultures induced FGF-2 production (Bourcier, Dockter et al. 1995;

DeLeo, Colburn et al. 1997). Production of IL-1 $\beta$  is seen early during HSV-1 infection (Aravalli, Hu et al. 2005) giving credence to the possibility that modulation of FGF-2 expression early during HSV-1 brain infection may be mediated through IL-1 $\beta$ . Given the association between immune cytokines and growth factor production during neuroinflammation (Kalluri and Dempsey 2008; Bellavance and Rivest 2012; Zhu, Xu et al. 2012), it is plausible that the dynamic nature of the inflammatory cytokine milieu generated during HSE (Aravalli, Hu et al. 2005; Marques, Hu et al. 2006; Armien, Hu et al. 2010) modulates the functional response of NSCs ultimately defining outcomes associated with injury-induced neurogenesis. We demonstrate that treatment of infected animals with FGF-2 stimulates proliferation of brain cells. Similar results have been described after ischemic brain injury and in Alzheimer's disease models, where exogenous FGF-2 administration stimulates proliferation of NSCs and improves behavioral outcomes. (Kiyota, Ingraham et al. 2011; Feng, Zhang et al. 2012). The present study demonstrates that a single FGF-2 pulse in the lateral ventricles during the chronic phase of infection is sufficient to promote proliferation of endogenous brain cells, indicating revitalization of neurogenesis suppressed by chronic inflammation.

### **Conclusions:**

Our findings demonstrate that HSV-1 infected brains exhibits temporally distinct proliferative responses including evidence of increased neurogenesis early in disease that is progressively arrested during the chronic phase, and that FGF-2 expression rises and falls concomitant with this proliferative response. These divergent responses coincide directly with the constituents of the inflammatory milieu, dominated by macrophages and

neutrophils acutely and activated T cells in the chronic phase (Marques, Cheeran et al. 2008; Armien, Hu et al. 2010). This implies that any intervention to enhance neurogenesis would need to consider the effect of the inflammatory milieu on NSCs. We demonstrate that supplementing FGF-2 in the chronic phase of HSE enhanced the proliferative response in the brain, a possible first step in the recovery process. Further studies are essential to determine if FGF-2 may be used to offset the neurological deficits seen in the wake of viral infections of the brain.

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**Chapter 4: Alternatively activated macrophages stimulate neural stem/progenitor cell proliferation through a Wnt5a dependent pathway: Implications for Herpes Simplex encephalitis.**

**Abstract:**

Activation of macrophages and microglia is a critical component of the host response to brain damage, regardless of the etiology. Previous studies in our laboratory demonstrated that HSV-1 brain infection in mice triggered an early increase in neural stem/progenitor cell (NSC) proliferation at 3 and 6 d p.i., concurrent with peak macrophage infiltration. In the present study, we examined the role of macrophages and their activation phenotypes on NSC proliferation. Examination of activation states showed that the number of macrophages (CD45(hi)CD11b(+)) expressing CD206, an M2 activation phenotype marker, was five-fold higher than those expressing CD86, an M1 phenotype marker. To further determine if M1 or M2 macrophage activation had a direct effect on NSC proliferation, cultured NSCs were treated with supernatants obtained from *ex vivo* polarized M1 or M2 bone-marrow derived macrophages (BMDM). NSC cultures treated with M2 conditioned medium (M2CM) had 4-fold higher numbers of proliferating cells compared to those cultured in control growth media. To examine if M2 polarization would alter NSCs *in vivo*, M2 macrophages were transplanted into the lateral ventricles of uninfected mice. At 5 d post-transplantation, M2 macrophages resulted in a 15% increase in Sox2(+) NSCs proliferation and doubled the total number of Sox2(+) NSCs in the brain, compared to animals treated with saline or heat-killed cells. Interestingly, M2 polarized macrophages also exhibited significant increase in Wnt5a expression, a factor known to promote stem cell proliferation. Pretreatment of NSC cultures with either dickkopf-1, a Wnt5a inhibitor, or Wnt5a neutralizing antibody suppressed M2 macrophage driven NSC proliferation, to levels observed in untreated cultures. Wnt5a

gene expression in HSV-1 infected brains increased at 3 d p.i., coinciding with the increase in neurogenesis and M2 macrophage infiltration into the brain. Results from these studies suggest that M2 macrophages promote NSC proliferation following HSE and may provide an avenue for therapeutic intervention utilizing the reparative properties of adult NSCs.

**Introduction:**

Monocytes and macrophages play a critical role in recovery from disease and are known for their important role in supporting tissue “clean-up” and regeneration post injury (Schwartz 2010). Consequent to tissue injury, inflammatory monocytes and macrophages migrate to injured sites and phagocytize pathogens and/or damaged cells. Depending on the etiology, innate sensor systems located on the macrophage detect molecular cues produced by damaged tissue that define the inflammatory milieu generated, either promoting regeneration or initiating immune cell activation and tissue destruction (Ekdahl, Kokaia et al. 2009; Kigerl, Gensel et al. 2009; Brancato and Albina 2011). Macrophage phenotypes are intricately connected with this inflammatory tissue environment, where their effector functions are defined, often influencing the outcomes of injury. Macrophage effector subsets are broadly classified as M1 (or “classically activated”) pro-inflammatory or M2 (or “alternatively activated”) anti-inflammatory cells, albeit their activation and functional profiles are spread across a continuum of activation states where the M1 and M2 phenotypes serve as bookends of the spectrum (Mosser and Edwards 2008).

M2 macrophages are often referred to as “healing or growth promoting” macrophages (Hu, Li et al. 2012). Although such functional phenotypes are associated with a specific subsets of cells, a blend of macrophage activation phenotypes are often found within injured tissue *in vivo* (Ka, Daumas et al. 2014). Studies in rodent models of spinal cord injury have shown that the predominance of macrophages in a specific activation state promotes different outcomes during recovery (Kigerl, Gensel et al. 2009; Schwartz 2010). M1 macrophage activation is associated with immunopathology (Colton 2009; Getts, Terry et al. 2012; Crespo, Bertolotti et al. 2013), while M2 macrophage activation has been shown to facilitate regenerative processes (Kigerl, Gensel et al. 2009; Hu, Li et al. 2012). Similarly, a predominance of M2 macrophages have been shown to promote reparative myogenesis following toxin-induced damage (Arnold, Henry et al. 2007; Brancato and Albina 2011). More recent studies show evidence that macrophages are essential for the complete and accurate regeneration of excised axolotl limbs, capable of influencing specific stages in the regenerative process occurring post-injury (Godwin, Pinto et al. 2013). In addition to their role in regeneration post injury, recent studies have implicated a role for microglia, the resident brain macrophage, in maintaining the size of the neural stem/progenitor pool during embryogenesis (Cunningham, Martínez-Cerdeño et al. 2013). Macrophages have also been found to be essential for the maintenance of gut homeostasis (Takada, Hisamatsu et al. 2010), indicating that macrophages, in addition to their unique roles in tissue clean-up, also directly promote regeneration.

In the brain, regeneration is thought to be mediated by a specific subset of cells called neural stem/progenitor cells (NSCs). This self-renewing cell population is

responsible for the generation of olfactory interneurons (Doetsch, García-Verdugo et al. 1997) and promotes development of new neurons in the dentate gyrus of the hippocampus (Eriksson, Perfilieva et al. 1998) under homeostatic conditions. NSCs have been shown to migrate to sites of damage in response to specific cues in the brain associated with injury, such as chemokines (Brown, Couillard-Després et al. 2003; Imitola, Raddassi et al. 2004; Schuette-Nuetgen, Strecker et al. 2012). However, this system is seemingly less efficient in its ability to repair damaged brain tissue and long lasting neurological deficits are common outcomes following brain damage.

Emerging evidence suggests that while NSC respond to cues originating at the injury site, the inflammatory milieu generated by resident and infiltrating immune cells play a prominent role in directing the reparative functions of NSCs. During ischemic stroke and other models of non-infectious brain injury, neurogenic mechanisms involved in generating new neurons are influenced by the recruitment of macrophages (Kernie and Parent 2010; Zhou, Cheng et al. 2011; Hu, Li et al. 2012). Based on the cytokines involved, macrophage activation can be polarized to specific molecular and functional phenotypes (Martinez, Helming et al. 2013), which have distinct effects on neurogenesis. Infections of the brain, such as Japanese encephalitis (Das and Basu 2008) and West Nile encephalitis (Wang, Lobigs et al. 2003), typically induce a strong Th1 response which produces high levels of IFN- $\gamma$ . IFN- $\gamma$  is known to promote an M1 macrophage activation state that is associated with decreased levels of neurogenesis (Das and Basu 2008). Interestingly, sterile (non-infectious) models of brain damage, such as ischemic stroke (Hu, Li et al. 2012) and traumatic brain injury (Kernie and Parent 2010), appear to

promote an M2 macrophage activation state and are associated with less severe neurological outcomes (Colton 2009; Martino, Pluchino et al. 2011). While the role of cytokines in modulating NSC function have been studied, there is hitherto limited information on how macrophage activation alters NSCs.

Previous studies in our laboratory (described in chapter 3) demonstrated that peak adult neural stem cell (NSC) proliferation occurs concurrent with peak macrophage infiltration, early during infection (Rotschafer, Hu et al. 2013). Using a mouse model of Herpes Simplex encephalitis (HSE) that closely resembles the human condition (Armien, Hu et al. 2010), we investigated the role of macrophages in enhancing adult neurogenesis during viral encephalitis. Given the concurrent increases in NSC proliferation and macrophage infiltration, we hypothesized that an M2 like polarization of infiltrating macrophages stimulates NSC proliferation during the acute phase of infection.

## **Methods:**

### **Animals and Infection**

All experimental procedures using animals were conducted per the guidelines set forth by the Guide for the Care and use of animals and is approved by the UMN IACUC. Briefly,  $2.5 \times 10^5$  plaque forming units (PFU) HSV-1 strain Syn17+ was instilled intranasally into anesthetized eight to ten-week old female BALB/c mice (Charles River, Wilmington, MA) as described previously (Marques et al., 2006).

### **Flow Cytometric Evaluation of Immune Cell Infiltration**

Infected and uninfected mouse brains were isolated and homogenized by mechanical dissociation. This homogenate was then centrifuged on a discontinuous 70-

30% Percoll gradient (Sigma Aldrich, St. Louis, MO). Total brain leukocytes were isolated, washed, and enumerated for immunostaining.  $5 \times 10^5$  cells were used for immunostaining for CD45 (PE-Cy7, PerCP-Cy5.5, APC, or FITC; eBioscience, San Diego, CA), CD11b (APC, APC-Cy7 or eFluor 405; eBioscience), Ly6C (PerCP-eFluor710 or APC; eBioscience), CD206 (APC; BD Biosciences, San Jose, CA), CD86 (PE or FITC; BD Biosciences), and MHC class II (PerCP-eFluor 710 or PE; BD Biosciences). Immunostaining samples were blocked for five minutes with anti-CD16/CD32 antibody (BioXCell, West Lebanon, NH) prior to addition of fluorophore conjugated cell surface antibodies. Absolute quantification was achieved by adding 50uL AccuCount blank particles prior to sample acquisition on a BD FACSCanto cytometer within 24 hours of sample collection.

### **Flow Cytometric Evaluation of Endogenous Neural Stem Cells**

The neurogenic regions of infected and uninfected mice were isolated (coronal -1 to +3mm bregma). A papain-based Neural Tissue Dissociation Kit (Miltenyi Biotec, Auburn, CA) was used to generate a single cell suspension. Myelin was depleted using Myelin Removal Beads from Miltenyi and live cells were counted for immunostaining.  $5 \times 10^5$  cells were used for immunostaining for CD45 (PerCP-Cy5.5; eBioscience), Sox2 (eFluor 660; eBioscience), and Ki-67 (Abcam, Cambridge, MA). Secondary antibody used for Ki-67 was donkey anti-rat F'ab PE (BD Biosciences). Quantification of endogenous stem cells was performed by adding 50 $\mu$ L blank AccuCount particles (Spherotech, Lake Forest, IL) immediately prior to reading on a BD FACSCanto.



Absolute numbers of NSCs and proliferating cells was calculated per the manufacturer's instructions as a ratio of positive events to particle events read.

### ***In vitro* culture and proliferation of NSCs**

Embryonic day 14.5 cortices were isolated from embryos of pregnant BALB/c mice. A single cell suspension was generated by mechanical dissociation and cultured in DMEM:F12 medium supplemented with N2 culture supplement. Epidermal growth factor (EGF) and fibroblast growth factor-2 (FGF-2) were added every other day to 20 ng/mL. Cultures were grown as spheres and cells between passages 1 and 4 were used in experiments.

Proliferation of NSCs was measured by labeling the cells with a fixable proliferation dye. Briefly, NSCs were dissociated to a single cell suspension. The cells were then concentrated to  $1 \times 10^7$  cells per mL by centrifugation and suspension in 900  $\mu$ L complete NSC medium. 100  $\mu$ L 100nM diluted fixable Cell Proliferation Dye eFluor 670 (1:500 dilution of recommended stock) was added and cells were cultured in the dark at 37°C for five minutes. Labeling was quenched using 5 mL 1:1 7.5% bovine serum albumin (Sigma Aldrich) to complete NSC medium and incubated on ice for ten minutes. Cells were washed four times in the 1:1 BSA:NSC medium solution and once more in complete NSC medium.  $1 \times 10^5$  labeled NSCs were then cultured with or without conditioned macrophage media in 24 well plates. A time course of proliferation was evaluated by cell cycle analysis using 7-aminoactinomycin (7-AAD) DNA binding. Cells in the G<sub>2</sub>/M phase were quantified with AccuCount particles, added prior to data acquisition to ensure accurate cell quantification of proliferating cells.

The role of Wnt/ $\beta$ -catenin signaling in M2C induced NSC proliferation was evaluated by adding an endogenous Wnt signaling blocker, dickkopf-1 (DKK-1; 100 ng/mL, R&D Systems), to M2C treated NSCs and cultured for 72 hours (h). Wnt5a specificity in was demonstrated by blocking Wnt5a specific signaling using a polyclonal neutralizing antibody to Wnt-5a (5  $\mu$ g/mL; R&D Systems), and cultured with M2C treated NSCs for 72 h.

### ***In vitro* Macrophage Polarization**

Bone-marrow derived monocytes (BMDMs) were isolated from tibias of uninfected BALB/c mice by flushing serum-free macrophage culture media (Life Technologies, Grand Island, NY) through the marrow cavity. Bone marrow cells were differentiated in to macrophages by supplementing serum free macrophage media (Life Technologies) with macrophage-colony stimulating factor (M-CSF, eBioscience; 10 ng/mL) and allowed to grow for three days. Non-adherent cells were removed by washing and adherent macrophages were maintained as unstimulated, or were stimulated with either interleukin-4 (eBioscience; 20 ng/mL) or lipopolysaccharides (Sigma Aldrich; 50 ng/mL) and interferon- $\gamma$  (R&D Systems, Minneapolis, MN; 20 ng/mL) for 4 days to generate alternatively or classically activated macrophages respectively. Supernatants were collected at the end of the 4d period and stored at -80°C. Validation of classical or alternative activation was achieved by flow cytometry for CD45, CD11b, CD206 and CD86 as well as reverse transcription-PCR for gene products shown to be specific for each polarization including *interleukin-15*, *interleukin-6*, *arginase1*, and *CD206*.

### **Immunocytochemistry of Polarized Macrophages**

BMDMs were seeded onto glass coverslips in 24 well plates and polarized as described above. Unpolarized (M0) or IL-4 treated (M2) macrophages grown on coverslips were rinsed twice with PBS and fixed in 4% paraformaldehyde for 30 min at room temperature. Coverslips were washed three times with PBS and permeabilized using a 0.1% Triton-X 100 in PBS solution for 10 minutes at room temperature. Coverslips were washed three times in PBS and blocked for one hour at room temperature in 1% BSA. Primary antibody, goat anti-mouse Wnt5a (1:50 dilution, R&D Systems, Minneapolis, MN) was incubated overnight at 4°C. Coverslips were washed three times in PBS and anti-goat Ig conjugated to AlexaFluor 488 (1:500 dilution, Life Technologies, Carlsbad, CA) was applied for 30 min at room temp and protected from light. Coverslips were counterstained with DAPI and imaged using a Nikon fluorescent microscope.

### ***In vivo* Macrophage Supplementation**

Uninfected 8 to 10-week old female BALB/c mice were anesthetized and heat-killed or live M2 activated macrophages was administered by *intracerebroventricular* (*icv*) injection (-0.9mm anterior/posterior, +0.5mm midline, and -2.5mm dorsal/ventral relative to bregma) using a 27.5GA needle. Cells were heat-killed by incubation at 55°C for 15 minutes and cell death was verified by visual evaluation of the cells using a Trypan Blue (Sigma Aldrich) exclusion assay.  $3 \times 10^4$  heat-killed or live macrophages were stereotactically injected in 3  $\mu$ L saline at a rate of 1 $\mu$ L per minute into the right lateral ventricle.

### **Immunohistochemistry in infected mouse brains**

6 d p.i. mice infected with HSV-1 were perfused with 4% paraformaldehyde and their brains were isolated. These brains were post-fixed in 4% paraformaldehyde for 24 h and then cryopreserved by passaging through increasing amounts of 30% sucrose in phosphate buffered saline (PBS). Finally, brains were frozen in Optimal Cutting Temperature (OTC) medium (Sakura, Torrance, CA) and cryosectioned into 10  $\mu$ m coronal or sagittal sections. 12 sections were collected 250  $\mu$ m apart on each slide. Endogenous peroxidase was quenched in a 0.3% hydrogen peroxide solution for 10 min and washed 3 times in PBS. Sections were blocked in 10% bovine serum albumin (BSA) for 1 h at room temperature and CD11b primary antibody was applied (1:10, Pierce/ThermoScientific, Rockford, IL) overnight at 4°C. Slides were then washed three times in PBS and permeabilized in a 0.1% Triton-X 100 solution for 10 min. Slides were washed three times in PBS and anti-Wnt5a primary antibody was added (1:50, R&D Systems, Minneapolis, MN) overnight at 4°C. Following three PBS washes, chicken anti-goat Ig AlexaFluor 488 and mouse anti-rat IgG2b Biotin secondary antibodies were applied for two h at room temperature. Slides were washed three times in PBS and incubated with streptavidin AlexaFluor 555 (1:100, Life Technologies, Carlsbad, CA) for 10 min. Slides were washed and counterstained with DAPI. Sections were imaged using a Nikon fluorescent microscope. Images were collected at the same exposure times (within fluorophores) for all samples.

### **RT-PCR**

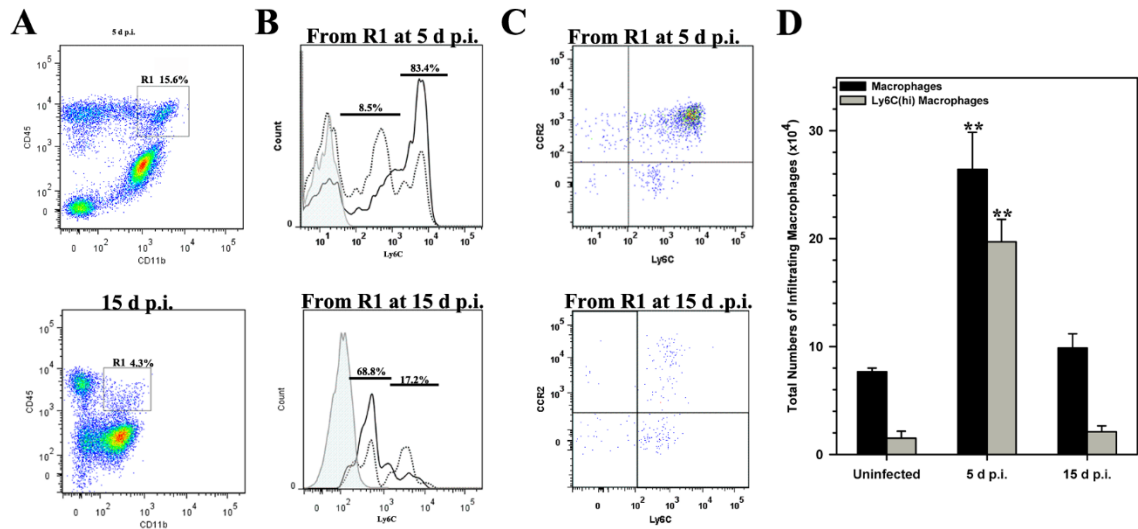
Total RNA was isolated from mouse brains using the BioRad Aurum Fatty/Fibrous Tissue kit (Hercules, CA) per manufacturer's recommendation. Total RNA

from polarized macrophages and cultured NSCs was isolated using the RNeasy Plus kit (Qiagen, Valencia, CA) and 2µg RNA was reverse transcribed to cDNA. cDNA was amplified using a SyBR based PCR reaction on a Roche LightCycler 480 using primers designed for specific genes: *Wnt5a*: Forward 5'-ATGAACTGGGGGCATCTTGG-3', Reverse 5'-CGAGGAATTCAAGCGAAGCG-3'; *Wnt7b*: Forward 5'-TCTCTGCTTTGGCGTCCTCTAC-3', Reverse 5'-GCCAGGCCAGGAATCTGTTG-3'; *Wnt10b*: Forward 5'-TTCTCTCGGGATTTCTTGGATTC-3', Reverse 5'-TGCACTTCCGCTTCAGGTTTTTC-3';  $\beta$ -*catenin*: Forward 5'-AAGGAAGCTTCCAGACATGC-3', Reverse 5'-AGCTTGCTCTCTTGATTGCC-3'; *IL-15* Forward 5'-CATCCATCTCGTGCTACTTGTGTT-3', Reverse 5'-CATCTATCCAGTTGGCCTCTGTTT-3'; *IL-6* Forward 5'-CAGAATTGCCATCGTACAACCTCTTTTCTCA-3', Reverse 5'-AAGTGCATCATCGTTGTTTCATACA-3'; *Arg1* Forward 5'-ACAAGACAGGGCTCCTTTCAGG-3', Reverse 5'-GGAGAAGGCGTTTGCTTAGTTCTG-3'; *CD206* Forward 5'-TCTTTGCCTTTCCCAGTCTCC-3', Reverse 5'-TGACACCCAGCGGAATTTTC-3'.

## **Results:**

### **Alternatively activated (M2) macrophages infiltrate the brain at 5 d p.i.**

To identify phenotypes of infiltrating macrophages in the brain during the course of Herpes Simplex encephalitis, brain leukocytes were stratified on a flow cytometer based on surface expression of CD45 and CD11b. Consistent with previous findings (Marques, Cheeran et al. 2008; Mutnal, Cheeran et al. 2011), the total infiltrating



**Figure 4.1: Infiltrating monocyte/macrophages are Ly6C(hi)CCR2(+) at 5 d p.i. (A)** Representative dot plots of CD45 versus CD11b immunostaining of brain immune cells showing several distinct populations of immune cells in infected brains. The infiltrating monocyte/macrophage population was defined as CD45(hi)CD11b(+) (gate R1). Representative dot plots from 10-15 animals at each time point are presented. **(B)** Ly6C expression on infiltrating monocyte/macrophages (R1) from uninfected (dotted line) or infected (black line) brain samples at 5 d p.i. (top plot) or 15 d p.i. (bottom plot) was compared to isotype-stained controls (gray shaded). Representative histograms obtained from 10-15 animals per group at the indicated time points showing percentages of Ly6C(hi) and Ly6C(int) monocyte/macrophage populations. **(C)** To further characterize R1 cells, CCR2 expression was evaluated at 5 and 15 d p.i. Representative dot plots obtained from 10-15 animals are presented. **(D)** Absolute numbers of infiltrating monocyte/macrophages [CD45(hi)CD11b(+); black bars] and those that were also Ly6C(hi) (gray bars) were quantified using AccuCount particles from uninfected and HSV-1 infected brains at 5 and 15 d p.i. Data presented as average number of cells ( $\pm$ SEM) per mouse brain. \*\*  $p \leq 0.01$  vs uninfected animals.

macrophage population (CD45(hi) CD11b(+); R1, Fig. 4.1A) was larger at 5 d p.i. during acute HSE, compared to macrophage numbers observed at 15 d p.i. ( $282,000 \pm 13,880$  cells vs  $40,800 \pm 1560$  cells;  $p \leq 0.01$ ) during the chronic phase of the disease (Fig. 4.1A). Presence of macrophages expressing high levels of the activation marker Ly6C significantly increased at 5 d p.i. However at 15 d p.i. a minor population of macrophages expressing intermediate levels of Ly6C was observed that was not seen at 5 d p.i. (Fig. 4.1B). At 5 d p.i., more than 90% of Ly6C(hi) R1 macrophages also expressed CCR2(+) whereas at 15 d p.i. CCR2(+)Ly6C(hi) macrophages were undetectable in the brain. Interestingly, CCR2 was expressed on Ly6C(int) cells at 15 d p.i. (Fig. 4.1C). Quantification of these cell populations reveals that at 5 d p.i. approximately 83% of the total CD45(hi)CD11b(+) macrophage population also expressed Ly6C(hi); while at 15 d p.i. this population was greatly reduced (Fig. 4.1D).

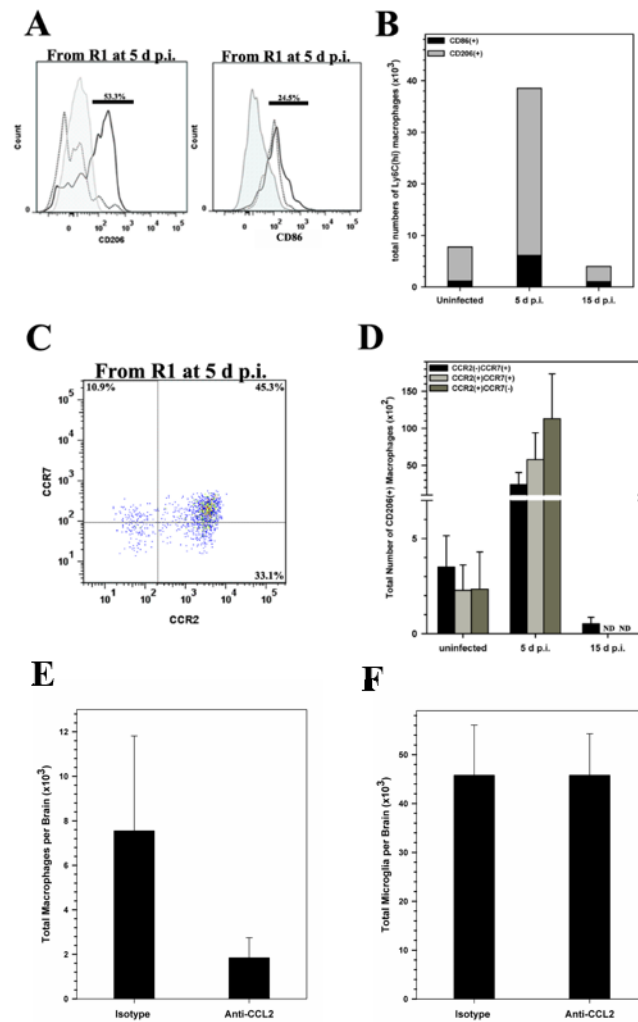
To examine if discreet macrophage activation states correlated with increases in NSC proliferation, expression of CD206 (the mannose binding receptor and an alternative activation marker) and CD86 (a beta-seven integrin and a classical activation marker), which represent two distinct activation states of the cell were evaluated. At 5 d p.i., an increase in macrophages expressing CD206 was observed, whereas a minimal change in CD86 expressing macrophages was seen at this time point (Fig. 4.2A). Furthermore total numbers of CD206(+)Ly6C(hi) macrophages outnumbered CD86(+)Ly6C(hi) macrophages by a ratio of approximately 5:1 ( $38,436 \pm 2385$  CD206(+) macrophages vs  $4983 \pm 543$  CD86(+) macrophages) compared to a 2:1 ratio in infected brains at 15 d p.i. and uninfected controls (Fig. 4.2B). In addition, more than half of the CD206(+) cells

expressed both CCR2 and CCR7 (Fig. 4.2C). This observation is congruent with observation that blocking macrophage infiltration using a CCL2 antibody alone does not abrogate macrophage infiltration into the brain at 5 d p.i (data not shown). Quantification of CCR2 and CCR7 expression on the CD206(+) infiltrating macrophage population revealed that in addition to a robust population of CCR2(+) CCR7(+) double positive Ly6C(hi) cells, there is significantly more CCR2(-)CCR7(+), single positive, CD206(+) cells than in uninfected control mice (Fig. 4.2D). Surprisingly, at 15 d p.i., CCR7 was absent in the CD206(+) macrophage population (Fig 4.2D). On the other hand, blocking CCR2 utilizing a CCL2 neutralizing antibody was unsuccessful (Fig 4.2E) in preventing infiltration of macrophages or total numbers of microglia (Fig 4.2F) present in the brain.

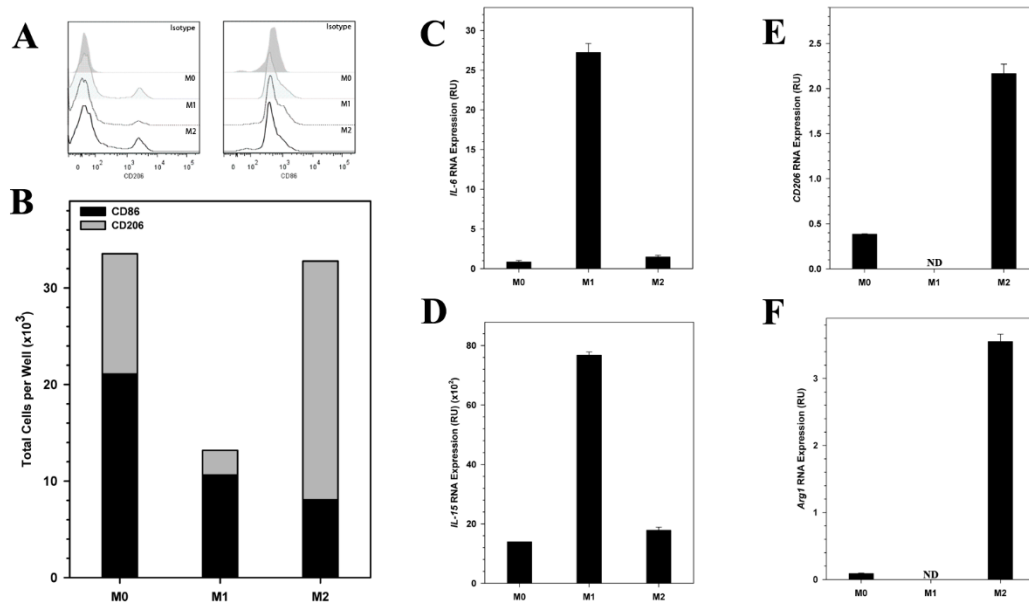
***In vitro* polarization of BMDMs results in distinct M1 and M2 phenotypes.**

To determine if macrophage polarization altered NSC function, cultured BMDMs were differentiated with either IL-4 or LPS and IFN- $\gamma$  as previously described (Hu, Li et al. 2012) to generate M2 and M1 macrophages, respectively. *In vitro* polarized macrophage activation profiles were validated using flow cytometry and RT-PCR (Fig. 4.3). While CD206 and CD86 expression were observed in macrophages in all treatment groups (Fig. 4.3A), the ratio of CD206 expression was 4-fold greater than CD86 under M2 polarizing conditions than M1 (2471.63 $\pm$ 226.26 CD206(+) cells vs. 214.66 $\pm$ 59.98 CD86(+) cells,  $p \leq 0.01$ ). Fig. 4.3A). Similarly M1 macrophages expressed approximately 4-fold more CD86 than CD206 (1063.03  $\pm$  39.28 CD86(+) cells vs. 255.57  $\pm$  32.58 CD206(+) cells,  $p \leq 0.01$ ). The M0 “unpolarized” treatment group had approximately a





**Figure 4.2: Increased infiltration of alternatively activated (M2) monocyte/macrophages at 5 d p.i.** (A) Representative histograms of CD206 (left plot) and CD86 (right plot) expression on the infiltrating monocyte/macrophage population (R1 in Figure 1) from infected (solid black line) and uninfected (dotted black line) animals were compared to isotype stained controls (shaded line) at 5 d p.i. (B) Average numbers of cells per brain obtained from 10-15 animals per time point at 5 and 15 d p.i. are presented. (C) Further characterization of Ly6C(hi)CD206(+) M2 macrophages demonstrated that these cells expressed CCR7, in addition to CCR2. Representative dot plot showing CCR2 and CCR7 expression on Ly6C(hi) infiltrating monocyte/macrophages. (D) Total numbers of CCR7(+)CCR2(-) (black bar), CCR7(+)CCR2(+) (light gray bar), and CCR7(-)CCR2(+) (dark gray bar) cells from the CD206(+) infiltrating macrophage population were quantified using AccuCount particles from uninfected and infected brains at 5 and 15 d p.i. Total numbers of macrophages infiltrating the brain (E) or microglia (F) of infected mice given a CCL-2 (Anti-CCL2; CCR2 ligand) neutralizing antibody during acute HSE was not altered compared to infected mice given an isotype antibody (Isotype). Data are presented as average ( $\pm$ SEM) cells per brain.



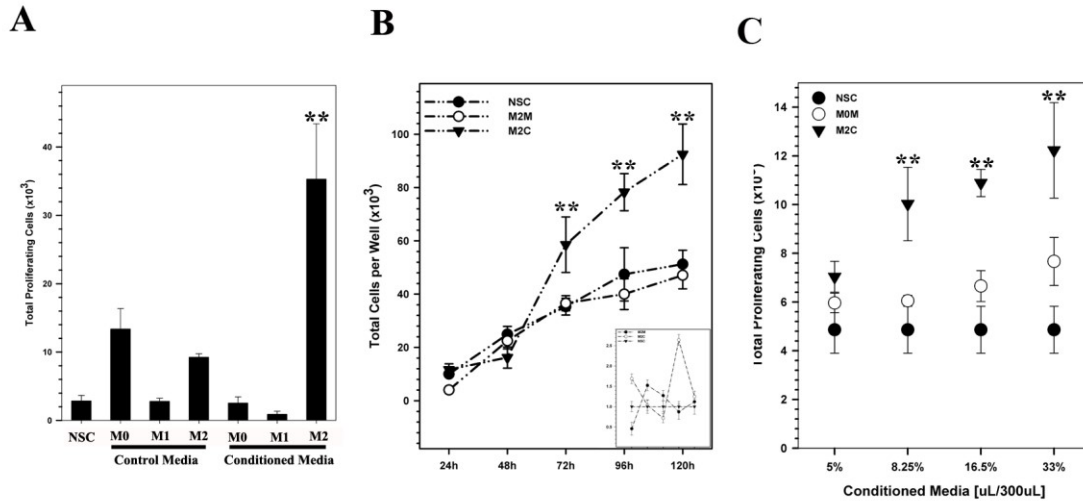
**Figure 4.3: *In vitro* polarization of bone marrow-derived macrophages (BMDMs).** (A) Flow cytometry analysis of unpolarized (M0) and polarized M1 or M2 BMDMs for CD206 and CD86 expression in the cultured CD11b(+) BMDMs is presented. Histograms obtained from CD11b(+) BMDM (top panel) immunostained for CD206 (top left panel) and CD86 (top right panel) show differential expression of CD206/CD86 on polarized BMDMs. (B) The ratios of absolute numbers of CD11b(+)CD206(+) macrophages to CD11b(+)CD86(+) macrophages are presented (bottom panel) as average values obtained from 2 independent experiments using pooled bone marrow from 6 animals per experiment that were polarized in triplicate cultures. (C-F) Quantitative RT-PCR analysis of *in vitro* polarized BMDMs from M0, M1, or M2 polarized macrophages, 24 hours post-polarization, is presented. Data are compiled from 3 independent experiments using pooled BMDMs from 6 animals per experiment and each polarization paradigm was tested in triplicate. PCR analysis of each sample was performed in triplicate and average relative gene expression, normalized to RPL27 housekeeping gene, is shown (average  $\pm$  SEM). Gene expression profiles indicative of an M1 phenotype was demonstrated by (C) interleukin-6 (*IL-6*) and (D) interleukin-15 (*IL-15*) expression levels and those of an M2 phenotype was demonstrated by (E) CD206 and (F) arginase-1 (*Arg1*) expression levels.

two to one ratio of CD86 to CD206 ( $2109.52 \pm 339.00$  CD86(+) cells to  $1245.53 \pm 128.70$  CD206(+) cells; (Fig. 4.3B).

RT-PCR analysis of M1 and M2 macrophages revealed that expression of the M1 markers IL-6 and IL-15 were up-regulated in the M1 treatment group after 24 hours of stimulation (Fig. 4.3C&D), and expression of the M2 markers CD206 and Arginase1 were up-regulated in the M2 group (Fig. 3E&F). CD206 and Arginase1 were not detectable in the M1 polarized group. These data validated the polarized activation phenotypes of the macrophages used in the following experiments.

### **M2 macrophage stimulation of proliferation is cell contact independent**

Unstimulated (M0C), M1 (M1C) and M2 (M2C) polarized macrophage culture supernatants or corresponding media controls (M0M, and M2M respectively) were added to embryonic (E14.5) neural stem cell cultures. Impressively, NSC grown in a 1:1 supplemented M2 conditioned medium (M2C) exhibited a three-fold increase in proliferation over NSC only medium ( $35,281 \pm 8,093$  cells vs  $2,843 \pm 808$  cells in NSC control medium;  $p \leq 0.01$ ; Fig. 4.4A). NSCs in all treatment groups expressed high levels of nestin (experiment average  $92.3 \pm 3.46\%$  nestin(+)) suggesting that none of the treatments induced differentiation of these cells. The proliferative effect of M2 macrophage conditioned medium increased over time, with cultures having significantly higher total number of cells than control media at 72h ( $58,333.58 \pm 834.65$  cells vs  $33,397.03 \pm 398.89$  cells,  $p \leq 0.01$ ), 96h ( $77,701 \pm 2227.91$  total cells vs  $40,739.54 \pm 1823.12$  cells) and 120h ( $87,701.03 \pm 2274.45$  cells vs  $43,548 \pm 504.73$ ,  $p \leq 0.01$ ; Fig. 4.4B). Increases in NSC proliferation by M2 conditioned media were robust and could be



**Figure 4.4: Soluble mediators produced by M2 polarized macrophages stimulate NSC proliferation.** (A) At 60 h post-treatment, dye dilution within dividing cells was measured by flow cytometry to determine changes in NSC proliferation following treatment with polarized macrophage supernatants. Increase in NSC division suggests that M2 macrophage supernatants secrete soluble factors that enhance NSC proliferation. (B) The kinetics of M2 macrophage driven NSC proliferation was measured by assessing the total numbers of NSCs treated with M2 control media (M2M), M2 polarized supernatant (M2C), or complete NSC control media (NSC) over the indicated time points. (Inset) Total DNA content per cell was also analyzed using 7-AAD and is presented as fold-change in the number of cells showing DNA doubling compared to untreated NSC control. Data are representative of three independent experiments done in triplicate and presented as average ( $\pm$  SEM) cells per well.  $**p \leq 0.01$  vs. untreated NSC media. (C) To evaluate the potency of M2 macrophage supernatants in enhancing NSC proliferation, M2 supernatants (M2C) and M0 media (M0M) were titrated against NSC media (NSC) to assess the dilution at which cells no longer respond to stimulation.

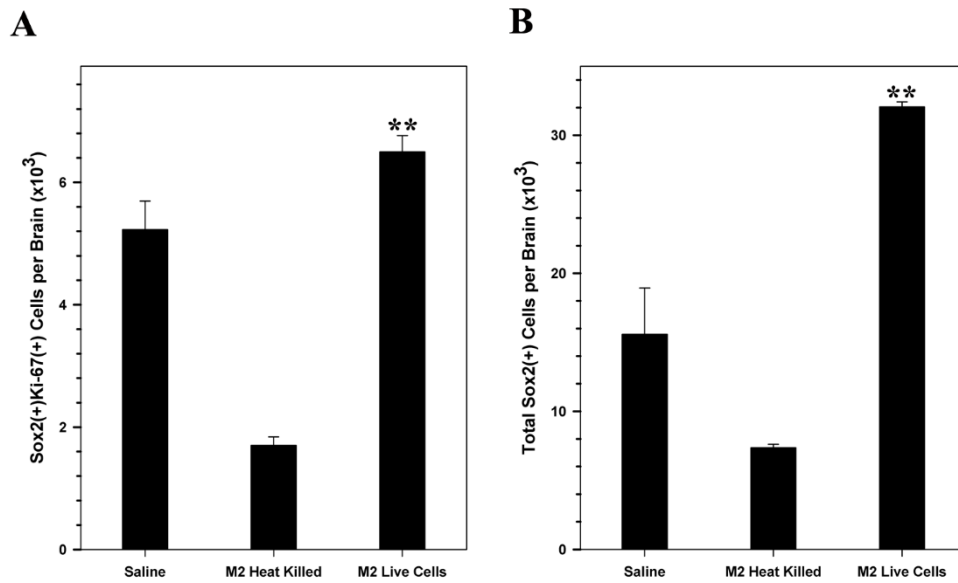
titrated with increasing dilutions of M2 macrophage supernatants, suggesting that soluble mediators with high potency mediate this effect (Fig. 4.4C).

### **M2 polarized macrophages increase NSC proliferation *in vivo*.**

Previous reports have suggested that M2 polarized macrophages promote myogenesis (Arnold, Henry et al. 2007). To test the possibility that M2 macrophages might promote adult neurogenesis, M2 activated BDBMs were transplanted into the lateral ventricles of uninfected BALB/c mice by *icv* injection. Brains collected 5 d later were analyzed by flow cytometry for Sox2, a neural stem cell marker, and Ki-67, a proliferation marker. Quantification of the endogenous brain cell population (CD45(-)) revealed a significant increase in the total number of proliferating NSCs (Sox2(+)Ki-67(+)) cells in recipients receiving live M2 macrophages ( $6,498 \pm 265$ ) when compared to recipients receiving heat-killed ( $1,702 \pm 138$ ) cells or saline-treated sham controls (vs  $5,228 \pm 465$ ; Fig. 4.5A). Correspondingly, the total numbers of Sox2(+) NSCs in the animals receiving live M2 activated cells were also significantly increased ( $32,054 \pm 365$  cells;  $p \leq 0.01$  to saline and heat-killed controls) compared to either the animals receiving saline or heat-killed M2 activated macrophages ( $15,586 \pm 3343$  cells or  $7,366 \pm 251$  cells respectively; Fig 4.5B).

### **Wnt5a mediates M2 macrophage induced NSC proliferation**

To identify the soluble factor(s) mediating M2 macrophage induced NSC proliferation, changes in gene expression of selected soluble factors were evaluated. Initially, common gene pathways associated with both macrophage activation profiles and NSC proliferation were evaluated for candidate gene selection with IPA (Ingenuity



**Figure 4.5: NSC numbers increase following M2 macrophage transplant *in vivo*.** Absolute numbers of (A) proliferating CD45(-)Sox2(+)|Ki-67(+) NSCs and (B) total CD45(-) Sox2(+) NSCs was assessed using AccuCount particles by flow cytometry. A significant increase in the number of proliferating Sox2(+) NSCs was observed. A total of five animals were used per treatment group and data presented are average values ( $\pm$  SEM) per brain. \*\*  $p \leq 0.01$ .

Altered Gene Expression in Polarized Macrophages (RU)

Gene	M0 Macrophages	M1 Macrophages	M2 Macrophages
<i>Wnt5a</i>	135.10±34.08	ND	<b>1156.06±417.35</b>
<i>Wnt7b</i>	9.07±3.19	0.52±0.15	<b>41.31±9.56</b>
<i>Wnt10b</i>	4.72±0.73	ND	1.74±0.24
<i>BDNF</i>	123.65±39.3	132.88±32.05	93.41±5.17
<i>NT-3</i>	117.04±0.35	652.19±60.50	<b>404.98±10.82</b>
<i>CSF1</i>	77.70±3.77	31.91±0.51	70.69±0.98
<i>IL-1<math>\beta</math></i>	0.15±0.01	0.86±0.03	<b>0.70±0.03</b>
<i>IL-6</i>	0.84±0.18	27.23±1.13	1.47±0.22
<i>IL-15</i>	139.72±29.12	767.49±10.64	178.39±10.50
<i>IL-23</i>	13.86±3.39	38.04±8.68	<b>71.62±14.21</b>
<i>Lif</i>	0.09±0.01	6.37±0.40	<b>0.87±0.054</b>

**Bold numbers indicate genes with significantly ( $p \leq 0.05$ ) different expression between M0 and M2 polarized macrophages.**

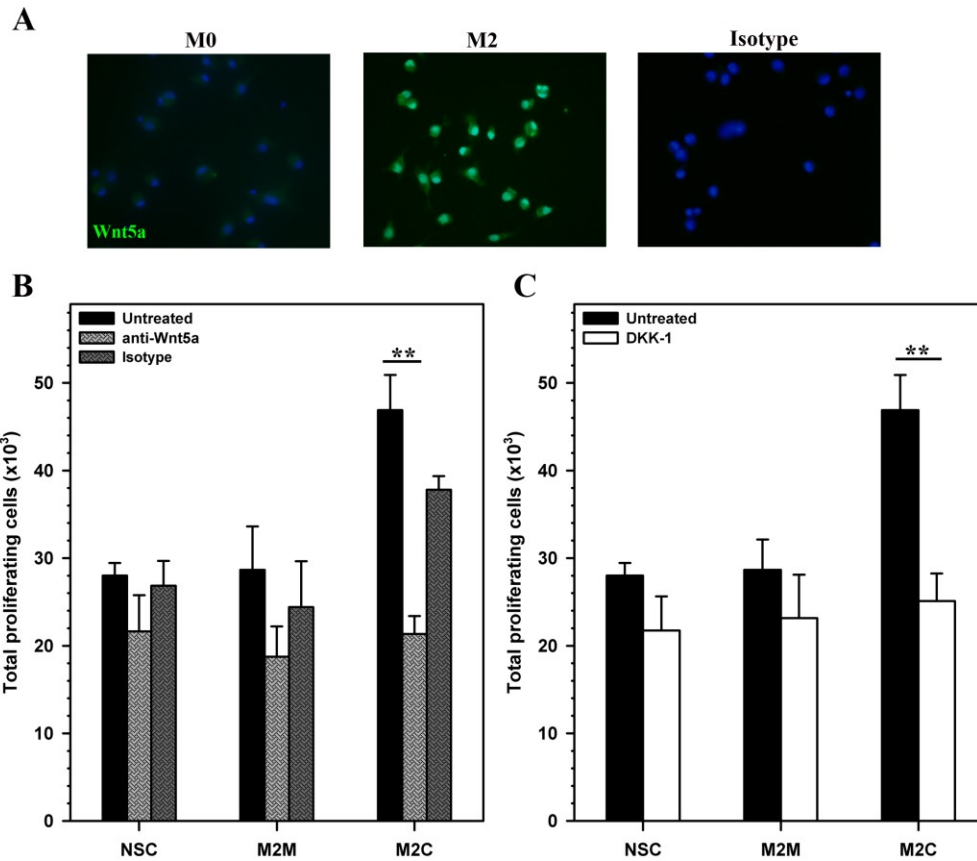
**Table 4.1: Wnt family growth factors are expressed in M2 macrophages.** Total RNA from unpolarized (M0), classically activated (M1), and alternatively activated (M2) macrophages was isolated and amplified by RT-PCR for various growth factors known to affect NSC proliferation. While several factors had significantly increased gene expression in M2 macrophages, *Wnt5a* had the largest increase.

Pathway Analysis, Ingenuity, Redwood City, CA) using data generated by (Martinez, Helming et al. 2013). Selected gene transcripts were then analyzed by quantitative RT-PCR. Data obtained from the PCR analysis of M1 and M2 macrophages are summarized in Table 1. Notably, transcription of *Wnt5a* was significantly increased in M2 polarized macrophages compared to M0 (unpolarized) or M1 macrophages. This finding was confirmed by immunocytochemistry for *Wnt5a* expression in *in vitro* polarized M2 macrophages, showing that M2 macrophages had greater *Wnt5a* specific staining (Fig 4.6A) compared to unpolarized (M0) macrophages. The involvement of *Wnt5a* was confirmed by abrogating the effect of M2 macrophage on NSCs using either a *Wnt5a* neutralizing antibody or dickkopf-1 (DKK), an antagonist of low density lipoprotein receptor-related protein-5 (LRP-5) involved in Wnt signaling. Treatment with *Wnt5a* neutralizing antibody resulted in reduction in levels of NSC proliferation in M2 conditioned media compared to cells grown in control medium or treatment with heat-inactivated goat serum (isotype control; Fig. 4.6B). Similarly, addition of 5 µg/mL DKK resulted in a significant reduction in proliferation in NSCs cultured in M2 macrophage conditioned media (Fig. 4.6C), whereas DKK in either the NSC medium or M2M controls had no significant effect on the numbers of proliferating NSC.

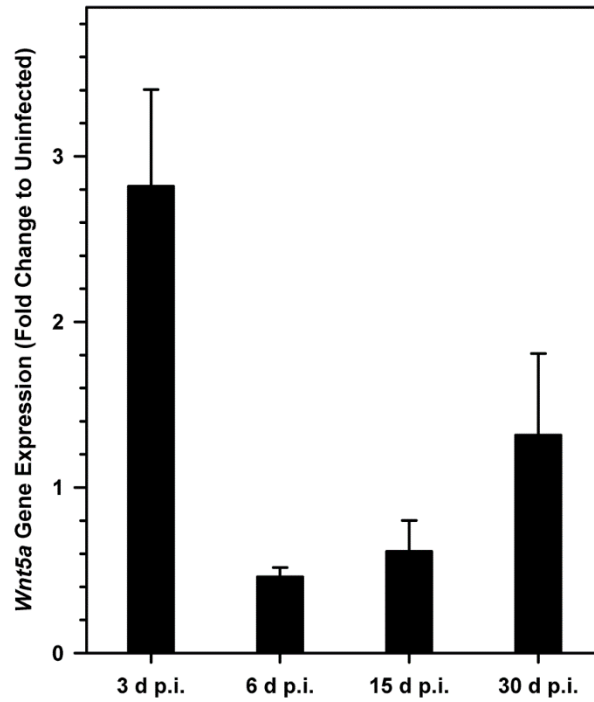
### ***Wnt5a* is up-regulated during acute HSE**

To determine if *Wnt5a* was involved in mediating the increase in NSC proliferation during acute HSE *in vivo*, *Wnt5a* gene expression was measured using RT-PCR from total RNA isolated from whole brain homogenates at various time points post infection. At 3 d p.i. *Wnt5a* gene expression increased by approximately three-fold (Fig.





**Figure 4.6: M2 macrophage effects are dependent on Wnt5a.** Polarized macrophages were assessed for expression of growth factors previously identified to alter NSC proliferation. **(A)** Only M2 macrophages expressed Wnt5a in culture. NSCs were cultured with M2 media control (M2M), M2 polarized macrophage supernatants (M2C), or NSC control media (NSC) as previously described (Figure 4) with or without **(B)** anti-Wnt5a neutralizing antibody (5 $\mu$ g/mL) or **(C)** the Wnt signaling antagonist, dickkopf-1 (DKK-1; 100ng/mL). Data are presented as average ( $\pm$ SEM) per well of 2 independent experiments with triplicate treatment groups. \*\* $p \leq 0.01$ .

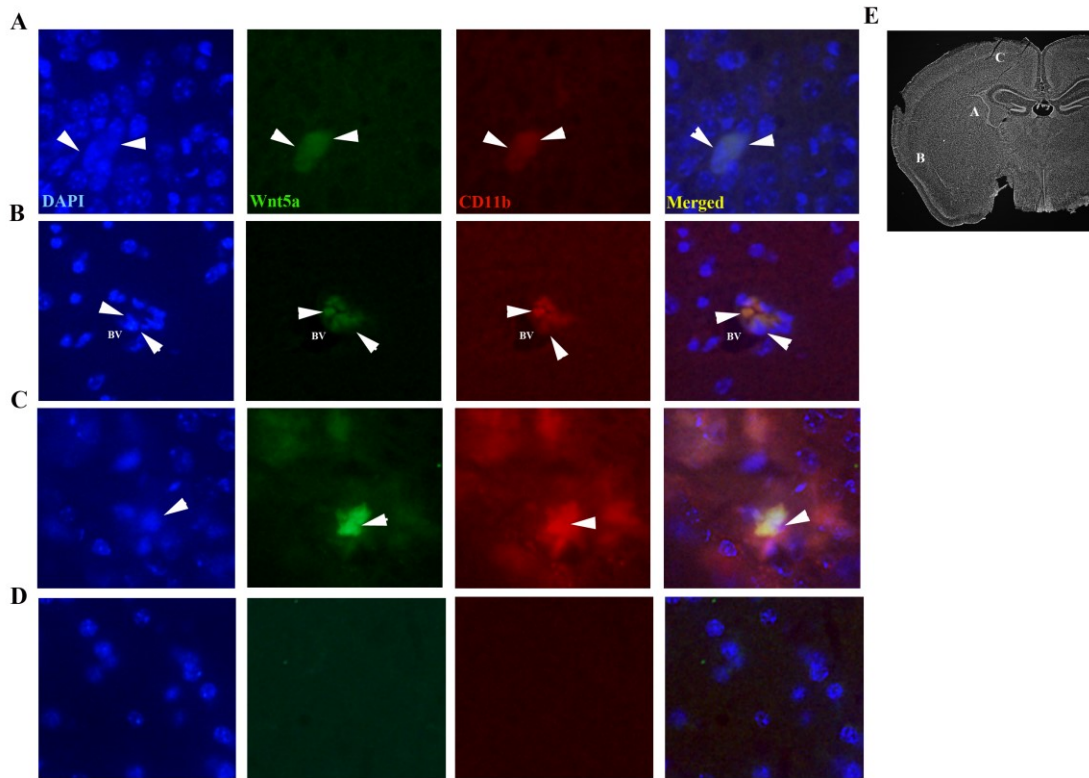


**Figure 4.7: Increased *Wnt5a* gene expression in HSV-1 infected brains at 3 d p.i.** Average fold increases ( $\pm$  SEM) measured in triplicate from RNA obtained from 3-5 animals at each time point are presented. *Wnt5a* gene expression was found to increase acutely by 3-fold at 3 d p.i., which is concurrent with M2 macrophage infiltration and increased endogenous brain cell proliferation.

4.7). The increase in Wnt5a at 3 d p.i is consistent with previous work in our lab showing that endogenous NSC cell proliferation begins as early as 3 d p.i. (30), suggesting the involvement of Wnt5a in HSE induced neurogenesis. Limited Wnt5a protein expression was detected in CD11b(+) macrophages of the brain at 3 d p.i. (data not shown). However by 6 d p.i., Wnt5a production was observed in CD11b(+) macrophages at several brain regions (Fig. 4.8). Notably, Wnt5a(+) macrophages were found in the entorhinal cortex near the lateral ventricles (Fig. 4.8A) as well as in periventricular spaces (Fig. 4.8B) and in cortical regions where HSV-1 induced lesions are observed (Fig. 4.8C). The presence of Wnt5a positive macrophages in the brain suggests that brain macrophages produce Wnt5a in response to HSV-1 infection, which temporally corresponds to the increase in Sox2(+) NSC proliferation observed at 6 d p.i. in previous studies (Rotschafer, Hu et al. 2013).

### **Discussion:**

The present study demonstrates that majority of infiltrating macrophages in HSV-1 infected mouse brains, exhibited an alternatively activated phenotype at 6 d p.i., at a time when NSC proliferation is significantly increased (Rotschafer, Hu et al. 2013). In addition, M2 macrophages seen during the acute phase of HSE expressed high levels of Ly6C (Ly6C<sup>hi</sup> cells) and expressed both CCR2 and CCR7 on their cell membranes, indicating the potential redundancy in chemokine use by these cells for trafficking into the HSV-1 infected brain. *Ex vivo* analysis of revealed that M2 macrophages stimulated both potent and long-lasting proliferation of NSCs, without inducing stem cell



**Figure 4.8: Wnt5a(+)CD11b(+) macrophages are present during acute HSE.** Sections from 6 d p.i. infected mice were stained for Wnt5a and CD11b. Arrowheads denote double positive macrophages were observed in three brain regions (A-C) identified in (E). Isotype antibody stained sections are observed in (D). All images are representative of 5 animals.

differentiation. Furthermore, transplantation of alternatively activated M2 macrophages into the lateral ventricles was sufficient to stimulate NSC proliferation and to double the population size in the adult mouse brain. Analysis of soluble factors expressed by macrophages, known to induce cell proliferation, suggested that select molecules of the Wnt family may be involved in mediating NSC proliferation. Gene expression analysis of M2 polarized macrophages showed a dramatic increase in Wnt5a, indicating this molecule was a likely soluble factor mediating proliferation of NSC. The involvement of activated macrophage induced Wnt5a both *in vivo* and *in vitro* was further confirmed by showing increased expression of Wnt5a at 6 d p.i. in HSV-1 infected brains and loss of proliferative activity in co-cultures of NSC and M2 macrophage supernatants treated with neutralizing antibodies to Wnt5a.

Activated macrophages are known to exhibit distinct functional phenotypes that lie along a spectrum of activity ranging from toxic pro-inflammatory macrophages to healing macrophages essential for tissue regeneration (Brancato and Albina 2011). Simplistically, activated macrophages may be categorized into two distinct phenotypes usually observed at opposing ends of this functional spectrum: the classical, pro-inflammatory M1 phenotype and the alternatively activated, healing M2 phenotype (Hu, Li et al. 2012; Crespo, Bertolotti et al. 2013; Hong, Chung et al. 2014). While the M1 phenotype is a potent stimulator of inflammation, the M2 phenotype has been shown to promote tissue repair. Mechanisms by which M2 macrophage induced tissue repair include the production of growth factor, as seen during skeletal muscle (Arnold, Henry et al. 2007) and brain injury (Hu, Li et al. 2012). In the present study, majority of

infiltrating macrophages responding to experimental HSV-1 infection expressed one of the markers found on M2 polarized macrophages, CD206. The increase in CD206(+) infiltrating macrophages coincided with peak NSC proliferation. This study is the first to demonstrate a role for M2 macrophages in stimulating NSC proliferation during viral encephalitis. M2 polarized macrophages are essential in heart tissue regeneration after ischemia (Aurora, Porrello et al. 2014) and salamander limb regeneration after amputation (Godwin, Pinto et al. 2013). Our findings suggest that M2 macrophages may facilitate tissue repair after brain injury by stimulating adult NSC proliferation. Further experimentation is necessary to establish a role for infiltrating M2 macrophages in reparative processes and associated neurological outcomes during HSE.

NSC proliferation is the first step in promoting resolution of neurological deficits associated with brain damage. Murine models of cerebral ischemia have demonstrated that in the presence of M2 polarized macrophages, proliferation of neuroblasts, a NSC derived cell type, is significantly elevated with improvement in balance and coordination following ischemia in mice (Jin, Cheng et al. 2014). The data presented in this chapter demonstrate that M2 macrophages increase NSC proliferation via a cell contact independent mechanism. Transplantation of M2 polarized macrophages into uninfected mice significantly increased the total number of NSCs 5 days after transplantation. Studies in Japanese encephalitis have shown that suppression of the M1 macrophage activation phenotype using minocycline results in robust increases in NSC proliferation (Das, Dutta et al. 2011). The M2 macrophage activation effects observed in the present study may be preserved in other models of viral encephalitis.

Previous studies using mouse models of HSE have demonstrated that CCR2(+) macrophages are essential to resolve HSV-1 brain infection (Boivin, Menasria et al. 2012). However, in several models of viral encephalitis, infiltrating CCR2(+) macrophages largely possess a pro-inflammatory phenotype. In West Nile virus encephalitis, infiltration of Ly6C(hi)CCR2(+) macrophages results in worsened disease and increased mortality in infected mice (Getts, Terry et al. 2008). On the other hand, the present study demonstrated that at the early time points (5 d p.i.), majority of the CCR2(+) macrophages exhibited an alternatively activated phenotype, which later (at 15 d p.i.) shifted to a predominance of classically activated phenotype in the brain. This finding suggests that while macrophage activation may change, chemotaxis signals that induce migration of different macrophage polarizations may be similar. The use of CCR2 by M2 macrophages as a migratory mechanism was observed in mice infected with schistosomiasis, where it was shown that M2 macrophages were absent in CCR2 knockout mice (Nascimento, Huang et al. 2014). In the present study, total number of infiltrating macrophages present in HSV-1 infected mice brains subsequent to treatment with a CCL2 neutralizing antibody did not change. However, the findings in the present study show that these M2 macrophages also express CCR7, suggesting an alternate mechanism for chemotaxis into the brain.

The mechanism of M2 macrophage supernatant induced increase in NSC proliferation *in vitro* was dependent on Wnt5a, which is a prototypical activator of the non-classical Wnt signaling pathway (Pourreyon, Reilly et al. 2012). Wnt5a is most commonly associated with proliferation of tumor cells; however, Wnt5a also plays an

important role in cell turnover in healthy tissues, such as human periodontal ligament cells (Hasegawa, Wada et al. 2015), epithelial cell wound healing of colonic myofibroblasts (Raymond, Marchbank et al. 2012), and regeneration of colonic crypts after experimental crypt excision (Miyoshi, Ajima et al. 2012). Importantly, the fact that Wnt5a mediated its effects on villus epithelium replacement from progenitor cells in the crypt bed, well beneath the wound, suggests that Wnt5a can induce tissue regeneration from sites distal to the areas of tissue damage (Miyoshi, Ajima et al. 2012). Similarly, this present study identified Wnt5a(+)CD11b(+) cells present in brain lesions of mice at 6 d p.i. that were distant from the sub-ventricular zone of the lateral ventricles.

In conclusion, the present study shows that at peak NSC proliferation, the immune response to HSE is dominated by alternatively activated, M2 macrophages and that these macrophages have the capacity to induce NSC proliferation. *In vitro* these macrophages mediated their effect through Wnt5a and observation of Wnt5a(+)CD11b(+) cells in the brains of mice at 6 d p.i. suggests a possible mechanism by which M2 macrophages induce NSC proliferation *in vivo*. However, further studies are required to characterize M2 macrophages mediating increased NSC proliferation and to further evaluate the potential of Wnt5a to improve neurological deficits in humans. Together these findings suggest that alternatively activated M2 macrophages and the associated Wnt5a production may prove to be a useful point of intervention to stimulate NSC proliferation and subsequent neurogenesis in patients suffering life-altering deficits following viral encephalitis.



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**Chapter 5: Activated CD8(+) T lymphocytes inhibit neural stem/progenitor cell  
proliferation: Role of interferon- $\gamma$ .**

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**Abstract:**

The ability of neural stem/progenitor cells (NSCs) to self-renew, migrate to damaged sites, and differentiate into neurons has renewed interest in using them in therapies for neurodegenerative disorders. Neurological diseases, including viral infections of the brain, are often accompanied by chronic inflammation. The interactions of neuroinflammation and neuroregenerative processes are poorly understood. We have previously shown that chronic neuroinflammation during HSE is dominated by brain-infiltrating activated CD8(+) T cells. In the present study, activated CD8(+) T cells were found to suppress NSC proliferation *in vitro*. Total numbers of proliferating Sox2(+) NSCs were decreased by half in wells cultured with either stimulated CD8(+) T cells or IFN- $\gamma$ . Analysis of these cells for apoptosis revealed that CD8(+) T cell mediated decrease in NSC numbers are not due to apoptosis. Inhibition of NSC proliferation by CD8(+) T cells was found to be dependent on IFN- $\gamma$  as NSCs from IFN- $\gamma$  receptor knockout mice proliferated normally in the presence of either stimulated CD8(+) T cells or IFN- $\gamma$ . Pre-treatment of NSCs with IFN- $\gamma$  resulted in significant reduction of NSC proliferation even in the presence of growth stimuli, shown to produce four to five-fold increase in numbers of cultured NSCs. In addition, activated CD8(+) T cells increased GFAP expression in NSCs suggesting possible induction of an altered differentiation state. Taken together, the studies presented here demonstrate a role for activated CD8(+) T cells in regulating NSC function mediated through the production of IFN- $\gamma$ . This cytokine may influence neuro-restorative processes and ultimately contribute to the long-term sequelae commonly seen following herpes encephalitis.



**Introduction:**

Over the past decade it has become well established that adult mammalian brains possess an inherent ability to regenerate (Gage 2000). This property of neurogenesis is realized by a small population of undifferentiated, self-renewing cells called neural stem/progenitor cells (NSC). The neurogenic cell population is housed predominantly in two well-described, discrete germinal centers (niches) within the brain, the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the hippocampus. These neurogenic niches are composed of multiple cell types (Merkle, Mirzadeh et al. 2007), which when examined at the population level exhibit distinct morphological and functional properties. Importantly, all cell types present in the niche retain the ability to proliferate and differentiate into distinct cell types (Kempermann, Jessberger et al. 2004; Kempermann 2011). Astroglial-like B cells are the top-level stem/precursor population and are capable of symmetrical and asymmetrical division which generates C cells. These intermediate C, or transit-amplifying, cells retain expression of stem/progenitor markers (like Sox2) and begin to express lineage specific markers, like doublecortin (DCX) (Kempermann, Jessberger et al. 2004) before they become A cells or neuroblasts.

Although several models have been proposed to define lineage relationships of different neural stem/progenitor cell types (Ming and Song 2011), it is very evident that the germinal niches provide complex and dynamic microenvironments necessary to sustain the NSC population in multiple stages of differentiation (Alvarez-Buylla and Lim 2004). On the other hand, the niche is influenced by both intrinsic and extrinsic signals that modulate neurogenic processes in the brain (reviewed in Russo, Barlati et al. 2011).

The inflamed brain microenvironment consequent to infection (Das and Basu 2008), ischemic stroke (Arvidsson, Collin et al. 2002), or in neurodegenerative disease states, like Huntington's and Parkinson's disease (Winner, Jappelli et al. 2011), affects the composition and architecture of the SVZ resulting in changes in proliferation, migration and differentiation of NSCs (reviewed in Kaneko and Sawamoto 2009). Neurological injury, associated with concomitant acute and/or chronic inflammation, alters neurogenesis within these NSC niches. Hence, understanding the role and influence of inflammation on these germinal centers in the brain is essential to garner the potential for NSCs to regenerate/repair damaged brains (Gonzalez-Perez, Jauregui-Huerta et al. 2010; Gonzalez-Perez, Quinones-Hinojosa et al. 2010; Kernie and Parent 2010).

T cells have been postulated to play a major role in neurogenesis. Activated T cells influence the recovery process regardless of the type of brain injury (Yirmiya and Goshen 2011; Lynch and Mills 2012). In fact, it has been postulated that CD4(+) T cells may have a physiological role in maintenance of memory and learning. Impairment in CD4(+) T cells, either by genetic manipulation or immunological depletion, results in decreased hippocampal neurogenesis and significant impairment in performance on memory tests (Kipnis, Cohen et al. 2004; Ziv, Ron et al. 2006; Brynskikh, Warren et al. 2008; McGowan, Hope et al. 2011), suggesting that peripheral immune cells and mediators may play a role in maintaining cognitive function under physiological conditions (Yirmiya and Goshen 2011).

Contrary to their physiological role in promoting neurogenesis, T cells in pathological conditions are known to inhibit neurogenesis and altering T cell function is often

associated with favorable outcomes (Wei, Yemisci et al. 2011). Infiltration of myelin-specific T cells during experimental autoimmune encephalitis (EAE) have been associated with dysfunctional alteration in both the composition and the architecture of the SVZ, the NSC niche (Pluchino, Muzio et al. 2008). Similarly, larger infarct volumes resulting from ischemic brain injury have been linked to presence of activated T cells in the brain (Hurn, Subramanian et al. 2007; Shichita, Sugiyama et al. 2009; Kleinschnitz, Schwab et al. 2010), presumably driven by the neurotoxic effects of immune mediators generated by Th1 and Th2 T cells (Gu, Xiong et al. 2012). Interestingly, T cell deficiency also increased neural stem/progenitor proliferation during the acute stages of stroke (Saino, Taguchi et al. 2010). This dichotomous function of activated T cells may be related to distinct cytokine profiles generated during the inflammatory process following brain injury (Whitney, Eidem et al. 2009) and suggests that immune cells play an critical role in both the initiation and progression of neurogenesis.

Inflammation is an essential component of the host response that protects against brain infections. Our laboratory has demonstrated that the inflammatory response to HSV-1 infection clears virus from the brain, but leaves in its wake a chronic activated T (Marques, Cheeran et al. 2008; Mutnal, Hu et al.) and B lymphocyte (Mutnal, Hu et al. 2012) response, potentially serving critical surveillance functions. Persistent cytotoxic CD8(+) T cells produce IFN- $\gamma$ , a cytokine that both our laboratory and others have shown to alter NSC functions (Cheeran, Jiang et al. 2008). While a substantial number of studies have investigated the role of the inflammatory response in defense of and damage to the brain, little if anything is known about the impact of neuroimmune responses on

NSCs in the context of viral encephalitis. In the present study, we utilized a co-culture system to determine the impact of activated T cells on NSC proliferation and began to decipher the molecular cues associated with their effects on NSCs.

## **Materials and Methods:**

### **Ethics Statement**

All experiments in this study using animals were conducted under protocols approved by the University of Minnesota Institutional Animal Care and Use Committee and in concordance with the Guide for the Care and Use of Laboratory Animals.

### **Neural stem/progenitor cell culture:**

Murine NSCs were cultured from the brains of E14.5 BALB/c mice, or IFN- $\gamma$ R1 KO mice (CByJ.129S7(B6)-*Ifngr1<sup>tm1Agt</sup>/J*; Jackson Laboratory, Bar Harbor Maine) as previously described (Ni, Hu et al. 2004; Cheeran, Hu et al. 2005) with few minor modifications. Briefly, timed-pregnant mouse embryos (E14.5) were dissected in Hank's buffer (Sigma, St. Louis, MO). After decapitation and removal of skin, skull, and meninges, cerebral cortices were mechanically triturated in Hank's buffer and dissociated cells were collected and resuspended in serum-free NSC medium (DMEM/F12, 8 mM glucose, glutamine, 20 mM sodium bicarbonate and N2 Plus supplement, R&D Systems, Minneapolis, MN). Cells ( $2 \times 10^5$ ) were then plated as neurospheres in 10-cm diameter dishes or as monolayers on 10-cm diameter dishes, pre-coated with 15  $\mu$ g/ml poly-L-ornithine and 1  $\mu$ g/ml bovine fibronectin. Media for all culture conditions was NSC medium supplemented with bFGF (20 ng/ml; R&D Systems) and EGF (20 ng/ml; R&D Systems). NSC monolayer cultures, maintained at 37°C (5% CO<sub>2</sub>) in NSC growth

medium (with bFGF and EGF), were sub-cultured when 60-70% confluence was attained and re-plated at a density of  $2 \times 10^5$  cells per 10 cm dish or  $2 \times 10^3$  cells per well of 24-well plates. This culture was considered as passage 1. NSC cultures between passages 1-3 were used throughout this study. Near homogeneity (>98% positive) in Sox2 and Nestin (stem cell markers) expression was observed in these cultures, with a proportion ( $41.2 \pm 5.1\%$ ) of the cells expressing doublecortin (neuroblast marker) in addition to Sox2. GFAP expression was not detected in cultured NSCs.

### **T cell isolation and stimulation**

Spleens and lymph nodes were collected from naïve BALB/C mice, raised under SPF conditions, as previously described (Marques, Cheeran et al. 2008). Briefly, single cell suspensions of splenocytes were depleted of red blood cells by treatment with 0.87% ammonium chloride, washed twice, and cell viability was confirmed using Trypan blue. T cell subsets were isolated from RBC free, lymph node cells and splenocytes using a ferrofluid based system (R&D Systems Inc., Minneapolis, MN), according to the manufacturer's instructions. Untouched CD4(+) and CD8(+) T cells were enriched from lymph node and spleen using a negative selection based antibody cocktail that enriches for each T cell population. Enrichment of T cell subtypes were >90% in all experiments.

Enriched CD8(+) T cells were co-cultured with proliferation dye labelled NSCs (methods below) 24 h after seeding the stem cells. Cell ratios of NSC and T cells were calculated based on number of NSC seeded in each well. All experiments were conducted using passage 1 (P1) -P3 NSC cultures, which were determined to have similar doubling rates. T cells were stimulated in co-cultures using a mixture of monoclonal

antibodies to CD3 (5 µg/ml; clone 17A2) and CD28 (5 µg/ml; clone 37.5; BioXCell, West Lebanon, NH). Appropriate controls with and without activating antibodies or T cells were used to compare NSC proliferation in the presence of T cells. T cell conditioned medium was prepared from cell free supernatants of stimulated or unstimulated T cells cultured in NSC growth medium for 72 h. Half of the NSC growth medium was replaced with T cell conditioned medium to investigate the role of soluble factors in T cell-mediated effects on NSCs.

### **NSC Proliferation Assay**

E14.5 embryonic NSCs were labeled with 5nM fixable proliferation dye eFluor450 (eBioscience, San Diego, CA) for 5 minutes at 37°C and quenched with 5mL one to one ratio of complete NSC media (DMEM/F12 with N2 supplement) and 7.5% bovine serum albumin.  $1 \times 10^5$  labeled cells were cultured in NSC media or in the presence of 20 pg/mL interferon-gamma (IFN- $\gamma$ ),  $5 \times 10^5$  CD8(+) T cells either left unstimulated, or stimulated overnight (with 10 µg/mL each of anti-CD3 and anti-CD28) prior to co-culture for 72 hours. Cells were immunostained for CD45 (PerCP-Cy5.5; eBioscience) and Sox2 (eFluor 670; eBioscience), and assayed on a BD FACSCanto for cell numbers with AccuCount blank particles for absolute quantification. Quantification of cell proliferation was performed by measuring dye dilution in the eFluor450 labeled cells, per the manufacturer's instructions. Cell proliferation analysis was done using FlowJo software (Treestar Inc. Ashland OR)

### **Analysis of NSC by Flow Cytometry**

NSCs were stained for nestin expression by fixing cells in 4% paraformaldehyde (in PBS) for 20 min and incubating in SAP buffer (2% FCS, 0.5% saponin, and 0.1% sodium azide in PBS) containing anti-rat nestin-PE monoclonal antibody or IgG-PE isotype antibody (R&D Systems, MN). Following incubation for 30 min at room temperature the cells were washed once with SAP buffer, once with PBS, resuspended in 400  $\mu$ l of PBS and analyzed on a FACS Canto flow cytometer (Becton-Dickinson, Mountain View, CA). At least ten thousand events were analyzed using FlowJo software (Tree Star Inc. Ashland OR). Doublets were eliminated from the analysis of live cell gated events using data obtained on their scatter height and width geometry.

NSCs were immunostained by fixing and permeabilizing cells (BD Cytotfix/Cytoperm kit (Becton-Dickinson) prior to staining with anti-Human/Mouse Sox2-eFluor<sup>®</sup>660 (Clone-Btjce; eBioscience, San Diego, CA) and rabbit anti-mouse Ki-67 (Abcam, Cambridge, MA). To visualize Ki67 staining, a PE labeled goat anti-rabbit IgG (Becton-Dickinson) secondary antibody was used. Antibodies were incubated at 4°C and 50  $\mu$ L blank AccuCount particles (Spherotech, Lake Forest, IL) were added immediately prior to data acquisition for cell quantification, per manufacturer's directions. A minimum of 30,000 events was collected on a FACS Canto flow cytometer (BD, Mountain View, CA).

#### **Cytotoxicity analysis in NSC-T cell co-cultures.**

To identify NSC apoptosis in co-cultured cells, the monolayer was removed from the plate gently using Ca<sup>++</sup>/Mg<sup>++</sup> free Hank's balanced solution for dissociation. T cells were identified by staining with APC-conjugated anti-CD45 antibody (BD Biosciences)

in FACS blocking buffer (1  $\mu$ g Fc block, 2% normal mouse and rat serum). The cell suspension was washed twice with cold PBS and re-suspended in 100  $\mu$ L of Annexin V binding buffer with FITC-conjugated Annexin V (Invitrogen, Carlsbad, CA). Cells were then stained with propidium iodide to identify dead cells and read on a FACS Canto within 1 hour of the assay. Data were analyzed using FlowJo software.

### **Immunocytochemistry for stem cell differentiation.**

Murine NSCs were cultured in 24-well tissue culture plates alone, with unstimulated CD8(+) T cells, or with pan-stimulated CD8(+) T cells as described in the T cell Isolation and Stimulation methods section. Cells were cultured for 72 h. NSCs were fixed with 2% paraformaldehyde and permeabilized in PBS with 0.3% Triton-X 100 (Sigma-Aldrich, St. Louis, MO). Cells were then blocked in PBS with 5% bovine serum albumin and 5% goat serum. Cells were incubated with primary GFAP (Dako Laboratories, Carpinteria, CA),  $\beta$ -III tubulin (R&D Systems, Minneapolis, MN), or O4 (R&D Systems) antibodies. Secondary antibodies were as follows: goat anti-rabbit AlexaFluor 488 for GFAP, and biotinylated anti-mouse Ig for  $\beta$ -III tubulin and O4 (Life Technologies, Grand Island, NY). DAPI was used as a counterstain. Cells were imaged on an Olympus IX70 inverted fluorescence microscope.

### **Results:**

#### **Activated CD8(+) T cells inhibit neural stem cell proliferation**

To determine if activated T cells altered NSC functions, stem cells isolated from the cortices of BALB/c embryos (E14.5) were co-cultured with stimulated or unstimulated CD8 T cells. Previous experiments demonstrated that peak proliferation was

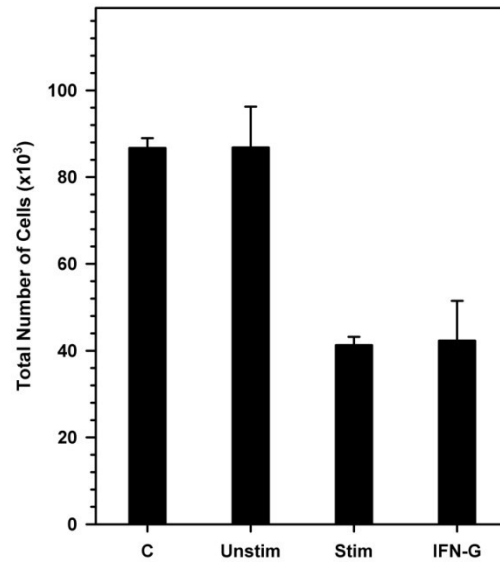


observed 48-72 h post seeding. CD8(+) T cells were activated by crosslinking CD3 and CD28 with monoclonal antibodies (Mab) and seeded at a 5 to 1 ratio CD8(+) T cells to NSC. At 72hrs post co-culture proliferation of NSC cultured with stimulated CD8(+) T cells was reduced two-fold compared to NSCs cultured without any CD8(+) T cells (41297.4±1918.6 cells per well vs 86712.5±2253.4 cells per well,  $p < 0.01$ ; Fig. 5.1). This reduction in NSC proliferation was consistent with the reduction observed in NSCs cultured in the presence of 10pg/mL IFN- $\gamma$  (42324.5±9147.4 cells per well). Unstimulated CD8(+) T cells had no effect on NSC proliferation (86845.8±9391.1 cells per well).

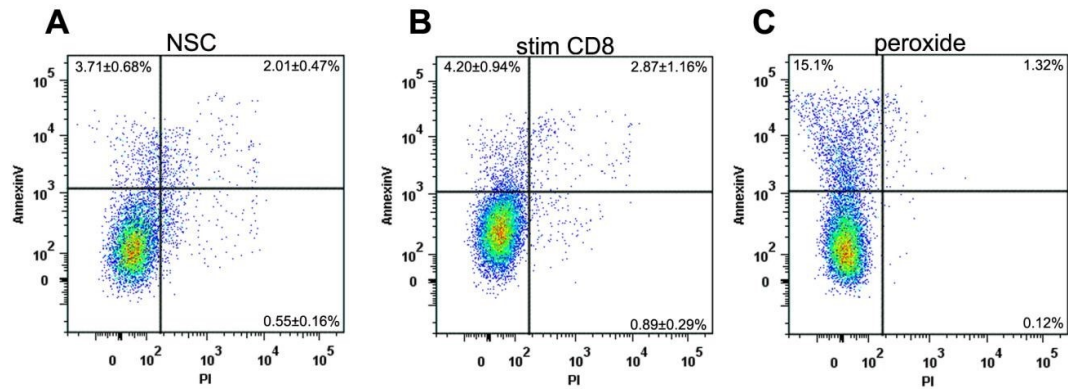
To exclude the possibility that CD8(+) T cell inhibition of NSC growth was mediated by cytotoxicity, NSCs co-cultured with activated and unstimulated CD8(+) T cells were assessed for Annexin V staining, along with changes in cell permeability to propidium iodide (PI), to evaluate cell death. CD45(+) staining was used to separate T cells and NSC for flow cytometry analysis. No differences in Annexin V binding or PI incorporation were observed in NSCs after 72 h co-culture (Fig 5.2A). On average, AnnexinV staining was seen in 3-5% of NSC cultured with or without T cells compared to 15% AnnexinV binding to NSC treated with hydrogen peroxide for 3 h prior to analysis (Fig 5.2B&C).

#### **Activated CD8(+) T cell effects are mediated through IFN- $\gamma$ signaling.**

The IFN- $\gamma$  receptor is composed of two chains, the alpha (R1 subunit) and beta chains (R2 subunit; Bach, Aguet et al. 1997). Previous studies have shown that murine



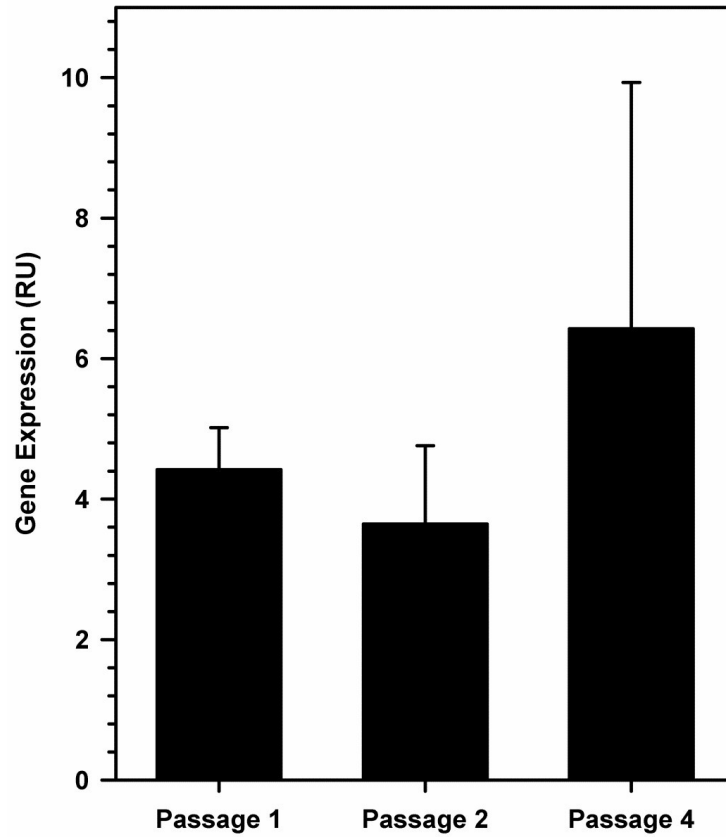
**Figure 5.1: CD8(+) T cells inhibit NSC proliferation.** BALB/c NSCs obtained from E14.5 d mice were co-cultured with stimulated or unstimulated CD8(+) T cells 24 h after labelling with proliferation dye. T cells in the co-cultures were either left unstimulated or were stimulated with a mixture of anti-CD3 and anti-CD28 antibodies (5 $\mu$ g/ml). NSCs treated with IFN- $\gamma$  (10pg/mL) were used as positive controls. After 72 h of stimulation, cells were immunostained for Sox2 and read on a flow cytometer. Data presented are pooled from 3 separate experiments with each treatment performed in triplicate (mean  $\pm$  SEM).



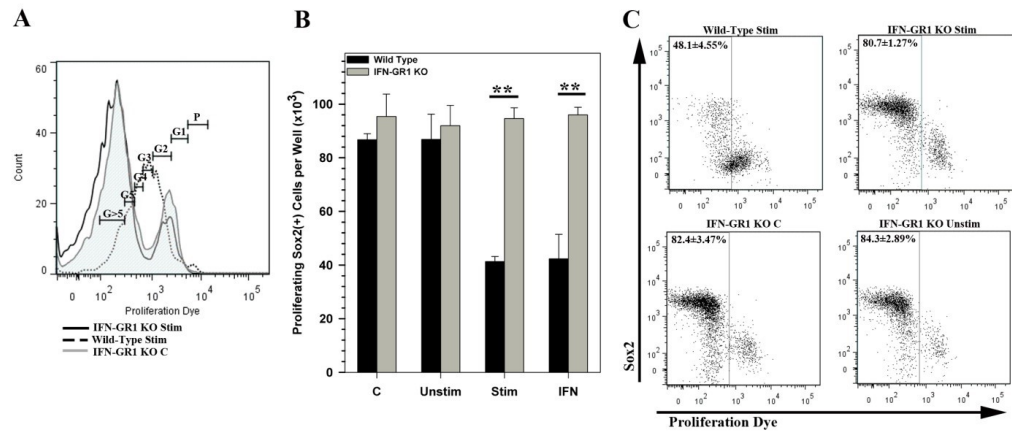
**Figure 5.2: Stimulated CD8(+) T cells do not induce apoptosis.** Using the same culture groups as in Figure 1, cells were analyzed for increased apoptosis and cell death by flow cytometry. Representative dot plots showing Annexin-V binding and propidium iodide uptake by (A) untreated NSCs (NSC), (B) NSCs cultured with antibody-stimulated CD8(+) T cells (stim CD8), and (C) NSCs treated with hydrogen peroxide for 1 hour (peroxide).

NSCs express functional IFN- $\gamma$  receptors on the cell membrane (Walter, Honsek et al. 2011). We verified using a quantitative RT-PCR that IFN- $\gamma$ R1 was expressed in NSC from all passages of culture used in this study (Fig 5.3). To further confirm that activated CD8(+) T cells mediated their effects on NSCs through the IFN- $\gamma$  receptor, NSC cultures derived from IFN- $\gamma$  R1 KO mice were assessed for both proliferation and expression of Sox2 in the presence or absence of stimulated CD8(+) T cells. In a dye dilution proliferation assay, eFluor670 labeled NSCs showed a graded decrease in the fluorescence intensity, with cells reaching up to >5 divisions in 72 h under proliferative culture conditions (Fig 5.4A; untreated). The majority of the wild-type NSCs co-cultured with stimulated CD8(+) T cells showed an arrest in proliferation at 2 to 4 cell divisions. However, this arrest in proliferation was not observed in IFN- $\gamma$ R1 KO NSCs, which proliferated to the same extent as untreated cells (Fig 5.4A). Wild-type and IFN $\gamma$ R1KO cells that were untreated or treated with unstimulated CD8(+) T cells showed similar patterns of proliferation in that majority of the cells (~80%) had undergone >5 cell divisions.

When co-labeled for Sox2 expression, similar results were obtained demonstrating the loss of the inhibitory effect of activated CD8(+) T cells on IFN- $\gamma$  R1 KO NSC proliferation (Fig 5.4B). Total numbers of proliferating NSCs cultured with stimulated CD8(+) T cells were significantly increased in IFN- $\gamma$  R1 KO NSC (94648.0 $\pm$ 3970.3 cells per well) compared to wild-type BALB/c NSCs (41297.4 $\pm$ 1918.6



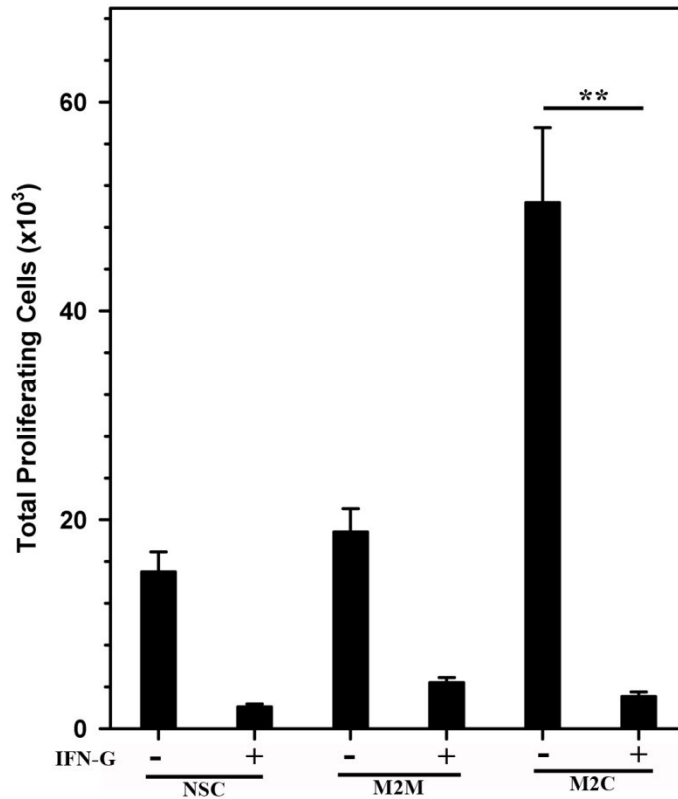
**Figure 5.3: IFN- $\gamma$  receptor expression is consistent between passages 1 and 3.** NSCs were cultured in the presence of IFN- $\gamma$  for one hour prior to lysing and isolation of whole cell RNA. 2 $\mu$ g of total RNA were used for cDNA synthesis and PCR was performed for expression of the IFN- $\gamma$  receptor subunit 1. No significant differences were observed between the passages.



**Figure 5.4: IFN- $\gamma$ R1 knockout NSCs are resistant to CD8(+) T cell mediated inhibition of proliferation.** NSCs were labeled with eFluor670 proliferation dye and were cultured alone (untreated), with 20pg/mL IFN- $\gamma$  (IFN) or in the presence of unstimulated (Unstim) or stimulated CD8(+) T cells (Stim). **(A)** Representative flow histograms of stimulated CD8(+) T cell treated and untreated NSCs demonstrating dilution of labeling dye fluorescence corresponding to cell divisions. Gates for generation of progeny was allocated based on progressive halving of the fluorescence measured in dividing NSCs (calculated using the cell proliferation algorithm in the FlowJo software). Proliferation of IFN- $\gamma$ R1 KO NSCs when co-cultured with stimulated CD8(+) T cells (black line) compared to untreated NSCs (untreated, gray tinted line), or wild-type NSCs cultured with stimulated CD8(+) T cells (dotted line) is shown. **(B)** Quantification of total number of proliferating Sox2(+) NSCs in shown under each culture condition for wild type and IFN- $\gamma$ R1 KO NSCs. Data are presented as an average of three replicates  $\pm$  SEM. \*\*  $p < 0.01$ . **(C)** Representative dot plots showing Sox2 immunolabeling vs dilution of proliferation dye fluorescence observed in wild type (upper left) and IFN-GR1 KO NSCs (upper right) co-cultured with stimulated CD8(+) T cells. Untreated NSC cultures (lower left) and those co-cultured with unstimulated CD8(+) T cells (lower right) show no difference in proliferation or Sox2 expression. Gates are drawn to indicate cells that have undergone five to seven rounds of division (or proliferation dye dilution) post culture and the percentages of proliferating cells are presented as average  $\pm$  SEM.

cells per well,  $p < 0.01$ ) (Fig. 5.4B). Furthermore, there was no significant difference between any of the treatment groups in the IFN- $\gamma$  R1 KO NSCs while the wild-type cells had significantly decreased NSC proliferation with both stimulated CD8(+) T cells and IFN- $\gamma$ . Interestingly, arrested proliferation occurred in cells with decreased Sox2 expression suggesting that Sox2 expression in NSCs may directly correlate with its proliferative ability.

Previous studies in the laboratory demonstrated that co-culture of NSCs with alternatively activated macrophage supernatants result in four to five-fold increases in total numbers of NSCs per well. To evaluate the extent of IFN- $\gamma$  mediated inhibition of NSC proliferation, we pretreated proliferation dye labelled NSCs with 20pg/mL IFN- $\gamma$  for six hours prior to applying alternatively activated (M2) macrophage culture supernatants (M2C; Fig. 5.5). While NSCs not treated with IFN- $\gamma$  increased proliferation in response to M2 polarized macrophage supernatants ( $50378.1 \pm 7175.3$  cells per well vs  $15014.93 \pm 1917.2$  cells per well untreated,  $p < 0.01$ ), proliferation of IFN- $\gamma$  treated NSCs was not significantly different from controls ( $3080.9 \pm 434.1$  cells per well M2C with IFN- $\gamma$  vs  $2090.1 \pm 296.5$  cells per well NSC with IFN- $\gamma$ ). Furthermore, there was no significant difference in the total number of proliferating cells per well in any of the IFN- $\gamma$  treated groups, suggesting that pre-treatment with IFN- $\gamma$  makes NSCs refractory to the growth promoting effects of M2 macrophages.



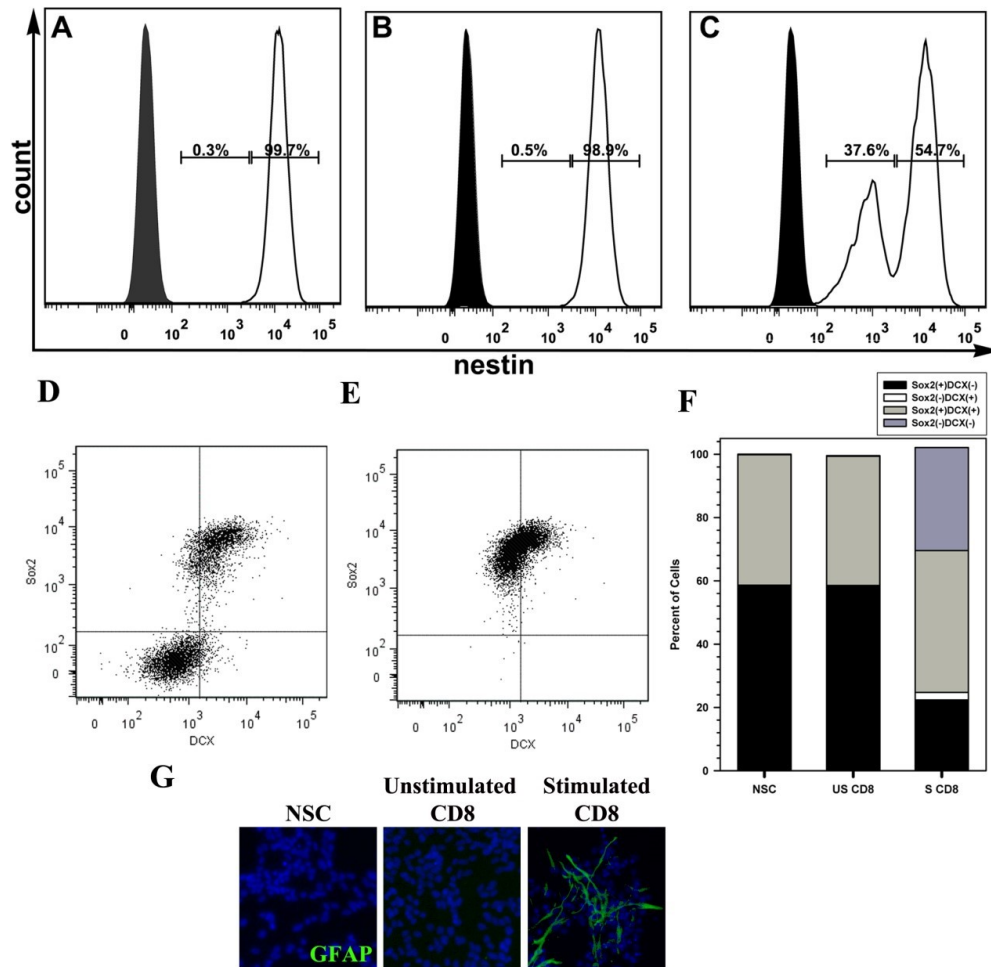
**Figure 5.5: Pre-treatment with IFN- $\gamma$  renders NSCs non-responsive to M2 induced proliferation.** NSCs were labeled with a cell membrane bound proliferation dye and cultured with 20pg/mL IFN- $\gamma$  for six hours prior to addition of conditioned or control (M2M or NSC alone) media at a 1:1 ratio with NSC culture media, for 60 hours. Absolute cell counts were quantified using AccuCount particles added to each sample. Notably, IFN- $\gamma$  pre-treatment resulted in a significant reduction in proliferation, without change consequent to treatment with M2C. Data presented are averages  $\pm$ SEM of 3 independent experiments performed in triplicate. \*\*  $p \leq 0.01$ .



### **Stimulated CD8(+) T cells inhibit nestin and SOX2 expression in NSC**

Finally, to determine if activated CD8(+) T cells altered NSC phenotypes, expression of the stem cell and progenitor cell markers, nestin, Sox2 and doublecortin (DCX), were evaluated by flow cytometry. All NSC cultures used during these experiments had >98% of cells that expressed nestin and Sox2. Upon co-culture with activated CD8(+) T cells, nestin expression on NSCs decreased in magnitude. Nestin expression was high in all untreated NSCs (Fig 5.6A), and its expression was not altered following culture with unstimulated CD8(+) T cells (Fig. 5.6B). In contrast, CD8(+) T cell-treated cells expressed both high and intermediate levels of nestin (Fig. 5.6C). Although total number of NSCs expressing nestin did not alter significantly, approximately 38% of the cells showed lower fluorescence intensities of nestin immunoreactivity and ~55% continued to express the protein at high levels.

Similarly, about 50% decrease in Sox2(+) expression was observed when NSCs were co-cultured with stimulated CD8(+) T cells (Fig. 5.6D). NSC cultures that were left untreated or co-cultured with unstimulated CD8(+) T cells showed two distinct CD45(-) cell populations when immunostained for both Sox2 and DCX; the Sox2(+)DCX(-) NSCs and Sox2(+)DCX(+) neuroblasts (Fig. 5.6E & F). CD45 immunostaining was used to exclude T cells from the analysis. When co-cultured with activated CD8(+) T cells, Sox2 expression decreased in the Sox2(+)DCX(-) population but the proportion of Sox2(+)DCX(+) cells remained unaltered, further indicating that CD8(+) T cells may induce differentiation in a distinct subset of stem cells. To further validate that NSCs enter an altered differentiation state in response to activated CD8(+) T cells, co-cultures



**Figure 5.6: Stimulated CD8(+) T cells initiate NSC differentiation.** Co-cultures of NSC and T cells were assessed for nestin (a stem cell marker) expression by flow cytometry. NSCs were analyzed on a live cell gate after exclusion of CD45(+) lymphocytes. **(A)** Histogram showing nestin immunoreactivity in untreated NSCs (open), compared to isotype antibody staining (shaded). Nestin expression in NSCs was measured 72h after co-culture with **(B)** un-stimulated CD8(+) T cells and **(C)** with activated CD8(+) T cells. The amplitude of nestin expression and proportion of nestin-expressing cells decreased when NSC were co-cultured with activated CD8(+) T cells. Representative data from 2 separate experiments are presented. **(D)** Representative dot plots showing expression of Sox2 and doublecortin (DCX) on untreated NSCs or **(E)** NSCs cultured with antibody-stimulated CD8(+) T cells. **(F)** Stacked bars demonstrating changes in ratios of NSCs phenotypes, defined by Sox2 and doublecortin (DCX) expression, in untreated (NSC) cultures, NSCs cultured with unstimulated CD8(+) T cells (US CD8), and NSCs cultured with antibody-stimulated CD8(+) T cells (S CD8). **(G)** Immunohistochemistry of cultured NSCs showing differentiation into GFAP(+) cells when treated with stimulated CD8(+) T cell (stim). Untreated (NSC) NSCs and those co-cultured with unstimulated CD8(+) T cells show no GFAP immunostaining (green fluorescence). Nuclei were counterstained with DAPI (blue).

were stained for Tuj1, O4, and GFAP to assess NSC differentiation towards neurons, oligodendrocytes, and astrocyte-like cells, respectively. Untreated NSC cultures did not express GFAP or O4 but showed very limited numbers of cells with Tuj-1 immunostaining (<1% of cells). However, a large proportion of cells expressed GFAP when co-cultured with stimulated CD8(+) T cells compared to untreated (NSC) cultures or co-cultures with unstimulated CD8(+) T cells (Fig. 5.6G). There were no observed differences in the limited Tuj-1 immunostaining in NSC cultures after 3 days in co-culture with activated CD8(+) T cells. O4 immunostaining was not detected in CD8(+) T cell treated NSC cultures.

In contrast NSCs co-cultured with stimulated CD4(+) T cells underwent minimal differentiation with approximately 10% of cells losing their Sox2 expression (Fig. 5.7). As with the unstimulated CD8(+) T cells, unstimulated CD4(+) T cells had no effect on NSC differentiation. Additional studies done in the lab using NSC constitutively expressing luciferase demonstrated that there was no significant difference in the total number of cells between control and stimulated CD4(+) T cell-treated groups. The differential effects of CD4(+) and CD8(+) T cell populations on NSC proliferation may perhaps be due to the robust expression of IFN- $\gamma$  in CD8(+) T cells (data not shown). In contrast to CD8(+) T cells, CD4(+) T cells stimulated with antibodies to CD3 and CD28 had no measureable production of IFN- $\gamma$ . Addition of an IFN- $\gamma$  neutralizing antibody to stimulated CD8(+) T cell-NSC co-cultures restored NSC proliferation to control levels.

**Discussion:**

Data presented in this study demonstrate that activated CD8(+) T cells inhibit NSC proliferation. Proliferation of NSCs was reduced by approximately 50% in the presence of stimulated CD8(+) T cells. This decrease in cell number was not associated with an increase in cytotoxicity, a well-known CD8(+) T cell effector function. On the contrary, the inhibition of NSC proliferation was mediated via soluble factors produced by activated CD8(+) cells, effects of which could be abolished by eliminating IFN- $\gamma$  signaling in the co-culture system. These effects of IFN- $\gamma$  were robust with IFN- $\gamma$  pre-treatment rendering NSCs resistant to proliferation despite exposure to the potent stimulus of alternatively activated macrophage supernatants. Furthermore, co-culture with activated CD8(+) T cells drives NSCs into a differentiation pathway, an effect that has been previously attributed to IFN- $\gamma$  (Kim, Son et al. 2007; Lum, Croze et al. 2009). Co-culture of NSCs with stimulated CD4(+) T cells did not have any effect on NSC proliferation and had minimal effects on NSC differentiation.

Brain injury resulting from either infectious (Marques, Cheeran et al. 2008; Mutnal, Hu et al. 2011) or non-infectious insults (Chaitanya, Schwaninger et al. 2010; Kleinschnitz, Schwab et al. 2010), has been shown to result in CD8(+) T cell recruitment to sites of brain damage. Our laboratory has previously demonstrated that in response to herpesvirus infections, activated CD8(+) T cells accumulate and are retained in the brain long after the infection has been resolved (Marques, Cheeran et al. 2008; Mutnal, Hu et al. 2011). Retention and maintenance of activated CD8(+) T cells in damaged CNS tissue is not unique to infectious etiology, but is observed in models of ischemic stroke

(Sekeljic, Bataveljic et al. 2012) and aseptic cerebral injury (Ling, Sandor et al. 2003). Notably, this accumulation of CD8(+) T cells occurs without apparent replenishment from peripheral lymphoid tissues and is mediated through both antigen-dependent and -independent mechanisms (Ling, Sandor et al. 2006). Several studies have demonstrated that CD8(+) T cells contribute to tissue damage, largely mediated by their cytotoxic effector functions (Chaitanya, Schwaninger et al. 2010; Kleinschnitz, Schwab et al. 2010). We demonstrate that antigen-independent activation of CD8(+) T cells robustly inhibits NSC proliferation, without associated cytotoxicity. Recently, we reported that endogenous NSC proliferation is markedly reduced during chronic herpes encephalitis (Rotschafer, Hu et al. 2013). This decrease in NSC proliferation coincides with the accumulation of activated CD8(+) T cells are the present in the brain (Marques, Cheeran et al. 2008; Armien, Hu et al. 2010). Results from the present study suggest that activated CD8(+) T cells in the brain could foster an inflammatory milieu that inhibits neurogenesis, mediated in a large part by IFN- $\gamma$ .

Several studies have shown that IFN- $\gamma$  alters NSC properties; including inhibition of cell proliferation (Cheeran, Jiang et al. 2008; Walter, Honsek et al. 2011), and driving differentiation into neurons or oligodendrocytes (Wong, Goldshmit et al. 2004; Ricci-Vitiani, Casalbore et al. 2006; Kim, Son et al. 2007; Wang, Imitola et al. 2008; Lum, Croze et al. 2009). These effects are initiated by IFN- $\gamma$  binding to its cognate receptor, which is widely expressed on NSCs and its derivative cells (Walter, Honsek et al. 2011). Data presented here demonstrate that by blocking IFN- $\gamma$ R1, using IFN- $\gamma$  R1 KO NSCs, effectively abrogates the inhibitory effects produced by activated CD8(+) T cells,

indicating that these effects are likely mediated through the IFN- $\gamma$  receptor. The downstream signaling pathways initiated by IFN- $\gamma$  binding that drives these changes in NSCs are poorly understood. NSCs treated with IFN- $\gamma$  show increased Stat 1 phosphorylation (Lum, Croze et al. 2009), which in turn induces the expression of sonic hedgehog (Shh), a major brain morphogen (Wang, Imitola et al. 2008; Lum, Croze et al. 2009; Li, Walker et al. 2010). These downstream signaling events are speculated to be involved in both dysregulation of NSC proliferation (Wang, Imitola et al. 2008; Li, Walker et al. 2010) and the generation of a unique neuronal phenotype with irregular electrophysiological properties (Lum, Croze et al. 2009). Data presented in our current study show that co-culture with activated CD8(+) T cells down regulates nestin and Sox2 expression in NSCs, suggesting that activated CD8(+) T cells may initiate a differentiation program in cultured NSCs through the IFN- $\gamma$  signaling pathway. However, decreased Sox2 expression in NSCs is directly correlated with decreased proliferation, a phenomenon that is abrogated in cells that lack IFN- $\gamma$ R1 expression. This direct relationship between Sox2 expression with cell proliferation and differentiation has been shown in several cell types, including NSCs (Tompkins 2011; Julian LM, 2013; Cimadamore, 2013; Thiel G, 2013). In addition, our results demonstrate that activated CD8(+) T cells may direct differentiation of NSC, driving them towards an astrocyte-like phenotype. Similar studies have shown that when induced to differentiate, IFN- $\gamma$  drives NSC into a dysfunctional neuronal phenotype that express GFAP potentially skewing the reparative processes in response to brain injury (Walter, Honsek et al. 2011).

Activated CD8(+) T cells affect NSC function through different mechanisms. In the present study, we have demonstrated that antigen independent activation of CD8(+) T cells suppresses NSC proliferation through an IFN- $\gamma$  mediated mechanism. Wang et al (2010) report that soluble mediators like Granzyme B (GrB), produced by human CD8(+) T cells activated by crosslinking CD3/CD28, inhibit NSC proliferation and drive differentiation of cells to an astrocyte phenotype (Wang, Lee et al. 2010). This inhibitory effect of GrB is mediated through the induction of a voltage-dependent potassium channel, Kv1.3, on NSC. GrB signaling in NSC is sensitive to Pertussis toxin, indicating that it may be mediated through a G protein-coupled receptor (Wang, Lee et al. 2010). It is noteworthy that the effects of T cells on NSC function are dependent on both the inflammatory context and developmental stage at which these cells interact (reviewed in Kokaia, Martino et al. 2012). In fact, IFN- $\gamma$  treatment of human embryonic stem cell derived neural progenitors fosters different differentiation profiles compared to murine NSCs (Walter and Dihne 2012). Similarly, T cell derived IFN- $\gamma$  production during acute HSV-1 brain infection is significantly higher than that expressed during the chronic phase (Armien, Hu et al. 2010). Studies on the effect of various concentrations of IFN- $\gamma$  have indicated that higher concentrations of IFN- $\gamma$  may activate caspases and potentially induce NSC apoptosis (Walter and Honsek 2011). Previous studies in our lab have shown that at low concentration, IFN- $\gamma$  can inhibit NSC proliferation without inducing apoptosis (Cheeran, Jiang et al. 2008). In the present study, we show that at the ratios of T cells tested, NSC proliferation is suppressed without the overt induction of apoptosis. In several brain diseases (like Multiple Sclerosis, and ischemic stroke) among humans, IFN-

$\gamma$  is present at relatively high concentrations (~1000 pg/ml) in the cerebrospinal fluid (Walter and Döhne) and understanding the interactions between soluble mediators produced by T cells and is critical to developing therapeutic approaches, particularly when using stem cells.

In summary, the impact of CD8(+) T cells on reparative processes in the inflamed brain is both complex and poorly understood. Our study demonstrates the antigen-independent effects of activated CD8(+) T cells on NSCs is mediated by IFN- $\gamma$ , through its effect on the IFN $\gamma$ R1 on NSCs. Antigen-dependent effects of CD8(+) T cells could be facilitated by IFN- $\gamma$ -induced increases in MHC-1 expression on NSCs (Cheeran, Jiang et al. 2008; Johansson, Price et al. 2008) and may be unique to the antigens involved in the insult. Numerous immune mediators have been shown to modulate the reparative processes initiated at the NSC niches (reviewed in Gonzalez-Perez, Gutierrez-Fernandez et al. 2012), suggesting that understanding the inflammatory context is critical for evaluating the impact of immune cells on neurogenesis. The data presented in this study elucidate one of many mechanisms that may be involved in immune modulation of adult neurogenesis.

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**Chapter 6: Persistent CD8(+) T cells impair adult neurogenesis during chronic  
Herpes Simplex encephalitis via an interferon- $\gamma$ -mediated mechanism.**



**Abstract:**

CD8(+) T cells are the dominant immune cell type present in the inflammatory milieu following experimental Herpes Simplex encephalitis (HSE). At 15 d p.i., 49.4±2.76% of CD8(+) T cells isolated from HSV-1-infected mouse brains produced interferon-gamma (IFN- $\gamma$ ) in response to viral antigen. The objective of the present study was to evaluate the role of CD8(+) T cells in inhibiting NSC proliferation during HSE at 15 d p.i..

Depletion of CD8(+) T cells from the HSV-1 infected brain resulted in a 5-fold increase in CD45(-)nestin(+) NSCs, compared to isotype antibody-treated controls. CD4(+) T cell numbers and activation phenotype did not change significantly following CD8(+) T cell depletion. Interestingly, in the absence of CD8(+) T cells, total numbers of CD45(hi)CD11b(+) macrophages increased concurrent with a shift in activation phenotypes from 1:2 CD206 to CD86 to 4:1. Macrophages have been shown to preferentially polarize to a classical CD86(+) phenotype in the presence of IFN- $\gamma$ . As expected, gene transcription analysis demonstrated a six-fold reduction in IFN- $\gamma$  expression in CD8(+) depleted animals, compared to isotype-treated mice. To assess the contribution of IFN- $\gamma$  signaling on the observed changes in NSC proliferation and macrophage polarization, IFN- $\gamma$  receptor knockout (IFN- $\gamma$ R1KO) mice were infected. At 15 d p.i., total numbers of Sox2(+) NSCs were significantly increased in infected IFN- $\gamma$ R1KO mice compared to infected wild-type mice. Furthermore the total number of NSCs in 15 d p.i. IFN- $\gamma$ R1KO mice was not significantly different from uninfected mice. Taken together, these data suggest that in the experimental HSE model, CD8(+) T cells impair endogenous NSC proliferation in an IFN- $\gamma$  dependent manner. Future directions of

these studies may identify crucial steps for therapeutic intervention in patients with brain damage resulting from viral encephalitis.

## **Introduction**

Herpes Simplex virus type 1 (HSV-1) is the most common cause of sporadic viral encephalitis (HSE) in the United States, and more than fifty percent of human patients surviving HSE develop life altering neurological deficits (McGrath, Anderson et al. 1997; Kimberlin 2007). The murine model of experimental HSE, which closely resembles the human disease, is characterized by robust T cell infiltration that begins at 6 d p.i. and peaks between 14 and 16 d p.i.. These T cells persist in the brain beyond 30 d p.i. (Marques, Cheeran et al. 2008; Armien, Hu et al. 2010). Notably, the phenotypes of infiltrating T cells changes as the inflammation progresses, being largely CD4(+) at 8 d p.i. and shifting to a primarily CD8(+) phenotype at 15 and 30 d p.i. (Marques, Cheeran et al. 2008). Furthermore, T cells present in the brain at 15 d p.i. express high levels of IFN- $\gamma$ , despite a lack of detectable viral antigen (Marques, Cheeran et al. 2008).

CD8(+) T cells are essential for clearance of viral infections (Wang, Lobigs et al. 2003; Lang and Nikolich-Zugich 2005; Kreutzfeldt, Bergthaler et al. 2013). However, these cells have been shown to promote neuronal degeneration and tissue damage subsequent to viral infections, like West Nile virus (WNV) (Wang, Lobigs et al. 2003). In addition, adoptive transfer of myelin-specific CD8(+) T cells, obtained from mice with experimental autoimmune encephalitis (EAE), into naïve animals resulted in development of clinical signs including ataxia, spastic reflexes, loss of coordination and spinning behaviors (Huseby, Liggitt et al. 2001). In mouse models of Japanese

encephalitis and EAE, the resultant T cell mediated neuroinflammation has also been shown to disrupt proliferation and migration of adult neural stem cells (NSCs) (Pluchino, Muzio et al. 2008; Das, Dutta et al. 2011; Sekeljic, Bataveljic et al. 2012). In mice with ischemic brain injury, increased numbers of T cells are associated with decreased numbers of NSCs and increased lesion volume several days post infection resulting in more severe neurological deficits (Takata, Nakagomi et al. 2012; Schuhmann, Kraft et al. 2015). Similarly, depletion of T cells from ischemic mice was associated with increased numbers of NSCs and decreased lesion volumes, suggesting that activated T cells play an critical role in development of brain damage.

IFN- $\gamma$  has been implicated in promoting CD8(+) T cell associated central nervous system (CNS) immunopathology in numerous model systems. Pneumococcal meningitis in IFN- $\gamma$  knockout mice results in decreased neuronal damage of the hippocampus and cerebral cortices, associated with significantly improved clinical disease scores (Too, Ball et al. 2014 ). Adoptive transfer of malaria-specific IFN- $\gamma$ (+) CD4(+) T cells induced significant increases in clinical symptoms of cerebral malaria in IFN- $\gamma$  knockout mice manifesting as disruption of the blood-brain barrier and increased cerebral edema (Villegas-Mendez, Greig et al. 2012). Similarly, in experimental lymphocytic choriomeningitis (LCMV) encephalitis, IFN- $\gamma$  inhibited adult neurogenesis and worsened coordination deficits seen during the disease (Kreutzfeldt, Bergthaler et al. 2013). Furthermore, CD8(+) T cells have been shown to significantly impair NSC proliferation *in vitro* via an IFN- $\gamma$  dependent mechanism (Hu, Rotschafer et al. 2014), which suggests that CD8(+) T cells may influence reparative functions in the brain.

Adult neurogenesis is mediated by a persistent, small population of endogenous brain cells called neural stem/progenitor cells (NSCs) which proliferate in response to brain damage including viral encephalitis (Lichtenwalner and Parent 2005; Parent, Elliott et al. 2006; Das, Dutta et al. 2011; Rotschafer, Hu et al. 2013). NSCs are maintained within distinct germinal niches in the brain that support their multipotent phenotype and self-renewal properties. Brain injury and the resultant inflammation initiate NSC proliferation by altering the niche to activate neurogenesis (Merkle, Tramontin et al. 2004). Increased NSC proliferation is associated with reduced inflammation and has been shown to replace damaged neurons following brain injury in several models, including EAE (Aharoni, Arnon et al. 2005), stroke (Arvidsson, Collin et al. 2002) and Japanese encephalitis (Das, Dutta et al. 2011). The reduction in inflammation combined with increased neurogenesis results in improvement of brain function following damage.

The objective of the present study was to evaluate the contribution of CD8(+) T cells and IFN- $\gamma$  to NSC proliferation during chronic HSE. Given the persistent inflammatory response observed during HSE, we hypothesized that CD8(+) T cells impair NSC proliferation through IFN- $\gamma$  dependent mechanisms.

### **Materials and Methods:**

#### **Ethics Statement:**

All animal procedures were done in accordance with the policies and practices of the animal care and use committee of the University of Minnesota.

#### **HSV-1 Infection:**

Cannulated and uncannulated female BALB/c and IFN- $\gamma$ R1 knock out mice (eight and ten weeks of age) were anesthetized using ketamine and xylazine.  $1.25 \times 10^5$  plaque forming units of Herpes Simplex Virus-1 (HSV-1) strain Syn17+, a neurovirulent virus, was instilled into both nares in a volume not exceeding 2  $\mu$ L per nostril. Disease progression and cannula stability was evaluated daily for the duration of the experiment. Peak mortality was observed between 5 and 12 d p.i. and only animals surviving the infection were used for CD8(+) depletion.

### ***Ex vivo* Evaluation of IFN- $\gamma$ Production**

BALB/c mice were infected as described above. At 15 d p.i. brains, cervical lymph nodes, and spleens were isolated from infected and uninfected mice. The tissues were processed aseptically to generate a single cell suspensions mechanical dissociation and passed through 100, 70, and 40 $\mu$ m cell strainers. Red blood cells were chemically lysed and the cell suspension was washed three times.

Isolated cells were plated in six-well tissue culture plates and left unstimulated or stimulated with 2 $\mu$ g/mL heat inactivated, sucrose purified HSV-1 Syn17+, 2 $\mu$ g heat inactivated, sucrose purified Swine Influenza Virus (SIV H1N1; non-specific stimulation control), 10ng/mL phorbol myristate acetate with 200ng/mL ionomycin (Sigma Aldrich, St. Louis, MO; positive stimulation control). Viruses were heat inactivated by incubation at 55°C for 15 minutes. HSV-1 inactivation was confirmed by plaque assay on rabbit skin epithelial cells (ATCC, Manassas, VA). SIV inactivation was measured by TCID<sub>50</sub> on MDCK cells (ATCC). No virus growth was observed at any virus dilution. Isolated brain leukocytes were incubated for 20 hours with or without antigen. 1 $\mu$ g Brefeldin A per mL

culture medium as GolgiPlug (BD Biosciences) was added to inhibit protein transport for the last four hours of incubation. Cells were collected for immunostained with CD45 (BD Biosciences, 1:50 dilution APC), CD3e (eBioscience, 1:50, PE-Cy7), CD4 (BD Biosciences; 1:50 dilution, conjugated to APC-Cy7), CD8b (eBioscience; 1:50, conjugated to APC), and IFN- $\gamma$  (BD Biosciences; 1:25 dilution, conjugated to PE). Cells were analyzed by flow cytometry within 24 hours of immunostaining.

### **Mouse Cannulations:**

To facilitate depletion of CD8(+) T cells from the brain, an *intracerebroventricular* (*icv*) cannula were placed in the right lateral ventricle of BALB/c mice prior to infection with HSV-1. Briefly, six to seven week old BALB/c mice were anesthetized using ketamine/xylazine and placed on a mouse stereotactic device (Stoelting, Wood Dale, IL) prior to surgery. The precise location of the right lateral ventricle was identified as -0.9mm anterior-posterior and +0.5mm midline relative to bregma. A single 28GA cannula was implanted to a depth of -2.5mm from the skull and affixed to the skull using cyanoacrylate, dental cement and wound clips which remained in place for the duration of the experiment. Cannulas were capped using dummy guides and mice were allowed to recover for seven to ten days prior to infection with HSV-1. Cannula guides were cleaned daily. Only mice with intact, patent cannulas were used in all experiments.

### **CD8(+) T Cell Depletion and Flow Cytometric Evaluation of Immune Milieu**

Infected and uninfected animals were treated with 10 $\mu$ g anti-CD8a antibody (clone 2.43, rat IgG2b, BioXCell, West Lebanon, NH), isotype antibody or were left

untreated on days 15, 17 and 19 post infection (d p.i.) into the brain via the indwelling *icv* cannula. Location of the cannula was verified at sacrifice by injection of 2.5uL isotonic Trypan Blue (0.1% solution in phosphate buffered saline). Brains from mice in all treatment groups were harvested and mechanically dissociated to generate a single cell suspension. Brain leukocytes were isolated using a discontinuous Percoll gradient (30-70%) and washed twice. Isolated cells were immunostained for CD45 (BD Biosciences, conjugated to FITC, PerCP-Cy5.5, APC, V500), CD11b (eBioscience (an Affymetrix Company), San Diego, CA; 1:50 dilution, conjugated to APC, APC-Cy7, eFluor 450), CD3e (eBioscience, 1:50, PE-Cy7), CD4 (BD Biosciences; 1:50 dilution, conjugated to FITC and APC-Cy7), CD8b (eBioscience; 1:50, conjugated to APC), CD62L (BD Biosciences; 1:50, conjugated to PE), CD103 (BD Biosciences; 1:50, conjugated to FITC), CD69 (BD Biosciences; 1:50, conjugated to FITC), CD25 (BD Biosciences; 1:50, conjugated to PE), Major Histocompatibility Class II (eBioscience; 1:50, conjugated to PerCP-eFluor 780), Ly6C (eBioscience; 1:50, conjugated to APC, eFluor 450, or PerCP-eFluor 780), CD206 (BioLegend, San Diego, CA; 1:25, conjugated to APC), CD80 (BD Biosciences; 1:50, conjugated to PE), and CD86 (BD Biosciences; 1:50, conjugated to FITC).

### ***In vitro* M2 Macrophage Polarization**

Bone-marrow derived monocytes (BMDMs) were isolated from tibias of uninfected BALB/c mice by flushing serum-free macrophage culture media (Life Technologies, Grand Island, NY) through the marrow cavity. Bone marrow cells were differentiated in to macrophages by supplementing serum free macrophage media with

macrophage-colony stimulating factor (M-CSF, eBioscience; 10 ng/mL) and allowed to grow for three days. Non-adherent cells were removed by washing and adherent macrophages were stimulated with interleukin-4 (eBioscience; 20 ng/mL) for 4 days to generate alternatively activated M2 macrophages. Validation of alternative activation was achieved by flow cytometry for CD45, CD11b, CD206 and CD86 as well as reverse transcription-PCR for gene products shown to be specific for each polarization including *interleukin-15*, *interleukin-6*, *arginase1*, and *CD206*.

### ***In vivo* M2 Macrophage Supplementation**

Uninfected 8 to 10-week old female BALB/c mice were anesthetized and heat-killed or live M2 activated macrophages was administered by *icv* injection (-0.9mm anterior/posterior, +0.5mm midline, and -2.5mm dorsal/ventral relative to bregma) using a 27.5GA needle. Macrophages were heat-killed by incubation at 55°C for 15 minutes and cell death was verified by visual evaluation of the cells using a Trypan Blue (Sigma Aldrich) exclusion assay.  $3 \times 10^4$  heat-killed or live macrophages were stereotactically injected in 3  $\mu$ L saline at a rate of 1  $\mu$ L per minute into the right lateral ventricle.

### **Flow Cytometric Analysis Endogenous NSC Proliferation**

Brains of infected and uninfected BALB/c and IFN- $\gamma$ R1 KO mice were isolated and trimmed to include the area around the lateral ventricles (+1 to -3 relative to bregma) using a coronal brain matrix with 1mm graduations (Braintree Scientific, Braintree, MA). A single cell suspension was generated using the papain-based neural tissue dissociation kit (Miltenyi Biotec, Auburn, CA) and myelin was removed from the suspension using myelin removal beads (Miltenyi) as per the manufacturer's instructions. Cells were then

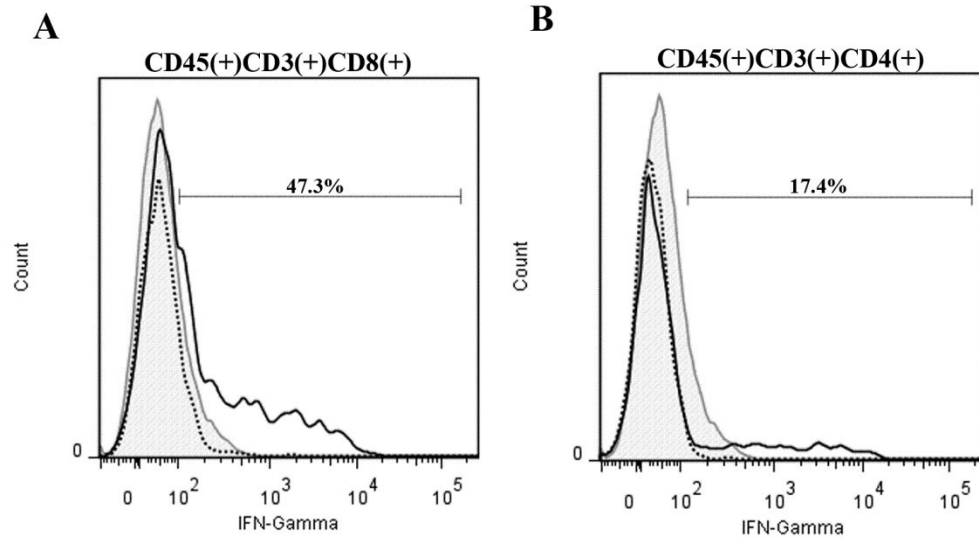


immunostained for CD45 (BD Biosciences, San Jose, CA; 1:50, conjugated to PerCP-Cy5.5), a myeloid marker and nestin (BD Biosciences; 1 test, PE conjugated), a neural stem cell marker, or CD45 and Ki-67 (Abcam, Cambridge, MA; 1:25), a proliferation marker. PE conjugated high affinity donkey anti-rabbit F<sup>2</sup>(ab) fragments were used as the secondary antibody for Ki-67. Alternatively, cells were stained with CD45 and Sox2 (eFluor 660, eBioscience, San Diego, CA, 1:50). To assess proliferation in the NSC population, brain cells were immunostained with CD45, Ki-67 and Sox2 an alternative NSC marker.

## **Results:**

### **Antigen Specific CD8(+) T cells produce IFN- $\gamma$ at 15 d p.i..**

To determine if T cells present in the brain at 15 d p.i. were HSV-1 specific, total brain leukocytes were isolated and stimulated with either HSV-1 antigens, SIV antigens, or left unstimulated. After 20 hours of *ex vivo* stimulation, total brain leukocytes were immunostained for IFN- $\gamma$  and analyzed by flow cytometry. Among T cell populations (CD45(hi)CD3(+)), 49.4 $\pm$ 2.76% of HSV-1 specific CD8(+) T cells produced IFN- $\gamma$  (Fig. 6.1A) whereas production of IFN- $\gamma$  by non-specifically stimulated cells was negligible and not significantly different from unstimulated cells. In contrast, only 19.7 $\pm$ 3.5% of CD4(+) T cells produced IFN- $\gamma$  in response to HSV-1 antigens at 15 d p.i. (Fig. 6.1B). Concurrent with the robust IFN- $\gamma$  production, CD8(+) T cells have been shown to outnumber CD4(+) T cells two to one at 15 d p.i. (Marques, Cheeran et al. 2008; Rotschafer, Hu et al. 2013) which demonstrates that CD8(+) T cells



**Figure 6.1: HSV-1 specific IFN- $\gamma$  production by CD8(+) T cells in the brain at 15 dpi.** (A) CD8(+) and (B) CD4(+) subpopulations of CD45(+)CD3(+) T cells were analyzed for IFN- $\gamma$  production subsequent to *ex vivo* stimulation with HSV-1 antigen for 20h. Shown in each graph is staining isotype (tinted gray line), non-specifically stimulated cells (dotted black line) and HSV-1 stimulated cells (black line). Representative dot plots are shown of three independent experiments.

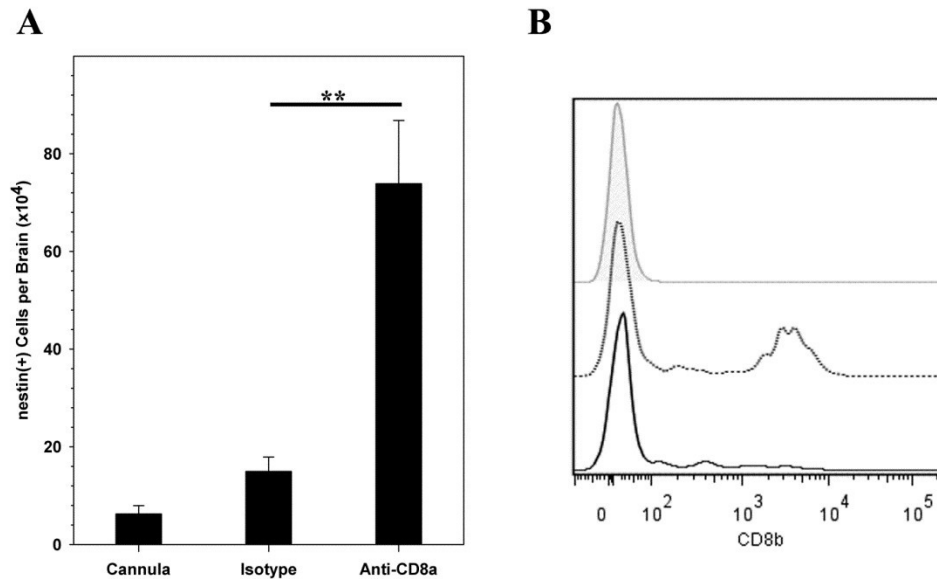
are the most significant contributor to IFN- $\gamma$  in the brains of HSV-1 infected mice at this time point during infection.

### **CD8(+) T cell depleted mice have increased total numbers of NSCs.**

Previous findings in the laboratory have demonstrated that total numbers of endogenous NSCs significantly decreased with peak T cell infiltration and IFN- $\gamma$  production at 15 d p.i. (Marques, Cheeran et al. 2008; Rotschafer, Hu et al. 2013). Depletion of CD8(+) T cells significantly increased total numbers of CD45(-)nestin(+) NSCs (73,884.34 $\pm$ 12,923 total cells,  $p\leq 0.01$ ) in the brains of infected mice compared to isotype antibody treated animals (14,983.98 $\pm$ 2915.95 total cells; Fig. 6.2A). While addition of either antibody to uninfected mice resulted in moderate increases in total numbers of CD45(-)nestin(+) NSCs, this increase was not significantly different from untreated controls (Fig. 6.2A). Success of CD8(+) T cell depletion in the brain was confirmed by immunostaining with a CD8b specific antibody which revealed an absence of CD8(+) T cell in depleted mice while demonstrating a robust population of CD8(+) T cells in infected isotype antibody treated animals, .

### **CD8(+) T cell depletion alters brain macrophage activation phenotypes.**

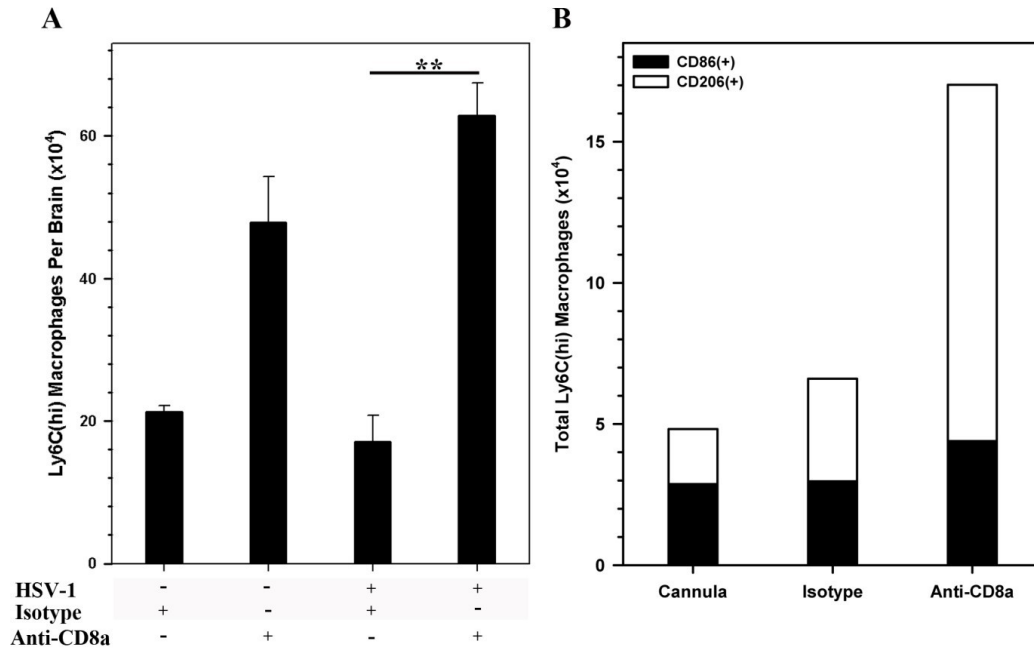
To assess the effect of CD8(+) T cell depletion on other immune cell populations in the brain, activation phenotypes of infiltrating macrophage (CD45(hi)CD11b(+)) and microglia (CD45(int)CD11b(+)) was assessed. In the macrophage compartment, a significant increase in the total number of Ly6C(hi) infiltrating macrophages was observed following CD8(+) T cell depletion (629,062.42 $\pm$ 45436.83 total cells;  $p\leq 0.01$ )



**Figure 6.2: The NSC population increases following CD8(+) T cell depletion.** (A) The total number of nestin(+) NSCs were significantly increased in CD8(+) depleted mice (Anti-CD8a) compared to untreated (Cannula) or isotype antibody treated (Isotype) controls. (B) Representative flow plot showing CD8b immunostaining in HSV-1 infected isotype-treated (middle, dotted black line) and CD8a depleted mice (bottom, solid black line) compared to staining isotype (top, gray line), confirming the success of CD8(+) T cell depletion. Data presented are averages  $\pm$  SEM of 8 animals per group. \*\*  $p \leq 0.01$ .

compared to infected isotype antibody treated animals ( $170,575.08 \pm 8692.41$  total cells; Fig. 6.3A). Further evaluation of the CD45(hi)CD11b(+) macrophage population revealed a shift in the abundance of CD86(+) vs CD206(+) macrophages in CD8(+) T cell depleted, HSV-1 infected mice compared to isotype antibody treated infected controls. Alteration in this ratio suggests a shift in macrophage activation. Isotype treated infected mice had CD86 to CD206 cells at a one to one ratio ( $29,749.99 \pm 3589.02$  CD86(+) macrophages to  $36,306.25 \pm 6769.62$  CD206(+) macrophages; Fig. 6.3B) whereas the CD8a depleted mice had a CD86 to CD206 ratio of one to three ( $43,975.26 \pm 4862.06$  total CD86(+) macrophages to  $126,191.32 \pm 24,136.54$  CD206(+) macrophages). Untreated infected mice did not have significant alteration in the CD86 to CD206 ratio compared to infected isotype antibody treated mice. The change in ratio resulted from a significant increase in the total number of CD206(+) macrophages in CD8a depleted mice ( $126,191.32 \pm 24,136.54$  total CD206(+) macrophages,  $p \leq 0.01$ ) compared to isotype controls ( $36,306.25 \pm 6769.62$  total CD206(+) macrophages). While administration of the CD8(+) depleting antibody resulted in an increase in the total number of infiltrating macrophages in uninfected mice compared to isotype treated animals, the total magnitude of the change was smaller. Uninfected mice had two-fold greater infiltrating macrophages compared to a four-fold change in population size in infected mice brains (Fig. 6.3A).

To further investigate changes in microglia activation, major histocompatibility class II (MHCII) was evaluated subsequent to CD8(+) T cell depletion. Infected CD8a

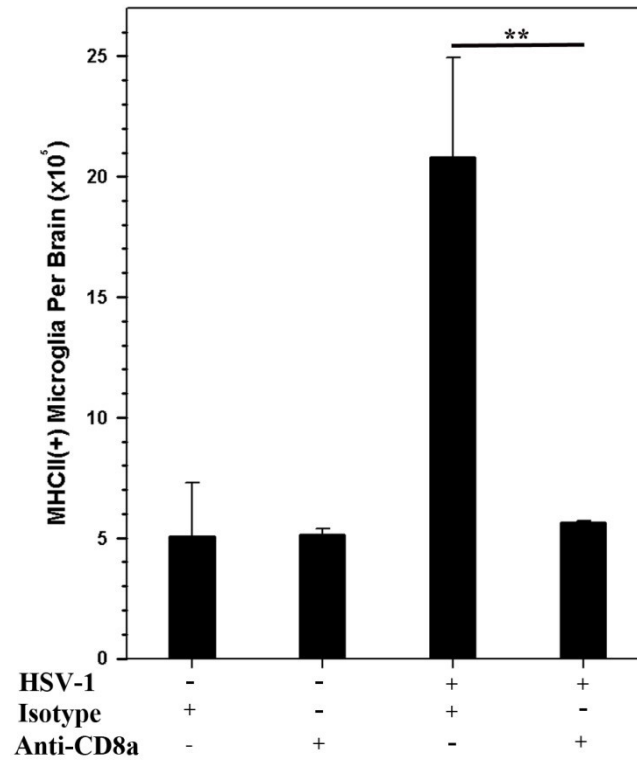


**Figure 6.3: Depletion of CD8(+) T cells increases Ly6C(hi) macrophage migration into the brain.** (A) Total number of CD45(hi)CD11b(+)Ly6C(hi) infiltrating macrophages increased by 3 fold in the infected depleted group compared to infected isotype-treated animals. (B) Interestingly, among the Ly6C(hi) macrophages, there was a shift in the ratio of M1 (black bar) and M2 macrophages (white bar) towards a net M2 polarization phenotype. Data presented are averages  $\pm$ SEM of 8 animals per group. \*\*  $p \leq 0.01$ .

depleted mice ( $562,412.78 \pm 8945.40$  total microglia,  $p \leq 0.01$ ) had significantly less expression of MHCII compared to infected isotype treated animals ( $2,079,460.47 \pm 414,555.70$  total microglia; Fig. 6.4). Additionally, MHCII expression on microglia in infected depleted mice was reduced to levels observed in uninfected mouse levels (Fig. 6.4). In combination, reductions in microglial activation and changes in macrophage activation suggests that depletion of CD8(+) T cells resulted in an altered inflammatory milieu potentially leading to increases in NSC proliferation.

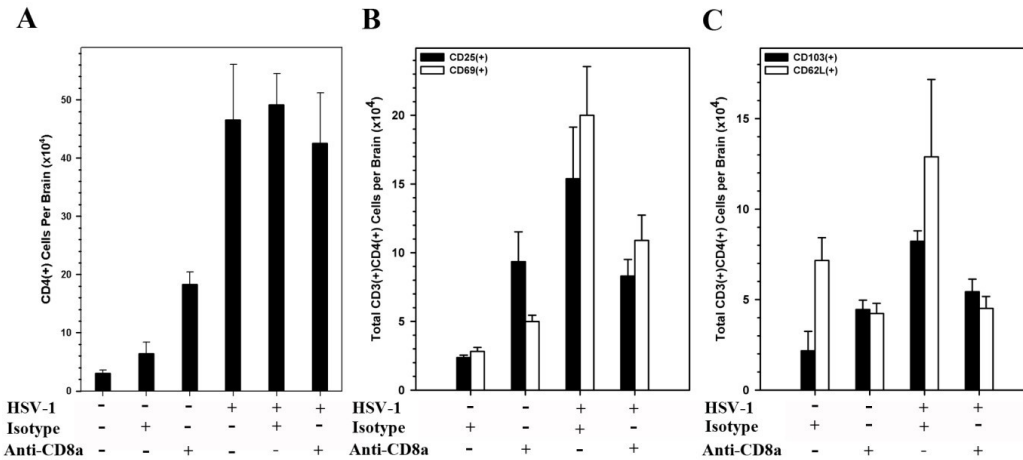
#### **CD4(+) T cell phenotypes are not altered in CD8a depleted, HSV-1 infected animals**

To identify changes in the CD4(+) T cell compartment associated with the depletion of CD8(+) T cells, CD4(+) T cell phenotypes were evaluated. Surprisingly, total numbers of CD4(+) T cells were not altered in CD8a depleted animals, compared to other infected groups (Fig. 6.5A). CD4 T cell expression of CD69 was lower in depleted animals ( $108,945.47 \pm 18451.81$  cells,  $p=0.054$ ; Fig. 6.5B) compared to isotype treated controls ( $200,028.29 \pm 25518.64$  CD69(+) cells); however this difference was not statistically significant. Neither was there a significant difference in decreased expression of CD25 in infected depleted mice ( $82,977.66 \pm 12,108.54$  CD25(+) cells) compared to infected isotype antibody treated controls ( $153,923.68 \pm 37514.44$  CD25(+) cells). CD4(+) T cells also did not show a significant change in expression of the resident memory cell phenotype marker, CD103, between CD8a depleted infected mice ( $54,373.65 \pm 6915.09$  CD103(+) cells; Fig. 6.5C) and isotype infected controls ( $82,222.50 \pm 5745.33$  CD103(+) cells). CD44 and CD62L were also evaluated for alteration in memory phenotypes but there were no significant differences observed (data not shown).



**Figure 6.4: Microglia activation is decreased after depletion of CD8(+) T cells.** Infected, CD8(+) T cell depleted mice expressed uninfected mouse levels of MHCII. Data presented are averages  $\pm$ SEM of 8 animals per group. \*\*  $p \leq 0.01$ .



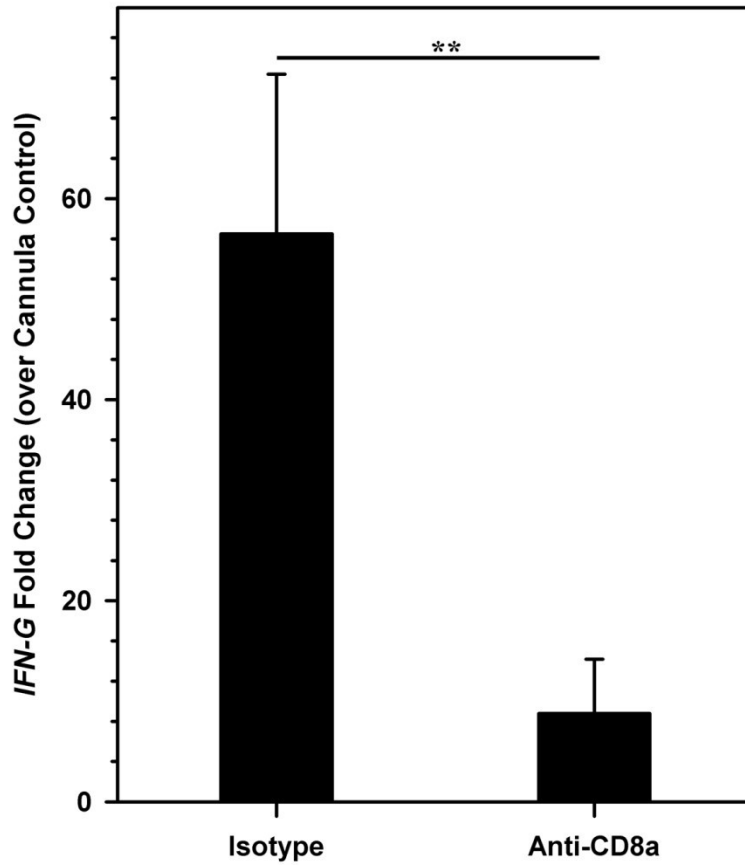


**Figure 6.5: CD4(+) T cells are not affected by CD8(+) T cell depletion.** (A) The total number of CD4(+) T cells was not significantly different between any of the infected groups. (B) Among the effector CD4(+) T cell subset there were no significant changes in activation markers, CD25 (black bars) and CD69 (white bars) in the infected groups. (C) Furthermore, no significant difference in the expression of memory T cell markers was observed in the CD4(+) T cell compartment, CD103 (black bars) and CD62L (white bars) between the infected groups. Data presented are averages  $\pm$ SEM of 8 animals per group.

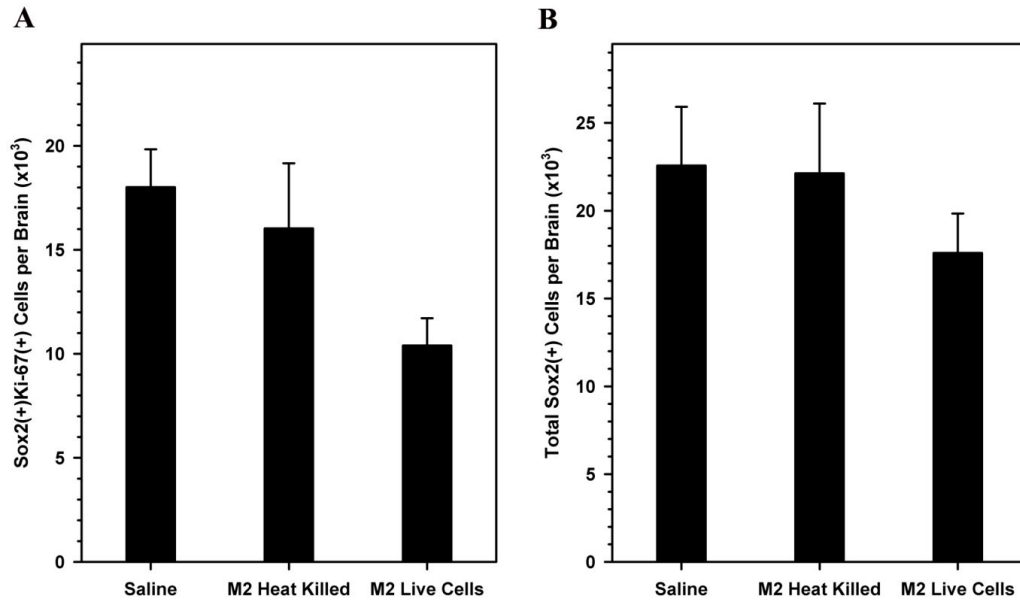
To assess alterations in the soluble factors expressed in the brain during chronic HSE, expression of T cell cytokines was evaluated. As expected, infected CD8(+) T cell depleted mice had significantly less IFN- $\gamma$  gene expression (8.79 $\pm$ 5.38 fold change,  $p\leq 0.05$ ) compared to infected isotype controls (56.49 $\pm$ 15.89 fold change; Fig. 6.6). No significant changes in interleukin-4 or interleukin-10 gene expression was observed in chronically infected mice (data not shown). RT-PCR analysis of HSV-1 glycoprotein D gene expression showed no signs of viral reactivation after CD8(+) T cells were depleted (data not shown). These findings suggest that removal of CD8(+) T cells alters the inflammatory milieu such that NSC proliferation may be promoted.

**IFN- $\gamma$  inhibits NSC response to proliferative stimuli.**

Previous studies, discussed in Chapter 4, demonstrated that transplantation of *ex vivo* M2 stimulated macrophages into the lateral ventricles of uninfected mice have the capability to increase NSC proliferation in an uninfected brain. However, transplantation of the same number of *ex vivo* M2 polarized macrophages into the lateral ventricles of 15 d p.i. mice had no significant impact on proliferation of Sox2(+) NSCs (Fig. 6.7A) or on total numbers of Sox2(+) NSCs (Fig. 6.7B). This observation is congruent with studies that show ovine macrophages polarized *ex vivo* to an M2 polarized phenotype can readily acquire the M1 phenotype in the presence of M1 polarizing stimuli *in vivo* (Crespo, Bertolotti et al. 2013). The robust production of IFN- $\gamma$  in the brains of HSV-1 infected mice may repolarized transplanted M2 macrophages to an M1 phenotype.



**Figure 6.6: IFN- $\gamma$  gene transcription decreases with CD8(+) T cell depletion.** Isotype antibody treated mice (isotype) had a significant increase in IFN- $\gamma$  gene expression compared to CD8(+) depleted mice (anti-CD8a). Persistent IFN- $\gamma$  expression in the brains of depleted mice may be explained by the continued presence of CD4(+) T cells in the brain. Data presented as fold-change over uninfected, cannulated control animals  $\pm$  SEM of five animals per treatment group. \*\*  $p \leq 0.01$ .

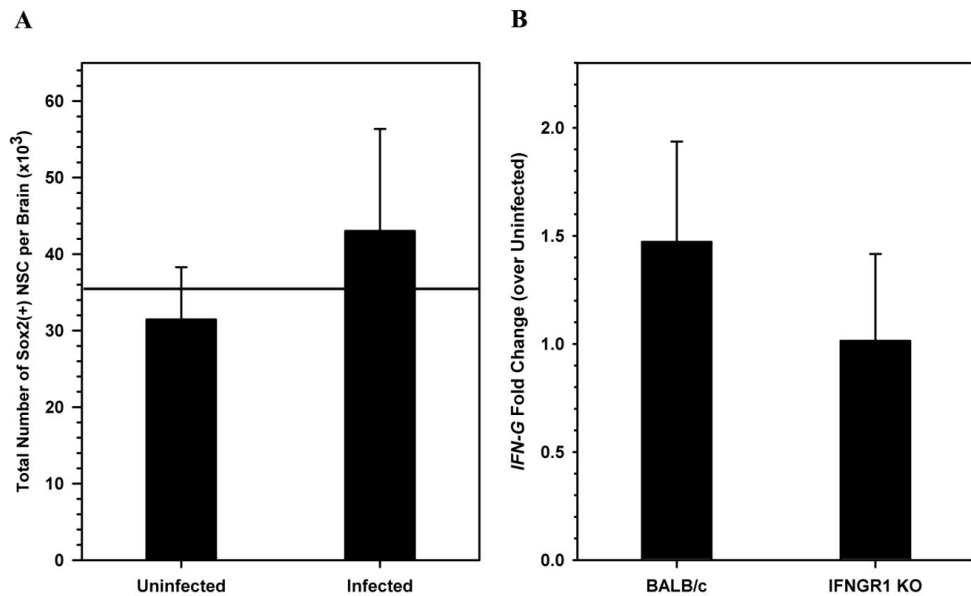


**Figure 6.7: Transplantation of M2 macrophages does not enhance NSC proliferation in chronically infected mice.** Brains of infected mice were injected with saline, heat-killed M2 polarized bone-marrow derived macrophages (BMDMs), or live M2 polarized BMDMs via *intracerebroventricular* injection. **(A)** There was no significant difference among treatment groups in numbers of proliferating Sox2(+) NSCs nor was there a significant difference in **(B)** the total number of Sox2(+) NSCs in treated brains. Data presented as average  $\pm$  SEM of four animals per treatment group.

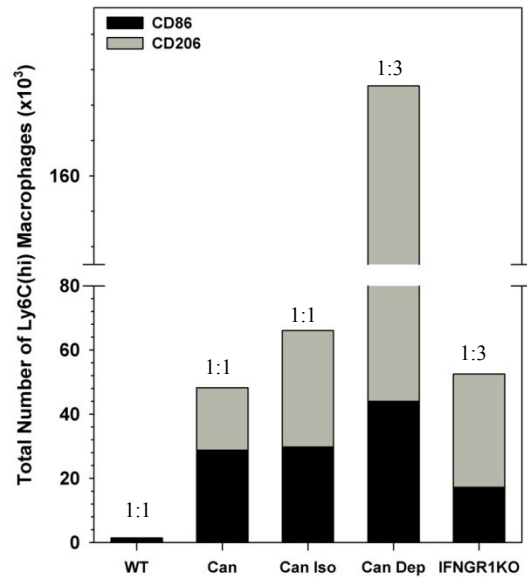
An alternate approach was employed to ascertain the impact of IFN- $\gamma$  on NSC proliferation during chronic HSE. IFN- $\gamma$ R1 KO mice on a BALB/c genetic background were used to ablate IFN- $\gamma$  signaling in the brains of infected mice. Evaluation of proliferation in the endogenous brain cell (CD45(-)) compartment demonstrated a significant increase in the total numbers of Sox2(+) NSCs in infected IFN- $\gamma$ R1 KO mice compared to wild-type BALB/c controls (Fig. 6.8A). Additionally, the total numbers of Sox2(+) NSCs were not significantly different than uninfected, age-matched controls (43035.4 $\pm$ 6828.4 total Sox2(+) cells per infected brain vs 31450.9 $\pm$ 8335.9 total Sox2(+) cells per uninfected IFN- $\gamma$ R1 KO mouse brain; Fig. 6.8A). Surprisingly, the total numbers of NSCs in infected IFN- $\gamma$ R1 KO mice was not significantly different than uninfected BALB/c mice in spite of robust expression of IFN- $\gamma$  at 15 d p.i. (Fig. 6.8B).

**Discussion:**

The present study demonstrated that more than half of CD8(+) T cells persisting in the brains of HSV-1 infected mice, at 15 d p.i., produce IFN- $\gamma$  in response to the virus. This phenomenon occurred concurrent with significantly decreased NSC proliferation. In contrast, CD8(+) T cell depleted mice had significantly increased numbers of NSCs, and significantly reduced IFN- $\gamma$  expression compared to isotype antibody treated controls. This improvement is associated with a change in brain macrophage activation, progressing from a M1-like phenotype to an M2-like phenotype. The depletion of CD8(+) T cells abrogated microglial activation, as measured by MHC class II expression.



**Figure 6.8: Total numbers of NSC in 15 d p.i. infected IFN- $\gamma$  receptor knockout mice increased.** (A) Total numbers of Sox2(+) NSCs was not significantly different in uninfected or infected IFN- $\gamma$ R1 KO mice nor was it different from uninfected wild-type BALB/c mice (mean=solid line across graph). (B) There was no significant difference in the amount of IFN- $\gamma$  produced at 15 d p.i. between BALB/c and IFN- $\gamma$ R1 KO mice. Data as presented as mean $\pm$ SEM of four animals.



**Figure 6.9: Macrophage polarization of experimental groups with higher NSC numbers skews to M2-like.** CD206 (gray bars) and CD86 (black bars) from the CD45(hi)CD11b(+)Ly6C(hi) infiltrating macrophage population in infected mice given different treatments are expressed as averages (SEM can be found in the text) of between five and eight animals per treatment group. Numerical ratios express the total number of CD86(+) cells to CD206(+) macrophages. Notably, the unmanipulated wild-type (WT), cannulated (Can), and isotype antibody treated (Can Iso) groups had CD86 to CD206 ratios of one to one, while CD8(+) T cell depleted (Can Dep) and IFN- $\gamma$ R1 KO (IFN- $\gamma$ R1KO) mice had ratios closer to one to three.

Surprisingly, there was no significant change in CD4(+) T cell numbers or activation following CD8(+) T cell depletion. The findings in the present chapter correlate to those described in Chapter 4, which demonstrate that an M2 dominated immune milieu promotes NSCs proliferation. We further confirmed this hypothesis by evaluating NSC proliferation in IFN- $\gamma$ R1 KO mice, demonstrating that mice lacking functional IFN- $\gamma$  signaling showed increased numbers of Sox2(+) NSCs compared to wild-type BALB/c mice at the same time point. Moreover, IFN- $\gamma$ R1 KO mice maintained an M2-like macrophage activation profile at 15 d p.i..

Collectively these findings point to an interplay between macrophage activation and NSC proliferation. Comparison of macrophage activation profiles of infiltrating macrophages at 15 d p.i. between uncannulated, cannulated untreated, or isotype antibody treated and CD8(+) T cell depleted or IFN- $\gamma$ R1 KO infected mice demonstrates that decreases in IFN- $\gamma$  expression or loss of IFN- $\gamma$  signaling results in increased numbers of NSC and altered macrophage polarization (Fig. 6.9). Despite variability in total numbers of Ly6C(hi) macrophages, only those animals lacking functional IFN- $\gamma$  signaling had CD86 to CD206 ratios of one to three or higher whereas wild-type, cannulated, and isotype antibody treated mice had CD206 to CD86 ratios of one to one.

Antigen specific CD8(+) T cells have been shown to remain in the tissue after resolution of infection in numerous model systems, including coronavirus-induced encephalitis (Phares, Stohlman et al. 2012) and allergic autoimmune encephalitis (Steinman 2001). In humans, memory CD8(+) T cells have been shown to circulate in the reproductive tract of women infected with Herpes Simplex virus-2 (Koelle, Posavad et al.



1998). The present study demonstrated that approximately half of CD8(+) T cells present in the brains of infected mice were HSV-1 specific and produced IFN- $\gamma$  in response to viral antigen, demonstrating the possibility for development of a memory response by the persistent CD8(+) T cells. In other models of viral disease such as influenza, memory CD8(+) T cells remain in the tissue to accentuate the local immune response should the pathogen be reintroduced to the host (van de Sandt, Hillaire et al. 2015). Despite undetectable viral antigen in our model of HSE at 15 d p.i., viral DNA from latent HSV-1 was detected, suggesting that HSV-1 specific CD8(+) T cells present after the onset of viral latency may be re-stimulated by viral antigen associated with reactivation events in the host.

On the other hand, CD8(+) T cells present in the brain have been linked to alteration of neurogenesis. In mouse models of viral encephalitis, viz. Japanese encephalitis (Das, Dutta et al. 2011) and West Nile encephalitis (Wang, Lobigs et al. 2003), activated CD8(+) T are associated with decreased NSC proliferation. In this present study, depletion of CD8(+) T cells resulted in an increase in total numbers of NSCs suggesting that CD8(+) T cells can affect the processes responsible for inducing NSC proliferation. *In vitro* co-culture with activated CD8(+) T cells inhibits NSC proliferation in an IFN- $\gamma$  dependent manner, as addition of an IFN- $\gamma$  neutralizing antibody restores NSC proliferation (Hu, Rotschafer et al. 2014). The role of IFN- $\gamma$  in the inhibition of NSC proliferation may be preserved *in vivo* as depletion of CD8(+) T cells was associated with a significant reduction in IFN- $\gamma$  expression and increased NSC proliferation. Furthermore, the size of the NSC population in infected IFN- $\gamma$ R1 knockout

mice remained unaffected by infection with HSV-1, which suggests that inhibition of NSC proliferation during chronic HSE is likely mediated by IFN- $\gamma$ .

In Chapter 5, IFN- $\gamma$  was shown to directly inhibit NSC proliferation in response to growth stimuli. However, indirect effects of IFN- $\gamma$ , such as inducing the M1 phenotype in macrophages, may contribute to its ability to impair NSC proliferation. IFN- $\gamma$  inhibits development of the M2 phenotype in macrophages, instead stimulating an M1 phenotype (Crespo, Bertolotti et al. 2013). M2 polarized macrophages have been shown to decrease infarct volume in mouse models of ischemic stroke (Hu, Li et al. 2012) and are essential for limb regeneration in salamanders (Godwin, Pinto et al. 2013). In Chapter 4, M2 macrophages were demonstrated to increase total numbers of NSCs in the brains of BALB/c mice and were the dominant macrophage phenotype in the brain at 6 d p.i., at a time when the NSC population size was significantly increased. In treatment groups with intact IFN- $\gamma$  signaling, NSC proliferation is significantly decreased at 15 d p.i. and the macrophage population is polarized to an M1 phenotype (shown in Chapter 4). Data presented in the present study suggests that IFN- $\gamma$  present in the brain contributes both directly and indirectly, via macrophage polarization, to the inhibition of NSC proliferation at 15 d p.i.. However, these experiments do not identify the relative importance of direct or indirect mechanisms in diminishing neurogenesis and their role in development of neurological deficits.

In summary, this present study shows that CD8(+) T cells negatively impact NSC proliferation during experimental HSE via an IFN- $\gamma$  dependent mechanism. While previous studies have shown that IFN- $\gamma$  has direct inhibitory effect on NSC proliferation,

we also demonstrate that removal of IFN- $\gamma$  alters the polarization of macrophages responding to the disease and promotes a healing phenotype in those macrophages. These findings suggest that the removal of CD8(+) T cells or IFN- $\gamma$  from the brains of infected mice may promote brain repair, ultimately improve neurological function in the wake of brain damage. Although further studies are needed to determine if alteration in macrophage profile in combination with improved neurogenesis is sufficient to ameliorate the devastating neurological deficits associated with viral encephalitis.

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**Chapter 7: Experimental Herpes Simplex encephalitis is associated with major differences in neural stem/progenitor cell proliferation and chronic inflammation in BALB/c and C57BL/6 mice.**



**Abstract:**

Brain infection with Herpes Simplex Virus-1 (HSV-1) is a devastating disease that results in robust and prolonged immune cell activation. Current mouse models of HSE either induce an alternative immune activation during the acute phase of infection (BALB/c) or are ubiquitously fatal and lack a chronic phase of the disease (C57BL/6). The objective of this study was to develop a C57BL/6 mouse model that is more reflective of acute human HSE while retaining the viral spread and persistent inflammation observed in human patients and the BALB/c mouse model. This study found that the spread of virus was similar in the traditional BALB/c mouse model and new C57BL/6 model. Furthermore, the kinetics of immune cell infiltration were similar between BALB/c and C57BL/6 mice. On the other hand, acutely infiltrating macrophages in the C57BL/6 mouse model consistently maintain a classically activated phenotype in contrast to the acute alternative activation of BALB/c mice. In both models, there was marked duration of T cell activation in the brain similar to the human condition. However, persistent T cells in C57BL/6 mice were predominantly CD4(+), not CD8(+) as in BALB/c mice. NSC proliferation increased during acute HSE and decreased significantly during chronic HSE in both C57BL/6 and BALB/c mice. The robust inflammatory response generated by C57BL/6 mice to HSV-1 infection may provide additional insights into the development of disease and subsequent failure of the brain to repair virus-induced damage.

**Introduction:**

Herpes simplex virus-1 (HSV-1) is the most common cause of sporadic viral encephalitis in the United States (Whitley 2006; Kimberlin 2007). While the specific insult resulting in HSV-1 migration into the brain is unknown, the progression of the virus through the mouse and human brain results in severe neurological deficits that are thought to be the result of combined viral and immune-mediated damage (Kastrukoff, Lau et al. 2010). Models that specifically address individual elements of the viral and immune components contributing to the development of neurological deficits are required to identify specific therapeutic interventions.

Despite being two strains of the same species, the immune responses of BALB/c and C57BL/6 mice have measurable differences which must be considered in evaluating data. A concise summary of the differences among immune cell types between the two strains may be observed in Table 7.1. BALB/c mouse T cells have a preferential shift towards the Th2 polarization during immune responses whereas C57BL/6 mouse T cells produce a more prominent Th1 polarization (Schulte, Sukhova et al. 2008). In mouse models of leishmaniasis, BALB/c derived T cells produced significantly more interleukin-4 than C57BL/6 T cells. In contrast, C57BL/6 derived T cells produced significantly more interferon- $\gamma$  (Hsieh, Macatonia et al. 1995). However, it has also been shown that failure to induce a strong Th1 immune response from BALB/c T cells does not result in heightened Th2 responses in mouse models of pulmonary mycobacterial infection (Wakeham, Wang et al. 2000). BALB/c mice also have significantly larger numbers of regulatory T cells than their C57BL/6 counterparts, which is thought to

Cell Type	C57BL/6	BALB/c	Reference
Macrophages	Increased production of TNF- $\alpha$ and IL-12	Faster response to <i>Mycobacterium bovis</i> BCG	Wakeham et al. 2000 Watanabe et al. 2004
T Cells	Increased production of IFN- $\gamma$ and IL-6 Decreased IL-13	Increased IL-4 production and B cell activation	Schulte et al. 2008 Müller et al. 1993 Hsieh et al. 1995
T regulatory cells	Fewer natural Treg	Increased natural Treg Faster production of inducible Treg	Hsieh et al. 1995 Chen et al. 2005 Jiang et al. 2010
NK cells	NK1.1 positive High IFN- $\gamma$ production	NK1.1 negative Low IFN- $\gamma$ production	Stenström et al. 2005 Arase et al. 2001
Dendritic Cells	Higher expression of TLR-9 and TLR-4; Increased production of IL-12p40	Higher expression of TLR-2, -5, and -6	Liu et al. 2002

**“Increases” and “decreases” are direct comparisons between the mouse strains.**

**Table 7.1: Differences in immune cell activities between C57BL/6 and BALB/c mouse strains.** This table presents observed differences in immune cell types between mouse strains.

confer resistance to autoimmune disorders in BALB/c mice (Chen, Oppenheim et al. 2005).

Macrophages from BALB/c and C57BL/6 mice have different responses to the same activating stimuli. In mouse macrophages activated with LPS, a potent toll-like receptor-4 (TLR-4) agonist, C57BL/6 derived mouse macrophages produced higher levels of tumor necrosis factor- $\alpha$  and interleukin-12 than BALB/c derived macrophages. Furthermore, C57BL/6 derived macrophages produced measurable amounts of lysosomal enzymes and nitric oxide in response to experimental bacterial peritonitis, whereas BALB/c mice did not (Watanabe, Numata et al. 2004), which suggests that the two different strains of macrophages had different effector functions during disease. Dendritic cells also differed between the two mouse strains. Dendritic cells (DCs) derived from BALB/c mice expressed significantly more TLR-2, TLR-4-6 mRNA than DCs from C57BL/6 mice (Liu, Matsuguchi et al. 2002). These differences in the innate immune responses between BALB/c and C57BL/6 mice are particularly relevant to assessing NSC proliferation during HSE as Chapters 4, 5, and 6 of this dissertation suggest that macrophage activation affects the level of NSC proliferation during disease.

The kinetics of immune cell infiltration into the brains of BALB/c mice infected with HSV-1 are strongly reminiscent of the human condition, with T cells persisting in both humans and mice long after the virus has become latent (Aurelius, Andersson et al. 1994; Marques, Cheeran et al. 2008). While the chronic immune response to HSE is consistent between BALB/c mice and humans, there are substantial differences during the acute phase of infection. Particularly, infiltrating macrophages during acute HSE are

predominantly alternatively activated in the BALB/c mouse model and are only classically activated during chronic disease (Chapter 4). Classical macrophage activation through Toll-like receptor (TLR) activation has been shown to be essential to development of human HSE (Medzhitov and Janeway Jr 2002; Conrady, Drevets et al. 2010). Similarly, TLR activation has been shown to be essential to development of lethal HSE in an intracerebral injection C57BL/6 mouse model of HSE (Kurt-Jones, Chan et al. 2004). However, this C57BL/6 mouse model results in diffuse HSV-1 infection that is not consistent with the human disease.

The overall objective of this dissertation chapter was to establish a C57BL/6 mouse model that retained the natural, human-like progression of the virus through the brain and the more human-like immediate classical macrophage activation phenotype during acute HSE. The model was assessed in terms of NSC proliferation, immune cell infiltration and disease progression.

## **Materials and Methods:**

### **Ethics Statement:**

All studies were done in accordance with the policies of the University of Minnesota College of Veterinary Medicine animal care and use committee.

### **HSV-1 Infection of BALB/c mice**

Briefly, eight to ten week old female BALB/c mice were anesthetized with a ketamine/xylazine solution and  $1.25 \times 10^5$  PFU of HSV-1 strain Syn17+ was instilled into each nostril in no more than 2 $\mu$ L per nostril. Animals were placed laid on their backs for 5 minutes and then righted and returned to their home cages. Infected mice were

observed every day during the entire course of the disease and animals meeting moribund criteria were euthanized.

### **HSV-1 Infection of C57BL/6 mice**

C57BL/6 mice exhibit the HSV-1 resistance NK1.1 gene loci and previous studies have shown that NK cells and CD8(+) T cells must be removed to induce HSE (Kastrukoff, Lau et al. 2010). To remove these cells, six to eight week old C57BL/6 female mice (Charles River Laboratories, Richmond, VA) were depleted of their NK and CD8(+) T cells using 200 µg anti-NK1.1 and 10ug anti-CD8a (both from BioXCell, Lebanon, NH) by intraperitoneal injection three times at -2, 0, and +2 days post infection (d p.i.). On infection day the mice were anesthetized using ketamine and xylazine to achieve plane 3 anesthesia and a 32GA sterilized stainless steel wire was threaded up their nasal passages and scraped against the nasal epithelium. Mice were righted and any blood collecting in the nares was collected on a cotton swab. Once blood flow had stopped for one minute,  $7.5 \times 10^5$  plaque forming units (PFU) of HSV-1 (Syn17+) was instilled into each nostril in  $>2 \mu\text{L}$  ( $1.5 \times 10^6$  PFU total). Mice were laid on their backs for 3 minutes and 2% lidocaine gel was applied to their nose. Mice were then returned to their home cages. Sham mice received the above procedure minus the virus instillation into the nares. Progression of clinical disease was scored using a previously established scale (Marques, Hu et al. 2006) from 0 (no visible signs) to 4 (ataxia and/or seizures are present). 1 indicates the presence of nasal lesions without neurological signs, 2 indicates ruffled fur, and decreased mobility, and 3 is indicative of spinning behaviors and/or lack of coordinated movement. Mice meeting euthanasia criteria at a clinical score of 4.

### **HSV-1 localization in the brain**

Infected mice were collected each day on days 4-11 post infection (n=3 per day) and perfused using 4% paraformaldehyde. Sham animals (n=3) were collected at 11 d p.i. and perfused as with the infected animals. Brains were isolated from the perfused animals and cryopreserved by passaging through a 30% sucrose solution and frozen in optimal cutting temperature (OCT) medium (Sakura, Torrance, CA). Brains were sectioned into 10 $\mu$ m longitudinal sections and sections were collected every 200 $\mu$ m throughout the brain. 10 sections were collected per slide with 15 slides collected per brain.

For immunohistochemistry for HSV-1, sections were quenched of endogenous peroxidases using 0.3% hydrogen peroxide in phosphate buffered saline (PBS) for 10 minutes at room temperature. Slides were then washed three times for five minutes in PBS before permeabilizing using 0.5% Triton-X 100 (Sigma Aldrich, St. Louis, MO) in PBS for 10 minutes at room temperature. Slides were washed as before. Non-specific antibody binding was blocked using 10% bovine serum albumin and 5% goat serum for one hour at room temperature, followed by application of anti-HSV-1 early/immediate early proteins (polyclonal rabbit IgG; 1:100 DAKO Technologies, Carpinteria, CA) in blocking solution. The antibody was incubated overnight at 4°C and washed three times as before. Following washing, anti-rabbit IgG conjugated to AlexaFluor 488 (1:500, Life Technologies, Carlsbad, CA) was applied for 30 minutes. Finally cells were washed and counterstained with DAPI immediately prior to imaging on a Nikon fluorescent microscope.

### **Immune Cell Phenotyping**

Infected and sham infected mice were collected at 3, 5, 8, 15 and 30 d p.i. for flow cytometry. Brains and cervical lymph nodes were isolated and mechanically dissociated to generate a single cell suspension. 100 $\mu$ L of tissue was collected and preserved in RNAlater for further gene analysis at each time point. The single cell suspension was then depleted of myelin using Myelin Removal Beads II (Miltenyi Biotec, Auburn, CA) as per the manufacturer's instructions. Following myelin removal, brain and lymph node cells were immunostained for CD45, CD11b, CD86, CD4, CD8, CD3, MHC II, CD25, FoxP3 (eBioscience, San Diego, CA) and CD206 (BioLegend, San Diego, CA) for 15 minutes at 4°C. Following two wash steps, the cells were fixed in a 1% paraformaldehyde solution for 15 minutes at room temperature. Cells were then resuspended in phosphate buffered saline for flow cytometry. AccuCount blank particles (Spherotech, Lake Forest, IL) were added for absolute quantification and samples were read on a BD FACSCanto within 24 hours of collection.

### **Quantification of NSC proliferation**

Infected and sham-infected brains for quantification of the NSC population were prepared as for immune cell phenotyping described above. Following myelin depletion brains were immunostained for CD45 for 15 minutes at 4°C. Samples were then fixed and permeabilized using the BD CytoFix/CytoPerm kit (BD Biosciences) as per the manufacturer's instructions for 20 minutes at 4°C. Cells were washed two times in CytoFix/CytoPerm wash buffer and stained with anti-nestin PE (BD Biosciences), an



NSC marker, and anti-Ki-67 (Abcam, Cambridge, MA), a proliferation marker, or anti-Sox2 eFluor 670, an NSC marker, and anti-Ki-67 (Abcam) for 20 minutes at 4°C. Use of nestin or Sox2 to identify the NSC population was dependent on antibody availability at time of experimentation. Samples were washed twice in the CytoFix/CytoPerm wash solution and anti-rabbit IgG PE (Ki-67 secondary antibody; Life Technologies) was applied for 20 minutes at 4°C. Finally cells were washed twice more as above and resuspended in PBS. Blank AccuCount particles (Spherotech) were added and the samples were read on a BD FACSCanto within 24 hours.

### **Morris T-modified Water Maze**

Changes in short term memory were measured as described previously (Ganguly and Poo 2013). Briefly, mice entered the T maze in one set arm. Relative position of that arm to visual cues in the pool was unaltered. During the initial training run one arm of the T-maze was blocked using transparent Plexiglas and an accessible platform was found on the opposite arm relative to the one that was blocked off. Test runs immediately followed the training and were conducted identically except the platform was then removed from the previously unblocked arm, such that the animal had to correctly make the opposite turn from the training run in the test run to find the platform. Animals were given 60 seconds to find the platform and then were shown the location. In our paradigm, 30 d p.i. infected and 30 d p.i. sham infected animals were tested and deficits were identified as failure to improve the frequency of correct answers.

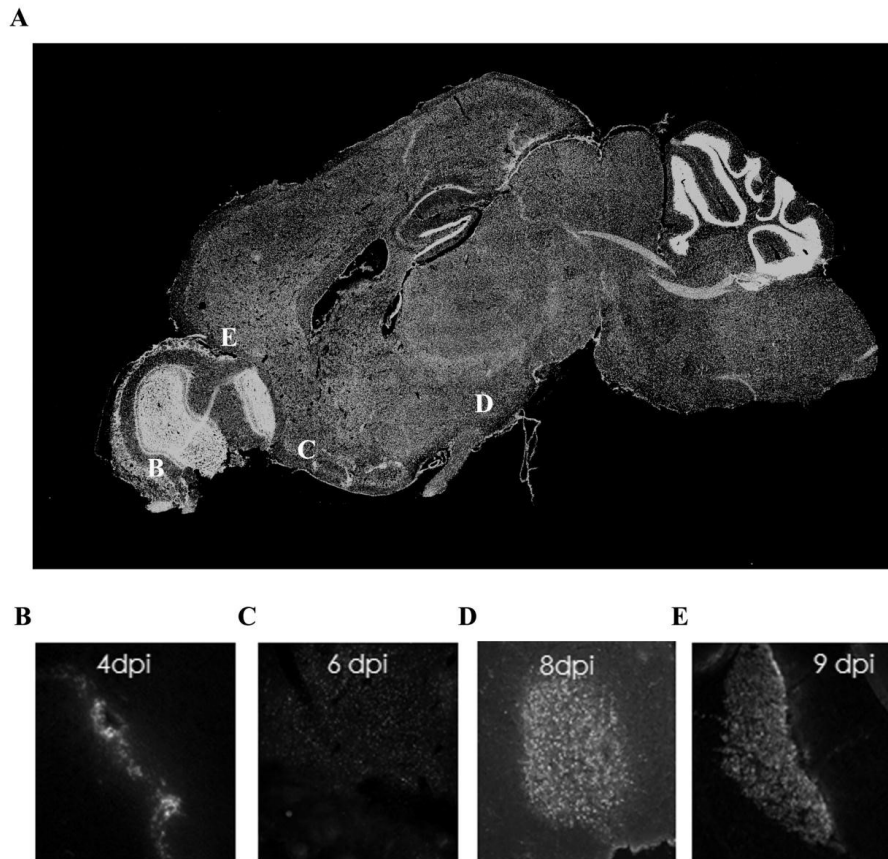
### **Statistics:**

All experiments were analyzed for statistical significance using the Student's T test. Significance is reported at the  $p < 0.05$  and  $p < 0.01$  levels.

## **Results:**

### **HSV-1 Progression through the Brain**

Extensive work looking at the progression of virus in the brains of infected BALB/c mice was done by Armien et al. which demonstrated that the virus has two routes of entry into the brain. The virus first moves through the cribriform plate and into the olfactory bulb as early as 2 days post infection (d p.i.). From the olfactory bulb, the virus progresses through the entorhinal cortex at 5 d p.i. and into the temporal cortex by 7 d p.i.. Secondly, HSV-1 antigen can be seen in the trigeminal ganglion at 5 d p.i. and progresses into the brainstem by 7 d p.i. (Armien, Hu et al. 2010). Both routes of infection result in lesions in the temporal lobe cortex and hippocampus by 7 d p.i.. In the C57BL/6 mouse model, multiple foci were observed as the infection progressed and can be observed schematically in Fig. 1A. Robust labeling of HSV-1 in the mouse brain was observed as early as four days post infection in the olfactory bulb (Fig. 1B). From the olfactory bulb the virus progressed into the cerebral cortex via the anterior olfactory nucleus by 6 d p.i. (Fig. 1C). Infection in the hypothalamus was observed between 7 and 9 days post infection (Fig. 1D) with cortical involvement evident by 9 d p.i. (Fig. 1E). Gene expression data of the HSV-1 gD glycoprotein was elevated more than 3 fold higher than uninfected mice from 3 d p.i. through day 12 (data not shown). Importantly, as in the BALB/c mouse model of HSE, viral antigen in the brains of infected C57BL/6

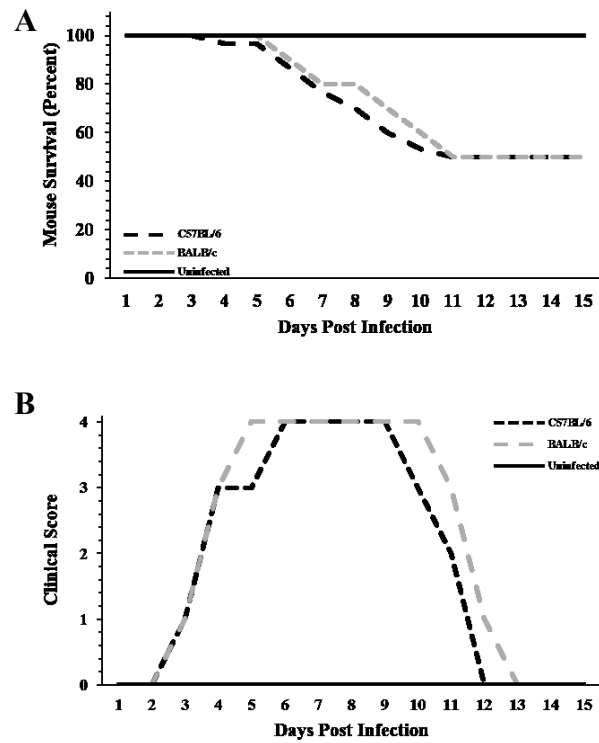


**Figure 7.1: Localization of HSV-1 in C57BL/6 mouse brains.** Conserved regions of HSV-1 intermediate-early protein staining are schematically diagramed on (A) a representative longitudinal brain section. White letters indicate sites of HSV-1 staining shown in (B-E). (B) HSV-1 was first observed in the olfactory bulb prior to progressing into the (C) ventral striatum by 6 d p.i.. Significant ingress was observed by 8 d p.i. when HSV-1 could be found in the (D) hypothalamus and (E) cerebral cortex. Representative images are shown of 5 sections per animal with three animals collected at each time point.

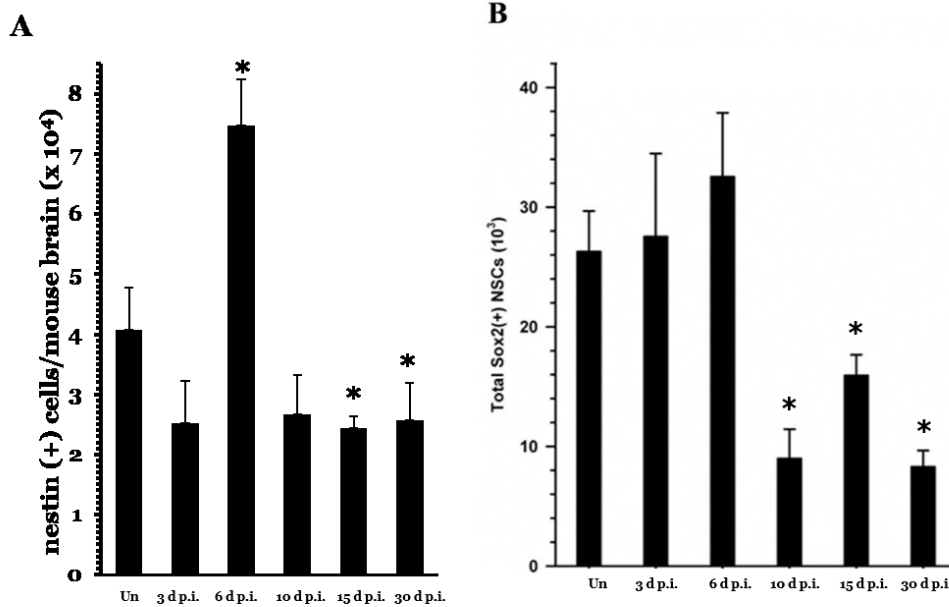
mice became undetectable by 15 d p.i. which demonstrates that immune cell activity continues well after the disease is resolved. Previous reports in the BALB/c mouse model have established that the mortality rate expected is approximately 50% (Marques, Hu et al. 2006; Marques, Cheeran et al. 2008; Armien, Hu et al. 2010; Rotschafer, Hu et al. 2013), which is consistent with the mortality observed in this study (Fig 2A). Peak morbidity was observed in both mouse strains between 5 and 11 d p.i.. Consistent with peak lethality at those time points, clinical disease scores also peaked between 4 and 10 d p.i. in both mouse model systems (Fig. 2B). Importantly the progression of the virus through the brain was localized to discrete regions in both model systems and followed the same morbidity and mortality kinetics as BALB/c mice infected with HSV-1 by intranasal inoculation as presentation of virus lesions as discrete viral lesions are characteristic of the human disease (Margaret 1982).

#### **HSE is associated with alterations in NSC proliferation and learning.**

NSC proliferation in the C57BL/6 mouse model responded dynamically to the infection. Peak NSC numbers (CD45(-)nestin(+)) occurred at 6 d p.i. ( $74733.6 \pm 7730.8$ ,  $p < 0.01$ ) in infected BALB/c mice and total NSC numbers were significantly decreased at 10 d p.i. ( $26680.6 \pm 6604.0$ ,  $p < 0.05$ ; Fig. 3A). Similarly, the C57BL/6 mouse model had peak NSC numbers at 6 d p.i. ( $32548.3 \pm 5332.9$  cells per brain; Fig. 3B) and the NSC population was significantly decreased by 10 d p.i. ( $8987.9 \pm 2451.7$  cells per brain,  $p < 0.01$ ). In contrast to the BALB/c mouse model, the increase observed at 6 d p.i. in C57BL/6 mice was not significantly increased compared to sham infected controls. However, significant ( $p < 0.01$ ) depression of the NSC population persists in infected mice



**Figure 7.2: Survival and clinical scores of HSV-1 infected BALB/c and C57BL/6 mice.** Infected and sham infected mouse survival was charted. **(A)** In both model systems peak mortality occurred between 5 d p.i. and 11 d p.i.. **(B)** Clinical disease scores were consistent between the mouse models. Data presented for three experiments with ten animals per background.



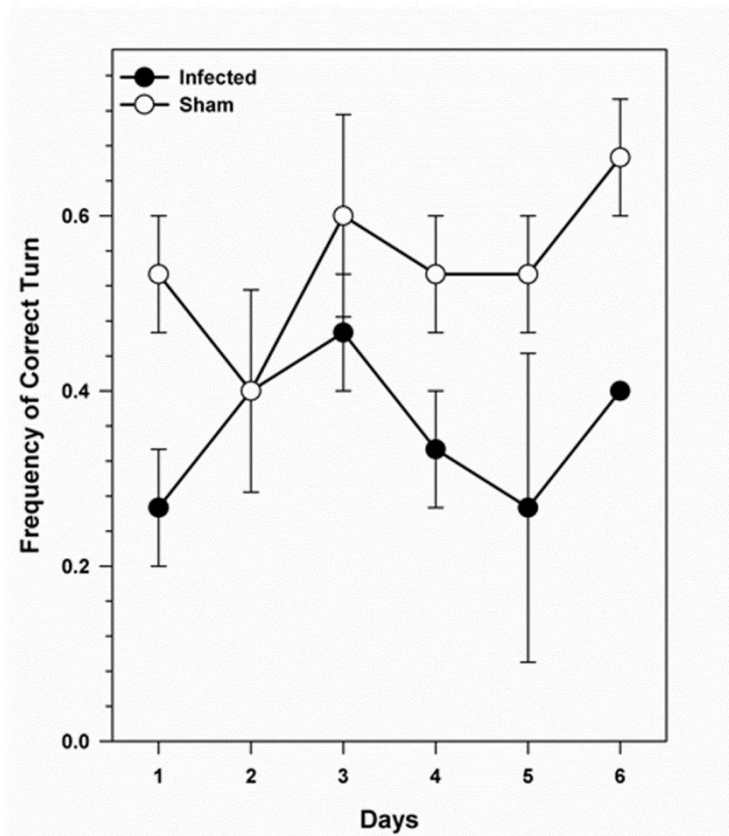
**Figure 7.3: Kinetics of NSC proliferation in response to HSE in BALB/c and C57BL/6 mice.** (A) Total numbers of NSCs in BALB/c mice. (B) In C57BL/6 mice, there is no significant increase in cell numbers. Total numbers of NSCs become significantly decreased at 10 d p.i. and remain decreased through the last time-point measured, 30 d p.i.. Data presented are the average $\pm$ SEM of between five and eight animals per group, \*  $p < 0.01$ .

out to 30 d p.i. in both mouse models which suggests that viral infection has significant impacts on adult neurogenesis consequent to HSE.

Infection of BALB/c mice has been previously demonstrated to result in substantial spatial memory deficits following infection (Armien, Hu et al. 2010). To assess neurological deficits in HSV-1 infected C57BL/6 mice, learning abnormalities were assessed in this model. The level of neurological impairment in infected C57BL/6 mice was assessed using a modified T-Morris water maze task which tests the short term spatial memory in infected animals. Infected mice had a trend of worsened spatial memory as these animals performed poorly in the behavior test compared to their sham-infected C57BL/6 controls. Specifically, infected mice failed to increase their incidence of correct turning in the testing trials whereas sham-infected mice showed a trend of increased frequency of correct turning (Fig. 4), which suggests that the C57BL/6 model is producing neurological deficits consistent both with the BALB/c model as well as the human condition of HSE.

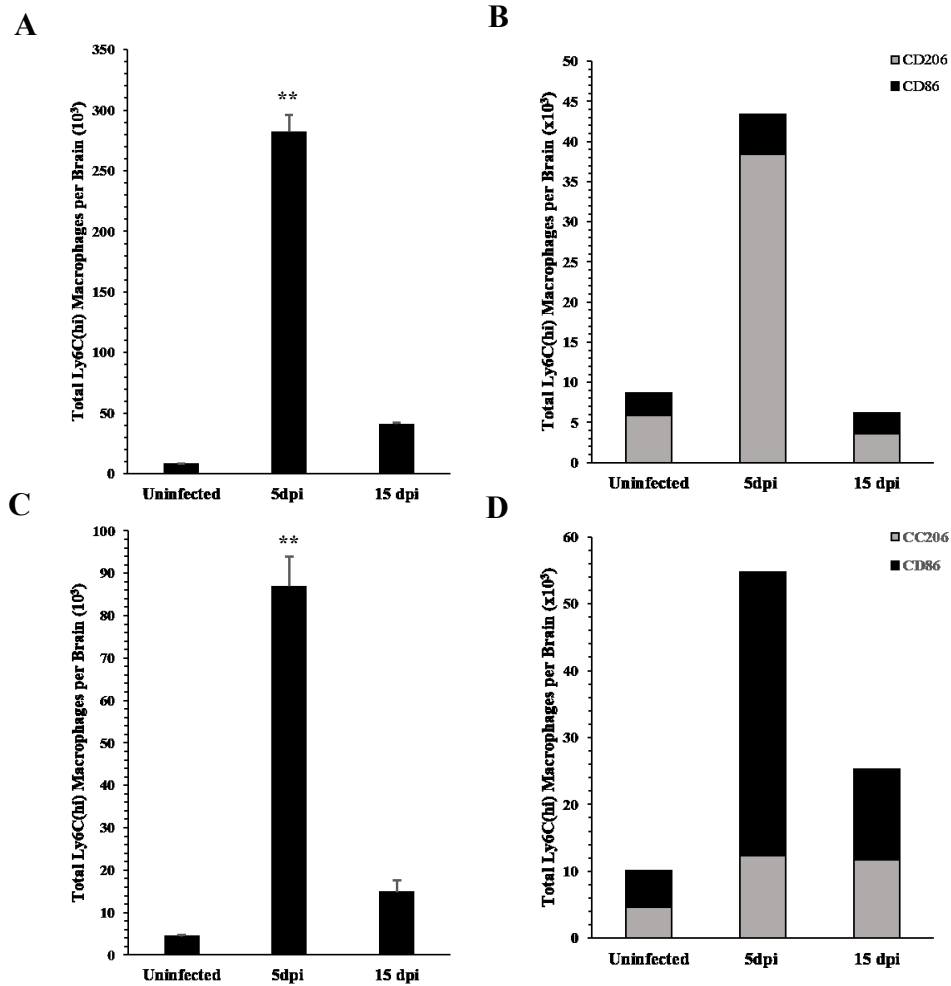
### **Immune Cell Infiltration in the Infected Brain**

Macrophage infiltration had similar trends in both BALB/c and C57BL/6 mice. Total numbers of Ly6C(hi) macrophages peaked at 6 d p.i (86835.1±7100.8 cells per C57BL/6 brain and 282,000 ± 13,880 cells per BALB/c brain,  $p < 0.01$ ; Fig. 5A&C). However, there was a significant ( $p < 0.1$ ) difference in the magnitude of macrophage infiltration between the mouse models. Despite the difference in population size, both populations were reduced by five-fold at 15 d p.i. which suggests that cellular



**Figure 7.4: Infected C57BL/6 mice have decreased short-term memory.** Infected and uninfected animals at 30 d p.i. had their short-term memory tested in a T modified Morris water maze task. Sham (open circles) and infected (black circles) were tested and the frequency of correct turns was measured. Data presented are the average $\pm$ SEM of three animals per group.

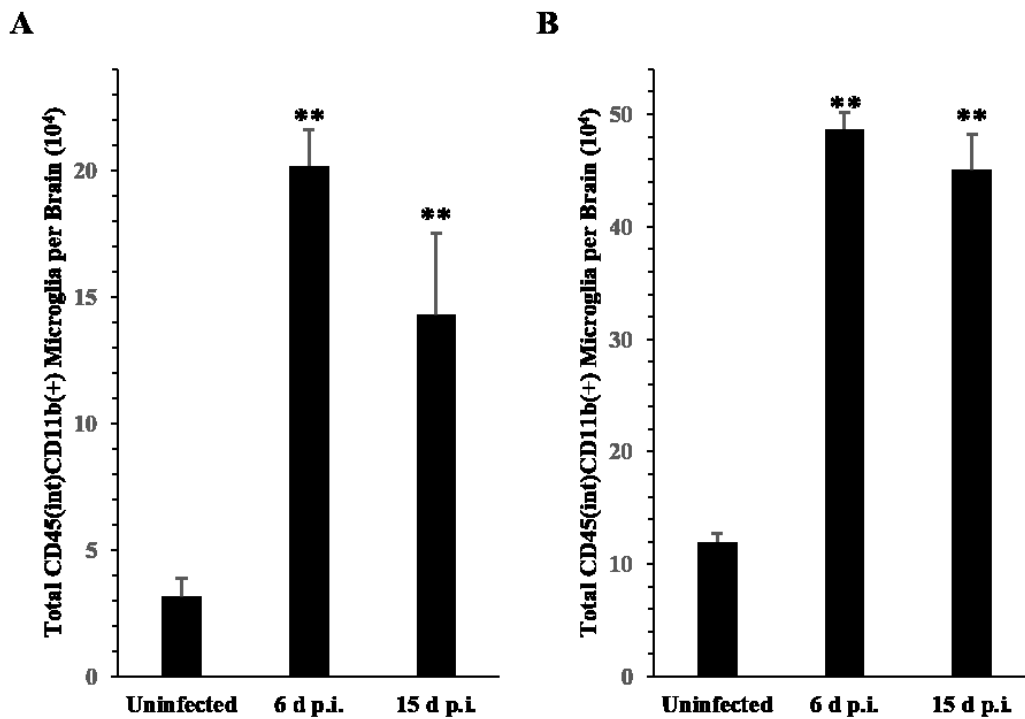




**Figure 7.5: Kinetics of macrophage infiltration and polarization into HSV-1 infected BALB/c and C57BL/6 mouse brains.** Total number of CD45(hi)CD11b(+)Ly6C(hi) infiltrating macrophages peaked at 6 d p.i. in both (A) BALB/c and (C) C57BL/6 mice. M1 polarized macrophages (black bar) was quantified and compared to M2 polarized macrophages (gray bar) in (B) BALB/c mice and (D) C57BL/6 mice. Data presented are averages  $\pm$ SEM of 5 animals per time-point. \*\*  $p \leq 0.01$ .

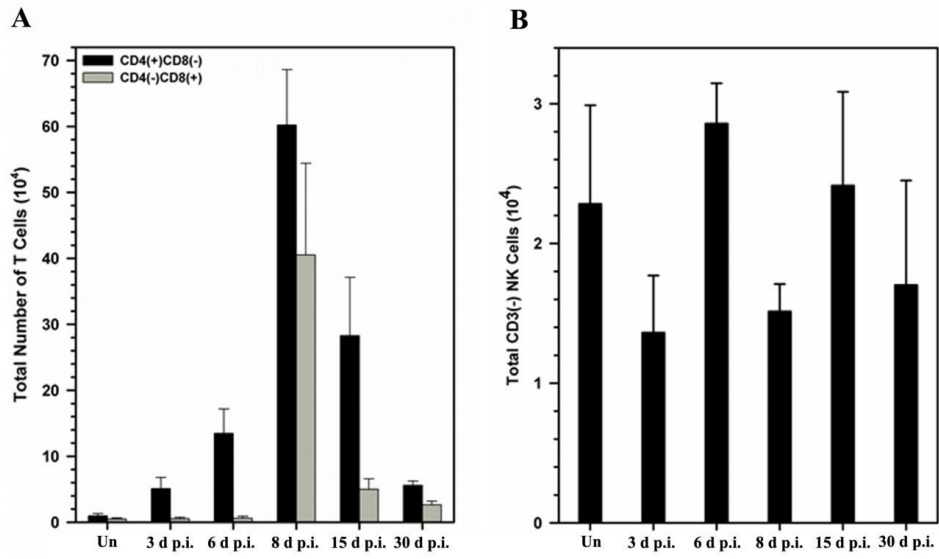
mechanisms controlling macrophage infiltration may be preserved in both model systems. Specific evaluation of the polarization of the infiltrating macrophages revealed notable differences in the population of CD86(+) classically activated macrophages between the two mouse models. BALB/c mice at 6 d p.i. had a ratio of CD86(+) M1 macrophages to CD206(+) M2 macrophages of one to five (Fig 5B) whereas C57BL/6 mice had a ratio of five to one at 6 d p.i. (Fig 5D). The difference in macrophage polarization between mouse models at 5 and 15 d p.i. was statistically significant at the  $p<0.01$  level.

Previous studies in the BALB/c mouse model have demonstrated that microglial cells are robustly activated after mice are infected with HSV-1 (Marques, Cheeran et al. 2008). Activation manifests as significant increases in chemokine production and significant up regulation of major histocompatibility class II (MHCII). As early as 8 d p.i. approximately 80% of microglia (CD45(int)CD11b(+)) in the brain are expressing MHCII whereas uninfected mice had no measureable expression of MHCII. Furthermore, microglia continued to express MHCII past 30 d p.i. (Marques, Cheeran et al. 2008). In the C57BL/6 mouse model system, microglial activation began at 3 d p.i. with a modest increase in the total numbers of MHCII(+) microglia ( $62031.2\pm17556.1$  cells per brain, ns; Fig. 6). Significant increases in numbers of activated microglia activation began at 6 d p.i. ( $201662.7\pm14161.7$  cells per brain,  $p<0.01$ ) and remained significantly elevated at 15 and 30 d p.i. ( $142993.9\pm31846.7$  cells per brain at 15 d p.i.,  $p<0.01$ ;  $103930.0\pm18081.6$  cells per brain at 30 d p.i.,  $p<0.01$ ) which is consistent with what is observed in the BALB/c model system.

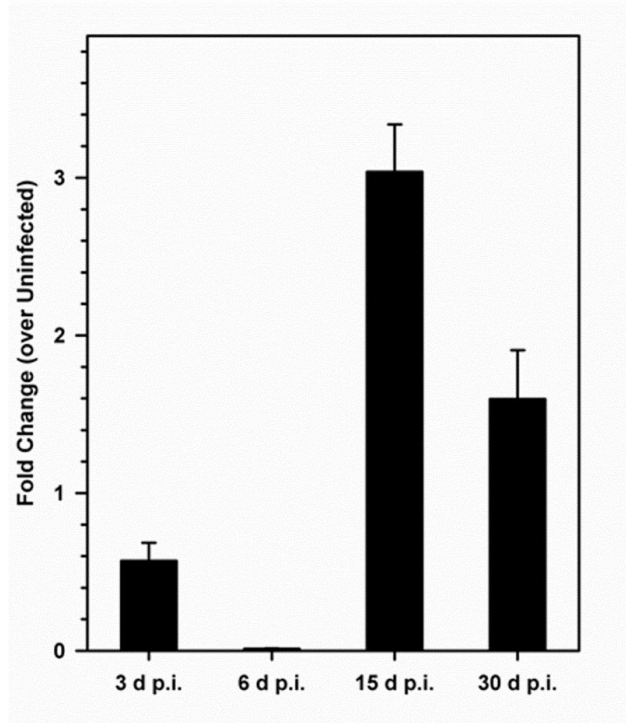


**Figure 7.6: Microglial activation is dynamic during HSE.** Total brain microglia were analyzed for MHCII expression. Notably in (A) C57BL/6 and (B) BALB/c microglial activation stayed elevated at 15 d p.i.. Data presented are averages  $\pm$ SEM of 5 animals per time point,  $p < 0.01$

Kinetics of T cell infiltration were similar between the BALB/c and C57BL/6 mouse model systems. In BALB/c mice notable numbers of T cells (CD45(hi)CD3(+)) infiltrate the infected brain by 8 d p.i. with peak T cell infiltration occurring at 15 d p.i. (Marques, Cheeran et al. 2008). Notably, as the T cell infiltration progressed, the phenotype shifted from one to one CD4(+) to CD8(+) at 8 d p.i. to one to two at 15 d p.i. (Marques, Cheeran et al. 2008). A marked difference between BALB/c and C57BL/6 mice infected with HSV-1 was the timing of peak T cell infiltration. Total numbers of T cells peaked at 8 d p.i. ( $1007353.6 \pm 222926.5$  cells per brain,  $p < 0.01$ ) and although T cells remained elevated through 30 d p.i., total numbers decreased dramatically at 15 d p.i. (Fig. 7A). Furthermore, CD4(+) T cells outnumbered CD8(+) T cells persistently throughout the infection. At 8 d p.i., CD4(+) T cells outnumbered CD8(+) T cells in a three to two ratio ( $601998.8 \pm 84059.0$  CD4(+) T cells per brain vs  $405354.8 \pm 138867.4$  CD8(+) T cells per brain) and by 15 d p.i. CD4(+) T cells outnumbered CD8(+) T cells almost six to one ( $282777.2 \pm 88385.5$  CD4(+) T cells per brain vs  $50028.9 \pm 15916.1$  CD8(+) T cells per brain). On the other hand, interferon- $\gamma$  (IFN- $\gamma$ ) expression was significantly elevated during T cell infiltration and peaked at 15 d p.i. in the C57BL/6 model. IFN- $\gamma$  remained significantly elevated at 30 d p.i. (Fig. 8). IFN- $\gamma$  has been well documented to persist after 15 d p.i. in BALB/c mice and has been shown to inhibit NSC proliferation in Chapters 5 and 7 of this dissertation (Marques, Cheeran et al. 2008). While the kinetics of T cell infiltration was significantly ( $p < 0.01$ ) different in C57BL/6 mice compared to BALB/c mice, the timing and duration of IFN- $\gamma$  expression was not significantly altered between the strains.



**Figure 7.7: CD4(+) T cells dominate the immune response at 15 d p.i.** Total brain T cells were evaluated in uninfected and HSV-1 infected C57BL/6 mice. **(A)** The total number of T cells increased at 8 d p.i. **(B)** Among the NK cell subset there were no significant changes in total numbers. Data presented are averages  $\pm$ SEM of 5 animals per time-point.



**Figure 7.8: IFN- $\gamma$  gene transcription peaks at 15 d p.i.** IFN- $\gamma$  gene expression was undetectable at 6 d p.i. and peaked at 15 d p.i.. Furthermore, IFN-  $\gamma$  expression was elevated past 30 d p.i. Data presented as fold-change over uninfected control animals  $\pm$  SEM of five animals per time-point.

## **NK cells do not affect the course of HSE in C57BL/6 mice**

Given the importance of the NK1.1 gene loci in resistance of C57BL/6 mice to infection with HSV-1 (Kastrukoff, Lau et al. 2010), changes in the NK and NK T cell populations was measured. Surprisingly, numbers of NK and NK T cells did not change significantly at any time post infection including the 3 d p.i. time point which occurred 1 day after the final injection with anti-NK1.1 and anti-CD8a antibodies (Fig. 7B) which suggests that NK cells are consistent throughout the course of infection and do not significantly contribute to the immune response in the brain.

### **Discussion:**

The objective of this dissertation chapter was to develop a new C57BL/6 mouse model of HSE that maintained the viral spread observed in the BALB/c model while altering the acute macrophage phenotype to more closely mirror the human condition. The C57BL/6 mouse infection with HSV-1 resulted in a robust inflammatory response and dynamic alteration in NSC proliferation. HSV-1 viral proteins were observed in the same brain regions of infected C57BL/6 mice as in the BALB/c mouse model at similar time points (Table 7.2). No significant differences in mortality ( $p=0.9378$ ) or clinical disease scores ( $p=0.8734$ ) were observed between infected BALB/c and C57BL/6 mice. Similar to the BALB/c mouse model, total numbers of NSCs were increased at 6 d p.i. and decreased by 10 d p.i. in C57BL/6 mice. Macrophage infiltration kinetics were similar between the strains ( $p=0.2645$ ); however, macrophage phenotype was consistently polarized to classical

Criteria	BALB/c Model	C57BL/6 Model	P-value
<b><i>Gross Findings</i></b>			
HSV-1 Antigen Present	Olfactory bulb at 2 d p.i.,	Olfactory bulb at 4 d p.i.	0.0837
	Entorhinal cortex at 5 d p.i.	Entorhinal cortex at 6 d p.i.	0.2843
	Temporal cortex at 7 d p.i.	Cerebral cortex at 8 d p.i.	0.5786
Viral antigen not detectable	ND at 15 d p.i.	ND at 15 d p.i.	0.8258
Clinical Scores	8 days at 4; mortality at 50%,	7 days at 4; mortality at 50%	0.8734
			0.9378
<b><i>Inflammatory Milieu Findings</i></b>			
Kinetics of NSC proliferation	Peak at 6 d p.i.	Peak at 6 d p.i.	0.2374
	Depressed at 10 d p.i.	Depressed at 10 d p.i.	0.9534
Macrophage Infiltration and Activation	Infiltration peak at 6 d p.i.	Infiltration peak at 6 d p.i.	0.2645
	<b>M1: M2 ratio of 5:1</b>	<b>M1: M2 ratio of 1:5</b>	<b>0.0023</b>
	<b>Persistently M1 dominant</b>	<b>M2 dominant at 6 d p.i.</b> <b>M1 dominant at 15 d p.i.</b>	<b>0.0042</b>
T cell Infiltration and Activation	<b>Peak infiltration at 15 d p.i.</b>	<b>Peak infiltration at 8 d p.i.</b>	<b>0.0062</b>
	<b>CD8(+) dominant at 15 d p.i.</b>	<b>CD4(+) dominant at 15 d p.i.</b>	<b>0.0034</b>
	Robust IFN- $\gamma$ expression at 15 d p.i.	Robust IFN- $\gamma$ expression at 15 d p.i.	<b>0.5963</b>

**Bold indicates significant differences between mouse model systems. P-values measure between the two strains.**

**Table 7.2: Summary of HSE Model Systems.** Brief description of HSE progression in BALB/c and C57BL/6 mouse model systems. While there were many similarities between the model systems, significant differences emerged.



(M1) activation ( $p=0.0042$ ). T cell infiltration was composed primarily of CD4(+) T cells, with peak T cell infiltration occurring at 10 d p.i. ( $p=0.0062$ ). On the other hand, there was robust production of IFN- $\gamma$  at 15 and 30 d p.i. ( $p=0.5963$ ), which suggests that the immune response induced in C57BL/6 was primarily a Th1 polarized response.

Adult neurogenesis in C57BL/6 mice had marked similarities to the BALB/c mouse model of HSE. NSCs have been shown to proliferate robustly in a C57BL/6 mouse model of Japanese encephalitis; however proliferation in this model lasts less than three days before total numbers of NSCs are significantly depressed. This depression lasts through the last time point measured at 9 d p.i. (Das and Basu 2008). This study demonstrated that NSCs in infected C57BL/6 mouse brains respond to infection with HSV-1 with a slight increase in total numbers of cells prior to an abrupt decrease that remains significantly lower than uninfected mice through 30 d p.i.. In contrast, BALB/c experience a more robust increase in proliferation at 6 d p.i. that is significant ( $p<0.01$ ) prior to a notable drop in the total number of NSCs at 10 d p.i. which suggests that, while C57BL/6 NSCs have a dynamic response to HSE, the response is different than the BALB/c mice.

Differences in the NSC response to HSE between the models may be explained by persistent M1 activation of brain infiltrating macrophages during normal NSC proliferation. While the M1 phenotype of macrophages is observed in humans during acute HSE (Conrady, Drevets et al. 2010), previous studies in this dissertation have shown that M1 macrophages are only present during chronic HSE when NSC proliferation is impaired and there is robust IFN- $\gamma$  present (Chapters 5&6). During acute

HSE in BALB/c mice, M2 macrophages are present and NSC proliferation is increased (see chapter 4). In a similar manner, NSC proliferation was reversibly associated with macrophage activation in a C57BL/6 mouse model of Japanese Encephalitis, as treatment with minocycline, an alternative phenotype inducing stimulus, induced a significant increase in NSC proliferation and concurrent decrease in M1 macrophage activation and IFN- $\gamma$  production (Dutta, Mishra et al. 2010). This study found that NSC proliferation was impaired under M1 macrophage dominant conditions in the presence of IFN- $\gamma$  similar to the Japanese Encephalitis, which suggests that the C57BL/6 mouse model of HSE may provide more relevant information on the interactions of macrophages and NSCs during acute viral encephalitis in humans than the BALB/c mouse model.

T cell kinetics during HSE are similar between the two strains despite difference in T cell phenotype. Previous studies in the BALB/c mouse model have demonstrated that T cells begin to infiltrate the infected brain around 6 d p.i. with peak infiltration occurring around 15 d p.i. (Marques, Cheeran et al. 2008). Infiltration in the C57BL/6 mouse model of HSE presented here also was measurable at 8 d p.i., however, peak T cell infiltration also occurred at 8 d p.i.. Moreover, the phenotype of infiltrating T cells was CD4(+) dominant at 8, 15, and 30 d p.i. in C57BL/6 mice. In contrast, the BALB/c mice have equal numbers of infiltrating CD4(+) and CD8(+) T cells at 8 d p.i. and gradual increase in the CD8(+) T cell compartment such that at 15 d p.i. CD8(+) T cells outnumber CD4(+) T cells two to one (Marques, Cheeran et al. 2008). While C57BL/6 mice infected in this study were CD8(+) T cell depleted, pilot studies using the CD8(+) T cell depleting antibody demonstrated that the splenic CD8(+) T cell compartment is

normally restored within 5 days of the last depletion treatment (data not shown), indicating that the CD4(+) dominance is due to the lack of available CD8(+) T cells. Furthermore, despite the preponderance of CD4(+) T cells, there was still robust production of IFN- $\gamma$  in the brains of infected C57BL/6 mice at 15 and 30 d p.i. suggesting that the T cell polarization was Th1 as in the BALB/c mouse model.

Progression of the virus through the brain in infected mice was consistent between BALB/c and C57BL/6 mouse strains. Previous studies using the BALB/c mouse model of disease demonstrated that the primary movement of the virus occurs in a retrograde manner with viral infection limited to specific tracts in the brain even while inflammation occurs at sites distant from actual viral lesions (Webb SJ 1989; Armien, Hu et al. 2010). The present study suggests that intranasal instillation of virus with nasal scarification results in the same progression of the virus in C57BL/6 mice. Gross measures of disease, such as clinical scores were consistent between the mouse strains and suggested that this route of infection resulted in similar kinetics of disease. Furthermore, evaluation of neurological deficits reveal measureable losses in spatial memory in BALB/c mice (Armien, Hu et al. 2010). Similarly, HSV-1 infection in C57BL/6 mice results deficits in spatial memory albeit less severe deficits than those observed in BALB/c mice.

This dissertation chapter describes the use of a C57BL/6 mouse model of HSE which provides a tool for assessing NSC-macrophage interactions during acute HSE. Furthermore, the trends observed in NSC proliferation in the C57BL/6 mouse model affirm the findings of the BALB/c mouse model (Chapters 4&6) which suggest that an M1 dominant macrophage milieu in the presence of IFN- $\gamma$  is connected to impaired NSC

proliferation. Both BALB/c and C57BL/6 mouse models of HSE suggest that the IFN- $\gamma$  is the key mediator of the observed reduction of NSC proliferation. This effect of IFN- $\gamma$  on NSC proliferation may be preserved in human HSE patients, as IFN- $\gamma$  has been found in the cerebrospinal fluid of HSE patients after disease resolution and during peak brain remodeling (Lellouch-Tubiana, Fohlen et al. 2000; James, Kimberlin et al. 2009). Identifying mechanisms to control the production of IFN- $\gamma$  in the brains of human patients may provide an avenue for future studies to improve adult neurogenesis following HSE.

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## **Chapter 8: Thesis Discussion**

## **Conclusions and Limitations:**

Several significant findings related to reparative processes in the HSV-1 infected mouse brain have been described in this dissertation. These studies provide the first documentation that neural stem/progenitor cells (NSCs) proliferate in response to HSV-1 brain infection. Increased NSC proliferation is associated with changes in the macrophage activation phenotype because M2 polarized macrophages were shown to increase NSC proliferation both *in vivo* and *in vitro*, whereas M1 macrophages were associated with decreased NSC proliferation. CD8(+) T cells were found to impair NSC proliferation both *in vivo* and *in vitro* via production of interferon- $\gamma$  (IFN- $\gamma$ ). IFN- $\gamma$  is a potent stimulator of the M1 macrophage phenotype, which is the dominant phenotype observed at 15 d p.i., when NSC proliferation declines below control levels. Expanding beyond the original BALB/c mouse system, a C57BL/6 mouse model of Herpes Simplex encephalitis (HSE) was developed to aid in further assessing the impact of inflammation in altering adult neurogenesis, specifically by exploiting pre-existing knockout mice that have been constructed on the C57/BL/6 genetic background.

## **Kinetics of NSC proliferation**

The kinetic response of NSCs to HSE was dynamic over the course of infection. Total NSC proliferation in this dissertation was defined as the change in numbers of any one of the three cell populations in the brain that are capable of proliferation. The proliferating NSC cell types include: B cells, primordial astroglia-like cells, C cells, transit amplifying cells, and A cells, migratory neuroblasts. The different possible phenotypes of proliferating cells in response to brain damage highlights the complexity of

adult neurogenesis. Reactive neurogenesis, in response to damage, begins when B cells differentiate into transit amplifying cells to generate more C cells and migratory neuroblasts (Doetsch, García-Verdugo et al. 1997; Doetsch, Caillé et al. 1999). All three populations proliferate in close proximity to the neurogenic niche. However, this study did not address each proliferating cell population individually. It is possible that each of these populations responds to different immune cues and contribute disproportionately to brain repair. Additionally, the functional outcomes of NSCs proliferating during acute HSE were not investigated in these studies. NSCs have the capacity to differentiate into neurons, astrocytes and oligodendrocytes under physiological and inflammatory conditions (Arvidsson, Collin et al. 2002). As terminally differentiated neurons and astrocytes cannot innately be reprogrammed into other brain cell types (Brami-Cherrier, Anzalone et al. 2014; Paşca, Panagiotakos et al. 2014), the functional recovery of neuronal circuitry following viral encephalitis requires that adult neurogenesis not only produces neurons, but also successfully replaces damaged neurons by integration into the existing neuronal circuitry.

Alterations in NSC proliferation were associated with concurrent change in fibroblast growth factor-2 (FGF-2) gene expression. NSCs have been shown to proliferate in response to FGF-2 in models of experimental autoimmune encephalitis (EAE) (Brown, Couillard-Després et al. 2003). Similarly, NSCs were found to be responsive to FGF-2 during HSE in Chapter 3 of this dissertation. Similar studies utilizing mouse models of Alzheimer's disease have demonstrated that supplementation of FGF-2 to the damaged brain increases NSC proliferation, concurrent with improved memory development

(Kiyota, Ingraham et al. 2011). In addition, the studies presented in this dissertation also demonstrated that expression of neurotrophin-3 (NT-3) was differentially regulated during acute and chronic HSE, suggesting that NT-3 may also have a role in temporal regulation of NSC proliferation during HSE. While the role of NT-3 was not elucidated in this study, FGF-2 supplementation into the brain increased proliferation of NSCs within 3 days after treatment. Further supporting a role of FGF-2 in neurogenesis initiated during HSE, NSC proliferation was suppressed at time points where FGF-2 was down regulated.

### **Macrophage interactions increase NSC proliferation during acute HSE**

Alternatively activated, M2 macrophages, comprised the largest fraction of infiltrating immune cells at 5 d p.i.. Furthermore, M2 macrophages increased NSC proliferation both *in vivo* and *in vitro*. Increased NSC proliferation in response to M2 macrophages *in vivo* and *in vitro* is consistent with previously established restorative functions of alternatively activated macrophages because M2 polarized macrophages have been shown to promote tissue regeneration in mouse models of stroke (Ekdahl, Kokaia et al. 2009; Zhou, Cheng et al. 2011; Hu, Li et al. 2012), West Nile encephalitis (Getts, Terry et al. 2012), skeletal muscle damage (Arnold, Henry et al. 2007), and allergic asthma (Hong, Chung et al. 2014). However, macrophage polarization is not rigidly fixed but, instead, reflects a spectrum of continuous activation phenotypes making the definitions of “classical” and “alternative” activation intrinsically artificial (Crespo, Bertolotti et al. 2013). Therefore *in vitro* M2-polarized CD206(+) macrophages may not perfectly mimic cells designated as M2 polarized by *in vivo* polarization based solely on

their CD206(+) expression. In a study of Alzheimer's patients classification of macrophage phenotypes in the cerebrospinal fluid into M1 or M2 phenotypes, based on gene expression, concluded that identifying macrophage polarization states in the human was impossible using current known markers (Wilcock 2014). However, regardless of the categories of discernable macrophage phenotypes in the brain, their effects on NSC proliferation based on CD206 expression is similar *in vivo* and *in vitro*, suggesting that the signals associated with macrophage activation play a critical role in modulating neurogenesis.

M2 macrophage induced NSC proliferation was found to be dependent on Wnt5a. The identification of Wnt5a as a possible soluble mediator produced by the immune system that drives NSC proliferation, both *in vivo* and *in vitro*, represents a major new finding. Involvement of the Wnt pathway suggests that embryonic mechanisms used during brain development, including Wnt signaling, may be preserved in adult brains (Masckauchán, Agalliu et al. 2006; Halleskog, Dijksterhuis et al. 2012; Cunningham, Martínez-Cerdeño et al. 2013; Halleskog and Schulte 2013) and that Wnt signaling may potentially be harnessed for therapeutic purposes. Wnt3a has been shown to increase numbers of immature neurons and decrease brain lesion volume in mouse stroke models (Shruster, Ben-Zur et al. 2012). Wnt3a was not found to have a significant impact on neurogenesis in HSE, however, this may explained by the reciprocal inhibition of Wnt3a and Wnt5a expression in the brain (Sato, Yamamoto et al. 2010). While Wnt5a is a prototypical activator of the non-canonical Wnt/ $\beta$ -catenin pathway (Pourreyon, Reilly et al. 2012), Wnt5a has also been shown to interact with different proteins in the canonical

pathway to promote midbrain dopaminergic neuron development (Andersson, Saltó et al. 2013). These findings suggest that the mechanism of NSC proliferation mediated by Wnt5a is more complex than elucidated in the current work and further studies are required to understand the intricacies of the pathway responsible for inducing neurogenesis.

### **CD8(+) T cells impair NSC proliferation during chronic HSE**

In this dissertation, CD8(+) T cells were shown to impair NSC proliferation, both *in vivo* and *in vitro*, through production of IFN- $\gamma$ . The increase in total number of NSCs observed in the absence of CD8(+) T cells in the HSV-1 infected mouse brain suggests that these cells contribute to inhibiting NSC proliferation during chronic HSE.

Concurrent reduction of IFN- $\gamma$  expression in CD8(+) T cell depleted mice and loss of the inhibitory effect in IFN- $\gamma$  receptor 1 knockout mice (despite the presence of IFN- $\gamma$  producing CD8(+) T cells), suggests that the effect on NSC proliferation is mediated by IFN- $\gamma$ . In the present studies, depletion of CD8(+) T cells required the installation of *intracerebroventricular* cannulas into the lateral ventricles as the blood-brain barrier is intact at 15 d p.i. (data not shown). CD8(+) T cells are required to clear the active HSV-1 infection (Mackay, Wakim et al. 2012), however cannula placement resulted in the transection of the right hippocampus which complicates interpretation of behavioral testing data in treated mice. Even though behavioral assessments were not fully interpretable in these studies, an increase in NSC proliferation was observed in CD8(+) T cell depleted mice, suggesting that an improvement in neurological deficits may be associated with depleting CD8(+) cells during the chronic phase of HSE. An important

future direction of these studies is to assess learning and memory in mice lacking CD8(+) T cells using a less intrusive method to remove these cells from the brain.

Perhaps the most important finding of these studies is the role of IFN- $\gamma$  in impairing NSC proliferation *in vivo* and *in vitro*, as IFN- $\gamma$ 's effects may be seen in many types of brain damage. In these studies, the NSC population in infected IFN- $\gamma$ R1 knockout mice was unaffected by infection with HSV-1, which suggests that inhibition of NSC proliferation is mediated by IFN- $\gamma$ . On the other hand, these findings also raise questions of whether IFN- $\gamma$ 's effects on NSC proliferation are mediated by direct interaction with NSCs or indirectly through macrophage activation during HSE. Additional research may be needed to tease out the contribution of direct and indirect mechanisms by which IFN- $\gamma$  inhibits NSC proliferation. Understanding the mechanisms of neurological damage following HSE may be dependent on identifying the specific contributions of IFN- $\gamma$  to inhibition of neurogenesis and development of brain damage at sites distal to the neurogenic niches. However, these studies only utilize the BALB/c mouse genetic background. In-bred mouse strains are known to have significant differences in generating immune responses to similar stimulation (Fiorentino, Bond et al. 1989) that decreases the applicability of these findings to out-bred populations, such as humans.

### **Alterations in NSC proliferation dependent on mouse genetic background**

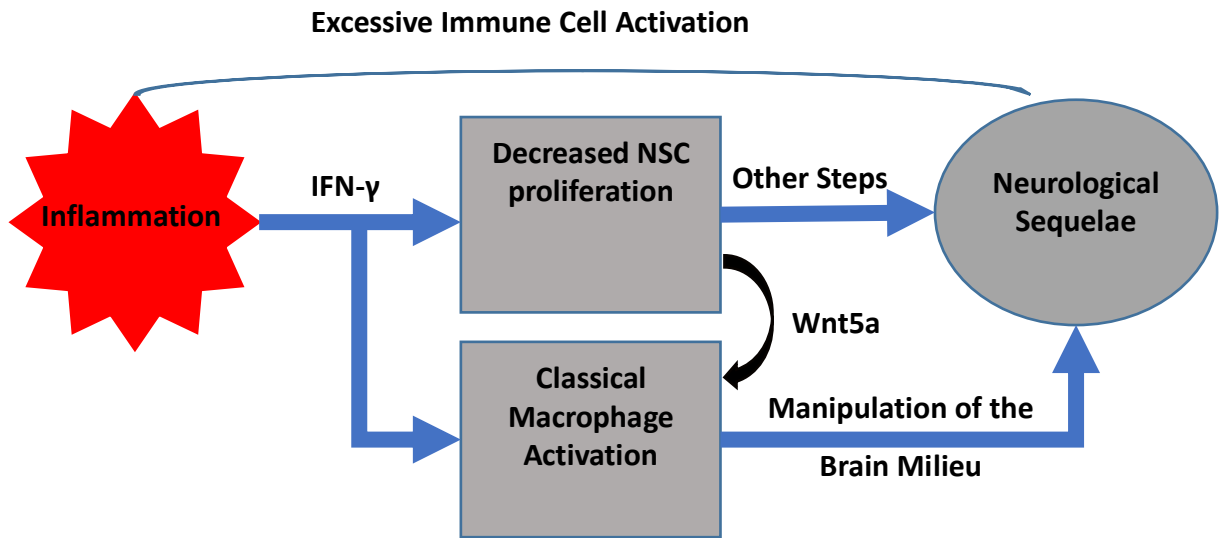
To begin to address the impact of genetic background on neurogenesis discussed in this chapter, a C57BL/6 mouse model for HSE was developed. Despite notable differences in the peak of T cell infiltration and the phenotype of infiltrating T cells,

many other similarities in the immune response was observed between the two strains. Particularly, the prominent presence of M1 macrophages during the chronic phase of infection, the robust decrease in NSC proliferation, and the prominent production of IFN- $\gamma$  in the chronic phase of HSE are similar in both models. This finding suggests that the C57BL/6 model may be useful for further assessment of the role of IFN- $\gamma$  in the brains of mice surviving HSE.

### **Summary**

Collectively, the findings in this dissertation demonstrate that the inflammatory milieu has a profound influence on the brain's reparative processes, both by direct and indirect mechanisms. A summary of the conclusions and future directions of these studies is represented diagrammatically in Figure 8.1. These studies demonstrated that inflammation resulting from HSV-1 brain infection decreases NSC proliferation under conditions that promote classical macrophage activation phenotype. In this context, the ongoing inflammatory processes may contribute directly to the development of neurological sequelae. Future directions for this research should primarily focus on the steps in adult neurogenesis that follow NSC proliferation. In addition, further manipulation of the brain milieu to direct inflammatory processes that drive neurogenesis will be critical to understand the contribution of the interaction between the two systems, viz. immune system, and neurogenesis. Specific points of interventions identified in this dissertation as having therapeutic potential are mechanisms that reduce IFN- $\gamma$  levels in the post-encephalitic brain and mechanisms to increase the concentration of Wnt5a in the





**Figure 8.1: Summary of thesis limitations and future directions.** Previous studies have established that existing immune cell activation contributes to the development of neurological sequelae. The major findings of this dissertation are found to the left of the boxes and items to the right of the boxes detail the limitations of the current studies as well as important future directions suggested by the findings of this dissertation (Wnt5a and IFN- $\gamma$ ).

brain to increase adult neurogenesis. These interventions, while promising, require further evaluation to assess safety and efficacy. The studies presented in this dissertation clearly establish that controlling neuroinflammation is a prerequisite for any successful attempt to induce NSC proliferation or brain repair. Ultimately, these insights may facilitate the development of therapeutic interventions to achieve life altering improvements for viral encephalitis survivors.

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