Impact of anti-viral immunity on neural stem/progenitor cell activity and implications for CNS development

Apurva Kulkarni

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IMPACT OF ANTI-VIRAL IMMUNITY ON NEURAL STEM/PROGENITOR CELL ACTIVITY AND IMPLICATIONS FOR CNS DEVELOPMENT

A Dissertation
Submitted to the Graduate School of Pharmaceutical Sciences

Duquesne University

In partial fulfillment of the requirements for
the degree of Doctor of Philosophy

By
Apurva Kulkarni

August 2017
IMPACT OF ANTI-VIRAL IMMUNITY ON NEURAL STEM/PROGENITOR CELL ACTIVITY AND IMPLICATIONS FOR CNS DEVELOPMENT

By

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Approved 5th June 2017

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ABSTRACT

IMPACT OF ANTI-VIRAL IMMUNITY ON NEURAL STEM/PROGENITOR CELL ACTIVITY AND IMPLICATIONS FOR CNS DEVELOPMENT

By
Apurva Kulkarni
August 2017

Dissertation supervised by Lauren A. O’Donnell

Viral infection and inflammation in the central nervous system (CNS) can cause neuropathology, particularly in the prenatal and neonatal stages. Severe damage to the CNS may result from cytopathic effects of viral infection or from the immune response that may lyse virally-infected cells or release inflammatory mediators to mediate viral clearance. Neural stem/progenitor cells (NPSCs) are multipotent cells in the CNS that are often disrupted by neurotropic viral infections. They may be directly infected by the virus or respond to inflammatory cytokines released from resident as well as infiltrating immune cells. This bystander effect may affect NSPC differentiation and proliferation depending on the milieu of inflammatory mediators. Interferon gamma (IFNγ), a potent antiviral cytokine required for the control and clearance of many CNS infections, can differentially affect cell survival and cell cycle progression depending upon the cell type and the profile of activated intracellular signaling molecules. Here, we show that IFNγ
inhibits proliferation of primary NSPCs through dephosphorylation of the tumor suppressor Retinoblastoma protein (pRb), which is dependent on activation of Signal Transducers and Activators of Transcription-1 (STAT1) signaling pathways. We observed inhibition of proliferation in wild type NSPCs (WT/NSPCs) as well as a decrease in neurosphere growth. IFN\(\gamma\) restricted cell cycle progression by inhibiting the G1- to S-phase transition. Cell cycle restriction was associated with decreases in the G1–phase specific cyclin E/CDK2 proteins and in pRb phosphorylation at serine 795 (S795). Together, these results indicate that the NSPC cell cycle was restricted in the late G1-phase. In STAT1-deficient (STAT1-KO) NSPCs, the effects of IFN\(\gamma\) on NSPC proliferation were lost, demonstrating that IFN\(\gamma\) signaling is STAT1-dependent. These data define a mechanism by which IFN\(\gamma\) could contribute to a reduction in NSPC proliferation in inflammatory conditions. Furthermore, this was the first study to implicate the pRb protein in mediating anti-proliferative effects of IFN\(\gamma\) on NSPCs.

The cellular tropism of neurotropic viruses varies, with NSPCs being targeted by some viruses and spared by others. During a viral infection, microglia are typically the first immune cells to become activated in the brain. Microglia may contribute to the anti-viral program generated against the virus and/or alter other neural cells through the release of inflammatory mediators. Evidence in neonatal brains suggest that microglia can also influence NSPC numbers and differentiation under basal conditions. However, whether microglia affect NSPCs during an anti-viral immune response is an outstanding question. To evaluate the effects of microglial activation on NSPCs, we used a mouse model for measles virus (MV) infection in neurons. In this model, MV infection is restricted to mature CNS neurons expressing the human isoform of CD46, a receptor for MV. NSPCs and microglia are spared from infection. In order to examine the interactions between infected neurons, microglia, and NSPCs, primary microglia were co-cultured with MV-infected CD46+ neurons and
the conditioned medium was used to treat primary NSPCs in culture. We found that factors released from the infected neuron/microglia co-cultures increased BrdU-incorporation and neuronal differentiation in NSPCs. Thus, even though the NSPCs are not infected in this model, the cells respond by generating young neurons that could serve as potential replacements for the mature neurons damaged by the virus. These studies provide a novel model system for identifying the signals that microglia use to communicate between infected neurons and responding NSPCs.
I thank Lauren A. O’Donnell, my advisor and mentor, for giving me the opportunity to work with her, for her constant support and allowing me grow as a researcher. Her advice and encouragement has been pivotal to my journey as a graduate student. Her enthusiasm and drive for research was ‘infectious’.

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>iv</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>vii</td>
</tr>
<tr>
<td>Chapter 1: Literature Review</td>
<td>1</td>
</tr>
<tr>
<td>NSPCs in the developing and adult CNS</td>
<td>1</td>
</tr>
<tr>
<td>Viral infections in the CNS</td>
<td>5</td>
</tr>
<tr>
<td>Human Cytomegalovirus (HCMV)</td>
<td>5</td>
</tr>
<tr>
<td>Herpes Simplex Virus-1 (HSV-1)</td>
<td>6</td>
</tr>
<tr>
<td>Zika Virus (ZIKV)</td>
<td>7</td>
</tr>
<tr>
<td>West Nile Virus (WNV)</td>
<td>8</td>
</tr>
<tr>
<td>Japanese Encephalitis Virus (JEV)</td>
<td>9</td>
</tr>
<tr>
<td>Borna Disease Virus (BDV)</td>
<td>9</td>
</tr>
<tr>
<td>Measles Virus (MV)</td>
<td>10</td>
</tr>
<tr>
<td>Effects of antiviral immunity on NSPC activity</td>
<td>11</td>
</tr>
<tr>
<td>Herpes Simplex Virus-1 (HSV-1)</td>
<td>14</td>
</tr>
<tr>
<td>Cytomegalovirus (CMV)</td>
<td>15</td>
</tr>
<tr>
<td>Zika Virus (ZIKV)</td>
<td>17</td>
</tr>
<tr>
<td>Measles Virus (MV)</td>
<td>18</td>
</tr>
</tbody>
</table>
Borna Disease Virus (BDV) .................................................................................................................. 21
NSPCs in other neurodegenerative and neuroinflammatory disease........................................... 23
IFNγ and its role in mediating NSPC activity....................................................................................... 28
IFNγ: canonical and non-canonical signaling pathways................................................................. 28
IFNγ-mediated signaling in NSPCs................................................................................................. 30
Role of IFNγ in viral infections and effects on NSPCs................................................................. 33

Chapter 2: Materials and Methods.................................................................................................. 37

Cell culture......................................................................................................................................... 37
Neural stem/progenitor cells (NSPCs) ......................................................................................... 37
Microglia-Neuron co-culture system............................................................................................... 38
Neurosphere assay.......................................................................................................................... 42
Bromodeoxyuridine (BrdU) Assay.................................................................................................... 43
Single pulse BrdU assay in neurospheres....................................................................................... 43
BrdU pulse-chase assay.................................................................................................................... 44

Terminal Deoxynucleotidyl Transferase dUTP Nick End

Labeling (TUNEL) Assay................................................................................................................. 45
Carboxyfluorescein succinimidyl ester (CFSE) assay..................................................................... 45
Immunofluorescence assay............................................................................................................... 46
Western blot....................................................................................................................................... 47
Quantitative reverse transcription polymerase chain reaction (qRT-PCR) ..... 48
RNA extraction.................................................................................................................. 48
Reverse transcription......................................................................................................... 49
Quantitative reverse transcription polymerase chain reaction (qRT-PCR) 49
NSPC characterization........................................................................................................ 50
Immunocytochemistry (ICC) ............................................................................................ 50
Flow cytometry.................................................................................................................. 51
In-cell Western (ICW) assay.............................................................................................. 54
Statistical analyses............................................................................................................ 55

Chapter 3: Assessment of the role of interferon-gamma signaling in neural stem/progenitor cell proliferation and differentiation................................................................................................................................. 58
Rationale........................................................................................................................... 58
Characterization of mouse E 12.5 NSPC: Neurosphere and monolayer cultures. 60
IFNγ inhibits neurosphere growth.................................................................................... 68
NSPCs proliferation is restricted at the G1/S checkpoint in response to IFNγ. 73
IFNγ alters activation and expression of STAT1 and STAT3 in NSPCs...................... 75
IFNγ decreases site-specific phosphorylation of pRb and expression of late G1/S cyclin/cdk complexes............................................................................................................................... 81
STAT1 is crucial for IFNγ-mediated inhibition of NSPC proliferation....................... 85
STAT1 mediates the effects of IFNγ on pRb phosphorylation and cyclin/cdk expression

IFNγ reduces neuronal differentiation in NSPCs

Discussion

Chapter 4: Effects of neuron-microglia interactions on NSPC activity

Rationale

Neuron-Microglia co-culture model

Conditioned medium from MV-infected neuron-microglia co-culture increases NSPC differentiation

Media from MV-infected neuron-microglia co-culture causes a modest Increase in DNA synthesis

Discussion

Conclusion

References
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Current hypotheses for IFNγ-mediated effect on NSPC activity</td>
<td>26</td>
</tr>
<tr>
<td>Figure 2</td>
<td>In vitro NSPC cultures contain low levels of differentiated neuronal and astrocytic cells.</td>
<td>62</td>
</tr>
<tr>
<td>Figure 3</td>
<td>NSPC cell density and Nestin levels increase over time</td>
<td>65</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Monolayer cultures form E 12.5 CNS express classical biomarkers of NSPCs</td>
<td>66</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Neurosphere cells express IFNGγR1.</td>
<td>67</td>
</tr>
<tr>
<td>Figure 6</td>
<td>IFNγ inhibits neurosphere growth in a concentration-dependent manner</td>
<td>70</td>
</tr>
<tr>
<td>Figure 7</td>
<td>IFNγ restricts cell cycle progression in NSPCs and induces minimal apoptosis</td>
<td>72</td>
</tr>
<tr>
<td>Figure 8</td>
<td>IFNγ decreases NSPC cell cycle progression</td>
<td>77</td>
</tr>
<tr>
<td>Figure 9</td>
<td>IFNγ reduces proliferation rate in NSPCs</td>
<td>78</td>
</tr>
<tr>
<td>Figure 10</td>
<td>NSPCs activate STAT1 and STAT3 upon IFNγ stimulation</td>
<td>79</td>
</tr>
<tr>
<td>Figure 11</td>
<td>IFNγ modulates the expression of cell cycle checkpoint proteins and the phosphorylation of pRb in NSPCs.</td>
<td>83</td>
</tr>
<tr>
<td>Figure 12</td>
<td>STAT1 is required for IFNγ-mediated inhibition of neurosphere growth</td>
<td>87</td>
</tr>
<tr>
<td>Figure 13</td>
<td>STAT1 loss leads to faster growth of neurospheres</td>
<td>89</td>
</tr>
<tr>
<td>Figure 14</td>
<td>IFNγ-mediated regulation of cell cycle progression is STAT1-dependent in NSPCs</td>
<td>90</td>
</tr>
<tr>
<td>Figure 15</td>
<td>IFNγ-mediated decrease in NSPC proliferation rate is STAT1-dependent</td>
<td>95</td>
</tr>
<tr>
<td>Figure 16</td>
<td>STAT1 is required for IFNγ-mediated decrease in cell cycle progression in astrocytes.</td>
<td>99</td>
</tr>
</tbody>
</table>
Figure 17: IFNγ activates STAT3, but does not inhibit cyclin E expression or pRb phosphorylation at S795, in the absence of STAT1......................................................... 100

Figure 18: IFNγ inhibits neuronal differentiation................................................................. 104

Figure 19: Measles virus (MV) infects mature neurons but spares nestin expressing neural stem/progenitor cells (NSPCs). ................................................................. 115

Figure 20: Neuron-microglia co-culture and treatment of NSPCs with conditioned medium......................................................... 119

Figure 21: MV infected CD46+ neurons in vitro. ................................................................. 120

Figure 22: Microglia in MV-infected co-cultures demonstrate changes in morphology......................................................... 121

Figure 23: Measurement of changes in NSPC differentiation and cell cycle progression........ 127

Figure 24: Enhanced NSPCs differentiation and BrdU incorporation upon treatment with conditioned medium......................................................... 128

Figure 25: Enhanced neuronal differentiation in response to treatment with MV-infected neurons co-cultured with microglia......................................................... 129

Figure 26: Reduced GFAP expression in NSPCs post conditioned medium treatment.......... 130

Figure 27: Changes in NSPC morphology upon treatment with conditioned medium......... 135

Figure 28: Immune response to neurotropic viral infections mediates changes in NSPC activity.................................................................................................................................................. 141
LIST OF TABLES

Table 1: Neurotropic Viruses and associated neuropathologies................................................. 4
Table 2: Neurotropic viruses and immune-mediated changes in NSPC activity......................... 13
Table 3: Antibodies for ICC.................................................................................................................. 52
Table 4: Antibodies for flow cytometric characterization of NSPCs. ....................................... 53
Table 5: Antibodies for ICW characterization of NSPCs. .............................................................. 56
Table 6: Summary of DCX+ cells post conditioned medium treatment..................................... 125
Chapter 1: Literature Review

I. NSPCs in the CNS

Neural stem cells are stem cells of the nervous system that can self-renew and generate both neurons and glia. They can self-renew and proliferate without limit, to produce progeny cells which terminally differentiate into neurons, astrocytes and oligodendrocytes. The term ‘progenitor cell’ refers to the cells that can proliferate and differentiate into more than one cell type. Neural progenitor cells can therefore be unipotent, bipotent, or multipotent. A distinguishing feature of neural progenitor cells is that, unlike a stem cell, it has a limited proliferative ability and does not exhibit self-renewal. For the purpose of this study we will refer to them collectively as neural stem/progenitor cells (NSPCs), which are the only multipotent population of cells in the CNS. Together, these cells bring about normal CNS development in the embryo where they populate the CNS broadly, while their anatomical localization becomes restricted as the brain matures\textsuperscript{1,2}.

NSPCs in developing and adult CNS

CNS development begins with the differentiation of neuroepithelial cells (NECs) from the ectodermal layer. These cells line the cerebral ventricles early in embryonic development. NECs switch from symmetric to asymmetric division, which results in the generation of committed progenitor cells. Cortical neurogenesis begins around embryonic day (E) 9–10 in the mouse, and neuroepithelial cells at this stage acquire features associated with glial cells, called radial glial (RG) cells\textsuperscript{3}. Formation of the cortical layers and thickening of the cortex increases the length of the pial-directed radial processes of RG. These processes are formed as result of extensive cytoskeletal changes in the radial glia,
and ultimately serve as scaffolds to direct newly born neurons to the edge of the cortex. The RG express a number of glial markers such as astrocyte-specific glutamate transporter (GLAST) and brain lipid-binding protein (BLBP). Like neuroepithelial cells, RG cells maintain apical-basal polarity, line the lateral ventricles, and undergo interkinetic nuclear migration, thereby maintaining a pseudostratified epithelium within the ventricular zone. A unique characteristic of RG cells is the interkinetic nuclear migration seen in different phases of the cell cycle. Nuclei undergoing S phase of the cell cycle form a layer several cell-body diameters from the ventricle, at the apical side of the VZ, while nuclei in M-phase line up along the surface of the ventricle, and nuclei in G1 and G2 phases are transitioning between the S and M phases in the mid-region. The function of this phenomenon is still not clear. Regardless, RG either generate daughter neurons directly or produce a second, more restricted intermediate progenitor cells (IPCs), also referred to as a basal progenitor cell, which populates the embryonic subventricular zone (SVZ).

As CNS development proceeds, the number of NSPCs progressively declines. In mice at E10.5, NSPCs amount to about 50% of the total cell number. This number declines to about 1% on postnatal day 1 (P1). A small population of NSPCs persists in specific niches in the adult brain, namely within the SVZ and the subgranular zone (SGZ) of the dentate gyrus (DG). The SVZ is located along the lateral wall of the lateral ventricle. NSPCs in the SVZ differentiate into neuroblasts that migrate along the rostral migratory stream into the olfactory bulb. In the olfactory bulb, these neuroblasts terminally differentiate into granule and periglomerular neurons. NSPCs in the SGZ of the DG give rise to granule neurons, which are important in learning and memory. However, the number of NSPCs in the adult SVZ are limited and therefore, for the purposes of this study,
NSPCs were derived from mouse E12.5 cortical tissue. Adult neurogenesis from the SGZ is affected in many pathologies such as epilepsy and mood-related disorders. Natural aging and age-related neurodegenerative diseases may also result in changes in neurogenesis\textsuperscript{11}. Comparative studies between embryonic, neonatal, and adult SVZ-derived NSPCs showed that these cells significantly differ in their gene expression profiles\textsuperscript{12}. The authors found changes in gene expression patterns in NSPCs from E13, E17, and adult SVZ NSPCs. As an example, the expression pattern of the neurogenic and gliogenic genes followed a specific pattern. At mid gestation (E13), neurogenic genes were highly expressed. This expression went down in late gestation (E17) and increased in the adult SVZ. During mid-gestation, genes that induce gliogenesis were highly expressed. Moreover, NSPCs at different ages differ in their proliferative potential and differentiation owing the differences in their gene expression profiles and epigenetic differences\textsuperscript{13}. Considering that these cells change in their gene expression profiles at different ages, how age-dependent changes in these cells contribute to disease progression at different stages of development needs to be studied.
Table 1

<table>
<thead>
<tr>
<th>Virus</th>
<th>CNS pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytomegalovirus</td>
<td>Deafness, encephalitis, epilepsy, blindness</td>
</tr>
<tr>
<td>Herpes Simplex Virus</td>
<td>Encephalitis, blindness, herpetic neuralgia</td>
</tr>
<tr>
<td>Zika Virus</td>
<td>Microcephaly, agyria</td>
</tr>
<tr>
<td>Lymphocytic choriomeningitis virus</td>
<td>Developmental deficits, hydrocephalus</td>
</tr>
<tr>
<td>West Nile Virus</td>
<td>Encephalitis, meningitis, myopathy, paralysis</td>
</tr>
<tr>
<td>Human Immunodeficiency virus</td>
<td>Dementia, loss of motor function, encephalitis, myelitis</td>
</tr>
<tr>
<td>Borna Disease Virus</td>
<td>Schizophrenia, depression</td>
</tr>
<tr>
<td>Measles Virus</td>
<td>Encephalitis (ADME, SSPE, MIBE)</td>
</tr>
<tr>
<td>Japanese Encephalitis Virus</td>
<td>Seizures, acute flaccid paralysis</td>
</tr>
<tr>
<td>Coxsackie B virus</td>
<td>Epilepsy</td>
</tr>
<tr>
<td>Varicella Zoster Virus</td>
<td>Vasculopathy of small and large cerebral vessels.</td>
</tr>
</tbody>
</table>

Table 1: Neurotropic Viruses and associated neuropathologies.
II. Viral infections in the CNS

Among the 61 known viral families, twelve families are known to infect the CNS. There is a substantial diversity in the neurotropic mechanisms of these viruses\textsuperscript{14}. In the human host, they may enter the CNS by blood and cerebrospinal fluid (CSF) or within infected leukocytes. The neuropathologic manifestations of most viral infections depend on the specific virus, the age and immune status of the patient, and the coexistence of other neurologic diseases and infections altering the blood-brain barrier. The characteristic neuropathologies of some of the viruses relevant to this study are briefly described here and summarized in Table 1.

**Human Cytomegalovirus (HCMV)**

HCMV is a double stranded DNA virus (dsDNA) and belongs to the *herpesviridae* family. In adults, HMCV is observed in immunocompromised patients, and most commonly in acquired immunodeficiency syndrome (AIDS) patients. HCMV-induced neurological disorders include longitudinal extensive transverse myelitis and encephalitis and subcortical dementia\textsuperscript{15}. HCMV neuropathology may manifest as encephalitis with microglial nodules frequently associated with cytomegalic cells or as necrotizing ventriculoencephalitis with intranuclear inclusion bodies. Congenital CMV infections are a major cause of birth defects in the US, with approximately 0.5% newborns infected by transplacental transfer from the mother\textsuperscript{16}. Ten to fifteen percent of these infections develop neurological sequelae such as hearing loss, mental impairments, and microcephaly\textsuperscript{17}. Thus, the developing brain is especially susceptible to the pathogenic effects of CMV infection. Imaging studies in developing fetuses reveal major structural abnormalities including focal
necrosis, astrogliosis, calcifications and brain atrophy. Similar abnormalities were also found in infected neonates. HCMV has shown tropism towards all cell types in the brain. *In vitro* studies have shown that CMV could infect microvascular endothelial cells, astrocytes, neurons, microglia and NSPCs. Primary postnatal astrocyte cultures show CMV-induced cytopathic cell death. In the CNS, CMV infection in the astrocytes induced apoptotic cell death when infection was at its peak. This could be possibly to allow for completion of viral replication cycle\(^{18}\). NSPCs also are permissible to HCMV infection. HCMV induces apoptosis in NSPCs through the activation of endoplasmic reticulum stress and mitochondrial dysfunction, which may partially explain the brain atrophy observed in many cases of HCMV infection.

*Herpes Simplex Virus-1 (HSV-1)*

HSV-1, also a dsDNA virus belonging to the *herpesviridae* family, infects approximately 67% of adults in the USA\(^{19}\). In most cases, the virus resides latently in sensory neurons of the trigeminal ganglion with intermittent bouts of reactivation\(^{19}\). During reactivation, new infectious viral particles are produced that travel down the axon and infect epithelial cells at the site of neuronal innervation, leading to the typical lesions associated with HSV-1. However, in some cases, HSV-1 may spread from the trigeminal ganglia to the temporal and inferior frontal lobes to establish a more severe, widespread CNS infection. The viral particles can be detected both in cytoplasm and nucleus of the infected neurons, astrocytes, and oligodendrocytes. The virus spreads via cell-to-cell contact and cytolytic cell death is observed in infected cells\(^{20}\). Apoptotic neuronal and glial death was observed in patients with acute viral CNS infection, specifically in patients with herpes simplex encephalitis\(^{21}\).
The resultant herpes simplex encephalitis (HSE) can be fatal or cause long-term cognitive deficits. The factors that lead to HSE and unrestricted HSV-1 spread in the brain are unknown. However, genetic factors including deficits in the type I interferons, have been implicated.

**Zika Virus (ZIKV)**

ZIKV is a single-stranded RNA (ssRNA) virus belonging to the *Flaviviridae* family, which includes other mosquito-borne viruses such as Dengue Virus and Chikungunya virus. The first human cases of ZIKV infections were detected in Uganda and Tanzania in 1952, with the presence of neutralizing antibodies in human sera. Significant interest and research has been carried out on the neurological sequelae associated with ZIKV after the 2015 outbreak in the American continent. Prior to this, sporadic outbreaks were reported in humans on the Yap Island in the Federated State of Micronesia in 2007 and French Polynesia in 2013–2014. However, in Brazil, a 20-fold increase in microcephaly prevalence in neonates indicated a possible role of ZIKV infection during pregnancy. The virus enters the human host after they are bitten by a mosquito vector (*Aedes aegypti* or *Aedes albopictus*). Infections in human adults are often asymptomatic or associated with mild illness, but the virus can cross the placenta and cause severe harm to the fetus. Recently, conclusive evidence was found that ZIKV infection in pregnant mothers was directly associated with neurological defects in neonates. ZIKV was found in the amniotic fluid of pregnant mothers and in brains of aborted fetuses. ZIKV-associated neuropathology was found to be mainly microcephaly but also included agyria, calcifications in the cortex and subcortical white matter, and ventriculomegaly. Studies
have focused on the mechanisms through which the ZIKV may be causing these CNS pathologies. ZIKV is known to infect human NSPCs as well as astrocytes\textsuperscript{27}. Of importance are the mechanisms of NSPC infection and consequences on CNS development.

**West Nile Virus (WNV)**

Also, a member of the *Flaviviridae* family, WNV is known to have sporadic outbreaks in the world\textsuperscript{28}. On average, one in 140 infected individuals may develop meningoencephalitis, but the prevalence increases in the older population\textsuperscript{29}. Patients with West Nile encephalitis (WNE) may also develop coarse tremors, particularly in the upper extremities. Other symptoms include weakness in either or all the limbs and acute flaccid paralysis\textsuperscript{30}. In FVB/N mice that are susceptible to WNV disease and mortality, it was also observed that WNV could cross the placenta during early gestation but not late gestation\textsuperscript{31}. The first human case of transuterine WNV transfer showed elevated levels of IgM antibodies. Elevated fetal IgM signifies prepartum infection in the newborn because IgM cannot cross the placental barrier and must be produced *de novo* by the fetus. The neonate also had severe chorioretinal and CNS malformations\textsuperscript{32}. A larger study in WNV-infected mothers found that only one of the 55 neonates born were found to have anti-WNV IgM, suggesting that WNV does not commonly cross the placental barrier. However, many of the fetuses had varying degrees of neurological sequelae including microcephaly and lissencephaly, suggesting that the maternal immune response against the infection may have an impact on neurodevelopment\textsuperscript{33}. Moreover, WNV can cause severe damage in neonates whether the virus is acquired before or after delivery. Therefore, it is important to study the mechanisms through which WNV may affect both the adult and developing CNS.
Japanese Encephalitis Virus (JEV)

JEV is another a member of the *Flaviviridae* family that is associate with neurological disease. JEV is endemic in South and Southeast Asia and transmitted to humans by mosquito bites. Transplacental transmission of the virus has also been observed. The virus spreads from the skin to other organs (kidney, liver, and spleen) and subsequently crosses the blood-brain barrier to enter the CNS. Severe encephalitis has been associated with JEV infections, which ultimately results in neuropathologies include poliomyelitis-like flaccid paralysis. Severe seizures are common in children in up to 85% JEV-infected children. To understand the immune mediated pathology *in vivo*, an intranasal JEV infection induced encephalitis model in macaques was used. Infiltration of CD4+ and CD8+ T-cells caused vascular damage and blood brain barrier (BBB) leakage. Neurons are the predominant target of JEV in the brain, and infected neurons are often surrounded by activated microglia. Although astrocytic infection is not observed, macrophage infiltration along with astrocyte and microglia activation are seen near sites of infection. In JEV brains, both infected and uninfected neurons undergo apoptotic cell death. This finding suggests that mechanisms other than JEV infection, such as immune-mediated cytotoxicity could also contribute to neuropathology.

Borna Disease Virus (BDV)

Bornaviruses belong to the family *Bornaviridae*, which are a family of negative sense RNA viruses. The prototypical mammalian Bornavirus-1, (BDV), causes a typically fatal neurological disease in horses and sheep. There is ample evidence of BDV-induced encephalitis and resulting behavioral changes in experimental animals such as rats, mice,
and tree shrews\textsuperscript{39}. The role of BDV in human neuropathology is controversial. A lack of reliable diagnostic tools for BDV in humans further complicates studies into BDV-dependent neuropathologies. Nevertheless, studies have shown presence of BDV RNA in human subjects with known behavioral deficits and hippocampal pathology\textsuperscript{40}. Serologic and molecular epidemiologic evidence suggest that BDV may also be associated with neuropsychiatric disorders\textsuperscript{41}. For example, a subset of psychiatric patients with either unipolar depression or biopolar disorder tested positive for BDV by detection of BDV-specific antibodies\textsuperscript{41}. These studies have relied on antibody titers, indirect immunofluorescence, and qRT-PCR measurements, which can be prone to artifacts and false positives. Thus, although there is significant interest in understanding the contribution of BDV to neurological disorders, the field is currently hampered by challenges in specific detection of the virus in human samples and a lack of epidemiological data.

\textit{Measles Virus (MV)}

MV is a negative sense RNA virus and a member of the \textit{Paramyxoviridae} family. It has been the a major cause of deaths in the developing world with an estimated 122,000 deaths in 2012\textsuperscript{42}. Upon exposure to respiratory droplets, the virus enters the upper respiratory tract and is taken up by dendritic cells, B cells, and T cells expressing the CD150 receptor\textsuperscript{43}. MV infection in the CNS, although rare, can cause severe neurological disorders: primary measles encephalitis (PME), measles inclusion body encephalitis (MIBE), and subacute sclerosing panencephalitis (SSPE). PME occurs concurrently with primary measles infection. Measles inclusion body encephalitis (MIBE) occurs in
immunosuppressed individuals 3–6 months following an acute MV infection. These cases occur in patients who are immunosuppressed (HIV infection) or those on immunosuppressive therapy\textsuperscript{44}. SSPE can manifest 4–10 years following acute MV infection with a frequency of approximately one in 1700 to one in 3300\textsuperscript{45}. The neuropathology of SSPE is characterized by extensive infection of predominantly neurons and oligodendrocytes throughout the brain including the frontal cortex, occipital cortex, basal ganglia, thalamus pons, medulla and parietal cortex\textsuperscript{46}. Presence of viral antigen in astrocytes and infiltrating macrophages has also been reported in SSPE patients\textsuperscript{47}. The delay between primary infection and CNS disease is not understood in SSPE, but hypotheses include a potential latent phase of viral replication in the brain, or a slow progression of the virus through CNS tissue via transynaptic spread. Regardless, because MV infection typically occurs in the first two years of life, MV-associated CNS disease is found almost exclusively in young children.

III. Effects of antiviral immunity on NSPC activity

The above viral neurotropic infections have diverse mechanisms of infection and neuropathology at different ages. Neurotropic viruses may damage CNS tissue by a cytopathic effect, where infected cells are killed directly by the virus, or because of the immune response to the virally-infected cells\textsuperscript{48}. In addition to neurons, NSPCs are also altered during viral CNS infections. NSPCs are permissible to several viruses including murine and human cytomegalovirus, herpes simplex virus, Japanese encephalitic virus, and Zika virus\textsuperscript{49-52}. These infections result in reduced NSPC proliferation and increased apoptosis, which could impair neurogenesis, neuronal development, and neural repair\textsuperscript{51,52}. 
In this section, we discuss the interactions between specific viruses and NSPCs. A summary of the aforementioned viruses and their effects on NSPC activity is described in Table 2.
<table>
<thead>
<tr>
<th>Virus</th>
<th>Immune response</th>
<th>Effect on NSPCs</th>
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| **Herpes simplex Virus** | • Infiltration of peripheral macrophages, CD8+ T-cells and neutrophils into the CNS leading to increased mortality independent of viral load<sup>53</sup>  
  • Microglial activation caused IL-6/STAT3-mediated increase in neuronal differentiation<sup>54</sup> | • NSPC, astrocytes, neurons infected  
  • Decreased NSPC proliferation  
  • Increased glial differentiation  
  • Less infection in the neuronal pool  
  • IFNγ dependent effects |
| **Cytomegalovirus**      | • CD8+ T-cell infiltration: major source of IFNγ; Essential for viral control  
  • IFNγ increased MHC I and II expression on NSPCs. CMV decreased IFNγ-induced MHC I expression  
  • Activation of infected microglia. Increased IL-6 and TNFα secretion<sup>55</sup> | • NSPCs are primarily infected  
  • ER stress-mediated decrease in NSPC proliferation, survival, and neuronal differentiation<sup>56</sup>  
  • Possible viral evasion from immune system and IFNγ<sup>57</sup>  
  • Persistent infection |
| **Zika Virus**           | • Increased secretion of proinflammatory cytokines in embryonic brain  
  • Increased cytokine levels in maternal amniotic fluid<sup>58</sup> | • Infection and persistence in NSPCs and radial glia  
  • Decreased pool and survival in these cells<sup>59</sup> |
| **Measles Virus**        | • Increased CD4+ and CD8+ T-cell infiltration. Critical for viral control<sup>60</sup>  
  • IFNγ-mediated viral control on neurons<sup>61</sup> | • Decreased immature neuron pool<sup>62</sup>  
  • No evidence of NSPC infection<sup>62</sup> |
| **Borna disease virus**  | • CD4+ and CD8+ infiltration into the CNS. CD8+ T-cells associated with neurotoxicity leading to neuronal loss in the hippocampus<sup>63</sup>  
  • Increased microglial activation and associated MHC I, MHCII and IL-6 expression | • Direct NSPC infection  
  • No change in NSPC survival and proliferation  
  • Decreased neuronal differentiation<sup>64</sup> |

Table 2: Neurotropic viruses and immune-mediated changes in NSPC activity.
**Herpes simplex virus-1**

NSPCs, astrocytes, young and mature neurons are all permissible to HSV-1 infection\(^65\). *In vivo* studies involving nasal HSV-1 inoculation in mice implicate two mechanisms of damage to the CNS. First is the infection and lysis of neurons that control critical physiological functions\(^66\). In addition, Lundberg et al showed that the anti-viral immune response against HSV-1 played a major role in CNS pathology\(^67\). In this study, mice susceptible to HSV-1 developed fatal focal lesions in the brain that consisted on infiltrating macrophages and neutrophils. When the macrophages and neutrophils were depleted, the mice showed delayed mortality as compared to non-depleted animals. Moreover, treatment with acyclovir decreased viral load to undetectable levels, but did not reduce mortality. Therefore, these studies show that the inflammatory response in the brain contributes to pathology and death in HSV-1 infected mice.

Studies using intranasal delivery of HSV to the brain also demonstrate an initial increase in NSPC numbers during the acute phase (6 days post-infection; dpi) of HSV infection. However, NSPC numbers decline during the chronic phase (10-30 dpi), wherein the adaptive immune response is active\(^68\). The NSPCs are not infected by HSV-1 in the intranasal model, suggesting that changes in NSPC function are due to the inflammatory environment. However, *in vitro* NSPC cultures derived from induced pluripotent stem cells (iPSCs) were permissible to HSV-1 infections and demonstrated HSV-1 mediated cell lysis\(^69\). Chucair-Elliott and colleagues observed that intraocular HSV-1 inoculation in C57/BL6 mice resulted in infection and loss of nestin+ NSPCs in the SVZ. Moreover, 8 days post-infection, the NSPC pool in the SVZ of these animals was depleted. *In vitro* cultures of NSPCs from C57/BL6 mice were susceptible to HSV-1 infection and resulted
in NSPC cell loss. The number of doublecortin+ (DCX+) immature neurons also declined, which was associated with a loss in cell survival and not as a result of deceased differentiation. The discrepancy in results between Chucair-Elliott et al and Rotschafer et al could be because differences in viral (Syn 17+ v/s McRae) or mouse (Balb/c v/s C57BL6) strains used. Surprisingly, co-culture with microglia prevented this loss, which suggests that microglia may play a neuroprotective role for immature neurons. Treatment with conditioned medium from NSPC-microglia co-cultures alleviated neuronal loss in an IL-6/STAT3 dependent manner. This study also looked at the effects of microglial activation during HSV-1 infection on NSPC activity. Treatment with conditioned medium from HSV-1 infected NSPCs caused decreased neuronal differentiation. Together, these studies suggest that NSPCs are affected by both viral infections and the immune response to it, and microglial cells play an important role in mediating the effects of the immune response.

Cytomegalovirus (CMV)

CMV preferentially targets NSPCs in brain tissue, leading to apoptosis of infected cells and inhibition of proliferation and neurogenesis. These observations have been made both in human NSPCs and in mouse models of murine CMV (mCMV) infection. Studies with hCMV also show that, when infected, human NSPCs undergo apoptosis due to improper folding of proteins in the endoplasmic reticulum (ER), triggering the ER stress response. These observations indicate that CMV not only inhibits NSPC function but also results in cell death. Moreover, Rolland et al observed that hCMV infection decreased neuronal differentiation in embryonic stem cell-derived NSPCs. hCMV infection led to
increased Peroxisome Proliferator-Activated Receptor gamma (PPARγ) activation in NSPCs. Importantly, hCMV-mediated PPARγ activation was seen in both uninfected and infected cells, suggesting that the infected NSPCs released soluble factors that resulted in PPARγ activation in other cells in the vicinity. This observation of a bystander effect was confirmed by treating uninfected NSPCs with virus-free supernatants from infected cultures, which produced a similar induction of PPARγ. Upon further evaluation, this soluble mediator was found to be the PPARγ agonist 9-hydroxyoctadecadienoic acid (9-HODE). Many studies have shown that mCMV infection of NSPCs induced apoptosis in the cells. In the developing brain, NSPCs have high proliferation rates. Therefore, they could be attractive hosts to viruses like HCMV, which need proliferating cells to produce viral progeny. The hCMV-mediated decrease in neuronal differentiation may also support the above hypothesis as neurons do not proliferate, and may not function as productive hosts for the virus.

mCMV models have been used to study the effect of viral infection on NSPCs in vivo. Much like hCMV, mCMV demonstrates a strong cellular tropism for NSPCs. Thus, most mCMV models involve direct infection of NSPCs by the virus. mCMV infection of newborn mice leads to extensive infection of NSPCs and a loss of the NSPC pool and newly-born neurons. The immune response against the virus begins with infiltration of macrophages and NK cells and the activation of CNS resident microglia. CD8+ T cells, which are critical in controlling mCMV infection, follow the activation of innate immune cells. CD8+ T cells mediate clearance of CMV through cytolytic and non-cytolytic mechanisms, including IFNγ production. HCMV-infected macrophages and microglia show enhanced activation enhance secretion of IL-6 and TNFα. In addition, the immune

16
response may also affect NSPCs. Thus, the effects on NSPCs may be a result of a combined effect of the viral infection itself as well as the immune mediators released from different types of immune cells.

**Zika Virus**

ZIKV has recently received considerable attention for its association with neurodevelopmental deficits and tropism in NSPCs. In embryonic brains, ZIKV can infect most neural cell types including NSPCs, intermediate progenitors, microglia and astrocytes\(^{72}\). However, it only has limited tropism in differentiated but immature neurons\(^{52}\). Much of the understanding of ZIKV’s effects on NSPCs has come from human NSPCs in vitro or non-human models\(^{72,73}\). Mouse models for research into various aspects of ZIKV infection suggested that ZIKV infected human NSPCs in vitro\(^{52,59}\). These studies suggested that ZIKV infect NSPCs and induced apoptosis. An intraventricular injection of ZIKV into an embryonic mouse brain also revealed that ZIKV could cross the placenta, infect NSPCs and radial glial cells causing apoptotic cell death\(^{72}\). The mechanism of viral entry into the NSPCs is still under active research. The receptor tyrosine kinase AXL was the first receptor found to mediate ZIKV entry into NSPCs; however, other receptors are likely to be involved\(^{74}\).

The role of the embryonic immune response to ZIKV infection is still poorly understood. Intrauterine inoculation of ZIKV in mice showed increased microglial activation, which correlated with decreased cortical thickness\(^{59}\). Gene expression analysis in embryonic mice showed that there was an upregulation in genes associated with the anti-viral immune response, demonstrating that the fetus is capable of activating at least some
of the classical anti-viral pathways\textsuperscript{72}. Microglial cells in the fetal brain were also infected with the virus. This led to increased secretion of proinflammatory cytokines including IL-6, TNFα, IL-1β, and monocyte chemotactic protein-1 (MCP-1) from the microglia\textsuperscript{75}. These cytokines have been shown to play a major role in modulating NSPC activity. Hyperplasia of ZIKV-infected Hoffbauer (macrophage) cells in the placenta, which may also contribute to the cytokine pool in the fetus was also reported \textsuperscript{76}. Another potential source of immune-mediated damage to the embryonic brain is the maternal immune system. qRT-PCR analyses of amniotic fluid of human subjects that had tested positive for ZIKV infections revealed elevated levels of proinflammatory cytokines including IFNγ, IL-6, IL4, IL-1β, and TNFα\textsuperscript{58}. Transplacental infiltration of ZIKV and induction of apoptosis of NPSCs is responsible for many of the pathological outcomes in the embryos. However, the relative roles of maternal and embryonic immune response in ZIKV infection is still unclear.

\textbf{Measles Virus}

The passage of MV into the CNS is thought to be either through direct infection of the cerebral endothelial cells, or through infected leukocytes across the BBB. Much of the neurotropism has been understood by studying the biology of the receptors used by MV. CD46 and signaling lymphocyte-activation molecule (SLAM), also known as CD150, are the two known receptors used by MV for infection of cells in humans. CD46 receptor is widely expressed by most nucleated cells in the body. However, it is utilized predominately by the vaccine strains. On the other hand, CD150 is mostly expressed by T- and B-cells
and is utilized by both wildtype and vaccine strains of the virus. Thus, how wildtype strains of MV are able to establish an infection in neural cells remains controversial.

Transgenic mice expressing the human isoforms of the MV receptors are available. These mouse models include global or tissue-specific expression of the human receptors used by circulating MV strains (CD150/SLAM) or by vaccine strains (CD46)\textsuperscript{77,78}. Our laboratory uses a mouse model that expresses human CD46 under the control of the neuron-specific enolase promoter (CD46+ mice), thereby restricting MV infection to mature CNS neurons\textsuperscript{78}. In the CD46+ model of MV infection, neonatal mice succumb to MV despite immune cell infiltration in the brain, whereas adults survive the infection with effective viral control\textsuperscript{60}. This provides an opportunity to examine how age-dependent differences in the immune response effect NSPC activity as well as how the anti-viral response impacts on brain development. Neonatal mice infected with MV demonstrated focal infection in the hippocampus, cortex, inferior colliculus, and paraventricular regions and in the Purkinje neurons of the cerebellum. Both neonatal and adult CD46+ mice show CD4+ and CD8+ T-cell infiltration into the CNS. However, only adult mice successfully clear the infection. In fact, in adult mice that lack T- and B-cells did not clear the infection and both neonatal (90%) and adult (71%) showed signs of MV-related sickness. Therefore it is clear that T-cells are critical for MV control\textsuperscript{60}. Additionally, mice deficient in IFNγ also show greater MV-related illness and fail to control the virus in the brain. IFNγ also exerted a direct antiviral effect on MV-infected primary neuronal cultures form CD46+ mice\textsuperscript{61}. It is hypothesized that IFNγ secreted from T-cells specifically is crucial for control of MV replication in neurons. Infection of CD46+ mice lacking the IFN type I and type II signal transducer, STAT1 (STAT1-KO mice), results in the survival of approximately 75% of
animals. Those mice that did not survive the infection died earlier than both T-cell deficient and IFNγ-KO mice. Moreover, the symptoms that they showed were different than the other genotypes, including “popcorn” seizures and earlier death within the first 6 dpi. Those mice that survived the initial 6-day period showed no signs of neurological damage. These mice also had normal T-cell infiltration and was not statistically different compared to wild type CD46+ mice. Thus, CD46+ mice depend upon IFNγ production from T cells in order to control the virus and limit pathology, but IFNγ may utilize STAT1-independent pathways in order mediate its anti-viral effects in neurons.

In neonatal CD46+ mice, we explored how the anti-viral immune in the brain affected NSPCs, which are spared from MV infection in the CD46+. We observed that MV infection did not affect the pool of NSPCs, mature neurons, or astrocytes in wildtype pups. However, a significant loss in the pool of differentiating but immature neurons (doublecortin+/CD24+) was observed. Moreover, there was significant increase in apoptotic cells in the neonatal CNS, although the lineage of the apoptotic cells remains to be identified. We also observed a decrease in neurogenesis (CD24+/BrdU+). The pattern of decrease in immature neuronal pool did not match that of actively proliferating immature neurons in that the loss in the cell pool was observed much before the decrease in actively synthesizing cells. These results indicate that the MV-induced immune response created bystander effects on immature neurons. We have observed extensive microglial activation in the neonatal CNS post-MV infection (Ganesan.P, unpublished data).
**Borna Disease Virus**

BDV neuropathology has been studied predominately through experimental animal models of BDV because human samples have been challenging to confirm. However, adult rats infected intracerebrally or intranasally with BDV usually develop an immune-mediated biphasic behavioral disease. Rats display clinical signs and a histopathological that described for the naturally infected horse\(^8^0\). Experimental intra nasal infection of the rat has allowed the study of CNS spread of BDV. After initial replication in the neurons located at the site of entry (olfactory bulb)\(^,\) BDV likely migrates intra-axonally in an anterograde or retrograde direction towards the CNS and ultimately disseminates into different regions of the brain\(^8^1\). Within the CNS, BDV exhibit a preferential tropism for the limbic system, including the hippocampus which carries the highest viral load. Despite widespread persistent infection, BDV does not exert cytolysis in infected neuronal cells\(^8^2\).

The cell-mediated immune response to BDV plays an essential role in the development of neuropathology\(^8^3\). This was shown by studies on animals with antibiotic-treated or immunocompromised animals. BDV infection of rats treated with cyclophosphamide, cyclosporin A, or of athymic rats did not lead to inflammation and development of disease\(^8^4\). Both CD4+ and CD8+ T-cells infiltrated the CNS during the immune-mediated pathology associated with BDV\(^6^3\). Adoptive transfer studies showed that cytotoxic CD8+ T-cells infiltrated the brain parenchyma and contributed to cortical atrophy through the release of perforin. CD4+ T-cells were only found in perivascular areas in the CNS and did not contribute disease or neuropathology\(^8^5\). Cytokines and free radicals also contribute to BDV-mediated immunopathology. Levels of IL-6, TNF\(\alpha\), and IL-1 mRNAs
are significantly increased in BDV-infected brains and their expression correlates with the severity of neurological signs\textsuperscript{86,87}.

BDV is considered as a noncytolytic virus. BDV-infected neuronal cells cultured \textit{in-vitro} do not exhibit impaired growth or survival\textsuperscript{88}. Nevertheless, BDV-infected organotypic slice cultures of the hippocampus from neonatal rats show a reduction in granular neurons. This loss is characterized by axonal retraction, decreased synaptic plasticity, and increased apoptosis. \textit{In vivo} analysis of BDV-mediated neuropathology is correlated with the inflammatory response accompanying the infection. Thus, it is difficult to attribute cell and tissue damage directly to BDV, but rather suggests that damage is due to the anti-viral immune response.

Brnic et al and Scordel et al have shown that BDV can also infect human NSPCs \textit{in vitro}\textsuperscript{64,89}. Brnic et al reported that even though the NSPCs were infected, the NSPC proliferation and survival was not altered. When the NSPCs were allowed to differentiate into other neural cells, differentiation in the neuronal lineage was impaired by increased apoptosis of immature neurons. Scordel et al also observed a similar decrease in neuronal differentiation, specifically in GABAergic neurons. Evaluation of the expression of pro-neuronal factors such as Noggin and ApoE showed a decrease in their expression. These results suggest that even though they are permissible to BDV, NSPCs are refractory BDV-induced cell death. However, immature neurons become susceptible as they differentiate. These studies still do not address the role of immune cells on NSPC proliferation, survival, or differentiation, which seems to be a key factor in \textit{in vivo} models. Ovanesov et al, observed that treating mixed neuron-microglia co-cultures with conditioned media from BDV-infected neurons (in the presence of astrocytes) caused microglial activation.
Microglial activation was characterized by changes in microglial morphology (rounded shape) with increase expression of major histocompatibility complex-I (MHC I), MHC-II, and IL-6 expression\textsuperscript{90}. Therefore, we wanted understand if BDV-activated microglia also affect NSPC activity through a similar bystander effect.

IV. NSPCs in other neurodegenerative and neuroinflammatory disease

The role of NSPCs under in neurodegenerative diseases is an active area of study. In addition to normal NSPC functions such as CNS development, learning and memory, and maintenance of olfaction, NSPCs are altered in CNS diseases such as epilepsy, depression, and even natural aging\textsuperscript{91-93}. Whether such changes in NSPCs helps to delay or accelerate the neuropathology of disease is an open question.

Conflicting evidence for changes in NSPC activity have been noted in age-related neurocognitive disorders such as Alzheimer’s disease (AD). In severely-affected Alzheimer’s brains, expression of neurogenic markers (doublecortin, NeuroD) is enhanced in the dentate gyrus and CA1 region of the hippocampus\textsuperscript{94}. In mouse models of AD, accumulation of amyloid β (Aβ) through transgene expression or intraventricular injection reduces NSPCs and newly-born neurons in the hippocampus\textsuperscript{95}. Aβ treatment of primary NSPCs \textit{in vitro} also inhibits NSPC proliferation and production of new neurons. These investigations show that NSPCs are responsive to the types of insults that occur in AD, although it is unclear how such changes in NSPC activity impact on disease progression in humans. Although the initiating factors that lead to AD pathology are not completely understood, inflammation plays a role in the progression of the disease\textsuperscript{96}. Elevated levels of inflammatory cytokines such as IL-1β, IL-6, and tumor necrosis factor α (TNFα) are
found in proximity to amyloid plaques and in the plasma and CSF of AD patients\textsuperscript{97-100}. Studies have found that IL-1\(\beta\)+ expressing microglia in the vicinity of A\(\beta\) plaques are necessary for formation of neuritic plaques\textsuperscript{101}. Moreover, transplanting NSPCs that over express IL-1 receptor antagonist (IL-1ra) rescued impairment in memory and neurogenesis in a mouse model of AD that had elevated levels of IL-1\textsuperscript{102}. These studies indicate that immune activation affects NSPCs which may contribute to disease pathology.

Multiple sclerosis (MS) is a chronic inflammatory autoimmune disease of the brain and spinal cord characterized by demyelination of axons and eventual neuronal death. Experimental autoimmune encephalitis (EAE) is a mouse model of MS currently widely used to model EAE in mice. Studies suggest that in EAE, there is induction of NSPC and oligodendrocyte progenitor cells (OPCs) proliferation in the SVZ. The subsequent migration of mitotically-active SVZ cells to the olfactory bulb and regions of demyelinated white matter is also enhanced\textsuperscript{103}. Evidence from recent studies suggests that immune response in MS and experimental mouse models causes apoptosis of oligodendrocytes, which contributes to demyelination and ultimately neurodegeneration\textsuperscript{104,105}. In the \textit{taiep} model of MS, autosomal recessive genetic defects leads to an accumulation of microtubules in oligodendrocytes, which leads to decreased myelination on neurons. The authors found transplanted OPCs could repopulate areas of demyelination but were unable to induce remyelination of axons. However, when coupled with acute inflammation, these OPCs were not only able to repopulate the demyelinated areas, they also successfully induced axonal remyelination\textsuperscript{106}. This indicates that in this model, inflammation played a supportive role and supported the reversal of pathology in the mice.
Studies from such disease states indicate that NSPCs are able to respond differentially in these neuropathologies. It is also important to note that the differential response is also influenced by the nature of the immune response. This suggests that the immune response plays an important role in modulating NSPCs and it is important to understand the role of cytokine/chemokine or trophic factors that may mediate these effects.
Figure 1: *Current hypotheses for IFNγ-mediated effect on NSPC activity.* IFNγ binds to the IFNγ receptor (IFNGR1 and 2) and activates Janus-activated kinases 1 and 2 and Signal transducers and activators of transcription-1 (JAK-STAT1) pathway. In NSPCs, STAT1 activation blocks NSPC proliferation through decreased expression of cyclin/cyclin-dependent kinase (CDK) complexes. Studies with adult NSPCs report that IFNγ-mediated STAT1 activation results in increased neuronal differentiation. STAT1 activation leads to increased expression and secretion of the sonic hedgehog (Shh) protein, which in turn causes anomalous and concurrent expression of both glial and neuronal markers in the same cell107. Moreover, IFNγ-mediated STAT1 activation and Shh expression in granule precursor cells leads to increased NSPC proliferation. In Paju cells, IFNγ-induced activation of the extracellular-signal regulated kinase-1/2 (ERK-1/2) increases neuronal differentiation, which may be independent of STAT1 activation108. In C17.2 cells, IFNγ-mediated activation of c-Jun N-terminal kinase (JNK) pathway caused neuronal differentiation, without the activation of ERK1/2 pathway109. Developmental
cytokines such as Leukemia inhibitory factor and Ciliary neurotropic factor mediate glial differentiation through STAT3. IFNγ-mediated activation of STAT3 is observed in fetal NSPCs; however, the role of this pathway in NSPC differentiation is yet unclear.
V. IFNγ and its role in mediating NSPC activity

Viral infections of the CNS lead to inflammation mediated by both resident microglia as well as infiltrating innate and adaptive immune cells. Viruses have diverse tropisms and capable of infecting multiple cell types in the CNS. The ensuing immune response helps in controlling the viral infection. However, as seen in the previous sections, anti-viral immunity may affect uninfected cells through cytokine release. Moreover, they can also express Major histocompatibility complex (MHC) I and II molecules, which make them targets for immune cells. IFNγ is a major anti-viral cytokine that is necessary for control of several viral infections in the CNS. Both type I and II interferons play important roles mediating antiviral activities in the CNS. Type I interferons mediate immediate antiviral activities whereas immunomodulatory functions of IFNγ are crucial later in the viral infections in establishing an antiviral state. IFNγ increases the expression of antiviral genes such as protein kinase R (PKR), the dsRNA-specific adenosine deaminase (ADAR), and guanylate-binding proteins (GBP1 and 2). PKR inhibits viral dsRNA replication, whereas ADAR causes mistranslation of viral dsDNA. GBP1 and 2 promote oxidative viral killing and helps in delivery of antimicrobial molecules to autophagolysozomes. In addition to these antiviral effects on infected cells, the bystander effects of IFNγ on NSPCs needs to be explored during viral infections. However, the outcomes and mechanisms through which IFNγ affects NSPCs are unclear.

**IFNγ: canonical and non-canonical signaling pathways**

To understand the effects of IFNγ, we need to acknowledge the diversity of signaling pathways that it initiates. IFNγ binds to the IFNγ receptor (IFNGR), which
consists of two IFNGR1 subunits and two IFNGR2 subunits. Binding of IFNγ to IFNGR1 causes heterotetramerization of the receptor, which then leads to activation of downstream kinases\textsuperscript{118,119}. IFNγ predominately activates the Janus associated kinase/Signal transducer and activator of transcription-1 (JAK/STAT) signaling pathway (Figure 1). Activation of JAKs results in the recruitment and activation (phosphorylation) of STATs at the receptor. Out of the seven STAT family members, STAT1 is the main downstream effector of IFNγ. Upon phosphorylation by JAKs, STAT1 homodimerizes and translocates to the nucleus, where it initiates the transcription of IFNγ-stimulated genes (ISGs). There are approximately 500 ISGs that can be stimulated by IFNγ, including genes involved in viral clearance, cell cycle control, and inflammatory signaling\textsuperscript{118}. For example, IFNγ increases major histocompatibility complex (MHC) expression in a STAT1-dependent manner, leading to recognition of tumor cells by the immune system\textsuperscript{120}. IFNγ also inhibits proliferation of fibroblasts by reducing cyclin and cyclin-dependent kinase (CDK) expression, particularly that of cyclin D/CDK4\textsuperscript{121}. The profile of ISGs is dependent both on cell type and on other inflammatory signals that are received by the target cell (reviewed in \textsuperscript{122}). Thus, the phenotypic response to IFNγ also varies depending upon cell type, which is reflected in conflicting reports of neuroprotection and toxicity with IFNγ treatment\textsuperscript{123-125}.

To evade clearance from the body, many viruses inhibit STAT1 function and expression, thereby abrogating the anti-viral response of the cell\textsuperscript{126,127}. However, IFNγ also signals through STAT1-independent mechanisms, which may be activated alone or in parallel with STAT1-dependent pathways\textsuperscript{128}. These pathways result in protective as well as pathological outcomes. Primary hippocampal neurons utilize STAT1-independent
pathways for viral control, possibly because endogenous STAT1 expression is inherently low in these cells\cite{79}. Neurons also activate extracellular regulated kinase-1/2 (ERK-1/2) signaling in response to IFNγ, which confers neuroprotection against apoptotic insults. In contrast, primary astrocytes activate STAT3 upon IFNγ treatment, which leads to the production of neurotoxic factors\cite{129}. IFNγ stimulation may also lead to recruitment of adaptor molecules such as the c-Cbl proto-oncogene and the GTPases Ras and Rap1\cite{130}. Like STAT1-dependent signals, activation of Rap1 signaling inhibits cell proliferation in human embryonic kidney cells\cite{131}. Therefore, IFNγ may activate multiple pathways that limit cell growth, which could be advantageous for controlling viral replication in a rapidly dividing cell. In lung epithelial cells, where STAT1 is activated through the activation of the Phospholipase C-gamma2/Protein Kinase C/Src (PLCγ2-PKCα-src) pathway in a JAK-dependent manner. This pathway causes increased expression of Intercellular Adhesion Molecule (ICAM)-1\cite{132} and facilitates binding and transmigration of immune cells into tissues. Therefore, in addition to pathways that are STAT1-independent, other signaling proteins and adaptor proteins may link JAK and STAT1 indirectly in certain cell types.

**IFNγ-mediated signaling in NSPCs**

The diversity of IFNγ-mediated signaling pathways raises the question of how IFNγ may affect NSPCs. The role of IFNγ is particularly important because the JAK-STAT family of proteins has been implicated in NSPC proliferation and differentiation\cite{133-135}. Several reports show that IFNγ inhibits proliferation of murine NSPCs derived from the adult SVZ in vitro\cite{136-140}. Moreover, NSPCs derived from mice lacking IFNγ show enhanced neurogenesis and proliferation\cite{141}. There is evidence that IFNγ can alter NSPC
function through activation of the STAT family of proteins. Lum, et al showed that when adult SVZ-derived NSPCs were treated with IFNγ, there was activation of both STAT1 and STAT3\textsuperscript{137}. Conversely, Pereira et al observed that IFNγ-treated NSPCs derived from postnatal brains (P7-P9) showed increased STAT1 activation but no change in STAT3 activation\textsuperscript{138}. One reason for this discrepancy could be the time point at which STAT3 activation was measured; the former study assessed STAT3 activation four days post-treatment whereas the latter measured activation at 15 minutes post-treatment. Studies in many cell types indicate that STAT1 and STAT3 play opposing roles in cell proliferation; STAT1 is generally anti-proliferative and STAT3 is pro-proliferative\textsuperscript{142}. This explains the role of STAT1 in mediating anti-proliferative effects of IFNγ on NSPCs, but the role of STAT3 is yet unclear. Previous studies in mouse embryonic fibroblasts have also shown that both STAT1 and STAT3 may be activated in a cell at the same time. However, STAT3 activation rapidly declines due the activity of inhibitors such as suppressor of cytokine signaling-3 (SOCS3)\textsuperscript{143}.

The sonic hedgehog (Shh) protein also plays an important role in NSPC proliferation and fate specification\textsuperscript{144}. In NSPCs, IFNγ induces Shh expression. These studies show that IFNγ-mediated Shh expression and signaling results in increased proliferation of cerebellar neural precursor cells\textsuperscript{145}. Other studies demonstrate that induction of Shh by IFNγ results in a dysregulated cell-fate characterized by expression of glial and neuronal markers in the same cell\textsuperscript{107}. IFNγ also increases Shh expression in adipocyte precursors, suggesting that IFNγ may act broadly on undifferentiated cells to induce differentiation or growth\textsuperscript{146}.
One outstanding question is how NSPC differentiation is affected by IFNγ during pathological insults. *In vitro* studies on adult murine NSPCs indicate that IFNγ induces neuronal differentiation\textsuperscript{136,137,139}. Pereira et al showed that infusion of IFNγ into the mouse SVZ decreased neuronal differentiation in a STAT1-dependent manner\textsuperscript{138}. However, Ben-Hur et al did not observe any changes in differentiation in IFNγ-treated NSPCs derived from neonatal rat striatum\textsuperscript{140}. In contrast to many studies on adult NSPCs, embryonic NSPCs exhibit decreased neuronal differentiation in response to IFNγ\textsuperscript{147}. Together, these studies suggest that NSPCs may differ in their responsiveness to IFNγ depending upon anatomical location and the age of the host.

A number of other cytokines, including Leukemia inhibitory factor (LIF) and Ciliary neurotropic factor (CNTF), activate JAK-STAT signaling and regulate NSPC cell fate. During CNS development, these cytokines trigger glial differentiation in NSPCs through activation of STAT1 and STAT3. However, they cause glial differentiation only during late gestational periods (embryonic day 16 and later). STAT-dependent gliogenic activity is repressed during the neurogenic period (embryonic days 10-14) through epigenetic inhibition of glial gene expression\textsuperscript{135,148}. Whether IFNγ synergistically affects LIF and CNTF signaling during development is unknown. However, one could conjecture that IFNγ may augment glial differentiation at later stages in development through activation of JAK-STAT signaling.

In addition to STAT1, mitogen-activated protein kinases (MAPK) have also been implicated in mediating neuronal differentiation in cell lines. IFNγ induces neuronal differentiation through the ERK-1/2 pathway in the human neuroblastoma Paju cell line\textsuperscript{108}. In a murine cerebellar cell line, IFNγ causes neuronal differentiation through the activation
of c-jun N-terminal kinase (JNK) pathway.\textsuperscript{109} Moreover, inhibition of the JNK pathway but not the ERK-1/2 pathway reversed the effects of IFNγ. Other MAPKs, such as the p38, have also been implicated in mediating neuronal differentiation\textsuperscript{149}. Admittedly, these studies were conducted in transformed cell lines and not in primary NSPCs\textsuperscript{108}. However, they highlight the importance of MAPK signaling as an alternative pathway for influencing cell fate decisions downstream of IFNγ. A summary of these pathways is depicted in Figure 1.

The fact that IFNγ affects NSPC proliferation and cell fate specification is well established, although the necessary signaling pathways are still being defined. Variables such as the age of the host, brain region, and species may impact on the NSPC response to IFNγ. Regardless of the differences in model systems, STATs play a major role in mediating the effects of IFNγ on NSPC activity. Activation of non-canonical pathways, such as MAPKs, and crosstalk with other STAT signaling pathways may also be involved. It will be important to account for the mutable responses of NSPCs to IFNγ when considering how these cells react in in vivo disease models. Moreover, the mechanisms that act downstream of these signaling pathways such as IFNγ-mediated cell cycle control and survival pathways are still undefined. This dissertation will also attempt to define these pathways which will further the understand of IFNγ-mediated control NSPC activity.

Role of IFNγ in viral infections and effects on NSPCs

In addition to viral infection, NSPC activity may be affected through a bystander effect from anti-viral cytokines\textsuperscript{150,151}. IFNγ is critical in controlling the spread of many neurotropic viruses including measles virus, Theiler's virus, herpes simplex virus and
Sindbis virus\textsuperscript{61,152-154}. Even though the anti-viral and immunomodulatory roles of IFN\(\gamma\) are well documented, its role in affecting NSPC activity in the context of viral infections is less clear. Recent research indicates that IFN\(\gamma\) may affect NSPC survival, proliferation, and neurogenic potential during infections, depending on the model system and on the cellular tropism of the virus. Here, we examine the specific role of IFN\(\gamma\) on NSPCs in different models of neurotropic viral infections.

Studies using intranasal delivery of HSV NSPCs are not infected by HSV-1, suggesting that changes in NSPC function are due to the inflammatory environment \textsuperscript{68}. During the chronic phase of HSV-1 infection, activated CD8+ T cells are the major source of IFN\(\gamma\) in the CNS. Co-culture of virus-activated CD8+ T cells and NSPCs showed reduced NSPC proliferation and differentiation into glial cells\textsuperscript{53}. When antibodies blocking IFN\(\gamma\) binding to its receptor were used, the decrease in proliferation was abrogated. Together, these findings demonstrate that IFN\(\gamma\) may be a key factor in dictating how NSPCs respond to cytotoxic T cells. These studies further suggest that the IFN\(\gamma\)-mediated effects on NSPCs may affect functional recovery post-HSV-1 infection.

In a murine model of mCMV infection, IFN\(\gamma\) increases the expression of MHC class I and II on NSPCs, which would allow recognition by infiltrating T cells\textsuperscript{155}. mCMV, however, is able to counteract the IFN\(\gamma\)-mediated induction of MHC class I in NSPCs\textsuperscript{156}. These mechanisms may allow the virus to evade immune clearance and establish latency in the NSPCs. Moreover, MHC expression is important for neuronal development and synaptic refinement\textsuperscript{157}. Therefore, a CMV-mediated decrease in MHC expression could hamper the development of neuronal networks, particularly during the formative stages of brain development.
Our laboratory uses the CD46 mouse model, where MV infection is restricted to mature CNS neurons. In CD46+ adult mice, IFNγ is required for non-cytolytic clearance of MV from infected neurons. In neonatal CD46+ mice, we explored how the anti-viral immune in the brain affected NSPCs, which were spared from MV infection. IFNγ preserved the NSPC pool during MV infection, but could not prevent a decline in neurogenesis. Moreover, newly-differentiated neurons (doublecortin+) were lost regardless of IFNγ expression. These data indicate that IFNγ may be critical in protecting uninfected NSPCs during an anti-viral immune response, but cannot protect new neurons from the effects of neuroinflammation.

A central role for inflammation has been acknowledged in many CNS diseases. However, the role of inflammation, and specifically of IFNγ, in modulating NSPC functions is under active study. IFNγ is one variable that affects how NSPCs respond in inflammatory environments. Because IFNγ is a pleiotropic cytokine, alterations in IFNγ expression often affect multiple neural and immune cells, which can further impact on NSPC function. Taking into account the diversity of signaling pathways activated by IFNγ, and variability of its effects on NSPCs in different systems, IFNγ may exert subtle alterations in pathological outcomes in neuroinflammatory conditions. The discovery of multipotent NSPCs in the adult brain has also generated interest in how these NSPCs are affected by inflammation in the mature brain, particularly during neurodegenerative disease.

Some outstanding questions remain. How does IFNγ mediate its effects in NSPCs? Which immune cells mediate the bystander effect? What role do microglia, which are the resident immune cells in the CNS and can be protective in some inflammatory
environments, play in this model? Which immune mediators are released by infiltrating and resident immune cells? In this thesis, we will address the effect of immune mediators, such as IFNγ, on NSPCs. Microglial activation and their role in CNS pathology during viral infections have been studied in other models. However, how the result of microglial interactions with infected neural cells and the subsequent effects on NSPCs are not known. To study these interactions, we have developed neuron-microglia co-culture model to explore the effects of virus-induced microglial activation on NSPC proliferation and differentiation.

Therefore, this study aims to define mechanisms through which the immune response influences NSPCs and the implications of these effects on pathology and brain development during neurotropic viral infections.
Chapter 2: Materials and Methods

I. Cell culture

*Neural stem/progenitor cells (NSPCs)*

Animal use protocols were reviewed and approved by the Duquesne University Institutional Animal Care and Use Committee. CD46+/WT (wildtype) and CD46+/STAT1-knockout (STAT1-KO) mice were a generous gift from Dr. Glenn Rall (Fox Chase Cancer Center)\(^1\). The genotype of the STAT1-KO mice used in these experiments was confirmed by PCR analysis of tail biopsy DNA\(^7\). NSPC cultures were prepared from wildtype CD46+/WT or STAT1-KO mouse embryos on embryonic day 12.5 (E12.5) as described previously with modifications\(^1\)\(^6\). Briefly, cortical tissue was digested using trypsin-EDTA solution (0.05%, Mediatech, Inc., Manassas, VA) to obtain a cell suspension. The trypsin was neutralized using an equal volume of soybean trypsin inhibitor (1mg/mL, Sigma-Aldrich, St. Louis, MO), and the cell suspension was passed through a 70\(\mu\)m cell strainer (Thermo Fisher Scientific, Waltham, MA). Cells were centrifuged (1150 rpm for 5 min) and the cell pellet was resuspended in 1 mL NSPC culture medium consisting of DMEM (Mediatech, Inc., Manassas, VA), 1X B27 supplement without vitamin A (Thermo Fisher Scientific, Waltham, MA), 1X N2 supplement (Thermo Fisher Scientific, Waltham, MA), heparin (2\(\mu\)g/mL, Sigma-Aldrich, St. Louis, MO), murine epidermal growth factor (20ng/mL, Peprotech, Rocky Hill, NJ) and murine fibroblast growth factor-2 (20ng/mL, Peprotech, Rocky Hill, NJ). The NSPCs were cultured in suspension at 2*10^6 cells in 5 mL NSPC culture medium and maintained at 37°C/5% CO\(_2\) in a T-25 flask (Thermo Scientific-Nunc, Waltham, MA Cat# 12-565-49).
After 3 days *in vitro* (DIV), neurospheres were disassociated with 0.25% trypsin-EDTA (Thermo Scientific, Cat# 25200056), counted, and used for subsequent experiments.

*Microglia-Neuron co-culture system*

To study the effects of immune activation following measles virus neuronal infection on NSPC activity, primary microglia were cultured first with primary neurons in a co-culture system. Conditioned medium from the co-culture was used to treat NSPCs cultured *in vitro* as a monolayer.

*Primary Embryonic neuronal culture*

Primary neurons were cultured from E. 15.5 mice as previously described. Briefly, Cortexes from E15.5 were treated with prewarmed with 0.05% Trypsin-EDTA for 15 mins at 37°C. The tissue was washed 3 times with 5 mL DMEM supplemented with 10% FBS (FBS; 10%, Atlanta Biologics, Lawrenceville, GA, Cat# K11050), 1% L-glutamine (200 mM, Cellgro, Manassas, VA, Cat# 25-005-C1) and 5% Penicillin-Streptomycin (Cellgro, Manassas, VA, Cat#30-002-C1). Trypsin-EDTA was inactivated with FBS. The tissue was dissociated with a flame polished pipets and passed through a 70μm cell strainer to remove debris. The suspension was transferred to a 15mL tube and underlaid with 2mL FBS and centrifuged at 1300 rpm for 5 mins at 21°C. The supernatant was removed and the pellet was resuspended in neurobasal medium (NBM; Thermo Fisher Scientific, Waltham, MA, Cat# 21103) supplemented with 1X B27 (Thermo Fisher Scientific, Waltham, MA; Cat# 17504-44), 1% L-glutamine, and 25 μM glutamic acid. The cells were grown in Poly-D-
lysine-coated 24-well or six-well plates (Corning Costar, Corning Incorporated, Corning, NY) for four days at 37°C.

**Primary microglia culture**

Stage 1: Mixed microglia-astrocyte culture

The base of a 75 cm² culture flask was coated with 10ml poly-D-lysine hydrobromide (100 μg/ml, MP Biomedicals, Solon, OH; Cat# 0215017525) and allowed to set overnight at 20°C prior to dissection. Three to four pups (1-3 days old) were culled by cutting both carotid arteries (without decapitating the mouse; otherwise subsequent handling is made difficult). The skin over the skull was cleared under aseptic technique. This was achieved by making a crucifix incision over the cranium (transverse incision at the base of the skull and the vertical incision along the midline of the skull up to the anterior part of the skull). New straight forceps were then used to peel away the skin on the scalp to fully expose the skull. A pair of corneal scissors were used to cut along the suture lines. A 3rd pair of straight forceps were then used to ‘peel’ the skull away and to fully expose the brain. New curved forceps were then inserted under the base of the brain and the brain was gently lifted away from the skull base. The brain tissue was minced using a #10 scalpel and the cleaned brain is then placed in a new culture dish with 2 ml of tissue digestion enzyme (Papain 20 U/mL activated with 5mM L-cysteine). The brain/enzyme suspension were transferred gently into a 15 mL centrifuge tube using a 5 ml serological pipette. The tube was maintained in the incubator at 37 °C for 20 min to allow for enzymatic disaggregation. After the allocated time, DMEM with 10% fetal bovine serum (FBS)/1% Penicillin/streptomycin (P/S) is added to the suspension (1:1 ratio) to neutralize the
enzyme. The single cell suspension was gently agitated by moving the solution up and down 20 times using a 1 ml plastic transfer pipette with care being taken to avoid bubble formation. This suspension was passed through a 70 μm nylon cell strainer into another 50 mL tube. The suspension is then centrifuged in a cooled (4 °C) centrifuge at 1000 x g for 5 min. The cells were resuspended in 6 mL of 30% Percoll solution (GE Healthcare Bio-Sciences, Pittsburgh, PA; Cat#17-0891-01) and centrifuged at 500 x g for 20 min at 4°C. The cell pellet was washed by resuspending with 6mL DMEM and centrifuged again at 1000 x g for 10 min at 4°C. The cells were suspended in 1 mL DMEM and counted. The poly-D-lysine was removed and the flasks were washed once with PBS. All of the cells from the three brains was added to the flask in 20 mL of DMEM incubated at 37 °C. After 2 days, the DMEM is replaced with a fresh DMEM. When the cells are approximately 80-90% confluent, they are used for purification (Stage 2).

Stage 2: Purification of murine microglia cells from the mixed glia cell culture

At this stage, the culture is a mixture of microglia and astrocytes. Once the flask reaches a 90% confluence level (after 10-14 days), the microglial cells are detached from the flask using the heated orbital shaker machine at 240 rpm for 1.5 - 2 h (37°C). The suspension was transferred into a 50 mL centrifuge tube and then centrifuged at 190 x g for 8 min at 21°C. The supernatant was discarded and the cell pellet is resuspended in one mL DMEM. The microglia are counted by trypan blue exclusion and prepared for neuronal co-culture.
**MV infection of primary neurons**

To prepare stocks of measles virus (MV), Vero cells were maintained in Dulbecco’s modified Eagle media (DMEM) supplemented with 10% FBS, 2 mM L-glutamine, 100 U of penicillin per ml, and 100 ng of streptomycin per ml. MV-Edmonston (MV) was purchased from American Type Culture Collection (Manassas, Va). The virus was passaged in Vero cells (three passages), titered in Vero cells via plaque assay, and stored at -80°C until further use.

On the fourth day post-plating of neurons, 75% (1.5 mL/well from 24-well plate or 3 mL/well from 6-well plates) of NBM was removed from each medium and saved in a centrifuge tube. The neurons were infected with Measles virus (MV) by incubating with 1 MOI (200 μL/well for 24-well plates and 500 uL/well for 6-well plate) of MV at 37°C for one hour. Meanwhile, the volume of saved NBM was made up to 100% by adding fresh NBM (without glutamic acid). The virus was removed, cells washed with 1X PBS (without Ca²⁺/Mg²⁺), and the saved NBM was added back to the wells. The cells were incubated at 37°C for four days.

**Primary neuron and microglia co-culture**

Four days post-infection of primary neurons, microglia were processed as described (stage 2). After resuspension in DMEM, the microglia were counted and appropriate number of cells were added to neuronal culture (100,000 cells/well for 6-well plates and 25,000 cells/well for 24-well plates). Four days post-co-culturing, the medium was saved (for ELISA) or added to NSPCs monolayer in the differentiation phase. The co-cultured cells in 24-well plates were fixed by treating with 4% paraformaldehyde (PFA) in PBS for
20 minutes, permeabilized with Triton X-100 (0.2% in PBS) for 15 minutes, washed with 1X PBS (without Ca\(^{2+}\)/Mg\(^{2+}\)), and stored in sterile PBS (without Ca\(^{2+}\)/Mg\(^{2+}\)) at 4°C until further use. Cells in the six-well plate were used for RNA extraction to be used in qRT-PCR and processed as described below.

**NSPC monolayer culture**

Twenty-four well plates with 12mm coverslips or six-well plates were coated with poly-D-lysine (100 μg/mL) and Laminin (1μg/mL). NSPCs (Passage 1; 20,000 cells/well for 24 well plate and 200,000 cells for 6-well plate) were grown in NSPC growth medium in the plates and allowed to attached for one day. The medium was changed with differentiation medium (growth medium w/o growth factors) or neuron-microglia conditioned medium and allowed to differentiate for 4 days. The cells were fixed with 4% PFA for and permeabilized using 0.2% Triton X-100 for 15 minutes at 20°C, washed with 1X PBS (without Ca\(^{2+}\)/Mg\(^{2+}\)), and stored at 4°C until further use. NSPCs in the six-well plates were used for BrdU assay or flow cytometry to measure NSPC differentiation.

**II. Neurosphere assay**

NSPCs (passage 1) were cultured in T-25 flasks at 20,000 cells/mL with different concentrations of interferon-gamma (IFN\(\gamma\); 1-1000 U/ml). IFN\(\gamma\) solutions were prepared by making 10X solutions in NSPC culture medium from a 1000 U/μL stock solution (BD Biosciences, San Jose, CA). As a negative control, heat-inactivated IFN\(\gamma\) (ΔH-IFN\(\gamma\)) was prepared by boiling IFN\(\gamma\) (1000U/mL) at 100°C for 5 min. After IFN\(\gamma\) treatment, the neurospheres were imaged after 3, 5, or 7 DIV using an EVOS FL microscope (Thermo
Fisher Scientific; Waltham, MA). Five fields were taken from each flask at 2X magnification by an observer blinded to experimental conditions. The neurosphere diameter (µm) and area (pixel$^2$) was measured for each neurosphere using Image J software (Version 1.6.0_65). The average diameter for each treatment group was calculated and graphed as percent of the untreated group. Neurosphere area was graphed as a histogram plot using pixel$^2$/neurosphere for each treatment condition.

III. Bromodeoxyuridine (BrdU) Assay

*Single-pulse BrdU assay in neurospheres*

WT and STAT1-KO NSPCs (100,000 cells/mL) were cultured in T-25 flasks and treated with IFNγ (1-1000 U/ml) for 3 DIV. BrdU staining was performed following manufacturer’s instructions using the BD Pharmingen™-FITC BrdU Flow Kit (BD Biosciences, San Jose, CA). On DIV 3, the neurospheres were treated with BrdU (25µM) solution for 90 min. at 37°C. The neurospheres were dissociated using 0.05% trypsin solution (3 min at 37°C) into a cell suspension. BrdU content was determined by incubating the cells with a FITC-conjugated anti-BrdU antibody (1:50; 559619, BD Biosciences, San Jose, CA) for 30 min. A FITC-conjugated isotype control antibody (1:50; 556649, BD Biosciences, San Jose, CA) was used as a negative control for BrdU staining. The cells were counterstained with 7-Aminoactinomycin D (25µM) for 15 min at 20°C. Single cells (100,000 events per sample) were analyzed by flow cytometry (Accuri C6, BD Biosciences, San Jose, CA) using an initial gate to exclude debris. Signals for BrdU-FITC (FL1) and 7-AAD (FL3) were compensated by subtracting FL3 from FL1 (2%) and FL1 from FL3 (10%) as per manufacturer’s guidelines prior to analyses. Co-labeling with BrdU
and 7-AAD staining allowed for discrimination of cell cycle phases: G0/G1 (7-AAD low, BrdU low), S-phase (BrdU high, 7-AAD intermediate), and G2/M-phase (BrdU low, 7-AAD high).

**BrdU pulse-chase assay**

For the BrdU pulse-chase assay, NSPCs were grown in T-25 flasks with or without IFNγ (100 or 1000 U/mL) treatment as described for the BrdU assay. On DIV 3, the neurospheres were pulsed with BrdU for one hour and washed with 1X PBS and stored in 1X PBS until further use. Neurospheres were harvested at 0, 3, 6 and 9 hours post-BrdU washout. Neurospheres for the zero-hour time point were harvested immediately post-BrdU washout. For the other time points, the neurospheres were resuspended in NSPC culture medium and harvested at the respective time points. Following harvest, the protocol for the BrdU assay described above was followed for flow cytometric analysis.

**Single-pulse BrdU assay in cell monolayers**

WT-CD46+ NSPCs (20,000 cells/well) or astrocytes (250,000 cells/well) grown in 6-well plates from conditioned medium treatments were pulsed with BrdU (25μM) and incubated at 37°C for 90 mins. Post-BrdU treatment, the medium was discarded and the cells were detached using with 1mL/well pre-warmed 0.05% Trypsin-EDTA. The trypsin was neutralized using an equal volume of soybean trypsin inhibitor. The detached cells were collected in 1.5 mL microcentrifuge tubes and centrifuged at 300g for 5 mins at 21°C. Mixed glial cultures were incubated with IFNγ for 24 or 72 h, and BrdU (25 mM) was added for 1 h prior to harvest. Cells were trypsinized, fixed and permeabilized using 19
cytofix/cytoperm solution (BD biosciences). BrdU assay was performed as described in single pulse BrdU assay protocol.

IV. Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay

The TUNEL assay was performed using the Trivigen Flow TACS Apoptosis kit (R&D Systems, Minneapolis, MN) according to manufacturer’s protocol. Briefly, 50,000 cells/mL of NSPCs (WT or STAT1-KO) were cultured with or without IFNγ (100 and 1000 U/ml) for 3 DIV. Neurospheres were collected by centrifuging at 400 rpm and trypsinized as described above. The cells were fixed in 3.7% PFA for 10 min, permeabilized using 100 μL cytonin solution (Trevigen, Gaithersburg, MD, #4876-60-01) for 30 min, and treated with 25μL/sample TdT labeling mixture for 1 hr at 37°C. The cells were then treated with 25μL/sample of strep-fluorescein for 10 min in the dark and washed 1x in PBS. Neurosphere cells (1x10^5 cells/sample) were analyzed by flow cytometry to quantify TUNEL positive cells (FL1 channel) with gating to exclude cellular debris. DNase-treated and TdT enzyme-omitted groups were used as positive and negative controls for the assay, respectively.

V. Carboxyfluorescein succinimidyl ester (CFSE) assay

CellTrace CFSE Cell Proliferation Kit (Invitrogen, Grand Island, NY) was used to perform CFSE labeling. A single cell suspension of WT or STAT1-KO NSPCs (100,000 cells/mL) were labeled with 5μM CFSE in pre-warmed PBS at 37°C for 10 mins. The cells were washed with cold NSPC culture medium, centrifuged at 1500 rpm, and resuspended in fresh NSPC culture medium. The NSPCs were added to T-25 flasks, treated with 100
U/mL IFNγ NSPC culture medium, incubated at 37°C/5% CO2. After 2, 3, or 5 DIV, the neurospheres were dissociated using 0.05% trypsin (3 min at 37°C) and fixed using 3.7% paraformaldehyde (PFA) in PBS (5 min at 20°C). The fixed cell suspension was analyzed by flow cytometry to measure the intensity of the CFSE signal (FL1 channel) per cell (22,500 cells per condition in each experiment; n=3).

VI. Immunofluorescence assay

NSPCs were grown as neurospheres and passaged as indicated above for plating onto coverslips. The cells were grown in 24-well plates on poly-d-lysine/laminin-coated coverslips (10,000 cells/well) in NSPC culture medium (with EGF and FGF). On DIV2, NSPC culture medium was replaced with differentiation medium (47.4 mL DMEM, 1mL B27 without Vit A, 0.5 mL N2, 100 uL heparin; 1.8 mL/well). The cells were treated with 100 U/mL IFNγ. Two hundred uL of 10X solution of IFNγ (1000U/ml) was added to each well.

On 2 and 6 days-post IFNγ treatment, cells were washed with PBS and fixed in a solution of 4% PFA for 20 minutes at room temperature. The cells were permeabilized with 0.2% Triton X-100 solution. The NSPCs were stained for nestin, MAP2 and GFAP using primary antibodies at the same concentration indicated in Table 3. The nuclei were stained using Hoechst nuclear stain (1:10,000). The coverslips were mounted on glass slides with 5 μL of prolong gold mounting medium (Thermo-Fisher Scientific, Waltham, MA). The mounting medium was allowed to dry and the cells were imaged using the EVOS FL microscope (Thermo Fisher Scientific; Waltham, MA).
VII. Western blot

NSPCs were cultured in T-75 flasks by seeding 50,000 cells/mL in NSPC culture medium. The cells were treated with IFNγ (100 U/mL) or left untreated. On DIV 2, 3, or 5, the neurospheres were collected by centrifugation at 400 rpm and lysed using 200μL 1x Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA) with protease inhibitor cocktail (10 uL/10^6 cells; Sigma-Aldrich, St. Louis, MO). The lysate was centrifuged at 11,000 rpm and the supernatant was stored at -80°C until analysis. The protein concentration of each lysate was measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). For each sample, 20μg of lysate was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis on NuPAGE 7% Tris-acetate gels (Thermo Fisher Scientific, Waltham, MA). The gel was blotted onto Immobilon-FL Membrane (Millipore, Billerica, MA) and the membranes were blocked using a 1:1 mixture of 1X phosphate buffered saline/Tween-20 solution (Sigma-Aldrich, St. Louis, MO) and Odyssey blocking buffer (Licor Biosciences, Lincoln, NE) for 60 min at 20°C. The membranes were treated with primary antibody solutions diluted in Odyssey blocking buffer overnight at 4°C on a rocker. The membranes were washed thrice with PBS-Tween for 10 min each and incubated in secondary antibody solutions (goat anti-rabbit 680 or donkey anti-mouse 800 (1:10000); Licor Biosciences, Lincoln, NE) for 60 min at 20°C. The membranes were washed thrice in PBS-Tween and imaged on the Odyssey Infrared Imaging System (Licor Biosciences, Lincoln, NE). Individual bands were quantified using Image Studio software (Licor Biosciences, Lincoln, NE, version 4.0.21). The signal from each band(s) was normalized against the GAPDH signal as a loading control. Primary antibodies used were as follows: anti-phospho STAT1 (Y701,


VIII. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

**RNA extraction**

Cells from co-cultured 6-well plates were detached with 1mL/well pre-warmed 0.05% Trypsin-EDTA. The trypsin was neutralized using an equal volume of soybean trypsin inhibitor. The detached cells were collected in 1.5 mL microcentrifuge tubes and centrifuged at 300g for 5 mins at 21°C. The supernatant was discarded. RNA extraction was performed using Qiagen RNeasy kit (Qiagen; Hilden, Germany; Cat# ID: 74104) as per the manufacturer’s protocol. RNA was quantified using NanoDrop (ThermoFisher
Scientific, Waltham, MA). The RNA concentration for each sample was recorded and stored at -20°C until further use. The volume needed for 1μg RNA was calculated.

**Reverse transcription**

Reverse transcription was performed using the Quantitect Reverse transcription kit (Qiagen, Cat# 205311). Stored RNA was thawed immediately before use and kept on ice. Sample RNA (1μg) was pipetted in an RNase free microcentrifuge tube. Genomic DNA elimination reaction and reverse transcription was performed as per the kit directions. This reaction yielded 20μL of cDNA (50ng/μL) which would be used for qRT-PCR experiments.

**Quantitative reverse transcription polymerase chain reaction (qRT-PCR)**

The reverse transcription product (50 ng/μL, 20μL) was diluted to 6.25 ng/μL by adding 160μL of RNase free water. The master mix was prepared by diluting the forward and reverse primers for each gene with Eve green dye (Midsci, Valley Park, MO; Cat# BEQPCR-R). The master mix (37 μL/tube) was added to a microcentrifuge tube and the diluted cDNA (24μL/tube) was added to it. Seventeen μL of the prepared solution for each sample was added to a 96-well plate in triplicate. RNA was quantified by detection using the StepOnePlus Real-Time PCR System (ThermoFisher Scientific). ΔΔCT values were calculated for each sample. The fold change value for all the sample were calculated in comparison to uninfected+ no microglia group. The fold change values were graphed.
IX. NSPC characterization

To ascertain that the cultured E12.5 NSPCs were at the desired developmental stage, the cultures were characterized for co-expression of the developmental markers, nestin, SOX2, and PAX6 (Table 4) using immunocytochemistry (ICC). Cellular composition of neurosphere cultures was determined by staining for neural markers (Table 3) and quantifying their cellular expression using flow cytometry. Levels of marker expression (Table 5) and their temporal changes in an in vitro culture were determined using in-cell western assay (ICW). These assays and techniques are described below in detail. For all the characterization methods, NSPCs were grown as neurospheres first, On DIV3, these neurospheres were passaged and cultured as monolayers in tissue culture plates.

Immunocytochemistry (ICC)

NSPCs were grown in Poly-D-lysine/laminin coated coverslips in 24-well plates at 20,000 cells/well. The cells were cultured in NSPC culture medium Coverslips were blocked for one hour at 20°C using blocking solution (4% goat serum in 1X PBS without Ca2+/Mg2+). All primary and secondary antibody solutions were prepared by diluting in blocking solution. Coverslips were incubated in the primary antibody overnight at 4°C. The coverslips were washed by dipping them gently in 1X PBS without Ca2+/Mg2+ and incubated in secondary antibody solution for one hour at 20°C. The coverslips were washed again in 1X PBS without Ca2+/Mg2+. Five μL of prolong gold mounting medium (Thermo-Fisher Scientific, Waltham, MA) was placed on a glass slide and the coverslips were mounted on it. Cells were imaged on the Olympus epifluorescence microscope at 20X magnification. Cells expressing neural markers were quantified using Image J software.
(Version 1.6.0_65) and graphed. A detailed description of the primary and secondary antibodies is provided in Table 3

**Flow cytometry**

Cells grown in six-well plates and treated with conditioned medium were allowed to differentiate for four days. The cells were then detached as described above. The cells were collected in a microcentrifuge tube and centrifuged at 300xg. The supernatant was discarded and the cells were fixed using 100μL/sample cytofix-cytoperm (Bd biosciences, Cat# 554714) solution for 30 minutes at 20°C. The cytofix-cytoperm solution was washed by adding 500μL/sample perm-wash solution (BD biosciences Cat# 554723) and centrifuging at 300xg. The pellet was resuspended in 50μL/sample primary antibody solution for 30 mins at 20°C. All antibody solutions were made by diluting stock solution in perm/wash buffer. The cells were washed with 500μL/sample of perm-wash buffer and centrifuged at 300xg for 5 mins at 20°C. The cells were treated with secondary antibody for 30 mins at 20°C and washed with cytofix-cytoperm solution as the previous step. The pellet was resuspended in staining solution (3% FBS in 1X PBS). The cells were run through the flow cytometer using the gate to exclude debris as described in the single pulse BrdU assay protocol. Signals for neural markers were measured and the percent live cells were graphed. A detailed description of the antibodies is provided in Table 4.
### Table 3

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Primary Antibody (Concentration, Supplier, Cat#)</th>
<th>Secondary Antibody (Concentration, Supplier, Cat#)</th>
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</thead>
<tbody>
<tr>
<td>Nestin</td>
<td>Mouse anti-Nestin (1:200; EMD Millipore, Cat# MAB353)</td>
<td>Anti-mouse Alexa Fluor 488 (1:1000; Thermo Fisher Scientific, A11034)</td>
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<tr>
<td>BIII-tubulin</td>
<td>Rabbit anti-BIII-tubulin (1:500; Cell Signaling Technology; Cat# 5568)</td>
<td>Anti rabbit-Alexa Fluor 555 (1:1000; Thermo Fisher Scientific, A21428)</td>
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<tr>
<td>Doublecortin (DCX)</td>
<td>Guinea pig anti-doublecortin (1:1000; EMD Millipore, Cat# AB2253)</td>
<td>Anti guinea pig-Alexa Fluor 555 (1:2000 Thermo Fisher Scientific, A11073)</td>
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<td>Microtubule associated protein 2 (MAP2)</td>
<td>Rabbit anti-MAP2 (1:500, EMD Millipore Cat# AB5622)</td>
<td>Anti rabbit- Alexa Fluor 555 (1:1000; Thermo Fisher Scientific, A21428)</td>
</tr>
<tr>
<td>Glial fibrillary acidic protein (GFAP)</td>
<td>Rabbit anti-GFAP (1:500; DAKO, Cat# Z0334)</td>
<td>Anti rabbit- Alexa Fluor 555 (1:1000; Thermo Fisher Scientific, A21428)</td>
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<tr>
<td>SOX2</td>
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<td>Anti-rabbit- Alexa Fluor 555 (1:1000; BD biosciences, 558416)</td>
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<tr>
<td>PAX6</td>
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<td>Anti-rabbit-Alexa Fluor 555 (1:1000; BD biosciences, 558416)</td>
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</table>

Table 3: Antibodies for ICC.
Table 4

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Primary Antibody (Concentration, Supplier, Cat#)</th>
<th>Secondary Antibody (Concentration, Supplier, Cat#)</th>
<th>BD Accuri Channel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nestin</td>
<td>Mouse anti-nestin-PE (1:50; RandD systems, IC1259P)</td>
<td>-</td>
<td>FL2</td>
</tr>
<tr>
<td>SOX2</td>
<td>Rabbit anti-SOX2 (1:50; EMD Millipore, Ab5603)</td>
<td>Anti-rabbit-PE (1:100; BD biosciences, 558416)</td>
<td>FL2</td>
</tr>
<tr>
<td>BIII-tubulin</td>
<td>Rabbit anti-BIII-tubulin (1:50, Cell Signaling Technology Cat#5568)</td>
<td>Anti rabbit-Alexa Fluor 488 (1:100; Thermo Fisher Scientific, A11034)</td>
<td>FL1</td>
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<tr>
<td>DCX</td>
<td>Goat anti-doublecortin (1:50; Santa Cruz biotechnology, sc-8066)</td>
<td>Anti-goat-Alexa Fluor 647 (1:100; Jackson immunochemicals, 705-605-147)</td>
<td>FL4</td>
</tr>
<tr>
<td>GFAP</td>
<td>Rabbit anti-GFAP (1:50 DAKO, Cat# Z0334)</td>
<td>Anti rabbit-PE (1:100; BD biosciences, 558416)</td>
<td>FL1</td>
</tr>
</tbody>
</table>

Table 4: Antibodies for flow cytometric characterization of NSPCs.
**In-cell Western (ICW) assay**

Neurospheres were passaged as described above. NSPCs were seeded in 96-well plates at 3386 cells/well in NSPC growth medium. The cells were allowed to grow for 5 or 7 DIV. On these days, the medium was removed and the cells were fixed with 4% PFA for 20 minutes and permeabilized using 0.2% Triton X-100 for 15 minutes. The plates may be stored in 1X PBS until further use. For ICW, the cells were blocked using 1:1 solution (odyssey buffer:PBS without Ca\(^{2+}/\)Mg\(^{2+}\)) of blocking buffer for 1 hour at 20°C. Concentration-matched isotype control antibodies (IgG) were also prepared in blocking buffer. Primary antibodies were prepared in 1:1 blocking buffer at indicated concentrations and kept at 4°C until further use (Table 5). Blocking solution was discarded and primary antibody solution was added to the plates. The plates were incubated overnight at 4°C. Primary antibody solution was removed and the plates were washed twice with PBS. Secondary antibody solutions were prepared in 1:1 blocking buffer at indicated concentrations in Table 5. DRAQ5 (Biostatus Limited, Leicestershire UK; Cat# DR50050), a DNA intercalating probe, was added at a final concentration of 5 μM (stock concentration 5mM) to the secondary antibody solutions at indicated concentration. The secondary antibody solutions were added to the cells and incubated at 4°C for one hour at 20°C. The secondary antibody solution was removed and the cells were washed twice with PBS. The plates were imaged on Odyssey Infrared Imaging System. Integrated fluorescence intensity values for each neural marker were normalized with the corresponding DRAQ5 values. Normalized values for each neural marker were subtracted from its corresponding concentration matched isotype control values. The adjusted values ± SEM of each marker were graphed using Microsoft Excel.
X. Statistical analyses

Data are presented as the mean ± SEM from 3-4 technical replicates for all experiments. For the neurosphere assay, a one-way ANOVA was performed. To compare the IFNγ-treated groups with the untreated group, a Dunnett’s multiple comparison test was applied. For the BrdU assay and western blot analyses, a one-way ANOVA was performed and a Bonferroni multiple comparisons post-hoc test was applied for pair-wise comparisons between the untreated and IFNγ-treated groups. For the BrdU pulse-chase assay, a two-way ANOVA was performed to compare the differences between the different treatment groups across different time points, with Bonferroni multiple comparisons post-hoc analysis. Flow cytometry data (BrdU incorporation and NSPC differentiation) as well as ICC data from conditioned medium treatment experiments were analyzed using two-way ANOVA with Holm-Sidak post-hoc test for multiple comparisons. For the CFSE and TUNEL assays, a two-tailed Student’s t-test was applied for statistical comparisons. For all other assays, a one-way ANOVA was performed. Differences were deemed significant when p ≤ 0.05. For p values between 0.0001 and 0.05, actual p values are reported. For any values <0.0001, Graphpad software reports the values as “p<0.0001”, which we list as appropriate. All statistical analysis was performed using Graphpad Prism (Version 6.0b).
<table>
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<tr>
<th>Antigen</th>
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<th>Secondary Antibody</th>
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<tbody>
<tr>
<td></td>
<td>(<em>Concentration, Supplier, Cat#</em>)</td>
<td>(<em>Concentration, Supplier, Cat#</em>)</td>
</tr>
<tr>
<td>Nestin</td>
<td>Mouse anti-nestin (1:500; EMD Millipore, MAB353)</td>
<td>IRDye 800CW Donkey anti-mouse (1:10000; Licor Biosciences, 926-32213)</td>
</tr>
<tr>
<td>Microtubule associated protein-2 (MAP2)</td>
<td>Rabbit anti-MAP2 (1:500, Millipore Cat#AB5622)</td>
<td>IRDye 800CW Goat anti-Rabbit (1:10000, Licor Biosciences, 926-32211)</td>
</tr>
<tr>
<td>GFAP</td>
<td>Rabbit anti-GFAP (1:1000 DAKO, Cat# Z0334)</td>
<td>IRDye 800CW Goat anti-Rabbit (1:10000, Licor Biosciences, 926-32211)</td>
</tr>
<tr>
<td>Mouse Isotype control</td>
<td>normal mouse IgG (1:200, Santa Cruz biotechnology, sc-2025)</td>
<td>IRDye 800CW Donkey anti-mouse (1:10000; Licor Biosciences, 926-32213)</td>
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<tr>
<td>Rabbit Isotype control</td>
<td>normal rabbit IgG (1:130 for GFAP, 1:200 for MAP2, Santa Cruz biotechnology, sc-2027)</td>
<td>IRDye 800CW Goat anti-Rabbit (1:10000, Licor Biosciences, 926-32211)</td>
</tr>
</tbody>
</table>

Table 5: **Antibodies for ICW characterization of NSPCs.**
Outliers were identified using the Grubb’s method and the alpha level was set to 0.05 using Graphpad Prism. In the neurosphere assay for WT NSPCs, five outlier data points were reported corresponding to individual neurospheres. Upon statistical analysis of the cleaned data, the comparisons retained significance. Technical replicates were obtained from at least 3 separate set of dissections. Each replicate was defined as embryonic cortical NSPCs from obtained from one female dam. Typically, each replicate involved NSPCs derived from 6-8 embryos.
Chapter 3: Assessment of the role of interferon-gamma signaling in neural stem/progenitor cell proliferation and differentiation

I. Rationale

Neural stem/progenitor cells (NSPCs) are multipotent cells in the CNS. During CNS insults (viral infection, stroke, protein misfolding stress), the NSPC pool can expand or contract. During many neurotropic infections, NSPC proliferation and neurogenesis declines. These cytostatic responses by NSPCs may be attributed to direct infection by the virus and/or the anti-proliferative effect of inflammatory cytokines. Our previous work demonstrated that neural cells display tailored cellular responses to IFNγ, depending upon the availability of JAK/STAT signaling molecules. For example, in primary neurons, IFNγ mediates control of MV infection. However, STAT1, which is the canonical transcription factor downstream of IFNγ, is not required for IFNγ-mediated MV control. Moreover, STAT1 activation and expression is delayed in neurons unlike astrocytes, where it is activated immediately post-IFNγ treatment. However, the effect of IFNγ on proliferation and cell cycle control of neural cells, and the resultant changes in neurodevelopment or differentiation, is largely undefined. IFNγ is required for viral clearance of many neurotropic viral infections. IFNγ protected neurons from Sindbis virus-mediated cell death in a non-cytolytic fashion. Similar observations have been made in IFNγ-mediated clearance of MV and hepatitis virus among others. This is significant as mature neurons are largely a non-renewable cell type and control of virus without cell loss is imperative for effective protection of CNS functions. Given the critical
role that IFNγ plays during CNS infections and the pluripotent effects of the cytokine of different neural cells, we examined IFNγ-mediated signaling pathways in primary NSPCs and the consequences on proliferation and survival.

To study the effects of IFNγ on NSPC activity, we cultured NSPCs in vitro form E.12.5 embryos as has been described in Materials and Methods. For the purposes of this study, we wanted to elucidate the immune-mediated effects on growth and cell fate choice of NSPCs. Therefore, NSPCs were derived from E12.5 mice were used in order to provide sufficient cell numbers for experiments and to capture cells that were still in a proliferative state. When cultured as a suspension, the NSPCs form clusters called neurospheres. When cultured on poly-D-lysine/laminin-coated plates, the NSPCs will attach and grow as monolayers. The neurosphere culture system is used extensively as an in vitro model system to study neural development, proliferation, and self-renewal of NSPCs. Neurosphere cultures derived from E12.5 embryos also have been characterized in the literature. These neurospheres consist of a heterogeneous mixture of neural stem and progenitor cells and recapitulate the spatiotemporal characteristics of the developing CNS at that age\textsuperscript{171}. The neurosphere cultures, when dissociated and cultured as monolayers, differentiated into all the neural cell types depending upon culture conditions. Moreover, GABAergic and glutamatergic neuronal sub-types types were observed that formed functional synaptic connections. The NSPCs when allowed to differentiate, gradually differentiate into neuro and glioblasts and finally into terminally differentiated neurons and glia. Therefore, monolayer cultures may be used as effective tools to measure NSPC differentiation. To determine whether our NSPC cultures from CD46+ mice conformed to
the above observations, the cells were characterized to determine purity of the cells in both neurosphere and monolayer culture.

II. Characterization of mouse E 12.5 NSPC: Neurosphere and monolayer cultures.

Primary NSPCs can be cultured as neurospheres and monolayers. These models were used to answer specific questions pertaining to proliferation/cell cycle progression and differentiation, respectively. To understand the effects of IFNγ on NSPCs, it was important to determine the cellular composition of NSPC cultures *in vitro*. Neurospheres (~150μm in diameter) were formed by three days of growth *in vitro* (DIV3) in growth media designed to limit differentiation but maintain proliferative potential of the cells. After 3 DIV, the neurospheres were dissociated and the NSPCs were either plated as monolayers to measure neural markers by IFA/ICW, or grown in suspension again to determine neural marker and the IFNγ receptor. These experiments are described below in detail.

The expression of neural markers in monolayer cultures was determined. Post-dissection, cortices were processed as described in materials and methods, NSPCs were cultured initially as neurospheres to expand the cell number for future experiments. On DIV3, the neurospheres were passaged and cultured as monolayers on poly-D-lysine/laminin coated 24-well plates with coverslips for ICC (Figure 2), or 96-well plates for in-cell western assay (ICW, Figure 3). For ICC and ICW, the cells were grown for seven days and cellular purity was determined on DIV 3, 5, and 7 with ICC and on DIV 5 and 7 with ICW.
On DIV 3, the monolayers had more than 90% of cells expressing nestin, whereas 4% expressed MAP2 and, 1% cells expressed GFAP. This cellular composition stayed consistent over 5 and 7 DIV. The expression levels of these markers also were quantified using ICW to measure the fluorescence intensity of each marker in the culture. The fluorescence intensity for each antibody was normalized against a nuclear stain (DRAQ5) to account for the total cell number in each well. Normalized ICW data showed that the cultures expressed low levels of MAP2 and GFAP on DIV 5, which is consistent with the ICC data. The levels of nestin increased over time from DIV 5 (0.03±0.05) to 7 (0.1±0.01). The expression of MAP2 increased on DIV 7 (0 to 0.02±0.001), but GFAP was not detected at any time point. This showed that that over time the composition of NSPC cultures remained constant, with very few terminally differentiated neurons or astrocytes.
Figure 2: In vitro NSPC cultures contain low levels of differentiated neuronal and astrocytic cells. NSPCs were grown in 24-well plates on poly-D-lysine/laminin coated coverslips. The coverslips were collected on DIV 3, 5 and 7 fixed, permeabilized and co-stained for (A) Nestin (red) and MAP2 (green), and (B) Nestin (red) and GFAP (green). Nestin+ (NSPCs), MAP2+ (neurons) and GFAP+ (astrocytes) cells were counted and graphed for all time points (C). Green arrows denote differentiating neurons (MAP2+, top row) and astrocytes (GFAP+, bottom row). The total cell number...
was counted using Hoechst as nuclear stain. Each cell type was quantified for all three time points in vitro and graphed as a percentage of total cells ± SEM. Scale bar=100μm

NSPCs derived from E12.5 mouse telencephalons have been shown to express sex determining region Y-box 2 (SOX2) and Paired box protein (PAX6) in vivo and when grown as neurospheres\textsuperscript{172}. SOX2 and PAX6 are transcription factors that play an important role in the neural specification of ectodermal cells\textsuperscript{173} and maintain the proliferation of NSPCs once they differentiate from ectodermal lineage into the neural lineage\textsuperscript{174}. Together, they coordinate the expression of genes such as \textit{Fgf4}, \textit{Utf1}, and \textit{Fbx15} that maintain NSPC proliferation and bring about CNS development\textsuperscript{175-177}. Loss of SOX2 and/or PAX6 decreases the clonogenicity of NSPCs, a measure of proliferative potential of the cells\textsuperscript{178}. Therefore, to ascertain the purity of the NSPC cultures and the developmental stage of the stem cells, we co-labeled the NSPCs with nestin and SOX2 or PAX6. In the monolayer cultures, the NSPCs were found to be double-positive for nestin+/PAX6+ (\textbf{Figure 4A}) and nestin+/SOX2 (\textbf{Figure 4B}), suggesting that in nestin+ cells in our model are positive for the standard biomarkers of NSPCs.

In order to study the effects of IFN$\gamma$ on the NSPC cultures, we first determined whether the cells expressed the receptor for IFN$\gamma$ (IFN$\gamma$R). We measured the cell surface expression of IFN$\gamma$R1, one of the components of the heterotrameric receptor, on NSPCs using flow cytometry (\textbf{Figure 5}). IFN$\gamma$R1 was detectable on 81.6% of the cells in the neurospheres (\textbf{Figure 5A}). The neurospheres were comprised of a total of 95.3% nestin+ cells (\textbf{Figure 5B}), suggesting that some NSPCs may not express the receptor or may express the receptor at levels that are below the limit of detection of the assay. Twenty-five
percent of the cells expressed both DCX, a marker for immature neurons, and the IFNγR1 subunit (Figure 5C). Although we were not able to triple-label the cells for nestin, DCX, and IFNγR1, we suspect that there is a subset of nestin+/DCX+ cells that represent cells transitioning from NSPCs to immature neurons, and that these developing cells express IFNγR1. No GFAP+/IFNγR1+ cells were found (data not shown), which is consistent with the low to undetectable levels of GFAP found in the characterization studies.
Figure 3: NSPC cell density and Nestin levels increase over time: WT/NSPCs were seeded on Poly-d-lysine/laminin coated 96-well plates at 3368 cells/well. Cells were grown in NSPC culture medium for (A) 5, or (B) 7 DIV and ICW assay was performed. Plates were fixed on DIV 5 or 7 and NSPCs were stained for (A and B, left panel) Nestin, MAP2, GFAP, and (A and B, right panel) DRAQ5. (C) Fluorescence values of neural markers were normalized against DRAQ5 and graphed for DIV 5 and 7. Data is presented as mean fluorescence (A.U) ± SEM.
Figure 4:

Monolayer cultures form E 12.5 CNS express classical biomarkers of NSPCs: WT/NSPCs were seeded on Poly-d-lysine/laminin coated 24-well plates at 20,000 cells/well. Cells were grown in NSPC culture medium for 3 DIV. Plates were fixed and cells were stained for (A) PAX6 (red) and (B) SOX2 (red). Both biomarkers were co-stained with nestin (green). Hoechst 33342 (blue) was used as nuclear marker. Because PAX6 and SOX2 are transcription factors, staining for both proteins often appears in the nucleus, giving a pink color in areas of co-localization with Hoechst. Scale bar=50μm.
Figure 5.  *Neurosphere cells express IFNγR1.* Neurospheres were cultured for 3 days *in vitro,* dissociated into a single cell suspension, and stained for neural cell markers and for the R1 subunit of the IFNγ receptor (IFNγR1). Antibodies for IFNγR1 (α-IFNγR1), the NSPC marker nestin (α-Nestin), and the immature neuron marker doublecortin (α-DCX) were used along with appropriate isotype controls. The cells were analyzed by flow cytometry and debris was excluded using the gate in (A). IFNγR1 expression was measured for NSPCs. (B). To co-label the neurosphere cells with the receptor and nestin or DCX, cells were stained for nestin and IFNγR1 (C) or DCX and IFNγR1 (D). Nestin+ cells were
also positive for IFN\(_{\gamma}R1\), and DCX+ cells were also positive for IFN\(_{\gamma}R1\). NSPCs (nestin+) and immature neurons (DCX+), are the predominant cell populations in the neurospheres. Co-labeling with nestin and DCX reveals that 55.2% of the nestin+ cells and 75.5% of DCX+ cells expressed IFN\(_{\gamma}R1\).

### III. IFN\(_{\gamma}\) inhibits neurosphere growth

As NSPCs express the IFN\(_{\gamma}\) receptor, we reasoned that NSPCs would respond to elevated levels of IFN\(_{\gamma}\) during a CNS infection, whether the cells were infected by the virus. Specifically, we hypothesized that IFN\(_{\gamma}\) would decrease NSPC proliferation by inhibiting cell cycle progression. To determine the effects of IFN\(_{\gamma}\) on NSPC activity, we subjected the NSPCs to IFN\(_{\gamma}\) treatment. NSPCs were allowed to form neurospheres and NSPC diameter was measured as a marker of proliferation. Neurosphere diameter was significantly smaller in IFN\(_{\gamma}\)-treated cultures in comparison to untreated cells or to cells treated with heat-inactivated IFN\(_{\gamma}\) (ΔH IFN\(_{\gamma}\); 1000 U/ml) at all concentrations tested (Figure 6). These concentrations were chosen based upon physiological concentrations observed in vivo post MV administration\(^{179}\). At DIV 3, IFN\(_{\gamma}\) limited neurosphere diameter in comparison to untreated controls at both low (1 U/ml IFN\(_{\gamma}\)) and high (1000 U/ml IFN\(_{\gamma}\)) concentrations of IFN\(_{\gamma}\). Neurospheres were restricted to 89.5%±3.3 of untreated controls at 1 U/ml IFN\(_{\gamma}\) (n=3, p=0.0063) and 59.4%±3.0 of untreated controls at 1000 U/ml (n=3, p<0.0001) (Figure 6B). By 7 days post-IFN\(_{\gamma}\) treatment, neurosphere diameter was less than half of the untreated cells at 100 and 1000 U/ml of IFN\(_{\gamma}\) (44.6%±3.2 of untreated; n=3, p<0.0001 and 43.7%±3.2 of untreated; n=3, p<0.0001, respectively). These results show that IFN\(_{\gamma}\) treatment was associated with a prolonged reduction in neurosphere proliferation.
We next determined the distribution of neurosphere sizes during IFNγ treatment using a histogram analysis of neurosphere area. We observed a decrease in median neurosphere area with IFNγ treatment (100 U/mL, DIV 5) as measured by the number of pixels² in each neurosphere (Figure 6C). The median neurosphere area was reduced 3-fold from 2054.4 pixel² in untreated cells to 656.5 pixel² in IFNγ-treated NSPCs (100 U/ml).

Furthermore, the distribution of neurosphere sizes shifted toward a smaller-sized population of neurospheres with the addition of IFNγ, as shown by the leftward shift of the curve in the IFNγ-treated (100 U/ml) group (Figure 6C) versus untreated cells or cells treated with 1 U/ml IFNγ.
Figure 6

A

DIV 5

Untreated
ΔH IFN
IFN (100 U/mL)
IFN (1000 U/mL)

B

DIV 3
DIV 5
DIV 7

Neurosphere diameter (% Untreated)
IFN (U/mL)

C

Untreated
1 U/mL
100 U/mL

Number of neurospheres
Area (Pixel²)
Figure 6: IFNγ inhibits neurosphere growth in a concentration-dependent manner. Wildtype NSPCs (WT/NSPCs) were treated with IFNγ (1-1000 U/ml) or with heat-inactivated IFNγ (ΔH IFNγ; 1000 U/ml) as a negative control for 3, 5, or 7 days in vitro (DIV). (A) Representative images of neurospheres imaged 5 days post-IFNγ treatment at 2x magnification for different concentrations of IFNγ (Scale bar=250 μm). (B) Quantitation of neurosphere diameter at 3, 5, and 7 days post-IFNγ treatment. The longest diameter of each neurosphere was measured using Image J software. Data was collected from neurospheres in five fields/condition from three biological replicates and graphed as a percentage of untreated controls. Statistical analysis was applied by one-way ANOVA (*** p<0.01, **** p<0.0001). (C) Histogram plots of neurosphere area for different concentrations of IFNγ on DIV 5 as measured by pixel² for each neurosphere. For each condition, five fields/condition were measured using cells from three biological replicates. Frequency distribution of the number of neurospheres corresponding to the indicated neurosphere area in pixel² was plotted for each condition.
**Figure 7**

(A) TUNEL assay was performed on untreated and IFNγ-treated (100 and 1000 U/ml; 72h) NSPCs. The average percentage of total TUNEL+ cells from 3 independent experiments is plotted with SEM (one-way ANOVA, ** p<0.01).

(B) WT/NSPCs were treated with IFNγ (1, 100, 1000 U/ml) for 72h and labeled with BrdU and 7-AAD. The intensity of BrdU and 7-AAD staining per cell was assayed by flow cytometry. Cell populations were gated in different phases of cell cycle (S = synthesis phase, G1= gap phase 1, M= mitosis phase, G2= gap phase 2). Representative plots for untreated and IFNγ-treated NSPCs (1000 U/ml) are shown.

(C) Quantitation of NSPCs in each cell cycle phase. The average percentage of cells in each cell cycle was plotted with SEM (n=3). Statistical analysis was applied using one-way ANOVA (****p<0.0001; ***p<0.001).

(D) Cells in the S-phase gates were analyzed for mean fluorescence intensity (MFI, arbitrary units, A.U.) of the BrdU signal for all treatment groups. The MFI for the BrdU signal of IFNγ-treated cells was compared with untreated controls using a one-way ANOVA (*p<0.05).
IV. NSPCs proliferation is restricted at the G1/S checkpoint in response to IFNγ

Based on the inhibition of neurosphere growth that we observed with IFNγ treatment, we reasoned that cell death and/or changes in the cell cycle could contribute to the reduction in neurosphere size. IFNγ can induce apoptotic or pro-survival pathways depending on the cell type and on the context of other inflammatory mediators in the system \(^{180-183}\). To determine if apoptosis contributed to the IFNγ-mediated restriction in neurosphere size, we quantified the percentage of apoptotic cells in the neurospheres using the TUNEL assay (Figure 7A). A significant but modest increase in apoptosis was seen at the highest concentration of IFNγ used in the study (0.45%±0.08 TUNEL+ cells in untreated versus 3%±0.5 TUNEL+ cells at 1000 U/mL IFNγ (n=4, p=0.0037), demonstrating that IFNγ can lead to limited cell death.

We next performed BrdU/7-AAD staining in IFNγ-treated neurospheres to identify the stages of the cell cycle. The thymidine analog BrdU is incorporated into newly synthesized DNA, which marks cells in the S (synthesis) phase of the cell cycle. The cells are also counterstained with 7-AAD to measure the total DNA content \(^{184}\). We analyzed BrdU/7-AAD-stained NSPCs by flow cytometry, and gated single cells by cell cycle stage (representative plots are shown in Figure 7B). With IFNγ treatment, there was a dose-dependent decrease in the percentage of cells in the S-phase (48.8%±1.0 untreated versus 33.9%±0.5 IFNγ-treated (1000 U/ml; n=4 p<0.0001)), suggesting that fewer cells were actively synthesizing DNA (Figure 7C, left panel). Moreover, we observed a dose-dependent increase in the number of cells in the G0/G1phase (38.7%±1.0 untreated versus 48.9%±1.0 IFNγ-treated (1000 U/ml; n=4, p<0.0001)) and G2/M phase (0.36%±0.1 untreated versus 3.7%±0.6 IFNγ-treated (1000 U/ml; n=4, p=0.001)) (Figure 7C, middle
These findings indicate that IFNγ induces a major restriction in cell cycle progression at the G1/S checkpoint, and a significant, but relatively minor, restriction at the G2/M checkpoint.

The changes in cell cycle progression could be explained by a longer G0/G1 phase and/or by a shorter S phase with IFNγ treatment. To determine if IFNγ treatment also altered the duration of the cell cycle phases, we first compared the mean fluorescence intensities (MFI) of NSPCs in S phase (Figure 7D). The MFI of IFNγ-treated NSPCs in S-phase was higher than the untreated group (n=4; p=0.0491 for 100 U/ml and p=0.0357 for 1000 U/ml). Moreover, when comparing the number of BrdU+ cells in the G2/M phase, we found that the IFNγ-treated groups (100 and 1000 U/ml) had higher numbers of BrdU+ cells than the untreated control (Figure 7C), suggesting that more NSPCs were transitioning from the S phase to G2/M with IFNγ treatment. Together, these findings support the notion that IFNγ induces a shorter S phase in NSPCs.

To further investigate IFNγ-mediated changes in the cell cycle progression, we also performed a BrdU pulse chase experiment. In this experiment, IFNγ-treated neurospheres (72h) were pulsed with BrdU for one hour and cell cycle progression was tracked in cells for nine hours post-BrdU washout (Figure 9) This allows the cells to complete one cell cycle and IFNγ-mediated effects can be detected by looking at the number of cells in each cell cycle phase over time. At all time points, IFNγ treatment decreased the proportion of cells transitioning from G1 to S phase (G1/S) and from S-phase to G2 (S/G2) (Figure 9A and B). However, the number of cells BrdU+ cells in the G0/G1 and G2/M phases increased with IFNγ treatment. This observation corroborates the increased MFI of BrdU+ S-phase cells seen with IFNγ treatment (Figure 7D), suggesting that IFNγ leads to a
shortened S-phase. These studies also suggest that IFNγ treatment lengthens the G0/G1 phase, as the percentage of cells increased in G0/G1 in the presence of IFNγ.

To directly test whether IFNγ controls the rate of proliferation, we conducted a CFSE proliferation assay. CFSE [5(6)-carboxyfluorescein diacetate succinyl ester] is a fluorescent dye that penetrates cell membranes, undergoes metabolism, and becomes trapped within the cell. During cell division, CFSE is equally distributed to daughter cells with fluorescence intensity decreasing by half with each cell division, thereby acting as a marker for the number of cell divisions that a cell has undergone. NSPCs were pulse-labeled with CFSE and treated with IFNγ (100U/ml) for 2, 3, and 5 days. IFNγ-treated NSPCs had higher mean fluorescence intensity (MFI) of the CFSE label (red lines, Figure 9) in contrast to untreated control cells at all time points (black lines, Figure 9), which indicates that the untreated cells had progressed through more cell divisions during the same time period. The MFI of IFNγ-treated NSPCs was 1.3-fold greater than untreated cells at two days post-treatment (Figure 9, right panel) and 2-fold greater than untreated cells by five days post-treatment (Figure 9, left panel). Thus, IFNγ reduced the rate of proliferation by limiting the number of NSPC cell divisions over the 5-day time course.

V. IFNγ alters activation and expression of STAT1 and STAT3 in NSPCs

IFNγ primarily signals through the JAK/STAT1 pathway. However, alternative signaling via STAT3 has also been reported, and activation of both STAT1 and STAT3 has been demonstrated in primary hippocampal neurons after IFNγ stimulation. STAT1 and STAT3 also are associated with inhibition and activation of cell proliferation, respectively. Thus, based upon the cell cycle blockade observed in IFNγ-treated
NSPCs, we next analyzed the activation of STAT1 and STAT3 (Figure 10A). Both STAT1 and STAT3 were phosphorylated upon IFNγ treatment (Figure 10B). Phosphorylation of STAT1 on tyrosine 701 (Y701) and serine 727 (S727) was elevated in all IFNγ-treated groups, with phosphorylation of Y701 and S727 peaking at three days post-treatment when normalized to GAPDH (348-fold increase above untreated for Y701 and 184-fold above untreated for S727; n=3, p<0.0001). Phosphorylation of STAT3 was less pronounced than STAT1, with a 2.4-fold increase in phosphorylation versus untreated NSPCs at DIV 2 (n=4, p=0.0018), followed by a decline in phosphorylation below the untreated group by DIV 5. Basal levels of phosphorylated STAT3 also increased over time in the untreated groups (10-fold increase in phosphorylated STAT3 in the untreated group at day 5 versus day 0).

We also observed an increase in the total expression levels of STAT1 and STAT3, regardless of phosphorylation status, in all IFNγ-treated groups. These findings indicate that NSPCs respond to IFNγ with a sustained activation of STAT1 and a transient, but significant, activation of STAT3.
Figure 8: IFN$\gamma$ decreases NSPC cell cycle progression. WT NSPCs were treated with IFN$\gamma$ (100 U/ml) 3 days or left untreated. On day 3, the cells were pulsed with BrdU for one hour. The BrdU was washed out and the cells were harvested at 0, 3, 6, and 9h post-washout. Cells were processed as described in materials and methods for BrdU/7-AAD staining. (A) Representative plots for untreated (top row) and IFN$\gamma$-treated (1000 U/mL, bottom row) NSPCs with gates for G0/G1, G1/S, S/G2 and G2/M phases at different time points. (B) Averages of cell percentages in each of cell cycle gate were quantified for untreated, and IFN$\gamma$-treated (100 and 1000 U/mL) NSPCs. Error bars represent SEM. Statistical
analysis was applied using two-way ANOVA with Bonferroni post-hoc analysis (n=3), * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

Figure 9

A

Figure 9: IFNγ reduces proliferation rate in NSPCs. Untreated NSPCs were loaded with CFSE and treated with IFNγ (100 U/ml; red line) or were left untreated (black line). (A) CFSE signal was measured by flow cytometry and assayed mean fluorescence intensity (MFI) on DIV 2, 3, and 5 post-IFNγ treatment. Representative histograms for each time point are shown. (B) The MFI for each condition was averaged and statistical analysis was applied using student’s t-test (*p<0.05; n=5).
**Figure 10:** NSPCs activate STAT1 and STAT3 upon IFNγ stimulation.

IFNγ-treated NSPCs (100U/ml) were collected on DIV 2, 3, and 5, and lysed for western blot analysis. 

(A) Representative blots are shown for phosphorylated STAT1 (Y701 and S727), total STAT1, phosphorylated STAT3 (Y705), and total STAT3. GAPDH is shown as the loading control. (B) The fluorescence signal for each protein was quantified and normalized to GAPDH. (C) For the phosphorylated STAT1 and STAT3 bands, the fluorescence signals were also normalized to the levels...
of the total STAT1 and STAT3, respectively. The average of 3-5 biological replicates is plotted with SEM. Statistical analysis was applied using repeated measures one-way ANOVA with Bonferroni multiple comparisons post-hoc analysis (** **p<0.0001, *** p<0.001, ** p<0.01 * p<0.5).

We also observed an increase in the total expression levels of STAT1 and STAT3, regardless of phosphorylation status, in all IFNγ-treated groups. These findings indicate that NSPCs respond to IFNγ with increased expression of STAT1 and STAT3. Normalization of phosphorylated STAT1 and 3 with their respective total protein levels was also performed (Figure 10C). The protein ratio of STAT1-P/STAT1 at both phosphorylation sites was consistent with that seen with GAPDH normalization. However, the protein ratio of STAT3-P/STAT3 did not show increased activation with IFNγ at DIV 2 or 3. These data indicate that increased STAT1 activation is not only attributed to greater total STAT1 expression but also to increased activation of the STAT1 pathway. However, increased STAT3 phosphorylation corresponded with greater expression of total STAT3 protein levels post-IFNγ treatment. The STAT3 phosphorylation decreased on DIV5 with IFNγ treatment, while the total STAT3 expression continued to increase. This suggests that the NSPCs may be activating mechanisms that lead to decreased phosphorylation of STAT3, such as through (SOCS3) or protein tyrosine phosphatases (PTPs). SOCS3 prevents JAK-mediated STAT3 activation by inhibiting binding through the with tyrosine kinase SH2 domains\textsuperscript{190}. PTPs remove phosphate groups from STAT3, thereby reducing activation\textsuperscript{191}. Thus, one possibility is that long-term IFNγ treatment leads dephosphorylation of STAT3 despite the elevated expression of the protein.
VI. IFNγ decreases site-specific phosphorylation of pRb and expression of late G1/S cyclin/cdk complexes

Growth arrest is mediated by decreased expression of positive regulators of the cell cycle, including particular cyclins and cyclin-dependent kinases (cdks) depending on the cell cycle checkpoint. In order to progress through the G1/S checkpoint, both cyclin D1/cdk4 and cyclin E/cdk2 phosphorylate the retinoblastoma protein (pRb) at serine 795 (S795), which is a rate-limiting step in the progression from the G1 phase to the S phase. Phosphorylation at S795 is critical for pRb inactivation and dissociation from the transcription factor, E2F1; whereas, phosphorylation at serine 807/811 (S807/811) is not required for progression to the S phase. Because we observed that IFNγ restricted cell cycle progression at G1/S checkpoint, we next analyzed the protein expression of cyclin D/cdk4 (early G1 phase) and cyclin E/cdk2 (late G1 phase) complexes in NSPCs post-IFNγ treatment (Figure 11). Cyclin D1 expression did not significantly change after IFNγ treatment until DIV 5, where cyclin D1 levels increased transiently (n=4, p=0.0011). Cyclin D1 also undergoes cyclical changes in phosphorylation and subcellular localization. This includes nuclear to cytoplasmic redistribution and phosphorylation, with the highest levels of phosphorylation occurring after the G1/S transition. Therefore, if IFNγ treatment slowed the transition of NSPCs into the S-phase, we would expect a decrease in the phosphorylated form of cyclin D1. In Figure 5, the upper cyclin D band (open arrowhead) corresponds to the phosphorylated form of the protein; the lower band corresponds to the unphosphorylated form (closed arrowhead) (Figure 11A). When quantified separately, the phosphorylated form of cyclin D1 decreases in the IFNγ-treated groups at DIV 2 and 3, consistent with a G0/G1 restriction. Cdk4 expression was also
unchanged in the presence of IFNγ (data not shown). Expression of cyclin D3 decreased with IFNγ treatment only at DIV 3, suggesting that the regulation of expression of D-type cyclins do not play a major role in IFNγ-mediated control of cell cycle progression. In contrast, the expression of both cyclin E and cdk2 decreased at all time points post-IFNγ treatment, suggesting that IFNγ acts at the late G1 phase. We also examined multiple phosphorylation sites on pRb (Figure 11B). Expression levels of total pRb did not change with IFNγ treatment. However, phosphorylation of pRb at S795 was decreased at both DIV 2 (2.25-fold decrease versus untreated) and DIV 3 (2-fold decrease versus untreated) with IFNγ treatment (n=4, p=0.0002), while S780 and S807/811 were not affected by IFNγ. In untreated NSPCs, pRb phosphorylation at each of the phosphorylation sites increased at DIV 2 and 3, and declined by DIV 5, suggesting that the untreated neurospheres undergo a period of sustained growth early in culture that slows by DIV 5. Together, these results show that IFNγ interferes with G1/S cell cycle progression by decreasing the expression of cyclin/cdk complexes that control the late G1 phase transition, and ultimately decrease pRb phosphorylation. However, the expression of early G1 phase cyclin/cdk complexes are unaffected by IFNγ.
**Figure 11**  

IFNγ modulates the expression of cell cycle checkpoint proteins and the phosphorylation of pRb in NSPCs.
(A) Expression of cyclins D1, D2, D3, E and cdk2 were measured using western blot and fluorescence signals were normalized to GAPDH as a loading control. For cyclin D1, the top band (open arrowhead) corresponds to the phosphorylated form of cyclin D1 and the bottom band (closed arrowhead) corresponds to the unphosphorylated form of cyclin D1. (B) Expression of total retinoblastoma protein (pRb) and associated pRb phosphorylation at different serine residues (S780, S795, and S807/811) was measured. The fluorescence signal for each band was normalized to GAPDH as a loading control. For pRb S795, normalization was also performed against total pRb. Quantitation of samples is shown as the average with SEM. Statistical analysis was applied using repeated measures one-way ANOVA with Bonferroni multiple comparisons post-hoc analysis (****p<0.0001, *** p<0.001, ** p<0.01 *p<0.5; n=3-5).
VII. STAT1 is crucial for IFNγ-mediated inhibition of NSPC proliferation

IFNγ induced an anti-proliferative phenotype in NSPCs that was characterized by strong, persistent STAT1 activation. To study the role of STAT1 in IFNγ-induced growth arrest in NSPCs, we analyzed primary NSPCs from STAT1-KO mice, which express a truncated form of STAT1 that is devoid of the first three translated exons and is functionally deficient in the ability to bind to GAS elements. The impact of IFNγ on NSPC proliferation was abrogated by the loss of functional STAT1 (Figure 12). We first determined the diameter and area of STAT1-KO neurospheres treated with IFNγ. Neurosphere diameter was decreased at the highest concentration of IFNγ (1000 U/ml) (Figure 12B), but not at the other concentrations of IFNγ that had an impact on the growth of wildtype neurospheres (1, 10, and 100 U/ml, (Figure 12B). At 1000 U/ml IFNγ, there was a 27% decrease in neurosphere diameter in STAT1-KO NSPCs (n=3, p<0.0001) as compared to a 53% decrease in the WT NSPCs at the same time point (DIV 5). Histogram plots of the area of the STAT1-KO neurospheres showed that IFNγ treatment (1 and 100 U/ml, (Figure 12C) reduced neurosphere area to a lesser extent than in wildtype NSPCs. The median area decreased from 2547.5 pixel² in untreated STAT1-KO neurospheres to 2247 pixel² in neurospheres treated with 100 U/ml IFNγ; a 300 pixel² decrease as compared to a 1400 pixel² decrease in WT NSPCs. Notably, the area of the untreated STAT1-KO neurospheres (2547.5 pixel², DIV 5) was greater than untreated WT neurospheres (2054 pixel²) at the same time point (Figure 6 and 12). Moreover, the diameter in all treatment groups of the STAT1-KO NSPCs was greater than in the corresponding treatment groups of WT NSPCs (Figure 13), suggesting that deletion of STAT1 permitted greater growth of the neurospheres regardless of IFNγ treatment. The higher growth rate of the STAT1-
KO NSPCs limited data collection at DIV 7, as the larger neurospheres began to clump and settle at the bottom of the flask, with the appearance of a darker center by phase-contrast microscopy. We suspect that the larger size of the STAT1-KO neurospheres prevented necessary growth factors and nutrients from reaching the cells located in the core at later time points.
Figure 12

A

DIV 5

Untreated

ΔH IFN

IFN (100 U/mL)

IFN (1000 U/mL)

B

DIV 3

DIV 5

Neurosphere diameter (% Untreated)

IFN (U/mL)

- 1 10 100 1000

- 1 10 100 1000

C

Untreated

1 U/mL

100 U/mL

Number of neurospheres

Area (Pixel²)

Area (Pixel²)

Area (Pixel²)
**Figure 12:** *STAT1 is required for IFNγ-mediated inhibition of neurosphere growth.* STAT1-KO NSPCs were treated with IFNγ (1-1000 U/ml) or with heat-inactivated IFNγ (ΔH IFNγ; 1000 U/ml) as a negative control for DIV 3 and 5 post-IFNγ treatment (A) Representative images of neurospheres imaged 5 days post-IFNγ treatment at 2x magnification for different concentrations of IFNγ (Scale bar=250 μm). (B) Quantitation of neurosphere diameter at 3 and 5 days post-IFNγ treatment. The longest diameter of each neurosphere was measured using Image J software. Data was collected from neurospheres in five fields/condition from three biological replicates and graphed as a percentage of untreated controls. Statistical analysis was applied by one-way ANOVA (** p<0.001, **** p<0.0001). (C) Histogram plots of neurosphere area for different concentrations IFNγ on DIV 5 as measured by pixel² for each neurosphere. Frequency distribution of the number of neurospheres corresponding to the indicated neurosphere area in pixel² was plotted for each condition. Note that the range of the x-axis is greater than in Figure 6C.
Figure 13

A Wildtype NSPCs

**DIV 3**

**DIV 5**

**DIV 7**

B STAT1-KO NSPCs

**DIV 3**

**DIV 5**

**Figure 13**: *STAT1 loss leads to faster growth of neurospheres*. Absolute values of (A) WT and (B) STAT1-KO NSPCs were graphed for controls (untreated, and ΔH), and IFNγ-treated (1, 10, 100, 1000 U/mL) NSPCs at different time points. Data was collected from neurospheres in five fields/condition from three biological replicates and the diameters of all groups were compared with untreated group using one-way ANOVA and Dunnett’s post-hoc corrections. *p<0.05, ****p<0.0001.
Figure 14: IFN-γ-mediated regulation of cell cycle progression is STAT1-dependent in NSPCs.  (A) STAT1-KO NSPCs were treated with IFNγ (1, 100, 1000 U/mL) for 72h and labeled with BrdU and 7-AAD. The BrdU and 7-AAD intensities per cell were assayed by flow cytometry. Cell populations were gated in different phases of cell cycle (S = synthesis phase, G1= gap phase 1, M= mitosis phase, G2= gap phase 2). Representative plots for untreated and IFNγ-treated NSPCs (1000 U/mL) are shown. (B) Quantitation of NSPCs in each cell cycle phase. The average percentage of cells in each cell cycle phase was plotted with SEM (n=3). Statistical analysis was applied using one-way ANOVA (** p<0.01).
We also measured the cell cycle progression in STAT1-KO NSPCs during IFNγ treatment. We found that the cytostatic effect in WT NSPCs was lost at all but the highest concentration of IFNγ (1000 U/ml) (Figure 14A and B). There was no change in the number of cells in the S, G0/G1, and G2/M phases with 1-100 U/ml IFNγ treatment at any time point. A modest effect was observed with 1000U/mL IFNγ, with only a 4%±0.9 decrease (n= 4, p=0.005) in S phase cells in STAT1-KO NSPCs as compared to 15%±0.5 decrease for WT NSPCs at the same concentration (Figure 14B). These findings demonstrate that STAT1 mediates the cytostatic effect of IFNγ on NSPCs, though STAT1-independent pathways may also contribute. Furthermore, there was no change in the proliferation rate of STAT1-KO NSPCs in the presence of IFNγ when measured by CFSE labeling (Figure 15). These findings demonstrate that STAT1 mediates the cytostatic effect of IFNγ on NSPCs, though STAT1-independent pathways may also contribute.

IFNγ-treated WT NSPCs transitioned faster into the G2/M phase than untreated cells (Figure 6). This effect was not observed in the STAT1-KO NSPCs (Figure 14), where IFNγ did not affect the percentage of cells in G2/M. However, when comparing the untreated controls of WT and STAT1-KO NSPCs, the latter had significantly higher cells in the G2/M phase. Collectively, these data indicate that STAT1 is responsible for mediating much of the anti-proliferative effects of IFNγ on NSPCs. However, STAT1-KO NSPCs may also be inherently cycling a faster rate than WT NSPCs, as indicated by the greater neurosphere sizes of untreated STAT1-KO cells.

To compare the effects of IFNγ on cell cycle progression in NSPCs with other neural cell types, we also performed the BrdU assay on primary murine astrocytes in vitro. Astrocytes (Figure 16) were treated with IFNγ (100 U/mL) for 24 or 72 h. Cultures were
assayed by flow cytometry. In WT/astrocytes, IFNγ-induced a decrease in S-phase cells by 72 h, but also caused a decrease in cells in G2/M phase and an increase in the percentage of cells in the resting G0/G1 phase (Figure 16A). Together, these results suggest that IFNγ induces a cytostatic effect in astrocytes, with fewer cells actively progressing through the cell cycle. To determine if STAT1 was responsible for the cytostatic effects of IFNγ in these cultures over time, astrocytes (Figure 16B) from STAT1-KO mice with IFNγ. In the absence of functional STAT1, IFNγ did not affect cell cycle progression in MEFs or astrocytes at either time point. Thus, the cytostatic effects of IFNγ are STAT1-dependent in astrocytes.

VIII. STAT1 mediates the effects of IFNγ on pRb phosphorylation and cyclin/cdk expression

We further explored whether the absence of STAT1 abrogated changes in the expression of cell cycle regulatory proteins. Western blot analysis on STAT1-KO NSPCs showed that STAT3 activation increased at DIV 2 (Figure 17A; n=3, p<0.0001). IFNγ-mediated STAT3 activation was greater in the absence of STAT1 than in WT NSPCs (10-fold vs. 2.4-fold induction, respectively, on DIV 2). Cyclin E expression was unaffected by IFNγ treatment at DIV 2 and 3, but increased at DIV 5, in contrast to WT NSPCs where cyclin E expression is reduced at all time points. Cdk2 expression did not change at any time point. IFNγ treatment also did not alter the expression of cyclin D1 and D3, similar to WT NSPCs (Figure 17B). The dephosphorylation of pRb at S795 seen in WT NSPCs was not observed with IFNγ treatment in STAT1-KO cells. Rather, a significant increase in pRb S795 phosphorylation was observed on DIV 3 (Figure 17C; n=4, p=0.0406). As with
WT NSPCs, the phosphorylation of pRb at S780 and S807/811 was unaffected in STAT1-KO cells during IFNγ treatment. Together, these studies further suggest that functional STAT1 is required for IFNγ-mediated modulation of cyclin E/cdk2 expression and pRb dephosphorylation during inhibition of cell cycle progression.

IX. IFNγ reduces neuronal differentiation in NSPCs

In addition to proliferation, altered NSPC differentiation may also play an important role in CNS development. IFNγ may induce differentiation into several cell types through the activation of the JAK-STAT pathway. Other signaling pathways such as the ERK1/2 signaling pathway may also play an important role in cellular differentiation. The role of IFNγ-activated STAT and other alternative pathways have been discussed in detail in the introduction. We wanted to determine whether differentiation in embryonic NSPCs was also affected by IFNγ. To study the same, NSPCs were grown as monolayers on poly-d-lysine/laminin-coated coverslips. Differentiation was induced in NSPCs by withdrawal of growth factors from the medium and the cells were treated with IFNγ or left untreated. Differentiation was measured using immunofluorescence assay (Figure 18). Two days post-IFNγ treatment, both neuronal and astrocytic differentiation was low (Figure 18A), with 6% of total cells expressing neuronal marker, MAP2; and 1.7% of total cells expressed the astrocytic marker, GFAP (Figure 18C). On day 2, neuronal differentiation did not change with IFNγ treatment remaining at 6%. The astrocytic differentiation increased from 1.7 to 5% but was not statistically significant (p=0.6) (Figure 18B and C). On day 6 post-IFNγ treatment, there was an overall increase in both neuronal and glial differentiation in comparison to day 2 (neuronal, 6% to 43% and astrocytic, 1.7 to 30%). However, in IFNγ-
treated NSPCs, neuronal differentiation was significantly reduced (31±2.8%) whereas astrocytic differentiation increased (41.06 ±3.6%). These data indicate that IFNγ increased NSPC differentiation into astrocytic lineage whereas decreased differentiation into neuronal lineage, likely through induction of the STAT1 signaling pathway.
Untreated STAT1 KO NSPCs were loaded with CFSE and treated with IFNγ (100 U/ml; red line) or were left untreated (black line). (A) CFSE signal was measured by flow cytometry and mean fluorescence intensity (MFI) was calculated for each treatment group on DIV 2, 3, and 5 post-IFNγ treatment. Representative histograms for each time point are shown. (B) The MFI for each treatment group was averaged and statistical analysis was applied using student’s t-test (ns=not significant; n=5)
X. Discussion

Characterization studies showed that both in neurosphere cultures and monolayers, more than 90% cells expressed nestin, which is a classical marker of NSPCs. The percent expression of the receptor for IFNγ, IFNγR1 was also high (~82% of total neurosphere cells). Therefore, IFNγ may bind to the IFNγR and NSPC are likely to respond to its effects. Almost one-third of the cells also expressed DCX, a marker of immature neurons, suggesting that a subset of the NSPCs were committing to a neuronal lineage. SOX2 and PAX6 co-expression with nestin also corroborates results from other studies that have defined the expression of stem cell markers in NSPCs derived from E. 12.5 mice. In monolayer cultures, more than 90% of the cells expressed nestin. They also expressed markers for mature neurons (MAP2) and astrocytes (GFAP). However, both the number of cells and expression levels of mature neuronal and astrocytic markers remained below 5%. Both ICC and ICW data indicated that the cells were highly proliferative with increases in cellular density and NSPC marker expression over time. Taken together, the characterization data indicates that our in vitro model mostly consisted of NSPCs with few fully differentiated mature neural cells. However, the high proportion of DCX expressing cells indicates that the NSPCs, although highly proliferative, are of a heterogeneous mixture of cells in varying stages of differentiation.

We found that IFNγ inhibited NSPC proliferation as characterized by a decrease in neurosphere diameter and median neurosphere area (Figure 6). Cell cycle analysis showed that IFNγ restricted the transition of NSPCs into the S-phase and shortened the transition from S to the G2/M-phase (Figure 7). We also observed a modest but significant increase in apoptosis at the highest concentration of IFNγ tested (1000U/ml). Our results contrast
with studies on E14 NSPCs from C57BL/6J mice, where IFNγ substantially activated caspase-3/7 and only slightly decreased proliferation \(^{199}\). However, studies on striatal NSPCs from post-natal rats showed a similar increase in TUNEL+ cells with IFNγ treatment (~2-fold increase above untreated controls), albeit with a higher basal level of apoptosis in untreated neurospheres (15% TUNEL+ cells versus 0.5% TUNEL+ cells in our experiments, (Figure 7)\(^ {140}\). The cytostatic effects we observed are also consistent with other studies on E14 NSPCs from BALB/c mice and on post-natal day 2 and adult murine NSPCs from the subventricular zone, where IFNγ was shown to inhibit NSPC proliferation \(^{141,156}\). Thus, whether IFNγ induced a cytotoxic or cytostatic effect on NSPCs may be dependent, at least in part, on the stage of development during which the NSPCs are harvested or on differential susceptibility of NSPCs in different brain regions.

BrdU-labeling demonstrated a major restriction in the G1 phase of the cell cycle in the presence of IFNγ (Figure 7). In the G1 phase, hypophosphorylated pRb binds to the transcription factor E2F1, which prevents nuclear translocation of E2F1 and in turn inhibits E2F1-mediated gene expression of other cell cycle regulators \(^{200}\). The C-terminal domain of pRb is sequentially and differentially phosphorylated at specific amino acid residues by cyclin/cdk complexes, which then regulate pRb-E2F1 binding. We observed only transient changes in cyclin D1 and D3 expression and no changes in cdk4 expression at all time points following IFNγ treatment (Figure 8). These cyclin/cdk complexes control the early G1 phase checkpoint \(^{201}\), which suggests that IFNγ does not restrict cell cycle progression at the early G1 phase. In contrast, the cyclin E/cdk2 complex controls the late G1 phase checkpoint \(^{201}\). IFNγ reduced cyclin E and cdk2 expression at all time points examined (Figure 8), suggesting that IFNγ acts at the late G1 phase in NSPCs, prior to entry into S
phase. IFNγ also caused a minor restriction in G2/M phase (Figure 7), which may be due to early exit from S phase or delayed exit from G2/M. In conjunction with the moderate increase in TUNEL+ cells (Figure 7), these data suggest that DNA damage signaling pathways may be activated at high concentrations of IFNγ. Induction of DNA damage signaling has been noted in other cell types during IFNγ treatment, with similar effects on apoptosis and G2/M arrest\textsuperscript{202,203}.

pRb phosphorylation at S795 is necessary for disruption of pRb-E2F1 binding\textsuperscript{192,194}. Consistent with reports in human fibrosarcoma cells, we found that IFNγ led to dephosphorylation of pRb at S795\textsuperscript{121}. In primary cortical neurons, phosphorylation of pRb at S795 is also associated with cell death in a model of HIV-induced neurotoxicity\textsuperscript{204}. Thus, the low levels of apoptosis (\textasciitilde3%) seen in IFNγ-treated neurospheres may have been limited because of the loss of phosphorylation at S795. We did not observe any effect on other pRb C-terminal phosphorylation sites (S780 and S807/811) (Figure 11). This is consistent with previous studies that show the S807/811 residues are important for binding to the proto-oncogene c-Abl, but do not implicate them in affecting pRb binding with E2F1\textsuperscript{182}. pRb is also phosphorylated at S780 by the cyclin D1/cdk4 complex but not by the cyclin E/cdk2 complex\textsuperscript{205,206}. Therefore, the lack of change in phosphorylation of S780 by IFNγ at any time point further reinforces the role of cyclin E/cdk2 in mediating effects of IFNγ.
Figure 16

A  Wildtype astrocytes

B  STAT1-KO astrocytes

Figure 16: STAT1 is required for IFN-γ-mediated decrease in cell cycle progression in astrocytes. Primary (A) WT and (B) STAT1-KO astrocytes were treated with IFN-γ (100 U/mL) for 24 or 72 h. Stages of the cell cycle were measured by BrdU assay. Samples were then assayed by flow cytometry. Experiments with cells from individual dissections were averaged (n = 3–4) and plotted with SEM. Statistical significance was assessed via paired t-test (*p < 0.01).
**Figure 17**

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**Figure 17:** IFNγ activates STAT3, but does not inhibit cyclin E expression or pRb phosphorylation at S795, in the absence of STAT1. STAT1-KO NSPCs were treated with IFNγ (100U/ml) and collected on DIV 2, 3, and 5 for western blot analysis. Representative blots for phosphorylated and total STAT3 (A); cyclins D1, D2, D3, E and cdk2, (B); and for total pRb and associated pRb phosphorylation sites (C) are shown. Signal intensity for each band was normalized to GAPDH as a loading control. Quantitation of signal intensity is shown as the average from 3 independent biological replicates with SEM. Statistical
analysis was applied using repeated measures one-way ANOVA with Bonferroni multiple comparisons post-hoc analysis (*p<0.5, **** p<0.0001).
IFNγ mediates unique signaling pathways in neural cells depending upon the availability of intracellular signaling molecules. Our previous work has shown that astrocytes express relatively high levels of endogenous STAT1 and respond to IFNγ with rapid but transient STAT1 phosphorylation. IFNγ also blocks cell cycle progression in the G0/G1 phase in astrocytes in a STAT1-dependent manner, with minimal induction of apoptosis. In contrast, primary hippocampal neurons express low endogenous levels of STAT1, which is associated with delayed but sustained engagement of JAKs with IFNγR and prolonged STAT1 phosphorylation. IFNγ also induces protective signaling pathways in neurons that are STAT1-independent. Similar to neurons, NSPCs express low basal levels of STAT1, and STAT1 expression and activation is sustained (over 72h) after IFNγ treatment. However, IFNγ induces similar cytostatic effects in astrocytes and NSPCs that are STAT1-dependent, despite different kinetics of STAT1 expression and activation (Figure 15). Thus, STAT1 is central to the anti-proliferative effects of IFNγ in neural cells, although the profile of STAT1 activation is not predictive of changes in cell cycle progression. IFNγ also inhibits neuronal differentiation through the JAK/STAT1 pathway, suggesting that STAT1 activation is likely to impact on multiple aspects of NSPC function.

STAT1-KO NSPCs show a minor but significant decrease in neurosphere diameter and BrdU incorporation at the highest concentration of IFNγ tested (1000 U/mL) (Figure 13). This result could be attributed to residual STAT1 activity in the STAT1-KO mouse model (the truncated STAT1 protein retains 2% of DNA binding activity compared to full length STAT1). Nevertheless, we observed that a functional loss of STAT1 abrogated most of the cytostatic effects of IFNγ. In cells lacking STAT1, IFNγ caused increased
phosphorylation of pRb on S795 as opposed to the dephosphorylation at S795 seen in the WT NSPCs. STAT1-KO NSPCs also did not reduce cyclin E/cdk2 expression in response to IFNγ, but instead increased expression of cyclin E. These observations indicate that the effects of IFNγ on proliferation, pRb phosphorylation, and cyclin/cdk expression are dependent on STAT1 (Figure 16).

In the absence of STAT1, IFNγ induced greater STAT3 activation as compared to WT NSPCs. STAT3 can function as an alternative signaling pathway in response to IFNγ, as has been observed in embryonic fibroblasts. Moreover, STAT1 and STAT3 show opposing effects on cell proliferation; STAT1 being anti-proliferative and STAT3 being pro-proliferative. STAT1 inhibits cell proliferation in epithelial carcinoma cells through the upregulation of cyclin-dependent kinase inhibitors such as p21 and p27, and primary rat NSPCs upregulate p21 in response to IFNγ. Conversely, heightened STAT3 expression and activation has been observed in B-cell lymphomas, which increases proliferation and survival. The substantial increase in STAT3 activation in STAT1-KO NSPCs could also explain the enhanced neurosphere growth compared to WT NSPCs (Figure 13). Therefore, it is possible that the absence of STAT1 leads to a loss of cell cycle inhibitory proteins, such as p21, as well as compensatory changes in other signaling pathways (e.g. STAT3), leading to greater cell cycle progression and more rapid neurosphere growth.
Figure 18. *IFNγ inhibits neuronal differentiation*. Effect of IFNγ on NSPC differentiation was studied by treating NSPCs with 100U/mL IFNγ and staining the cells for Nestin (NSPCs), MAP2 (neurons), and GFAP (astrocytes). (A and B) show the effect of IFNγ on neuronal and astrocytic differentiation at different time points respectively. On 2 days post-IFNγ treatment, both neuronal and astrocytic differentiation was low in untreated and IFNγ-treated groups. Differentiation into both lineages increased at 6 days post-IFNγ treatment. However, with IFNγ treatment there was significantly higher astrocytic differentiation and lower neuronal differentiation as compared to untreated control. (C) Quantified
results of panels A and B. ** p<0.01 *p<0.05 vs. untreated; two-way ANOVA with Sidak’s multiple comparison’s test.
Besides IFNγ, other cytokines signal through the JAK/STAT pathway and are expressed during neurodevelopment and immune activation. Cytokines such as ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF) maintain the NSPC pool and initiate glial differentiation in vivo through the STAT3 pathway. Acute maternal administration of IL-6 in pregnant mice increased NSPC number in the forebrains of adult offspring and resulted in perturbations in the localization of NSPCs in the SVZ and cortex. In this study, we describe the STAT1-dependent role of IFNγ in controlling NSPC proliferation, which not only supports recent studies on NSPCs in the adult SVZ, but also identifies a mechanism for mediating NSPC cell cycle control. However, an outstanding question is how the inflammatory cytokine milieu would affect NSPC function. Since IFNγ, IL-6, and other inflammatory cytokines may be expressed concurrently during an anti-viral immune response, NSPC proliferation may be dictated by multiple signals. Moreover, IFNγ itself induces expression of multiple chemokines in adult NSPCs in vitro with downstream effects on differentiation. Further studies to explore the combinatory effects of multiple cytokines will be necessary to fully understand how NSPCs respond to an inflammatory environment.

This was the first study that describes STAT1-mediated control of pRb in the NSPC cell cycle. Our data show that IFNγ-mediated activation of STAT1 may directly or indirectly control pRb activity in NSPCs, and suggests that immune mediators can affect NSPC growth and the phosphorylation status of pRb. Further studies are necessary to determine whether IFNγ mediates similar effects on NSPCs in the context of an anti-viral immune response in vivo, and whether communication between NSPCs and other brain cells by exchange of IFNγ-rich extracellular vesicles influences the anti-proliferative
Moreover, the long-term impact of alterations in NSPC proliferation on the infected host remains to be explored. Our studies, and others like them, underscore the importance of considering the influence of the immune response in developing CNS tissue and whether inflammatory mediators bear upon neurodevelopmental disorders.

We also determined whether IFN\(\gamma\) affected differentiation in NSPCs derived from embryonic mice (Figure 18). We observed reduced neuronal and increased astrocytic differentiation. Studies in adult NSPCs treated with IFN\(\gamma\) increased neuronal differentiation\(^2\)\(^1\)\(^6\). However, Pereira et al have observed that intraventricular administration of IFN\(\gamma\) lead to a decrease in neuronal differentiation and neurogenesis\(^1\(^3\)\(^8\). These data, in addition to our observations, indicate that NSPC response to IFN\(\gamma\) is different at different stages of neurodevelopment. Moreover, direct effects of IFN\(\gamma\) on NSPCs may be different than indirect effects. IFN\(\gamma\), may also activate immune cells in the CNS. These cells may in turn affect NSPCs proliferation and differentiation. Currently, our results do not reflect the effects of other immune cells. Therefore, these may have to be considered in designing future experiments.

Our model focuses on the effects of anti-viral cytokines on uninfected NSPCs because many CNS viruses show tropism for neurons and other neural cells over NSPCs (e.g. herpes simplex viruses, measles viruses, Human Immunodeficiency Virus). Thus, an outstanding question is how the cytokine milieu would affect NSPCs that are concurrently infected, as would be seen in many cases of CMV and ZIKV infection. A number of DNA and RNA viruses encode proteins that target critical cell cycle regulators to achieve cellular conditions that are beneficial for viral replication. Many DNA viruses induce quiescent cells to enter the cell cycle, which is thought to increase pools of deoxynucleotides and
facilitate replication of viral genomes. Human papilloma virus (HPV), Adenoviruses (Ad), and polyomaviruses such as the Simian virus 40 (SV40) drive cells into the S-phase\textsuperscript{217}. The E6 and E7 protein of HPV up-regulate the expression of cyclins A, B, and E and inhibits cyclin D1 expression, which is important in the Rb pathway\textsuperscript{218}. In contrast, some viruses can arrest cells in a particular phase of the cell cycle that is favorable for replication of a specific virus. Cell cycle arrest may inhibit early cell death of infected cells, allow the cells to evade immune defenses, or help promote virus assembly. For example, JEV inhibits G1 to S-phase cell cycle progression by decreasing cyclin D1 expression in NSPCs\textsuperscript{219}. ZIKV and Sindbis virus also decreases cell cycle progression of NSPCs by forcing the cells into an apoptotic pathway\textsuperscript{220}. MV causes decreased T-cell proliferation though cell cycle arrest in the G0/G1 phase through decreased cyclin D1 and E expression. Together, these studies demonstrate that viruses have differential effects on cell cycle progression depending on the target cell and the eventual benefit for survival and propagation of the virus. This leads to the question of the how IFNγ-mediated effects on cell proliferation would play out in a virally infected cell. The data presented here suggests that IFNγ decreases NSPC proliferation by decreasing the expression of cyclin/CDK complexes and retinoblastoma protein phosphorylation. As discussed above, these mechanisms are also targeted by certain viruses. Therefore, the answer to the question would depend on what mechanism the virus employs when it infects NSPCs. If the inherent mechanism employed by the virus is to increase NSPC proliferation, then IFNγ would counter the same. However, those viruses that also inhibit cell cycle progression and thus proliferation, IFNγ would further contribute to this effect. The overall implication would also depend on the signaling mechanism that is activated by IFNγ in each cell type. For MV, previous data
from our lab showed that neurons control MV infection through a IFN$\gamma$-dependent mechanism. However, this viral control is not achieved through the canonical STAT1 signaling mechanism in neurons. IFN$\gamma$ also confers a neuroprotective effect on primary neurons, which is independent of STAT1 and dependent upon Extracellular-regulate kinase-1/2 (ERK1/2). Here, our data suggests that IFN$\gamma$ inhibits proliferation in NSPCs and astrocytes in a STAT1-dependent manner. Thus, neural cells are not monolithic in the signaling pathways and phenotypic responses that are induced by a single cytokine. These observations highlight the importance of the responding cell type in dictating the outcome of cytokine signaling and the diversity of pathways that can invoked by IFN$\gamma$. 
Chapter 4: Effects of neuron-microglia interactions on NSPC activity

I. Rationale

Microglia, as the resident immune cells of the brains, play important roles in the healthy and diseased CNS. They comprise 10–15% of all CNS cells in the adult brain, and are found interspersed throughout the brain parenchyma. In vivo studies in healthy brains reveal that microglia are constantly in motion, surveying their microenvironment and communicating with neurons and other glia through direct contact with their cellular projections. They play a major role in maintaining homeostasis in the adult CNS by releasing trophic factors such as nerve growth factor (NGF), brain-derived neurotropic factor (BDNF), glial cell-line derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF) and neurotrophin-3 (NT3), which mediate axonal growth. These functions include phagocytosis of apoptotic neurons, synaptic pruning, and general immune surveillance (reviewed in 225). Microglia also influence neurogenesis axonal growth though the release of factors such as insulin-like growth factor-I and BDNF. Microglia also affect NSPC activity during embryonic and neonatal brain development. Microglial cells colonize the developing cerebral cortex of prenatal and postnatal macaques and rats, phagocytosing NSPCs as neurogenesis nears completion. Moreover, it was observed that phagocytosed NSPCs were not apoptotic (negative for TUNEL reactivity and cleaved caspases). The authors concluded that during CNS development, excess NSPCs were produced and these were eliminated by microglia. Moreover, microglial activation by LPS administration in pregnant rats decreased the NSPC numbers (PAX6+) and numbers of newly differentiated neurons (TBR2+) in the embryos. Therefore,
microglia play varying roles in both CNS development and maintenance of normal functions once the CNS is formed.

Considerable research has been conducted in several diseases states to determine the role of microglia in disease progression or protection against pathogenesis. Microglia are activated in both acute and chronic neuroinflammatory diseases. In acute CNS injuries such as stroke, depletion of microglia during ischemia leads to exacerbation of injury characterized by increased infarct size, inflammatory cytokine release, and neurodegeneration\(^{230}\). Increased microglial activation is noted in traumatic brain injury, where activation was not only present in lesioned areas but also in areas remote to the focal damage\(^{231}\). This finding implies that inflammatory mediators released from injured cells affect healthy cells in the vicinity of the injured cells and in other distal parts of the brain. Studies have also shown that activated microglia play a significant role in the course of neurodegenerative diseases such as Parkinson’s Disease, through the release of toxic inflammatory mediators\(^{232-234}\).

There is ample evidence that microglia are activated in neurotropic infections in the CNS. HCMV infections of the CNS can cause severe neurodevelopmental deficits. Microglia are permissive to hCMV infections, but do not show signs of productive infection or cytopathic changes\(^ {18}\). hCMV-infected astrocytes secrete chemokines, primarily CCL2/MCP-1, that recruit microglial cells to the area of infection\(^ {235}\). The microglial cells, but not astrocytes, produce the antiviral cytokine TNF-\(\alpha\), which suppresses hCMV replication in astrocytes\(^ {18}\). \textit{In utero} infection with MCMV leads to increased microglial activation in the fetal CNS along with infiltration of peripheral immune cells\(^ {236}\). HSV-1 causes a CNS infection in neonates and immunocompetent adults,
which results in acute focal necrotizing encephalitis. Microglial cells in HSV-1 brains express elevated MHC class I and class II molecules and have a wide distribution throughout the brain, including areas distinct from the productive infection\textsuperscript{237}. Increased microglial activation resulted in increased TNF-\(\alpha\) and IL-1\(\beta\) expression from these cells. Both TNF-\(\alpha\) and IL-1\(\beta\) are known to cause increased NSPC differentiation and affect their proliferation\textsuperscript{238,239}. Similarly, BDV infection is associated with microglial activation and release of pro-inflammatory cytokines such as IL-1\(\beta\), TNF-\(\alpha\), and IFN\(\gamma\). However, when the mice were treated with ribavirin, an antiviral agent, there was reduced microglial activation, a decrease in the expression of pro-inflammatory cytokines and improvement in body weight and clinical score\textsuperscript{87}. These examples show that microglia are important mediators of the host immune response against viruses, which involves the release of proinflammatory cytokines and chemokines that may also cause bystander effects on neighboring cells.

The cytokines released during a viral infection can bind to any cell expressing the cognate receptor, regardless of whether the cell is infected. NSPCs express a wide array of chemokine receptors, which likely means that the NSPCs would respond to the inflammatory milieu. Because microglia are a major producer of inflammatory cytokines during viral infections in the brain, it is likely that microglia produce factors that impact on NSPC function. Shigemoto-Mogami et al found that inhibition of basal microglial activation using minocycline decreases the number of proliferative cells in the developing brain. Moreover, the decline in the pool of NSPCs and young neurons correlated with depressed levels of the pro-inflammatory cytokines IL-1\(\beta\), TNF-\(\alpha\), and IL-6. The levels of anti-inflammatory cytokines (IL-4 and IL-10) remained unchanged. This suggested that a
basal activation level may be necessary for homeostasis and normal development of CNS. Butovsky, *et al* observed that NSPCs co-cultured with differentially activated microglial *in vitro* induced opposing effects on NSPCs. LPS-activated microglia inhibited neurogenesis, whereas co-culture with IFN\(\gamma\)- or IL-4-activated microglia increased neurogenesis\(^{240}\). Thus, the effect of microglia on NSPC activity may depend on the type of insult or stimuli received by the microglia and the pool of cytokines released by the microglia. Moreover, Carr et al observed that co-culturing microglia with HSV-1-infected NSPCs reduced the loss of newly differentiated neurons. This protection is afforded by IL-6 secreted by the microglia\(^{54}\). This finding demonstrates that microglia can be protective during CNS infections through the release of cytokines. These studies highlight the need for a better understanding of the interaction between microglia and NSPCs during viral CNS infections.

In this chapter, we establish an *in vitro* model system to define the interactions between infected CNS neurons, responding microglia, and the ultimate impact on NSPC function. Using the CD46+ model of neuron-restricted MV infection, we infect primary hippocampal neurons with MV in a co-culture with primary microglia. Supernatants with secreted factors from the neuron/microglia co-culture are applied to monolayers of primary NSPCs. We found that the MV-infected neuron/microglia co-cultures led to an increase in neurogenesis and a loss of pluripotency in the NSPCs, which was not observed with infected neurons alone or uninfected neuron/microglia co-culture supernatants. Surprisingly, there was not a significant effect of NSPC proliferation or cell cycle progression, suggesting that the microglia may not be dominate producers of IFN\(\gamma\) or may produce other cytokines that maintain NSPC growth. These results suggest that microglia
respond to infected neurons in such a manner that encourages the differentiation of new neurons from uninfected NSPCs.
Figure 19: Measles virus (MV) infects mature neurons but spares nestin expressing neural stem/progenitor cells (NSPCs). Sagittal brain sections from MV-infected CD46+ pups were collected 10 days post-infection and stained for (A-I) MV (red) and nestin (green) or (J-L) MV (red) and NeuN (green). Representative images were taken using the Olympus epifluorescence microscope in different areas of the brain; (A-C) Thalamus and, (D-F) CA1 region of the hippocampus. (G-I) Fiber tracts of the medial forebrain bundle system. Magnification: 20X and scale bar: 50 μm. (J-L) Dentate gyrus of the
hippocampus. Magnification: 40X and scale bar: 25 μm. All sections were also stained with the nuclear stain, Hoechst (blue)

II. Neuron-Microglia co-culture model

There is ample evidence in the literature of crosstalk between microglia and infected or damaged neurons or NSPCs\textsuperscript{241,242}. However, there are few studies that address the communication that occurs between infected neurons, microglia, and the resultant message that is delivered to NSPCs. One could hypothesize that the interaction between activated microglia and infected neurons affect NSPC proliferation and differentiation. To address this question, we take advantage of a mouse model of neuron-restricted measles virus (MV) infection (CD46+ mice). The CD46+ model is discussed in detail in chapter 1. Briefly, in this model, MV selectively infects neurons as the human CD46 receptor is only expressed on mature neurons of the central nervous system, while NSPCs are spared from the infection (Figure 19). To demonstrate if mature neurons are selectively infected by MV and NSPCs are spared from infection, CD46+ mice were infected intracranially with MV and the brains were harvested. Brain sections from the thalamus (Figure 19 A-C), CA1 region (Figure 19 D-F) and the dentate gyrus of the hippocampus (Figure 19 G-I) were stained for MV (Figure 19 A, D, G) and nestin (Figure 19 B, E, H). No co-localization of MV and nestin was observed in any regions of the brain. On the other hand, staining for NeuN (marker for mature neurons) co-localized with MV. These results demonstrate that NSPCs are spared from infection and only mature neurons are infectable with MV in CD46+ mice. Additionally, ICC was performed to ensure that MV selectively infected neurons \textit{in vitro}. MV also selectively infected neurons \textit{in vitro} with consistent co-localization observed for MV and MAP2 staining (Figure 21).
To determine the effects of neuron-microglia interactions on NSPC activity, primary neurons were derived from E. 15.5 mice and co-cultured with primary microglia isolated from postnatal day 1-2 mice. The CD46+ neurons have been extensively characterized *in vitro* by Rall et al\(^{243}\). Neurons were infected with MV-Edmonston strain at multiplicity of infection (MOI) of 1 and the extent of infection increased with time. At 1 day post-infection (dpi), a low proportion of neurons (5% or less) showed evidence of MV antigen, but by 3 dpi the proportion of infected cells averaged 10 to 20% (Figure 21). Extracellular virus is not produced and the virus spreads through transynaptic spread between the neurons without formation of syncytia (Figure 21). Thus, the microglia and NSPCs do not become infected with MV in this model. A schematic of the co-culture model and timeline of experiments is depicted in Figure (20 A and B). In the co-cultures, microglia were positive for the marker IBA-1, a calcium binding protein that stains microglia *in vitro* and *in vivo*. The morphology the microglia was also was similar to previous studies of *in vitro* microglial cultures\(^{244}\). The neurons are plated first to allow for attachment and the formation of synapses between the neurons. After 4 days in vitro, the neurons are infected with MV and incubated for 3 days to allow the infection to spread through the culture. At 3 dpi, the microglia were purified from a mixed glial culture and added to the infected neuron cultures. Four days later, the supernatant from the neuron/microglia co-cultures was removed and applied to the NSPC cultures and incubated for 4 more days to allow for changes in differentiation and growth.

Microglia comprise 10-15% of the cells in the CNS, with a neuron to glia ratio of 3:1 \(^{245}\). To mimic the cellular ratio found in the brain, microglia were plated at 25,000 and 100,000 cells/well in 24-well and 6-well plates respectively in the co-culture. The CD46+
neurons were cultured at 250,000 cells/well for 24-well plates and 1,000,000 cells/well for 6-well plates. One drawback of this model is that we do not include astrocytes in the system, which are a major component of brain tissue. However, our goal is to dissect how the microglia react to infected neurons, and how subsequent signals from the microglia influence the NSPCs. We observed that in co-cultures with MV-infected neurons, there were higher number of microglia that had assumed an amoeboid shape. In the uninfected cultures, there were higher numbers of microglia with a rounded smaller morphology (Figure 22). Whether the amoeboid microglia have higher activation state or an M1/M2 phenotype is unknown. However, other glial cells, such as astrocytes, also develop an amoeboid morphology under stress (such as co-incubation with infected neurons). Peripheral monocytes also assume a flatter, more amoeboid morphology when migrating along a chemokine gradient. Thus, it is possible that the amoeboid morphology seen in the microglia represents cells that are trying to move towards infected neurons. Regardless of the activation status of the amoeboid microglia, it is apparent that at least some of microglia that are incubated with the virally-infected neurons trend toward a unique morphology.
Figure 20: Neuron-microglia co-culture and treatment of NSPCs with conditioned medium. (A) CD46+ primary neurons were cultured in poly-D-lysine coated plates. The neurons were infected with MV (1MOI). Microglia were co-cultured with the neurons and the conditioned medium was used to treat NSPCs grown on poly-D-lysine/laminin coated plates. (B) Timeline of co-culture and NSPC treatment. Microglia were cultured as mixed glial cultures. Neurons were cultured separately from E. 15.5 mice embryos and infected with MV or left untreated. On Day 11, microglia were shaken and detached from mixed glial cultures and co cultured with neurons. NSPCs were separately cultured as neurospheres, passaged and cultured as monolayers on Day 14. These cultures received the conditioned medium from the co-cultured (refer text for treatment groups). NSPCs were detached by trypsinization and processed accordingly.
Figure 21: *MV infected CD46+ neurons in vitro.* CD46+ neurons were isolated from cortices dissected from E.15.5 embryonic mice were cultured on poly-D-lysine coated coverslips in Neurobasal medium (NBM). On DIV3, the cells were infected with 1 MOI of MV (right) or left untreated (left). Four days post-infection, the coverslips were collected and the cells were fixed and stained for neuronal marker (MAP2, green) and measles virus matrix and hemagglutinin proteins (red). Cell nuclei were stained with Hoechst (blue). Both cell body and processes were observed to be infected with MV. Scale bar: 50 μm.
Figure 22

Uninfected Neurons +Microglia  MV-Infected Neuron +Microglia

Figure: 22: *Microglia in MV-infected co-cultures demonstrate changes in morphology.* CD46+ neurons were isolated and cultured (250,000 cells/well) as described previously. On DIV3, the cells were infected with 1 MOI of MV or left untreated. Four days post-infection, microglia (25,000 cells/well) were purified from mixed glial cultures and co-cultured with MV-infected (right) or uninfected neurons (left). (Cell nuclei were stained with Hoechst (blue). Four days-post co-culturing, the coverslips were fixed and stained for MV (matrix and hemagglutinin proteins, red), neuronal marker (NeuN, green), and microglial marker (IBA1, purple). Microglia in MV-infected cultures were observed to have a more amoeboid shape, whereas those in the uninfected cultured were small without and circular. Scale bar: 50 μm.
III. **Conditioned medium from MV-infected neuron-microglia co-culture increases NSPC differentiation**

To understand the effect of factors released from neuron-microglia co-cultures on NSPC differentiation, conditioned medium from the co-cultures was transferred to NSPC cultures, which were then allowed to differentiate for 4 days. NSPCs were treated with conditioned medium from the following groups:

a. Uninfected neurons only- Group I  
b. MV-infected neurons without microglia- Group II  
c. Uninfected neurons with microglia- Group III  
d. MV-infected neurons with microglia- Group IV

The first two groups would control for effects from factors released from uninfected neurons alone and from MV-infected neurons. The third group accounts for factors released due to co-culturing of uninfected neurons with the microglia. Finally, Group IV would demonstrate the effects of mediators released due to interactions between microglia and MV-infected neurons.

NSPCs were cultured in the conditioned medium and collected at the end of treatment period (4 DIV). NSPC cultures were stained with neural markers for NSPCs (nestin and SOX2) to determine effects on the NSPC pool, or with DCX, βIII tubulin (immature and mature neurons) and GFAP (mature glia) to measure differentiation by flow cytometry. Representative graphs of cell count versus fluorescence intensity for each marker (**Figure 23A-E, quantification Figure 24A-E**) are shown. DCX+ cells increased in the NSPCs treated with media from the Group IV compared to all three controls (**Figure 23, 24A**). The percentage of DCX+ cells in each group is listed in (**Table 6**). A representative ICC
image of NSPCs treated with conditioned medium form group II and IV is shown in Figure 25. This data indicates that media from Group IV leads to an increase in differentiation into the neuronal lineage. No statistically significant changes were observed with the more mature neural markers: βIII tubulin and GFAP (Figure 23, 24B and C). Longer incubation with the conditioned media would have allowed for some of the DCX+ cells to transition into more mature neurons (βIII tubulin). The magnitude of change in the number of cells expressing βIII tubulin and GFAP between groups I-II and groups III-IV was similar (~2-fold). This suggested that MV-infected neurons themselves may be releasing factors that resulted in increased neuronal and glial differentiation.

No significant differences were observed in the pool of nestin+ NSPCs (Figure 23, 24D). On the other hand, a significant decrease was seen in SOX2 expression in comparison to groups I, II and III (Figure 23, 24E; 92.0%±2.3 v/s 76.7%±4.7; p<0.05). These results may indicate that NSPC differentiation increases when NSPCs are treated with conditioned medium from MV-infected neurons co-cultured with microglia. In NSPCs, nestin expression is regulated by SOX2 which acts as a transcription factor. Therefore, the changes in SOX2 expression may preceded changes in Nestin expression. Additionally, we observed was with the morphology of the NSPCs (Figure 26). Nestin staining during the differentiation process revealed that NSPCs that were treated with conditioned medium from Groups III and IV (with microglia in co-culture), had a distinct morphology. These cells had a more rounded shape in comparison to NSPCs treated with group I and II conditioned medium, which had thin cellular processes that were elongated from end-to-end. Whether these changes in morphology are associated with differentiation, changes in cytoskeletal structure, or both is still to be determined. To determine whether
differentiation was associated with changes in levels of protein expression, mean fluorescence intensity (MFI) was used as a measure neural marker expression. We did not observe any change in the protein expression corresponding to those seen with cell numbers seen in Figure 24. In fact, GFAP expression was reduced in groups II, III and IV. For DCX, even though more cells were being formed in Group IV, there was no change in the net protein expression within the groups. For GFAP, group I which showed lowest differentiation had highest GFAP expression as compared to other groups. Astrocytes may increase GFAP expression when stressed or activated\textsuperscript{249}. Therefore, it could be possible that in presence of conditioned medium with MV infection and microglia co-culture, there may be increased GFAP differentiation but lower stress/activation.

IV. Media from MV-infected neuron-microglia co-culture causes a modest increase in DNA synthesis

To determine whether the neuron-microglia supernatants affect NSPC proliferation, we measured BrdU incorporation in treated NSPCs. BrdU incorporation is an indicator of newly synthesized DNA and can be used to track cell cycle progression. NSPCs were cultured in the conditioned medium as with the differentiation experiments. Four days post-treatment with conditioned medium, the cells were pulsed with 25 μM BrdU for 90 minutes at 37°C. The cells were collected and BrdU incorporation was measured using flow cytometry. BrdU incorporation was increased in group IV compared to group I (Figure 23, 24F). However, group IV did not significantly differ when compared to groups II and III. Therefore, more cells were in the S-phase in group IV as compared to group I. This indicated that MV infection or microglia alone did not affect
NSPC proliferation. There had to be an interaction between the two to have a significant effect on NSPCs proliferation.

Changes in the cell cycle progression was also measured by counterstaining the NSPCs with 7-AAD along with BrdU staining. Cells in S, G0/G1 and G2/M phases of the cell cycle were measured by analyzing the cells graphed for BrDU v/s 7-AAD (Figure 23G, 24 F, G, H). We did not observe any changes in the cell cycle progression. There were no significant differences observed in the G0/G1 or G2/M phases with any of the groups (Figure 24G and H).

Table 6

<table>
<thead>
<tr>
<th>Conditioned medium treatment</th>
<th>DCX+cells (%Live cells)</th>
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<tbody>
<tr>
<td>Group I</td>
<td>19.40±0.948</td>
</tr>
<tr>
<td>Group II</td>
<td>19.29±0.986</td>
</tr>
<tr>
<td>Group III</td>
<td>19.64±1.122</td>
</tr>
<tr>
<td>Group IV</td>
<td>24.13±1.452</td>
</tr>
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Table 6: Summary of DCX+ cells post-conditioned medium treatment
Figure 23

A  DCX

B  βIII Tubulin

C  GFAP

D  Nestin

E  SOX2

F  BrdU

Group I  Group II  Group III  Group IV
Figure 23: Measurement of changes in NSPC differentiation and cell cycle progression. NSPCs were treated with conditioned medium from co-cultures on DIV1. Treatment groups were: uninfected without microglia (group I), MV-infected without microglia (group II), uninfected with microglia (group III), and MV-infected with microglia (group IV). Cells from each treatment groups were detached from culture plates, fixed, permeabilized, and stained for (A) DCX, (B) βIII tubulin, (C) GFAP, (D) Nestin, (ESOX2 and passed through the flow cytometer. Live cells were first gated to exclude debris. Cell Cycle progression was measured by subjecting the NSPC cultures from different groups to the BrdU assay. (F) The number of BrdU+ NSPCs (S-phase) were graphed separately as number of cells v/s fluorescence intensity (A.U). (G) Representative graphs of NSPCs form groups I, II, III and IV in G0/G1, S and G2/M phases of the cell cycle prepared by measuring BrdU incorporation v/s 7-AAD staining.
Figure 24: Enhanced NSPCs differentiation and BrdU incorporation upon treatment with conditioned medium. (A-E) Percentage of live cells positive for (A) DCX, (B) βIII tubulin, (C) GFAP, (D) Nestin (E) SOX2, were graphed for each of the treatment groups. Data was presented as mean±SEM for each of the treatment groups and analyzed using a two-way ANOVA with Holm-Sidak multiple comparison test; n=3, * p<0.05. Cells were process as for neural markers and average percentage±SEM of cells in the (F) S phase (BrdU+), (G) G0/G1 phase (H) G2/M phase, were graphed analyzed using two-way ANOVA with Holm-Sidak multiple comparison test; *p<0.05
Figure 25: Reduced GFAP expression in NSPCs post conditioned medium treatment. (A-E) Mean fluorescence intensities (MFI) in arbitrary units were graphed for each of the treatment groups (A.U) for (A) DCX, (B) βIII tubulin, (C) GFAP, (D) Nestin (E) SOX2. Data was presented as mean±SEM for each of the treatment groups and analyzed using a two-way ANOVA with Holm-Sidak multiple comparison test; n=3, * p<0.05.
Figure 26: Enhanced neuronal differentiation in response to treatment with MV-infected neurons co-cultured with microglia. Representative ICC images of NSPCs treated with conditioned medium from groups III (left) and IV (right). NSPCs were allowed to differentiate as monolayer cultures and stained for Nestin (red) and DCX (green). Treatment with conditioned medium from group IV had higher DCX+ cells as compared to group III. Scale bar 50 μm.
V. Discussion

Microglia are an important source of cytokines during viral infections and play a major part in the host defense against viruses in the brain. They also play an important role in normal brain development, where they are responsible for controlling NSPCs numbers and pruning of neuronal networks. NSPCs may be infected by the viruses themselves which may cause changes in their survival and/or changes in differentiation. However, in addition to infections, NSPCs may also be affected by inflammatory mediators released from immune cells and infected cells. We hypothesized that microglial cells would affect NSPCs during a neurotropic viral infection through release of soluble mediators. To address this question, we have used a co-culture system of MV-infected neurons and microglia. The viral infection is restricted to the neurons and microglial cells are not infected. The inflammatory mediators released from both the neurons and microglia can then be applied to NSPC cultures to study how proliferation and differentiation of the NPSCs is affected.

Treatment with conditioned medium from MV-infected neurons/microglia cultures increased the immature neuronal cell pool (DCX+ cells). However, the same was not observed for mature neural markers, βIII tubulin (neurons) as well as GFAP (astrocytes). One reason for this effect could be that the time point at which the cells were collected may have not allowed for full differentiation and expression of more mature neural markers. At basal levels (uninfected neurons-No microglia group), approximately 2% of the total cells in the NSPC cultures express these markers, suggesting a low basal level of differentiation in the cultures.
BrdU incorporation is a measure of DNA synthesis that can represent cells that are in the S-phase of the cell cycle or cells that are actively repairing damaged DNA. Notably, there was increased BrdU incorporation when NSPCs were treated conditioned medium from MV-infected neurons co-cultured with microglia (group IV). One explanation for this could be that NSPCs, even though differentiating, may not be terminally differentiated. There may be in increase in the amounts of committed precursors that may still be in cooperating BrdU. This can be corroborated by the increase in the number of DCX+ cells in this group. If these DCX+ neurons accounted for an increase in BrdU incorporation could be confirmed by double staining for DCX and BrdU using ICC. Also, it is important to note that there was no significant increase in the numbers of terminally differentiated neural cells (neurons or astrocytes). Longer differentiation time points may be necessary to determine if there are any changes in the glial and neuronal differentiation. Moreover, elucidation of committed glial precursor cell numbers is needed to determine if early glial commitment is affected.

Another important question would be to determine which inflammatory mediators in the conditioned medium mediate the effects seen on NSPCs. In chapter 3, we have demonstrated that IFNγ, an important proinflammatory cytokine released during viral infections, reduced NSPC proliferation. Here, we observe an increase in BrdU incorporation, which suggests that IFNγ may not be expressed by the microglia or that other cytokines with a proliferative effect may dominate. Moreover, the role of other cytokines and chemokine such as IL-6, IL-1β, and TNF-α also needs to be elucidated. Yukari, et al studied the physiological roles of microglia in the early post-natal brain. They observed that activated microglia were present in the SVZ of the rat brain from P1 to
P10. When the activation of these microglia was attenuated, the expression of proinflammatory cytokines (IL-1β, IL-6, TNF-α, and IFNγ) was reduced. Attenuation of microglial activation also inhibited the number of NSPC and neurons *in vivo* and *in vitro*. These studies showed that a basal level of microglial activation brings about normal CNS development. In CNS diseases, however, how enhanced microglial activation affects the normal activity of NPSCs is unclear. Butovsky, *et al* suggest that the type of stimuli that activate the microglia may determine the ultimate effects on NSPC activity. They observed that co-culture of microglia activated by IL-4 or low levels of IFNγ with NSPCs increases neuronal differentiation, as we have observed in our cultures. On the other hand, microglia activated by the endotoxin LPS reduced neuronal differentiation251. Moreover, a recent study by Choi, *et al* demonstrated that differentially activated microglia (LPS- versus IL-4- treated) had distinct effects on NSPC proliferation and differentiation in organotypic cultures derived from ischemic mouse brain252. Conditioned medium from IL-4-treated microglia increased proliferation in the SVZ-NSPCs and increased neurogenesis (DCX+/BrdU+ cells). Moreover, the effects were mediated by IL-10 and TGFβ secreted from activated microglial cells. This study suggests that during CNS diseases, NSPCs respond to activated microglia by modulating production of new neurons, and the activating signal to the microglia determines the effects on NSPCs. In a study by Chucair-Elliott *et al*, microglia prevented a decrease neuronal differentiation caused by HSV-1 infection, either through transfer of supernatants from microglia-NSPC co-cultures or through direct co-culture with the microglia54. This finding highlights the fact that microglia may not only induce protective effects though direct contact with NSPCs, but also though factors released as a result of their interaction with other neural cell types.
Ovanesov et al, also examined the mechanisms of microglia activation in BDV-infected rat brains in a neuron-microglia co-culture. They observed that microglia are activated by conditioned medium from BDV-infected neurons, and released IL-6 in response. This reinforces the fact that microglia interact with injured neurons to stimulate cytokine release, which may affect NSPC activity.

Here, we have developed an in vitro model system to analyze the how microglia react to virally-infected neurons, and how subsequent release of factors impact on the responding NSPCs. We observed increased neuronal differentiation when NSPCs are exposed to conditioned medium from microglia co-cultured with MV-infected neurons. However, the cytokine profile release as a result of cross-talk between MV-infected neurons and microglia needs to be evaluated. Moreover, in this model, the role of other immune cells including infiltrating peripheral macrophages, T-cells (CD4+ and CD8+) is not addressed. Nevertheless, the observations that we define in vitro can be ultimately modeled in vivo in the CD46+ model through inhibition of microglial activation and through knockdown of specific cytokines that are produced by the activated microglia. Together, these studies will help to define how NSPCs respond to the inflammatory environment induced by an infection.
Figure 27

Changes in NSPC morphology upon treatment with conditioned medium. NSPCs were grown as monolayers on poly-D-lysine/laminin coated coverslips. Cells were treated with conditioned medium form (A) Uninfected neurons (group I) (B) MV-infected neurons without microglia (group II) (C) Uninfected neurons with microglia (group III) (D) MV-infected neurons with microglia (group IV). Four days post conditioned medium treatment, coverslips were fixed and stained for Nestin (green) and nuclear stain, Hoechst (blue). Cells treated with conditioned medium that were co-cultured with microglia (groups III and IV) showed altered morphology with thicker cellular processes as compared to those that were treated with conditioned medium from neurons that were not co-cultured with microglia (groups I and II). Scale bar= 50 μm.
Conclusion

Viral infections of the central nervous system are associated with devastating neurological consequences during brain development especially in the prenatal and neonatal stages. The aim of my thesis was to define how neural stem/progenitor cells (NSPCs) respond to key inflammatory mediators that are often expressed during viral infections. In addition, we wanted to elucidate whether immune-mediated changes in NSPC activity would contribute to protective or pathological outcome in the brain. We found that IFNγ, a cytokine critical for clearance of many virus from the CNS, also interferes with NSPC activity. Treatment of NSPCs with IFNγ decreased NSPC cell cycle progression by multiple cellular and molecular measures. We discovered a new mechanism for IFNγ-mediated control of NSPC proliferation involving restriction of the cell cycle at the late stage of G1/S transition through decreased expression of the Cyclin E/CDK2 complex. Moreover, to our knowledge, we are the first to report IFNγ-mediated in site-specific phosphorylation of the retinoblastoma protein, which plays a central role in cell cycle progression. The effects of IFNγ on the proliferation and cell cycle regulatory proteins were dependent upon STAT1, which contrasts with CNS neurons that respond to IFNγ through STAT1-independent mechanisms. These results suggest that IFNγ impedes NSPC proliferation and growth, but that the effects of IFNγ in the brain are unique to each cell neural cell type.

During a viral infection, both infected and uninfected neural cells will respond to the inflammatory environment in an attempt to limit viral spread. The ultimate effects of both responses may contribute to either pathology or protection from CNS damage. Our data demonstrate that uninfected NSPCs respond to IFNγ with decreased proliferation. This
cytostatic response of NSPCs could have both positive and negative effects for the host. If the NSPCs are targets of the virus, inhibiting proliferation could provide a beneficial effect to the host by slowing viral replication in the infected cells. In contrast, inhibition of NSPC growth could have a negative impact on brain development by limiting the pool of new neurons during critical stages of neurogenesis. If the virus displays tropism for immature neurons, inhibiting NSPC differentiation could further limit viral spread by reducing newly-differentiated neurons. Thus, the infected brain tissue must balance the benefits of preventing continued viral spread with the potential deficits to development of the brain. We did not observe high levels of IFNγ-induced apoptosis in NSPCs, despite evidence in other cell types for more extensive cell death. This finding suggests that decreased proliferation is not associated with programmed cell death.

Our model focused on the interactions between inflammatory mediators and uninfected NSPCs that may reside in the vicinity of the immune response. Ultimately, the effect of IFNγ on infected NSPCs may depend on the virus-induced changes in cellular proliferation mechanisms. The purpose of a virus infecting a host cell is to produce functional progeny that may then infect other cells and allow for viral persistence in the host. Depending on the type of virus, the host cell cycle may be modified by viral proteins to allow for increased persistence or survival of the virus\textsuperscript{253}. For example, HIV arrests cells in the G2-phase, which allows for an optimal environment for viral protein production for this virus\textsuperscript{254}. On the other hand, MV induces a cell cycle arrest in the G0 phase\textsuperscript{255}. Viruses such as the human papilloma virus (HPV) induce S-phase entry of infected cells in order to create an environment that is favorable for rapid viral replication\textsuperscript{256}. Given the pluripotent nature of IFNγ, a variety of anti-viral mechanisms are possible, which may be
partially dependent on the effects the virus has on the cell cycle. For those viruses that increase cell proliferation, IFNγ may counteract that effect by decreasing NSPC proliferation. However, with those viruses that favor cell cycle arrest, IFNγ may act through entirely different anti-viral pathways, such as activation of RNA degrading enzymes or through heightened sensitivity to Type I interferons. Regardless of whether the NSPCs are targets of a particular virus, the response of the NSPCs to the anti-viral immune response has the potential to severely alter brain development.

In addition to IFNγ, the cytokine milieu consists of a number of other inflammatory factors which that may affect NSPCs. During viral infections, there are several sources of the cytokines and trophic factors, including innate and adaptive immune cells, glial cells and even infected neurons. Among the immune cells that can respond to viruses, microglial cells are the first immune cell to encounter a virus in the brain. Microglia play an important role in the embryonic brain by contributing to CNS development by regulating the number of NSPCs and aiding the formation of neural networks\textsuperscript{250}. In neurotropic viral infections, activated microglia not only play a major role recruitment of immune cells during CNS pathology, but also function as effectors by secreting factors that may affect NSPC activity\textsuperscript{257}. Ongoing studies on the interaction between activated microglia and NSPCs show that microglia can direct NSPC proliferation and differentiation. Many groups have suggested that activated microglia may increase NSPC proliferation and induce their differentiation during neurodegenerative conditions\textsuperscript{258,259}. Because microglia are activated in response to viral infections in our CD46 model and others, we wanted to study how microglia that are responding to infected neurons could influence NSPC activity. Using the CD46 model, we selectively infected neurons with MV, sparing the microglia
and NSPCs from infection. To study the effects of the interaction between infected neurons and microglia on NSPCs, we developed a unique co-culture system of MV-infected CD46+ neurons and microglia, where media from the neuron/microglia cultures are incubated with NSPCs. We hypothesized that MV-infected neurons would activate microglia in vitro and immune mediators released from co-cultured cells would increase NSPC differentiation. Two aspects of NSPC activity are considered important with respect to their role in CNS development and response to CNS pathology, namely, NSPC proliferation and differentiation into neural lineages. We observed a modest increase in the number of NSPCs entering the S-phase when treated with conditioned medium from MV-infected neurons/microglia co-cultures in comparison to controls. In addition, NSPCs treated with MV-infected neurons/microglial conditioned medium demonstrated greater neuronal differentiation into immature neurons and reduced SOX2 expression, suggesting that the ‘stemness’ of the NSPCs was declining and were becoming more differentiated. Differentiation into mature astrocytic (GFAP+) and neuronal lineages (βIII-tubulin) was found to be generally low in all treatment conditions, which may reflect the time point at which differentiation was measured (4 DIV). The neuronal and microglial mediators that control NSPC proliferation and differentiation are an open question. Determining the cytokines and chemokines released from the microglia would add light on the type of microglial activation that may occur in response to neurotropic infection.

In this study, I have demonstrated that IFNγ may decrease NSPC proliferation, increase astrocytic differentiation and decrease neuronal differentiation. Viruses prefer actively proliferating cells to multiply. NSPCs, which also actively proliferate may are attractive targets for many of these viruses, which may affect NSPC survival an
differentiation and ultimately lead to neurodevelopmental deficits. The IFNγ-mediated decrease in NSPC proliferation and neuronal differentiation could potentially reduce NSPC from being attractive targets for viral infection. Neurons are non-renewable entities in the CNS and many viruses use them for spread and reservoirs during dormant phases. Therefore, reduced neuronal differentiation could reduce the cells available for viral spread and latency. On the other hand, cytokines released form potential changes in activation state of microglial cells in response to a neurotropic viral infection caused an increase in neuronal differentiation without changes in NSPC proliferation (Figure 27A). These results suggest that NSPC response to cytokines depends upon the types of cytokines acting on them. Infected neurons may produce chemokines such as CX3CL1. Microglial cells express CX3CR1, the receptor that binds the ligand CX3CL1. Upon CX3CL1 binding, microglial cells reduce production of pro-inflammatory cytokines such as IL-β, IL-6, and TNFα. Moreover, CX3CL1 mediates increased expression of TGF-β, which in turn may mediate neuronal differentiation in NSPCs. Considering the contrasting effects that we observe between microglia and IFNγ alone, one could predict that IFNγ might not be part of the cytokine milieu released by microglia during neurotropic viral infections. However, in vivo other immune cells such as T-cells and NK-cells which are sources of IFNγ may also affect NSPC activity. Moreover, these cells may affect the activation state of microglia which will also affect NSPC activity. Therefore, in vivo studies are needed to understand the effects of different parts of immune system on NSPC activity, and the ultimate contribution protection or pathology in viral infection.
The immune response to neurotropic viral infections mediates changes in NSPC activity. Microglia may respond to cytokines and chemokines such as CX3CL1 released from MV-infected neurons. In response to CX3CL1 stimulation, microglia upregulate expression of growth factors such as TGF-β and downregulate proinflammatory cytokines like IL-1β, IL-6 and TNF-α. Moreover, TGF-β causes neuronal differentiation in NSPCs. Therefore, results from our data indicate that neuronal differentiation observed in NSPCs treated with conditioned medium from MV-infected neurons co-cultured with microglia could be, in part, due to increased TGF-β section and decreased proinflammatory cytokine section from microglia. Other immune cells, such as T-cells and NK-cells, are major sources of IFNγ, which decrease NSPC proliferation through reduced expression of cyclin E/CDK2 complexes and inhibition of cell cycle progression. Moreover, microglia also respond to cytokine stimuli from these immune cells, particularly to IFNγ, which may drive the microglia into a more protective phenotype. Therefore, decreased NSPC proliferation would make NSPCs less attractive targets for viruses that prefer infecting actively proliferating cells. Increased neuronal differentiation would possibly replace the pool of neurons that may be lost or damaged due to the infection. However, whether increased neuronal differentiation also leads to functional integration or alterations in behavior or learning needs to be investigated.
In this study, my goal was to understand how anti-viral immunity may affect neural development, specifically focusing on the effects of soluble inflammatory mediators. NSPCs, being the only pluripotent cells in the CNS, are an especially important cell type when considering the various neurodevelopmental disorders that can result from viral infections. We defined an IFNγ-mediated mechanisms of NSPC cell cycle control that demonstrates that part of the anti-viral immune response in the brain could include prohibiting NSPC growth. Moreover, using a neuron restricted MV-infections model, we determined that inflammatory mediators released from infected neurons, and the responding microglia, can steer differentiation of the uninfected NSPCs.
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156


