UPPER-RESPIRATORY INFECTION TRIGGERS EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS ONSET IN AUTOIMMUNE PRONE T-CELL RECEPTOR TRANSGENIC MICE

BY

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THESIS

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II

Abstract

Multiple Sclerosis (MS) is an autoimmune-mediated demyelinating and neurodegenerative disease of the central nervous system. Most MS patients experience a disease course characterized by periods of symptom exacerbation (relapses) followed by periods of partial recovery (remission). Relapse contributes to disability but the processes that trigger relapses are poorly understood. Upper-respiratory viral infection increases the risk for relapse. Here, we tested the hypothesis that upper respiratory infection is sufficient to cause glial activation, promote immune cell trafficking to the CNS and trigger pathology in an autoimmune-prone T cell receptor transgenic mouse line. To test this hypothesis we infected 2D2 mice and monitored for symptoms of inflammatory demyelination (EAE). Clinical and histological EAE was observed in ~29.2% of infected 2D2 mice which closely resembles the incidence of upper-respiratory infection-induced relapse in MS patients. Chemokine production in brain resident glial cells, primarily astrocytes was induced by TNF and IL-1β stimulation which may assist in the trafficking of immune cells to the CNS. Furthermore, pharmacological inhibitors of JNK, ERK1/2, p38, and NF-κB modulated the levels of chemokines secreted by astrocytes in response to cytokine stimulation. Increased levels of serum IFN-γ were observed in C57BL/6 mice infected with influenza as well as increased $CD4^+$ T-cells in the choroid plexus. Finally, we observed immunosurveillance of the brain by T-cells and $CD45^{high}CD11b^+$ cells in C57BL/6 mice after influenza inoculation. This immunosurveillance could prove detrimental to MS patients.

Table of Contents

1. Introduction

Multiple sclerosis (MS) is a chronic, inflammatory, demyelinating, autoimmune disease that affects the central nervous system. Multiple sclerosis afflicts 2.3 million people worldwide and has a prevalence rate of 135 and 133 affected per 100,000 in the United States and Canada, respectively [1]. Multiple sclerosis decreases life expectancy by 7-14 years [2] and incurs an estimated $6.8 billion annually in health care costs [3]. Multiple sclerosis is an extremely heterogeneous disease that includes an equally diverse list of symptoms [4, 5]. Some of the most prevalent symptoms include paralysis, blindness, and chronic pain [6]. Multiple sclerosis patients exhibit four distinct types of disease progressions [6]. The most common of these accounting for 85% of all patients, is termed relapsing-remitting MS (RRMS), which is characterized by rotating periods of disease exacerbation and asymptomatic periods of neurological repair and recovery of function. Primary progressive MS accounts for 10% of the population, in which patients suffer from a continual worsening of symptoms with no remission periods. Progressive relapsing MS affects approximately 5% of patients, and is similar to primary progressive, but includes flare-ups of disease exacerbation to go along with the constant gradual decline. The final clinical group is termed secondary progressive MS [7]. This stage of the disease is characterized by the diminution of relapses in relapsing remitting patients and a slow transition to a progressive disease state. The cause of disease onset is not known, but as studies probe, the potential culprits it has become clear that MS is a complex multifaceted disorder that results from multiple genetic and environmental triggers [8-14]. Some of the likely contributors are genetic predisposition (15-23), vitamin D deficiency [15, 16], smoking [17-21], obesity [22, 23], and certain viral infections (i.e. Measles, HHV-6, EBV) [24].

Peripheral Immune System

Many immune cells have been shown to contribute to either MS onset or relapse including B-cells, both $CD4^+$ and $CD8^+$ T-cells, and several antigen presenting cell types. The primary function of B-cells is antibody production, although they are also competent antigen presenting cells. Antibodies neutralize toxins, activate the complement pathway, act as opsonins, and mark infected cells for destruction by natural killer cells. B-cells have the capability of responding to a broad range of pathogens in an antigen specific manner. The role of B-cells in MS is indisputable. One of the criteria for MS diagnosis is increased intrathecal IgG synthesis and detection of autoantibodies in the CSF. Several drugs have passed clinical trial for MS and have shown effects on B-cells including Natalizumab and Rituximab (monoclonal antibody against CD20). With the exception of Natalizumab, no drug has shown the ability to lower intrathecal IgG synthesis. Even Rituximab, a drug known to effectively deplete B-cells in the CSF, had no effect on intrathecal IgG synthesis [25]. Complement fixing antibodies may cause destruction of the myelin membrane and lesion formation. Memory B-cells readily cross the blood brain barrier and upon antigen stimulation may subsequently become activated into antibody secreting plasma cells [26]. Additionally, memory B-cells are also more easily activated and produce more pro-inflammatory cytokines than primary effector B-cells. TNF, which is produced by activated B-cells, has been shown to detrimentally affect oligodendrocytes, which are responsible for myelination. B-cells home to the CNS in response to chemokine gradients. In MS patients, B-cells appear to accumulate in the subarachnoid space where they form intrameningeal follicle structures. Epstein Barr virus (EBV) has been proposed as a culprit for the formation of these structures. Epstein Barr virus infected B-cells accumulate in white matter lesions and this virus could be one of the stimuli for the constitutive activation state of lymphocytes in MS patients [27].

Rituximab causes a 95% reduction in circulating B-cells, which affects many immunological responses such as antibody production, cytokine networks, antigen presentation, and T-cell, monocyte activation [28]. Indeed, a phase 2 clinical trial of the drug rituximab showed that patients in the treatment group had a reduction in the number of new gadolinium-enhancing lesions at 12, 16, 20, and 24 weeks of the trial. Another effective MS therapy that affects B-cell function is β-interferon, which can inhibit B-cell activation and antigen presentation [29]. This therapy reduces expression of activation markers CD80 and CD40 on B-cells. Together, these data clearly identify an important role for B-cells in MS exacerbation. However, the role of B-cells in MS has only recently begun to be appreciated and understood.

Multiple sclerosis is thought to be a $CD4^+$ T-cell mediated disorder even though $CD8^+$ T-cells are the most common immune cell present in the brain of MS patients [30]. Autoreactive T-cells gain access to the CNS by crossing the blood brain barrier in an α4β1 integrin dependent manner [31]. It is thought that once in the CNS, autoreactive T-cells mediate damage against the myelin sheaths covering neurons, leading to eventual destruction of the neuron and a progressively increasing neurological deficit in patients. Although remyelination can occur in relapsing remitting MS once axonal degeneration occurs, permanent effects of the disorder become irreversible. Within the population of $CD4^+$ helper T-cells there are several different sub-types including Th1, Th2, Th17, Th9, and Treg. The two that are most relevant to MS pathogenesis are Th1 and Th17 cells. Effector Th1 cells were initially thought to be the primary T-cells responsible for inflammation in MS patients, but Th17 cells, named for their secretion of IL-17A and IL-17F, have emerged as a major contributor to the auto-inflammatory response, at least in experimental autoimmune encephalomyelitis (EAE) [32-34]. The reason is that Th1 cells were thought to be the most important to pathogenesis due to an experiment using IL-12p40-/-

mice [35]. IL-12 is needed to generate a Th1 response and without it, these mice were resistant to EAE [35]. Multiple sclerosis was at one point considered the prototypical Th1 mediated autoimmune disease supported by the exacerbation of MS patients when treated with IFN-γ, a common cytokine released by activated Th1 cells. Although these studies suggest Th1 cells are the initiator of disease, a follow up study determined IFN-$\gamma^{-/-}$ and $STAT1^{-/-}$ mice developed more serious disease. These mice do not have circulating Th1 cells, which led to the discovery that IL-23 and IL-12 share the p35 protein subunit and that IL-23 knockout mice were resistant to EAE [36].

The cytokine IL-23 is necessary for Th17 cell expansion and has been a primary argument in favor of the role played by Th17 cells in both EAE and MS. This argument is strengthened by IL-$1RI^{-/-}$ mice being resistant to EAE, as IL-1β signaling promotes Th17 cell polarization. Interestingly, CCR6 is a chemokine receptor present on Th17 cells, and mice deficient in CCR6 are resistant to EAE [37-39]. Nevertheless, recent evidence indicates that the development of EAE appears to be mediated by a complex association of several different cell types including both Th1 and Th17 T-cells. For instance, Th1 cells can passively transfer EAE from an affected mouse to a $RAG1^{-/-}$ mouse, whereas studies have shown conflicting reports on whether Th17 cells can induce EAE when passively transferred alone [40-42]. Interestingly, Th2 cells rather than being protective also passively transfer EAE from an afflicted animal to a naive animal [43]. Th1 cells produce a less severe pathology in IL17-/- mice, which may indicate that Th1 cells are needed for initial onset and recruitment of Th17 cells to the CNS, and that Th17 cells cause tissue destruction and increased pathology [44]. Both Th1 and Th17 cells stimulate the attraction of different immune cell subtypes and recruit them to different regions of the CNS. Th1 cells appear to recruit monocytes and macrophages in large numbers to the spinal cord in

EAE, whereas Th17 cells recruit neutrophils primarily to the brain [45]. Notably neutrophils have recently received considerable attention for their role in both EAE onset and MS disease exacerbation [46, 47].

Cytotoxic $CD8^+$ T-cells are also found in the lesions of MS patients and outnumber $CD4^+$ T-cells by 3:1 [48]. $CD8^+$ T-cells are well known for their destruction of virally infected cells. Moreover, it was clearly shown that $CD8^+$ T-cells isolated from MS patients have undergone clonal expansion [49]. Specifically, one study determined that as much as 35% of the $CD8^+$ T-cells in MS lesions arise from a single clone, suggesting that $CD8^+$ T-cells are associating with their specific antigen [49]. Interestingly, $CD8^+$ T-cells specific for CNS antigens have also been found in the periphery of both MS patients and controls [50, 51]. The role of $CD8^+$ T-cells in the pathogenesis of MS is clear as they have been shown to assert a regulatory function [30]. However, autoreactive (MBP specific) $CD8^+$ T-cells isolated from wild type mice have been shown to induce EAE, which further confounds what role they have in both EAE and MS [52]. $CD8^+$ T-cell mediated EAE more closely resembles MS pathology than the $CD4^+$ T-cell mediated variant [53] as $CD8^+$ T-cell mediated disease is primarily confined to the brain of mice whereas the $CD4^+$ mediated disease is primary localized to the spinal cord [54]. Monocytes, macrophages, and dendritic cells also play a role in MS and EAE disease pathology. T-cells need to be re-exposed to their antigen in the CNS to cause disease. Mature DCs inside perivascular cuffs have been shown to present antigens from myelin components in both active and inactive lesions [55] and are thought to contribute to lesion activation. Furthermore, an influx in inflammatory monocytes to the CNS is a pathological hallmark of both MS and EAE [56]. Indeed, $CD16^+$ monocytes show an increased production of IL-12 in MS patients, which further contributes to the inflammatory environment [57]. The chemokines CCL2 and CXCL2 are both

elevated in MS patients, and assist in recruiting pro-inflammatory monocytes [58]. The infiltrating monocytes along with the resident microglia are the cells responsible for phagocytosis, which is the mechanism responsible for tissue damage and repair.

Cells of the Central Nervous System

Glial cells (microglia, astrocytes, and oligodendrocytes) were once thought of as mere supporting cells of the brain. However, they have recently emerged as essential for maintaining homeostasis as well as providing assistance to the neurons, which receive and send signals to the rest of the body. There are four major glial cell populations in the brain; Pericytes, astrocytes, microglia, and oligodendrocytes. Pericytes are phagocytic cells that cover 22-32% of the brain capillaries. These cells regulate capillary contraction, endothelial proliferation, and angiogenesis. Depletion of pericytes leads to endothelial hyperplasia, vasculogenesis, and increased blood brain barrier permeability. Both pericytes and astrocytes make up an important glial component of the blood brain barrier.

Astrocytes are the most abundant cell type in the brain [59]. Astrocytes are named for their star like shape. Astrocytes have many projections and end feet that function in barrier formation, intracellular signaling, and trophic support [59]. Astrocytes almost completely ensheath the capillary walls that surround the brain and make up one of the key components of the blood brain barrier. Their barrier function effects blood flow, K^+ ion concentration, release of CNS waste products, and trafficking of glucose to brain resident cells [59]. Astrocytes are extremely important for structural support of the CNS and transport of ions in and out. They express high levels of the transporters glucose transporter type 1 (GLUT-1), aquaporin 4 (AQP4), connexin-43, and ATP-sensitive inward rectifier potassium channel 10 (Kir4.1). Another barrier function that astrocytes have been shown to be important for is walling off

sections of neurons to assist in better interaction between select groups of neurons. This allows neurotransmitters, hormones, and other signaling molecules to more easily reach target neurons in high concentrations. Recently it was discovered that astrocytes represent a major source of chemokines when stimulated with cytokines like IL-1β and TNF [60],[Figure 6]. These cytokines could be produced from immune cells in the meninges, the periphery, or from the CNS resident immune cell, the microglia and facilitate the attraction of autoimmune cells to the CNS.

Microglia are phagocytic glial cells of the CNS that act as tissue resident macrophage [61]. As the tissue resident macrophage they stimulate other cells when activated by the secretion of an array of cytokines [61]. They account for roughly 10-15% of cells in the CNS. Similar to astrocytes, microglia have many branched projections and are constantly sampling their microenvironment for debris or foreign pathogens. Microglia are extremely fast moving cells, being the fastest in the brain. The microglia of the brain can completely monitor the entire area of the brain every several hours shrinking and growing projections at speeds of 1.25 μm/min [62, 63]. Resting microglia have many projections constantly sampling the surround environment. When activated, microglia change phenotype, becoming an amoeboid-like cell with only a few robust projections. Some microglia also rapidly divide in order to increase numbers around the area of insult. Microglia also migrate using their activated amoeboid form and are attracted by secreted molecules such as ATP, cannabinoids, chemokines, lysophosphatidic acid and bradykinin. As phagocytes, they can remove debris and waste products [61, 64]. They also express low levels of MHCII, consistent with a role in antigen presentation. Both MHCII, and co stimulatory factors CD40 and CD86 are not measurable on resting microglia, but rapidly become upregulated following stimulation [65]. Such appears to be the case in MS [65]. Microglia are

thought to phagocytose damaged myelin and then present myelin derived antigens to autoreactive T-cells that have entered the brain.

Oligodendrocytes are responsible for the creation of new myelin surrounding neurons. This is important both in development, general maintenance, and during injury or degenerative diseases like MS. Myelin is important for allowing neural signal to travel quickly between neurons and reach their target destination by a process called saltatory conduction. Myelin coats most areas around neurons and is interrupted by sections of demyelinated space known as Nodes of Ranvier. Nodes of Ranvier are rich in sodium ion channels where action potentials are formed. Myelin allows signals to jump between nodes rather than traveling the full length of the neuron. This helps to save energy and allows the signal to travel much faster to its destination. When myelin is degraded, the propagation of the action potential is slowed. Myelin is mostly lipid (70%) the remainder being made up of protein. Some of the major proteins of myelin are myelin associated glycoprotein (MAG), myelin basic protein (MBP), and myelin oligodendrocyte glycoprotein (MOG).

Genetic Risk Factors

Many single nucleotide polymorphisms convey increased risk for MS. The most prominent include those in alleles of the human leukocyte antigen (HLA) within the major histocompatibility complex (MHC) [66]. Many HLA genes are linked to processes in immune cell function and signaling. A recent genome wide association study analysis of MS patients uncovered the genes; Lethal (3) malignant brain tumor-like protein 3 (*L3MBTL3***),** Myc-associated zinc finger protein **(***MAZ*), and Transcriptional regulator ERG (*ERG)* which all show increased risk for MS and are all important for immune regulation [67]. For instance, *L3MBTL3* is required for proper maturation of myeloid progenitor cells. The gene *SHMT1* was also found

to increase risk for developing MS [67]. The product of SHMT is responsible for methylation homeostasis [67]. Since epigenetic modification controls the activation levels of multiple target genes it is likely that *SHMT1* is responsible for altering expression of other at yet to be discovered risk genes [67]. Two other gene loci that have shown significant link to MS onset are HLA-DRB1 variant 15 (HLA-DRB1*15) in the MHCII region [68, 69] and the IL2RA [70, 71]. HLA genes are involved with antigen presentation and make up a component of the MHCII molecule while IL-2A is a cytokine receptor. Further evidence for a genetic link is uncovered by familial studies [9, 10, 67, 72, 73]. Specifically, studies by Ebers et al., conducted nearly 20 years ago demonstrated a 25% risk between monozygotic twins, 5% between siblings, and 2% between parents and their children [74]. These data demonstrate genetic association with risk of onset. However, a 25% concordance rate between monozygotic twins clearly implicates environmental factors are also involved in disease onset [72, 73]. Thus, while genotype likely predisposes an individual, one or several environmental factors are needed to initiate disease onset.

Vitamin D and Multiple Sclerosis

Vitamin D deficiency contributes to disease onset and perhaps relapse [75]. Exposure to UV irradiation from sunlight is needed for vitamin D production. Interestingly, MS incidence is lower in populations that live closer to the equator [16]. Conversely, the highest prevalence of MS occurs in Northern Europe, and North America [16], regions where UV exposure is likely insufficient to meet the demand for proper vitamin D production [75]. Most interestingly, the HLA-DRB1*15 locus includes a vitamin D response element in its promoter which shows high levels of genetic conservation in MS patients, but large levels of diversity in the general population [16] indicating that the at risk locus is important for MS onset.

Obesity and Smoking Cause Low Grade Inflammation

Smoking has been shown to increase susceptibility to MS onset and accelerates disease progression [17-19, 21]. Strikingly smoking has been shown to increase risk of MS by 1.8 fold [17, 18]. A separate study showed a 50% increase risk of MS when the patient had elevated levels of cotinine, a metabolite of nicotine [20]. Although smoking appears to cause an increased risk a form of smokeless tobacco has been shown to have a protective effect [21]. Nicotine signals to the alpha7 nicotinic acetylcholine receptor which is known to activate the cholinergic anti-inflammatory pathway [76]. Indeed, nicotine treatment effectively ameliorates EAE [77]. Smoking causes continual low-grade inflammation of the respiratory tract [21, 78]. These data indicates that inflammation, rather than nicotine increases the risk of MS in smokers [17, 18, 20, 21].

Similarly, obesity is known to cause systemic inflammation [22, 36]. Furthermore, recent evidence suggest that obesity is a disease characterized by chronic low grade inflammation [23]. Adipocytes have the ability to secrete a milieu of cytokines which can regulate inflammation among their other physiological roles [23]. Indeed, obese patients exhibit increased levels of TNF, CCL2 and IL-6), and decreased levels of IL-10 [23]. This cytokine milieu is indicative of systemic inflammation and a mobilization of immune cells that may contribute to MS relapse and/or progression. These also data indicate that peripheral inflammation can cause the trafficking of activated immune cells throughout the body including the CNS.

Pregnancy and Multiple Sclerosis

Sex dramatically affects the risk of autoimmunity in that females are more susceptible than males [79, 80]. As such, it is not surprising that MS has a significantly higher prevalence in woman than men [79-81]. In fact, the ratio of female to male is at least 2:1 and has been

suggested to be as high as 3.2:1 [80]. A higher risk for MS amongst women may suggest an effect of gonadal hormones in the pathogenesis of MS [82]. In support of this concept, woman who become pregnant after the onset of MS are protected from relapse during the later stages of the pregnancy and have a better future prognosis [83]. Specifically when 254 MS afflicted woman were examined over time relapse risk dropped by 90% during the third trimester of pregnancy [84]. Conversely, child birth, which induces inflammation, promotes relapse [84]. Intriguingly, breast feeding has shown to alleviate some of this post-partum risk [84].

Numerous hormones change during pregnancy including estrodiol, glucocorticoids, and progesterone, which all can influence the immune system [81, 85-87]. It has been shown that treatment with estrogen protects mice from generating EAE. [86, 87]. Although progesterone treatment alone has little to no benefit, it appears to act in synergy with estrogen to decrease MS progression [88]. Specifically, both estrogen and progesterone protect against axonal damage and demyelination [88]. Moreover, estrogen appears to enhance the protective effects of vitamin D during EAE [15]. This link between the immune system and pregnancy is also apparent during longitudinal studies that follow woman over the course of pregnancy and after parturition. For instance, during pregnancy the immune response shifts from a type 1 to a type 2. Whether or not hormones that are altered by pregnancy influences T cell polarization or function is controversial. However, Krementov et al., has recently indicated that macrophage p38 MAPK signaling is significantly altered in females compared with males decreased [89]. The most promising ongoing clinical trials indicate that estrogen receptor β based treatments elicit the most beneficial effects on ameliorating MS [90]. Hormone analogue drugs may start out effective, but they could lead to patient toleration through decreased cellular receptor expression.

Upper Respiratory Infection and Multiple Sclerosis

Upper respiratory viral infections are linked to MS relapse [91]. Specifically, there is an apparent at risk period, that begins one week prior to the occurrence of URI symptoms and lasts until five weeks after URI symptom arrival, that corresponds with increased risk for relapse [92-94]. Importantly, 30-40% of MS patients that have an URI suffer a relapse during this at risk period [3, 92, 94-96]. Relapse has been associated with picornaviral infection [97]. However, since rhinovirus is not the only cause of upper respiratory infections [98] it seems unlikely that infection with this virus represents the only source of infection based relapse. Importantly, influenza vaccination lowers relapse risk, which directly implicates influenza infection in precipitating relapse [95, 96]. Despite a recognized association between viral upper respiratory infection and relapse risk, how upper-respiratory infections trigger relapse in MS patients is unknown, but may be partially explained by very recent data emerging only within the last few months. Specifically, Louveau et. al., have demonstrated the presence of a CNS lymphatic system that drains into the cervical lymph nodes [99]. These lymph nodes also drain antigens from the respiratory tract, which allows immune cells in the lymph node to have access to both respiratory tract and CNS antigens. During an URI activated antigen presenting cells traffic to the cervical lymph nodes. The draining CNS antigens creates an opportunity for CNS specific self-antigens to be presented within an inflammatory milieu. This might represent a mechanism for how peripheral immune cells, that have escaped immune tolerance become primed to CNS antigens and eventually receive signals that traffic them to the brain in genetically prone individuals.

Viral Infections and Multiple Sclerosis

Viral infections have a substantial link to MS disease onset and relapse. Viral particles isolated from the CNS of MS patients induced altered immunological responses versus those

normally generated in the periphery in MS patients [100, 101]. Many viruses have been linked to MS onset, but none have been identified as a causative agent. Included in this list is measles, human herpesvirus-6 (HHV-6), Epstein Barr virus (EBV), as well as several other herpes viruses[24]. Measles was studied for two reasons. First it is known to cause post infectious encephalitis (PIE) and subacute sclerosing panencephalitis (SSPE) [102]. SSPE is of particular interest due to the characteristic brain inflammation it causes and because SSPE was previously thought of as an etiologic agent for MS and a demyelinating disease [102]. The second reason measles virus was researched in regards to MS was due to the longevity of measles antibodies. They have a long-lived immunological response that stay in the body for decades. However, the successful implementation of the measles vaccine indicated that measles is not the cause of MS.

The herpesvirus HHV-6 is a highly neurotropic virus that establishes a lifelong infection [103]. HHV-6 has a tropism for T-cells, B-cells, and glial cells and has been shown to alter immune responses. It has a global distribution and seroprevalence of >90%. The age of acquisition made HHV-6 a good target for MS studies due to its early life infection tendency. This is important because one of the environmental factors associated with MS occurs early on in life. Most people with HHV-6 are asymptomatic, although there can be serious health complications, such as encephalitis, in immunocompromised patients. There are two subsets of HHV-6 (HHV-6A, HHV-6B). HHV-6A by mechanisms not yet fully understood has a tendency to become reactivated in the CNS. Interestingly, HHV-6A DNA was found in the plaques of MS patients [104]. Staining against the viral HHV-6 proteins 101k and p41 were detected in oligodendrocytes of MS patients and not in controls [24]. HHV-6 infected relapsing remitting MS patients also showed increased levels of α-p41/38 early antigen IgM [104].

Epstein Barr virus, a gamma herpes virus, has been the most extensively studied of the viral etiologies for MS. While 95% of the human population is seropositive for EBV, nearly 100% of MS patients are seropositive [105]. Epstein Barr virus on its own has been known to cause neurological disorders including Alice in Wonderland syndrome (neurological condition that affects size perception) and progressive macrocephaly [105]. Epstein Barr virus reactivation and MS relapse show patterns that correlate with one another [106]. This indicates that EBV could be a viral trigger of MS, but potentially only one of a cascade of events that leads to MS disease onset. One mechanism whereby infection with EBV could trigger autoimmunity is molecular mimicry [107], which is supported by the findings that T-cell clones specific for MBP epitopes also recognize EBV viral peptides [108].

Influenza Vaccine and Multiple Sclerosis

Many studies have delved into the effects of the commonly given influenza vaccine and its effects on MS patients. It has been repeatedly shown that influenza vaccination is safe [109-111] and can actually lower the risk of relapse in relapsing remitting patients [96].While it has previously been established that upper respiratory infections, including influenza, increase risk of relapse in MS patients [93], it has been debated whether the influenza vaccine has a positive or negative effect on disease activity. For instance Karussis et. al. found no evidence for subclinical disease exacerbation by MRI following influenza vaccination [112]. There have also been studies that have not shown an effect either way [113]. A meta-analysis was performed that determined that there was no link between vaccinations and MS relapse [114-116].

Virus-Induced Models of Demyelination

There are several viral models that are used to mimic virus-induced inflammatory demyelination; Semiki Forest virus (SFV), mouse hepatitis virus (MHV), and Theiler's murine encephalomyelitis virus (TMEV). Inflammatory demyelination in these models are thought to be

triggered by either bystander activation or molecular mimicry. Bystander activation occurs when immune cells are activated in an area of inflammation and cause damage to the surrounding tissue. This damage is mediated by an abundance of inflammatory cytokines and chemokines as well as myelin antigens that are continually released as the damage progresses. Molecular mimicry is when a pathogen presents antigens that are similar to self-antigens. If the body attacks this pathogen and it has this similar peptide, the activated immune cells can cause damage to the surrounding tissue. Several epitopes within EBV proteins have been shown to mimic myelin antigens. HBV has high sequence homology with epitopes of myelin basic protein (MBP) and can cause MBP specific T-cells to proliferate and attack the myelin sheaths. Other viruses that utilize this mechanism are EBV, herpes simplex virus (HSV), cytomegalovirus (CMV), influenza, and papillomaviruses. This is interesting due to the large number of those viruses that have been linked to MS.

Semliki Forest Virus is a member of alphaviridae. Peripheral infection of neurotropic strains of SFV causes an acute viremia in C57Bl/6 mice that is followed by perivascular and periventricular inflammation and demyelination of the CNS [117]. As is thought to occur during MS lesion formation, SFV-induced demyelination is mediated by CD4+ T-cells, because CD4 T-cell depletion causes a reduction in CNS pathology [118]. It has been shown that SFV-specific T-cells become activated by epitopes contained within the E2 glycoprotein [119]. Importantly, Mokhtarian et. al. has shown that these epitopes share sequence homology with the myelin oligodendrocyte glycoprotein $(MOG)_{18-32}$ peptide [117]. These T-cells remain in the CNS following viral clearance and continue to react to MOG causing inflammatory mediated demyelination. These data provide evidence for infection induced molecular mimicry in facilitating inflammatory demyelination [117, 119].

The most commonly used viral induced animal model of MS is intracerebral infection with Theiler's encephalomyelitis virus (TMEV). TMEV was discovered by Max Theiler in 1931 [120] and is a highly prevalent picornavirus in mice [120]. In this model, intracerebral infection of genetically susceptible strains results in a chronic infection of CNS resident glia which is followed by progressive demyelination and neurodegeneration that resembles primary progressive MS. Interestingly resistance to TMEV-induced demyelination disease (TVIDD) is conferred by the H2-D locus [121]. Specifically, H-2D^{b} are resistant [121]. Indeed, that resistance maps to loci encoded on MHC class I antigens implicates CD8 cells in the control of infection. CD8^{+} T-cells are thought to be protective as they clear virus from the CNS [122]. However, inflammatory demyelination in this model is mediated by Th1 cells and is presented antigen by virus presenting macrophages and microglia. In EAE endogenous myelin specific Th1 cells are activated through a process known as epitope spreading. In SJL mice with PLP 178-191 induced EAE the mice were initially injected with PLP 139-151, but F4/80-CD11c+CD45hi dendritic cells are able to present endogenous PLP 178-191, which mediates the pathology [123]. McMahon et. al indicates that naïve T-cells enter the pro-inflammatory environment of the CNS and are presented with antigen from dendritic cells which allows for epitope spreading [123].

Murine Hepatitis virus (MHV) is a member of the family coronaviridae and was first discovered and isolated from a mouse that was suffering from hind limb paralysis [124, 125]. While all strains of MHV cause hepatitis several, including, A9 [126] and JHM are neurotropic and neurovirulent [124, 125]. Intracerebral injection of non-neurovirulent strains cause inflammatory mediated demyelination [127]. This model can be used in mice, rats, as well as non-human primates. Following injection, the virus replicates in the astrocytes, microglia, and oligodendrocytes. Unlike TMEV, demyelination is thought to result from the direct lysis of

infected oligodendrocytes resulting in a multifocal lesion phenotype [128]. Interestingly lymphocytes from MHV infected rats can induce EAE-like lesions when adoptively transferred into a naive host.

Mouse Models of Multiple Sclerosis

One of the most established animal models of MS is EAE. Experimental autoimmune encephalomyelitis is an inducible disease model for MS that is mediated by $CD4^+$ T-cells and causes demyelination and neuronal degradation [129, 130]. In this model, disease is typically induced following injection with an encephalitogenic peptide emulsified in Complete Freund's Adjuvant. The immunogenic epitope that can induce EAE onset differs between species and mouse strains, but for the commonly used C57BL/6 mouse, the epitope is myelin oligodendrocyte glycoprotein peptide 35-55 (MOG_{35-55}). What is consistent across species is the use of adjuvant, which unfortunately clouds the ability to deduce information about alternative sources of inflammation like peripheral infection in the pathogenesis of disease. In order investigate the effects of peripheral infection on EAE and CNS inflammation an alternative approach should be implemented. The MOG_{35-55} TCR transgenic 2D2 mouse is another established model relevant to the study of MS and other autoimmune demyelinating diseases. Greater than 90% of $CD4^+$ T-cells in this mouse strain are specific for a CNS derived auto-antigen[131]; (Figure 1) yet fewer than 4% develop disease. Therefore, 2D2 mice may present a useful model with which to study the mechanisms that are necessary to recruit auto reactive cells into the CNS to induce EAE without the use of strong adjuvants. Although EAE is a well-established model for MS, there are some prominent differences between the two diseases. Experimental autoimmune encephalomyelitis is a disease that primarily targets the spinal cord of mice whereas most MS pathology occurs in the brain. We hypothesize that peripheral infection with a mouse adapted human influenza virus can induce EAE onset in autoimmune prone 2D2

mice and may prove to be a relevant model to uncover the mechanisms controlling infection-induced relapse in MS patients.

2. Materials and Methods

Animal Husbandry

Eight to twelve week old male and female mice were used for these studies. All mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME). Mice used for these experiments included wild-type C57BL/6J and C57BL/6-Tg(Tcra2D2,Tcrb2D2)1Kuch/J received from Jackson Laboratory (Bar Harbor, ME). The majority of $CD4^+$ splenocytes in the 2D2 mouse strain express the transgenic TCR (Vα3.2 and Vβ11). In-vitro splenocytes respond to the MOG 35-55 peptide sequence. At three weeks of age mice were genotyped using standard practices. In brief, a small piece of tail was removed with scissors and placed in 40µL of digestion buffer containing 1M Tris pH 8.0 (Sigma Aldritch, St. Louis, MO), 5M NaCl (Thermo Fischer Scientific, Waltham, MA), 0.5M EDTA (Thermo Fischer Scientific, Waltham, MA), 10% SDS (Sigma Aldritch, St. Louis, MO), distilled water) containing Protease K (5mg/mL; Thermo Fisher Scientific, Waltham, MA) then incubated for up to 30 min. at 55°C. Genomic DNA was amplified by PCR using according to the manufacturer's instructions (New England BioLabs, Ipswich, MA) using the primers shown in table 1. Following electrophoresis gels were imaged using an LAS-4000 Luminescent Image Analyzer from Fujifilm Life Science (India). All animals were housed under constant 12-h light/dark cycles and constant temperature in covered cages and fed with a standard rodent diet ad libitum. The experimental procedures described herein were approved by the Institutional Animal Care and Use Committee at the

University of Illinois Urbana-Champaign and were performed in accordance with guidelines of the National Institutes of Health.

Infection

Working virus concentrations were achieved by diluting stock virus (mouse adapted human H1N1 Influenza A/PR/8) in sterile phosphate buffered saline (pH 7.4). Mice were anesthetized with 4.0% isoflurane (Clipper Distributing Company LLC, St. Joseph, MO), then inoculated intranasally with either sterile saline or influenza in 50 μL total volume per mouse.

Sickness Behavior and Experimental Autoimmune Encephalomyelitis Scoring

Mice were weighed daily on an Adventure Pro scale (OHAUS, Parsippany, New Jersey) and body conditioning scores (0-5) are determined as described previously [132], where 1 equates to muscular wasting, no fat deposits and visible skeletal structures, 2 is vertebral column and the dorsal pelvic bones remained distinct and mouse is obviously under conditioned, 3 is a healthy conditioning score, 4 is slightly over conditioned, vertebrae no longer visible and can be palpated with firm pressure, 5 is an obese mouse.

Mice were assessed for clinical EAE using a 5 point scale as described previously [133]. In brief, 0 is equated to no signs of EAE; 0.5 is tail weakness or hind limb weakness;1 is full tail paralysis; 1.5 is tail paralysis and uncoordinated gate; 2 is tail paralysis and loss of righting reflex; 2.5 includes partial fore/hind-limb paralysis; 3 is complete paralysis of one limb; 3.5 is one limb with complete paralysis and weakness of another; 4 is dual hind-limb paralysis; 4.5 is dual hind limb paralysis and fore-limb weakness; 5 is moribund or dead. Additionally, signs of atypical EAE included: spinning, tip-toe gate, hunching, and ruffling. These mice were not given a clinical score on the 0-5 scale unless they showed signs of classical EAE as well. All signs of atypical EAE were recorded.

Glial Isolation and Treatment

Neonatal C57BL/6 mice were dissected at post-natal day 0-3 in a biological safety cabinet. Pups were decapitated using surgical scissors, the brains removed and placed into sterile chilled HBSS media. Meninges were removed under a dissecting scope and digested with 200µg/mL DNase (Sigma Aldritch, St. Louis, MO) and 20uL .25% trypsin/EDTA (Gibco, Langley, OK) per brain were added to the tube and incubated at 37°C for 15 min. Brains were then broken apart by pipetting with a 10 mL pipet and washed using DMEM media (UIUC cell media facility, Urbana, IL) supplemented with L-glutamine (1 mM) (Sigma Aldritch, St. Louis, MO), sodium pyruvate (1 mM) (Sigma Aldritch, St. Louis, MO), 10% heat inactivated fetal bovine serum (FBS) (Corning, Corning, NY) and 1% penicillin/streptomycin (100U each Thermo Fischer Scientific, Waltham, MA) (D10S media) to create a single cell suspension. Cells were passed through a 70 µm filter (Corning, Corning, NY) and plated in poly-D-Lysine (Sigma Aldritch, St. Louis, MO) coated plastic ware. Cells were cultured at 37°C and 5.0% CO_2 until confluent. Once confluent the flasks were shaken at 150 rpm on a MaxQ 4000 (Thermo Fisher Scientific, Rochester, NY) for one hour. Microglia can then be isolated from the supernatant and astrocytes will remain attached. Once microglia containing media was removed a repeat of the shake step was performed to further enrich the astrocyte culture. Astrocytes were then treated with 50mM L-Leucine meythlester (Sigma Aldritch, St. Louis, MO) for 1 hour prior to trypsonization and subculture. Glia were then individually plated at a density of $5x10^5$ cells/well (astrocytes) and $1x10^6$ cells/well (microglia) in PDL coated 6-well plate in 3 mL of D10S media for 24 h. The cells were then washed twice with 3 mL of DMEM FBS free media (UIUC cell media facility, Urbana, IL). Cells were washed in serum free media containing 0.5% bovine serum albumin (BSA) and treated with 10ng of recombinant mouse IL-1β (eBioscience, San

Diego, CA) and/or recombinant mouse TNF-α (eBioscience, San Diego, CA) for 24 h. The supernatants were then collected and frozen at -80°C.

Astrocytes were plated on PDL-coated (Sigma Aldrich, St. Louis, MO) 6-well plates (Thermo Fisher Scientific, Rochester, NY) at a concentration of $1x10^6$ cells and cultured until confluent. Astrocytes were then treated with specific inhibitors of JNK (SP600125, 20 μM), ERK 1/2(PD98059, 20 μM), NF-κB (BAY 11-7085, 20 μM), and p38 (SB203580, 20 μM) for 30 minutes prior to cytokine stimulation (all from Cell Signaling Technologies, Danvers MA). After treatment, cells were stimulated with 10ng/mL of mouse rIL-1β (eBioscience, San Diego, CA) and mouse rTNF-α (eBioscience, San Diego, CA) for 15 min. Supernatants were isolated after 24 h of stimulation from sister cultures resulting from the same dissections that were identically treated on the same day as those cultures used for protein isolation and western blot.

Measurement of Cytokine/Chemokine Levels

Glial supernatants from each independent experiment were thawed. Then 0.2 ml aliquots from each experiment were pooled, and used to measure chemokine secretion using a chemokine proteome profiler array kit according to manufacturer's instructions (R&D systems, Minneapolis, MN). Resulting blots were imaged using an LAS-4000 Luminescent Image Analyzer from Fujifilm Life Science (India). The pixel density of each chemokine was quantified using ImageJ (National Institute of Health, Bethesda, MD). Where indicated glial supernatant levels of CCL2 (MCP-1) and CCL5 (RANTES) were measured using ELISA Ready-SET-Go! Kit (affymetrix eBioscience, San Diego, CA) following manufacturer's instructions. Optical density (OD) was determined using a Bio-Tek Synergy HT plate reader (Bio-Tek, Winooski, VT) at 450nm. Serum was isolated from whole blood by centrifugation (500x*g* for 5 minutes) and then measured for IL-6, IFN-γ, IL-1β, IL-10, IL-17, and TNF-α levels using a mouse cytokine/chemokine magnetic bead panel (Millipore, Billerica, MA) following manufacturer's instructions. Cytokine

concentrations were determined using a Bio-Rad Luminex Cytometric Bead Analyzer (Bio-Rad, Hercules, CA).

RNA Isolation and RT-qPCR

Total RNA was isolated using Tri-Reagent according to the manufacturer's instructions (Sigma Aldritch, St. Louis, MO), then quantitated using a NanoDrop spectrophotometer ND-1000 (Thermo Fischer Scientific, Waltham, MA). Subsequently1µg of RNA was converted in a 20µL reaction to cDNA using a reverse transcription system (Promega, Madison, WI). A no RT control was also utilized. 1.0µg of cDNA was analyzed using Sybergreen qPCR (Bio Rad, Hercules, CA) on a Step One Plus Real-Time PCR System and associated software (Applied Biosystem, Foster City, CA). $2^{(-\Delta\Delta CT)}$ formula was used to determine gene fold change. GAPDH was used as the housekeeping control gene.

Central nervous system infiltrating lymphocyte isolation

Mice were anesthetized by i.p. injection of ketamine (85-100mg/kg) and xylazine (10-13mg/kg) diluted in 0.1 ml of PBS, then transcardially perfused using 20 mL of sterile PBS. Tissues were dissected and placed into ice cold D10S (Brains and spinal cords) or RPMI1640 media (lungs)(UIUC cell media facility, Urbana, IL) supplemented with L-glutamine (1 mM) (Sigma Aldritch, St. Louis, MO), sodium pyruvate (1 mM) (Sigma Aldritch, St. Louis, MO), 10% heat inactivated FBS (Corning, Corning, NY) and 1% penicillin/streptomycin (100U each Thermo Fischer Scientific, Waltham, MA) (R10S media). Tissues were minced using sterile razor blades and digested using StemPro Accutase digestion buffer (Gibco, Langley, OK) for 15min. at 37°C. Samples were then passed through 70 µm filter (Corning, Corning NY) and suspended in 5 ml of 35% percoll/PBS solution. This solution was underlayed with, 3 ml of 75% percoll/PBS solution (Sigma Aldritch, St. Louis, MO). Cells were centrifuged at 2000x*g* for 20

min. with no break. Lymphocytes were extracted from the 35/75% interface, washed with RPMI and counted using a hemocytometer.

Flow Cytometry

The following antibodies were used for immunophenotyping by flow cytometry:CD3-eFluor450 (clone: 17A2), CD3-PE-Cy7 (clone: 145-2C11), CD4-eFluor450 (clone: GK1.5), CD4-APC (clone: GK1.5), CD4-FITC (clone: GK1.5) CD8-PE-cy5.5 (clone: 53-6.7), CD19-PE (clone: eBio1D3), CD19-APC (clone: eBio1D3), CD45-APC (clone: 30-F11), CD45-PE (clone:30-F11), CD11b-FITC (clone: M170), CD11c-eFluor450 (clone: N418), MHCII-eFluor450 (clone: M5/114.15.2), MHCII-APC (clone: H1519), Vα3.2-APC (clone: RR3-16), Vα3.2-eFluor450 (clone: RR3-16), Vβ5.1-PerCP-eFluor710 (clone: MR9-4), IFN-γ-APC (clone: XMG1.2), IL-17a-PE (clone: eBio17B7) all from eBioscience (San Diego, CA) and CCR4-PE-cy7 (clone: 2612) (BioLegend, San Diego, CA) to identify the varying subtypes of lymphocytes and glia. In brief, $5x10^5$ cells were stained for 30min. suspended in PBS with 2% FBS. Cells were then centrifuged at 300x*g* for 5 min. and washed using PBS+2% FBS solution. Immunophenotyping was determined using a LSRII Flow cytometer (BD, San Jose, CA). Gates were determined using unstained and single stained samples resulting from the same tissue. Results were analyzed using FCS Express 4 Flow cytometry software (De Novo Software, Los Angeles, CA).

Choroid Plexus Isolation

The choroid plexus were isolated from saline and influenza inoculated mice at day 8 p.i. as previously described [134]. Following isolation, the entire choroid plexus of each mouse was blocked in PBS with 5% goat serum (Sigma Aldritch, St. Louis, MO) and 0.3% Triton X-100 (Sigma Aldritch, St. Louis, MO) for 1 hour. Tissues were incubated with a rat anti-mouse CD4 (1:1000 RM4-5 eBioscience San Diego, CA) and rabbit anti-Iba-1 (1:1000 Wako, Japan).

Tissues were washed three times for 5 minutes using PBS and then stained using species specific secondary antibodies (either Alexa Fluor 488 goat anti-rabbit 1:1000 Life Technologies, Eugene OR, or Alexa Fluor 594 goat anti-rat 1:1000 Life Technologies, Eugene OR). Nuclei were then stained using Hoechst 33342 trihydrochloride (1:4000 Life Technologies, Carlsbad, CA) for 1 min. Slides were then mounted using flouromount-G (Southern Biotech) and imaged using a fluorescent microscope and associated Zen software.

Statistics

Data were analyzed using Student's T-tests and multi level analysis of variance followed by Bonferonni post-hoc analysis. Chi-square analysis were used to determine the establish elevated incidence of EAE in 2D2 mice. Significance was set at $p \leq 0.05$. Data are reported as means $\pm$ standard error (S.E.). Analysis was performed using Prism 6 software (GraphPad Software, San Diego, CA). Quantity of animals used for the following experiments are shown in the figure legends.

3. Results

3.1: Non-Neurotrophic Influenza virus Induces Experimental Autoimmune Encephalomyelitis in autoimmune prone 2D2 mice

Transgenic 2D2 mice possess an inherent reduction in $CD8^+$ T-cells, which might impede virus-specific immune responses [135]. Indeed, following intranasal inoculation with a normally non-lethal dose of influenza, 2D2 mice lost more weight, and a higher percentage succumbed to infection compared to controls (Figure 1A-B). However, when inoculated with a lower viral titer the mortality rates of infected 2D2 mice decreased and the majority of mice survived infection.

Because 2D2 mice exhibit exacerbated disease following infection, we questioned if viral tropism was altered in this strain. Viral *M1* was neither detectible in the olfactory bulbs,

cerebellum, nor spinal cords of infected 2D2 mice at day 8 p.i (Figure 1C). Since the virus is not present in the CNS of the 2D2 mice, we performed flow cytometry to determine why 2D2 mice had reduced ability to combat influenza infection. First, C57BL/6 mice were used to determine a baseline of what proportions of $CD4^+$ and $CD8^+$ T-cells are present in the lung at day 2, 4, and 8 p.i. (Figure 2A). We found that $CD8^+$ T-cells were increased in the lungs of C57BL/6 mice at day 8 p.i. (Figure 2B). C57BL/6 mice T-cell proportions at day 8 p.i. were then compared to 2D2 mice at day 8 p.i. to see if these populations were altered and 2D2 mice were found to have a lower $CD8^+$ T-cell population, which explains the diminished ability for this strain of mice to fend off viral infections (Figure 2C). Lung infiltrating cells from 2D2 mice were isolated to determine if encephalogenic T-cells ($V\alpha3.2^+$) were trafficking to the lungs following infection (Figure 2D) and what proportion of these cells were activated ($V\alpha3.2^+CD62L^-$) versus naïve ($V\alpha3.2^+CD62L^+$) (Figure 2E) and found that both naïve and activated encephalogenic T-cells were in the lungs at day 8 p.i.

Although no virus is present in the brain of 2D2 mice, ~29% of virus-inoculated 2D2 mice developed clinical EAE (7/24) within 2 weeks of infection, while saline inoculated 2D2 mice did not develop disease (0/13). This incidence is similar to the effect of upper-respiratory infection on relapse in MS and is significantly higher than would be anticipated to occur in this strain ($\chi2=37.565$; $p<0.001$). Maximal disease severity of influenza-induced EAE was variable, ranging from tail weakness to unilateral paralysis (Table 2). Moreover, a high percentage of influenza inoculated mice developed symptoms of atypical EAE characterized by unilateral spinning, ruffling and hunching (Table 3), however diagnosis of atypical EAE is inconclusive due to the similarities in symptoms between influenza sickness behavior and atypical EAE symptoms. Flow cytometry results from a subset of 2D2 mice exhibiting signs of EAE showed

increased percentages of CNS infiltrated leukocytes and lymphocytes, including the presence of autoreactive $CD45^{hi}V\alpha3.2^{+}$ T cells when compared with saline inoculated controls (Figure 3). Additionally, histopathological analysis confirmed the presence of inflammation (Figure 4A). In contrast, saline inoculated mice showed fewer pathological signs of EAE (Figure 4). Together, these data indicate that upper-respiratory infection with a live virus is capable of triggering inflammatory demyelination in TCR transgenic 2D2 mice.

3.2: IFN-γ is increased in the serum at day 8 p.i.

Infections cause the secretion of cytokines into circulation. Using a multiplex array we established what cytokines (IL-6, IFN-γ, IL-1β, IL-10, IL-17, and TNF-α) were in the serum of C57BL/6 mice at day 2, 4, 8, and 16 p.i. with influenza. Influenza infected C57BL/6 mice had increased concentrations of serum IL-6 at day 2 p.i. (Figure 5A) and increased concentrations of IFN-γ at day 8 p.i. (Figure 5B). Circulating concentrations of IL-1β, IL-10, IL-17, and TNF-α were not detected (Figure 5C-F).

3.3 TNF-α and IL-1β induce chemokine secretion from astrocytes and microglia

Using primary glial cultures we examined both astrocytes and microglia for production of chemokines following cytokine stimulation. The chemokine secretion profile of glial cell types following stimulation with IL-1β and TNF-α was needed to determine which glial cell type was needed to promote immune cell trafficking. First, the purity of each culture was assessed by immunocytochemistry using antibodies specific for Iba-1 (microglia) and GFAP (astrocytes) which demonstrated that the cultures were 94% astrocytes (Figure 6A) and >99% microglia respectively. Cultured astrocytes stimulated for 24h with IL-1β, TNF-α, or IL-1β and TNF-α together showed different chemokine secretion profiles (Figure 6). TNF alone stimulated a much stronger response than IL-1β, although TNF-α did not stimulate any chemokines unique from IL-1β. Only IL-1β stimulation caused increased secretion in CXCL1 and CXCL5 (Figure 6 B-C),

but when astrocytes are stimulated with both cytokines the effect is synergistic in some cases or slightly depressed compared to the initial TNF-α profile. CCL2, CCL5, CCL12, CXCL5, and CXCL1 were synergistically increased following stimulation with both TNF-α and IL-1β (Figure 6B-C). Interestingly the chemokine secretion profile differed between astrocytes and microglia following pro-inflammatory cytokine stimulation. Stimulated astrocytes produced an abundance of CCL2, CCL5, CCL12, CXCL5, and CXCL10, whereas stimulated microglia produced CCL2, but in lower amounts than astrocytes and constitutively secreted CCL9/10 regardless of stimulation (Figure 6D-E). These chemokines are responsible for the trafficking of a variety of immune cells including monocytes, neutrophils, and T-cells. The results of the chemokine array were validated using an ELISA for CCL2 (Figure 6F).

Given that astrocytes were the primary producers of chemokines following cytokine stimulation, we questioned which signaling pathway was needed to promote chemokine production. Therefore we treated cultured astrocytes with inhibitors of JNK, ERK1/2, p38, and NF-κB to determine the downstream signaling mechanisms for these chemokines. Pre-treatment with the JNK inhibitor SP600125 decreased secretion of CCL9/10, CCL11, CCL12, CXCL9, CXCL12, and RARRES2 by greater than 50% (Figure 7B-F). Treatment with the ERK inhibitor PD98059 decreased CCL9/10, CCL12, and CXCL2 by greater than 50%, while p38 and NF-κB only decreased CCL12 and CXCL12 respectively (Figure 7B-F). Interestingly, constitutive expression of CCL6 was decreased by IL-1β and TNF-α treatment, an effect that was reversed following retreatment with SP600125, SB203580, and BAY 11-7085 inhibitors, while CCL3/4 was increased only during NF-κB inhibition (Figure 7F-G).

3.4 Peripheral infection causes increased $CD4^+$ T-cells in the choroid plexus

The choroid plexus has previously been established as a portal of entry for immune cells into the brain. We found that the elevation of serum IFN-γ peaked at the same time as peak immune infiltration (Figure 5B) and IFN-γ has previously been connected to the stimulation of the choroid plexus epithelium, which aides in immune infiltration into the CNS. Interestingly $CD4^+$ T-cells were increased in choroid plexus isolations from infected C57BL/6 mice at day 8 p.i. versus saline inoculated animals (Figure 8) further establishing the choroid plexus as an immune portal of entry and serum IFN-γ as a trigger following peripheral infection.

3.5 Peripheral infection causes immune infiltration and surveillance of the CNS at day 8 p.i.

Peripheral infection upregulates TNF-α and IL-1β in the CNS [136]. Because we found that both TNF-α and IL-1β promote chemokine secretion from glia we questioned whether peripheral infection cause immune cell attraction to the CNS. Using flow cytometry (Figure 9A) and monitoring mouse body weights (Figure 9B) we found that infection of C57BL/6 mice increased the percent of T-cells ($CD19^-CD3^+$) (Figure 9C), but not B-cells ($CD19^+CD3^-$) (Figure 9D) in the CNS at day 8 p.i. No differences in either population was observed at day 2, 4, or 16 p.i. (Figure 9). In contrast to what was seen in the brain, no significant effect of infection was observed on $CD3^+$ or $CD19^+$ cells in the spinal cord (Figure 10).

A follow up experiment was performed to determine which sub-type of T-cells were increase at day 8 p.i. (Figure 11). Compared to control, infection increased percentages of both cytotoxic T-cells ($CD3^+CD4^-$) and helper T-cells (Figure 11D) ($CD3^+CD4^+$) were increased (Figure 11B), while B-cells remained unchanged (Figure 11F). To determine what other types of immune cells traffic to the brain during peripheral infection we examined the percentage of

$CD11b^{+}CD45^{high}CD11c^{-}$ (monocyte/neutrophil) (Figure 12C), $CD11b^{+}CD45^{int}$ (microglia) (Figure 12 D), $CD11c^{+}$ (Dendritic cells)(Figure 12E) and $CD11b^{-}CD45^{high}$ (lymphocytes)(Figure 12F). In this study an increased monocyte/neutrophil population ($CD11b^{+}CD45^{+}CD11c^{-}$) was observed at day 8 p.i. (Figure 12C). Lymphocytes described as $CD11b^{-}$ $CD45^{+}$ were also increased in the brain during this experiment (Figure 12F).

At day 8 p.i. immune cell populations were isolated to determine if the populations increased in the brain were the same cell types that were increased in the circulation (Figure 13). This comparison was gated stringently on the lymphocyte population only, to ensure accuracy between the two groups of experimental data. The monocyte/neutrophil and B-cell populations were significantly different between brain and circulating populations whereas increases in T-cell population was similar (Figure 13).

4. Discussion

4.1 Findings

First, it was necessary to establish a dose of influenza that did not result in high mortality. We found that inoculation with a lower dose of virus mitigated mortality while maintaining symptoms of infection (Figure 1B). Furthermore, this study demonstrates that peripheral infection with influenza can induce histological and clinical symptoms of EAE in an autoimmune prone mouse strain (Figure 3-4, Table 2). We also found that, infection of C57BL/6 mice with influenza stimulated immune infiltration and surveillance of the CNS by T-cells, as well as monocyte/neutrophils. Influenza infection of C57BL/6mice caused transcriptomic changes to occur in the cerebellum and spinal cord at day 8 p.i. (unpublished). The genes that have the most altered expression were associated with IFN-γ stimulation (unpublished), which was elevated in circulation at day 8 p.i. IFN-γ has been shown to stimulate the choroid plexus

epithelium and induce the release of chemokines [137]. This stimulation may have caused the increase in $CD4^+$ T-cells that was observed in the choroid plexus of infected animals when compared to saline controls (Figure 6). Our findings indicate that immune cell surveillance of the CNS is associated with the expression of IFN-γ in the periphery. Chemokine signaling between the CNS and the immune system is critical in recruiting immune cells to the site of infection and injury. Following stimulation with pro-inflammatory cytokines IL-1β and TNF-α cultured astrocytes and microglia both secreted chemokines, although astrocytes were the primary producer. This suggests that immune cells may also be recruited to the CNS during peripheral infection by the glia.

4.2 Infection and Experimental Autoimmune Encephalomyelitis onset/ Multiple Sclerosis relapse

Although infection induced EAE in some mice, only ~29% of mice showed clinical symptoms of disease. It is not understood why the incidence of EAE is low, but there could be some threshold or of activation that needs to occur before clinical symptoms are observed. Classical EAE is primarily a disease of the spinal cord, but atypical symptoms have been shown to be caused by inflammation in the cerebellum. It has been established that an increased Th17/Th1 T-cell ratio is important for brain parenchymal infiltration, while no specific ratio has been observed for spinal cord infiltration [138]. One reason for the discrepancy between classical score and atypical score could be a disruption in IFN-γ signaling. IFN-γ disruption has shown an increase in atypical EAE symptoms and inflammatory loci containing high amounts of neutrophils [139]. Although confounding, control mice also had some foci of inflammation, but significantly fewer than the infected animals. Upper-respiratory viral infections have had a strong link with MS relapse [3, 92-97, 140, 141]. There is an at risk period of one week prior to URI symptoms to 5 weeks after that corresponds with risk for relapse [92-95]. Twenty seven –

forty eight percent of MS patients that have an URI suffer a relapse during this at risk period [92, 94, 95, 97, 140-142]. The EAE incidence rate of 2D2 mice following infection closely mimics this relapse rate.

4.3 Peripheral infection causes CNS immune infiltration and surveillance

Previous studies have used Poly I:C and TLR7 agonists as mimics of viral infection to induce CNS immune surveillance, but these do not establish a replicating viral infection that stimulates an adaptive immune response making our finding in regards to the adaptive immune response novel [143-145]. Peripheral infection with influenza caused an increase in $CD4^+$ and $CD8^+$ T-cells as well as an increased $CD45^{high}CD11b^+$ (monocyte/neutrophil) population. Using immunofluorescence T-cells were increased in the choroid plexus of C57BL/6 mice at day 8 post influenza infection. These results are in agreement with the findings of others that have demonstrated immune cell homing to the brain following peripheral infection of CFA or cutaneous administration of imiquimod, a TLR7 agonist [146, 147]. While it is known that meninges surrounding the brain contain populations of immune cells that are constitutively survailling the brain [148-150] including monocytes, macrophages and T-cells exactly how peripheral infection increases immune cell surveillance of the CNS is not known. It is tempting to speculate that glial activation following peripheral infection is a requisite for increased immune cell surveillance. Indeed, infection with the non-neurotropic pandemic influenza A/CA/04/2009 strain of influenza stimulated microgliosis in the hippocampus and substantia nigra [151]. Furthermore, Jurgens et. al. found that the same H1N1 A/PR/8 virus used in our experiments induced neuroinflammation and changed neuronal morphology in the hippocampus [136]. These results establish that influenza infection can induce an inflammatory response in the brain without the presence of virus.

The blood brain barrier is effective at controlling immune access to the CNS, but the choroid plexus has been established as an immune portal of entry. The choroid plexus can be activated by IFN-γ to allow immune cells greater access to the CSF and to stimulate further chemokine secretion from choroid plexus epithelial cells. In-vitro culture recreations of the choroid plexus epithelial layer have given a glimpse into how memory T-cells cross the blood cerebrospinal fluid barrier (BSCB) [152]. Memory CD4 T-cells constitutively reside in the choroid plexus [152] and are thought to be the source of IFN-γ necessary to control choroid plexus permeability [150]. T-cells have been found in the choroid plexus of untreated humans and animals [147, 153] and their numbers increase to a small degree during peripheral infection [137, 147]. The onset of cerebellar EAE (atypical) has been shown to be stimulated by the infiltration of Th17 cells through the choroid plexus which acts in a CCR6/CCL20 dependent manner [37].

4.4 IFN-γ stimulation of CNS associated tissue

We previously performed a RNA-seq experiment to determine if changes occur in the cerebellum and spinal cord over the course of infection. At day 8 bioinformatic analysis indicated that shared immune response gene upregulation was mediated by IFN-γ signaling. The up-regulation of these genes found by the RNA-seq of the cerebellum and spinal cord correlated with increased serum IFN-γ. Interferon gamma has a previously established role in immune cell infiltration into the CNS by enhancing expression of cell adhesion molecules and chemokines in the endothelial cells of the choroid plexus [154]. Furthermore, IFN-γ is also an important anti-viral cytokine that was initially thought to be a potential treatment option for MS patients. However, Panitch et. al. found that intravenously administrated IFN-γ caused relapse in 7/18 (38.89 %) MS patients. Notably, all relapses occurred within 4 weeks of treatment [155, 156]. This increased exacerbation rate was suspected to be immunologically mediated since it

coincided with an increase in monocytes with class II (HLA-DR) surface antigen, proliferative responses of peripheral blood leukocytes, and in natural killer cell activity [155, 156]. As might be anticipated MS patients were shown to have increased concentrations of circulating IFN-γ within the established 6 week "at risk" period for virus-induced relapse [157]. Together, these data indicate that in MS patients IFN-γ has a pivotal role in disease exacerbation. However, the effect of IFN-γ on EAE is not as clear-cut and, in fact, this cytokine appears to exhibit protective effects. The same has been shown when mice have a disruption in the gene that encodes IFN-γ. Specifically, In B10.PL crossed with IFN-$\gamma^{-/-}$ mice are more susceptible to disease onset [158]. This contradiction between the role of IFN-γ in MS and EAE has been hypothesized to be time dependent [159]. For instance, IFN-γ treatment during the induction phase of EAE mice exacerbates disease, whereas treatment during the effector phase is protective [159, 160]. Therefore, the timing of IFN-γ levels could be the crucial element to the otherwise paradoxical results of past studies.

4.5 CNS stimulation causes the secretion of glial specific chemokine profiles

A major finding of this study was that treatment of primary microglia and astrocytes with recombinant IL-1β and TNF-α elicits different chemokine secretion profiles. Specifically, we demonstrated that astrocytes produce more chemokines than microglia in response to IL-1β and TNF-α stimulation. This study also observed that astrocytes produce an altered chemokine profile when stimulated with IL-1β and TNF-α separately as well as synergistic secretion of some when treated with both (Figure 6B). Both microglia and astrocytes are known to secrete numerous chemokines in response to poly (I:C), TNF-α, and/or IL-1β stimulation [161, 162]. Both microglia and astrocytes secrete the CCL2 and CCL5 in response to IL-1β and TNF-α [162, 163]. Cultured human astrocytes additionally secrete CCL3, CCL4, CCL5, CCL20, and CXCL12 in response to TNF-α and IL-1β [163]. CCL3, CCL4, and CCL5 have shown in-vitro abilities to

recruit immature dendritic cells [163]. Astrocyte stimulation could be one way by which immune cells are recruited to the CNS during infection. Interestingly, CCL12 has also been shown to be upregulated in MS patients and was secreted in dramatically increased levels after cytokine stimulation [164] (Figure 6).

Once the secretion profiles of microglia and astrocytes were established, we determined the downstream signaling required for the secretion of our previously discussed panel of chemokines from primary astrocytes. The BAY 11-7082 inhibitor of IκB has previously been shown to stimulate both p38 and JNK-1 signaling therefore explaining some of the increased secretion levels seen during this treatment. All inhibitors (SP600125, PD98059, SB203580, and BAY 11-7085) modulated the secretion profile to a certain degree, but inhibition of JNK showed the largest effect. The expression of CCL9/10, CCL11, CCL12, CXCL2, CXCL9, CXCL12, and RARRES2 was decreased by greater than 50% by SP600125. CCL9/10 and RARRES2 both recruit dendritic cells [165, 166], CXCL2 and CXCL12 recruit neutrophils and B-cells[167], and CXCL9 is responsible for the recruitment of Th1 cells [168]. The chemokines CCL11, CXCL2, CXCL9, and CXCL12 all have been linked to demyelination or MS [165, 167, 169, 170]. The receptor for CXCL9 (CXCR3) is increased on the surface of Th1 cells in the CSF of MS patients while CXCL12 has increased expression in the lesions of MS patients and it has been found in their CSF as well [170]. CXCL2 was found to be increased during peak demyelination of TMEV infection in mice [171]. Together these data suggest that JNK is a key kinase in the downstream signaling of pro-inflammatory cytokines connected to MS, but our evidence strongly suggests that redundancies in the signaling networks exists to prevent a stop to chemokine secretion in the event of JNK inhibition. However, inhibition of other pathways affected chemokine secretion indicating that there is some redundancy in this response.

5. Summary

In conclusion, T-cells and $CD45^{high}CD11b^{+}$ cells are increased in the brain, at day 8 p.i., in C57BL/6 mice (Figure 9, Figure 11-12). Influenza infection induces EAE in autoimmune prone 2D2 mice, which may be connected to the infiltration of immune cells observed in C57BL/6 mice (Figure 3, Table 2). Finally, chemokine production in brain resident glial cells, primarily astrocytes are induced via TNF-α and IL-1β which could assist in the trafficking of immune cells to the CNS (Figure 6). Chemokine secretion is stimulated through the downstream signaling of JNK, ERK, p38, and NF-κB to varying degrees, although JNK inhibition was the most effective treatment at lowering chemokine secretion levels.

6. Tables and Figures

Gene	Primer	Sequence
2D2 transgene	FWD	CCC GGG CAA GGC TCA GCC ATG CTC CTG
	REV	GCG GCC GCA ATT CCC AGA GAC ATC CCT
M1 (Influenza)	FWD	AAG ACC AAT CCT GTC ACC TCT GA
	REV	CAA AGC GTC TAC GCT GCA GTC C
GAPDH	FWD	GCA TCT TCT TGT GCA GTG CC
	REV	TAC GGC CAA ATC CGT TCA CA

Table 1: Forward (FWD) and reverse (REV) primers sequences used during PCR and qPCR

Mouse strain	Treatment	Clinical EAE incidence	Clinical EAE (%)	Highest Score achieved	Day of onset
C57Bl/6	Flu	0/5	0	N/A	N/A
2D2	Saline	0/13	0	N/A	N/A
2D2	Flu	7/24	29.2	0.5-3.5	10-14

Table 2: C57BL/6 mice and 2D2 mice were inoculated with either saline or influenza and evaluated for symptoms of clinical EAE. Analysis of disease severity, day of onset, number of animals exhibiting symptoms and the percentage of each mouse population that displayed disease.

Mouse strain	Treatment	Atypical EAE incidence	Atypical EAE (%)	Day of onset
C57Bl/6	Flu	0/5	0	N/A
2D2	Saline	1/13	7.7	10
2D2	Flu	17/24	70.8	9-17

Table 3: C57BL/6 mice and 2D2 mice were inoculated with either saline or influenza and evaluated for symptoms of atypical EAE. Analysis of disease severity, day of onset, number of animals exhibiting symptoms and the percentage of each mouse population that displayed disease.

Item/Reagent	Company	Catalogue #	Item/Reagent	Company	Catalogue #
α-mouse VCAM	Abcam	ab134047	Vβ5.1-PerCP-eFluor710	eBioscience	46-5796-80
Precision Plus Protein™	Bio Rad	1610385	(clone: MR9-4)		
WesternC™ Standards			α-mouse CD4 RM4-5	eBioscience	14-0042-81
Syber Green	Bio Rad	1725270	StemPro Accutase	Gibco	A1110501
CCR4-PE-cy7 (clone: 2612)	BioLegend	131213	digestion buffer		
ERK 1/2 inhibitor PD98059	Cell Signaling Tech.	9900S	trypsin/EDTA	Gibco	25200072
ERK Ab	Cell Signaling Tech.	4370S	Hoechst 33342 trihydrochloride	Life Technologies	H3570
JNK Ab	Cell Signaling Tech.	9252S	mouse cytokine/chemokine	Millipore	MCYTOMAG
JNK inhibitor SP600125	Cell Signaling Tech.	8177S	magnetic bead panel		70K-PMX
NfkB Ab	Cell Signaling Tech.	8242S	Nf-kB inhibitor Bay 11-7085	Millipore	196872-10MG
p38 Ab	Cell Signaling Tech.	8690S	Phusion® High-Fidelity PCR Kit	NE BioLabs	E0553L
p38 inhibitor SB203580	Cell Signaling Tech.	5633S	reverse transcription system	Promega	A3500
p-ERK Ab	Cell Signaling Tech.	4094S	proteome profiler array	R&D systems	ARY020
p-JNK Ab	Cell Signaling Tech.	9255S	proteinase inhibitor mixture I	Roche Applied Sci.	04693116001
p-NfkB Ab	Cell Signaling Tech.	3033S	10% SDS	Sigma Aldritch	L4522
p-p38 Ab	Cell Signaling Tech.	4511S	1M Tris pH 8.0	Sigma Aldritch	T3038-1L
70μm filter	Corning	35235	200μg/mL Dnase	Sigma Aldritch	AMPD1-1KT
heat inactivated FBS	Corning	35-016-CV	goat serum	Sigma Aldritch	G9023-5ML
CD19-APC (clone: eBio1D3)	eBioscience	17-0193	L-glutamine	Sigma Aldritch	G8540-100G
CD3-PE-Cy7 (clone: 145-2C11)	eBioscience	25-0031-81	L-Leucine meythlester	Sigma Aldritch	L1002-10G
CD45-APC (clone: 30-F11)	eBioscience	17-0451-82	Na2P2O7	Sigma Aldritch	P8010-500G
IFN-γ-APC (clone: XMG1.2)	eBioscience	17-7311-81	Na3VO4	Sigma Aldritch	S6508-10G
CCL2 ELISA Ready-SET-Go! Kit	eBioscience	88-7391-22	NaF	Sigma Aldritch	71522
CD11b-FITC (clone: M170)	eBioscience	11-0112	percoll	Sigma Aldritch	P1644-500mL
CD11c-eFluor450 (clone: N418)	eBioscience	48-0114-80	PMSF	Sigma Aldritch	10837091001
CD19-PE (clone: eBio1D3)	eBioscience	12-0193-81	Poly-D-Lysine	Sigma Aldritch	P4957
CD3-eFluor450 (clone: 17A2)	eBioscience	48-0032-80	sodium pyruvate	Sigma Aldritch	P5280-100G
CD45-PE (clone:30-F11)	eBioscience	12-0451	Tri-Reagent	Sigma Aldritch	T9424-100mL
CD4-APC (clone: GK1.5)	eBioscience	17-0041	Tris, pH 7.5	Sigma Aldritch	T1503-1kg
CD4-eFluor450 (clone: GK1.5)	eBioscience	48-0041-80	Triton X-100	Sigma Aldritch	X100-500mL
CD4-FITC (clone: GK1.5)	eBioscience	11-0041	Tween 20	Sigma Aldritch	BP337-500
CD8-PE-cy5.5 (clone: 53-6.7)	eBioscience	15-0081-81	flouromount-G	Southern Biotech	0100-01
IL-17a-PE (clone: eBio17B7)	eBioscience	12-7182	0.5M EDTA	Thermo Fisher Sci.	15575020
MHCII-eFluor450 (clone: M5/114.15.2)	eBioscience	48-5321-80	6-well plates	Thermo Fisher Sci.	130184
rIL-1β	eBioscience	14-8012-62	NaCl	Thermo Fisher Sci.	BP358-10
rTNF-α	eBioscience	14-8321-62	penicillin/streptomycin	Thermo Fisher Sci.	15140122
Vα3.2-APC (clone: RR3-16)	eBioscience	17-5799-82	Protease K 5mg/mL	Thermo Fisher Sci.	25530-015
Vα3.2-FITC (clone: RR3-16)	eBioscience	11-5799-80	α-mouse Iba-1	Wako	019-19741

Table 4: List of all supplies necessary to complete the previously described experiments, manufacturer of each supply, and catalogue number.

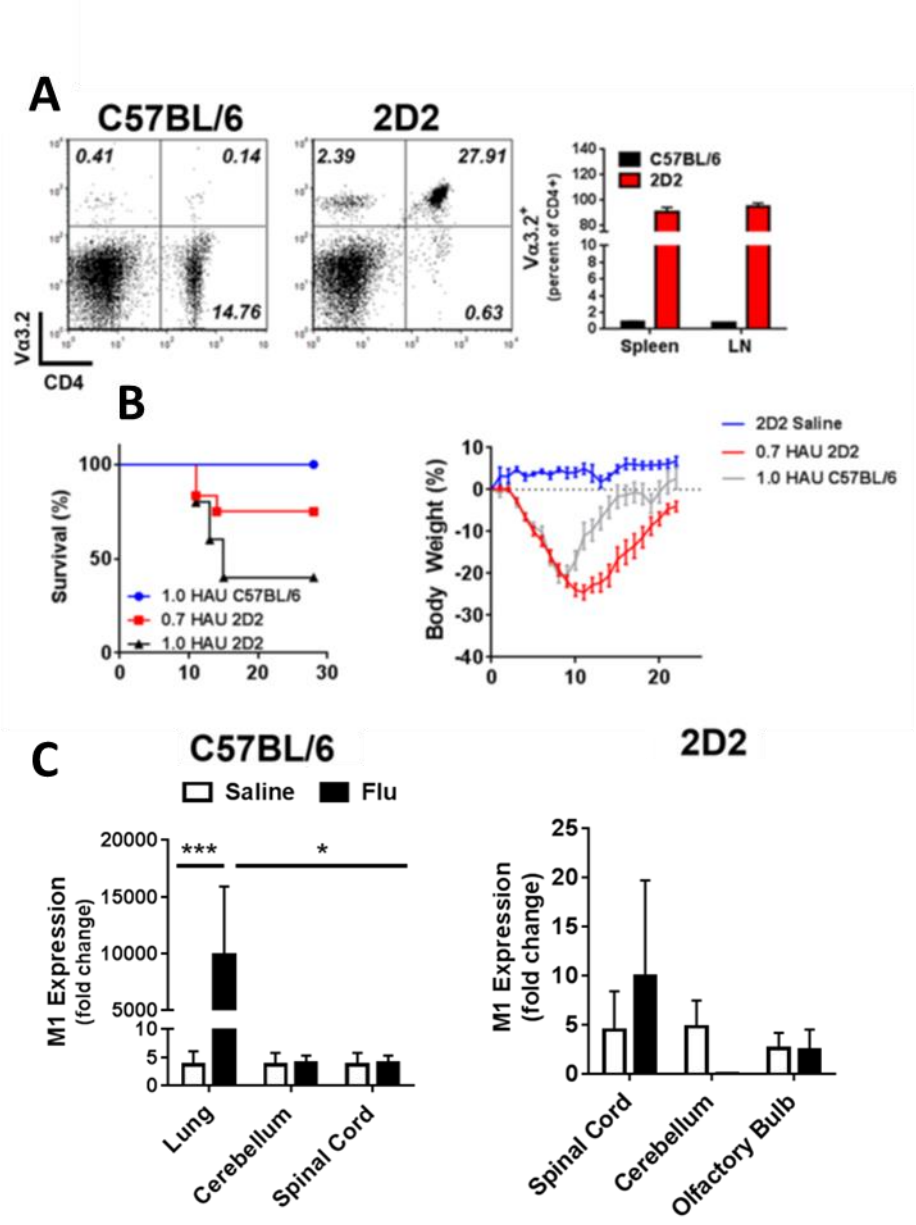

Figure 1: A) Vα3.2^{+} T-cell percentage and comparison of 2D2 versus C57BL/6 T-cell populations. **B)** Survival curve and dose comparison for H1N1 Influenza A PR/8 in 2D2 mice at 0.7 and 1.0 HAU and C57BL/6 mice at 1.0 HAU as well as weight loss for each mouse breed at ideal infectious dose. **C)** M1 RNA expression fold change in C57BL/6 mice in the lungs, cerebellum, and spinal cord for saline and flu infected mice as well as spinal cord, cerebellum, and olfactory bulb for 2D2 mice.

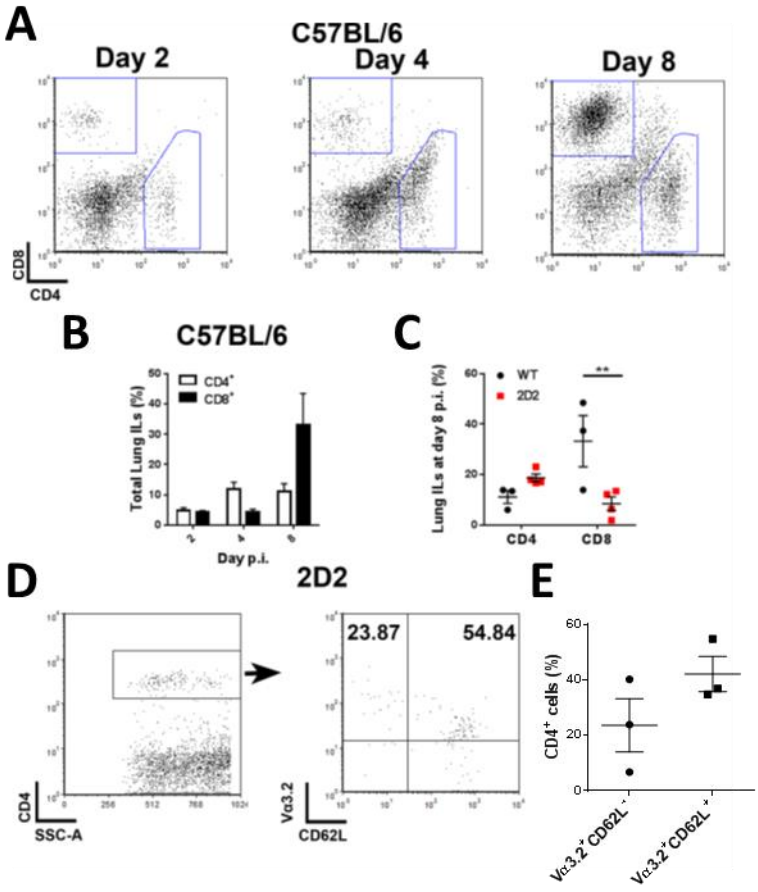

Figure 2: A) Gating strategy for CD4/CD8 cells at day 2, 4, and 8 p.i. with influenza or inoculation with saline in C57BL/6 mice. **B)** The percentage of $CD4^+$ versus $CD8^+$ T-cells in the lungs of C57BL/6 mice at day 2, 4, and 8 p.i. **C)** The comparison of $CD4^+$ and $CD8^+$ T-cells in the lungs of C57BL/6 mice versus 2D2 mice at day 8 p.i. **D)** An antibody against CD62L was used to determine activation status of encephalogenic T-cells in the lungs of 2D2 mice. **E)** $CD62L^+$ versus $CD62L^-$ T-cell comparison in the lungs at day p.i. (n=3 C57BL/6, n= 3-4 2D2, results are means ± SE, *$p<0.01$)

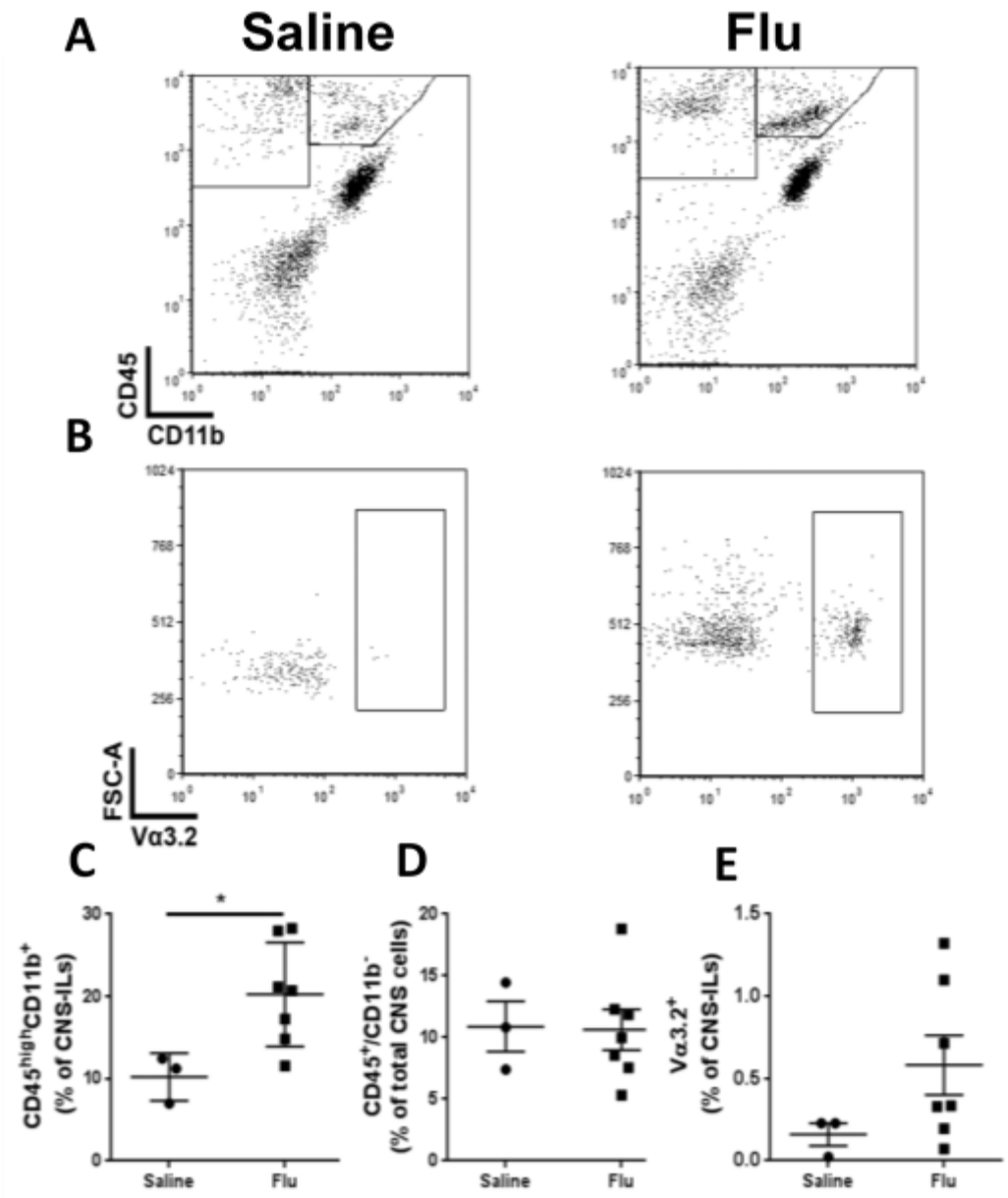

Figure 3: A-B) Flow cytometry gating strategy for mouse brains **C)** Mouse brain lymphocytes were isolated (day 8 p.i.) and stained to determine the percentage of $CD45^{high}CD11b^{+}$ in saline inoculated animals versus influenza infected animals at day of EAE onset (n=3) or day 8 for animals that were sacked before EAE onset period (10-14) (n=4). **D-E)** the same cells were analyzed for the percentage of $CD45^{high}CD11b^{-}$ cells and $V\alpha3.2^{+}$ cells (n= 3 saline n=7 flu, results are means ± SE, *$p<0.05$).

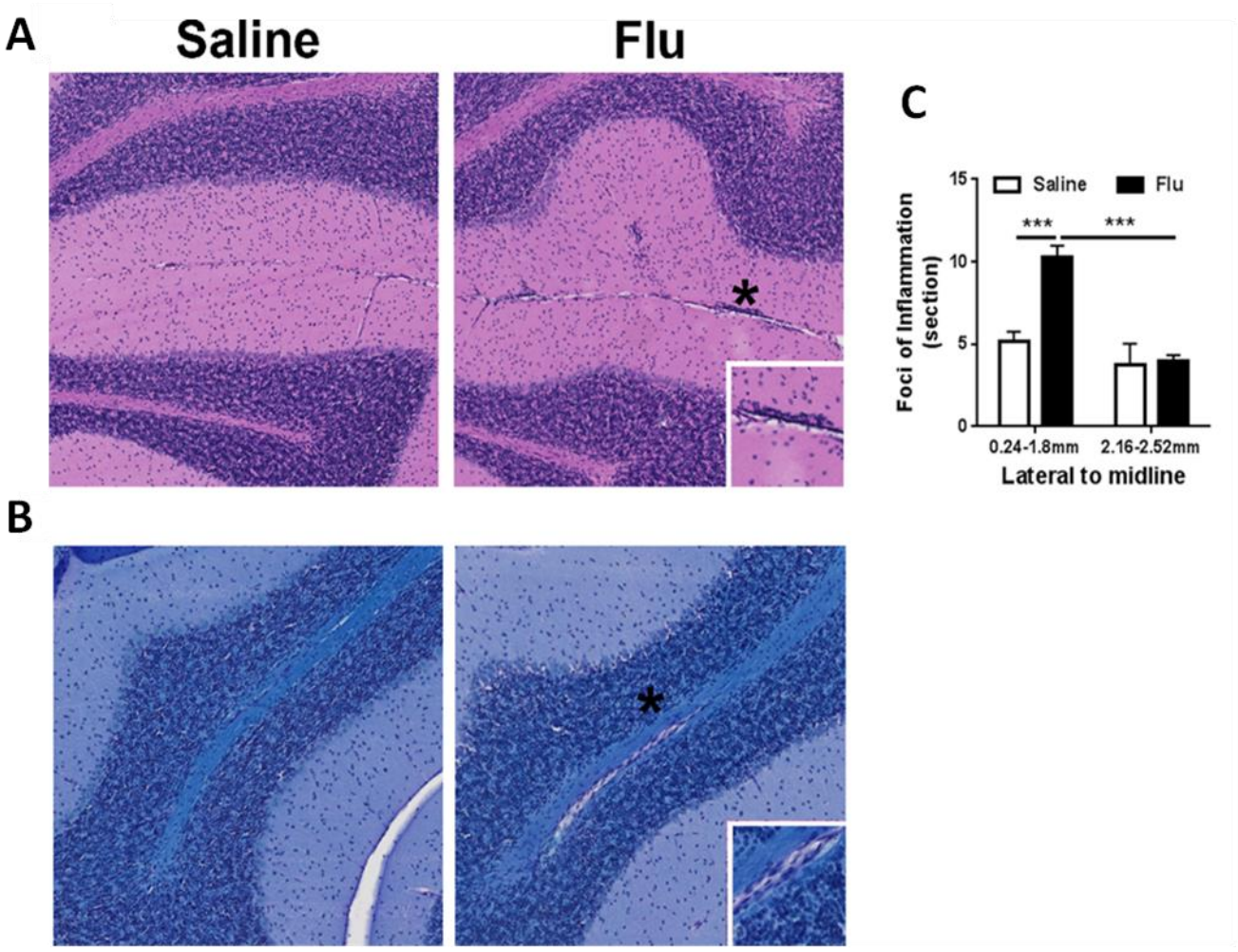

Figure 4: A) Brains were extracted from mice inoculated with saline and infected with influenza (i.n) at day 8 p.i. Sections were stained with hematoxylin and eosin to determine regions of hypercellularity in the meninges and parenchyma of the brain. **B)** Mouse brains were stained for myelin (luxol fast blue stain) and analyzed for regions of demyelination. **C)** Regions of hypercellularity were quantified and sorted based on distance from brain midline. (n=3 mice per group, results are means ± SE, ***$p<0.001$).

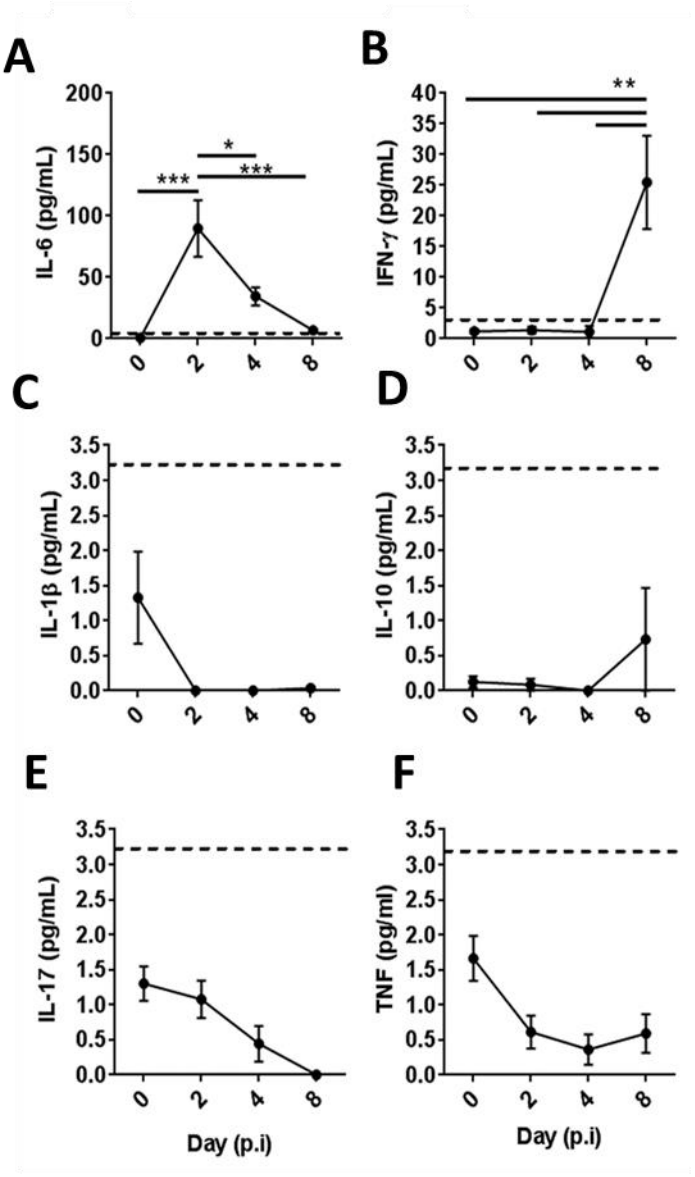

Figure 5: A-F) C57BL/6 mice were inoculated with saline or influenza virus i.n. (1.0 HAU). At days 0, 2, 4, and 8 p.i. serum was isolated and cytokine levels of IL-6 **(A)**, IFN-γ **(B)**, IL-1β **(C)**, IL-10 **(D)**, IL-17 **(E)**, and TNF-α **(F)** were determined by multiplex analysis (n=7-8 mice per group, results are means ± SE, *p<0.05, **p<0.01, ***p<0.001) Hashed lines signify the assay detection limit.

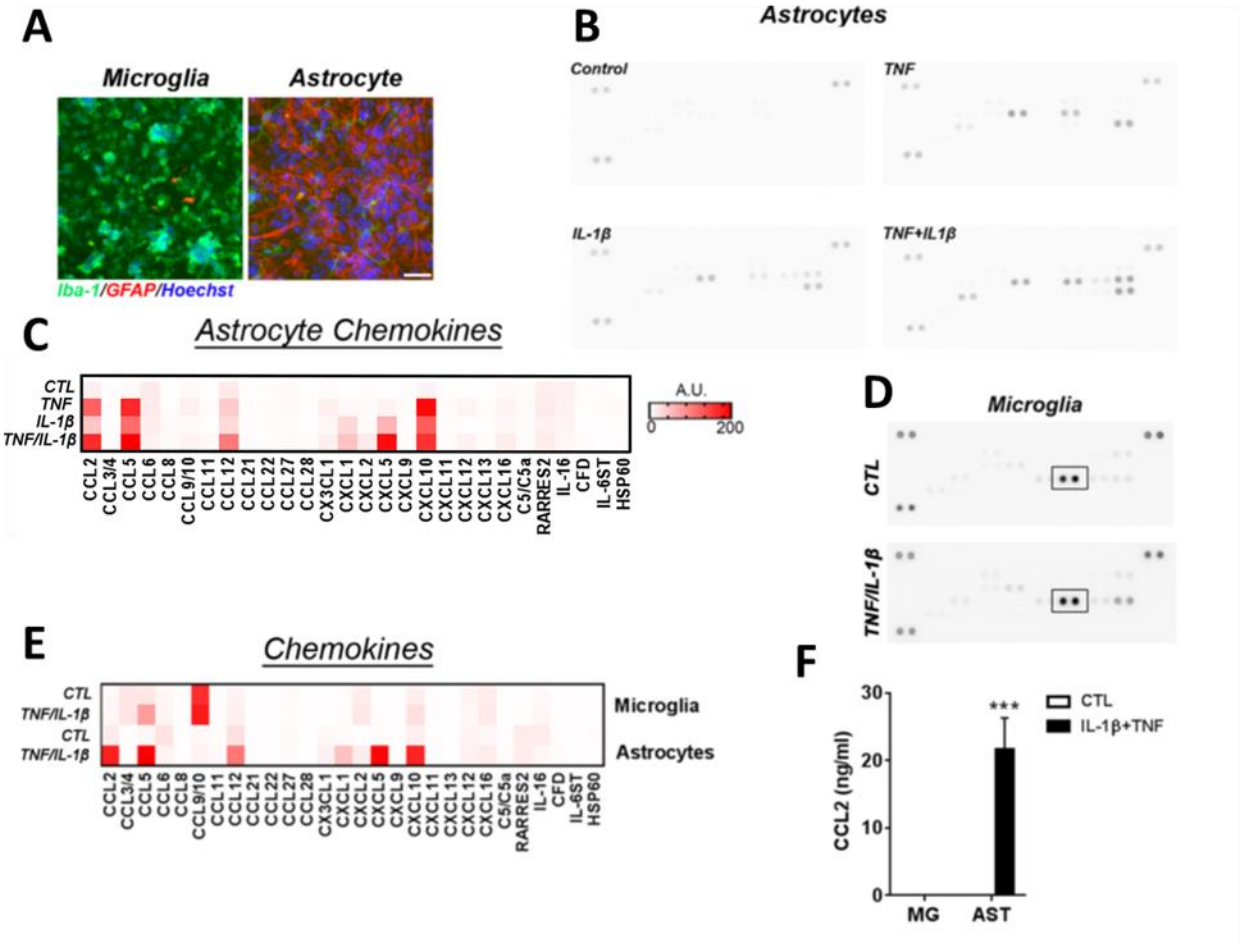

Figure 6: A) Microglia and astrocytes were isolated from fetal day 3 C57BL/6 mouse pups and plated at a density of $5x10^5$ cells per well (6-well plate). Cells were stained for Iba-1 (Microglia) and GFAP (Astrocytes) to determine culture purity. **B)** Astrocyte cultures were either treated with 0.5% BSA serum free media (control) or IL-1β and TNF-α for 24 hours. Supernatants were then removed, three separate experiments were pooled and relative levels of secreted chemokines were determined using ImageJ. **C)** Astrocyte chemokine secretion (AU) was plotted on a heat map. **D)** Microglial cultures were treated and analyzed as explained in part B. Boxes surrounding CCL9/10 dots highlight a major difference between the chemokine secretion profile of microglia **(D)** and astrocytes **(B)**. **E)** Microglia versus astrocyte chemokine secretion (AU) was plotted onto heat maps. **F)** Chemokine array was validated by CCL2 ELISA (n=3, results are means ± SE, $p<0.001$).

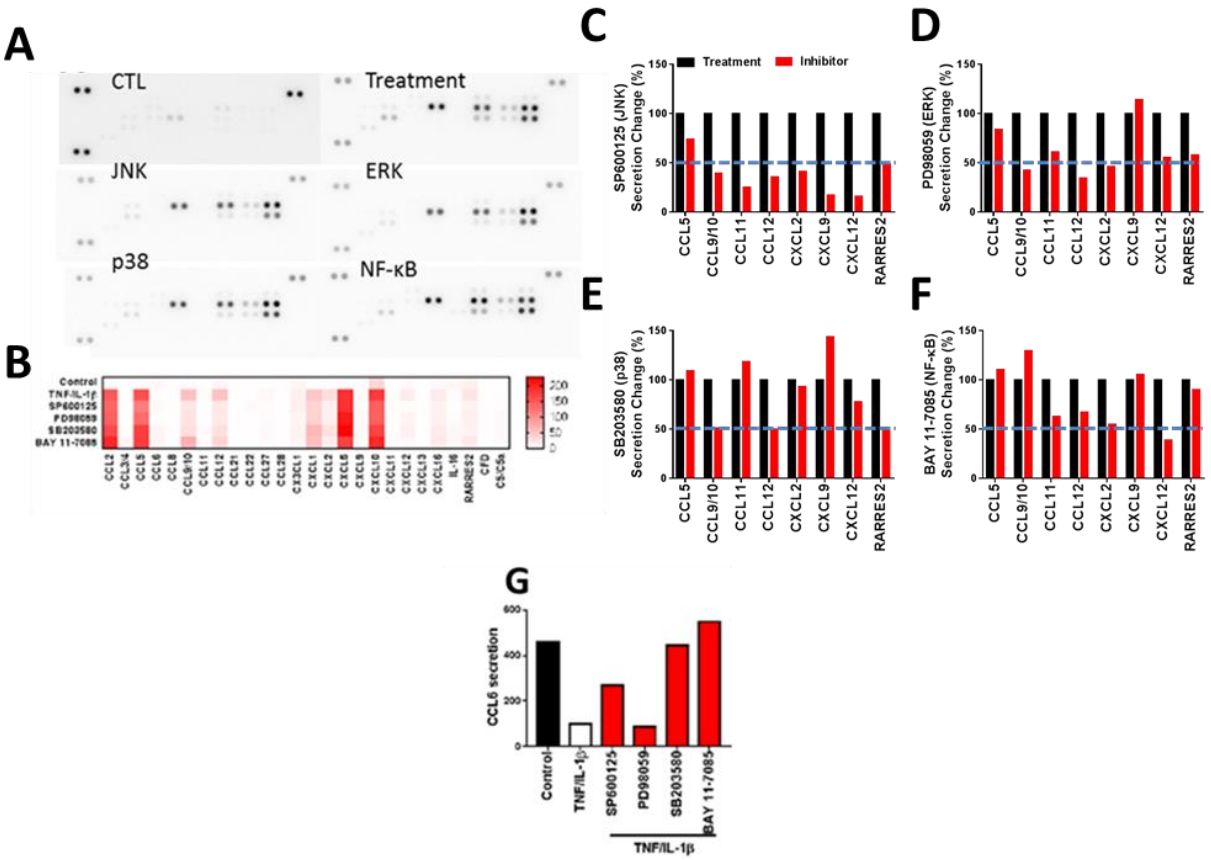

Figure 7: A) Chemokine array blots from primary astrocyte supernatants treated with 20 µM of SP600125 (JNK inhibitor), PD98059 (ERK inhibitor), SB203580 (p38 inhibitor), or BAY 11-7085 (NF-κB inhibitor) for 30 minutes before 24 hour treatments with IL-1β and TNF-α. **B)** Chemokine levels (AU) were plotted onto a heat map. **C-F)** Secretion of chemokines with each inhibitor with the greatest change relative to cytokine treatment control. Blue dotted line signifies 50% reduction. **G)** Secretion of CCL6 with each inhibitor when compared to both control and treatment groups. Supernatants were pooled from three separate experiments.

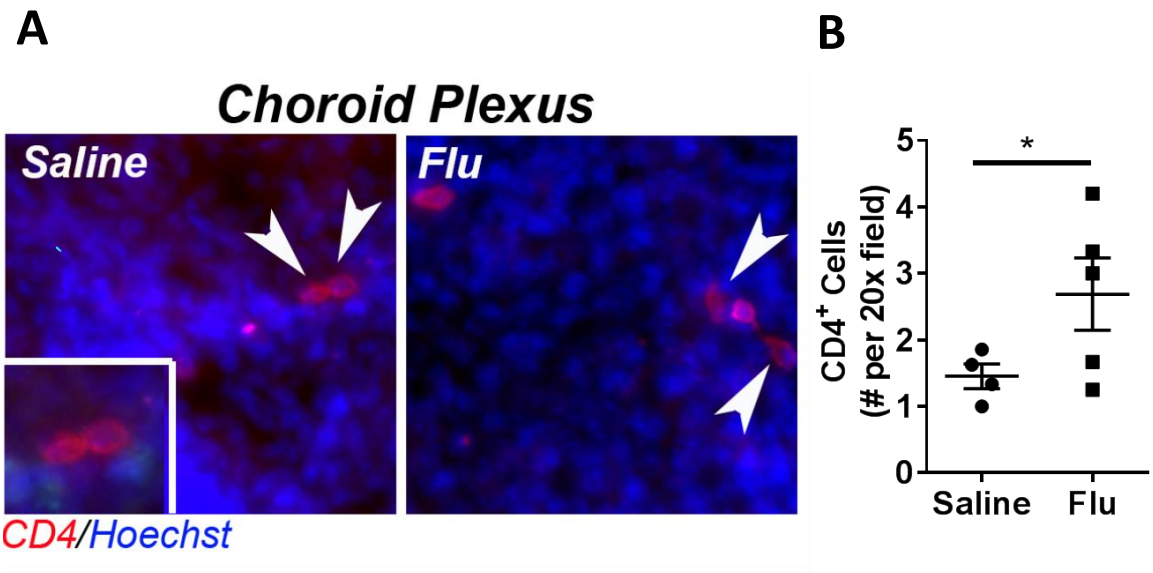

Figure 8: A) C57BL/6 mice were innoculated with saline or 1.0 HAU of influenza. At day 8 p.i. brains were fixed in 4% PFA for 24 hours and the choroid plexus was dissected out. Choroid plexus were stained for nuclei and CD4. **B)** The number of $CD4^+$ cells in saline versus infected mice (n= 4 saline, n= 5 flu, results are means ± SE, *$p<0.05$).

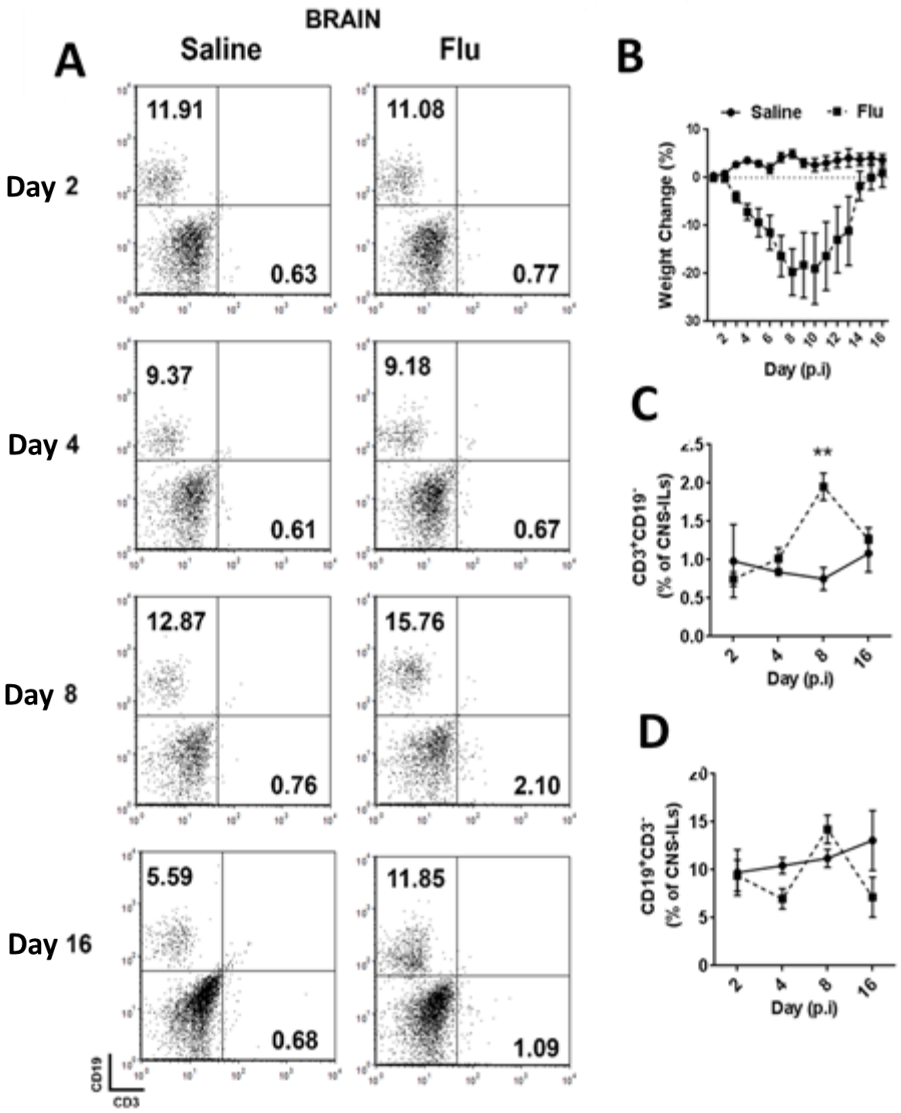

Figure 9: A) Flow cytometry gating strategy for CD3/CD19 cells in saline versus 1.0 HAU infected C57BL/6 mouse brains at day 2, 4, 8, and 16 p.i. **B)** Weight change for saline versus infected mice. **C)** $CD3^{+}CD19^{-}$ cell population in saline versus infected animals at day 2, 4, 8, and 16 p.i. **D)** $CD19^{+}CD3^{-}$ cell population in saline versus infected animals at day 2, 4, 8, and 16 p.i. (n=3-5 mice per group, results are means ± SE, **p<0.01).

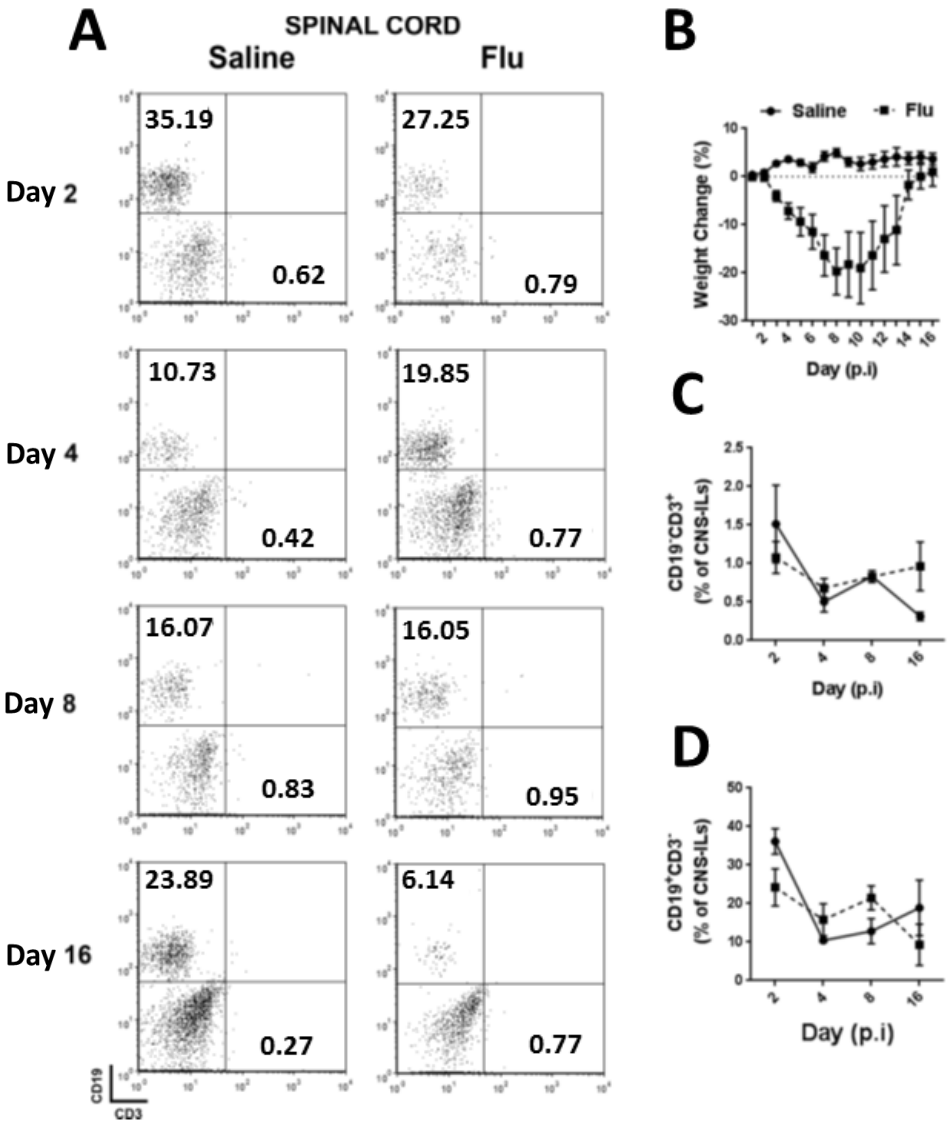

Figure 10: A) Flow cytometry gating strategy for CD3/CD19 cells in saline versus 1.0 HAU infected C57BL/6 mouse spinal cords at day 2, 4, 8, and 16 p.i. **B)** Weight change for saline versus infected mice. **C)** $CD3^{+}CD19^{-}$ cell population in saline versus infected animals at day 2, 4, 8, and 16 p.i. **D)** $CD3^{-}CD19^{+}$ cell population in saline versus infected animals at day 2, 4, 8, and 16 p.i. (n=3-5 mice per group, results are means ± SE,**$p<0.01$).

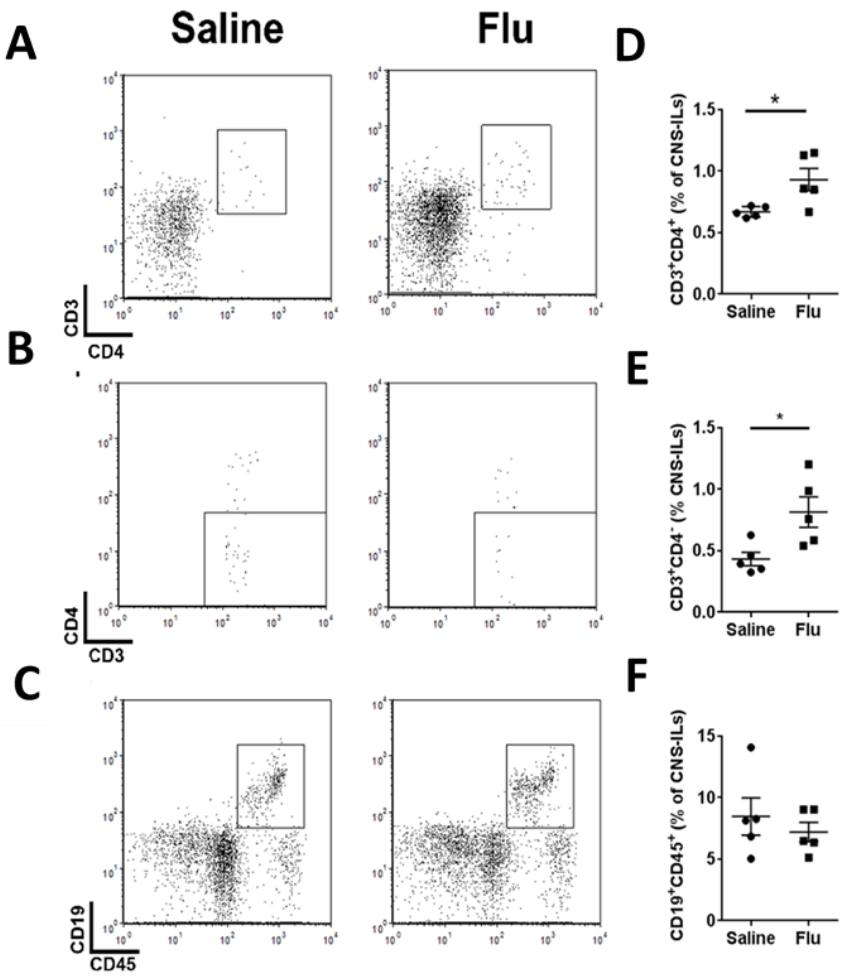

Figure 11: A) Flow cytometry gating strategy for CD3/CD4 in the brain, CD4/CD3 (used to determine CD4- population) **(B)**, and CD19/CD45 **(C)** cells on saline inoculated or influenza infected C57BL/6 mice at d8 p.i. **D)** $CD3^+CD4^+$ T-cells at day 8 p.i. in infected versus saline C57BL/6 mice. **E)** $CD3^+CD4^-$ T-cells at day 8 p.i. in infected versus saline C57BL/6 mice. **F)** $CD19^+CD45^+$ cells at day 8 p.i. versus saline (n=5 mice per group, results are means ± SE, *p<.05)

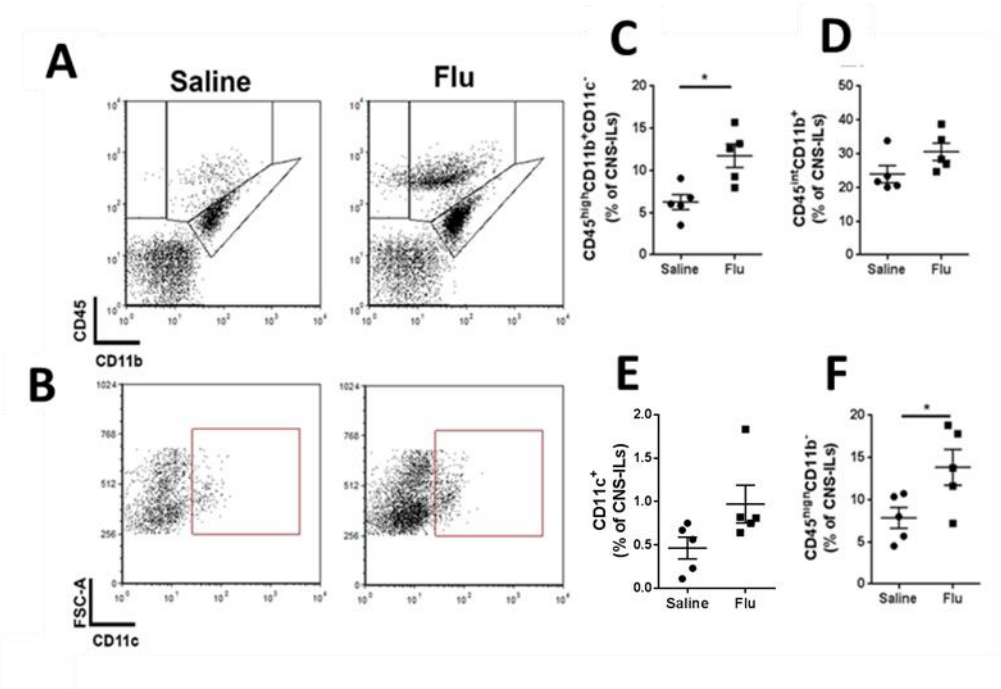

Figure 12: A) Flow cytometry gating strategy for CD45/CD11b and CD11c **(B)** cell populations. **C-F)** Quantitation of $CD^{high}CD11b^{+}CD11c^{-}$ **(C)**, $CD45^{int}CD11b^{+}$ **(D)**, $CD11c^{+}$ **(E)**, and $CD45^{high}CD11b^{-}$ **(F)** cell populations in the brain of saline inoculated and influenza infected animals at day 8 p.i. (n=5 mice per group, results are means ± SE, *p<0.05, **p<0.01)

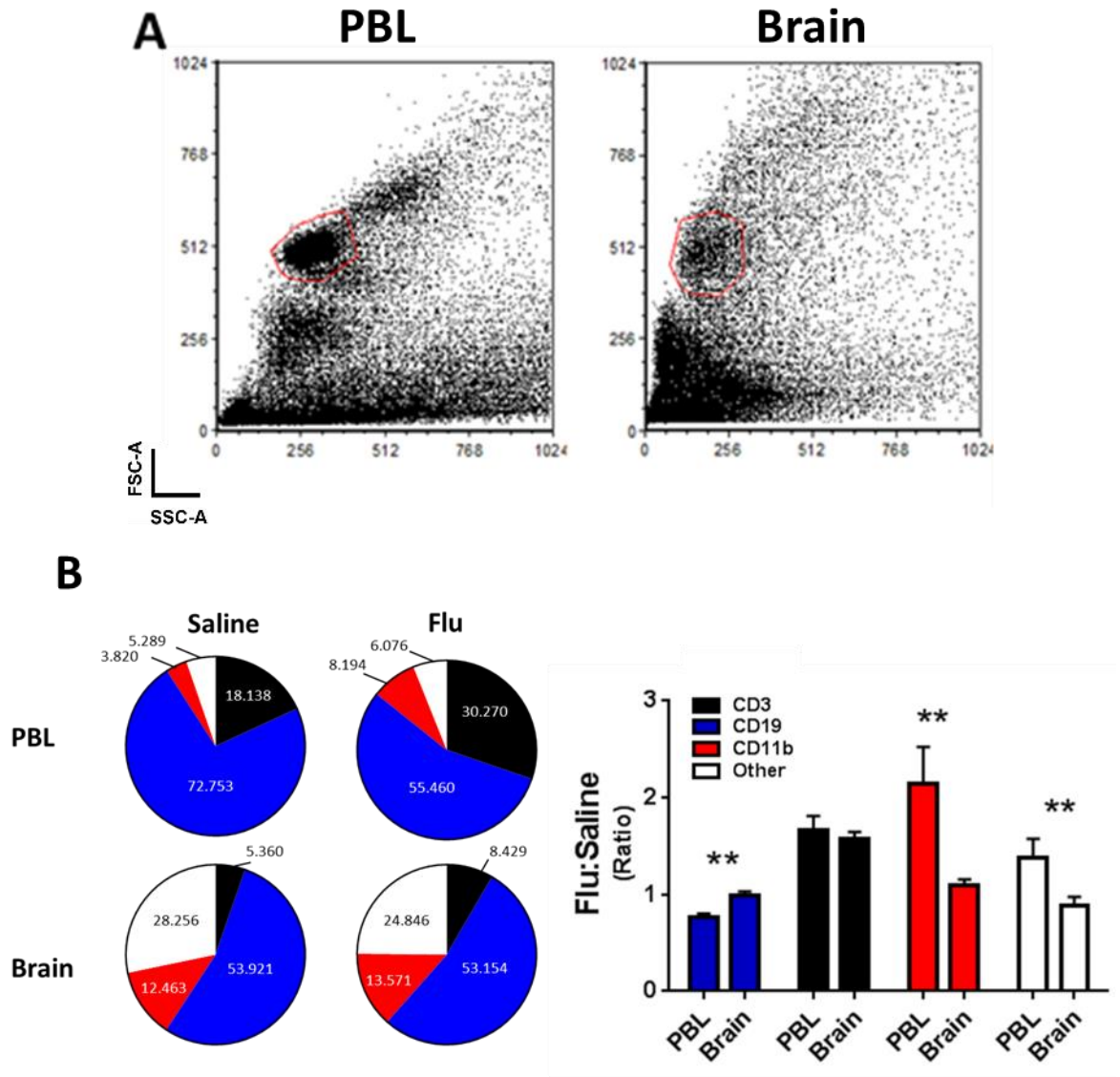

Figure 13: A) Brain and peripheral blood leukocytes (PBL) were isolated from C57BL/6 mice and the ratios of various immune cell populations were analyzed for similarities. **B)** The monocyte/neutrophil population and B-cells had different fold changes, while T-cell levels had no difference in the PBL versus the brain (n=5 PBL, n=8 Brain, results are means ± SE, **p<0.01).

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