Immune-mediated pathogenesis and immune regulation during fatal alphavirus encephalomyelitis

By

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Abstract

Encephalitic viral infections are emerging as an important cause of morbidity and mortality worldwide. The mechanisms by which these infections cause disease, however, are poorly understood. To better understand the pathogenesis of severe encephalitic viral infections, these studies focused on determining the mechanisms of immune-mediated pathogenesis and the regulation of this response during Neuroadapted Sindbis virus (NSV) infection. The first set of experiments characterized the role of the immunoregulatory cytokine, interleukin-10 (IL-10), during NSV infection. These experiments showed that IL-10 is important in regulating the Th17 response to NSV infection. The next set of experiments characterized the Th17 populations in WT and Il10-/- mice. The Th17 cells found in WT and IL-10 KO mice had a pathogenic phenotype. Furthermore, in the absence of IL-10 the Th17 cells had increased GM-CSF expression and there was an expansion of the pathogenic Th1/17 population. The last set of experiments identified which cell types produce IL-10, as well as which cells express the IL-10R in order to determine possible mechanisms of action for IL-10 regulation of the Th17 response during NSV infection. These studies contribute to understanding the mechanisms of immune-mediated pathogenesis and regulation during encephalitic viral infections and other inflammatory diseases in the central nervous system.
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To my family

Kevin, Paulette, and Katie Kulcsar

For all of their love and support

And to my grandparents

Annette and William Kulcsar

For all of their love and support and teaching me what it means to
work hard, persevere, and laugh
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General Introduction
Arbovirus encephalitides

Arthropod-borne viral infections are a major cause of morbidity and mortality worldwide. Arbovirus infections are emerging as a major public health problem and constitute approximately 30% of all emerging infections in the past decade (1). These viruses can cause a wide range of diseases including rash, arthritis, fever, hemorrhage, and encephalitis (2). The main groups of viruses that cause encephalitis are Flaviviruses (e.g. Japanese encephalitis and West Nile), Alphaviruses (Venezuelan, eastern, and western equine encephalitis), and the family Bunyaviridae (e.g. La Crosse encephalitis). All of these viruses are maintained in natural cycles between vertebrate and invertebrate hosts and are geographically restricted.

In particular, alphaviruses are a major cause of veterinary and human infections. Alphaviruses are split into two main categories depending on disease manifestation and geographical location. The Old World alphaviruses include Sindbis virus (SINV), Chikungunya virus (CHIKV), Ross River virus (RRV), and Semliki Forest virus (SFV). These viruses typically present as febrile illness, rash, and arthritis in humans. The Old World alphaviruses typically do not cause encephalitis, although neurological infection has been reported for SINV and CHIKV (3). These viruses are widespread, particularly throughout Europe, Asia, Africa, and Australia (3). The New World alphaviruses include Venezuelan equine
encephalitis virus (VEEV), eastern equine encephalitis virus (EEEV), and western equine encephalitis virus (WEEV). Infection with all of these viruses can result in encephalitis primarily in horses and humans. The case fatality rate and severity of disease varies with EEEV typically being the most virulent, followed by WEEV, then VEEV; however, VEEV is the most transmissible of the three equine encephalitic viruses (2). Infants and children are generally more susceptible to developing encephalitis after alphavirus infection than adults (3). Survivors of alphavirus encephalitis, both humans and horses, are typically left with neurological sequlae including cognitive disorders, epilepsy, and paralysis (3). These viruses circulate primarily in North and South America and are causes of seasonal outbreaks of encephalitis in horses and humans (2, 3).

The transmission cycle for arboviruses is dependent on a reservoir host in which the virus replicates to high titers while causing little to no disease, as well as a competent vector. The reservoir hosts for alphaviruses include birds, rodents, and non-human primates (2). A variety of mosquito species can transmit alphaviruses including *Aedes aegypti, Aedes albopictus*, and *Culex pipiens*. Horses and humans are considered to be dead-end hosts in the transmission cycle because viremia is typically not high enough to propagate transmission after a mosquito takes a blood meal. VEEV and CHIKV, however, can replicate
to high enough titers in horses and humans, respectively, to allow for the continuation of transmission (3).

The emergence and re-emergence of these viruses is influenced by socio-economic, environmental, and ecological factors (2, 3). The rapid urbanization of areas with endemic alphavirus circulation has increased contact between humans, mosquitoes, and reservoirs increasing the likelihood of transmission (2). Increased trade and travel have spread vectors and the viruses farther and more quickly than before (4, 5). Climate change resulting in increased temperature in endemic areas has increased the vector population and provided the opportunity for changes in vector range, vertebrate hosts, and vector composition and dynamics (2, 6, 7).

There are few prevention and therapeutic options for alphavirus encephalitides. The vaccines that are available are licensed for veterinary use and approved only for limited use in military personnel or those with a high risk of exposure. The efficacy of these vaccines is relatively poor and requires multiple administrations due to low immunogenicity (3). No therapeutics exist for alphavirus infections, the only option is supportive care (3). The increasing prevalence of alphavirus infections, particularly those causing severe and debilitating encephalitic disease, has introduced a great need in understanding viral pathogenesis in order to prevent and treat disease.
**Sindbis virus**

Sindbis virus (SINV) is the prototypic alphavirus and amongst the best studied. SINV is an Old World alphavirus and infection results in mild rash, arthritis, and febrile illness in humans, but induces encephalomyelitis in mice (8, 9). Alphaviruses are enveloped, plus-strand RNA viruses in the family *Togaviridae*. Alphavirus genomes are approximately 11-12kB in size and contain four nonstructural proteins encoded at the 5’ end of the genome and up to five structural proteins encoded at the 3’ end of the genome under control of a subgenomic promoter (Figure 1) (10).

SINV, in particular, is 11.7kB in size. The nonstructural proteins nsp1, nsp2, nsp3, and nsp4 are translated from genomic viral RNA. These proteins are necessary for efficient replication of the virus genome and include the RNA-dependent RNA polymerase, viral proteases, and the replication complex anchor (10). The structural proteins are transcribed from a subgenomic viral RNA and are capsid, E1, E2, E3, 6k, and the transframe protein TF (Figure 1). E1 and E2 are the viral glycoproteins and form heterodimeric trimers to make up the viral envelope (Figure 2) (10). E2 is responsible for receptor binding and E1 contains the fusion peptide. E2 also contains the most neutralizing epitopes for antibody-mediated prevention of infection. The receptor required for entry is unknown. Most likely, SINV can use a variety of
receptors, including heparan sulfate, to bind and enter cells for infection (10).

The virulence of SINV is dependent on the strain of the virus used, as well as the age of the animal, and the genetic background of the mice studied. Neonatal mice are more susceptible to SINV-induced disease and become less susceptible as the animal matures (11-13). The increased susceptibility in young animals is independent of the maturation of the immune response and appears to be mediated more by neuronal maturation and the increased susceptibility of immature neurons to viral-induced apoptosis (13, 14).

A variety of strains of SINV are used in the laboratory setting. The least virulent is the tissue culture-adapted HRSP strain (15), the original isolate from mosquitoes, AR339, is of moderate virulence, and the most virulent is neuroadapted Sindbis virus (NSV). NSV was created by serially passaging AR339 through the brains of neonatal and weanling mice (16). Virulence has been mapped to a variety of mutations throughout the genome; however, the most prominent mutations conferring virulence lie in the 5’ non-translated region (NTR) and in the E2 glycoprotein. Neuroinvasion is affected by residues 55 and 190 of E2. Changing residue 55 of E2 from a glutamine to a histidine increases the efficiency of virus replication in neurons and confers virulence in weanling and adult mice (11, 12, 17).
The genetic background of the animal is also an important factor in SINV pathogenesis. NSV has 100% penetrance of lethal infection in weanling and adult mice on the C57Bl/6 background; however, mice of the comparable age on the Balb/c background are resistant to lethal disease induced by NSV (18). The genetic determinants of this susceptibility have been mapped to chromosome 2, but are still unknown (19).

**Neuroadapted Sindbis virus pathogenesis**

Neurons can experience different outcomes upon virus infection. During some virus infections, infected neurons are not killed, but virus replication is controlled via antibody and IFNγ production. During other viral infections, the virus can induce neuronal cell death directly via apoptosis. Finally, in other infections, such as neuroadapted Sindbis virus (NSV), neurons die as a consequence of glutamate excitotoxicity and neurotoxic inflammation (20). If too many neurons are killed, the animal succumbs to lethal disease. NSV can be inoculated via the intranasal route or intracerebral route and induces encephalomyelitis in both instances. After intranasal inoculation, the virus binds to olfactory neurons found in the nasal cavity and travels into the olfactory lobes of the central nervous system (CNS) by approximately 24 hours post infection. NSV then spreads in a cell-to-cell dependent manner caudally
throughout the brain and into the spinal cord. The only cell type found to be infected with NSV is neurons. Virus replication in the CNS reaches peak titers 3-5 days post infection (Figure 3C). Clearance begins around 5 days post infection and coincides with the entry of T cells, as well as the onset of clinical symptoms (Figure 3). Symptoms first appear as ruffling of the fur, hunched back, and altered hind-limb posture. These progress to unilateral hind limb paralysis, bilateral hind limb paralysis, full body paralysis, and eventually death around 7-10 days post infection in C57Bl/6 mice. Infection in mice results in the death of neurons that can be necrotic, in the case of motor neurons, or apoptotic and necrotic, in the case of neurons found in the hippocampus (21, 22).

Previous studies have identified T cells as the primary mediators of NSV-induced pathogenesis. In these studies, survival was improved in mice that were deficient in αβT cells, β2-microglobulin, TAP, or CD4+ T cells, which suggests that both CD4+ and possibly CD8+ T cells are important for NSV pathogenesis (23). Furthermore, a variety of T cell effector molecules were investigated in order to try to identify what function of the T cells was pathogenic. These studies showed that mice deficient in Fas, perforin, and the TNFα-receptor were not protected during NSV infection, suggesting that the mechanisms of pathogenesis are independent of cytolytic effector function of CD8+ T cells (23). Additionally, mice deficient for IFNγ, the IFNγ-receptor, and IL-6 also
showed no improvement in survival during NSV infection suggesting that
certain functions and/or subsets of T cells are mediating
immunopathogenesis (23, 24). Although a variety of cytokines present in
the CNS during NSV infection have been identified, the subsets and
functionality of CD4+ T cells have not been investigated. Furthermore,
the role of immunoregulatory molecules, particularly those with effects
on T cells, has not been investigated during NSV infection.

Role of T cells mediating immunopathology during neurotropic viral
infections

T cell-mediated damage is implicated in the pathogenesis of a
variety of neurotropic viral infections. The most well characterized role
for T cells causing immunopathology comes from studies using
lymphocytic choriomeningitis virus (LCMV). In mice, LCMV infects the
meninges, choroid plexus, and ependyma inducing lethal meningeal
inflammation in 6-8 days post intracerebral (i.c.) infection (25). LCMV is
not cytolytic which led to the idea that the pathogenesis of this virus
infection is due to the immune response (26). T cells were shown to be
the primary mediators of pathogenesis when thymectomized mice
displayed protection from lethal disease after i.c. inoculation (27). Since,
virus-specific CD8+ T cells have been identified as the primary mediators
of fatal disease (27-36). The mechanism by which CD8+ T cells are
mediating damage is still unclear. Mice deficient for granzyme B (37) or perforin (38, 39) display no protection during infection suggesting it is a cytolytic-independent mechanism. CD8+ T cell damage also appears to be independent of TNFα (40, 41) and IFNγ production (42). LCMV has proven to be an incredibly useful model for delineating the importance of the immune response in viral pathogenesis.

The pathogenesis of neurotropic coronavirus JHMV, Borna virus, and VEEV all involve a detrimental T cell response as well. JHMV can cause a lethal acute infection or a persistent demyelinating disease. JHMV infection of Rag1-/- mice leads to no demyelination despite a high viral load and widespread inflammation (43). When these mice are reconstituted with immunocompetent splenocytes, demyelination returns. If CD4+ and CD8+ T cells are then depleted, demyelination is once again suppressed (44). Furthermore, it was shown that CD4+ and CD8+ T cells result in different pathogenic consequences. CD4+ T cells are associated with the severe, acute pathology observed during some JHMV infections (44, 45); whereas, CD8+ T cells contribute to the demyelination observed during persistent infections (43). CD4+ T cells also contribute to disease during Borna disease virus (BDV) infection. BDV- specific CD4+ T cells are protective when adoptively transferred prior to infection, presumably by limiting BDV spread; however, when
BDV-specific cells were adoptively transferred after infection, an acceleration of encephalitis occurs (46).

A role of T cells during infections with arboviruses were described early on. In a study comparing the effects of immunosuppression between Langat and West Nile virus (WNV) after i.c. infection, it was found that cyclophosphamide treatment results in a delay in Langat virus pathogenesis, but does not protect during WNV infection (47). It has since been shown that CD4+ and CD8+ T cells are protective during WNV infection, except in the case of attenuated WNV strains in which CD8+ depletion decreased mortality (48, 49). A detrimental role of T cells during VEEV infection has also been described. SCID mice, thymectomized mice, and T cell-depleted mice all have improved survival after VEEV infection compared to immunologically normal mice (50, 51). Although T cells are implicated as a major mediator of pathogenesis during viral encephalitis, the mechanisms responsible and the regulation of these responses are largely unknown.

**Interleukin-10 biology**

Interleukin-10 (IL-10) was first described as a Th2 cytokine that could inhibit Th1 cytokine production (52); however, it has since been shown to be a potent and wide-ranging immunoregulatory molecule (53). Bioactive IL-10 is a homodimer and binds to the heterodimeric receptor
complex consisting of IL-10Rα and IL-10Rβ (52, 54, 55). The IL-10Rα subunit contains the ligand-binding domain for IL-10 and is necessary for IL-10 signaling (54, 55). IL-10Rα is expressed primarily in hematopoietic cell types (54-57) although induced expression in other cell types, such as keratinocytes (58) and colonic epithelium (59, 60), has been observed. The IL-10Rβ subunit is constitutively expressed in a wide variety of cell types and is shared with the IL-22 receptor complex (53, 61, 62). Thus, expression of the IL-10Rα subunit, not the IL-10Rβ subunit, determines which cell types are responsive to IL-10.

Hematopoietic cells are the primary sources of IL-10 and include macrophages, monocytes, DCs, neutrophils, natural killer (NK) cells, B cells, CD8+ T cells, and a variety of CD4+ T helper cell subsets (53, 63).

IL-10 signaling occurs via the Jak/STAT pathway. Specifically, Jak1 is associated with the IL-10Rα chain and binding of the receptor to IL-10 activates Jak1 phosphorylation of tyrosine residues on the IL-10Rα subunit providing for docking sites of the SH2 domains of STAT3 (53, 64). STAT3 is required for the anti-inflammatory effects of IL-10 (57, 65-67); however, STAT1 and STAT5 also associate with the IL-10Rα chain (68-70). Interestingly, STAT3 activation also occurs via other cytokine-receptor interactions, such as the IL-6R; however, this IL-6 signaling does not result in an anti-inflammatory response. The reason for this is that the suppressor of cytokine synthesis 3 (SOCS3) binds to the IL-6R.
and inhibits signaling, whereas SOCS3 cannot bind to the IL-10R, thus signaling via IL-10 is refractory to inhibition by SOCS3 (65). STAT3 is not directly responsible for the anti-inflammatory effects of IL-10, but activates other genes, such as ETV3, SBNO2, and ZFIL3, to further dampen the inflammatory response (71, 72). The anti-inflammatory effects of IL-10 are mediated primarily at the transcriptional level, but can also be at the posttranscriptional level (53, 65). IL-10 regulates a subset of inflammatory genes, most of which are associated with Toll-like receptor (TLR) activation (65), including NFkB. IL-10 also regulates a micro RNA, miR-155, which plays a role in the inflammatory response (73).

IL-10 plays a largely non-redundant role in regulating the immune response. Its effects are primarily anti-inflammatory and can be either direct or indirect (53). The best characterized IL-10-mediated regulation acts on antigen presenting cells, such as dendritic cells (DCs) and macrophages, to down regulate inflammatory cytokine and chemokine secretion, as well as MHC class II and co-stimulatory molecule expression thus inhibiting T cell responses (53). In activated monocytes and macrophages, IL-10 inhibits production of cytokines including IL-1α, IL-1β, IL-6, IL-10, IL-12, IL-18, GM-CSF, G-CSF, M-CSF, and TNF (74-78). IL-10 also inhibits the production of a wide variety of chemokines including CCL2, CCL3, CCL4, CCL5, CCL19, CCL20, IL-8, CXCL10,
CXCL1, and CXCL2 (79-82), thus inhibiting the recruitment of monocytes, DCs, neutrophils, and T cells. IL-10 also alters the phenotype of monocytes and macrophages to become more phagocytic to increase clearance of infected or apoptotic cells (83, 84). The effect of IL-10 on DCs is to promote an anti-inflammatory response, primarily by affecting cytokine production that alters T cell activation and skewing (53). IL-10 can also act directly on neutrophils to inhibit inflammatory cytokine production as well as limiting survival (53).

The effects of IL-10 on CD4+ T cell function are important for understanding how IL-10 regulates immunopathology. The importance of IL-10 for CD4+ T cells is exemplified in the development of spontaneous colitis in Il10−/− mice, which is mediated primarily by CD4+ T cells (85-87). IL-10 restricts Th1 and Th17 cell development in a variety of infection models (63, 88, 89). One mechanism for controlling these responses is that IL-10 can potently inhibit Il12b transcription via ZFIL3 promoter suppression (72). This reduces levels of IL-12p40, which is necessary to produce bioactive IL-12 and IL-23 by forming a heterodimer with IL-12p35 or IL-23p19, thus limiting Th1 and Th17 differentiation factors. IL-10 production from regulatory T cells is important in restricting Th1 and Th17 mediated pathologies (90, 91).

In addition to its anti-inflammatory effects, IL-10 can also promote certain aspects of the immune response. B cell survival, proliferation,
and maturation are promoted by IL-10 secretion (53). IL-10 promotes B cell differentiation and class switching, thus having a positive consequence on the humoral response (53). Although IL-10 displays inhibitory effects on CD4+ T cells, it actually has stimulatory effects on CD8+ T cells. IL-10 promotes the recruitment and proliferation of CD8+ T cells, as well as inducing cytotoxic activity in this population (92-95).

**Interleukin-10 and viral infections in the central nervous system**

The role that IL-10 plays in regulating immune responses to infectious diseases varies depending on the pathogen, the timing and source of IL-10 production, and the site of infection. In most cases, neutralization or absence of IL-10 results in an exaggerated immune response that can lead to increased pathogen clearance, but also increased tissue damage and pathology. With less virulent pathogens that induce a mild inflammatory response IL-10 production allows for persistence of a typically asymptomatic or mild infection; however, highly virulent infections induce a robust immune response which requires IL-10 to inhibit further inflammation and immunopathology (63). In the case of viral infections, the majority of studies looking at the effects of IL-10 focus on its role in inducing persistence leading to chronic infections with little emphasis on the role of IL-10 during acute infections. Even less is known about the role of IL-10 during viral infections in the central
nervous system (CNS), which requires an extra level of immunopathogenic regulation due to the limited renewal capacity of neurons.

IL-10 deficiency typically results in increased inflammation during viral infections in the CNS with variable effects on viral replication (96-101). During neurotropic mouse hepatitis coronavirus infection (JHMV), IL-10 deficiency leads to an increase in inflammation resulting in greater mortality with no effect on viral replication (97). During Japanese encephalitis virus (JEV) infection, IL-10 levels peak early and then decline during the course of infection, which correlated with an increase in proinflammatory cytokine production and an increase in viral titers (99-101). During herpes simplex virus (HSV) encephalitis, IL-10 decreases microglial production of IL-1β, TNFα, and CCL5, without an effect on CXCL10 (98). \textit{Il10}\textsuperscript{-/-} mice infected with murine cytomegalovirus (MCMV) exhibit an increase in mortality that is independent of viral titers, but is associated with increased expression of IL-6, IFNγ, CXCL9, and CXCL10, as well as decreased lymphocyte, but increased neutrophil counts (96, 102). It is becoming clear that in the context of the CNS, IL-10 plays an important role in limiting immunopathology; however, the mechanism by which these inflammatory mediators are causing damage remains unknown. In particular, the role of IL-10 in regulating the skewing of the various CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells, the primary mediators of
pathology in many of these neurotropic or gliatropic infections, has been largely overlooked.

**Th17 cell biology**

Upon activation, naïve CD4+ T cells can differentiate into a variety of T helper cell subsets. The most widely recognized and best characterized subsets are Th1, Th2, Th17, and Treg cells (Figure 4). Th1 cell differentiation occurs in the presence of IL-12 and IFN\(\gamma\) via STAT3 and STAT1 signaling, respectively, express the transcription factor Tbet, and produce the characteristic Th1 cytokine IFN\(\gamma\). Th2 cells differentiate in the presence of IL-13 and IL-4 via STAT6 signaling, express the transcription factor Gata3, and produce the characteristic Th2 cytokines IL-4, IL-5, and IL-13. Th17 cells differentiate in the presence of IL-6 and TGF\(\beta\) via STAT3 and Smad signaling, respectively, express the transcription factor ROR\(\gamma\)t, and produce the characteristic Th17 cytokines IL-17A, IL-17F, IL-21, and IL-22. Tregs differentiate in the presence of TGF\(\beta\) via Smad signaling, express the transcription factor foxp3, and produce the regulatory cytokines IL-10 and TGF\(\beta\) (103, 104) (Figure 4).

Traditional roles for each of these T helper subsets have been defined, although fluidity between these roles occurs (Figure 4). Th1 cells are important for controlling intracellular pathogens, such as
viruses, as well as promoting cellular immunity. Th2 cells are important in the development of humoral immunity and control of parasitic infections. Th17 cells are important in autoimmunity and the immune response to extracellular pathogens and fungi. Tregs are important in regulating the Th1, Th2, and Th17 responses (103-105) (Figure 4). Although, Th1 cells have been the primary T helper cell type implicated in immunopathology during viral infections, the contribution of Th17 cells in virus clearance and immune-mediated pathology requires more attention.

Th17 cell differentiation in mice is driven by a combination of various cytokines as is described in Figure 5. Activation of naïve CD4+ T cells in the presence of TGFβ along with some combination of IL-6, IL-21, and IL-1β results in the induction of RORγt in a STAT3-dependent manner (104, 106). RORγt is the characteristic Th17 cell transcription factor and is important for IL-17 expression (107-109). The expansion of Th17 cells is sustained by IL-21 (110-113) and the maturation and stabilization of the Th17 population is maintained by IL-23 (104). In the absence of IL-23, Th17 cell function is not sustained (104, 106, 114). Study of Th17 cells in EAE has led to the identification of subsets that are pathogenic and nonpathogenic. TGFβ3 is important in the development of GM-CSF- and granzyme B-producing pathogenic Th17 cells, whereas TGFβ1 results in the differentiation of IL-10-producing,
nonpathogenic Th17 cells (115). In these studies, IL-23 induced TGFβ3 expression in a Tbet-dependent manner. TGFβ3 then upregulated expression of the IL-23R, thus promoting a positive feedback loop on pathogenic Th17 cell differentiation (115). This suggests a long sought after mechanism for why Tbet continues to be considered a pathogenic marker for the Th17 cell lineage (106).

Th17 cells can have a detrimental or protective role depending on the context of disease. During bacterial and fungal infections, Th17 cells are protective (116). In the case of autoimmunity disorders, Th17 cells are detrimental (114, 117). The best characterized model focused on the role of pathogenic Th17 cells is the murine model of multiple sclerosis (MS), experimental autoimmune encephalitis (EAE). Some characteristics of pathogenic Th17 cells during EAE are Tbet expression, production of IL-17, IL-22, granzyme B, and GM-CSF, and expression of the IL-23R (115, 118-125) (Figure 5). The mechanism by which these Th17 cells induce disease, however, remains unclear. IL-17 has neurotoxic effects; however, Il17−/− mice still develop EAE suggesting that its role during pathogenesis is limited (126). Granzyme B is an important cytolytic effector molecule and can induce neuronal cell death (127, 128). GM-CSF activates microglia and recruits monocytes to the site of inflammation (129-133). Mice deficient in GM-CSF are protected
from the development of EAE (134). The role of Th17 cells during viral infections, particularly those in the CNS, remains to be determined.

**Th17 cells and viral infections**

Although Th1 cells are primarily associated with viral infections, there is emerging evidence that IL-17 and Th17 cells also play a role, although the exact role is not clear. IL-17 production from innate and adaptive sources has been reported following a wide variety of viral infections in mice (influenza virus, respiratory syncytial virus (RSV), adenovirus), nonhuman primates (simian immunodeficiency virus, measles virus), and humans (RSV, hepatitis B and C viruses, and HIV). The role of this IL-17 production, however, varies from protective to detrimental depending on the virus and the tissue affected (135-148).

Th17 cells have been studied to some extent in the context of two viral infections whose pathologies are highly associated with inflammation, HSV-1 stromal keratitis and coxsackievirus-B3 myocarditis. IL-17 producing γδT cells and CD4+ T cells infiltrate the cornea during HSV-1 infection and ablation of IL-17 signaling using \( Il17R^{-/-} \) mice showed a decrease in disease severity which was associated with decreased neutrophil infiltration (149, 150). IL-17 expression and Th17 cells are also increased in the hearts of coxsackievirus B3-infected mice and are associated with higher viral titers and increased
autoantibody production (151, 152). Neutralization of IL-17A decreased viral titers and improved the disease outcome in these mice (153).

During viral infections in the central nervous system, there have been reports of IL-17 production and the presence of Th17 cells. During gliatropic murine coronavirus (JHMV) infection, a role for Th1 cells, but not Th17 cells, was observed using \( \text{Il}12\text{p}35^{-/-}, \text{Il}12\text{p}40^{-/-}, \text{and Il}23\text{p}19^{-/-} \) mice (154). If mice were deficient in IFN\( \gamma \), however, an increase in IL-17 was observed along with neutrophil accumulation, which correlated with an increase in disease severity (155). Neutralization of IL-17 improved the outcome in these JHMV-infected mice suggesting a detrimental role for this subset of cells (155). During Theiler’s murine encephalomyelitis virus (TMEV) infection, DCs promote Th17 cell development, which contributes to viral persistence and demyelinating disease (156, 157). Furthermore, rabies virus infection in mice upregulates \( \text{Il}17 \) mRNA expression, but the consequence of this for disease pathogenesis is unknown (131). Ultimately, the role of Th17 cells and viral infections is in the early stages of investigation and requires further study.

There have been few reports of the effects of IL-10 in regulating Th17 cell responses during viral infections. One report looked at acute RSV infection in mice and saw that with IL-10R blockade, there was an increase in the amount of CD4^+IL-17^+ T cells which was associated with increased severity of disease (158). During lethal influenza infection,
*Il10*−/− mice showed a decrease in morbidity and mortality associated with a specific increase in Th17 cells and neutrophil infiltration (88). These data suggest that during severe, acute infections IL-10 may play a regulatory role in limiting a Th17 response, which in the context of the CNS, most likely would have a detrimental outcome.
Figure 1. Alphavirus genome organization. The four nonstructural proteins (nsP1, nsP2, nsP3, and nsP4) are located at the 5’ end of the genome and the structural proteins (capsid, E1, E2, E3, and 6k) are located at the 3’ end of the genome. The nonstructural proteins are translated from full-length genomic RNA and the structural proteins are translated from subgenomic RNA. Full-length viral RNA is translated and nsP2 processes the nonstructural polyproteins to create the plus- and minus-strand replicase complexes. These are responsible to transcribing more full-length viral RNA, as well as subgenomic RNA for structural protein synthesis. (Reproduced from Diane Griffin, Fundamentals of Virology, 2013).
Figure 2. Cryo-EM of the Sindbis virus virion structure. Surface-shaded view of Sindbis virus as determined by cryo-electron microscopy (cryo-EM) at 7.0 Å. The trimeric petal-shaped spikes are visible, with solid triangles representing the threefold axes, and white triangles representing quasi-threefold axes. One of the asymmetric units is highlighted by green shading. B: The same view as shown in A but with the front half of the reconstructed structure removed. The outer layer containing spikes is shown in blue, whereas the underlying skirt density is in magenta. Crossing the lipid bilayer (cyan) reveals the ordered capsid protein (green; residues 114–264), a disordered region containing a mix of protein and RNA (yellow), and a region containing the remainder of the RNA genome (red). The transmembrane densities of E1 and E2 are seen spanning the outer and inner leaflets of the lipid bilayer (cyan).

**Figure 3. Morbidity, mortality, and virus titers in 4-6 week old C57Bl/6 mice infected intranasally with NSV.** C57Bl/6 mice were infected intranasally with 1x10^5 PFU of NSV. Mice were monitored daily for A) the appearance of clinical symptoms. Clinical scores were defined as follows: 0) no clinical symptoms, 1) abnormal hind-limb and tail posture, ruffled fur, hunched back, 2) unilateral hind-limb paralysis, 3) bilateral hind-limb paralysis or full body paralysis, and 4) death. Data are expressed as the mean ± SEM of the clinical scores from 3 independent experiments with a total n= 27. B) Survival of C57Bl/6
mice during NSV infection is presented as a Kaplan-Meier curve and statistics were calculated using the log-rank test. Data are pooled from 3 independent experiments with an n=35. The mean day of death in C57Bl/6 mice is 10 days post infection. C) Infectious virus titers in C57Bl/6 mice were determined using 10% (w/v) clarified brain homogenates plated on a BHK-21 monolayer. The data are representative of 3 independent experiments with a total of n=6-9 for each time point. The red arrow indicates the beginning of viral clearance and the onset of clinical symptoms.
Figure 4. Summary of T helper cell subsets. Naïve CD4+ T cells can be skewed into a variety of T helper cell subsets depending on the differentiation factors present at the time of activation. Th1 cells develop in the presence of IL-12 and IFNγ, Th2 cells develop in the presence of IL-4, Th17 cells develop in the presence of TGFβ and IL-6, amongst other cytokines, and Tregs develop in the presence of TGFβ. Each of these subsets also expresses a lineage specific transcription factor. Th1 cells express Tbet, Th2 cells express Gata3, Th17 cells express RORγt, and Tregs express foxp3. These subsets also produce one or more signature cytokines. Th1 cells produce IFNγ, Th2 cells produce IL-4, IL-5, and IL-13, Th17 cells produce IL-17A, IL-17F, IL-21, and IL-22, and Tregs produce IL-10 and TGFβ. These subsets also have prototypic roles
during infection and inflammation. Th1 cells are important for cellular immunity and control of intracellular pathogens. Th2 cells are important for control of parasitic infections and the development of humoral immunity. Th17 cells are implicated in autoimmune disease and are important in control of extracellular pathogen and fungal infections. Tregs play a role in regulating the Th1, Th2, and Th17 responses during inflammation.
Figure 5. Current understanding of factors contributing to and inhibiting the differentiation of Th17 cells that are pathogenic during EAE. Naïve CD4+ T cells differentiate into Th17 cells in the presence of TGFβ, IL-6, and IL-1β. These cells express the Th17 lineage transcription factor RORγt and produce IL-17a. IL-21 is produced primarily by Th17 cells and promotes proliferation of this population. Pathogenic Th17 cells develop in the presence of IL-23 and TGFβ3, whereas nonpathogenic Th17 cells develop in the absence of IL-23 and presence of TGFβ1. Pathogenic Th17 cells are characterized by the expression of a variety of molecules including Tbet, GM-CSF, IL-22, granzyme B, and IFNγ. Nonpathogenic Th17 cells are characterized by the expression of IL-10. IL-27 and IL-10 are negative regulators of Th17 differentiation.
Chapter 1: Characterization of the role of interleukin-10 during Neuroadapted Sindbis virus infection
Introduction

Encephalitic arthropod-borne viruses are an important cause of morbidity and mortality worldwide (2, 3). Alphaviruses are mosquito-borne viruses that can induce encephalomyelitis in humans. Venezuelan equine encephalitis (VEE) and eastern equine encephalitis (EEE) viruses have been the cause of recent major outbreaks in the Americas (159, 160). As the emergence and spread of these viruses rises, so does the need to better understand the mechanisms of pathogenesis for encephalitic viruses.

Sindbis virus (SINV) is the prototypical alphavirus and is closely related to VEEV and EEEV. It induces mild disease in humans in the form of rash and arthritis; however, it induces encephalomyelitis in mice due to its tropism for neurons (8, 9). The virulence of SINV is dependent on the age of the animal, the genetic background of the animal, and the strain of virus used (11-16, 18, 19). Neuroadapted Sindbis virus (NSV) is the most lethal strain of SINV. NSV induces fatal, paralytic disease in adult mice on the C57Bl/6 background and serves as a model for fatal viral encephalomyelitis. Pathogenesis of NSV infection is mediated by the immune response, not the virus itself, as shown by the survival of SCID mice (161). In particular, T cells have been implicated as the major cell type responsible for immune-mediated damage (23), although the pathogenic function of the T cells remains elusive.
Because the immune response plays a large role in NSV pathogenesis, understanding the regulation of such a response is key to determining targets for therapeutic intervention. Interleukin-10 (IL-10) is a key regulator of the immune response. IL-10 was first defined as a Th2 cytokine that regulates Th1 responses (52), but is produced by a variety of cell types including macrophages, dendritic cells, natural killer (NK) cells, and a variety of T cell subtypes, including Tregs (53, 63). The classic function of IL-10 is to inhibit MHC II and co-stimulatory molecule expression on antigen presenting cells (APCs), which leads to a reduction of T cell activation, but it can also directly inhibit pro-inflammatory cytokine production from APCs and T cells (53). IL-10 has also been shown to affect the skewing of CD4+ T cell responses, particularly the Th1 and Th17 response during infections and models of autoimmune disease (63, 88, 89). Altered IL-10 expression patterns have been associated with susceptibility to infection and autoimmune disease in mice (53, 85, 86, 162) and humans (53, 163-168), and with neurodegenerative diseases in humans (169, 170). The role that IL-10 plays during NSV infection is unknown.

To determine the role of IL-10 during fatal alphavirus encephalomyelitis, we characterized the response to NSV infection in wild-type C57Bl/6 (WT) and Il10−/− C57Bl/6 (IL-10 KO) mice. We show that in the absence of IL-10, the course of disease is accelerated during
NSV infection. This accelerated pathogenesis is not due to increased virus replication, exacerbated inflammation, or increased production of pro-inflammatory cytokines and chemokines. Examination of CD4+ T cells showed that Th1 and Th17 responses are induced during NSV infection coincident with the onset of paralysis. In the CNS of IL-10 KO mice, the Th17 response, but not Th1 response, is increased. These data support the conclusion that Th17 cells contribute to the immunopathology of NSV infection in mice and that this immunopathologic process is exacerbated in the absence of IL-10.

**Materials and Methods**

**Animals and virus**

C57Bl/6J wild-type (WT) and B6.129P2-Il10<sup>tm1Cgn</sup>/J (C57Bl/6J IL-10 KO) mice were purchased from Jackson Laboratories and bred in house. Mice in all experiments were sex-matched and intranasally infected at 4-6 weeks of age with $1 \times 10^5$ PFU of neuroadapted Sindbis virus (NSV) diluted in HBSS. For assessment of morbidity and mortality, mice were infected and monitored once daily for the appearance of clinical symptoms and death. The scoring system used was: 0) no clinical signs observed, 1) abnormal hind-limb and tail posture, ruffled fur, and/or hunched back, 2) unilateral hind-limb paralysis, 3) bilateral hind-limb paralysis or full-body paralysis, and 4) dead. For tissue
collection, mice were anesthetized with isoflurane and blood was collected via cardiac puncture. The animals were then perfused with ice-cold PBS and cervical lymph nodes, brains, and spinal cords were extracted, used fresh or snap frozen and stored at -80°C. All experiments were performed according to guidelines approved by the Johns Hopkins University Institutional Animal Care and Use Committee.

**Determination of viral titer**

Snap frozen tissue was thawed and homogenized in ice-cold PBS to make 10% (w/v) brain and spinal cord homogenates. Viral titers were determined by serially-diluting the homogenate in DMEM+1% FBS and plating 200 μl on a BHK-21 cell monolayer. The infected monolayer was incubated for 1 hour, overlayed with 1.2% bactoagar, and incubated at 37°C with 5% CO₂ for 48 hours. After 48 hours, the wells were stained with neutral red for 2 hours and plaques were counted. Data are plotted as the mean of the log₁₀ value of plaque forming units found in each sample ± SEM. Samples in which no virus was detected at a 1:10 dilution were assigned a value of 0.85, which is halfway between the limit of detection and 0, for quantitation purposes.
Mononuclear cell isolation

Brains, spinal cords, and cervical lymph nodes were collected from uninfected and infected mice at 3, 5, and 7 days post infection. For cervical lymph nodes, single-cell suspensions were made by pooling the lymph nodes from 5 mice per group in 5 mL of RPMI+1% FBS and homogenizing them in C tubes using the GentleMACS system (Miltenyi) spleen program 1 for 2 cycles. The suspension was then filtered through a 70 μm filter and cells pelleted. Red blood cells were lysed using 2 mL of red blood cell lysis buffer (Sigma #R-7757) for 3 minutes and then washed with PBS + 2mM EDTA. The pellet was resuspended in PBS + 2 mM EDTA and live cells were counted using trypan blue exclusion.

For the brain and spinal cord, tissue was collected from 6-10 mice per group and placed in HBSS on ice. Once all tissue was collected, up to 2 brains or 5 spinal cords were pooled in a C tube containing 4 mL of enzyme digest mix made up of RPMI + 1% FBS, 1 mg/mL collagenase (Roche #11088858001), and 0.1 mg/mL DNase (Roche #04536282001) and a gross dissociation was performed with scissors. The tissue was then run 2 times on the GentleMACS (Miltenyi) brain program 3, incubated for 15 minutes at 37°C with intermittent rocking, this process was repeated once more, and then the samples were homogenized again using one run on brain program 3. The homogenate was filtered using a 70 μm filter rinsing with RPMI +1% FBS and centrifuged at 1400 rpm for
10 minutes. To remove myelin debris, the pellet was suspended in 30% percoll and under-layed with 70% percoll for a 30/70% gradient in a 15 mL conical tube with 1 brain or up to 2.5 spinal cords per gradient. The gradient was spun for 30 minutes at 850xg at 4°C. The myelin coat in the top layer was aspirated off and the mononuclear cells at the interface were collected and washed with PBS + 2 mM EDTA. The pellet was resuspended in PBS + 2 mM EDTA and live cells were counted using trypan blue exclusion.

Flow cytometry to define cellular infiltrates

To determine the number and types of cells infiltrating the tissue, 1-2x10⁶ cells were used for each panel. The cells were stained with a violet live/dead stain (Invitrogen #L345955), blocked using rat anti-mouse CD16/CD32 Block (BD #553142), surface stained for phenotyping, and then fixed using BD CytoFix/CytoPerm solution (BD #5546722), resuspended in 200 μl of PBS + 2 mM EDTA+ 0.5% BSA, and data acquired. The surface markers used were CD45-PerCPCy5.5 (clone 30-F11, BD #561869), CD11b-PE (clone M1-70, BD #557397), Ly6G-PECy7 (clone 1A8, BD #560601), Ly6C-APC (clone HK1.4, BD #17-5932-80), CD3-APCCy7 (clone 17A2, BD #560590), CD4-PerCpCy5.5 (clone RM4-5, eBioscience #45-0042-80), CD8-PECy7 (clone 53-6.7, BD #552877), CD25-PE (clone PC61.5, eBioscience #12-0251-81), NK1.1-
APCCy7 (clone PK136, BD #560618), and γδTCR-APC (clone eBioGL3, eBiocience #17-5711-81). Cell types were defined as follows: microglia (CD45loCD11b+Ly6G-Ly6C-), macrophages/monocytes (CD45hiCD11b+Ly6G-Ly6C+), neutrophils (CD45+CD11b+Ly6G+Ly6Cint), NK cells (CD45+CD3-NK1.1+), T cells (CD3+), CD4 T cells (CD3+CD4+), CD8 T cells (CD3+CD8+), NKT cells (CD45+CD3+NK1.1+), and γδ T cells (CD3+γδTCR+). Data were acquired using the BD FACS Canto II and FACS Diva software, and analyzed using FlowJo 8.8.7 (TreeStar Inc.).

**Intracellular cytokine staining**

For determination of T cell cytokine production, 2-3x10⁶ cells were stimulated with RPMI + 1% FBS containing 50ng/mL of phorbol-12-myristate 13-acetate (PMA) and 1 μg/mL of ionomycin in the presence of GolgiPlug (BD #555029) for 4 hours. Cells were then washed and stained with the violet live/dead marker (Invitrogen #L345955), blocked with rat anti-mouse CD16/CD32 Block (BD #553142), and then stained for the surface markers CD4-FITC (clone RM4-5, eBioscience #11-0042) and/or CD8-PerCPCy5.5 (clone 53-6.7, eBioscience, #45-0081-82). After surface staining, cells were fixed and permeabilized using the BD CytoFix/CytoPerm kit (BD #554714). For CD4⁺ T cells, cytokine staining used antibody to IFNγ-PECy7 (clone XMG1.2, eBioscience #25-7311-82) and IL-17a-APC (clone eBio17B7, eBioscience #17-7177-81). For CD8⁺ T
cells, cytokine staining used antibody to IFNγ-APC, (clone XMG1.2, eBioscience #17-7311-82), TNFα-PECy7 (clone MP6-XT22, BD #557644), and granzyme B-PE (clone NGZB, eBioscience #12-8898-80). Cells were washed, resuspended in PBS + 2mM EDTA + 0.5% BSA, and data acquired using the BD FACS Canto II and FACS Diva, and analyzed using FlowJo 8.8.7 (TreeStar Inc.).

*Gene expression analysis using real-time PCR*

Snap frozen tissue was homogenized in Qiazol buffer and RNA was extracted using the RNeasy Lipid Mini RNA Isolation Kit (Qiagen #74804). RNA was quantified using a nanodrop spectrophotometer and cDNA was prepared with the High Capacity cDNA Reverse Transcription Kit (Life Technologies #4368814) using 500ng of input RNA. Quantitative real-time PCR was performed using 2.5 μl of cDNA and TaqMan gene expression arrays in 2x Universal PCR Mastermix (Applied Biosystems, #4304437). The TaqMan gene expression arrays used were *Ifnγ, Cxcl10, Ccl2, IL-12a, IL-12b, IL-10, IL-1β, Tnfα*, and *IL-6*. *Gapdh* mRNA levels were determined using the rodent primer and probe set (Applied Biosystems, #4308313). All reactions were run on the Applied Biosystems 7500 Real-time PCR machine with the following conditions: 50°C for 2 minutes, 95°C for 10 minutes, 95°C for 15 seconds, and 60°C for 1 minute for 50 cycles. Transcript levels were determined by normalizing the target gene
Ct value to the Ct value of the endogenous housekeeping gene Gapdh. This normalized value was used to calculate the fold-change relative to the average of the uninfected control (ΔΔCt method).

Statistical analysis

Data from three independent experiments or at least 3 mice per group are used. All statistical analysis was done using Graph Prism 5 (GraphPad). Survival was compared using Kaplan-Meier survival curves (log rank test). Differences between groups during the course of infection are determined using a 2-way ANOVA and Bonferroni post-tests for comparison of the differences at each time point (morbidity, cellular infiltrates, cytokine analysis, and viral titers). Comparisons between groups for single parameter analysis were done using an unpaired, two-tailed Student’s test with a 95% confidence interval (CD4 and CD8 T cell function).

Results

Il10 mRNA levels increase during NSV infection

NSV pathogenesis is mediated by the immune response, primarily T cells (23). Therefore, understanding the regulation of the inflammatory response during NSV infection is critical. IL-10 is a key regulator of both the innate and adaptive arms of the immune response; however its
production during NSV infection of the CNS is unknown. To determine this, we looked at \textit{Il10} mRNA levels during the course of infection in the brains and spinal cords of C57Bl/6 (WT) mice. \textit{Il10} mRNA expression significantly increased in the brains and spinal cords of WT mice during NSV infection (****p<0.0001 for both). In the brain, \textit{Il10} gene expression began to increase in the brain at 5 days post infection (*p<0.05) and continued to increase through 7 days post infection (Figure 6a, ***p<0.001). In the spinal cords, \textit{Il10} gene expression increased by 7 days post infection (Figure 6b, ***p<0.001). Because \textit{Il10} mRNA does not significantly increase in the brain or spinal cord until the time of T cell infiltration, this time course suggests that T cells are the main producers of IL-10 in the CNS during NSV infection.

\textit{IL-10 deficiency leads to accelerated morbidity and mortality from NSV infection}

To determine if IL-10 would affect outcome, wild-type C57Bl/6 (WT) mice and \textit{Il10/-} C57Bl/6 (IL-10 KO) mice were infected intranasally with NSV and monitored for morbidity and mortality. We observed a significant difference in the course of disease as measured by the progression of clinical signs of disease between WT and IL-10 KO mice (Figure 7a) (****p<0.0001). Accelerated disease was incident in IL-10 KO mice by 6 days post infection (**p<0.001) and this difference remained
through 8 days post infection (****p<0.0001) by which time mice were succumbing to fatal infection (Figure 7a). The accelerated morbidity we observed correlated with an earlier mean day of death in IL-10 KO mice at 8 days post infection compared to 10 days post infection in WT mice (Figure 7b) (****p<0.0001). These data suggest that IL-10 plays a role in modulating disease pathogenesis, and in its absence immunopathogenic mechanisms are either amplified, occur earlier, or both. Because IL-10 is important in both a resistant and susceptible model of NSV infection, these data suggest that IL-10 is regulating a critical mechanism of NSV pathogenesis.

**Viral replication is similar in the absence of IL-10, but clearance is delayed**

The effects of IL-10 deficiency on virus replication and clearance are variable depending on the virus used (96-101). To determine if accelerated disease in IL-10 KO mice was related to differences in viral replication or clearance, brain and spinal cord homogenates were analyzed for infectious virus titers throughout the course of infection. In the brain production of infectious virus was similar; however, by 7 days post infection WT mice had mostly cleared virus, whereas IL-10 KO mice had not (Figure 8a, **p<0.01). This delay in clearance led to a difference in the amounts of virus observed over the course of infection (Figure 8a, p=0.0502).
In the spinal cord, infectious virus appeared earlier in the IL-10 KO (3 days) mice than WT mice (4 days) (Figure 8b, **p<0.01), although peak titers were similar between both groups. Similarly to the brain, IL-10 KO mice also exhibited a delay in virus clearance 7 days post infection compared to WT animals (Figure 8b). Overall, viral replication in the spinal cord was significantly different between WT and IL-10 KO mice (Figure 3b, **p=0.0022). The question of why IL-10 KO exhibit earlier virus replication in the spinal cord, but not brain, compared to WT mice during NSV infection was further investigated and will be discussed in the general discussion chapter. These data suggest that IL-10 deficiency does not affect peak viral titers, but does result in a delayed clearance throughout the CNS.

**IL-10 deficiency does not result in a general exacerbation of inflammation**

IL-10 is an important immunoregulatory cytokine and in its absence the magnitude of the immune response can increase due to the lack of negative inhibition (53, 63). Because NSV pathogenesis is mediated by the immune response, an increase in the magnitude of this response could lead to accelerated damage and disease. To determine if the immune response to NSV infection was increased in the absence of IL-10, we first determined the total number of cells isolated from the cervical lymph node, brain, and spinal cord of WT and IL-10 KO mice.
during the course of infection (Figure 9). The number of cells isolated from the cervical lymph nodes of infected mice was similar at baseline, 3, and 7 days post infection; however, at 5 days post infection, a time of peak response in the cervical lymph node, there were significantly more cells found in the lymph nodes of WT mice compared with IL-10 KO mice (Figure 9a, *p<0.05). In the brain, the number of cells isolated continued to increase through 7 days post infection, but the number of cells was similar at each time point between WT and IL-10 KO mice (Figure 9b). The number of cells isolated from the spinal cord did not show much of an increase until 7 days post infection, reflecting a slower increase in inflammation compared to the brain which correlates with the delay in virus spread to this tissue (Figure 9b and 9c). At all time points measured, the number of cells between WT and IL-10 KO mice was similar in the spinal cord (Figure 9c). These data suggest that a general increase in cellular infiltration into the CNS does not occur in the absence of IL-10.

Flow cytometry was used to characterize the relative frequency and number of both innate and adaptive immune cell types found in the cervical lymph nodes (Figure 10), brain (Figure 11), and spinal cord (Figure 12) during the course of NSV infection. T cells were the predominant cell type found in the cervical lymph nodes of infected mice (Figure 10a). Numbers of macrophages (CD45+CD11b+Ly6G-Ly6C+),
natural killer (NK) cells (CD45+CD3-NK1.1+), and T cells (CD3+) all peaked at 5 days post infection (Figure 10a). Numbers of neutrophils (CD45+CD11b+Ly6G+Ly6Cint), increased slightly during the course of infection (Figure 10a). There are no significant differences between WT and IL-10 KO observed in any of these cell types (Figure 10a).

In the brain, the number of microglia (CD45loCD11b-Ly6G-Ly6C-) remained relatively constant (Figure 11a). Macrophages/monocytes (CD45hiCD11b+Ly6G-Ly6Chi) were the primary infiltrating population through 7 days post infection (Figure 11a). The numbers of these cells began increasing by 3 days post infection and continued to increase through 7 days post infection. The number and frequency of this population was similar between WT and IL-10 KO except at 7 days post infection when more macrophages/monocytes were found in WT mice compared to IL-10 KO mice (**p<0.01) although there was no difference observed in the relative frequency of these cells (Figure 11a). NK cells (CD45+CD3-NK1.1+) peaked at 5 days post infection and both numbers and frequencies were similar between WT and IL-10 KO mice (Figure 11a). Neutrophil (CD45+CD11b+Ly6G+Ly6Cint) infiltration peaked at 7 days post infection in the brain, at which point there are significantly more neutrophils in IL-10 KO compared to WT mice (Figure 11a, **p<0.01). During the course of infection, the number of neutrophils was also significantly higher in IL-10 KO compared to WT mice (Figure 11a).
The frequency of neutrophils was also significantly elevated in IL-10 KO compared to WT mice during the course of infection (**p=0.0087) and significantly higher at 7 days post infection (*p<0.05) (Figure 11a). T cell infiltration began by 5 days post infection in the brain and continued to increase at 7 days (Figure 11a). The number of T cells in the brains of WT and IL-10 KO mice was similar at baseline, 3, and 5 days post infection, but by 7 days post infection there were more T cells found in WT compared to IL-10 KO mice (Figure 11a, *p<0.05).

The spinal cord mostly showed similar cellular infiltration patterns as the brain. The number of microglia remained consistent throughout the course of infection and the numbers and frequencies were similar between WT and IL-10 KO mice (Figure 12a). Macrophages/monocytes represented the predominant infiltrating cell population and continued to increase through 7 days post infection. The number and frequency of macrophages/monocytes was similar in the presence and absence of IL-10 (Figure 12a), unlike the increased number of macrophages/monocytes in the brains of WT mice at 7 days post infection (Figure 11a). NK cells also peaked in the spinal cord at 5 days post infection like they did in the brain and had similar numbers and frequencies between WT and IL-10 KO mice (Figure 12a). Neutrophil numbers peaked at 5 days post infection, but numbers were similar between WT and IL-10 KO mice, unlike in the brain where IL-10 KO had more neutrophils than WT mice.
The relative frequency of neutrophils at 5 days post infection, however, was significantly higher in IL-10 KO compared to WT mice (Figure 12a) (*p<0.05). T cells began infiltrating the spinal cord by 5 days post infection and increased at 7 days post infection (Figure 12a). The number and frequency of T cells was similar between WT and IL-10 KO mice at all time points measured (Figure 12a). These data suggest that in the absence of IL-10, the number and relative frequency of neutrophils in the brain and spinal cord are increased, with little effect on the infiltration of other cell types.

These data support the idea that inflammation in general is not exacerbated in the absence of IL-10, which could lead to accelerated pathogenesis. If anything, the numbers of various cell types was higher in WT mice compared to IL-10 KO mice as seen by higher numbers of macrophages/monocytes and T cells in WT compared to IL-10 KO mice. However, there were more neutrophils found in the brains of IL-10 KO mice compared to WT mice, and this cell type may contribute to accelerated disease or be a by-product of another immunological process.

**IL-10 deficiency has little effect on the T cell types present in the draining lymph nodes and CNS**

Although the number of CD3+ T cells was similar or higher in WT mice compared to IL-10 KO, we wanted to determine if the infiltration of
various T cell types were also similar. Therefore, we examined the number and frequency of CD4+, CD8+, γδ TCR+ (γδ T cells), and NK1.1+ T cells (NKT cells) in the cervical lymph nodes, brains, and spinal cords of WT and IL-10 KO mice during NSV infection. CD4+ and CD8+ T cells made up the majority of the cells present in the cervical lymph nodes, but γδ T cells and NKT cells were also present (Figure 10b). The number of NKT cells peaked at 3 days post infection, whereas CD4+, CD8+, and γδ T cell numbers all peaked at 5 days post infection (Figure 10b). The number of each of these T cell types was similar between WT and IL-10 KO mice at all time points (Figure 10b). The frequency of each of these cell types was also similar at all time points, except a higher frequency of γδ T cells at 5 days post infection in IL-10 KO mice (**p<0.01); however, the difference was very small (0.5 vs. 0.8%) and may have little biological significance (Figure 10b).

In the brains of mice infected with NSV, all four types of T cells were found during infection, with CD4+ and CD8+ T cells being the most common. The numbers of CD4+ and CD8+ T cells increased in the brain through 7 days post infection, but the number and frequency of these cells types in WT and IL-10 KO mice was similar during the course of infection (Figure 11b). The number of γδ T cells plateaued around 5 days post infection and was similar between WT and IL-10 KO mice; however, the overall frequency of these cells during infection was elevated in IL-10
KO mice (*p=0.0393), but the difference was small (0.3 to 0.6%) and may have little biological significance (Figure 11b). The number of NKT cells increased at 7 days post infection and more were found in the brains of WT compared to IL-10 KO mice (**p<0.001) with a significant difference throughout the course of infection (Figure 11b, *p=0.0219). The frequency of NKT cells between both groups was similar (Figure 11b).

T cells in the spinal cords of NSV-infected mice were harder to characterize due to their low numbers. All four types of T cells were detected in this tissue just as in the brain, and again, CD4+ and CD8+ T cells made up the majority of this population (Figure 12b). CD4+, CD8+, and NKT cells peaked at 7 days post infection with very few γδ T cells present and/or infiltrating by 7 days post infection (Figure 12b). The number and frequency of all T cell subtypes was similar in WT and IL-10 KO spinal cords during NSV infection (Figure 12b).

These data suggest that there is little to no difference in T cell types during NSV infection in the presence and absence of IL-10 in the periphery or infected CNS tissue.

_Pro-inflammatory cytokine and chemokine mRNAs are not increased in the absence of IL-10_

To determine if increased immunopathogenesis in the absence of IL-10 regulation could be related to an increased capacity of these cells
to produce pro-inflammatory molecules in the absence of IL-10 regulation, we analyzed the pro-inflammatory gene expression profiles in the brains and spinal cords of WT and IL-10 KO mice infected with NSV. In the brain, there was significantly higher expression of $Ifn_{\gamma}$ (**p=0.004), $Tnf_{\alpha}$ (**p=0.0006), $Il1_{\beta}$ (**p=0.0004), $Il12b$ (**p=0.001), and $Cxcl10$ (**p=0.0008) in WT mice compared to IL-10 KO mice (Figure 13a). Specifically, $Ifn_{\gamma}$ mRNA expression was higher in WT mice at 5 and 7 days post infection (**p<0.01 and *p<0.05, respectively) (Figure 13a). $Tnf_{\alpha}$, $Il1_{\beta}$, and $Il12b$ mRNA expression was higher in WT mice at 5 days post infection (**p<0.001 for all) (Figure 13a). $Cxcl10$ mRNA expression was higher in IL-10 KO mice at 7 days post infection (Figure 13a, ***p<0.001). No differences were observed between WT and IL-10 KO mice for $Il12a$, $Il6$, and CCL2 (Figure 13a).

In the spinal cords, the pattern was similar to the brain in that WT mice typically had higher levels of pro-inflammatory cytokine and chemokine mRNA expression. $Ifn_{\gamma}$ (**p=0.0007), $Tnf_{\alpha}$ (p=0.0026), $Il1_{\beta}$ (**p=0.0001), $Il12a$ (**p=0.0001), Ccl2 (**p<0.0001) and $Cxcl10$ (**p<0.0001) were higher over the course of infection in WT mice compared to IL-10 KO mice (Figure 14a). Specifically, $Ifn_{\gamma}$ mRNA expression was higher in WT mice at 7 days post infection (**p<0.001) (Figure 14a). $Tnf_{\alpha}$ mRNA expression was higher in WT mice at 5 and 7 days post infection (**p<0.01 and ***p<0.001, respectively) (Figure 14a).
Il1β mRNA expression was higher in WT mice at 4, 5, and 7 days post infection (**p<0.01, ***p<0.001, and ***p<0.001, respectively) (Figure 14a). Il12a expression was higher at 1, 2, 4, and 7 days post infection (***p<0.001, **p<0.01, *p<0.05, ***p<0.001 respectively) (Figure 14a). Ccl2 mRNA levels were higher in WT mice at 5 and 7 days post infection (**p<0.01 and ***p<0.001, respectively) (Figure 14a). Cxcl10 mRNA levels were higher in WT mice at 4, 5 and 7 days post infection (*p<0.05, ***p<0.001, ***p<0.001) (Figure 14a).

The higher levels of pro-inflammatory cytokine expression in WT mice compared to IL-10 KO mice again show that the immune response is not simply elevated across all parameters of inflammation in the absence of IL-10; suggesting that IL-10 is having a more specific effect on the immune response leading to accelerated disease progression.

**IL-10 deficiency does not affect CD8+ T cell effector function**

CD4+ and CD8+ T cells have both been implicated as primary mediators of immunopathology during NSV infection (23). To determine the function of these T cell subsets, we looked at CD8+ T cell effector function because of the defect observed in viral clearance in the CNS of IL-10 KO mice. To determine if the CD8+ cytotoxic T lymphocytes (CTLs) are behaving similarly between WT and IL-10 KO mice, we measured effector molecule production via intracellular cytokine staining in CD8+ T
cells isolated from the site of priming, the cervical lymph nodes, as well as the target tissue of infection, the brain and spinal cord. The effector molecules examined were IFN\(\gamma\), TNF\(\alpha\), and granzyme B. Viral clearance in the CNS begins around 5 days post infection with the influx of both CD4\(^+\) and CD8\(^+\) T cells. At this point in both the brain and spinal cord, we saw similar levels of infectious virus (Figure 8). CD8\(^+\) T cells in the cervical lymph nodes of mice infected with NSV at 5 days post infection showed no difference between WT and IL-10 KO mice in the frequency or number of CD8\(^+\) T cells expressing IFN\(\gamma\), TNF\(\alpha\), or granzyme B (Figure 15a). By 7 days post infection, however, the frequency of granzyme B\(^+\) CD8\(^+\) T cells was lower in the cervical lymph nodes of IL-10 KO mice (44.8%) compared to WT mice (79.4%, *p=0.0484, Figure 15b). This resulted in a lower number of granzyme B\(^+\) CD8\(^+\) T cells in IL-10 KO mice than WT mice (5.58x10\(^5\) vs. 1.81x10\(^6\), *p=0.0312, Figure 15b). A lower frequency of TNF\(\alpha\)\(^+\) CD8\(^+\) T cells was also observed in IL-10 KO mice compared to WT mice (53.1% vs. 82.7%, respectively) although this difference did not reach statistical significance (p=0.1127, Figure 15b). This also led to fewer, although not significant, CD8\(^+\) TNF\(\alpha\)\(^+\) cells in IL-10 KO mice compared to WT mice (6.67x10\(^5\) vs. 1.92x10\(^6\), *p=0.0841) (Figure 15b). The frequency and number of CD8\(^+\) IFN\(\gamma\)\(^+\) T cells was similar between WT and IL-10 KO mice (Figure 15b).
Although no differences were observed at 5 days post infection between WT and IL-10 KO mice in the cervical lymph node CD8\(^+\) T cells, in the brain the percentage of CD8\(^+\) T cells expressing granzyme B was lower in IL-10 KO (68.8\%) compared to WT mice (86.4\%, \(*p=0.0247\), Figure 16a). The frequency of CD8\(^+\) T cells producing IFN\(\gamma\) and TNF\(\alpha\) was similar in the presence and absence of IL-10 (Figure 16a). The numbers of CD8\(^+\) T cells producing each of these cytokines was also similar between the groups (Figure 16a). By 7 days post infection, however, no differences were observed between WT and IL-10 KO mice in the percentage or number of CD8\(^+\) T cells expressing any of these effector molecules (Figure 16b).

CD8\(^+\) T cells from the spinal cord were analyzed only at 7 days post infection due to the low yield of cells for intracellular cytokine staining at 5 days post infection. No differences were observed between WT and IL-10 KO mice in the either the frequency or number of CD8\(^+\) T cells expressing IFN\(\gamma\), TNF\(\alpha\), or granzyme B (Figure 17a).

The pattern of expression of these proteins during infection was interesting. The percentage of CD8\(^+\) T cells expressing granzyme B in WT mice was 80-93\% in the periphery as well as the brain and spinal cord at 5 and 7 days post infection (Table 1). IFN\(\gamma\) expression in CD8\(^+\) T cells, was 67-91\% in the cervical lymph node at 5 and 7 days post infection (Table 1); however, in the brain the frequency of CD8\(^+\) T cells expressing
IFNγ decreased between 5 and 7 days post infection (75.6% to 39.3% for IFNγ). The percentage of CD8+ T cells expressing TNFα in the brain decreased from 30.3% to 9.3% from 5 to 7 days post infection as well (Table 1). Thus, the dominant effector cytokine expressed in the CNS is granzyme B, followed by IFNγ, and then TNFα.

These data suggest that there some defects in the ability of CD8+ T cells to produce granzyme B in the absence of IL-10, which may affect their ability to aid in viral clearance, particularly as granzyme B was the most prominent effector molecule expressed by CD8+ T cells. Consistent with other parameters of inflammation, however, there appeared to be less pro-inflammatory and neurotoxic molecule production from CD8+ T cells in IL-10 KO mice during NSV infection compared to WT mice, so it is unlikely that increased CTL activity is contributing to the accelerated pathogenesis observed in IL-10 KO mice.

The magnitude of the TH17, but not the TH1, response to NSV infection is increased in the absence of IL-10

To assess a potential role for CD4+ T cells, T helper cell subsets present in the periphery and CNS during infection were analyzed. CD4+ T cells were isolated from tissue at 5 and 7 days post infection, stimulated with PMA/ionomycin, and assessed for production of IFNγ, IL-4, and IL-17a to characterize the Th1, Th2, and Th17 responses,
respectively. Very few CD4+IL-4+ Th2 cells were found in the cervical lymph node or brain (data not shown); however, CD4+IFNγ+ Th1 cells and CD4+IL-17a+ Th17 cells were present in the cervical lymph node and brain at 5 and 7 days post infection and the spinal cord at 7 days post infection (Figure 18a and 18b). At 5 days post infection, no differences were observed in the cervical lymph nodes between WT and IL-10 KO mice in the number or frequency of Th1 or Th17 cells (Figure 18a); however, in the brain there was a significant increase in the Th17 population, but not Th1 population in IL-10 KO mice (Figure 18b). This was characterized by ~5-fold increase in the frequency of IL-17a+ CD4+ T cells from 2.97% in WT mice to 14.62% in IL-10 KO (**p=0.0063) (Figure 18b). This also meant an increase in the number of CD4+IL-17a+ T cells from 639 cells per brain in WT mice to 5,173 cells per brain in IL-10 KO mice (*p=0.0153) (Figure 18b). The appearance of Th1 and Th17 cells in the CNS occurs at the onset of clinical symptoms, which suggest that both of these populations may be playing a pathogenic role during NSV infection.

We also looked at the Th1 and Th17 responses 7 days post infection in the cervical lymph nodes, brain, and spinal cord. At 7 days in the cervical lymph nodes there was a trend toward a higher frequency of Th17, but not Th1, cells in IL-10 KO mice compared to WT mice (2.76% vs. 0.75%, p=0.1197, Figure 19a). In the brain, the Th17
population remained increased in IL-10 KO mice compared to WT mice (1.94 vs. 0.41%, *p=0.0120); however, the difference was less than at 5 days post infection (Figure 19b). This results in more Th17 cells in the brains of IL-10 KO mice than WT mice (5363 cells vs. 1176 cells, p=0.0508) (Figure 19b). At 7 days post infection in the spinal cord the Th17 response, but not the Th1 response, was higher in the absence of IL-10 (Figure 19c). The frequency of Th17 cells was 2.89% in IL-10 KO mice compared to 0.3% in WT animals (p=0.0588) and the number of Th17 cells was 468 cells per animal in IL-10 KO mice vs. 48 cells per animal in WT mice (*p=0.0420) (Figure 19c). These data suggest that Th1 and Th17 cells are both present during NSV infection. In the absence of IL-10, disease is accelerated coincident with the entry of T cells into the CNS at 5 days post infection and this correlates with an increase in the Th17, but not Th1, population compared to WT animals suggesting a role for Th17 cells in the pathogenesis of NSV infection. This increase of Th17 cells continues through 7 days post infection; however, it is to a much lower extent.

**Discussion**

IL-10 is an important immunoregulatory molecule and its expression is upregulated in the CNS of C57Bl/6 mice during NSV infection. To determine the role of IL-10 during fatal alphavirus
encephalomyelitis, we characterized the response to NSV infection in WT and IL-10 KO mice. We found that IL-10 KO mice experienced accelerated morbidity and mortality compared to WT mice. Accelerated disease was not due to a difference in virus replication, although clearance was delayed in the CNS of IL-10 KO mice. We found that the accelerated death was also not associated with a general increase in the inflammatory response to NSV infection in the absence of IL-10 regulation as assessed by cellular infiltration and pro-inflammatory cytokine and chemokine expression, in fact, the response was typically higher in WT mice. The CD8+ T cell response was similar between groups during NSV infection in the cervical lymph nodes and brains, with only granzyme B production reduced in IL-10 KO compared to WT mice. However, examination of the CD4+ T cell response revealed an increase in the Th17 response, but not Th1 response. This also correlated with an increase in neutrophil recruitment into the brains of IL-10 KO mice. Our results show that IL-10 plays an important role in NSV pathogenesis by specifically regulating the Th17 response, with little effect on other parameters of inflammation during fatal alphavirus encephalomyelitis.

IL-10 is an important regulator of the immune response. It can dampen T cell activation, inhibit antigen presenting cell function, dampen inflammatory cytokine production, and skew T helper cell
responses (53, 63). The effects of IL-10 on viral infections have primarily focused on its role during chronic viral infections and persistence, with little work done on acute infections. In chronic infections, IL-10 typically promotes viral persistence, and in its absence the immune response is able to clear the virus and resolve infection thus improving outcome (63). During acute infections, particularly those of the CNS, IL-10 deficiency usually leads to increased inflammation with increased morbidity and mortality, and variable effects on viral replication (96-100). The pathology that occurs during NSV infection is primarily immune-mediated.

During NSV infection Il10 mRNA expression was increased at 5 and 7 days post infection, which also correlated with the beginning of accelerated development of clinical symptoms in IL-10 KO mice. The pattern of Il10 mRNA expression also correlates with the infiltration of T cells into the brain and spinal cord suggesting that the primary source of IL-10 may be T cell derived. Studies looking at the production of IL-10 in the CNS during a gliatropic coronavirus infection determined that CD4+ and CD8+ T cells were the dominant producers of IL-10, specifically highly activated CD8+ T cells at the peak of infection (171, 172). Future studies will focus on identification of the cells that are producing IL-10 in the CNS during NSV infection, as well as which of these cell types is important in restricting the Th17 response induced by NSV infection.
For other viral infections, IL-10 has variable effects on virus replication. During NSV infection, we saw no difference in virus replication in the brains of WT and IL-10 KO mice; however, clearance was delayed in the brains and spinal cords of IL-10 KO mice. For many infections, CD8+ T cells are primarily responsible for the clearance of infectious virus. IL-10 can affect CD8+ T cell proliferation and development (92-95); however, there was no difference in the number of CD8+ T cells present in WT and IL-10 KO mice. We also investigated the function of CD8+ T cells in order to determine if there was a deficiency in the ability of IL-10 KO CD8+ T cells to produce IFN\(\gamma\), TNF\(\alpha\), or granzyme B and only saw a transient deficiency in granzyme B production by CD8+ T cells of IL-10 KO mice. IL-10 can also have an effect on B cell maturation and antibody production (53). Levels of neutralizing antibody in the sera of WT and IL-10 KO mice were not different (data not shown); however, we have not looked at neutralizing antibody levels in the brain or NSV-specific IgG and IgM levels. Viral infections that involve a Th17 response have shown that IL-17 or Th17 cells can enhance virus replication and delay clearance (152, 156). The delay in virus clearance in the presence of an increased Th17 population, may be due to the effects of Th17 inhibition on the target cell response to CTL effector function, as is reported with TMEV (156), or an inhibition by Th17 cells
or IL-17a on virus specific antibody or IFNγ function, both of which are important in SINV clearance (161, 173, 174).

In previous studies where IL-10 deficiency leads to exacerbated disease, an increase in inflammation and pro-inflammatory cytokine production in IL-10 KO mice is typically the cause (53, 63, 96, 98). This is not the case during lethal alphavirus encephalomyelitis. Recent studies focusing on influenza and leishmania infection reported an increase in the Th17 response or both the Th1 and Th17 response, respectively (88, 89). In the case of influenza virus infection, the effect of Il10 deficiency was dependent on the model of infection used. In a sublethal infection, no difference was observed in the morbidity and mortality of IL-10 KO mice compared to WT mice; however, when a lethal dose of influenza virus was administered, IL-10 KO mice were protected compared to WT mice. The primary difference between WT and IL-10 KO mice was not due to a general increase in inflammation, but rather a specific increase in the Th17 response, which also led to an increase in neutrophil recruitment (88). This recapitulates what we observed during NSV infection, except in the context of viral infection in the CNS the increased Th17 response in the absence of IL-10 correlates with increased pathogenesis. In both the leishmania and influenza studies, however, the pathogenic or non-pathogenic phenotype of the Th17 cells was not characterized. In chapter 2, we investigate the pathogenic
potential of the Th1 vs. Th17 cells found during NSV infection, as well as the pathogenic potential of Th17 cells in the presence and absence of IL-10 will give more clear insight into how IL-10 affects Th17 cell development and function.

In conclusion, we determined that Il10 mRNA is expressed in the CNS during NSV infection and is important in modulating NSV-induced immunopathogenesis. This study identified the presence of Th1 and Th17 cells in the CNS during infection and the importance of IL-10 in specifically regulating this cell population. In the absence of this regulation, mice experience accelerated disease. These data support the idea that T cells are important in the pathogenesis of NSV infection. It also supports emerging evidence that IL-10 is an important regulator of T helper cell skewing, particularly during lethal infections.
**Figure 6. Il10 mRNA expression in the brain and spinal cord during NSV infection.** WT and IL-10 KO mice were infected intranasally with 1x10^5 PFU of NSV. Tissue was collected from uninfected and infected mice through 7 days post infection. RNA was isolated from tissue homogenates and real-time PCR was performed for Il10 mRNA in A) brain and B) spinal cord. Data are normalized to the housekeeping gene Gapdh and shown relative to uninfected mice (ΔΔCt method). Data are presented as the mean ± SEM from 2 independent experiments each with n=3-4 uninfected WT and IL-10 KO controls and 2-3 infected mice. Statistics were done using a one-way ANOVA and Dunn post tests comparing infected to uninfected samples, *p<0.05, **p<0.01, and ***p<0.001.
Figure 7. Morbidity and mortality in WT and IL-10 KO mice infected with NSV. WT and IL-10 KO mice were infected with 1x10^5 PFU of NSV intranasally and monitored once daily for the appearance of clinical signs and death.  A) Morbidity in WT and IL-10 KO mice during NSV infection. Scoring was as follows: 0) no clinical symptoms, 1) abnormal hind-limb and tail posture, ruffled fur, hunched back, 2) unilateral hind-limb paralysis, 3) bilateral hind-limb paralysis or full body paralysis, and 4) death. Data are presented as the mean ± SEM of the clinical scores from 3 independent experiments with a total n= 27 for WT and n=36 for IL-10 KO mice. Statistics were done using a 2-way ANOVA analysis for differences during the course of infection, as well as Bonferroni post-tests to determine significant differences between groups at each time point. ****p<0.0001. B) Survival of WT and IL-10 KO mice during NSV infection. Survival is represented as a Kaplan-Meier curve and statistics were calculated using the log-rank test. Data are pooled from 3 independent experiments with an n=35 for WT and n=37 for IL-10 KO
mice. The mean day of death in WT mice was 10 days post infection compared to 8 days post infection in IL-10 KO mice, ****p<0.0001. The data for WT mice are re-presented from the introduction section (Figure 3).
**Figure 8. Virus replication in the brains and spinal cords of WT and IL-10 KO mice during NSV infection.** Mice were infected intranasally with 1x10⁵ PFU of NSV. Tissue was collected and snap frozen through 7 days post infection. Infectious virus titers were determined by plaque formation using serial dilutions of 10% (w/v) clarified A) brain and B) spinal cord homogenates plated on a BHK-21 monolayer. The data are representative of 3 independent experiments with a total of n=6-9 for each time point. Statistical analysis was done using a 2-way ANOVA to determine the differences between groups during the course of infection and Bonferroni post-tests to determine significant differences between groups at each time point. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001. The data for WT mice are represented from the introduction section (Figure 3).
Figure 9. Total number of cells infiltrates isolated from peripheral and CNS tissue of WT and IL-10 KO mice during NSV infection.

Mice were infected intranasally with 1x10^5 PFU of NSV. A) Cervical lymph nodes, B) brains, and C) spinal cords were collected from uninfected and infected mice at 3, 5, and 7 days post infection for WT and IL-10 KO mice. Single-cell suspensions were made and total live cells were counted using trypan blue exclusion. Data are presented as the mean ± SEM of 1-4 independent experiments containing n=5 pooled cervical lymph nodes, and n=6-10 brains and spinal cords. Statistics were calculated using a 2-way ANOVA for differences during the course of infection and Bonferroni post-tests to determine differences between groups at each time point. *p<0.05.
Figure 10. Characterization of cells in the cervical lymph nodes of **WT and IL-10 KO mice during NSV infection.** Mice were infected intranasally with $1 \times 10^5$ PFU of NSV. Cells were isolated from pooled cervical lymph nodes (n=5) from uninfected and infected mice at 3, 5, and 7 days post infection for WT and IL-10 KO mice. A) Macrophage/monocytes (CD45$^+$CD11b$^+$Ly6G$^-$Ly6C$^+$), neutrophils (CD45$^+$CD11b$^+$Ly6G$^+$Ly6C$^+$), natural killer cells (CD45$^+$CD11b$^+$CD3$^-$,
NK1.1+), and T cells (CD3+) were identified. B) CD4 T cells (CD3+CD4+), CD8 T cells (CD3+CD8+), γδT cells (CD3+γδTCR+), and NKT cells (CD45+CD3+NK1.1+) were identified. Data are presented as the mean ± SEM for 1-4 independent experiments. Statistics were calculated using a 2-way ANOVA to determine differences during the course of infection with Bonferroni post-tests to determine differences between groups at each time point, *p<0.05, **p<0.01, and ***p<0.001.
Figure 11. Characterization of cellular infiltrates in the brains of WT and IL-10 KO mice during NSV infection. Mice were infected intranasally with 1x10^5 PFU of NSV. Cells were isolated from pooled brains (n=6-10) from uninfected and infected mice at 3, 5, and 7 days post infection for WT and IL-10 KO mice. A) Major cell types were identified as microglia (CD45^loCD11b^Ly6G^-Ly6C^-), macrophage/monocytes (CD45^hiCD11b^Ly6G^Ly6C^+), neutrophils
(CD45^CD11b^Ly6G^Ly6C^-), NK cells (CD45^CD11b^CD3^NK1.1^-), and T cells (CD3^-). B) Major T cell subsets were identified as CD4^ T cells (CD3^-CD4^-), CD8^ T cells (CD3^-CD8^-), γδ T cells (CD3^-γδTCR^-), and NKT cells (CD45^-CD3^-NK1.1^-). All data are plotted as the mean ± SEM for 1-4 independent experiments. Statistics were calculated using a 2-way ANOVA to determine differences between groups during the course of infection with Bonferroni post-tests to determine differences between groups at each time point, *p<0.05, **p<0.01, and ***p<0.001.
Figure 12. Characterization of cellular infiltrates in the spinal cords of WT and IL-10 KO mice during NSV infection. Mice were infected intranasally with 1x10^5 PFU of NSV. Cells were isolated from pooled brains (n=6-10) from uninfected and infected mice at 3, 5, and 7 days post infection for WT and IL-10 KO mice. A) Major cell types were identified as microglia (CD45^loCD11b^+Ly6G^-Ly6C^-), macrophage/monocytes (CD45^hiCD11b^+Ly6G^-Ly6C^+), neutrophils
(CD45+CD11b+Ly6G+Ly6C+), NK cells (CD45+CD11b+CD3-NK1.1+), and T cells (CD3+). B) Major T cell subsets were identified as CD4+ T cells (CD3+CD4+), CD8+ T cells (CD3+CD8+), γδT cells (CD3+γδTCR+), and NKT cells (CD45+CD3+NK1.1+). All data are plotted as the mean ± SEM for 1-4 independent experiments. Statistics were calculated using a 2-way ANOVA to determine differences between groups during the course of infection with Bonferroni post-tests to determine differences between groups at each time point, *p<0.05, **p<0.01, and ***p<0.001.
**Figure 13. Pro-inflammatory cytokine and chemokine gene expression in brains of WT and IL-10 KO mice during NSV infection.**

Mice were infected intranasally with 1x10⁵ PFU of NSV and brain tissue was collected from uninfected and infected WT and IL-10 KO mice during infection and snap frozen. RNA was isolated and mRNA expression was determined using TaqMan Gene Expression Arrays. The data are presented as the mean ± SEM pooled from 2 independent experiments with a total n=4-6 mice. Statistics were done using a 2-way ANOVA to determine the overall differences between groups during the course of infection and Bonferroni post-tests to determine the differences between
groups at each time point, *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.
Figure 14. **Pro-inflammatory cytokine and chemokine gene expression profiles in spinal cords of WT and IL-10 KO mice during NSV infection.** Mice were infected intranasally with 1x10^5 PFU of NSV and spinal cord tissue was collected from uninfected and infected WT and IL-10 KO mice during infection and snap frozen. RNA was isolated from tissue homogenates and mRNA expression was determined using TaqMan Gene Expression Arrays. The data are presented as the mean ± SEM from 2 independent experiments with a total n=4-6 mice. Statistics were done using a 2-way ANOVA to determine the overall differences between groups during the course of infection and Bonferroni post-tests.
to determine the differences between groups at each time point, *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.
Figure 15. CD8+ T cell function as measured by effector molecule production by cells in the cervical lymph nodes of WT and IL-10 KO mice during NSV infection. Mice were infected intranasally with 1x10^5 PFU of NSV. Cells were isolated from pooled cervical lymph nodes (n=5) from WT and IL-10 KO mice at A) 5 and B) 7 days post infection. CD8+ T cell function was assessed by flow cytometry and intracellular cytokine staining for the production of IFNγ, TNFα, and granzyme B after ex vivo stimulation with PMA/ionomycin. Flow plots and histograms are representative of 3 independent experiments. Data represent the mean ± SEM of 3 independent experiments. Statistics were calculated using unpaired, two-tailed, Student’s t tests with a 95% confidence interval, *p<0.05, *p<0.01, ***p<0.001, and ****p<0.0001.
**Figure 16. CD8+ T cell function as measured by effector molecule production by cells in the brains of WT and IL-10 KO mice during NSV infection.** Mice were infected intranasally with 1x10^5 PFU of NSV. Cells were isolated from pooled brains (n=6-10) from WT and IL-10 KO mice at A) 5 and B) 7 days post infection. CD8+ T cell function was assessed by the production of IFNγ, TNFα, and granzyme B after ex vivo stimulation using PMA/ionomycin using flow cytometry and intracellular cytokine staining. Flow plots and histograms are representative of 3 independent experiments. Data represent the mean ± SEM of 3 independent experiments. Statistics were calculated using unpaired, two-tailed Student’s t tests with a 95% confidence interval, *p<0.05, *p<0.01, ***p<0.001, and ****p<0.0001.
Figure 17. CD8+ T cell function as measured by effector molecule production by cells in the spinal cords of WT and IL-10 KO mice during NSV infection. Mice were infected intranasally with 1x10^5 PFU of NSV. Cells were isolated from pooled spinal cords (n=6-10) from WT and IL-10 KO mice at A) 7 days post infection. CD8+ T cell function was assessed by the production of IFNγ, TNFα, and granzyme B after ex vivo stimulation with PMA/ionomycin using flow cytometry and intracellular cytokine staining. Flow plots and histograms are representative of 3 independent experiments. Data represent the mean ± SEM of 3 independent experiments. Statistics were calculated using unpaired,
two-tailed Student’s t tests with a 95% confidence interval, *p<0.05, *p<0.01, ***p<0.001, and ****p<0.0001.
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<td>Granzyme B</td>
<td>89.4%</td>
<td>86.4%</td>
</tr>
<tr>
<td>Spinal Cord</td>
<td>IFNγ</td>
<td></td>
<td>78.0%</td>
</tr>
<tr>
<td></td>
<td>TNFα</td>
<td></td>
<td>30.3%</td>
</tr>
<tr>
<td></td>
<td>Granzyme B</td>
<td></td>
<td>92.3%</td>
</tr>
</tbody>
</table>

**Table 1.** Effector molecule expression by CD8⁺ T cells in the cervical lymph nodes, brains, and spinal cords of WT mice during *NSV* infection. The frequency of CD8⁺ T cells that express IFNγ, TNFα, and granzyme B at 5 and 7 days post infection in the cervical lymph nodes, brains, and spinal cords of WT mice. Data are presented as the mean percentage of CD8⁺ T cells that are positive for each marker from 2-3 independent experiments.
Figure 18. Th1 and Th17 responses at the onset of clinical symptoms, 5 days post infection, in the cervical lymph nodes and brains of WT and IL-10 KO mice. Mice were infected intranasally with 1x10^5 PFU of NSV. Cells were isolated from pooled A) cervical lymph nodes (n=5) and B) brains (n=6-10) from WT and IL-10 KO mice at 5 days
post infection. The Th1 and Th17 responses during NSV infection were evaluated by stimulating cells isolated from infected animals ex vivo with PMA/ionomycin for 4 hours and looking at the production of IFNγ (Th1) and IL-17a (Th17) in CD4+ T cells by flow cytometry at 5 days post infection. The data are presented as the mean ± SEM from 3 independent experiments. Statistics were calculated using an unpaired, two-tailed Student's t test with a 95% confidence interval, *p<0.05 and **p<0.01.
Figure 19. **Th1 and Th17 responses at 7 days post infection in the cervical lymph nodes, brains, and spinal cords of WT and IL-10 KO mice.** Mice were infected intranasally with 1x10^5 PFU of NSV. Cells were isolated from pooled A) cervical lymph nodes (n=5) and B) brains (n=6-10), and C) spinal cords (n=6-10) from WT and IL-10 KO mice at 7 days post infection. The Th1 and Th17 responses during NSV infection were evaluated by stimulating cells isolated from infected animals ex vivo with PMA/ionomycin for 4 hours and the production of IFNγ (Th1) and IL-17a (Th17) by CD4+ T cells using flow cytometry. The data are presented as the mean ± SEM from 3 independent experiments. Statistics were calculated using an unpaired, two-tailed Student’s t test with a 95% confidence interval, *p<0.05 and **p<0.01.
Chapter 2: Interleukin-10 restricts the pathogenic Th17 response to Neuroadapted Sindbis virus infection
**Introduction**

Sindbis virus (SINV) is the prototypical alphavirus and is closely related to the encephalitic alphaviruses VEEV and EEEV. SINV causes encephalomyelitis in mice, but mild disease in humans in the form of rash and arthritis (8, 9). The virulence of SINV for mice is dependent on the age of the animal, the genetic background of the animal, and the strain of virus used (11-16, 18, 19). Neuroadapted Sindbis virus (NSV) is the most lethal strain of SINV. NSV induces fatal, paralytic disease in adult mice on the C57Bl/6 background and serves as a model for fatal encephalomyelitis. Early studies in immune deficient mice have shown that the immune response is the primary mechanism of pathogenesis, not virus infection itself (161). In particular, T cells have been implicated as the major cell type responsible for immune-mediated damage (23). We have recently identified the presence of both Th1 and Th17 cells in the brains and spinal cords of NSV-infected mice (Chapter 1, unpublished); however the pathogenic potential of these cell types is unknown.

Interleukin-10 is an important regulator of T cell responses during infection and autoimmune disease (53, 85, 88, 89). We have previously shown that *Il10<sup>-/-</sup>* C57Bl/6 (IL-10 KO) mice have a specific increase in the Th17 response, with little effect on the Th1 response, compared to wild-type C57Bl/6 (WT) mice. This increase occurs coincident with the onset of clinical disease and is associated with accelerated morbidity and
mortality of IL-10 KO mice compared to WT mice, suggesting that the Th17 cells found during NSV infection are pathogenic (unpublished).

The development of Th17 cells in mice is dependent on a combination of IL-6, IL-1β, IL-23, and TGFβ (104, 106). Recently, it has been shown that TGFβ1 differentiates Th17 cells into a nonpathogenic phenotype characterized by upregulation of IL-10, whereas TGFβ3 promotes differentiation of pathogenic Th17 cells, which do not produce IL-10 (115). TGFβ3 expression is induced by IL-6 and maintained in Th17 cells via IL-23 signaling (115). IL-23 is important for sustaining and maturing Th17 cells and, in its absence, Th17 cells fail to develop (114). Another important cytokine involved in the enhancement and proliferation of Th17 cells is IL-21 (110-112) although its expression is not required (175, 176). Th17 cells are also the main producer of this cytokine, thus positively regulating their development (104, 111-113).

Th17 cells are characterized primarily by their expression of the cytokines IL-17A and IL-17F, as well as the Th17 lineage transcription factor RORγt (104). Th17 cells can be protective, such as in bacterial and fungal infections (116) or pathogenic, such as in autoimmune disorders (104, 114). In the context of the central nervous system (CNS), Th17 cells are often pathogenic, with the majority of research in this area focusing on their role during experimental autoimmune encephalomyelitis (EAE), the animal model for multiple sclerosis (MS).
Pathogenic Th17 cells are characterized by expression of a variety of transcription factors, cytokines, and receptors, but most notably by the expression of Tbet, IL-23R, GMCSF, IL-22, IFNγ, and granzyme B (115, 118-122, 124, 125, 177).

Tbet is classically known as a transcription factor characterizing Th1 cells; however, it has been shown to be important in EAE pathogenesis. Tbet-deficient (Tbx21⁻/⁻) mice are protected from developing EAE independent of IFNγ expression and signaling (178). Tbet is also expressed by pathogenic Th17 cells, but not nonpathogenic Th17 cells (115). The presence of IL-17a⁺IFNγ⁺CD4⁺ T cells, along with IL-17a⁺CD4⁺ T cells, has also been associated with pathogenesis in autoimmune disorders (179).

GMCSF is a potent marker of encephalitogenicity; in its absence, either by neutralization or genetic deficiency, mice are protected from developing EAE (133, 134). GMCSF is expressed primarily by T cells and is upregulated by IL-23 and RORγt (129, 130). The pathogenic function of GMCSF appears to primarily be its role in activation and recruitment of myeloid cells, including the activation of microglia (133).

IL-22 is produced primarily by differentiated Th17 cells and is dependent on IL-23 signaling (180). IL-22 acts on epithelial and endothelial cells to loosen tight junctions and allows for break down of the blood-brain barrier and cellular infiltration during EAE (181).
Granzyme B, a serine protease that is expressed by CD4+ and CD8+ T cells, is also a marker of pathogenic, but not nonpathogenic Th17 cells (115). Granzyme B is directly neurotoxic and can also break down extracellular matrix proteins allowing for cellular infiltration (179, 181, 182).

To determine if the Th17 cells found in WT mice are pathogenic and how IL-10 regulates the pathogenicity of Th17 cells during NSV infection, we characterized the phenotype of CD4+IL-17a+ T cells in WT and IL-10 KO mice at 5 days post infection, which is when the onset of clinical symptoms occurs and when the greatest difference in Th17 cell populations was observed.

**Materials and Methods**

*Animals and virus*

C57Bl/6J wild-type (WT) and B6.129P2-I10\textsuperscript{tm1Cgn}/J (C57Bl/6J IL-10 KO) mice were purchased from Jackson Laboratories and bred in house. Mice in all experiments were sex-matched and intranasally infected at 4-6 weeks of age with 1x10^5 PFU of neuroadapted Sindbis virus (NSV) diluted in HBSS. For tissue collection, mice were anesthetized with isoflurane and blood was collected via cardiac puncture. The animals were then perfused with ice-cold PBS and cervical lymph nodes, brains, and spinal cords were removed. All
experiments were performed according to guidelines approved by the Johns Hopkins University Institutional Animal Care and Use Committee.

**Mononuclear cell isolation**

Brains, spinal cords, and cervical lymph nodes were collected after perfusion from infected mice at 5 and 7 days post infection. For cervical lymph nodes, single-cell suspensions were made by pooling the lymph nodes from 5 mice per group in 5 mL of RPMI+1% FBS and homogenizing in C tubes using the GentleMACS system (Miltenyi) spleen program 1 for 2 runs. The cell suspension was then filtered through a 70 μm filter and centrifuged. Red blood cells were lysed using 2 mL of red blood cell lysis buffer (Sigma #R-7757) for 3 minutes and then washed with PBS + 2 mM EDTA. The cell pellet was resuspended in PBS + 2 mM EDTA and live cells were counted using trypan blue exclusion.

For the brain and spinal cord, tissue was collected from 6-8 mice per group and placed in HBSS on ice. Once all tissue was collected, up to 2 brains or 5 spinal cords were pooled in a C tube containing 4 mL of enzyme digest mix made up of RPMI + 1% FBS, 1 mg/mL collagenase D (Roche #11088858001), and 0.1 mg/mL DNase I (Roche #04536282001) and a gross dissociation was performed with scissors. The tissue was then run 2 times on the GentleMACS (Miltenyi) brain program 3, incubated for 15 minutes at 37°C with intermittent rocking, this process
was repeated once more, and then the samples were again homogenized with brain program 3. The homogenate was filtered using a 70 μm filter, rinsed with RPMI +1% FBS, and centrifuged at 1400rpm for 10 minutes. The pellet was resuspended in 30% percoll and under-layered with 70% percoll to form a 30/70% gradient with 1 brain or up to 2.5 spinal cords per 15mL conical tube. The gradient was spun for 30 minutes at 850xg at 4°C to remove myelin debris. The myelin coat in the top layer was aspirated off and the mononuclear cells at the interface were collected and washed with PBS + 2 mM EDTA. The pellet was resuspended in PBS + 2 mM EDTA and live cells counted using trypan blue exclusion.

**Intracellular cytokine staining**

For determination of T cell cytokine production, 2-3x10⁶ cells were stimulated with 50 ng/mL of phorbol-12-myristate 13-acetate (PMA) and 1 μg/mL of ionomycin in RPMI + 1% FBS in the presence of GolgiPlug (BD #555029) for 4 hours. Cells were then washed and stained with the violet live/dead marker (Invitrogen #L345955), blocked with rat anti-mouse CD16/CD32 Block (BD #553142), and then stained with antibody to the surface markers CD4-FITC (clone RM4-5, eBioscience #11-0042), and IL-23R-PE (clone 3C9, BD Bioscience #562648). After surface staining, cells were fixed and permeabilized using the BD CytoFix/CytoPerm kit (BD #554714) if antibodies to cytokines were in
the panel or the eBioscience Foxp3 Transcription Factor Fix/Perm kit (#00-5523-00) if the panel contained antibodies to transcription factors. Antibodies used for intracellular staining were IFN\(\gamma\)-PECy7 (clone XMG1.2, eBioscience #25-7311), IL-17a-APC (clone ebio17B7, eBioscience #17-7177), Tbet-PerCpCy5.5 (clone ebio4B10, eBioscience #45-5825), ROR\(\gamma\)t-PE (clone B2D, eBioscience #12-6981), GMCSF-PE (clone MP1-22E9, eBioscience #46-7221), Granzyme B-PerCPeFluor710 (clone NGZB, eBioscience #46-8898), and IL-22-PerCPeFluor710 (clone 1H8PWSR, eBioscience #46-7221). Cells were washed, suspended in PBS + 2 mM EDTA + 0.5% BSA, data were acquired using the FACS Canto II (BD Bioscience) and FACS Diva, and analyzed using FlowJo version 8.8.7 (TreeStar Inc.).

**Gene expression analysis using real-time PCR**

Snap frozen tissue was homogenized in Qiazol buffer and RNA was extracted using the RNeasy Lipid Mini RNA Isolation Kit (Qiagen #74804). RNA was quantified using a nanodrop spectrophotometer and cDNA was prepared using the High Capacity cDNA Reverse Transcription Kit (Life Technologies #4368814) and 2 \(\mu\)g of input RNA. Quantitative real-time PCR was performed using 2.5 \(\mu\)l of cDNA and TaqMan gene expression arrays in 2x Universal PCR Mastermix (Applied Biosystems, #4304437). The TaqMan gene expression arrays used were \(Il17a\), \(Il21\), \(Il22\), \(Csf2\),
Il23a, and Ccl20. Gapdh mRNA levels were determined using the rodent primer and probe set (Applied Biosystems, #4308313). All reactions were run on the Applied Biosystems 7500 Real-time PCR machine with the following conditions: 50°C for 2 minutes, 95°C for 10 minutes, 95°C for 15 seconds, and 60°C for 1 minute for 50 cycles. Transcript levels were determined by normalizing the target gene Ct value to the Ct value of the endogenous housekeeping gene Gapdh. This normalized value was used to calculate the fold-change relative to the average of the uninfected control (ΔΔCt method).

**Statistical analysis**

Data from three independent experiments or at least 3 mice per group was used. All statistical analysis was done using Graph Prism 5 (Graph Pad). The difference between groups during the course of infection was determined using a multi-variable 2-way ANOVA and Bonferroni post-tests to compare groups at each time point (cytokine analysis). Comparisons between groups at single time points were done using Student’s unpaired T test, two-tailed, 95% confidence interval.

**Results**

*Th17-related cytokine and chemokine mRNA expression was elevated in IL-10 KO mice during NSV infection*
Previously we have shown that mice infected with NSV develop a Th17 inflammatory response that is exacerbated in the absence of IL-10. To further investigate the role of Th17 cells, we determined the levels of Th17-related cytokines and chemokines during NSV infection and in the CNS of IL-10 KO mice compared to WT mice. In addition to \textit{Il17a} mRNA levels, we looked at the expression of the mRNAs for the differentiation cytokine, \textit{Il23a}, and the maintenance cytokine, \textit{Il21}, the Th17 effector cytokines \textit{Il22} and \textit{Csf2}, and the Th17 chemoattractant \textit{Ccl20} (104). \textit{Il17a, Il21, Il22, Csf2,} and \textit{Ccl20} mRNAs were all increased in the brains (Figure 20a) and spinal cords (Figure 20b) of WT and IL-10 KO mice during NSV infection. There was little upregulation of \textit{Il23a} mRNA in the brain and spinal cord during NSV infection (Figure 20a and 20b), which may be due to the limited expression and tight regulation of \textit{Il23a} transcription (183).

In the brain, only \textit{Il17a} mRNA levels were higher in IL-10 KO mice compared to WT animals over the course of infection (*p=0.0144) as well as at 7 days post infection (p<0.05) (Figure 20a), coincident with the largest difference in Th17 cells between WT and IL-10 KO mice (Chapter 1, Figure 18). Interestingly, \textit{Il22, Il23a,} and \textit{Ccl20} mRNAs were higher in the brains of WT mice compared to IL-10 KO mice during infection (****p<0.0001, **p=0.0039, and *p=0.0270, respectively, Figure 20a) even though fewer Th17 cells are present in these animals.
In the spinal cord, levels of *Il17a*, *Il22*, and *Ccl20* mRNAs were higher over the course of infection in IL-10 KO mice compared to WT mice (**p=0.0095, ****p<0.0001, and *p=0.0126 respectively, Figure 20b). In particular, *Il17a*, *Il22*, and *Ccl20* were significantly higher at 7 days post infection (**p<0.001, ***p<0.001, and **p<0.001 respectively, Figure 20b) when there are more Th17 cells in the spinal cords of IL-10 KO mice compared to WT mice. Interestingly, the induction of *Csf2* and *Il22* gene expression was greater in the spinal cord than in the brain (~50-fold vs. 400-fold and ~8-fold vs. 35-fold, respectively), whereas the induction of *Il21* was higher in the brain than in the spinal cord (~30-fold vs. ~10-fold) (Figure 20a and 20b), suggesting that the Th17 cells infiltrating the spinal cord may be different than those in the brain. These data show that Th17 cell recruitment, differentiation, and effector molecule expression is occurring during NSV infection of the CNS.

*Th1/Th17 cells are increased, along with Th17 cells, in the brains of IL-10 KO mice at the onset of disease*

Th1 and Th17 cells are both considered to have pathogenic potential within the CNS, particularly in models of EAE, although the relative contributions of each are somewhat disputed (178, 184, 185). A population of Th17 cells that express IFNγ, as well as IL-17a, have been identified in a variety of autoimmunity models (179, 186, 187). To
determine if this population exists during NSV infection, we looked at IFNγ and IL-17a production in CD4+ cells. At 5 days post infection in the cervical lymph nodes, where the population of CD4+IL-17a+ cells is small, there are very few IFNγ+IL-17a+ in WT or IL-10 KO mice. In the brains of NSV-infected mice, WT mice have predominantly IFNγ+ or IL-17a+ single positive CD4+ T cells, with very few double-positive cells (Figure 21a). In the absence of IL-10, however, we see a dramatic increase in the IL-17a+ single-positive population (2.53% WT mice vs. 8.98% IL-10 KO mice, ***p=0.0003, Figure 21a) and also in the IFNγ+IL-17a+ double-positive population (0.46% WT vs. 4.28% IL-10 KO, *p=0.0262, Figure 21a). Again, the IFNγ+ single-positive population is similar in the brains of WT mice and IL-10 KO mice (37.63% WT vs. 34.40% IL-10 KO) (Figure 21a). This expansion of the IFNγ+IL-17a+ population specifically in the CNS and not the periphery, was also seen in EAE (187). By 7 days post infection the Th1 response increases and the difference in the numbers of IL-17a+ and IFNγ+IL-17a+ cells in brains of WT and IL-10 KO mice is no longer significantly different (Figure 22a), although a trend remains. These data suggest that IL-10 regulates the development of Th17 and Th1/Th17 cells at the site of infection or inflammation and that this exaggerated response may lead to accelerated disease in IL-10 KO mice during NSV infection.
**Th17 and Th1/Th17 cells express Tbet and RORγt**

The canonical transcription factors dictating Th1 and Th17 cells are Tbet and RORγt, respectively; however, there is some plasticity in this. A marker of Th17 pathogenicity in EAE is the expression of Tbet (115, 122, 178). Also, Th1/17 cells are able to produce IFNγ by Tbet-dependent and -independent mechanisms (187). Because both of these transcription factors are important in Th17 lineage and CD4+ T cells that are IFNγ+IL-17a+ are present in the brain, we determined the pattern of transcription factor expression in IFNγ+, IL-17a+, and IFNγ+IL-17a+ cells in WT and IL-10 KO mice. At 5 days post infection in the cervical lymph nodes, there is no difference in the magnitude of the Th1 and Th17 response, so we focused on the CD4+ T cells in the brain. The IFNγ+ T cells are Tbet, but not RORγt, as would be expected for Th1 cells (Figure 21b). The IL-17a+ T cells express both Tbet and RORγt, which suggests that they are pathogenic Th17 cells (Figure 21b). The IFNγ+IL-17a+ T cells also express Tbet and RORγt much like the IL-17a+ only population (Figure 21b). Interestingly, the amount of Tbet expression in IFNγ+IL-17a+ T cells was slightly increased compared to cells that only express IL-17a. No differences in transcription factor expression patterns were observed in the IFNγ+ T cells or IL-17a+ T cells in the brains of WT and IL-10 KO mice (Figure 21b).
Preliminary studies at 7 days post infection showed that Th1 cells continue to express primarily Tbet, and that Th17 and Th1/Th17 cells express both Tbet and RORγt (Figure 22b). These data show that the Th17 and Th1/Th17 populations express RORγt, a marker of Th17 lineage differentiation, as well as the Th1 and pathogenic Th17 marker, Tbet suggesting that the Th17 cells found in the CNS of WT and IL-10 KO mice, as well as the Th1/17 cells found in the CNS of IL-10 KO mice are pathogenic.

CD4+IL-17a+ cells in the cervical lymph nodes and brains at the onset of disease exhibit a pathogenic phenotype

The pathogenicity of Th17 cells has been most extensively studied in EAE, but few studies have examined a role for Th17 cells during viral infections. An increase in the Th17 population is seen in IL-10 KO mice infected with Leishmania (89) and influenza virus (88); however, these studies did not characterize or compare the phenotypes of Th17 cells present in WT and IL-10 KO mice. In EAE, Tbet, GMCSF, IL-22, granzyme B, and IL-23R receptor expression are all associated with a pathogenic Th17 phenotype (104, 115). The Th17 cells present in WT and IL-10 KO mice express Tbet and some Th17 cells in IL-10 KO mice also express IFNγ (Figure 23 and 24). To further characterize the pathogenic phenotype, we used flow cytometry and intracellular cytokine
staining to examine surface expression of IL-23R and production of GMCSF, IL-22, and granzyme B in Th17 cells in the cervical lymph node and brain of NSV-infected WT and IL-10 KO mice at the onset of clinical disease. At 5 days post infection Th17 cells in the cervical lymph nodes and brains of WT and IL-10 KO mice displayed a pathogenic phenotype. The majority of Th17 cells in the cervical lymph nodes expressed granzyme B in WT (70.2%) and IL-10 KO (63.6%) (Figure 23a,b) resulting in similar numbers of Th17 cells that are granzyme B+ in WT (1.3x10^5) and IL-10 KO mice (5.4x10^5) (Figure 23a,b). However, in the brain at this time point, a significantly higher proportion of Th17 cells express granzyme B in WT mice (23%) compared to IL-10 KO mice (10.8%, *p=0.0282, Figure 24a,b). Although the frequency of granzyme B+ Th17 cells was less in IL-10 KO, the number was significantly higher due to higher numbers of Th17 cells in IL-10 KO compared to WT mice (646 vs. 79, *p=0.0328, Figure 23a,b). Interestingly, granzyme B expression in Th17 cells was generally higher in the cervical lymph nodes compared to the brains at 5 days post infection (Figure 22a,b and 23a,b).

Next we looked at IL-22 production by Th17 cells. There was a similar frequency of Th17 cells expressing IL-22 between WT (6.6%) and IL-10 KO mice (6.9%) in the cervical lymph nodes at 5 days post infection (Figure 22a,b) resulting in similar numbers of Th17 cells expressing IL-22 in WT mice (7.5x10^4 cells) and IL-10 KO mice (2.2x10^5 cells) (Figure
22a,b). In the brain, there was an increase in the frequency of IL-22+ Th17 cells in WT mice (41.5%) compared to IL-10 KO mice (22.6%), but this difference did not reach statistical significance (p=0.1039) (Figure 23a,b). Although the frequency of Th17 cells producing IL-22 tended to be higher in WT mice, the overall number of IL-22+IL-17a+ T cells was significantly higher in IL-10 KO mice due to the increased Th17 response (*p=0.0369) (Figure 23a,b). There was a higher frequency of IL-22+ Th17 cells in the brains of WT and IL-10 KO mice compared to the cervical lymph nodes for both groups (Figure 22a,b and 23a,b).

Next, we examined the expression of GMCSF in Th17 cells. In the cervical lymph nodes, there was a similar frequency of GMCSF+ Th17 cells between WT (15.7%) and IL-10 KO (23.8%) mice, which resulted in similar numbers of IL-17a+GMCSF+ T cells at this site (1.27x10^5 WT vs. 5.35x10^5 IL-10 KO) (Figure 22a,b). The amount of GMCSF produced by Th17 cells is similar in WT and IL-10 KO mice as determined by fluorescence intensity (Figure 22a,b). In the brain, however, there was a significant increase in the frequency of Th17 cells that are GMCSF+ in IL-10 KO mice (23.8%) compared to WT mice (15.7%, *p=0.0173, Figure 23a,b) and a higher number of GMCSF+IL-17a+ T cells in the brains of IL-10 KO mice at 5 days post infection (*p=0.0268, Figure 23a,b). Analysis of fluorescence intensity indicated a higher level of GMCSF production in Th17 cells of IL-10 KO mice (Figure 23a).
Using antibody to the IL-23R we were unable to detect the IL-23R protein on CD4+ T cells during NSV infection. Other investigators examining IL-23R expression on Th17 cells have looked at \textit{Il23r} mRNA expression, protein expression via immunostained Western blots of sorted Th17 cells, or IL-23R-eGFP reporter mice suggesting that surface staining with commercially available reagents may not be sensitive enough to detect surface expression (188-190). The expression of GMCSF, an IL-23 dependent cytokine (130), suggests that the IL-23R is expressed on these cells. Further investigation into IL-23R expression using more sensitive methods may be necessary.

Together, these data suggest that the Th17 cells infiltrating the CNS during NSV infection are pathogenic and may mediate neuronal death and fatal disease. Furthermore, the data suggest that granzyme B expression is downregulated in Th17 cells once they enter the CNS and IL-22 expression is upregulated at the site of infection in the Th17 cells found in WT and IL-10 KO mice. In the absence of IL-10, GMCSF expression was higher in the Th17 cell population suggesting that IL-10 may play a role in limiting GMCSF expression by Th17 cells.

**Discussion**

NSV infection causes a lethal, paralytic disease in C57Bl/6 mice. CD4+ and CD8+ T cells have previously been implicated as being primary
facilitators of immune-mediated pathogenesis; however, a mechanism for how these T cells are inducing disease remained unknown. Our studies have shown a Th1 and Th17 response with an exacerbated Th17, but not Th1, response in IL-10 KO mice, which correlated with accelerated disease progression and death. This suggested that the Th17 cells entering the CNS of mice infected with NSV might be pathogenic. We determined that the Th17 cells found in WT and IL-10 KO mice do exhibit a pathogenic phenotype similar to the pathogenic Th17 cells found during EAE. The CD4+IL-17a+ T cells found in the cervical lymph nodes of WT and IL-10 KO mice produced not only IL-17a, but also the Th17 effector cytokines IL-22, granzyme B, and GMCSF. These cells also expressed the Th17 lineage transcription factor, RORγt, as well as Tbet, another pathogenic marker of Th17 cells. This correlated with an increase in the mRNA levels of Il17a, Il21, Il22, Csf2, and Ccl20 in the brains and spinal cords of WT and IL-10 KO mice during the course of NSV infection relevant to various aspects of Th17 cell biology. Comparison of the profiles of Th17 cells showed a higher frequency of GMCSF+IL-17a+ T cells in IL-10 KO mice than WT mice, as well as higher levels of GMCSF expression in Th17 cells of IL-10 KO mice. There was also an IFNγ+IL-17a+ Th17/Th1 population in the brains of IL-10 KO mice, but not WT mice. These data support a pathogenic phenotype for
the Th17 cells infiltrating the CNS of WT and IL-10 KO mice during fatal NSV encephalomyelitis.

The effector function(s) of Th17 cells that cause disease is not clear. The Th17 cells found in the CNS of NSV-infected mice express IL-17a, IL-22, GMCSF, and granzyme B. The role that IL-17a plays in Th17-mediated pathogenesis is controversial. IL-17 has been reported to induce neuronal cell death (191); however, mice lacking IL-17 and the IL-17R have attenuated EAE, but are still susceptible to disease (126), which suggests a contribution, but not a requirement, for this cytokine for disease. GMCSF is a mediator of pathogenesis of Th17 cells. Mice lacking GMCSF (Csf2-/-) are completely protected from the induction of EAE (134). GMCSF is induced by IL-23 and exerts a positive feedback loop by inducing IL-23 expression as well as IL-6 expression to further enhance and sustain Th17 cells (129, 130, 134, 192). It also activates microglia and enhances myeloid cell recruitment (129-133). How these effects play out during NSV pathogenesis is unknown. Granzyme B is a serine protease that is typically thought of as a CD8+ cytotoxic lymphocyte effector molecule; however, CD4+ T cells can also express granzyme B. Granzyme B can induce neurotoxicity via activation of protease-activated G protein-coupled receptors (PARs) that are found on neurons (48, 127, 182). Granzyme B and IL-22 are also important mediators in disrupting the blood-brain barrier (181). Understanding the
important effector functions of Th17 cells during NSV infection is critical for identification of possible modulators of disease.

There was an expansion of the Th1/Th17 population during NSV infection in the absence of IL-10. IFNγ+IL-17a+CD4+ T cells have also been observed during EAE, MS, rheumatoid arthritis, and colitis and are associated with pathogenesis of disease (178, 186, 190). Consistent with previous observations in autoimmune diseases, the IFNγ+IL-17a+CD4+ T cells expressed the transcription factors RORγt and Tbet. Interestingly, the expansion of this population was specifically seen in the CNS, and not the cervical lymph nodes, which was also observed during EAE in which the population preferentially existed at the site of inflammation (187). Unlike EAE, however, these cells were only found in the absence of IL-10, suggesting that IL-10 is playing a regulatory role in limiting the Th1/Th17 phenotype during NSV infection. This may also be pathogen and/or tissue-specific because IL-10 KO mice infected with a lethal dose of influenza virus (88) or *Leishmania major* (89) showed no enhancement of an IL-17a+IFNγ+ CD4+ T cell population in any of the tissues examined. However, in a model of intestinal inflammation, IL-10 blockade led to an increase in the Th1/Th17 population (90).

Although an expansion of the Th17 population in the absence of IL-10 has been previously observed (88, 89), how IL-10 is restricting the development of this population is unclear. We previously looked at *Il12a*
and IL12b levels in the brains and spinal cords of WT and IL-10 KO mice and saw a very low-level of IL12a induction, whereas IL12b was expressed during infection. In this study, we also looked at IL23a levels and again induction of IL23a transcription were low after infection in both groups, similar to IL12a. A similar observation was made during gliatropic coronavirus infection, in which IL12b transcription peaked at 5 days post infection, but there was little to no induction of IL12a or IL23a transcription (154). Both IL12a and IL23a are ubiquitously expressed at low levels (183), so little detection of these cytokine mRNAs in whole tissue is not entirely surprising. Examination of IL-12 and IL-23 protein levels was unsuccessful. Looking at cell-type specific expression of IL12a, IL12b, and IL23a in CD11b+ cells isolated from the CNS of NSV infected mice, as was done during EAE (193), may yield better results.

Recent studies show that pathogenic Th17 cells in mice develop in the presence of TGFβ3, whereas nonpathogenic Th17 cells develop in the presence of TGFβ1 (115). Further investigation into the levels of these cytokines in WT and IL-10 KO mice during NSV infection may give more insight into the development and regulation of pathogenic Th17 cells in the CNS.

Th17 and IL-17 producing cells have been identified in a variety of viral infections; however, the contribution of this cell type to disease, particularly in comparison with Th1 cells, has rarely been investigated.
Using genetic models in which the differentiation of Th1 (Il12p35/−), Th17 (Il23p19/−), and both T helper subsets (Il12p40/−), have been used to deduce the relative importance of these cells in EAE will help to delineate the contribution of each cell type to NSV pathogenesis in future studies. In models of EAE Il12p35/− mice are susceptible to EAE, whereas Il23p19/− mice are resistant (114). During JMHV infection, a gliotropic coronavirus, Il23p19/− mice were as susceptible as WT mice to disease, whereas Il12p35/− and Il12p40/− mice were resistant, suggesting a dominant role for Th1 cells, rather than Th17 cells, during JMHV pathogenesis (154). During JMHV infection, however, there are fewer Th17 cells present in the CNS than are present during NSV infection. Furthermore, NSV infects neurons, whereas JMHV infects glial cells, which may also alter the mechanisms of pathogenesis. Future investigations into this subject will clarify the necessary mediators of CD4+ T cell pathogenesis.

In conclusion, we have determined that the Th17 cells found in the brains of NSV-infected mice have a pathogenic phenotype. The virus-infected CNS supports the recruitment and development of Th17 cells, and effector molecule expression occurs at the site of infection. Furthermore, in the absence of IL-10, these cells also expand into a Th1/Th17 population and the Th17 cells express higher levels of
GMCSF, which may contribute to the accelerated pathogenesis observed in IL-10 KO mice.
Figure 20. Th17-related cytokine and chemokine gene expression in the brains and spinal cords of WT and IL-10 KO mice during NSV infection. Mice were infected intranasally with 1x10^5 PFU of NSV and A) brain and B) spinal cord tissue was collected from uninfected and
infected WT and IL-10 KO mice during infection. RNA was isolated and mRNA expression was determined using TaqMan Gene Expression Arrays. The data are presented as the mean ± SEM from 2 independent experiments with a total n=4-6 mice/group at each time point. Statistics were done using a 2-way ANOVA to determine the overall differences between groups during the course of infection and Bonferroni post-tests to determine the differences between groups at each time point, *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.
Figure 21. Th1, Th17, and Th1/17 cells and transcription factor expression patterns in the cervical lymph nodes and brains of WT
**and IL-10 KO mice at 5 days post infection.** Mice were infected intranasally with 1x10^5 PFU of NSV. Mononuclear cells were isolated from pooled cervical lymph nodes (n=5) and brains (n=6-8) from WT and IL-10 KO mice at 5 days post infection. A) IFNγ and IL-17a production and B) Tbet and RORγt expression by CD4⁺ T cells was assessed by flow cytometry after stimulating the mononuclear cells with PMA/ionomycin for 4 hours and staining for surface and intracellular markers. Histograms show the intensity of expression in cells from WT (blue) and IL-10 KO (orange) compared to an isotype control (grey). Flow plots are representative of 3 independent experiments. The data are presented as the mean ± SEM from 3 independent experiments. Statistics of analysis used a Student’s unpaired, two-tailed t test with a 95% confidence interval, *p<0.05 and ***p<0.001.
Figure 22. Th1, Th17, and Th1/17 cells and transcription factor expression patterns in the brains and cervical lymph nodes of WT and IL-10 KO mice at 7 days post infection. Mice were infected intranasally with 1x10^5 PFU of NSV. Mononuclear cells were isolated from pooled cervical lymph nodes (n=5) and brains (n=6-8) from WT and IL-10 KO mice at 5 days post infection. A) IFNγ and IL-17a production and B) Tbet and RORγt expression by CD4+ T cells was assessed by flow cytometry after stimulating the mononuclear cells with PMA/ionomycin for 4 hours and staining for surface and intracellular markers. Histograms show the intensity of expression in cells from WT (blue) and IL-10 KO (orange) compared to an isotype control (grey). For IFNγ and IL-17a producing T cells, flow plots are representative of 3 independent experiments and the data are presented as the mean ± SEM from 3 independent experiments. Statistics were calculated using Student’s unpaired, two-tailed t test with a 95% confidence interval. Flow plots for Tbet and RORγt expression are representative of 1 experiment.
Figure 23. Characterization of pathogenic Th17 cells in the cervical lymph nodes of WT and IL-10 KO mice at 5 days post infection. Mice were infected intranasally with $1 \times 10^5$ PFU of NSV.
Mononuclear cells were isolated from pooled cervical lymph nodes (n=5) from WT and IL-10 KO mice at 5 days post infection. Isolated mononuclear cells from infected animals were stimulated ex vivo with PMA/ionomycin for 4 hours and stained for surface and intracellular markers. A) GMCSF, granzyme B, and IL-22 expression was assessed in IL-17a+ CD4+ T cells. The data are representative of 3 independent experiments. Histograms show the relative intensity of expression between WT (blue) and IL-10 KO (orange) mice compared to an isotype (grey). B) The frequency and number of IL-17a+ cells expressing GMCSF, granzyme B, and IL-22 frequency of IL-17a+ cells in WT (black) and IL-10 KO (white) mice. Data are presented as the mean ± SEM from 3 independent experiments. Statistics were calculated using Student’s unpaired, two tailed t test with a 95% confidence interval.
Figure 24. Characterization of pathogenic Th17 cells in the brains of WT and IL-10 KO mice at 5 days post infection. Mice were infected
intranasally with 1x10^5 PFU of NSV. Mononuclear cells were isolated from pooled brains (n=6-8) from WT and IL-10 KO mice at 5 days post infection. Isolated mononuclear cells from infected animals were stimulated ex vivo with PMA/ionomycin for 4 hours and stained for surface and intracellular markers. A) GMCSF, granzyme B, and IL-22 expression was assessed in IL-17a^+ CD4^+ T cells. The data are representative of 3 independent experiments. Histograms show the relative intensity of expression between WT (blue) and IL-10 KO (orange) mice compared to an isotype (grey). B) The number and frequency of IL-17a^+ cells expressing GMCSF, granzyme B, and IL-22 in WT (black) and IL-10 KO (white) mice. Data are presented as the mean ± SEM from 3 independent experiments. Statistics were calculated using Student’s unpaired, two tailed t test with a 95% confidence interval, *p<0.05.
Chapter 3: Identification of the cellular sources of interleukin-10 and the cellular targets for interleukin-10 during Neuroadapted Sindbis virus infection
Introduction

Encephalitic arboviruses are an emerging cause of morbidity and mortality worldwide. The alphaviruses belong to this class of viruses. Sindbis virus (SINV) is the prototypical alphavirus and is closely related to Venezuelan equine encephalitis (VEE) and eastern equine encephalitis (EEE) viruses that cause encephalitis in humans. Although Sindbis causes mild disease in the form of rash and arthritis in humans, it induces encephalomyelitis in mice due to its tropism for neurons (8, 9). The virulence of SINV is dependent on the age of the animal, the strain of virus used, and the genetic background of the animal (11-16, 18, 19). Neuroadapted Sindbis virus (NSV) is the most virulent strain and is typically 100% lethal in adult C57Bl/6 mice. NSV pathogenesis is mediated by the immune response and we have recently identified the presence of pathogenic Th17 cells in the CNS of infected mice at the onset of paralysis (Chapter 2).

The Th17 cells induced during NSV infection display a pathogenic phenotype. This includes the expression of effector cytokines such as GMCSF and granzyme B, as well as presence of factors in the brain necessary for recruitment and development including CCL20 and IL-21 (Chapter 2). To determine possible mechanisms of regulation, we showed that IL-10 restricts the development of Th17 cells and, in the absence of IL-10, Th17 cells, but not Th1 cells, increase in the CNS of NSV-infected
mice at the onset of clinical disease. This amplified Th17 response in IL-10 KO mice results in accelerated morbidity during the T cell phase, which also correlated with earlier death compared to WT mice (Chapter 1). Interestingly, we also saw an increase in Th1-Th17 cells in the CNS of IL-10 KO mice, suggesting that IL-10 also plays a role in regulating IFNγ in the context of Th17 cells, but not in Th1 cells (Chapter 2).

Although IL-10 has effects on regulation of Th17 development, it is unknown what cell types are producing IL-10 during NSV infection and which of these sources is important in restricting Th17 development in the context of NSV infection. A wide variety of immune cells are capable of producing IL-10 including macrophages, dendritic cells, neutrophils, microglia, NK cells, CD4+ T cells, CD8+ T cells, and B cells (53). In other models of viral encephalitis, particularly coronavirus-induced encephalitis, T cells are the primary producers of IL-10 (171, 172). During the acute phase of JHMV infection, CD4+ T cells show greater IL-10 production than CD8+ T cells. Furthermore, the majority of the IL-10-producing cells were CD4+CD25+ suggesting that they were regulatory T cells (171). Studies of infection with the J2.2 strain of MHV showed that the IL-10+CD8+ T cells were more cytolytic than the IL-10-CD8+ T cells, but conferred some protection from disease, although mortality was the same in the presence and absence of IL-10 producing CD8+ T cells (172). Interestingly, infection with a virus exogenously expressing IL-10 at the
site of infection showed the protective function of IL-10 in the CNS during J2.2 infection occurred at or before 5 days post infection. This suggests that even though T cells are the primary producers of IL-10, there might be a role for an earlier source of IL-10 in mediating protection (194). Neither of these studies, however, examined the presence of CD4+IL-17a+ T cells.

The IL-10R consists of two subunits, IL-10Rα and IL-10Rβ. IL-10Rα contains the ligand-binding domain for IL-10 and is required for its signaling activity (54, 55). The IL-10R is expressed on most hematopoietic cells, although at low levels, and can also be found on other cell types such as fibroblasts and epithelial cells (53-60). Signaling through the IL-10R activates the Jak/STAT pathway to promote the regulatory effects of the cytokine, which occur primarily at the transcriptional level (65).

IL-10 regulation is typically thought to occur via effects on antigen presenting cells, such as macrophages, monocytes, and dendritic cells or in the case of the CNS, microglia and astrocytes (53). These effects result in downregulation of factors promoting T cell activation, thus restricting the T cell response. IL-10 has, however, also acts directly on T cells to inhibit cytokine production (53).

Of most interest are recent studies focusing on the role of regulatory T cell-derived IL-10 and its ability to inhibit Th1 and Th17
responses. Regulatory Tr1 cells are CD25+foxp3- and express IFNγ and the Th1 transcription factor Tbet, as well as IL-10. Conventional regulatory T cells (Tregs) are CD25+foxp3+ and express the transcription factors STAT3 and foxp3 as well as IL-10. Tregs express the IL-10R and amplify the regulatory cues from IL-10 via STAT3 activation, which results in the inhibition of the Th17 cell response specifically, with no effect on the Th1 response (195). Furthermore, Th17 cells express the IL-10R and receptor expression was required for both Tr1- and Treg-mediated suppression of this population and restriction of disease development in a colitis model (196).

There are two primary hypotheses for how IL-10 is regulating Th17 cell development during NSV infection. It could be that regulatory T cells are responding to the initial IL-10 production, further amplifying this signal, and being licensed to inhibit Th17 cells directly via IL-10 production (195, 196). Another option is that IL-10 produced by myeloid cells or T cells is regulating the levels of Th1 and Th17 developmental factors by acting on monocytes or microglia found in the CNS during infection and controlling IL-12 and IL-23 production. To determine the important source and target of IL-10 for regulating the pathogenic Th17 response to NSV infection, we first determined which cell types are producing IL-10 using an IL-10 mRNA reporter mouse, VertX (197, 198). Not surprisingly, T cells were found to be the primary contributor to IL-
production, with minimal expression detected in myeloid cells. We also identified the presence of Tr1 cells and Tregs in the CNS at the onset of clinical disease when the Th17 response is exacerbated the most. Identification of cell expressing the IL-10R at 5 days post infection indicated that all cells express the receptor, but the levels of expression differ, most notably with Th1 cells expressing higher levels of the IL-10R than Th17 cells. These data lay the foundation for testing IL-10 and IL-10R conditional knockout mice to determine the functional role of cell type-specific expression of these molecules in regulating the pathogenic Th17 response during NSV infection.

Materials and Methods

Animals and virus

C57Bl/6J wild-type (WT) and B6.129P2-Il10tm1Cgn/J (C57Bl/6J IL-10 KO) mice were purchased from Jackson Laboratories and bred in house. C57Bl/6 mice that were engineered to express eGFP under control of the Il10 promoter (VertX, (197, 198)) were kindly provided by Christopher Karp at Cincinnati Children’s Hospital and have since been made commercially available from Jackson Laboratories (Bar Harbor, Maine). Mice in all experiments were sex-matched and intranasally infected at 4-6 weeks of age with 1x10^5 PFU of neuroadapted Sindbis virus (NSV) diluted in HBSS. For tissue collection, mice were
anesthetized with isoflurane and blood was collected via cardiac puncture. The animals were then perfused with ice-cold PBS and cervical lymph nodes, brains, and spinal cords were removed and used fresh or snap frozen and stored at -80°C. All experiments were performed according to guidelines approved by the Johns Hopkins University Institutional Animal Care and Use Committee.

**Mononuclear cell isolation**

Brains, spinal cords, and cervical lymph nodes were collected after perfusion from infected mice at 5 and 7 days post infection. For cervical lymph nodes, single-cell suspensions were made by pooling the lymph nodes from 5 mice per group in 5 mL of RPMI+1% FBS and homogenizing in C tubes using the GentleMACS system (Miltenyi) spleen program 1 for 2 runs. The suspension was then filtered through a 70 μm filter and centrifuged. Red blood cells were lysed using 2 mL of red blood cell lysis buffer (Sigma #R-7757) for 3 minutes, washed with PBS + 2 mM EDTA, and resuspended in PBS + 2 mM EDTA. Viable cells were counted using trypan blue exclusion.

Brain and spinal cord tissues were collected from 6-8 mice per group and placed in HBSS on ice. Once all tissue was collected, up to 2 brains or 5 spinal cords were pooled in a C tube containing 4mL of enzyme digest mix made up of RPMI + 1% FBS, 1mg/mL collagenase D
(Roche #11088580001), and 0.1 mg/mL DNase I (Roche #04536282001) and a gross dissociation was performed with scissors. The tissue was then run 2 times on the GentleMACS (Miltenyi) brain program 3, incubated for 15 minutes at 37°C with intermittent rocking, this process was repeated, and then the samples were homogenized once more using one run on brain program 3. The homogenate was filtered through a 70 μm filter, rinsed with RPMI +1% FBS, and centrifuged at 1400rpm for 10 minutes. The pellet was resuspended in 30% percoll and under-layered with 70% percoll to form a 30/70% gradient with 1 brain or up to 2.5 spinal cords per 15mL conical tube. The gradient was spun for 30 minutes at 850xg at 4°C to remove myelin debris. The myelin coat in the top layer was aspirated off and the mononuclear cells at the interface were collected, washed with PBS + 2 mM EDTA, and resuspended in PBS + 2 mM EDTA. Viable cells were counted using trypan blue exclusion.

**Intracellular cytokine staining**

For determination of T cell cytokine production, 2-3x10^6 cells were stimulated with 50ng/mL of phorbol-12-myristate 13-acetate (PMA) and 1 μg/mL of ionomycin in RPMI + 1% FBS in the presence of GolgiPlug (BD #555029) for 4 hours. Cells were then washed and stained with the violet live/dead marker (Invitrogen #L345955), blocked with rat anti-mouse CD16/CD32 Block (BD #553142), and then stained with
antibodies for the surface markers CD4-FITC (clone RM4-5, eBioscience #11-0042), IL-10R-PE (clone 1B1.3a, BD #559914), and CD25-APC (clone PC61.5, eBioscience #17-0251). After surface staining, cells were fixed and permeabilized using the eBioscience Foxp3 Transcription Factor Fix/Perm kit (#00-5523-00). Antibodies used for intracellular staining were anti-IL-10 PE (clone JES5-16E3, BD #554467) and anti-foxp3-alexa 647 (clone MF23, BD #560401). Cells were washed, resuspended in PBS + 2mM EDTA + 0.5% BSA, data were acquired using the FACS Canto II (BD Bioscience) and FACS Diva, and analyzed using FlowJo Version 8.8.7 (TreeStar Inc.).

**Gene expression analysis using real-time PCR**

Snap frozen tissue was homogenized in Qiazol buffer and RNA was extracted using the RNeasy Lipid Mini RNA Isolation Kit (Qiagen #74804). RNA was quantified using a nanodrop spectrophotometer and cDNA was prepared with the High Capacity cDNA Reverse Transcription Kit (Life Technologies #4368814) using 2 μg of input RNA. Quantitative real-time PCR was performed using 2.5 μl of cDNA and the TaqMan gene expression array for *Il10Rα* in 2x Universal PCR Mastermix (Applied Biosystems, #4304437). *Gapdh* mRNA levels were determined using the rodent primer and probe set (Applied Biosystems, #4308313). All reactions were run on the Applied Biosystems 7500 Real-time PCR
machine with the following conditions: 50°C for 2 minutes, 95°C for 10 minutes, 95°C for 15 seconds, and 60°C for 1 minute for 50 cycles. Transcript levels were determined by normalizing the target gene Ct value to the Ct value of the endogenous housekeeping gene Gapdh. This normalized value was used to calculate the fold-change relative to the average of the uninfected controls (ΔΔCt method).

**Statistical analysis**

Data from three independent experiments or at least 3 mice per group were used. All statistical analysis was done using Graph Prism 5 (Graph Pad). The difference between groups during the course of infection was performed using a multi-variable 2-way ANOVA and Bonferroni post-tests to compare groups at each time point (cytokine analysis). Comparisons between groups at single time points were done using Students’ unpaired T test, two-tailed, 95% confidence interval.

**Results**

*T cells are the primary source of IL-10 production during NSV infection*

To determine which cell type or cell types are the important source of IL-10 for restricting Th17 cell development during NSV infection, we must first identify which cell types are producing IL-10. To investigate lymphoid and myeloid cell sources of IL-10 we utilized the IL-10
transcriptional reporter VertX mouse (198, 199). VertX mice express eGFP under control of an internal ribosome entry site (IRES) inserted just past the stop codon for *Il10* and before the poly-A site (198). This allowed us to track IL-10 expression in cells during infection by flow cytometry without the need for *ex vivo* stimulation.

We characterized IL-10 production in the cervical lymph nodes, the site of priming, as well as in the brain at 3, 5, and 7 days post infection and in the spinal cord at 5 and 7 days post infection (Figure 25). Few myeloid cells are found in the cervical lymph nodes, so we focused on CD4+ and CD8+ T cells. Little to no IL-10 eGFP signal was detected in either T cell subset during NSV infection (Figure 25c). This is consistent with a previous studies of CNS viral infection with JHMV in which IL-10 production was not observed in cells isolated from cervical lymph nodes (171).

During NSV infection in the brain, very little IL-10 eGFP was expressed in any cell type at 3 days post infection (Figure 25d). By 5 and 7 days post infection, IL-10 eGFP was expressed in CD4+ and CD8+ T cells (Figure 25d). There was a slight increase in IL-10 eGFP in monocytes and microglia at 7 days post infection, but it was much less robust than that observed in the lymphoid cells (Figure 25d). In the spinal cord, IL-10 eGFP expression was slightly increased in CD4+ and CD8+ T cells at 7 days post infection (Figure 25e). These data correlated
with the \textit{Il10} mRNA levels previously detected in the brain and spinal cord during NSV infection in which \textit{Il10} mRNA increased in the brain at 5 (*p<0.05) and 7 (****p<0.0001) days post infection (Chapter 1, re-presented in Figure 25a) and in the spinal cord at 7 days post infection (***p<0.001) (Chapter 1, re-presented Figure 25b). It is also corroborates other studies in which T cells were the primary producers of IL-10 during viral encephalitis (171, 172).

Next, we examined the dynamics of IL-10 expression by CD4$^+$ and CD8$^+$ T cells during NSV infection. Both IL-10$^+$CD4$^+$ and IL-10$^+$CD8$^+$ T cells have regulatory functions during EAE. During NSV infection, there were similar numbers of IL-10$^+$CD4$^+$ T cells and IL-10$^+$CD8$^+$ T cells in the brain and spinal cord at 5 days post infection (Figure 26a, 26c, 26f), when we see the largest expansion in the Th17 population in IL-10 KO mice (Chapter 1). By 7 days post infection, the numbers of IL-10$^+$CD8$^+$ T cells in the brain and spinal cord are greater than IL-10$^+$CD4$^+$ T cells (Figure 26b, 26c, 26f). The frequency of CD4$^+$ and CD8$^+$ T cells that express IL-10 increases in the brain and spinal cord from 5 to 7 days post infection (Figure 26a-h), which suggests that regulatory cells are increasing at the peak of inflammation. These data support conclusions that T cells are the most robust producers of IL-10 during viral encephalitis and that both CD4$^+$ and CD8$^+$ T cells may serve a regulatory role during CNS inflammation.
CD25+foxp3+ and CD25+foxp3- regulatory T cells are present in the CNS during NSV infection

We identified IL-10+CD4+ and IL-10+CD8+ T cells in the CNS during NSV infection. Although both cell types have regulatory potential during CNS inflammation, regulatory CD4+ T cells, both foxp3+ Tregs and foxp3- Tr1 cells, are best characterized for their roles in limiting the pathogenic Th17 response during EAE (196, 200). To determine if Tregs and Tr1 cells are regulating Th17 cell development via IL-10 production, we first characterized the CD25+foxp3+ Treg and CD25+foxp3- Tr1 populations in the cervical lymph nodes and brains of WT and IL-10 KO mice at 5 and 7 days post infection.

In the cervical lymph nodes at 5 days post infection, CD25+foxp3+ Tregs are present, but there were no significant differences in the frequency or numbers of these cells in WT mice (22.5%, 2.0x10^4) compared to IL-10 KO mice (12.8%, 8.1x10^3) (Figure 27a, 27b). At 7 days post infection there were still no differences observed in the frequency (7.9% WT vs. 7.2% IL-10 KO) or numbers (4.2x10^3 WT vs. 3.3x10^3 IL-10 KO) of CD25+foxp3+ Tregs present in the site of priming (Figure 27a, 27b). CD25+foxp3+ Tregs were present in the brain at 5 and 7 days post infection (Figure 27a). At 5 days post infection, when the frequency of Th17 cells is most different between IL-10 KO and WT mice
(Chapter 1, Figure 18), there was a significantly lower frequency of CD25+foxp3+ Tregs in the CD4+ T cell population of IL-10 KO mice (6.2%) compared to WT mice (10.3%, *p=0.0107, Figure 27a, 27b). This difference in frequency; however, did not result in a difference in the numbers of CD25+foxp3+ Tregs present in the brains of WT (2.7x10^3) and IL-10 KO mice (3.6x10^3, Figure 27a, 27b). At 7 days post infection, CD25+foxp3+ Tregs continued to infiltrate the brain. No differences were observed between WT and IL-10 KO in the frequency (8.3% WT vs. 7.0% IL-10 KO) or numbers (2.57x10^4 WT vs. 2.96x10^4 IL-10 KO) of CD25+foxp3+ Tregs (Figure 27a, 27b). These data show that CD25+foxp3+ Tregs are present during NSV infection and infiltrate the CNS along with other T cell populations, but do not differ between WT and IL-10 KO mice.

CD25+foxp3- Tr1 cells also regulate Th17 cells during CNS inflammation. Similar to CD25+foxp3+ Tregs, there were no significant differences in the number or frequency of CD25+foxp3- Tr1 cells in the cervical lymph nodes between WT and IL-10 KO mice at 5 or 7 days post infection (Figure 27a, 27b). At 5 days post infection in the brain of NSV-infected animals, the frequency of CD25+foxp3- Tr1 cells between WT (6.7%) and IL-10 KO (7.1%) mice are similar; however there was a significant increase in the number of CD25+foxp3- cells in IL-10 KO mice (3.7x10^3) compared to WT mice (1.7x10^3, **p=0.0013, Figure 27a, 27b).
At 7 days post infection, this difference between WT and IL-10 KO mice was no longer present and the frequency and number of CD25⁺foxp3⁻ Tr1 cells was similar between both groups (Figure 27a, 27b). Interestingly, the frequency of CD25⁺foxp3⁻ Tr1 cells and CD25⁺foxp3⁺ Tregs are similar in the brains of infected animals at 5dpi (~5-10%, Figure 27b). By 7 days post infection in the brain, the frequency of CD25⁺foxp3⁺ Tregs are still ~5-10%; however, the frequency of CD25⁺foxp3⁻ Tr1 cells had increased to ~30-40% (Figure 27b).

The presence of regulatory T cells in the CNS during NSV infection suggests that they may be important sources of IL-10 for regulating the Th17 response as has been shown in other inflammatory CNS diseases (196, 200). To determine if this could be the case, we looked at IL-10 production in CD25⁺foxp3⁺ Tregs and CD25⁺foxp3⁻ Tr1 cells on the day of onset of clinical disease in NSV-infected mice. Cells were isolated from the cervical lymph nodes and brains of WT mice at 5 days post infection and stimulated ex vivo with PMA and ionomycin. In contrast to the VertX mice, IL-10 production was detected with ex vivo stimulation in the T cells isolated from the cervical lymph nodes (Figure 28a). In the cervical lymph nodes, a higher percentage of CD25⁺foxp3⁺ Tregs were IL-10⁺ (11.0%) compared to CD25⁺foxp3⁻ Tr1 cells (5.0%, *p=0.0204, Figure 28a, 28c). Similarly, the number of IL-10⁺CD25⁺foxp3⁺ Tregs was
elevated compared to IL-10+CD25+foxp3- Tr1 cells, although this was not significant (Figure 28a, 28c).

In the brain, a similar percentage of CD25+foxp3+ Tregs were IL-10+ (10.8%) compared to CD25+foxp3- Tr1 cells (8.8%) (Figure 28b, 28d). The number of IL-10+CD25+foxp3+ Tregs (303 cells) was elevated compared to IL-10+CD25+foxp3- Tr1 cells (148 cells), although this did not reach statistical significance (p=0.0986) (Figure 28b, 28d). These data show that regulatory T cells, both CD25+foxp3+ Tregs and CD25+foxp3- Tr1 cells, are present in the CNS when the Th17 response is occurring and that these cell types are making IL-10. This IL-10 may act either directly or indirectly to inhibit the Th17 response in WT mice so that, in its absence, the Th17 response is exacerbated leading to accelerated disease pathogenesis.

The IL-10Rα subunit is expressed on myeloid and lymphoid cells at the site of priming and the site of infection at the time Th17 cells infiltrate the CNS.

To determine how IL-10 is regulating the Th17 cell response during NSV infection, we must first identify which cell types express the IL-10R. The IL-10Rα subunit contains the ligand-binding domain for IL-10, whereas the IL-10Rβ subunit is shared with other members of the IL-10 cytokine family (53). Myeloid cells, including monocytes, microglia, and neutrophils, as well as T cells, including Th1, Th2, Th17, and CD8
subsets, all express IL-10Rα (53, 196). To verify that IL-10Rα is expressed during NSV infection we looked at the kinetics of Il10Rα mRNA expression in the brain (Figure 29a) and spinal cord (Figure 29b) of WT and IL-10 KO mice. We saw a significant increase in the amounts of Il10Rα mRNA during infection in the brain (**p<0.0001, Figure 29a) and spinal cord (**p<0.0001, Figure 29b) during the course of infection in WT and IL-10 KO mice; however, over the course of infection there was a significantly greater upregulation of Il10Rα expression in the brains of WT mice compared to IL-10 KO mice (**p=0.0038, Figure 29a).

Interestingly, Il10Rα mRNA expression continued to increase in WT mice through 7 days post infection in the brain and spinal cord, but plateaued in the CNS of IL-10 KO mice from around 4-5 days post infection through 7 days post infection (Figure 29a, 29b). This resulted in significantly less Il10Rα mRNA in IL-10 KO mice compared to WT mice at 5 (11.4-fold in WT mice vs. 6.8-fold in IL-10 KO mice, **p<0.01) and 7 (13.5-fold in WT mice vs. 9.3-fold in IL-10 KO mice, *p<0.05) days post infection in the brain (Figure 29a) and at 7 days post infection in the spinal cord (14.3-fold in WT mice vs. 7.6-fold in IL-10 KO mice, ***p<0.001, Figure 29b). The increase in Il10Rα mRNA is associated with an influx of hematopoietic cells into the CNS during infection (Chapter 1, Figures 9, 11, 12).
To determine the cell types in the brain expressing IL-10Rα as possible targets of IL-10 regulation, mice were infected with NSV and tissue was collected at 5 days post infection. We chose to focus on IL-10Rα expression at 5 days post infection when we see the largest increase in the Th17 cell population in the CNS of IL-10 KO mice compared to WT mice (Chapter 1, Figure 18). Mononuclear cells were isolated and stained for expression of IL-10Rα. To determine expression on Th1 and Th17 CD4+ T cell subsets, cells were stimulated \textit{ex vivo} with PMA and ionomycin. It is important to note that the expression level of IL-10Rα is relatively low with only a few hundred copies per cell (53) and, although its expression is upregulated on activated myeloid cells, it is downregulated on activated T cells (53).

In the cervical lymph nodes where priming occurs, IL-10Rα was expressed on neutrophils (CD45hiCD11b+Ly6G+Ly6Cint), macrophages (CD45hiCD11b+Ly6G-Ly6Clo), inflammatory monocytes (CD45hiCD11b+Ly6G-Ly6Chi), and CD4+ T cells (Figure 29c). No significant difference was observed between WT and IL-10 KO mice in the percentage of each cell type expressing the IL-10Rα or in the number of IL-10Rα+ cells for each population (Figure 29c). Neutrophils had the highest frequency of IL-10Rα+ cells (23.9% in WT and 30.4% in IL-10 KO) in the cervical lymph node (Figure 29d). Inflammatory monocytes were next with 14.7% of WT monocytes and 18.9% of IL-10 KO monocytes.
expressing the IL-10Rα (Figure 29d). CD4+ T cells and macrophages had similar frequencies of IL-10Rα+ cells (Figure 29d). Although it was not statistically significant, the percentage of CD4+ T cells that were IL-10Rα+ was higher in WT mice (12.2%) than IL-10 KO mice (7.9%) and correlated with more CD4+IL-10Rα+ cells in WT mice (1.1x10⁶ cells) than IL-10 KO mice (3.4x10⁵ cells, Figure 29d). This may be due to a higher activation status of T cells in the absence of IL-10, which would lead to a downregulation of IL-10Rα expression.

Next, we looked at IL-10Rα expression on myeloid and lymphoid cells in the brains of WT and IL-10 KO mice infected with NSV. Again, microglia, monocytes, neutrophils, and CD4+ T cells all expressed IL-10Rα (Figure 29e, 29f). In the brains of infected mice, microglial (CD45hiCD11b+Ly6G-Ly6C-) cells had the highest frequency of IL-10Rα+ cells (Figure 29f). There was also a significant difference in the frequency of IL-10Rα expression on microglia between WT and IL-10 KO mice. In WT mice, 11.3% of the microglial cells were IL-10Rα+ compared to 14.7% of microglial cells in IL-10 KO mice (*p=0.0364, Figure 29f). This correlated with an increase in the numbers of IL-10Rα+ microglial cells in IL-10 KO mice (2.7x10^4 cells) than WT mice (1.3x10^4 cells, *p=0.0246) (Figure 29f). This may be due to increased activation of microglial cells in the absence of IL-10, which would be expected to lead to an upregulation of IL-10Rα (53). Alternatively, because the IL-10Rα
antibody used binds to the same site as IL-10, the signal from WT cells may be reduced by the presence of IL-10 and competition for binding. Neutrophils (4.0% WT vs. 4.7% IL-10 KO), monocytes (5.5% WT vs. 4.1% IL-10 KO), and CD4+ T cells (4.2% WT vs. 3.1% IL-10 KO) all had a similar frequency of cells that were IL-10Rα+ (Figure 29f). These data suggest that myeloid or lymphoid cells may be targets of IL-10 regulation during NSV infection.

IL-10Rα is expressed predominately on Th1 cells, not Th17 cells, in the cervical lymph nodes and brains of WT and IL-10 KO mice infected with NSV

CD4+ T cells expressing the IL-10Rα could be Th1 or Th17 cells. In certain instances, Th17 cells express higher levels of the IL-10R compared to Th1 cells (201). To identify which T helper cell subsets are expressing this receptor and to determine if the Th17 cells found during NSV infection are capable of being directly affected by IL-10, we isolated mononuclear cells from the cervical lymph nodes and brains of WT and IL-10 KO mice at 5 days post infection. Cells were stimulated ex vivo with PMA and ionomycin and stained for surface expression of IL-10Rα and CD4, as well as intracellular staining for IFNγ and IL-17a, to delineate Th1 and Th17 cells.
In the cervical lymph nodes, Th1 and Th17 cells express IL-10Rα. A similar frequency of IL-10Rα+ Th1 and Th17 cells were found in WT (15.6% Th1 vs. 15.9% Th17) and IL-10 KO mice (15.6% Th1 and 13.2% Th17, Figure 30a, 30b). Although there was a similar frequency of Th1 and Th17 cells that were IL-10Rα+, there was higher expression of IL-10Rα on Th1 cells compared to Th17 cells (Figure 30a). In the brains of NSV-infected mice, there was a higher level of IL-10Rα expression on Th1 cells compared to Th17 cells in WT and IL-10 KO mice (Figure 30c). A higher percentage of Th17 cells (20.6%) in WT mice were IL-10Rα+ compared to Th1 cells (4.5%), although this was not statistically significant (p=0.0961, Figure 30c, 30d). However, there were few events that fell into the IL-17a+IL-10Rα+ gate making it hard to distinguish if this is a true representation, although it was reproducible. In the brains of IL-10 KO mice, there was a similar frequency of Th1 (3.8%) and Th17 (2.9%) cells that were IL-10Rα+ (Figure 30d). These data suggest that Th1 and Th17 cells express IL-10Rα; however, the frequency of IL-10Rα+ cells was low which may be a side effect of the ex vivo stimulation on these cells.

*IL-10Rα is expressed on CD25<sup>+</sup>foxp3<sup>-</sup> Tr1 cells and CD25<sup>+</sup>foxp3<sup>+</sup> Tregs in the cervical lymph nodes and brains of WT and IL-10 KO mice infected with NSV*
Regulatory T cells, both CD25^foxp3^ Tregs and CD25^foxp3^-Tr1 cells, express the IL-10R and respond to its signaling (196); however, CD25^foxp3^ Tregs have been studied much more in depth. IL-10 acts positively on Tregs to induce IL-10 production in these cells, which directly regulates Th17 cells (195, 196). Because regulatory T cells directly respond to IL-10 and dampen the Th17 response, particularly in the CNS, we wanted to determine if CD25^foxp3^ Tregs and CD25^foxp3^-Tr1 cells express IL-10Rα.

We isolated mononuclear cells from the cervical lymph nodes and brains of WT and IL-10 KO mice at 5 days post infection and stained Tr1 and Tregs for IL-10Rα expression. Importantly, these cells were not stimulated ex vivo. IL-10Rα was expressed on CD4^CD25^foxp3^ Tregs and CD4^CD25^foxp3^-Tr1 cells similarly in WT and IL-10 KO mice (Figure 31). There was an increase in the mean fluorescent intensity of IL-10Rα expression in foxp3^ and foxp3^- T cells in the brains compared to the cervical lymph nodes; however, expression was similar on both types of regulatory T cells (Figure 31). These data suggest that regulatory T cells may act to amplify the anti-inflammatory effects of IL-10. Furthermore, these data show that foxp3^ Tregs do express the IL-10R and are thus able to activate STAT3 along with foxp3 expression and dampen the Th17 response.
Discussion

IL-10 is important in regulating the pathogenic Th17 response during NSV infection. The cellular sources of IL-10 and the targets of IL-10 regulation, however, were unknown. To determine how IL-10 is regulating Th17 cells during NSV infection, we first identified the cell types producing IL-10 using an IL-10 mRNA reporter VertX mouse. CD4+ and CD8+ T cells were the primary producers of IL-10 at 5 and 7 days post infection; however, myeloid and lymphoid sources did produce IL-10 by 7 days post infection. IL-10 production was also observed in CD25+foxp3- and CD25+foxp3+ regulatory T cells.

There was an increase in Il10Rα mRNA expression in the CNS of WT and IL-10 KO mice during NSV infection, but there was a significant deficit in the amount of Il10Rα mRNA expressed beginning at 5 days post infection in IL-10 KO mice. When microglia, monocytes, neutrophils, and CD4+ T cells all expressed the IL-10Rα. Microglial cells had the highest frequency of IL-10Rα expression. Th1 and Th17 cells in the cervical lymph nodes and brains of infected WT and IL-10 KO mice expressed IL-10Rα; however, the level of expression was lower in Th17 cells compared to Th1 cells. Both Tr1 and Treg cells expressed the IL-10Rα, consistent with reports that these cells may help amplify the anti-inflammatory response.
T cells have been identified as the primary producers of IL-10 during CNS viral infections (63, 171, 172). It was previously reported that a similar percentage of CD4+ and CD8+ T cells expressed IL-10; however, there were more IL-10+CD8+ T cells than IL-10+CD4+ T cells during the acute phase of JHMV infection (171). These IL-10+CD8+ T cells were also more cytolytic than their IL-10-CD8+ T cell counterparts (172). During NSV infection, the VertX IL-10 reported mice indicated that T cells were the primary producers of IL-10. These data are consistent with Il10 mRNA expression levels, which increase upon T cell infiltration into the CNS. The increase in the frequency of IL-10+ T cells from 5 to 7 days post infection suggests that the CD4+ and CD8+ T cells are starting to show a more prominent regulatory phenotype at the peak of inflammation. Furthermore, it also suggests that the CD8+ T cell population is becoming more cytolytic.

There were some problems in detecting IL-10eGFP signal in myeloid cells. Once activated, these cells autofluoresce in the same channel as eGFP. This reduces the sensitivity of eGFP detection in these populations, so the signal may be an underestimate of the true intensity of eGFP. However, there was no increase in Il10 mRNA prior to the arrival of T cells in the CNS, suggesting that T cells were still the primary producers of IL-10. Low levels of IL-10 from myeloid cells may still
contribute to the regulation of the immune response and should continue to be investigated.

Regulatory T cells are important regulators of Th17 cells. Regulatory T cells expressing the transcription factors Tbet, IRF4, and Stat3 required for Th1, Th2, and Th17 differentiation, respectively, can inhibit each respective cell type (195, 202, 203). Tregs and Tr1 cells express the IL-10R and amplify an anti-inflammatory response by responding to IL-10 in the milieu (196). This results in the upregulation of IL-10 production in regulatory T cells, via STAT3 and foxp3, which then acts on Th17 cells to dampen the response during EAE and colitis, thus protecting the host (195). Tr1 cells and Tregs in the cervical lymph nodes and brains of NSV-infected mice expressed IL-10Rα and produced IL-10, suggesting that this mechanism of IL-10 regulation may be occurring during NSV infection.

Th17 cells are reported to express the IL-10R at higher levels than Th1 cells (201). During NSV infection, however, we saw low levels of IL-10Rα expression on Th17 cells and higher levels of expression on Th1 cells. The IL-10R can be down regulated on highly activated T cells (53). Because we stimulated these cells with PMA and ionomycin ex vivo in order to detect IL-17a and IFNγ to identify Th17 and Th1 cells, respectively, this may have altered IL-10Rα expression on these cells types. The use of IL-17a-eGFP reporter mice may give a better indication
of IL-10R expression on Th17 cells without the artifacts from ex vivo stimulation.

An alternative to direct inhibition of Th17 cells by IL-10 is that IL-10 is acting via its more classical mechanism and inhibiting the expression of Th17 differentiation and maintenance factors in myeloid cells during NSV infection. Microglia and monocytes encompassed the majority of IL-10Rα-expressing cells in the brain during NSV infection. We previously looked at Il6 and Il1β mRNA levels in the brains of WT and IL-10 KO mice and saw higher levels in WT mice than IL-10 KO mice (Chapter 1). Il23a mRNA expression in whole brain was not elevated in whole tissue during infection (Chapter 2). Cell-type specific expression of these cytokines, however, has not been examined and may give a more detailed view on the mechanisms of IL-10 regulation. Determining the expression of TGFβ1 and TGFβ3 in WT and IL-10 KO mice, which result in nonpathogenic and pathogenic Th17 cells (115), respectively, would also be advantageous. The low levels of IL-10Rα expression on Th17 cells suggests that it may be an indirect, rather than direct, inhibitory mechanism.

Determining the effects of cell type-specific deletion of IL-10 and IL-10Rα on the Th17 response during NSV infection and NSV pathogenesis will clarify the observations made during this study. Conditional knockouts using IL-10fl/fl mice, IL-10Rfl/fl mice, CD4-Cre
mice, and LysM-Cre mice have been created. These crosses created groups of mice that have IL-10 deficient T cells, IL-10 deficient myeloid cells, IL-10Rα deficient T cells, or IL-10Rα deficient myeloid cells. Future studies focusing on the progression of disease and the Th17 response in these models will aid in the elucidation of the important source(s) and target(s) of IL-10 during NSV infection.
Figure 25. IL-10 eGFP expression in myeloid and lymphoid cells isolated from the cervical lymph nodes, brains, and spinal cords of mice during NSV infection. The time course of *Il10* mRNA expression as characterized in the A) brain and B) spinal cords of WT mice infected intranasally with 1x10⁵ PFU of virus. RNA was isolated from collected tissue, transcribed into cDNA, and qPCR was performed to determine levels of *Il10* and *Gapdh* mRNAs in the samples. *Il10* Ct values were normalized to *Gapdh* levels and the fold-change was calculated relative to uninfected mice (ΔΔCt method). The data are presented as the mean of n=4-6 samples/group at each time point ± SEM. This data is re-presented from Chapter 1 (Figure 1a and 1b). Statistics were calculated using the 1-way ANOVA with Dunn post-tests. *p<0.05, **p<0.001, ***p<0.0001. To characterize IL-10 eGFP expression in myeloid and lymphoid cells during NSV infection, VertX mice were infected intranasally with 1x10⁵ PFU of NSV. Mononuclear cells were isolated from pooled B) cervical lymph nodes (n=5), C) brains (n=6-8), and D) spinal cords (n=6-8) from VertX mice at 3, 5, and/or 7 days post infection. Cells were stained using antibodies against surface markers and acquired without fixation. The histograms show IL-10 eGFP expression from VertX mice (black) relative to WT mice (grey) as the negative control. The data are representative from one experiment.
**Figure 26. IL-10 eGFP expression in CD4+ and CD8+ T cells from the cervical lymph nodes, brains, and spinal cords of VertX mice infected with NSV at 5 and 7 days post infection.** VertX and WT mice were infected intranasally with 1x10^5 PFU of NSV. Mononuclear cells were isolated from pooled cervical lymph nodes (n=5), brains (n=6-8), and spinal cords (n=6-8) from WT and IL-10 KO mice at A) 5 and B) 7 days post infection. Mononuclear cells were stained for surface CD4 and CD8 expression and acquired without fixation. Positive IL-10 eGFP signal was gated relative to WT (no eGFP) controls. The number of IL-10 eGFP+ CD4+ and CD8+ T cells was calculated in the C) brains and F) spinal cords of infected mice at 5 and 7 days post infection. The frequency of CD4+ T cells that expressed IL-10 eGFP in the D) brain and G) spinal cords of infected mice were calculated. CD8+ T cells that expressed IL-10 eGFP in the E) brains and H) spinal cords of infected mice were calculated. The data are from one experiment.
Figure 27. Characterization of foxp3+ Tregs and foxp3- Tr1 cells in the cervical lymph nodes and brains of WT and IL-10 KO mice infected with NSV at 5 and 7 days post infection. Mice were infected
intranasally with 1x10^5 PFU of NSV. Mononuclear cells were isolated from pooled cervical lymph nodes (n=5) and brains (n=6-8) from WT and IL-10 KO mice at 5 and 7 days post infection. Isolated mononuclear cells from infected animals were stimulated ex vivo with PMA and ionomycin for 4 hours and stained using antibodies for surface and intracellular markers. A) Contour plots show the distribution of CD25^+foxp3^+ Treg and CD25^+foxp3^-Tr1 populations in the cervical lymph nodes and brains of WT and IL-10 KO mice. The data are representative of 3 independent experiments. B) The numbers and frequency of CD25^+foxp3^+Tregs and CD25^+foxp3^-Tr1 cells in WT (black) and IL-10 KO (white) mice were calculated. Data are presented as the mean ± SEM from 3 independent experiments. Statistics were calculated using Students’ unpaired, two tailed t test with a 95% confidence interval, *p<0.05 and **p<0.01.
Figure 28. **IL-10 production in CD25^{+}Foxp3^{+} Tregs and CD25^{+}Foxp3^{-} Tr1 cells in the cervical lymph nodes and brains of WT mice infected with NSV at 5 days post infection.** Mice were infected intranasally with 1x10^5 PFU of NSV. Mononuclear cells were isolated from pooled cervical lymph nodes (n=5) and brains (n=6-8) from WT and IL-10 KO mice at 5 days post infection. Isolated mononuclear cells from infected animals were stimulated *ex vivo* with PMA and ionomycin for 4 hours and stained for surface and intracellular markers. IL-10
expression was determined in A) CD25^+foxp3^- and B) CD25^+foxp3^+ cells of WT mice at 5 days post infection. The data are representative of 3 independent experiments. The frequency of CD25^+foxp3^- and CD25^+foxp3^+ CD4^+ T cells that express IL-10 and the number of CD25^+foxp3^+IL-10^+ Tr1 cells and CD25^+foxp3^+IL-10^+ Tregs in the C) cervical lymph nodes and D) brains were calculated. The data represent the mean ± SEM from 3 independent experiments. Statistics were calculated using Students’ unpaired, two tailed t test with a 95% confidence interval, *p<0.05.
Figure 29. Il10Rα mRNA expression kinetics in the CNS during infection and IL-10Rα expression on myeloid and lymphoid cells in the cervical lymph nodes and brains of WT and IL-10 KO mice at 5 days post infection. The kinetics of Il10Rα mRNA expression in the A) brains and B) spinal cords of WT and IL-10 KO mice was assessed by isolating RNA from infected tissues, transcribing cDNA, and performing qPCR looking for Il10Rα and Gapdh expression. Il10Rα levels were normalized to Gapdh levels and are shown relative to uninfected controls (ΔΔCt method). The data are representative of 2 independent experiments with n=4-6 mice/time point for each group. Statistics were calculated using Students’ unpaired, two tailed t test with a 95% confidence interval, *p<0.05, **p<0.01, and ***p<0.001. IL-10Rα surface expression on immune cells was analyzed in the C) cervical lymph nodes and E) brains of WT (blue) and IL-10 KO (orange) mice at 5 days post infection. Mice were infected intranasally with 1x10⁵ PFU of NSV. Mononuclear cells were isolated from pooled cervical lymph nodes (n=5) and brains (n=6-8) from WT and IL-10 KO mice at 5 days post infection. IL-10Rα expression was gated relative to an isotype control. The flow plots and histograms are representative of 3 independent experiments. The frequency and number of cells expressing IL-10Rα in the D) cervical lymph nodes and F) brains of infected mice represents the mean ± SEM from 3 independent experiments. Statistics were calculated using
Students’ unpaired, two tailed t test with a 95% confidence interval,
*p<0.05.
**Figure 30.** IL-10Rα surface expression on Th1 and Th17 cells in the cervical lymph nodes and brains of WT and IL-10 KO mice at 5 days post infection. Mice were infected intranasally with 1x10^5 PFU of NSV. Mononuclear cells were isolated from pooled cervical lymph nodes (n=5) and brains (n=6-8) from WT and IL-10 KO mice at 5 days post infection. Isolated mononuclear cells from infected animals were stimulated *ex vivo* with PMA and ionomycin for 4 hours and stained using antibodies for surface and intracellular markers. IL-10Rα on CD4+ and CD8+ T cells from the A) cervical lymph nodes and C) brains of WT
and IL-10 KO mice relative to an isotype control. IL-10Rα expression on Th1 (purple) and Th17 (black) cells is shown relative to an isotype control (gray). The data are representative of 3 independent experiments. The number of IL-10Rα+ Th1 and Th17 cells and the frequency of Th1 and Th17 cells expressing IL-10Rα were calculated for the B) cervical lymph node and D) brain-derived cells from WT (black) and IL-10 KO (white) mice. The data represent the mean ± SEM from three independent experiments.
Figure 31. IL-10Rα expression on foxp3+ Tregs and foxp3- Tr1 cells in the cervical lymph nodes and brains of NSV infected mice at 5 days post infection. Mice were infected intranasally with 1x10^5 PFU of NSV. Mononuclear cells were isolated from pooled cervical lymph nodes (n=5) and brains (n=6-8) from WT (blue) and IL-10 KO (orange) mice at 5 days post infection. Isolated mononuclear cells from infected animals were stained using antibodies for surface and intracellular markers without stimulation. IL-10Rα expression on CD25+foxp3- and CD25+foxp3+ T cells was assessed and is shown relative to an isotype control (gray). Data are representative of 2 independent experiments.
General Conclusions and Discussion
Summary

Arbovirus infections leading to encephalitis are an emerging and re-emerging cause of morbidity and mortality worldwide. Few vaccines and treatment options are available for these viruses. Understanding the mechanisms of pathogenesis that lead to severe and fatal encephalitic infections provides a way of identifying therapeutic targets for effective intervention. Neuroadapted Sindbis virus provides an excellent way for studying mechanisms of pathogenesis in lethal alphavirus encephalomyelitis.

Previous studies have identified T cells as a major immune mediator of pathogenesis; however, the function of the CD4+ and CD8+ T cells remained unknown, as did any regulatory mechanisms to control the T cell response. My thesis work focused on determining the functionality of the T cell subsets during NSV infection and how the immunoregulatory molecule IL-10 affects this response and its ultimate outcome on pathogenesis. I determined that in the absence of IL-10, mice infected with NSV have accelerated morbidity occurring at the time of T cell infiltration and that this correlates with an earlier time of death. The accelerated pathogenesis observed appears to be independent of viral titers because no difference in viral replication was observed in the CNS of WT and IL-10 KO mice. I identified the presence of Th1 and Th17 cells at the onset of clinical symptoms in the CNS of NSV-infected mice.
Accelerated pathogenesis was associated with an increase in the Th17 response, but not the Th1 response, in the absence of IL-10 regulation. Furthermore, these Th17 cells displayed a pathogenic phenotype as observed during EAE mediated by Th17 cells. This included the expression of Tbet and RORγt and the production of IL-17a, IL-22, GM-CSF, and granzyme B. Interestingly, GM-CSF expression was higher in the Th17 cells found in IL-10 KO mice. An increase in the pathogenic Th1-17 cell population was also observed in the brains of IL-10 KO mice, but not WT mice.

To determine a possible mechanism for IL-10 regulation of Th17 cells, I identified which cell types produce IL-10 and which cell types express the IL-10R during NSV infection. These studies showed that CD4+ and CD8+ T cells were the predominant producers of IL-10 in the CNS during NSV infection and this occurred beginning at 5 days post infection. Macrophages, microglia, neutrophils, and T cells all expressed the IL-10R. Of particular interest was the presence of Tr1 and Treg cells in the CNS at the time of Th17 cell infiltration. These regulatory T cells expressed the IL-10R and also produced IL-10 during infection. These cells are known to inhibit Th17 cells and may provide a mechanism of IL-10 regulation of the Th17 cell population during NSV infection.
Implications for alphavirus encephalomyelitis pathogenesis- roles of host and virus

Our data build upon previous studies of the lethal model of NSV pathogenesis and provide new insight into the function and regulation of the immune response during infection. We now know that Th1 and Th17 cells are present in the CNS during NSV infection, that these Th17 cells have a pathogenic phenotype, and that IL-10 negatively regulates the Th17 response. To further characterize the consequence of Th17 cells during alphavirus encephalomyelitis, we looked to a model of NSV infection that does not induce lethal disease. Mice on a Balb/c genetic background are resistant to the development of lethal disease with NSV infection. The genetic determinants for this difference have been narrowed to a QTL on chromosome 2 (19) and the immune responses between these two groups of mice are quite different. To determine if the importance of IL-10 regulation is more universal, we looked at the effect of IL-10 on NSV pathogenesis in WT Balb/c and Il10−/− Balb/c mice. We observed a significant increase in NSV pathogenesis in IL-10 KO mice on a Balb/c background with a mean day of death of 9 days post infection and only a 28.5% survival rate, compared to a 91% survival rate in WT Balb/c mice (Figure 32). It remains to be determined if this is due to an exacerbated Th17 response or some other effect of IL-10 in Balb/c mice. Comparing the effects of Th17 cells and IL-10 regulation in a susceptible
mouse strain to a resistant mouse strain will further our understanding of the pathogenesis of encephalitic viral infections.

Alphavirus encephalomyelitis varies in disease severity depending on the virulence of the strain of virus used. NSV is the most virulent strain of SINV and induces a lethal, acute infection. TE, however, does not induce fatal disease in weanling and adult mice. TE replicates to a lower level in the CNS of C57Bl/6 mice than NSV; however the similarities and differences between the immune response to each virus is unknown. An interesting comparison would be to determine the CD4+ T cell response in the CNS during TE infection in C57Bl/6 mice to that of NSV infection. The absence of Th17 cells during this sub-lethal infection would further suggest their importance during lethal disease. Also determining the effects of IL-10 regulation in this system would be of interest because a lethal outcome of infection in humans is relatively rare.

T cells have been implicated in the pathogenesis of diseases due to a variety of neurotropic viral infections including LCMV, BDV, VEEV, and the coronavirus strain JHMV. In most of these studies, if a CD8+ component plays a role it appears to be via a noncytolytic mechanism, as is true with NSV pathogenesis. The mechanism by which CD4+ T cells are causing neural damage, however, has been largely overlooked. The majority of studies have focused on the role of the Th1 response with few,
if any, studies investigating IL-17 production and the role of Th17 cells. Revisiting the role of T helper cells and their functions during neurotropic virus infections since the identification of Th17 cells would be of great interest. The studies discussed here showed that the Th17 response is important during fatal alphavirus pathogenesis.

In models of autoimmune disease, such as EAE and colitis, Th17 cells have been implicated in pathogenesis. The cytokines and effector molecules produced by these Th17 cells have also been identified. A major question in the Th17 pathogenicity field, however, is how are these different molecules causing damage and which one or which ones are critical in mediating pathology. IL-17 is rarely implicated as the effector molecule of Th17-mediated pathogenesis, although it has been reported to be neurotoxic (191). In one study, neutralization of IL-17 during gliatropic coronavirus infection protected Ifnγ−/− mice (155). During EAE, mice deficient in either Tbet or GM-CSF are protected from developing disease (129, 178, 180). Investigating the role of these molecules during NSV pathogenesis would identify more specifically how the Th17 cells are causing pathology in the CNS during NSV infection.

**Th17 cells, viral replication, and viral clearance**

Th17 cells also inhibit virus clearance, thus promoting persistence and/or increased pathogenesis by an unknown mechanism (204, 205).
The effect of IL-10 on virus replication and clearance is variable. This may be due to an increased presence of Th17 cells or IL-17 in the absence of IL-10 regulation, although the Th17 response has rarely been determined in these studies. During NSV infection, we observed a delay in viral clearance in IL-10 KO mice compared to WT mice, which was associated with a higher Th17 response. We evaluated the number of CD8+ T cells present and found no differences between WT and IL-10 KO mice. We also evaluated the ability of CD8+ T cells to produce the effector molecules IFN\(\gamma\), TNF\(\alpha\), and granzyme B and saw no differences between both groups suggesting that the functionality of the cytotoxic lymphocytes (CTLs) is similar. IL-17 cells have been reported to inhibit CTL-mediated effects by inhibiting apoptosis of the target cell (204), but identification of the mechanisms by which Th17 cells and IL-17 inhibit virus clearance requires more study.

We observed an interesting affect of IL-10 on virus replication in the spinal cords of NSV-infected mice. In the absence of IL-10, earlier virus replication was observed in the spinal cord, but not in the brain; however titers reached similar levels at the peak of infection (Chapter 1, Figure 8). This effect occurs prior to the infiltration of T cells into the CNS, suggesting that there is a cell type-specific effect of IL-10 on virus replication. This would mean that IL-10 affects the antiviral response specifically in motor neurons, the cell type infected in the spinal cord.
We isolated RNA from spinal cords of WT and IL-10 KO mice prior to infection, at 3 days post infection when infectious virus is detectable in IL-10 KO mice, but not WT mice, and at 5 days post infection when titers are similar again, and compared the antiviral gene expression profiles of both groups at each time point. We analyzed the differences in gene expression using the Partek software. Using a principle components analysis (PCA) we observed few differences between WT and IL-10 KO mice at baseline and at 5 days post infection; however, at 3 days post infection a large difference was observed (Figure 33).

We further analyzed the differential gene expression to determine genes that were the most different between the groups (Table 2). KEGG pathway analysis was performed to identify possible anti-viral pathways that are altered in the absence of IL-10. The pathway that stood out the most was the RIG-I/MAVS interface. All of these genes fall into a variety of antiviral pathways making the KEGG pathway analysis somewhat difficult because all of them appear. The most significant difference observed was in the relative induction of *Ifnb1* which was induced ~170-fold in the spinal cords of WT mice, but only ~13-fold in IL-10 KO mice despite the presence of more virus (Table 2). IFNβ induces IL-27, thus upregulating IL-10 expression, but to the best of my knowledge there are no reports of IL-10 inducing IFNβ production. Future investigation into the role that IL-10 may play in the antiviral response is also necessary.
IL-10, Th17 cells, and viral infections

There have been few studies identifying the presence of IL-17 and Th17 cells during virus infections, let alone studies comparing the contributions of the Th1 response to the Th17 response. The importance of Th17 cells in controlling viral infections and their contribution to virus pathogenesis is not far fetched. Type-I IFNs are a critical part of the antiviral immune response. Recent studies have shown that IFNβ, used as a treatment for MS, induces IL-27 which upregulates IL-10 production, thus limiting IL-17 production and dampening the Th17 response (206, 207). The idea that IFNβ may indirectly upregulate IL-10 and restrict the Th17 response during a virus infection is plausible. Future investigations looking at the effect of IFNβ and IL-10 on T helper cell differentiation during viral infections, in the CNS or in other tissues, will help delineate the importance of an IFNβ/IL-10/Th17 axis.

Therapeutic development for viral encephalitides is becoming increasingly necessary. Three options that may arise from studying the role of IL-10 and Th17 response during viral infections are to 1) use recombinant IL-10 as a therapeutic and 2) use IFNβ as a therapeutic. Administration of IL-10 would decrease the pathogenic Th17 response and may increase survival in cases of severe virus infections. This would be most advantageous in situations where virus persistence is not itself detrimental to the host. This includes cases of viral encephalitis.
Persistent infection of neurons is a compromise in neuronal survival during infection. Recrudescence is inhibited by the immune response, primarily antibody and IFNγ. Thus, the protective effects of IL-10 would outweigh the consequence of virus persistence.

The use of IFNβ for severe virus encephalitis infections may also help. IFNβ is currently used to treat MS in humans. Studies have shown that a result of IFNβ treatment is an increase in IL-10 and a decrease in Th17 cells (208). IFNβ administration may help in controlling the pathogenic immune response during virus infections in the CNS and may also have a beneficial side effect to reduce virus load.
Figure 32. *Morbidity and mortality analysis of WT Balb/c and IL-10 KO Balb/c mice infected intranasally with NSV.* WT and IL-10 KO mice on a Balb/cJ background were infected with 1x10^5 PFU of NSV intranasally and monitored once daily for the appearance of clinical symptoms and death. A) Morbidity in WT and IL-10 KO mice during NSV infection. Scoring was as follows: 0) no clinical symptoms, 1) abnormal hind-limb and tail posture, ruffled fur, hunched back, 2) unilateral hind-
limb paralysis, 3) bilateral hind-limb paralysis or full body paralysis, and 4) death. Data are presented as the mean ± SEM of the clinical scores from 1 experiment with a total n=13 for WT and n=14 for IL-10 KO mice. Statistics used a 2-way ANOVA analysis for differences during the course of infection, as well as Bonferroni post-tests to determine significant differences between groups at each time point. ****p<0.0001. B) Survival of WT and IL-10 KO mice after NSV infection. Survival is represented as a Kaplan-Meier curve and significance was calculated using the log-rank test. Data are from 1 experiment with an n=13 for WT and n=14 for IL-10 KO mice. WT mice had a survival of 90% and IL-10 KO mice had a survival of 28.5%. The mean day of death in IL-10 KO mice was 9 days post infection, **p<0.0020.
Figure 33. Antiviral gene expression analysis of spinal cords from WT and IL-10 KO mice at baseline, 3, and 5 days post infection with NSV. WT and IL-10 KO mice were infected intranasally with 1x10^5 PFU of NSV. Whole spinal cords were collected at 3 and 5 days post infection, as well as from uninfected mice. RNA was isolated from these tissues and RNA was pooled from 3 animals/group at each time point. This RNA was then transcribed and gene expression analyzed by qRT-PCR using the SABiosciences Mouse Antiviral PCR array. The Ct values were normalized against a housekeeping gene and then analyzed using Partek software. A) Principle component analysis (PCA) of the antiviral gene expression of the spinal cords was performed. Gene expression patterns were similar between WT and IL-10 KO mice at baseline and 5 days post infection, but were different at 3 days post infection. B) A comparison of genes that had a ± 2 fold-change in expression at 3 and 5 days post infection, after being normalized to uninfected controls, was performed. This identified genes whose expression changed in a way unique to each group as well as genes that were changed in both groups. C) A cluster analysis was performed and a heat map created from the gene expression changes at baseline, 3, and 5 days post infection in WT and IL-10 KO mice. A value of -2.00 (red) represent an increase in gene expression compared to the average whereas a value of 2.00 (blue) is a decrease due to the inverse in the calculations.
Table 2. Gene lists from Partek analysis using an antiviral gene array on WT and IL-10 KO spinal cord RNA isolated at 3 days post infection. The gene lists from 3 days post infection are presented along with the fold-change value relative to the uninfected controls. The cut-off value was ± 2.0 fold-change. The tables on the left are the genes that
were unique to each group. The table on the right is the list of genes that changed more than 2-fold in each group and their fold-change values.
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Curriculum Vitae

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EDUCATION

Johns Hopkins University School of Medicine, Baltimore, MD 2008-present
Graduate Program in Cellular and Molecular Medicine
Thesis: Immune-mediated pathogenesis and regulation during fatal Alphavirus encephalomyelitis
Advisor: Diane E. Griffin, MD, PhD

Colorado State University, Fort Collins, CO 2004-2008
Biochemistry B.S.
Chemistry and Molecular Biology Minor

RESEARCH EXPERIENCE

Ruth L. Kirschstein National Research Service Award Predoctoral Fellow
Johns Hopkins School of Medicine and Bloomberg School of Public Health
Laboratory of Dr. Diane E. Griffin, Baltimore, MD 2011-present
o Fellowship work focused on the role interleukin-10 plays in regulating T cell function during fatal alphavirus encephalomyelitis
o Identified that interleukin-10 specifically dampens the Th17 response in the central nervous system during viral infection
o Gained expertise in animal models, immunological assays, multi-color flow cytometry, cellular biology, and molecular biology
o Mentored 5 PhD candidates in the laboratory

Graduate Research Assistant
Johns Hopkins School of Medicine and Bloomberg School of Public Health
Laboratory of Dr. Diane Griffin, Baltimore, MD 2008-present
o Research focused on mechanisms of immune mediated pathogenesis and the regulation of such mechanisms during fatal viral infections in the central nervous system
o Determined the role of interleukin-10 in regulating the immune response to fatal viral encephalitis and how it accomplishes this
o Assessed host determinants of virulence by comparing the immune response between susceptible and non-susceptible hosts
• Gained expertise in animal models, immunological assays, multicolor flow cytometry, cellular biology, molecular biology, and immunohistochemistry

**Howard Hughes Undergraduate Research Scholar**
**Colorado State University**
Laboratory of Dr. Sandra Quackenbush, Fort Collins, CO 2004-2008
• Conducted research looking at the transcriptional regulation of walleye dermal sarcoma virus by focusing on the transcriptional competency of unintegrated viral DNA products
• Determined that the unintegrated viral DNAs retained promoter integrity and were associated with histones that had modifications which are associated with active transcription
• Gained experience in cellular, molecular, and biochemical techniques

**PUBLICATIONS**

**Published**


**Submitted/In Preparation**

**Kulcsar KA** and Griffin DE. Interleukin-10 restricts the development of pathogenic Th17 cells in the central nervous system during fatal Alphavirus encephalomyelitis. (in preparation)

**INVITED TALKS**

St. Jude Children's Hospital National Graduate Student Symposia
Memphis, TN 2013
The role of interleukin-10 in modulating fatal alphavirus encephalomyelitis
• **IL-10 restricts Th17 cell development during NSV infection**

**ABSTRACTS (ORAL PRESENTATIONS)**

Annual Meeting for the American Society of Virology
Penn State University, University Park, PA 2013
The role of interleukin-10 in modulating fatal alphavirus encephalomyelitis
• **IL-10 restricts Th17 cell development during NSV infection**

Annual Meeting for the American Society of Virology
University of Wisconsin, Madison, WI 2012
The role of interleukin-10 during fatal alphavirus encephalomyelitis
• **IL-10 deficiency leads to altered homeostatic levels of inflammatory gene expression and earlier viral replication in the spinal cord, but not the brain**
Annual Meeting for the American Society of Virology  
University of Minnesota, Minneapolis, MN  
2011

The role of interleukin-10 during fatal alphavirus-induced encephalomyelitis

- IL-10 deficiency has little effect on the overall magnitude of the immune response to viral infection, but leads to earlier viral replication in the spinal cord and delayed viral clearance in the CNS

ABSTRACTS (POSTER PRESENTATIONS)

Keystone Symposia: Positive Strand RNA Viruses  
Boston, MA  
2013

The role of interleukin-10 in mediating fatal alphavirus encephalomyelitis

- IL-10 restricts the Th17 response during NSV infection

International Society of Neuroimmunology  
Boston, MA  
2012

The role of interleukin-10 in modulating fatal alphavirus encephalomyelitis

- IL-10 deficiency has little effect on the overall magnitude of the immune response to viral infection, but leads to earlier viral replication in the spinal cord and delayed viral clearance in the CNS

Keystone Symposia: Viral Immunity  
Keystone, CO  
2012

The role of interleukin-10 in modulating fatal alphavirus encephalomyelitis

- IL-10 deficiency has little effect on the overall magnitude of the immune response to viral infection, but leads to earlier viral replication in the spinal cord and delayed viral clearance in the CNS

Annual Meeting for the American Society of Virology  
Montana State University, Bozeman, MT  
2010

The role of interleukin-10 in fatal alphavirus-induced encephalomyelitis

- IL-10 deficiency has little effect on the overall magnitude of the immune response to viral infection, but leads to earlier viral replication in the spinal cord and delayed viral clearance in the CNS

Biochemistry Undergraduate Research Thesis  
Colorado State University, Fort Collins, CO  
2008

Analysis of unintegrated walleye dermal sarcoma virus DNA as a competent template for transcription

Celebrate Undergraduate Research and Creativity  
Colorado State University, Fort Collins, CO  
2008
Analysis of unintegrated walleye dermal sarcoma virus DNA as a competent template for transcription

**American Biomedical Research Conference for Minority Students**
*Austin, TX*
Analysis of unintegrated walleye dermal sarcoma virus DNA as a competent template for transcription

**Celebrate Undergraduate Research and Creativity**
*Colorado State University, Fort Collins, CO*
Analysis of unintegrated walleye dermal sarcoma virus DNA

**Rocky Mountain Virology Club**
*Colorado State University, Pingree Park, CO*
Analysis of unintegrated viral DNA in walleye dermal sarcomas

**LEADERSHIP EXPERIENCE**
- Laboratory Mentor (5 PhD students) 2012-2014
- Association for Women in Science, Vice President of Programs 2011-2012
- Cellular and Molecular Medicine Program Recruitment Coordinator 2009 & 2010

**TEACHING EXPERIENCE**
- Fundamentals of Virology- Teacher Assistant 2013
  Johns Hopkins University Bloomberg School of Public Health
  Baltimore, MD
- Cell Biology- Teacher Assistant 2010
  Johns Hopkins University School of Medicine
  Baltimore, MD

**AWARDS, SCHOLARSHIPS, AND FUNDING**
- Ruth L. Kirschstein National Research Service Predoctoral Fellowship 2011-2014
- Selected as a participant for the St. Jude Children’s Hospital Graduate Student Symposium 2013
- Celebrate Undergraduate Research and Creativity Highest Honors 2008
- David E. Fahrney Undergraduate Scholarship in Biochemistry 2007-2008
- College of Natural Sciences Undergraduate Scholarship 2007-2008
- Merit-Based Work Study Award 2005-2008
- Howard Hughes Undergraduate Research Scholar 2004-2008
- Colorado State University Distinguished Scholar Award 2004-2008
- Colorado State University Honors Scholar 2004-2008