ABSTRACT

CROOK, KRISTEN RAE. Inhibition of Pattern-Recognition Receptor Signaling by the Secreted Form of West Nile Virus Non-structural Protein 1. (Under the direction of Dr. Frank Scholle.)

West Nile virus (WNV) is a mosquito-borne flavivirus which can cause severe neurological disease if the proper immune response is not mounted upon infection. In any infection, the initial immune response is not pathogen specific, but rather makes use of pattern-recognition receptors (PRRs) designed to recognize pathogen-associated molecular patterns, or PAMPs. Following recognition of a PAMP a signaling cascade is initiated, resulting in the production of proinflammatory cytokines and interferons. These molecules function to limit the spread of infection by inducing an anti-viral state and also influence the formation of the adaptive immune response.

In order to circumvent these immune responses many pathogens have developed mechanisms to evade and/or inhibit innate immune detection. West Nile virus has developed a number of immune evasion and immunomodulatory mechanisms to counteract the host immune response. Work done previously by this lab identified the WNV non-structural protein 1 (NS1) as a potent immunomodulatory protein, capable of inhibiting signaling through the PRR, Toll-like receptor 3 (TLR3). The work presented in this dissertation characterizes the spectrum of NS1-mediated inhibition of PRR-signaling and describes a role for the secreted form of NS1 in modulating innate immune responses.

A survey of different TLR-overexpressing cell lines identified NS1-mediated inhibition of NFκB activation in response to stimulation by their respective ligands. These results were confirmed in the Raw 264.7 macrophage-like cell line where these TLRs are

expressed endogenously. Additionally, NS1 expression was able to inhibit innate immune signaling triggered by infection with Sendai virus. These results are presented in Chapter 2.

In Chapter 3 the immunomodulatory activity of the secreted form of the WNV NS1 protein is assessed. Co-culture experiments and experiments using NS1-containing cell supernatants show inhibition of TLR3 signaling by naïve cells. These results could be replicated using purified secreted NS1. The inhibitory activity of secreted NS1 was maintained in immune cell types such as bone marrow-derived macrophages and dendritic cells. Importantly, NS1 was able to inhibit the immune response to Poly(IC:LC) and infection by West Nile virus replicon particles *in vivo*.

Inhibition of Pattern-Recognition Receptor Signaling by the Secreted Form of West Nile Virus Non-structural Protein 1

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Dedication

I dedicate this work to my family. Mom and Dad, I wouldn't be here today if it wasn't for you pushing me to succeed. I also dedicate this work to my 'children'; Skeet, Cooter, and Veruca Salt, for always being happy to see me, for comforting me when I need it, and for loving me, pass or fail. Finally I dedicate this work to my husband, Paul. Thank you for sticking by me through the good times and the bad and thank you for all the sacrifices you have made so I could achieve my goals. I couldn't have done this without you.

Biography

Kristen Rae Crook was born December 10, 1981 to Debra and Richard Belanger. She grew up in Winslow, Maine and graduated from Winslow High School in 2000. Kristen earned a dual bachelor's degree in Medical Laboratory Sciences and Microbiology from the University of New Hampshire in 2004. After graduating she worked as a Medical Technologist in a clinical microbiology lab and also worked for the New Hampshire State Public Health Lab. Kristen moved to Raleigh, NC for graduate school in 2006 where she conducted her dissertation work in the laboratory of Dr. Frank Scholle.

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Returning to school was one of the best decisions I have made in my life, and even through the struggles, the hard work, and the sleepless nights spent thinking about experiments I have really enjoyed my time as a graduate student. Thank you to Dr. Scholle for accepting me into the lab and for giving me the freedom to explore my own ideas. I feel it has helped me to become a more independent scientist and I really appreciate that. I also thank my committee members; Dr. Dennis Brown, Dr. Tim Petty, and Dr. Barbara Sherry for being understanding and for all the insightful ideas and guidance.

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Chapter 1

Literature Review

Literature Review

Introduction

West Nile virus (WNV) is a mosquito-borne flavivirus introduced to the US in 1999 when an outbreak of WNV encephalitis occurred in New York City. Since its introduction into the United States, WNV has rapidly spread across North America and into Canada and South America and is now considered endemic to these areas. As an arbovirus, WNV is maintained in an enzootic transmission cycle between mosquitoes and birds. Infected humans and horses are considered dead-end-hosts as the level of viremia in these hosts is not high enough to contribute to the transmission cycle. Human infection by WNV can produce severe disease including; meningitis, encephalitis, and paralysis, and can even lead to death. Currently no specific treatment or WNV vaccine has been approved for use in humans.

Infection by WNV will induce an immune response aimed at clearing the virus. The earliest immune response is referred to as the innate response. This response is not pathogen specific, but rather makes use of receptors designed to recognize pathogen-associated molecular patterns, or PAMPs. The receptors recognizing PAMPs are termed pattern recognition receptors and can be divided into a number of different classes including; RNA helicases, NOD-like receptors, and Toll-like receptors (TLRs). Following PAMP recognition a PRR will initiate a signaling cascade resulting in the production of proinflammatory cytokines and interferons. These molecules function to limit the spread of infection by inducing an antiviral state and also influence the formation of the adaptive immune response.

In order to circumvent these immune responses many pathogens have developed mechanisms to evade and/or inhibit detection by PRRs. West Nile virus has developed a number of immune evasion and immunomodulatory mechanisms to counteract the host immune response. Work done previously by this lab identified the WNV non-structural protein 1 (NS1) as a potent immunomodulatory protein, capable of inhibiting signaling through the PRR, TLR3. The work presented here characterizes the spectrum of NS1-mediated PRR-signaling inhibition and identifies a role for the secreted form of NS1 in modulating innate immune responses.

West Nile Virus Origin and Phylogeny

Flaviviruses can be divided into four distinct groups, two are mosquito-borne, one is tick-borne, and the remaining group is composed of non-vectored species. The mosquito-borne flaviviruses are split between two groups; group I is comprised of both the 'Old World' species vectored by the *Aedes* genus and the 'New World' species associated with the *Culex* genus. Group II is believed to have diverged first and contains viruses associated primarily with the *Aedes* genus of mosquito [1-3]. West Nile virus is a member of the *Flaviviridae* family of viruses along with other clinically important viruses such as dengue virus, yellow fever virus, and Japanese encephalitis virus. WNV falls into the Japanese Encephalitis Antigenic Complex. Typically flaviviruses are classified according to sequence analysis of non-structural protein 5 (NS5), however, some authors have also analyzed NS2, E, or even the whole genome to classify flavivirus species [1, 3-5]. Flaviviruses can also be grouped based on the vector and host associated with the species, disease caused, known

antigenic relationships with other species, geographical distribution, and various ecological characteristics [6]. It has been shown that different flavivirus species are distributed in distinct geographical regions based on factors such as; the vertebrate or invertebrate host for the species, climate conditions, the ecology of the natural habitat of the species, as well as the impact of human urbanization [2, 7].

Tick-borne and mosquito-borne species of flaviviruses diverged early in the evolutionary history of the family [4, 5, 7]. Mosquito-borne species are known to have had a more discontinuous pattern of evolution as compared to the gradual pattern seen for tick-borne species [7, 8]. Mosquito-borne flaviviruses have evolved more than two times faster than tick-borne species [5], likely due to the many bloodmeals taken by mosquitoes over their life span versus only three bloodmeals taken during the lifespan of ticks.

West Nile virus Transmission and Disease

West Nile virus is believed to have originated in Africa and the strains identified there are the most divergent. WNV uses the *Culex* genus of mosquito as its vector in an infection cycle with birds, however, WNV has also been shown to infect other mosquito species, such as *Aedes albopictus*, as well as ticks [9]. Migratory birds are the primary mode of geographic dispersal for the virus [10-12]. In 1999, WNV was first identified in the United States [13], presumably after introduction by an infected mosquito gaining access to the North American continent by a form of human transportation. After its introduction into the US, WNV spread along the East coast in a pattern correlating with known migratory routes of birds [10]. WNV spread first along the eastern coast of the US then, the virus spread westward, and

currently it has been identified in all the continental states. Shortly after the spread of WNV to the West coast of the US the virus was identified in the Caribbean as well as in Central and South America, and Canada. Some of the most virulent strains of WNV have been isolated from Hungary and southern Africa [14].

Although WNV exists in an enzootic transmission cycle between mosquitoes and birds, humans and horses can be incidental, dead-end hosts. The vast majority of human infections by WNV are asymptomatic, however, about 20% of infected individuals may develop flu-like symptoms accompanied by markedly high fever. The most severe cases of WNV infection progress to meningitis, encephalitis, and/or paralysis often leading to death. At the peak of WNV activity in 2002 and 2003 there were over 14,000 cases and a total of 548 deaths (http://www.cdc.gov/ncidod/dvbid/westnile/). According to the CDC, in 2011 the number of states reporting human neuroinvasive cases of WNV increased to 43, from 38 states in 2010, with 49 states reporting WNV activity. In 2011 there were 43 human deaths due to WNV disease reported in the U.S. There is currently no antiviral treatment or vaccine available for WNV infections.

Flavivirus Infection and Replication Cycle

Flavivirus particles are small in size, around 50nm in diameter. They are spherical particles with an electron-dense core surrounded by host cell-derived lipid envelope. On the surface of the virus particle lie two proteins, the envelope protein (E) and the membrane protein (M). The E protein mediates host cell binding and fusion during entry. The M protein functions to stabilize the E protein and assist in the correct positioning of E dimers on the

surface of the viral particle. As the virus particle is smooth, the E protein dimers are believed to lie parallel to the lipid bilayer in a head-to-tail type configuration, forming a herring-bone pattern of 90 E dimer molecules [15, 16]. The M protein is positioned within the E dimers near the fusogenic domain of E [15]. Beneath the lipid bilayer of the virus particle is the nucleocapsid of which the C proteins form a cage-like arrangement around the genome [15].

The genome is positive sense single stranded RNA with a type I cap at the 5' end, but no polyadenylation of the 3' end [17, 18]. The 5' end of the genome contains a non-coding region (NCR) of bases that is not well conserved amongst flavivirus species [19]. However, the secondary stem-loop structure formed in this region is known to be important for virus replication and this feature is conserved within the family [20, 21]. There is also an NCR at the 3' end of the genome. Again, this region is variable within the flavivirus family, but there is a conserved stem-loop structure that, like the 5' NCR stem-loop, is required for virus replication [22-24]. This 3' stem-loop interacts with host factors and has also been shown to interact with the non-structural proteins, NS3 and NS5, *in vitro* [25, 26]. Importantly, the 5' and 3' NCRs contain well-conserved complementary sequences [27]. These sequences interact to circularize the genome and the 5' region serves as the initiation site for RNA replication. These regions are known as cyclization sequences and mutations ablating complementation of these regions are lethal for virus replication [28, 29].

WNV is transmitted to a mammalian host when an infected mosquito takes a blood meal. Recent evidence suggests mosquito saliva transferred during feeding will enhance virus infection [30], which could be the result of modulation of the immune response at the site of infection. Flaviviruses are known to enter cells by receptor-mediated endocytosis. There are

many putative receptors for flavivirus binding and infection of cells, however, a consensus has not been reached on the primary receptor target for these viruses, except for dengue virus which is widely believed to bind DC-SIGN for entry [31, 32]. Upon endocytosis the virus is contained in a clathrin-coated, prelysosomal endocytic compartment before low pH-induced fusion of the viral and endocytic membranes allows for release of the virus nucleocapsid [33-35]. The virus nucleocapsid consists of multiple copies of the capsid protein, C, and one copy of the positive sense, single stranded RNA genome.

Upon infection, a rearrangement of the perinuclear cytoplasmic membranes can be observed. This is followed by a proliferation of the endoplasmic reticulum (ER) [36, 37]. At this point virus 'vesicle packets' or VPs may be found [36-40]. These are smooth membrane structures containing clusters of vesicles and are the presumed site of viral replication [41-43]. At later points of infection convoluted membranes, or CMs, can be found adjacent to the VPs. These structures are randomly folded membranes running contiguous with the ER and may be the site of genome packaging [36, 39].

The flavivirus genome contains one open reading frame from which translation generates a single polyprotein precursor that is proteolytically cleaved by both host and viral proteases to produce ten individual proteins. Of these proteins, three are structural, C, prM, and E, and seven are non-structural, NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5 [44]. Host signal peptidases cleave apart C from prM, prM from E, and E from NS1. The virally encoded serine protease, NS2b/NS3, cleaves between C and C anchor, NS2a and NS2b, NS2b and NS3, NS3 and NS4a, NS4a and NS4b, and NS4b and NS5 (Figure 1) [45, 46]. The

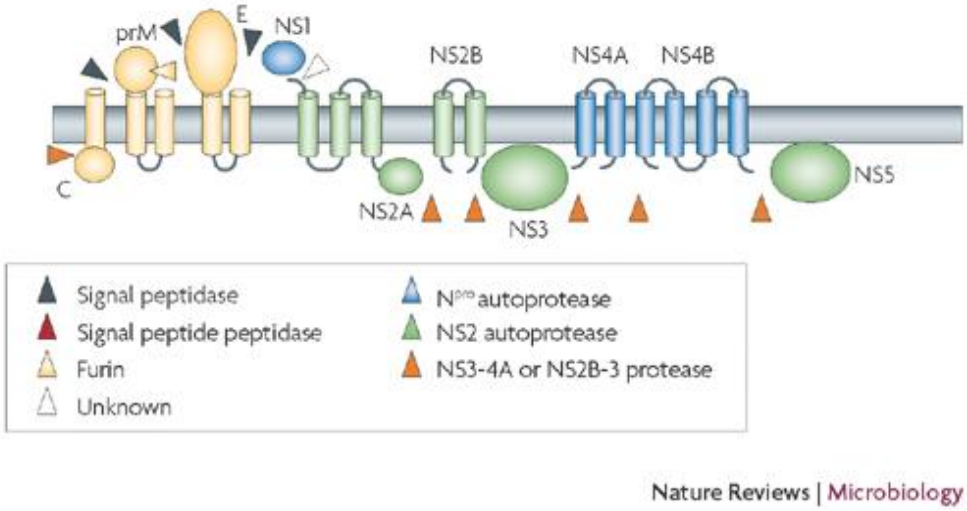

Figure 1: **Flavivirus protein topology**. The proposed topologies of the flavivirus proteins with respect to the ER membrane and the enzymes involved in polyprotein cleavage. Figure adapted from Murray et al [47].

The enzyme cleaving NS1 from NS2a is currently unknown, but may be an ER-resident protease. The functions of each non-structural protein will be discussed in more detail below.

RNA replication of the flavivirus genome is carried out by the RNA replicase complex of non-structural proteins. This process is believed to occur on the cytoplasmic membranes of VPs, however, these structures only form later in infection and therefore it is unknown where the earliest round(s) of viral replication occur [41, 42]. During genome replication, a negative-sense copy of the ssRNA genomic strand is first generated and then serves as a template for further positive-sense ssRNA synthesis. Genome packaging occurs in conjunction with the ER and progeny virions are assembled within the lumen of the ER. These immature virions are then transported through the Golgi where prM is cleaved by the

cellular protease, furin [48]. This cleavage produces M and completes virion maturation. The virus particle is then transported to the cell surface and released by exocytosis [49].

The capsid protein is a highly basic protein of only 11kD [50]. Both the N- and C-termini contain charged residues to facilitate interaction with viral RNA [51]. The internal region of the protein is hydrophobic, serving to mediate interactions with membranes [52]. Importantly, the C-terminus of the capsid protein contains a signal sequence for aiding the translocation of prM to the ER before they are cleaved apart [53].

The immature form of the M protein is called prM. This is the glycoprotein precursor for M, which remains bound to the C protein until its translocation into the ER [45]. Here prM serves to stabilize the E protein during passage through the early secretory pathway. The low pH of this pathway could induce E to rearrange into its trimeric, fusogenic form, the form it takes during virus entry [54-56]. As prM passes through the secretory pathway the host Golgi-resident enzyme, furin, cleaves pr to give the mature M form of the protein [57]. The pr region of the protein is then secreted [58].

The envelope protein, E, is around 53kD and contains twelve conserved disulfide-linked cysteine residues [59]. There are three distinct regions within the E protein. There is a β-barrel region, a region projecting between the two transmembrane regions of the E homodimer, and a region that mirrors the constant region of immunoglobulins and is believed to be involved in receptor-binding [16]. The E protein fusion peptide will mediate insertion into the host cell membrane after low pH exposure induces rearrangement of the E dimer into a trimeric fusogenic complex within the endosomal compartment [16, 60].

Flavivirus Non-structural Proteins

As previously mentioned, there are seven non-structural proteins produced during proteolytic cleavage of the polyprotein. The NS1 protein will be discussed in detail later in the chapter therefore only the remaining six non-structural proteins will be discussed here.

NS2a is a small, hydrophobic protein of around 22kD. This is a membrane-spanning protein whose N-terminus is cleaved in the ER and whose C-terminus is cleaved in the cytoplasm [61]. This protein localizes to VPs and is known to interact with NS3, NS5, and the 3' NCR [62, 63]. NS2a is believed to function in the shift between RNA replication and RNA packaging [64]. The interaction between the basic residue at position 190 of NS2a and acidic residue(s) of NS3 is required for assembly of infectious virus [63]. The NS2b protein is only 14kD and functions mainly as the cofactor for the serine protease activity of NS3 [65, 66]. NS2b contains a conserved hydrophilic region flanked by hydrophobic regions which mediate membrane association of the protein [67, 68]. It has been suggested that NS2b may also modulate membrane permeability of the host cell during infection [69].

NS3 is a large (70kD), multifunctional protein with enzyme activity involved in the processing of the polyprotein as well as in RNA replication [70]. This protein can localize to VPs where it functions as an RNA helicase, unwinding RNA duplexes [42, 71, 72]. NS3 can also localize to CMs via its interaction with NS2b [68, 73]. CMs are the presumed site of polyprotein processing and it is here that the NS3 serves its serine protease function [42].

NS4a and NS4b are hydrophobic proteins of small size, 16kD and 27kD respectively. NS4b is a transmembrane protein shown to localize to VPs as well as the nucleus [74]. NS4a may have a role in replication as it colocalizes with both VPs and CMs [62].

NS5 is a 103kD protein, well conserved among flavivirus species. The NS5 protein serves as the RdRp, or RNA-dependent RNA polymerase, for viral genome replication [44, 75, 76]. In VPs, this protein and the NS3 protein, bind to the 3' NCR stem-loop to initiate RNA synthesis de-novo [25, 76, 77]. NS5 also has methyltransferase activity for 5'cap-processing. As well as localizing to VPs, the NS5 protein of dengue and yellow fever viruses can also be found in the nucleus [77, 78]. The NS5 protein contains nuclear localization signals which are recognized by the host proteins, importin β1 and importin α/β [79, 80]. Research has shown that nuclear accumulation of NS5 reduces IL-8 production which in turn facilitates dengue virus replication [81, 82], though the precise mechanism of NS5 in inhibiting IL-8 production is currently unknown.

Non-structural Protein 1 (NS1) Maturation and Secretion

The flavivirus NS1 protein is approximately 50kD and contains twelve conserved cysteine residues. There are three glycosylation sites within the WNV NS1 protein, whereas the NS1 protein of dengue virus contains only two glycosylation sites [83, 84]. The NS1 protein is known to be required for flavivirus replication, however, the precise role played in the replication process has yet to be determined [40-42]. The protein can exist as a monomer, a dimer, a hexamer, and even higher order oligomer forms [83-85]. Upon initial polyprotein processing NS1 is inserted into the ER through a signal sequence at the C-terminus of the E protein. After cleavage of the signal sequence by signal peptidases the NS1 protein rapidly dimerizes and then is modified in the ER by the addition of N-linked glycans. The dimerization of NS1 is dependent on the presence of one or more of the three C-

terminal cysteine residues in the protein. As the protein travels through the Golgi a complex glycan is also added to the glycosylation site at position 130, however, the 207 glycosylation site remains a simple sugar, likely due to it being inaccessible after the dimerization event. Glycosylation of NS1 is not required for plasma membrane association of the protein as this is controlled by a region in the N-terminus of NS1. Glycosylation has been reported to be necessary for secretion of West Nile virus and Japanese encephalitis virus NS1 species[83]. However, recent studies from our lab have demonstrated that NS1 expressed from a construct with mutations at all three glycosylation sites is still secreted to levels equivalent to wild type (unpublished data).

The secreted form of NS1 is hexameric, composed of three joined dimers. Recent data suggest that the secreted hexamer form of dengue virus NS1 is a barrel-shaped high-density lipoprotein (HDL) [86]. These researchers demonstrated that three dimers compose the NS1 hexamer, held together by weak hydrophobic bonds. In this form the protein is soluble however separation into the dimer form indicates the protein is amphipathic, as the dimer is hydrophobic. The secreted NS1 hexamer is bound by 36 triglycerides, six per monomer, as well as multiple phospholipids, mono and diacylglycerol molecules, and cholesteryl esters [86]. The final composition of these moieties is similar to that seen for HDLs. Interaction of NS1 with these lipids, which occurs at distinct ER membrane microdomains, is necessary for formation of the hexamer form and its secretion. Stability of the hexamer form of dengue virus NS1 appears to be dependent on the presence of the N-linked glycan at the position 130 glycosylation site and glycosylation at position 207 appears to be required for secretion of the dengue virus NS1 protein [84].

Flavivirus NS1 is secreted during mammalian infection and accumulates to very high levels in the serum, peaking during the acute phase of the disease [87-90]. The levels of dengue virus NS1 in the serum have been shown to correlate with the severity of disease [91]. *In vitro*, WNV NS1 can be detected in the supernatant of BHK cells by 12 hours post infection, the time required for one round of virus replication [89]. High levels of NS1 were shown to be secreted from primary neuron and dendritic cell cultures. However, secreted NS1 was not detected in the supernatant of infected BMDM cells (bone marrow derived macrophages), which are classically less permissive to flavivirus infection, suggesting that the level of NS1 secretion correlates with the level of virus production/infectivity [89]. Experimental infection of mice with WNV showed high levels of NS1 in the plasma appearing at day three post infection and peaking 4-5 days post infection [89]. Another study using WNV-infected Syrian golden hamsters showed peak NS1 levels in the serum at five days post infection with concentrations up to 10μg/mL of NS1 [87]. The peak in NS1 secretion occurred after the peak in viral RNA production, indicating a delay in NS1 secretion. By infecting Vero cells these researchers found that only a small amount of the NS1 produced during infection is secreted, the majority is held within the cell, likely contributing to replication of the genome.

Experiments using purified NS1 have demonstrated the ability of the protein to bind uninfected cells and to be endocytosed, accumulating in late endosomes [92]. These researchers showed that pretreatment of cells with purified dengue virus NS1 protein resulted in enhanced virus production after dengue virus infection. Dengue virus NS1 appears to bind naïve cells, endothelial cells in particular, via the glycosaminoglycan (GAG) linkages,

heparan sulfate and chondroitin sulfate E [85]. The proper sulfation of these linkages is critical for NS1 binding as sodium chlorate treatment, which abolishes sulfation of GAG linkages, prevented NS1 binding.

Flavivirus Pathogenesis

During virus invasion there is considerable interplay between the virus and viral immune complexes, Fc receptors, major histocompatability complex antigens, and adhesion molecules. The result of these interactions determines virus pathogenicity and disease. Mutational analysis of the E protein, the viral receptor-binding protein, has shown adaptations to different cell types and different receptors [93, 94]. The flavivirus E proteins have considerable homology. The receptor binding site is believed to be located within domain III of the protein as monoclonal antibodies recognizing this region prevent infection [95, 96]. Mosquito-borne flavivirus species have a conserved RGD sequence within the E protein, which may bind integrins and has been implicated in virulence [16, 97, 98].

Dendritic cells are believed to be the initial site of flavivirus replication, namely Langerhans dendritic cells, located in the skin [99, 100]. These dendritic cells are thought to then transport the virus to the draining lymph nodes, initiating an influx of leukocytes into the lymph nodes [101]. In WNV infection, the initial rounds of replication in the draining lymph nodes induce the production of type 1 interferon as well as cytokines and chemokines [102]. WNV is also able to infect keratinocytes at the site of infection [103] and WNV RNA was detected in the skin of infected mice up to four months post infection [104]. Monocytes are also major targets of flavivirus infection and it has been proposed that circulating

monocytes play a role in dengue hemorrhagic fever [105-107]. Dengue virus-infected monocytes are a prominent source of TNFα and IL-1β, both vasodilating cytokines which mediate the migration of leukocytes across the endothelial barrier [108, 109]. Tumor necrosis factor α (TNFα) is known to be involved in a number of cellular processes including; immunoregulation, regulation of cell proliferation, cytotoxicity, and mediation of endotoxic shock. The elevated level of endothelial cell activation during dengue virus infections is known to be due, at least in part, to the TNFα secreted by infected monocytes [108]. Also, the levels of TNFα in the serum of patients with a severe case dengue virus disease are highly elevated [110, 111]. In WNV infection, one study using TLR3 knock out (KO) mice produced data suggesting TLR3-mediated production of TNFα led to increased blood-brain barrier (BBB) permeability and therefore increased development of lethal encephalitis [112].

Endothelial cells of the cerebral microvasculature provide a barrier to the CNS, however, neurotropic flaviviruses, namely WNV, circumvent this endothelial layer to gain entry into the brain. A number of mechanisms for this entry have been proposed. First, there may be transendothelial passage into the CNS by direct infection of the cerebral microvascular endothelial cells [113], however, there is currently no evidence that these cells are able to be infected by flaviviruses *in vivo* [105, 114]. Nevertheless, transcytosis of Japanese encephalitis virus across the endothelial layer has been documented [115]. Other proposed methods of entry into the CNS by flaviviruses include extravasation of infected cells of the immune system such as macrophages or dendritic cells, entry via the olfactory neurons, and the previously mentioned increase in BBB permeability due to TNFα

production [112, 116]. After entry into the CNS, WNV activates caspase-3 in neurons, leading to their apoptosis [117]. Neuronal apoptosis occurs in the anterior horn of the spinal cord [118], which leads to paralysis [119] as the anterior horn houses the motor neurons [120]. The transendothelial migration of leukocytes is regulated by the expression of adhesion molecules located on endothelial cells. Flaviviruses have been shown to activate the expression of endothelial adhesion molecules, which likely occurs by indirect mechanisms (i.e. cytokines) as, again, flaviviruses infection of endothelial cells has not been demonstrated [108, 121]. The increased expression of adhesion molecules likely contributes to virus dissemination throughout the tissues of the periphery.

Flaviviruses are known to infect many mammalian cell types. Flaviviruses can infect neurons and glial cells in the brain [122-124]. Dengue virus has been identified in B cells of acutely ill patients, however, dengue virus has never been identified in T cells *in vivo* [125]. Mast cells are found in high numbers in the tissues, closely associated with the vasculature [126]. Mast cells are a potent source of heparin and vasoactive mediators and may play a role in the pathogenesis of dengue infection as high levels of histamine in the urine and high levels of IgE in the serum of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) patients have been noted [127, 128]. Neutrophils are believed to be a major site for viral replication during the early stages of WNV infection. Researchers showed that pretreatment of mice with antibodies against neutrophils resulted in decreased viral load and decreased mortality rate [129]. However, neutrophils do play a critical role in viral clearance as treating mice with antibody to neutrophils post-WNV infection lead to an increase in viremia and mortality rate [129].

Platelets may play a prominent role in the pathogenesis seen during dengue infection. Platelet aggregation and adhesion, as well as vascular permeability are regulated by the communication between platelets and endothelial cells. Dengue virus infection is known to cause thrombocytopenia and viral immune complexes have been found on the platelets of dengue virus- infected patients [105, 130-132]. Research indicates that dengue infection will significantly reduce the half-life of platelets in circulation as well as increase their adhesiveness [133, 134]. Dengue infection of platelets has been found to be primarily through ADE, or antibody dependent enhancement [135]. In ADE infections the virus requires the presence of antibody on the surface of the cell in order to bind and infect the cell. It has been suggested that the antibody dependent binding of the virus to platelets may be a component of the profound disease found in patients secondarily infected with dengue virus.

A number of cell surface molecules are known to serve as receptors for flaviviruses. The cell surface DC-SIGN, found on dendritic cells, is a known receptor for dengue virus [31, 32]. GAGs and proteoglycans are cell surface molecules involved in ligand recognition and cell signaling [136]. These molecules are widely distributed among cell types and may vary in their composition. Flaviviruses have been shown to bind GAGs and putative GAG binding motifs have been identified in the E protein [137, 138]. The E protein binds GAGs with preference for heparan and heparan sulfate [137]. Repeated passage in cell culture confers dependence on GAG binding for virus infection, and interestingly, this mutation leads to a decrease in virulence and neurovirulence *in vivo* [138].

It has been demonstrated for a number of flaviviruses that virus-specific antibody, at subneutralizing concentrations, bound to Fc receptors can function as a entry mechanism for

virus infection on varying cell types [106, 107, 139-144]. This phenomenon is also true for antibody bound to complement components [145]. Upon binding the virus:antibody complex, the Fc receptor will signal through its cytoplasmic ITAM region in conjunction with accessory molecules to initiate endocytosis of the complex [146, 147]. Dengue virus infection of monocytes will induce the production of endothelial cell activators, however, this only occurs during ADE infection by dengue virus [108]. Monocytes, the primary target for flavivirus infection, express three types of Fc receptors; FcγRI, FcγRII, and FcγIII, otherwise known as CD64, CD32, and CD16, respectively [146, 148]. FcγRII is the most widely distributed Fc receptor among cell types and is known to recognize immune complexes rather than free immunoglobulins [146, 148]. Mast cells and basophils express FcγRII and ADE infection of the KU812 cell line (basophil and mast cell-like) by dengue virus induces the production of vasoactive cytokines [149, 150].

During infection, many flaviviruses are known to alter the distribution of certain cell surface molecules. For example MHC I expression is increased on a number of cell types during infection [151-156] and MHC II expression can be increased on macrophages, astrocytes, and myoblasts [151, 152, 156-158]. As previously mentioned, flavivirus infection can alter adhesion molecule expression. This effect includes increased expression of ICAM-1 (CD54), VCAM-1 (CD106), and E-selectin (CD62E) on endothelial cells of the vasculature [108]. The increase in expression is likely due to the type I IFN response elicited during flavivirus infection or in response to TNFα produced by virally infected monocytes [108, 154].

The complement system is known to contribute to the early response to flaviviruses by neutralizing the virus [159]. The complement pathway also stimulates adaptive immune responses to flavivirus infection. C3-/- mice have increased susceptibility to lethal WNV infection and a reduced level of antiviral antibodies produced [159]. Additionally, the mannose-binding lectin and the alternative complement activation pathways appear to be necessary for a functional CD8+ T cell response against WNV [160].

Both T-cell responses and humoral responses are implicated in the clearance of virus from the periphery. The antibody response to WNV infection is reliant on IgM initially, then switching to IgG, where the antibodies primarily recognize the viral envelope protein [161, 162]. These antibodies are neutralizing and recognition of the E protein allows for inhibition of viral attachment and membrane fusion. Mice incapable of producing these neutralizing antibodies succumb to WNV-induced encephalitis. In addition to antibody production against the E protein, antibodies recognizing secreted NS1 are produced and are protective in mice later challenged with WNV [163, 164]. Cytotoxic T cells are also important in clearing WNV from the periphery as CD8-/- mice infected with WNV have higher viral titers in the tissues and an increased rate of death. These effects were reversed by adoptive transfer of WNV-specific CD8+ T cells [165].

Innate Immunity

Upon infection, the innate immune response is the first line of defense against invading pathogens. Unlike the adaptive response, the innate response is not pathogen specific but rather the recognition of pathogen-associated molecular motifs, distinct from

those of the host cell. The receptors responsible for the recognition of these pathogen-associated molecular patterns (PAMPs) are collectively known as PRRs, or pattern-recognition receptors. Upon recognition of a pathogen these receptors signal the production of proinflammatory cytokines, chemokines, and interferons. There are several types of PRR responses, including RNA helicases, NOD-like receptors, and Toll-like receptors (TLRs). Each of these types of PRRs functions to prevent the spread of infection.

Toll-like Receptors and Signal Transduction

TLRs are a class of PRRs involved in innate immune detection of pathogens. TLRs are type-I integral membrane glycoproteins and can be found on the cell surface or within the endosomes of the cell depending on signal sequences within the transmembrane domains of the receptors [166]. The TLRs localized to the plasma membrane include; TLR1/2, TLR2/2, TLR2/6, TLR4, and TLR5. Endosomally located are TLR3, TLR7, TLR8, and TLR9. Each TLR recognizes a distinct ligand. TLR2 can homodimerize or can heterodimerize with TLR1 or TLR6 to recognize different lipoproteins, lipopeptides, and peptidoglycans [167, 168]. TLR4 will recognize lipopolysaccharide (LPS) [169], TLR3 will recognize double-stranded RNA [170], TLR7 and 8 both recognize single-stranded RNA [171]. TLR9 recognizes unmethylated CpG DNA (Figure 2) [172]. Ligand detection by TLRs occurs at the LRR, or leucine-rich repeat domain. Upon ligand recognition, adaptor molecule recruitment by the TLR occurs via the TIR domain (Toll/IL1-receptor homology domain) [166, 173, 174]. Upon ligand binding the TLR receptor molecule is activated to interact with an adaptor molecule. Most TLRs interact with the adaptor protein, MyD88, however, TLR3 interacts

solely with TRIF [175-177]. TLR4 can also signal through TRIF, but this occurs only in the presence of an additional adaptor molecule, TRAM, and only upon endocytosis of TLR4 [178-180].

TLR engagement by its respective adaptor molecule induces a signaling process that proceeds through recruitment of the E3 ubiquitin ligase, TRAF6. TRAF6 functions with the proteins UBC13 and UEV1A to form a ubiquitinating complex which catalyzes the production of K63-linked polyubiquitin (polyUb) chains [176, 181]. These polyUb chains recruit and activate TAK1 (TGFB-activated kinase 1) and its associated protein TAB2, or TAK-binding protein 2 [181]. After activation this protein complex can function in the activation of two different transcription factors. One signal will activate a second kinase complex composed of IKKα, IKKβ, and IKKγ [182]. This complex catalyzes the phosphorylation of IkB, an NFkB inhibitor. Phosphorylation initiates degradation of the inhibitor molecule allowing NFkB to translocate to the nucleus where it will initiate transcription. The second function of the activated TAK complex leads to AP-1 nuclear translocation [183]. TAK1 will activate the mitogen-activated protein kinases (MAPKs); p38, JNK (c-jun-N-terminal kinase), and ERK1 and 2. These kinases in turn activate the AP-1 transcription factor.

In addition to signaling which leads to NFκB activation, signaling through TLR adaptor molecules can also activate the interferon regulatory factors IRF3 and IRF7. Binding of the adaptor molecule to the TLR cytoplasmic tail will induce the recruitment of other pathway intermediates, facilitating the phosphorylation of IRF3 and IRF7. Phosphorylation

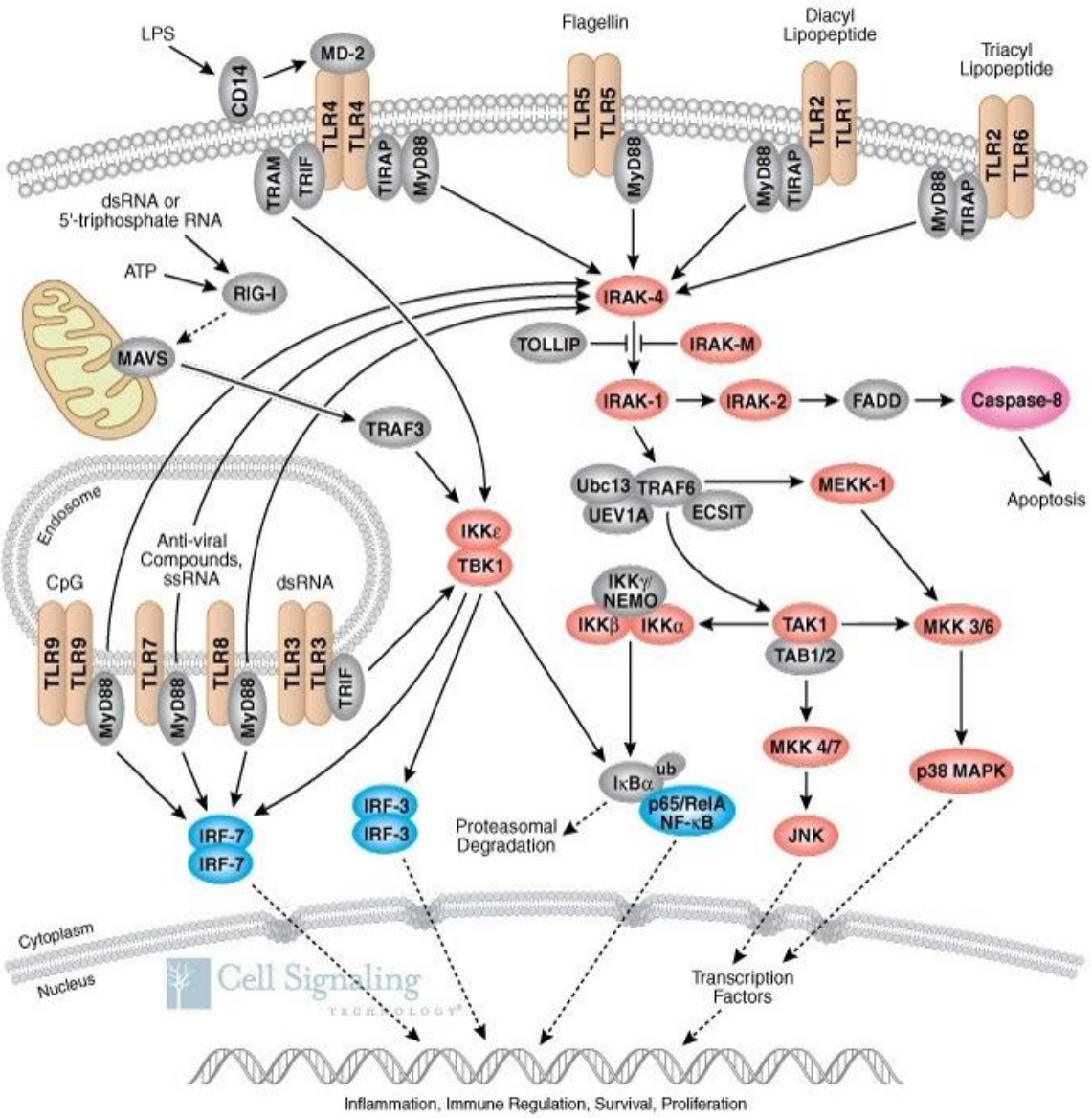

Figure 2: **TLR-mediated signaling pathways**. Image showing TLR cellular localization, adaptor molecule usage, signaling pathway intermediates, and transcription factors. http://www.cellsignal.com/reference/pathway/Toll_Like.html

of IRF7 appears to be dependent on its interaction with MyD88, IRAK1, and TRAF6 and leads to the production of IFNα [184, 185]. IRF7 activation is dependent on both TRAF6-mediated ubiquitination and IRAK4-induced phosphorylation of IRAK1 [184, 185].

Upon binding the TLR3 TIR domain, TRIF will recruit TRAF3, IKKe, and TBK1, forming a complex functioning to phosphorylate, and thereby activate, IRF3 [186, 187]. After phosphorylation, IRF3 will form a homodimer which is then able to translocate to the nucleus of the cell where it recruits CBP/p300 and binds either the IFNβ promoter [188] or the interferon stimulated response element (ISRE) to activate ISG15 transcription [189]. The IFNβ enhanceosome complex is composed of various transcription factors binding promoter enhancer elements in a coordinated fashion to ensure IFNβ transcription is tightly regulated (Figure 3) [187, 188]. NFκB and AP-1 are two transcription factors required for IFNβ enhanceosome assembly, however IRF3 and CBP/p300 (cAMP responsive element binding protein) are also required as part of the complex.

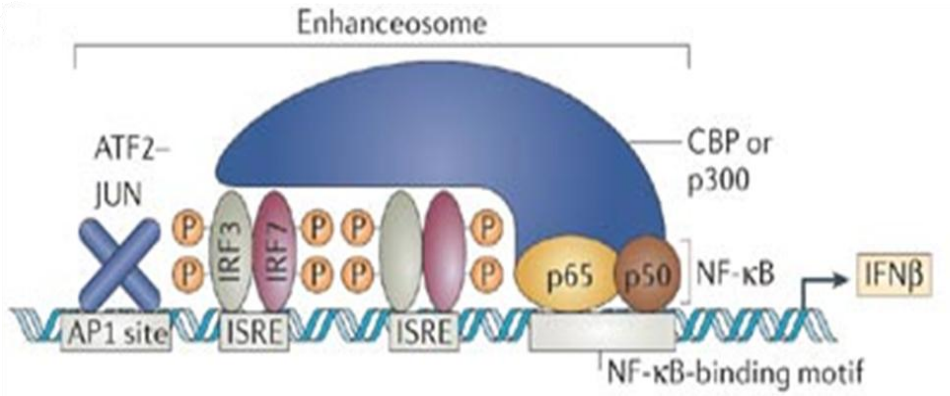

Figure 3: **IFNβ enhanceosome**. Enhancer elements of the IFNβ promoter are recognized by phosphorylated IRF3 with CBP/p300, NFκB, and AP-1 [190].

Comprising a second class of PRR are the RNA helicases, RIG-I-like receptors (RLRs), including RIG-I and MDA5. These receptors are located in the cytoplasm and will recognize 5'triphosphate RNA or dsRNA via their RNA helicase domain [191-195]. Recognition of ligand will recruit the adaptor protein IPS-1 (interferon beta promoter

stimulator-1) to the CARD-like domain of the receptor protein [196]. The IPS-1 protein, which is known by a number of different names (MAVS; mitochondrial antiviral signaling, Cardif; CARD adapter inducing IFNβ, VISA; virus-induced signaling adapter) will associate with RIG-I or MDA5 via its own CARD domain [196]. This association occurs in conjunction with the outer membrane of the mitochondria. Signaling originating from RLRs can lead to activation of TRAF3 by IPS-1. TRAF3 will then recruit TANK binding kinase 1 (TBK1) and IKKi (inducible IKK) to phosphorylate and activate IRF3 and IRF7 [186, 197]. RLR signaling through IPS-1 can also initiate TRAF6 to activate both AP1 and NFkB, in a manner congruent to TLR-mediated activation of these molecules, leading to the production of proinflammatory cytokines such as IL6, IL8, and RANTES [198-200]. The RLR-mediated induction of IFNβ production appears to be more reliant on full IFNβ enhanceosome assembly than that of TLRs as TRAF6 was shown to be required for full IFNβ production [198].

Interferon Response to Flavivirus Infection

The type I interferon response is a critical component in the host response to flavivirus infection. Interferon signaling works in an autocrine and paracrine fashion prompting cells to establish an antiviral state and thereby prevent viral spread. Interferon is secreted from activated cells and will bind the interferon α/β receptors (IFNR) on neighboring cells. Binding to the IFNR signals JAK (Janus Tyrosine Kinase) to phosphorylate STAT1 and 2 (signal transducer and activator of transcription). The phosphorylated STAT proteins then interact with IRF9 to form a complex called ISGF3. This

complex will then translocate to the nucleus and induce transcription of interferon-stimulated genes which regulate a number of cellular processes such as apoptosis and protein synthesis [201, 202]. For example, the antiviral protein PKR (RNA-dependent protein kinase R) will phosphorylate the host protein EIF2α upon recognition of dsRNA, thereby inhibiting mRNA translation [203-205]. RNAse L, another antiviral protein, can be activated by 2'-5'-oligoadenylate synthetase to induce degradation of viral RNA [206-210].

The type I IFN response is also important in facilitating the correct type II IFN response. The type II response involves IFNγ and the secretion of this IFN will impact the adaptive immune response to the virus. Type I interferon can stimulate CD8+ T cells to produce IFNγ, a cytokine important in the immune response to viral infections [211, 212]. However, type I IFN can also directly influence the adaptive response as IFNα/β-induced STAT4 activation is important in stimulating a Th1 immune response [213].

Role of Pattern Recognition Receptors in Flavivirus Detection

The response to flavivirus infection has been postulated to originate from a number of different PRRs, namely RIG-1, MDA5, TLR3, and TLR7. Increasing evidence suggests that there is much interplay between multiple PRRs during the innate immune response to flavivirus infection.

The RNA helicases, RIG-I and MDA5 appear to be the most critical components of the early response to flavivirus infection. IPS-1-/- MEFs (mouse embryonic fibroblasts) could not induce IFNβ production in response to dengue virus infection. However, upon infection by WNV the *in vivo* IFN response was actually higher in IPS1-/- mice, despite an

increased susceptibility to virus challenge, which the researchers attributed to the resulting decrease in the numbers of T regulatory cells that would normally serve to control the immune response [214].

The role of TLR3 in flavivirus detection remains controversial. TLR3 has been suggested to function as a PRR in the recognition of flavivirus infection as dengue virus-infected cells produced IFNβ and IL-8 via TLR3 stimulation [215]. In contrast, other studies using TRIF-/- MEFs reported normal viral replication of WNV. Another study using TLR3-/- mice suggested a detrimental role for this PRR in the response to WNV infection. Wild type (WT) mice showed an increase in blood-brain barrier permeability as compared to TLR3 knockout mice. This was suggested to be the result of TLR3-mediated TNFα production leading to an increase in blood-brain barrier permeability [112]. However, a later study produced opposing results, showing TLR3-/- mice were more susceptible to infection by WNV and had higher viral titers in the brain as compared to WT mice [216].

Experiments using MyD88-/- mice suggest a role for one or more MyD88-dependent TLRs in the response to WNV infection. Though the type I IFN response was unaffected by the absence of MyD88, the results of these studies suggested viral infection of the brain and spread in neurons may be limited by MyD88-dependent signaling [217]. The MyD88-dependent TLR7 does have a role in the early response to WNV infection. TLR7-/- mice displayed decreased recruitment of immune effector cells to the site of infection and increased mortality when WNV was delivered intraperitoneally. Studies later showed an IL-23 dependent homing defect in the TLR7-/- mice was responsible for the decreased cell recruitment [218]. Compared to WT mice, cutaneously administered WNV was able to more

efficiently infect keratinocytes of TLR7-/- mice with reduced production of interferon and cytokines in response to infection in these mice [219]. Moreover, these researchers determined that this TLR7 response was important in the induction of Langerhans dendritic cell migration in response to infection.

Flavivirus Immune Evasion Strategies

Like many viruses, flaviviruses have evolved many functions to circumvent the innate immune response. For example; WNV replicon-bearing cells, or cells expressing the coding region for only the non-structural proteins, were unable to activate STAT1 and STAT2 in response to IFNα treatment [220]. The NS4b proteins of WNV, YFV, and dengue virus were able to prevent signaling originating from the IFNR [221]. The NS5 protein of JEV and a tick-borne flavivirus, Langat virus, are able to prevent the phosphorylation of STAT1, thereby preventing IFN signaling and interrupting the antiviral state [222]. The NS5 protein of dengue virus can bind to and prevent the phosphorylation of STAT2 [223] as well as induce its degradation, blocking the interferon response [224]. Activation of STAT1 was also prevented by the NS5 protein of WNV however, the NS5 protein of Kunjin virus, an attenuated type of WNV, was unable to prevent STAT1 activation [225]. Other non-structural proteins of Kunjin virus were able to prevent signaling in response to IFNα treatment [226]. When expressed separately the Kunjin non-structural proteins NS2a, NS2b, NS3, NS4a, and NS4b were all able to inhibit STAT2 translocation after IFNα treatment.

Pathogenic strains of WNV are able to evade early recognition through RIG-I, preventing IRF3 activation until later times after infection [227]. In addition, the capsid

protein of WNV has been shown to increase the activity of PP2A, a protein able to down-regulate AP-1 dependent transcriptional activation [228]. Another structural protein with immune regulatory activity is the E protein of WNV. Recombinant E protein of insect origin was able to inhibit signaling originating from TLR3 when added to cells. These researchers showed that only E displaying insect-derived glycosylation patterns and not mammalian-derived E protein was able to inhibit TLR3 signaling [229].

The secreted form of WNV NS1 is able to bind components of the complement pathway, thus preventing complement-mediated killing of infected cells. Specifically, secreted NS1 is able to bind to the C4 protein of the complement pathway and induce its cleavage by bringing it in contact with C1s [230]. Additionally, NS1 can recruit the complement regulatory protein C4b binding protein (C4BP) which will inactivate C4b [231]. Finally, secreted NS1 can bind to the complement regulatory factor H and bring this cofactor in contact with factor I, thus promoting its protease activity and increasing the level of C3b cleavage [232]. Through these mechanisms NS1 is able to inhibit the classical and the mannose-binding lectin pathways of complement activation.

WNV-infected HeLa cells were found to be deficient in IFNβ promoter activation in response to poly(I:C) treatment. This was also true in HeLa WNV replicon-bearing cells, indicating this phenomenon was separate from that seen for the E protein described above [233]. Collectively, these data suggested the inhibition of TLR3 signal transduction was due either to the expression of one or more of the non-structural proteins or to the WNV RNA replication process. Later studies showed that the NS1 protein of WNV was able to inhibit TLR3-dependent signaling [234]. By additional pathway analysis, the point of NS1-mediated

inhibition was determined to be upstream of the activation of IRF3 and NFκB as translocation to the nucleus of both of these transcription factors was inhibited in the presence of intracellularly-expressed NS1.

The NS1 protein is clearly a multifunctional protein with activity in viral replication as well as multiple modes of immune inhibition. The secreted form of this protein has known immunomodulatory activities and has been shown to be endocytosed by uninfected cells. As the TLR-inhibitory activity of the NS1 protein is of particular interest, the focus of this dissertation is on the spectrum of NS1-mediated PRR signaling inhibition and the characterization of immune inhibition by the secreted form of the WNV NS1 protein.

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Chapter 2

The NS1 protein of WNV inhibits Toll-like receptor signal transduction

Abstract

The innate immune response is the first line of defense against foreign pathogens. Pattern recognition receptor (PRR) recognition of virus-associated molecular patterns such as double and single stranded RNA (dsRNA and ssRNA), initiates a signaling cascade resulting in the transcriptional upregulation and secretion of proinflammatory cytokines that induce an antiviral state. In order to establish a productive infection many viruses have evolved mechanisms to antagonize these immune responses. Work done previously by this lab has shown that expression of WNV NS1 protein inhibits TLR3-induced transcriptional activation of the IFNβ promoter and of an NFκB responsive promoter. In the presence of NS1, the TLR3 ligand poly(I:C) failed to induce nuclear translocation of IRF3 and NFκB, however the mechanism of NS1 inhibition of TLR3 signaling is currently unknown. To help answer this question the ability of NS1 to inhibit signaling through different TLRs was examined. In this study we demonstrate NS1-mediated inhibition of signaling originating from a range of TLRs. This inhibitory activity was not cell type specific as effects were seen in HEK 293 cells as well as Raw 264.7 cells. These data provide evidence suggesting that NS1-mediated inhibition occurs at a signaling molecule common to these TLR pathways.

Introduction

The innate immune system functions to recognize invading pathogens and initiate an immune response. Part of this innate response consists of type 1 interferon production as well as the production of proinflammatory cytokines. Recognition of pathogens is dependent on the presence of pathogen associated molecular patterns (PAMPs). The molecules that recognize these PAMPs are called pathogen recognition receptors, or PRRs, and upon binding of a PAMP will initiate a signaling cascade that leads to transcription factor activation and production of interferons and cytokines. Important in the recognition of virus infection are a class of PRRs called Toll-like receptors (TLRs). TLRs can recognize virus replication intermediates, viral genomic RNA, and even viral glycoproteins. The recognition and subsequent cytokine response serve to create an antiviral state in both the infected cell and the uninfected neighboring cells through paracrine effects. The PAMP recognized by TLR3 is double stranded RNA (dsRNA) [1], a replication intermediate for ssRNA viruses such as WNV. Other TLRs recognize other virally or bacterially-derived patterns. TLR2/6 will recognize triacylated peptides [2], TLR4 recognizes LPS [3], the ligand for TLR7 and TLR8 is ssRNA [4], and TLR9 is responsive to unmethylated CpG DNA [5]. Upon ligand recognition these PRRs will recruit adaptor molecules. For TLRs these adaptor molecules can be divided into two classes; MyD88-dependent and MyD88-independent. TLR3 signaling is exclusively MyD88-independent as TRIF is the adaptor molecule used in this signaling cascade [6]. TLR4 is able to recruit TRIF, but can also signal through MyD88 [6-8]. All the remaining TLRs signal exclusively in a MyD88-dependent manner [9]. In addition to specific ligand recognition and adaptor molecule usage, the TLRs can differ in

their cellular localization. TLR2/6 and TLR4 are located on the plasma membrane, whereas TLR3, TLR7/8, and TLR9 are all located within endosomes. TLR engagement of its respective adaptor molecule induces a signaling process whereby the E3 ubiquitin ligase, TRAF6, is recruited [10-13]. TRAF6 functions with the proteins UBC13 and UEV1A to form a ubiquitinating complex, catalyzing the production of K63-linked polyubiquitin (polyUb) chains. These polyUb chains recruit and activate TAK1 (TGFβ-activated kinase 1) as well as the associated protein TAB2, or TAK-binding protein 2 [13, 14]. After being activated this protein complex can function in the activation of two different transcription factors. First, TAK1 serves to activate a second kinase complex composed of IKKα, IKKβ, and IKKγ [15]. This complex catalyzes the phosphorylation of IκB, the NFκB inhibitor. Phosphorylation initiates degradation of the inhibitor molecule allowing NFκB to translocate to the nucleus where it will initiate transcription of inflammatory cytokine genes. The second function of the activated TAK complex leads to AP-1 nuclear translocation. TAK1 will activate the mitogen-activated protein kinases (MAPKs) p38, JNK (c-jun-N-terminal kinase), and ERK1 and 2. These kinases in turn activate the AP-1 transcription factor [16].

Another arm of signaling from a subset of TLRs leads to the phosphorylation of IRF3 or IRF7. IRF7 phosphorylation follows its interaction with MyD88 and TRAF6 and allows for its dimerization and subsequent translocation into the nucleus where it activates the IFNα promoter [11, 12]. Upon phosphorylation, IRF3 will form a homodimer which is then able to translocate to the nucleus of the cell and bind the interferon β promoter [17]. The IFNβ

enhanceosome complex is composed of various transcription factors that bind to promoter enhancer elements in a coordinated fashion to ensure IFNβ transcription is tightly regulated [17, 18]. NFκB and AP-1 are two transcription factors required for IFNβ enhanceosome assembly, however IRF3 and CBP/p300 (cAMP responsive element binding protein) are also required as part of the complex [17]. MyD88-independent, TRIF-dependent signaling leads to the activation of IRF3. Phosphorylated IRF3 will form a homodimer and translocate to the nucleus where it recruits CBP/p300 and binds the IFNβ promoter.

Many viruses have developed the ability to evade or inhibit the innate immune response, and WNV is no exception. Previous data identified the WNV non-structural protein NS1 as a potent inhibitor of TLR3 mediated signaling when expressed intracellularly. The WNV NS1 protein is a secreted glycoprotein, which is also required for virus replication, although its exact role in this process has yet to be elucidated. After infection, the flavivirus RNA genome is translated to produce a single polyprotein that is then proteolytically cleaved into ten individual proteins. Upon cleavage from the polyprotein, NS1 will translocate into the lumen of the endoplasmic reticulum (ER) where it rapidly dimerizes and becomes glycosylated [19]. Transit through the trans-Golgi network allows for further glycosylation of NS1 and then the protein can be secreted in a hexamer form or be retained inside the cell. The secreted form of this protein has been shown to modulate different aspects of the complement-mediated immune response [20-22]. In a previous study from our lab, NS1 was shown to inhibit the TLR3-mediated response to the dsRNA mimetic, poly-inosinic poly-cytidylic acid (poly I:C). NS1 was able to inhibit NFκB activation as well as IRF3

translocation to the nucleus [23]. Cells constitutively expressing the WNV NS1 protein showed a defective antiviral response when treated with poly(I:C) and subsequently infected with vesicular stomatitis virus (VSV) [23]. The precise mechanism of TLR3-mediated signaling inhibition by NS1 has yet to be elucidated, therefore it was the aim of this study to determine the range of inhibitory activity for NS1 with respect to different PRRs as well as different cell types. The data presented here show that in addition to inhibiting signaling through TLR3, NS1 is able to inhibit signaling through TLR2/6, TLR4, TLR7, TLR8, and TLR9, and suggests inhibition of signaling by other PRRs as well. This inhibitory effect was detected in Raw 264.7 mouse macrophage-like cells and HEK 293 TLR-overexpressing cell lines by monitoring NFκB activation. Taken together these results provide evidence that the inhibitory activity of NS1 extends beyond TLR3 and also suggests that the target for NS1 inhibitory activity may be an intermediate molecule common to these different PRR signaling pathways.

Materials and Methods

Cell lines, Viruses, and Chemicals

HEK 293 TLR overexpressing cell lines and Raw 264.7 cells were propagated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% Gemini FBS (Cellgro), 1% antibiotics, and 20μg/ml gentamicin. In addition, Raw 264.7 cell media contained 1.5 g/L sodium bicarbonate. The TLR2/6, TLR4/MD2, and TLR9 overexpressing cell lines were under G418 selection (40μg/mL) and were gifted by Golenbock, D., UMASS

via Nordone, S., NCSU. The TLR3, TLR7, and TLR8 cells were purchased from Invivogen and kept under blasticidin selection (1μg/mL). NS1 and vector control cell lines were constructed as described below. Cell lines were maintained in the above-stated media with the addition of puromycin (HeLa: 2 μg/mL, HEK 293 and Raw 264.7: 4 μg/mL). The stock of Sendai virus was obtained from Charles River Laboratories. LPS, R848, CpG DNA ODN 2006 G5, and Pam3CSK4 were purchased from Invivogen. Poly(I:C) was purchased from Calbiochem.

DNA constructs

The full length coding sequence of WNV NS1, including the final 30 amino acids of the E protein (amino-terminus) and a hexa-histidine epitope tag (carboxy-terminus), was cloned into the lentivirus expression vector pLEX (Thermo Scientific Open Biosystems) using SpeI and NotI restriction sites. Fidelity of the cloning was determined by sequencing of the plasmid (pLEX-NS1).

Lentiviral cell lines

pLEX-NS1 was packaged into VSV-G pseudotyped, replication defective, lentivirus particles through co-transfection of HEK 293T cells with vector, packaging (pREV and pMDLg), and envelope (pVSV-G) plasmids. Empty vector (pLEX-ctrl) was packaged identically. Lentivirus-containing supernatants were harvested and clarified 40h post-transfection, and stored at -80^{o}C until further use. 293 TLR cells or Raw 264.7 cells were transduced with pLEX-NS1 or pLEX-ctrl lentivirus for 24h in the presence of 8μg/mL

polybrene. Transduced cells were selected by puromycin treatment at 4μg/ml and maintained as a pool.

Reporter assays

Reporter assays using the luciferase (Luc) reporter gene under control of the NFκB response element (NFκB-Luc) (Stratagene) have been described previously[23]. Briefly, equal amounts of NFκB-Luc and pCMV-β-galactosidase (β-gal) (Invitrogen) plasmid were cotransfected into the HEK 293 TLR NS1 or MCS cells using the TransIT-LT1 transfection reagent (Mirus). pcDNA6 empty vector was used to keep the overall amount of DNA constant. Following ligand treatment, cells were lysed in reporter lysis buffer containing 0.1% Triton X-100, 10% glycerol, 2mM Dithiothreitol, 2mM trans 1,2 diamino cyclohexane-NNNN acetic acid, and 25mM Tris phosphate and assayed for Luc and β-Gal activities using a Promega Luc assay system and an ONPG (o-nitrophenyl-β-D-galactopyranoside)-based β-Gal assay. β-Gal activity was used to normalize the Luciferase data for all experiments. All data are expressed as relative light units/mU of β-Gal activity.

ELISA

IL-8 levels were assayed in the supernatants of HEK 293 TLR lentivirus-transduced cells after 8 hours of treatment with the appropriate ligands at the indicated amounts. IL-6 levels in the supernatants of Raw 264.7 cells were determined using an enzyme-linked immunosorbent assay (ELISA) kit (E-bioscience) according to the manufacturer's specifications.

Flow Cytometry

HEK 293 TLR overexpressing cells were washed with PBS before fixing and permeabilizing on ice for 20 minutes (BD Cytofix/Cytoperm kit). Cells were incubated with a primary antibody generated against the NS1 protein of Japanese Encephalitis virus and then a FITC-tagged secondary antibody. Analysis was performed on the Accuri C6 flow cytometer according to standard protocol.

Reverse transcription and quantitative PCR

Total RNA was extracted using a Qiagen RNeasy kit with DNase treatment according to the manufacturer's specifications. Reverse transcription (RT) was carried out with the ImProm II RT kit (Promega) using oligo dT as primer. Real-time PCR analysis was carried out using the SensiMix SYBR & Fluorescein kit (BioLine) and the following primers; Mouse: *GAPDH* 5'-TGCCCAGAACATCATCCCTG-3' and 5'-ATCCACGACGGACACATTGG-3', *IL-6* 5'GACTTCACAGAGGATACCACTCC-3' and 5'- TTCTGCAAGTGCATCATCGGT-3', *IFNβ* 5'- GGAGATGACGGAGAAGATGC-3' and 5'- CCCAGTGCTGGAGAAATTGT-3'. Reactions were set up in 96-well PCR plates (Genesee). Amplifications were carried out for 50 cycles, followed by a melt curve analysis of resulting products to confirm the specificity of the reactions. To construct standard curves, total RNA was isolated from the cells, and 300- to 600-bp fragments of the gene(s) of interest were amplified by RT-PCR using the appropriate primer sets. PCR fragments were gel purified and quantitated, and the copy number was calculated. Serial 10-fold dilutions

were prepared for use as templates to create standard curves in real-time PCRs. All data are expressed as the ratio of copy numbers of target gene per 10^3 copies of GAPDH for samples run in triplicate.

Results

WNV NS1 inhibits signaling through endosomal TLRs

Previous data generated in the lab demonstrated the ability of NS1 to inhibit signaling originating from TLR3, an endosomally located TLR. Therefore, we wanted to determine if this inhibitory capacity extended to signaling originating from other endosomally located TLRs. An NS1 expression construct or an empty vector was transfected into HEK 293 TLR 7, 8, or 9 overexpressing cells along with an NFκB reporter construct and β-gal expressing plasmid for normalizing transfection efficiency. Figure 1 shows that, upon ligand stimulation, NFκB activation was significantly inhibited in the presence of NS1 as compared to the control vector (pc6). These data indicate that the inhibitory mechanism of NS1 may come into play at the level of the endosome, or at a pathway intermediate common to the TLR signaling cascade.

WNV NS1 inhibits signaling through plasma membrane associated TLRs

To determine if NS1 inhibition is specific to the endosome, NS1 expressing plasmid or empty vector was transfected into HEK 293 TLR2/6 or HEK 293 TLR4/MD2 cells, its effect on TLRs which are located on the plasma membrane was tested. Using a reporter assay

to determine NFκB activation in response to the appropriate ligand treatment it was observed that NS1 also inhibited signaling originating from these TLRs (Figure 2). This suggests that NS1 inhibition is likely at a common pathway intermediate for MyD88 and TRIF-dependent signaling and is not restricted to endosomally localized TLRs.

NS1 inhibition of IL-8 production in select HEK 293 TLR overexpressing cell lines

As confirmation of the inhibitory activity of NS1 we decided to monitor cytokine production directly. We previously used IL-6 secretion as a readout to measure TLR stimulation; however, the HEK 293 TLR cells do not produce appreciable amounts of IL-6 in response to TLR ligand stimulation so we instead measured IL-8 secretion into the cell culture supernatant by ELISA. Unlike reporter assays which allow one to transfect the reporter construct and the effector construct into the same cell, an ELISA is a global measurement for the entire well of cells; therefore NS1 should be present in all cells to accurately determine inhibitory activity. To accomplish this, NS1 expressing HEK 293 TLR cell lines were generated using lentiviral transduction. The cells were infected with lentiviral particles containing the NS1 expression cassette or the empty vector control (MCS) for 72 hours before selecting for positively transduced cells with puromycin. Pools of puromycin resistant cells were then expanded. For each TLR the MCS or NS1 expressing cells were plated and stimulated with the appropriate ligands for 8 hours before harvesting the cell supernatants and preparing cell lysates for normalizing results to protein concentration. The results in Figure 3 demonstrate NS1-mediated signaling inhibition in TLR3, TLR4, TLR2/6,

and TLR9 cell lines. IL-8 production by TLR7 and TLR8 cells, however, was not inhibited in the presence of NS1.

To determine if the expression level of NS1 in these two cell lines could explain the results, flow cytometric analysis was performed to detect the level of NS1 expression. Figure 4 illustrates that NS1 expression in the TLR7 and TLR8 HEK 293 cell lines was comparable to that of the TLR4 cell line, where NS1 was able to inhibit the production of IL-8. Therefore, although NS1 was sufficiently expressed, the TLR7 and TLR8 cells were able to produce IL-8 in amounts equal to that of the MCS control cells after ligand stimulation. The reason for this discrepancy is unclear; however, the TLR7 and TLR8 overexpressing cell lines were generated using a different expression vector which contains genes other than the TLRs themselves which may be under the control of NFκB and would therefore explain differences in signaling. Overall the results indicate that the expression of NS1 in select TLR overexpressing cell lines will inhibit production of IL-8 after ligand stimulation of the TLR. Inhibition of IL-8 production was not seen in the TLR7 and TLR8 overexpressing cell lines regardless of NS1 expression level. Taken together, the pattern of NS1 inhibition of IL-8 production by various TLRs does not indicate that TLR localization or adaptor molecule usage are involved in the failure of NS1 to inhibit IL-8 production by TLR7 and TLR8, leading us to conclude that the lack of inhibition seen in those cell lines is specific to the cell line and not to the particular TLR.

NS1 inhibition of IL-6 production by Raw 264.7 cells

To extend the investigation of NS1-mediated signaling inhibition to a more immunologically relevant cell type, mouse macrophage-like Raw 264.7 cells were transduced with NS1-expressing lentivirus as previously described and pools of puromycin resistant cells were expanded. Raw 264.7 cells endogenously express all TLRs and therefore present a system which is more biologically relevant than HEK 293 cells. When ligands for TLR2/6, TLR3, TLR4, TLR7/8, and TLR9 were used to stimulate the NS1-expressing or MCS control Raw 264.7 cells statistically significant inhibition of IL-6 production was observed in the presence of NS1 for all ligands (Figure 5), further confirming the broad spectrum of TLR inhibition previously seen. These results also indicate a common TLR signaling pathway intermediate is likely the target of NS1-mediated signaling inhibition.

NS1 inhibits Sendai virus induced IL-6 production by Raw 264.7 cells

Sendai virus (SenV) has been shown to stimulate a number of different PRRs, which may vary by cell line. In HeLa cells, SenV infection is believed to solely trigger the RNA-helicase RIG-I, however, in cells of the immune system, such as Raw 264.7 macrophages-like cells, there are a number of PRRs potentially triggered by SenV infection [24]. Our previous reports demonstrate that in HeLa cells, SenV-induced IRF3 activation and IL-6 secretion were not inhibited by NS1 expression [23, 25]. Based on the broad spectrum of TLR inhibition in Raw 264.7 cells, however, it was of interest to determine whether NS1 expression might affect SenV- induced signaling, whether through RIG-I or other PRRs. We measured IL-6 transcript levels as well as IL-6 in the supernatants of Raw 264.7 cells

expressing NS1 or MCS control after SenV infection. The data indicate that indeed NS1 was able to inhibit PRR signaling in response to SenV infection (Figure 6). This inhibitory effect of NS1 could be seen even at later times after infection, indicating that even the higher levels of virus and viral RNA produced by viral replication were unable to produce an immune response in the presence of NS1. While it is still unclear whether SenV in this system signals IL-6 production through TLRs or through other PRRs, these findings illustrate that very different patterns of innate immune inhibition can be observed depending on the cell type in which the experiments are performed.

NS1 inhibits early IFNβ activation by Sendai virus in Raw 264.7 cells

As IL-6 induction requires only activation of the NFκB transcription factor all we could conclude from the previous experiments looking at SenV infection was that there was inhibition of NFκB activation. The IFNβ enhanceosome is composed of a number of different promoter elements and for full promoter activation the IFNβ enhanceosome must contain NFκB, AP-1, IRF3, and CBP/p300. As NFκB binding is required as part of the IFNβ promoter enhanceosome one might expect a reduction of IFNβ production in response to SenV infection. We infected the MCS- or NS1- expressing Raw 264.7 cell lines with SenV for a series of timepoints. Early IFNβ transcript levels as well as early IFNβ protein produced in response to SenV infection were lower in NS1-expressing cells; however, at later times in infection the inhibitory effect of NS1 was lost. As IFNβ works in an autocrine/paracrine fashion as part of a positive feedback loop, it is probable that the IFNβ produced at later

timepoints was the result of signaling from the IFNα/β receptor and not via a PRR. These results suggest NS1 is able to inhibit PRR signaling in response to SenV infection while it is not yet clear exactly which PRRs are involved and/or inhibited.

Discussion

Here we present evidence that WNV NS1, when expressed intracellularly, is able to inhibit signaling that originates from a number of different PRRs. This effect could be observed across different cell types such as HEK 293 TLR overexpressing cells as well as Raw 264.7 macrophage-like cells.

Baronti et al. have recently reported that the NS1 proteins of several different flaviviruses, including WNV, do not inhibit TLR3 signaling [26]. It is not clear why the results of these authors differ so significantly from ours. However, in their study the authors had to resort to overexpressing the IRF3 transcription factor in their cells in order to detect TLR3-induced IFNβ transcriptional activation and RT-PCR was the predominant readout used to assess TLR signaling. Our past and present studies use a combination of reporter assays, RT-PCR, and endogenous cytokine production as readouts and we demonstrate that TLR3 inhibition by NS1 is not limited to HeLa cells and HEK 293 cells but also occurs in more immunologically relevant cell types such as Raw 264.7 cells ([23, 25] and this report).

In our experiments the inhibitory effect of NS1 extended to signaling originating from both endosomally-located and plasma membrane-associated TLRs, as well as to TLRs which signal in a MyD88-dependent or MyD88-independent fashion. NS1 transfection into TLR-

overexpressing HEK 293 cell lines resulted in decreased NFκB activation in response to ligand stimulation. NS1 expression in HEK 293 TLR-overexpressing cells inhibited IL-8 production in response to ligand stimulation for all TLR cell lines tested except for the TLR7 and TLR8 cell lines. The reason for the failure of NS1to inhibit signaling in these cells is not clear. Analysis by flow cytometry confirmed a high level of NS1 expression for both TLR7 and TLR8 overexpressing HEK 293 cells; therefore the amount of NS1 expressed by the cells should have been sufficient to inhibit TLR signaling. The TLR7 and TLR8 overexpressing cell lines were generated using a vector expressing the Bcl-xL anti-apoptotic gene in addition to the TLR. This feature is unique to the TLR7 and 8 cell lines and was incorporated to prevent apoptosis of these cells. It was previously demonstrated in a number of different cell lines that Bcl-xL activation induces the production of IL-8 [27, 28]. The Bcl-xL gene is activated by NFκB [29, 30], therefore, activation of NFκB by TLR stimulation would lead to Bcl-xL-mediated IL-8 production, which could potentially override NS1-mediated inhibition. However, NS1 was able to inhibit signaling through these TLRs as well as all the other TLRs tested when measuring IL-6 production from Raw 264.7 cells, which express these TLRs endogenously. This further supports the idea that the lack of NS1-mediated inhibition in the HEK 293 TLR7 and 8 cell lines was cell line dependent and was not reflective of the inhibitory capacity of NS1.

Additionally, we found that NS1 was able to inhibit PRR signaling in response to SenV infection of Raw 264.7 cells. As mentioned previously, these cells express a number of TLRs and other PRR molecules endogenously. SenV has been shown to trigger different

PRRs in different cell lines. In non-immune cell types, such as HEK cells, recognition of SenV is believed to be dependent solely on RIG-I [24]. In immune system cell types, such as Raw 264.7 cells, however, SenV infection is believed to trigger TLR7 and TLR8, but not RIG-I, PKR, or TLR3 [24]. In one study, type 1 IFN production in response to SenV infection was dsRNA-dependent but appeared to be independent of all of the above mentioned PRRs, indicating that SenV may also be recognized by an unknown PRR in the Raw 264.7 cell line [24]. Therefore, the target of NS1 inhibition may be a signaling molecule common to other PRR signaling pathways in addition to TLR signaling.

Our experiments looking at IFNβ transcript levels and protein production in response to SenV infection suggest that NS1 is only able to down-modulate the early IFNβ response, but not the later response. This later IFNβ response is likely due to signaling through the IFNα/β receptor as part of the positive feedback loop, propagated by residual IFNβ produced during initial virus recognition. The IFNα/β receptor signals through the Jak/STAT pathway, which is quite different from the TLR signaling pathways. Therefore, the apparent inability of NS1 to inhibit the Jak/STAT signaling pathway indicates that NS1 is not a global signaling inhibitor, but rather has a more limited and specific function in signaling inhibition.

Other viral pathogens have developed strategies to inhibit TLR-mediated immune responses. For example, hepatitis C virus (HCV), another flavivirus, is able to inhibit innate immune signaling in a variety of ways. The NS3/4a protease has been shown to cleave TRIF [31] as well as the RIG-I adapter protein, IPS-1 [32]. It has been demonstrated that the HCV NS3/4a protease is able to prevent IRF3 phosphorylation in response to dsRNA stimulation [33] and NS3 is also able to bind the signaling molecule, TBK-1, preventing it from

interacting with IRF3 and thereby inhibiting further signal transduction [34]. Therefore, HCV has evolved mechanisms to evade both TLR as well as RLR-mediated innate immune stimulation. Herpes viruses encode the protein ICP0 (infected cell protein 0). This protein will cause the protein Ubc7 to translocate from the nucleus to deubiquitinate TRAF6 and IKKγ which are important signaling intermediates in the TLR pathway [35]. Deubiquitination of TRAF6/IKKγ prevents not only NFκB activation, but also prevents activation of other transcription factors such as IRF3 and AP-1. Whether a similar effect on TRAF6/IKKγ is the mechanism of action for NS1 as well remains to be determined. Based on the studies described here and other studies performed in the lab where overexpression of adapter molecules did not prevent NS1 inhibition, we believe that the point of NS1 inhibition occurs after the recruitment of the adapter molecule. It is also reasonable to assume that the point of inhibition is before activation of IRF3 and NFκB as these molecules represent a bifurcation of the signaling pathway, yet they are both affected by NS1 inhibition. In other studies, performed concurrently to these, NS1-mediated inhibition was shown to occur after adapter molecule recruitment and have indicated that NS1 inhibition occurs upstream of IKKs and MAP kinases (Wilson JR, manuscript in review). After adaptor molecule recruitment to the TLR there is recruitment of IL-1 receptor-associated kinase 1 or 4 (IRAK1 or 4), which will then bind TRAF6 [36]. From here TRAF6 will associate with Ubc13 and Uev1A to form K63-linked polyubiquitin chains [10, 13]. By a mechanism which is not completely understood, the TRAF6 polyubiquitin chain activates the TAK/TAB complex, without transfer of the ubiquitin [37]. This event is necessary for TAK1 kinase function and

further signaling. By breaking down the steps in the TLR signaling pathway which occur between the adaptor molecules and the IKKs and MAPKs, we identified three reasonable possibilities for the mode of NS1 inhibition. First, NS1 could somehow impair the association of IRAK with TRAF6. Secondly, NS1 could disrupt the formation of the K63-linked polyubiquitin chains by TRAF6. As a third possibility NS1 could prevent ubiquitin-mediated activation of the TAK/TAB complex, which is immediately upstream of the IKKs and MAPKs. Studies from the lab have identified TAK-1 as a potential target of NS1 inhibition; however the precise mechanism of inhibition remains elusive. NS1 could be acting either directly, or indirectly to perform its inhibitory function and may induce the degradation or destabilization of any of these intermediate molecules individually, or act in concert with another cellular molecule to inhibit the TLR signaling pathway.

The innate immune response to flavivirus infection is not completely understood. There is a clear role for the PRRs RIG-I and Mda-5, however the role for TLRs hasn't been fully described and remains somewhat controversial. A recent study has shown that TLR3 may work in concert with RIG-I and Mda-5 during the initial immune response to dengue virus infection [38], and another report suggests a protective role for TLR3 in flavivirus infection [39]. However, other data indicates that TLR3 may be detrimental during flavivirus neurotropic infection [40].

TLR7 has also been suggested to play a role in the innate immune response to flavivirus infection. Data generated by our own lab has shown an increased susceptibility to lethal WNV infection in TLR7-/- mice when virus is administered via the footpad (Miller-Kittrell, et al. unpublished data). Others have reported similar findings and have shown

increased WNV titers in the organs of TLR7-/- mice [41]. Yet a recent study has shown no difference in viral spread when WNV is administered cutaneously or during feeding by infected mosquitoes [42]. It was recently discovered that in response to WNV infection, TLR7 induces IL-23 production and subsequent homing of immune cells to sites of infection [41]. Additionally, it was shown that TLR7 signaling from keratinocytes infected with WNV resulted in cytokine production and subsequently, activation of Langerhans dendritic cell migration to the lymph nodes [42]. Still these somewhat limited roles for TLRs do not fully explain why a virus with a small genome would encode a protein with this inhibitory capability. As more data on the roles of TLRs in the immune response to flaviviruses become available the importance of the inhibitory function of NS1 should become more evident.

Acknowledgements

We would like to thank Dr. Eji Konishi for the anti-JEV NS1 monoclonal antibody, Clayton Morrison for construction of the pLEX lentiviral particles, and Jason Wilson for contributions to the IFNβ experiments.

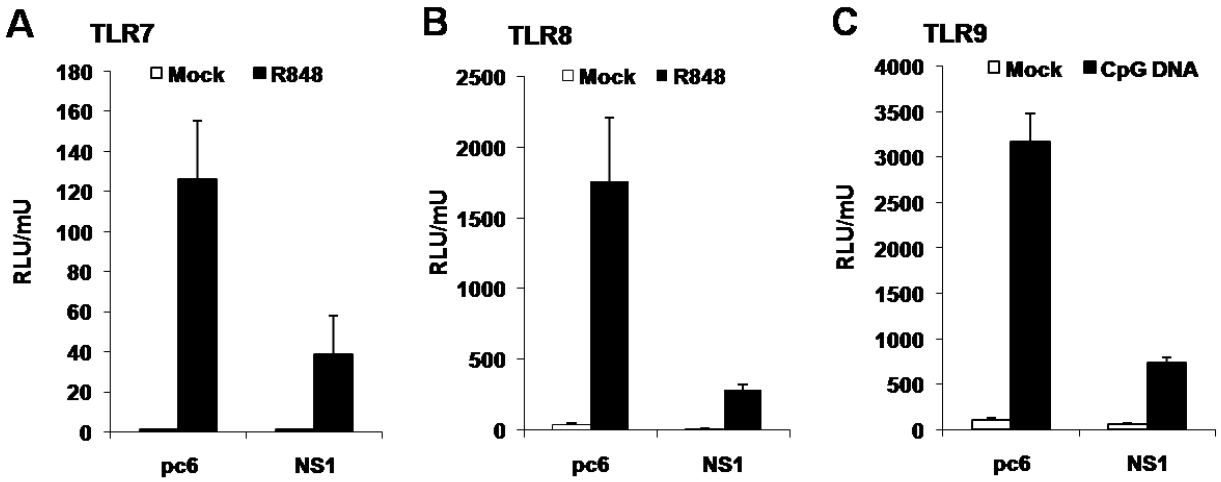

FIGURE 1: **WNV NS1 inhibits signaling through endosomal TLRs.** HEK 293 TLR7, 8, or 9 overexpressing cells were transfected with 500ng of WNV NS1, or vector control (pc6), and 150ng each of NFκB-dependent reporter and β-gal expression plasmids. 18h post-transfection cells were stimulated with the appropriate ligand (A, B) 1ug/mL R848, (C) 2.5μM CpG DNA. After 4 hours of incubation, lysates were prepared and assayed for NFκB activation by luciferase assay. Data are expressed as Luc activity normalized to β-gal activity. Error bars represent the standard deviations.

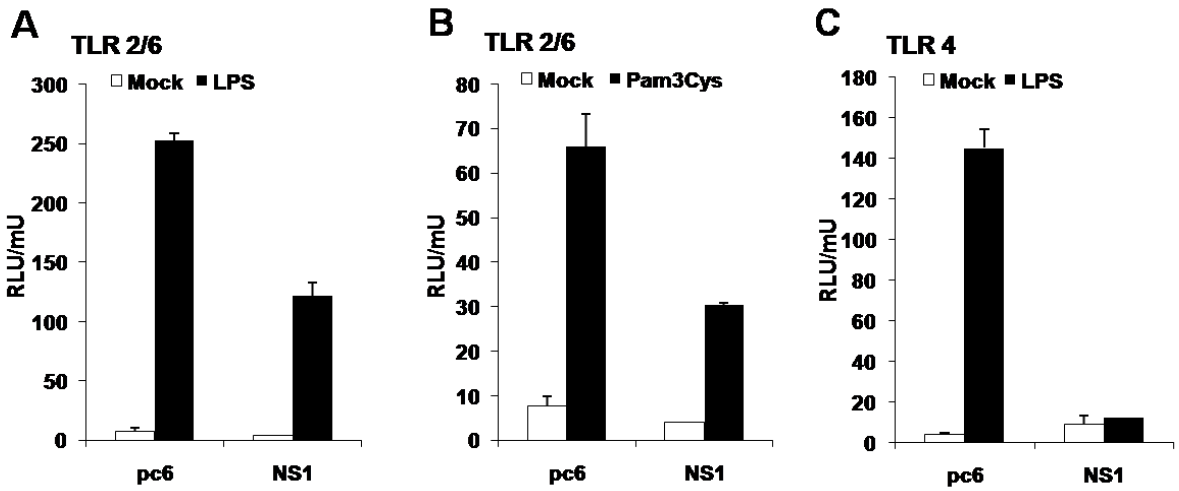

FIGURE 2: **WNV NS1 inhibits signaling through plasma membrane-associated TLRs.** HEK 293 TLR2/6, or TLR4/MD2 overexpressing cells were transfected with 500ng of WNV NS1-expressing plasmid, or vector control (pc6), and 150ng each of NFκB-dependent reporter and β-gal expression plasmids. 18h post-transfection cells were stimulated with (A) 1ug/mL LPS, (B) 100ng/mL Pam3CSK4, or (C) 1ug/mL LPS. After 4 hours of incubation lysates were prepared and assayed for NFκB activation by luciferase assay. Data are expressed as Luc activity and normalized to β-gal activity. Error bars represent standard deviations.

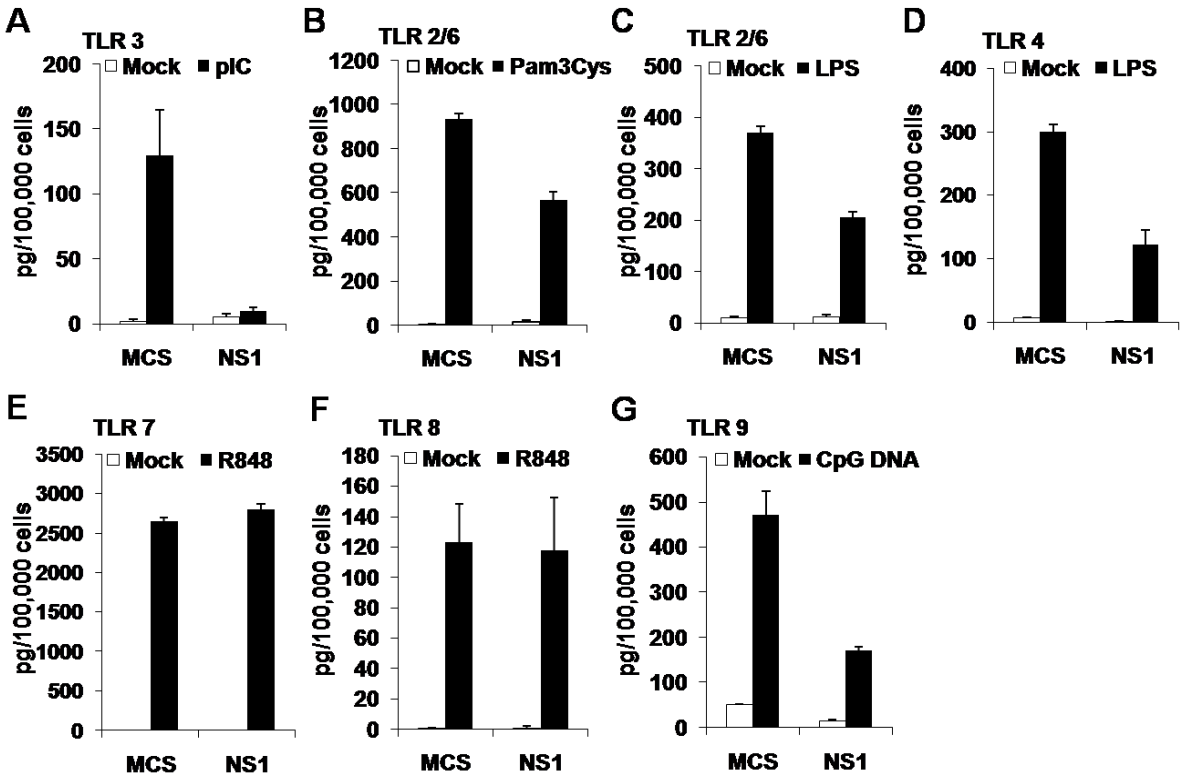

FIGURE 3: **WNV NS1 inhibits IL-8 production by select TLR-overexpressing cell lines.** HEK 293 TLR-overexpressing cells stably expressing WNV NS1 or MCS vector control were treated for 8 hours with the appropriate ligands (see Fig. 1 and 2). Supernatants were analyzed by ELISA and normalized to cell number per well for each cell line.

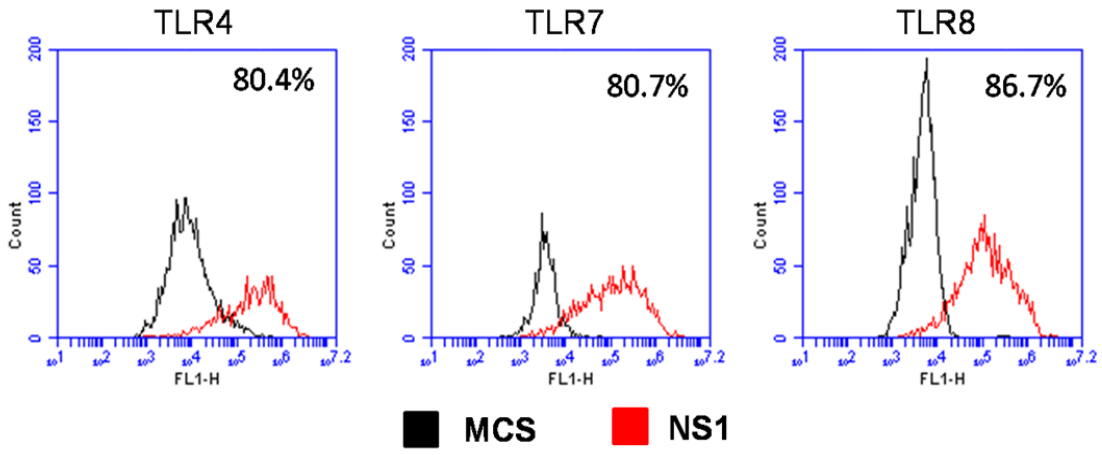

FIGURE 4: **NS1 expression levels in HEK 293 TLR-overexpressing lentiviral cell lines.** WNV NS1 expression was determined on fixed and permeabilized cells and compared to MCS control cells.

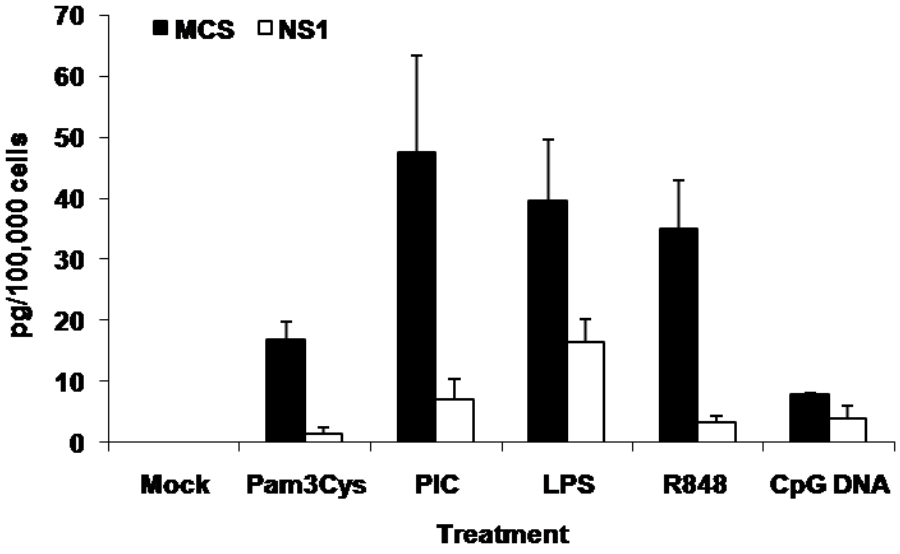

FIGURE 5: **WNV NS1 inhibition of IL-6 production by Raw 264.7 macrophage cells.** Raw 264.7 cells stably expressing NS1 or the MCS vector control were treated with the appropriate ligands (see Fig 1 and 2) for 16 hours before harvesting supernatants and analyzing IL-6 levels by ELISA. Results were normalized to cell number per well.

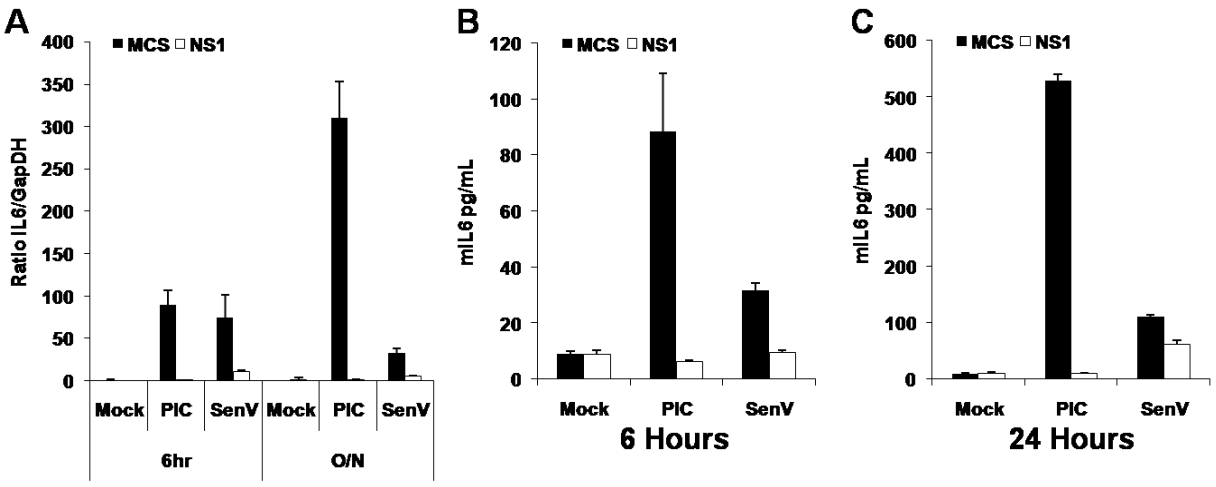

FIGURE 6: **WNV NS1 inhibits Sendai virus induced IL-6 production by Raw 264.7 macrophage-like cells.** (A) Raw 264.7 MCS or NS1-expressing cells were infected with 100HA units/mL Sendai virus or treated with 20μg/mL poly(I:C) (PIC) for 6 hours or 16 hours before analysis by qRT-PCR using SYBR green to detect IL-6 transcript levels, normalized to GAPDH. (B, C) Raw 264.7 MCS or NS1-expressing cells were infected with 100HA units/mL of Sendai virus for the indicated times before supernatants were harvested and IL-6 levels determined by ELISA.

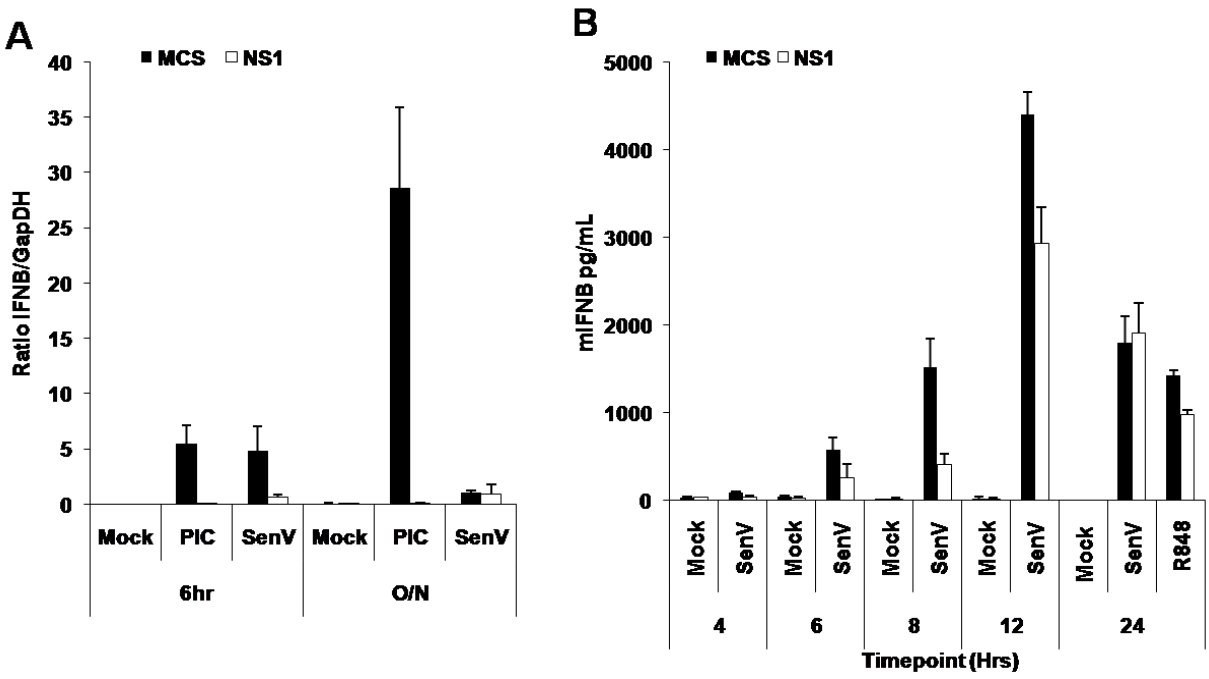

FIGURE 7: **WNV NS1 inhibits early Sendai virus-induced IFNβ production by Raw 264.7 macrophage cells.** (A) Raw 264.7 MCS or NS1-expressing cells were infected with 100HA units/mL Sendai virus or treated with 20μg/mL poly(I:C) (PIC) for 6 hours or 16 hours before analysis by qRT-PCR using SYBR green to detect IFNβ transcript levels, normalized to GapDH. (B, C) Raw 264.7 MCS or NS1-expressing cells were infected with 100HA units/mL of Sendai virus for the indicated times before supernatants were harvested and IFNβ levels determined by ELISA.

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Chapter 3

Toll-like receptor inhibition by the secreted form of the West Nile virus NS1 glycoprotein

Abstract

West Nile virus (WNV), a member of the family Flaviviridae, employs several different strategies to escape the innate immune response, including evading detection and actively repressing innate immune signaling pathways. A number of different nonstructural proteins of WNV have been shown to actively interfere with signal transduction from the type I interferon receptor and we have previously demonstrated that the WNV NS1 protein can interfere with signal transduction from toll-like receptor 3 (TLR3). NS1 is a glycoprotein that can be found intracellularly or associated with the plasma membrane. It is also known that NS1 is secreted to high levels during flavivirus infections. In this study we address the question of whether the secreted form of NS1 is able to inhibit toll-like receptor (TLR) signal transduction in uninfected cells. By these means, WNV could preemptively down-regulate TLR signaling in cells that are potential targets for future infection. We demonstrate here that co-culture of NS1-expressing cells with naïve cells results in inhibition of IFNβ promoter activation and show similar results using NS1-containing cell supernatants. To further define these effects, NS1 was purified from supernatants of NS1 expressing cells. Purified NS1 readily associated with uninfected cells and repressed IFNβ promoter activation and IL-6 secretion in response to TLR3 stimulation in HeLa cells. Purified NS1 was also able to inhibit TLR signaling in bone marrow-derived immune cell types. *In vivo* experiments showed that footpad administration of NS1 in a mouse model significantly reduced poly(I:C) and WNV replicon particle-mediated IL-6 and IFNβ transcription in draining lymph nodes.

Introduction

West Nile virus is a member of the family *Flaviviridae* whose clinically important members include yellow fever virus, dengue virus, and Japanese encephalitis virus, as well as a variety of tick-borne flaviviruses. WNV is transmitted by mosquitoes and humans are incidental hosts. Early flavivirus replication is believed to occur in Langerhans dendritic cells [1], which migrate to draining lymph nodes where further viral expansion will occur [2, 3]. Replication of WNV has also been reported in the skin [4, 5]. Upon entry into the bloodstream, the virus can spread to other organs, and/or enter the central nervous system (CNS) and lead to encephalitis. The immunocompetency of the host is critical in controlling the spread of WNV throughout the body. In recent years, significant progress has been made in identifying virus-host interactions particularly with regard to the innate immune system, the first line of defense against invading pathogens.

The innate immune response detects pathogen-associated molecular patterns (PAMPs) and elicits proinflammatory cytokine responses, production of type I interferon, and activation of NK cells and the complement system. Several different pattern recognition receptors (PRRs) have been implicated in signaling that leads to the production of proinflammatory cytokines and type I interferon (IFN) in response to flavivirus infections. Intracellularly replicating virus is detected through the RLH helicases RIG-I and Mda-5, which leads to activation of IRF3 and subsequent production of IFNβ [6, 7]. IFNβ production in response to infection has also been shown to depend on the activity of the dsRNA-activated protein kinase R (PKR) [8]. However, it is not clear whether PKR is acting as a PRR in this context or is acting mainly as an interferon-stimulated gene in

amplifying the response. Furthermore, several TLRs have been reported to be involved in innate responses to flaviviruses, although some controversy exists as to the exact effects and their significance in viral pathogenesis. On one hand, TLR3 has been reported to be detrimental to host survival in a mouse model of WNV encephalitis, [9] while in another study, TLR3 was found to have a protective function during WNV infection. Here, TLR3-deficient mice were shown to be more likely to develop WNV encephalitis and succumb to infection [10]. Dengue virus is reportedly recognized in HEK 293 cells overexpressing TLR3 [11], while TLR3 did not seem to mediate early recognition of WNV in other cell types [12]. Nasirudeen et al. reported that dengue is synergistically recognized by RIG-I, Mda-5 and TLR3 [13].

TLR7 is expressed to high levels in plasmacytoid dendritic cells (pDCs) and is thought to play a major role in IFNα production in response to infection [14, 15]. TLR7-recognition of flaviviruses has also been demonstrated in pDCs [16] [17]. Intraperitoneal infection of TLR7 deficient mice with WNV led to an increased morbidity/mortality that was traced back to a lymphocyte homing defect in the absence of TLR7 [18]. TLR7 might also be involved in regulating keratinocyte-mediated migration of WNV-infected cells to primary lymphoid organs [19]. MyD88, an adaptor protein in TLR signaling, was reported to predominantly affect WNV replication and spread in the CNS while not having a major effect on peripheral replication [20]

Our previous work has demonstrated that TLR3 signaling in WNV-infected cells is inhibited [21] and that this inhibition is due to expression of the viral NS1 protein [22]. NS1 is a glycoprotein that is co-translationally translocated into the lumen of the ER. NS1 is

required for RNA replication, where it is localized to replication complexes [23] and involved in early steps of RNA synthesis [24-26] although its exact mechanism of action in this process has yet to be elucidated. NS1 forms detergent-stable but heat-labile dimers and is secreted to high levels from infected cells [27, 28], after which it is detectable as a hexamer [29]. It was demonstrated that purified dengue virus NS1 administered to mice via the tail vein was targeted primarily to the liver and was endocytosed by hepatocytes [30]. Secreted NS1 (sNS1) can also associate with a variety of different cell types *in vitro* and *in vivo*, and dengue sNS1 has been shown to bind to endothelial cells through interactions with sulfated glycosaminoglycans [31]. Recent evidence identified sNS1 as an unusual high-density lipoprotein and it has been suggested that dengue virus sNS1 may exploit this trait to hijack lipid metabolism pathways, potentially contributing to the endothelial dysfunction observed during severe dengue disease [32].

In addition to these properties, several immunomodulatory effects of NS1 have been described. The lectin pathway of complement activation contributes to the restriction of WNV infection [33, 34]. Secreted NS1 binds to the C1s and C4 complement proteins and promotes cleavage of C4, preventing pathway activation [35]. Secreted NS1 also binds to C4b binding protein (C4BP), enhancing its cofactor activity, and preventing assembly of C3 and C5 convertases [36]. WNV sNS1 also modulates activation of the alternative complement pathway by binding to complement factor H and promoting cleavage of C3b [37]. This prevents opsonization of infected cells and therefore inhibits complement-mediated lysis. Interestingly, NS1 specific antibodies have been reported to be protective during flavivirus infection, leading to complement-mediated elimination of virally-infected

cells [38, 39]. Also, as previously mentioned; data from our own lab has shown intracellularly expressed WNV NS1 is able to inhibit TLR3 signal transduction [22], again demonstrating the multifunctional nature of the NS1 protein. These data demonstrate how the interplay between activation and inhibition of the complement system by sNS1 plays an important role during flavivirus pathogenesis.

Given the demonstrated interactions of sNS1 with naïve cells and its involvement in modulating the host response to infection, we tested the hypothesis that sNS1 modulates TLR signaling in uninfected cells. In this study we demonstrate that sNS1 purified from cell culture supernatants can inhibit TLR signaling in different cell types both of human and murine origin. The inhibition was detected both *in vitro* and *in vivo* and may therefore provide a mechanism for WNV to control innate immune signaling pathways in cells targeted for infection prior to virus entry.

Materials and Methods

Cell Lines

HeLa 1.1.1 cells, harboring a WNV replicon that expresses the WNV NS genes 1 to 5 and a neomycin resistance cassette (WNR NS1-5ET2AN) [40] were grown in Dulbecco's modification of minimal essential medium (DMEM; Cellgro) supplemented with 10% HyClone fetal bovine serum (Cellgro), 1% antibiotics, 20 µg/ml gentamicin, and 400 µg/ml G418 at 37°C. HeLa C cells are a "cured" derivative of HeLa 1.1.1, established by eliminating the WNV replicon through several passages in culture medium containing 200U/mL IFNα [21]. HeLa C cells were grown in the same medium as above without G418.

Human Embryonic Kidney (HEK 293T) cells were transduced with an NS1 encoding recombinant lentivirus. pLEX-NS1 was packaged into VSV-G pseudotyped, replication defective, lentivirus particles through co-transfection of HEK293T cells with vector, packaging (pREV and pMDLg), and envelope (pVSV-G) plasmids. Empty vector (pLEX-ctrl) was packaged identically. Lentivirus-containing supernatants were harvested and clarified 40h post-transfection, and stored at -80^{o}C until further use. 293T cells were transduced with pLEX-NS1 or pLEX-ctrl lentivirus for 24h in the presence of 8μg/mL polybrene. Polyclonal transduced cells were selected by puromycin treatment at 4μg/ml and selected as a pool. 293T NS1 cells were used for the production of sNS1 containing supernatants both for experiments and for sNS1 purification.

Primary Cells

Wild Type C57BL/6 mouse femurs were collected and bone marrow was harvested per standard protocols. Macrophages were grown in DMEM containing 10% fetal bovine serum, 1% non-essential amino acids, 1% antibiotics and differentiated in the presence of G-CSF. Myeloid dendritic cells were grown in RPMI containing 10% fetal bovine serum, 1% antibiotics, 55μM beta-mercaptoethanol and differentiated in the presence of IL-4.

Purification of NS1

A mouse monoclonal antibody to JEV NS1 (clone 6H4, a kind gift from Dr. Eji Konishi, Osaka University) was coupled to cyanogen bromide (CnBr) activated beads at 4˚C overnight. The remaining binding sites on the beads were then blocked. The column was

packed and prepared with oscillating pH washes followed by two additional low pH washes and 2 high pH washes. Clarified NS1 containing cell supernatant was then run over the column at a slow rate to ensure protein binding. The beads were washed at neutral pH, the protein was eluted in 0.5mL fractions with a high pH solution and immediately neutralized with 1M Tris buffer and assayed by ELISA for NS1 content. Fractions positive for NS1 by ELISA were run on 4-12% NuPage protein gels and subjected to silver stain (BioRad) to ensure purity. Pure NS1 fractions were then pooled and dialyzed against PBS overnight at 4ºC. The amount of protein present in the samples after dialysis was determined using a protein assay kit (Bio-Rad).

Immunodepletion of NS1

Flowthrough samples of NS1 supernatants were collected from the immunoaffinity column during purification. Depleted supernatant samples were analyzed by immunoblot to confirm the absence of NS1. Samples were dialyzed against PBS before use in cell culture assays.

Reporter Assays

Reporter assays using the luciferase (Luc) reporter gene under control of the IFNβ promoter (IFNβ pGL3; a gift from J. Hiscott) [21, 22]. Briefly, 150 ng each of IFNβ pGL3 and pCMV (where CMV is cytomegalovirus) β-galactosidase (β-Gal) (Invitrogen) was cotransfected into HeLa C cells, using the TransIT-LT1 transfection reagent (Mirus). At 4h posttransfection, clarified supernatant containing NS1 or clarified control supernatant was

added to the cells. Where purified NS1 was used, the appropriate concentration of sNS1 was prepared in full serum media. Control sample from purification was added at equal volumes in full serum media. Heat inactivation of sNS1 was completed by heating the sample at 95°C for 1h. Equal amounts of proteins were added for all experiments. After 16h cells were either left untreated or were treated for 4h with 20 μg/ml poly(I:C) (pIC; Calbiochem). Following treatment, cells were lysed in reporter lysis buffer containing 0.1% Triton X-100, 10% glycerol, 2mM Dithiothreitol, 2mM trans 1,2 diamino cyclohexane-NNNN acetic acid, and 25mM Tris phosphate and assayed for Luc and β-Gal activities using a Promega Luc assay system and an ONPG (o-nitrophenyl-β-D-galactopyranoside)-based β-Gal assay. β-Gal activity was used to normalize the Luciferase data for all experiments. All data are expressed as relative light units/mU of β-Gal activity.

Western blot analysis

Following treatment, protein extracts were prepared in cell lysis buffer (300 mM NaCl, 50 mM Tris-HCl, 0.1% Triton X-100, pH 7.6). Following a10-min incubation on ice, lysates were clarified by centrifugation, and protein concentrations were determined using a detergent-compatible protein assay kit (Bio-Rad). Equal amounts of protein were electrophoretically separated on 4 to 12% Nu-PAGE gels (Invitrogen) and electroblotted onto polyvinylidene difluoride membranes (Immobilon-P transfer membrane; Millipore). Following a blocking step with Tris-buffered saline-0.1% Tween-20 with 5% dry milk, NS1 was detected using a WNV-specific mouse hyperimmune serum (murine hyperimmune ascitic fluid [MHIAF]) as the primary antibody [21], followed by horseradish peroxidase-

conjugated secondary antibodies (KPL). Bound horseradish peroxidase was visualized with an ECL Plus kit (Santa Cruz). Membranes were stripped and reprobed with a mouse anti-β-actin antibody (Sigma) as a loading control.

Immunofluorescence

HeLa C cells were seeded onto LabTek chamber slides and incubated with supernatants from NS1 expressing cells or control for 16h at which time the slide was prepared for indirect immunofluorescence analysis (IFA) for identification of NS1 protein. Briefly, cells were washed with phosphate-buffered saline, fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked (2% bovine serum albumin, 5% normal horse serum, and 10 mM glycine in phosphate-buffered saline). The cells were incubated with a mouse anti-JE NS1 antibody, followed by incubation with AlexaFluor 568-conjugated anti-mouse antibody [Invitrogen]. The cells were then analyzed for presence of NS1 by immunofluorescence using a Zeiss Axioskop 2 microscope.

ELISA

IL-6 or IL12p70 levels in the supernatant of cells treated with poly(I:C) or LPS (Invivogen) for 8h or overnight or left untreated were determined using an enzyme-linked immunosorbent assay (ELISA) kit (E-bioscience) of appropriate specificity according to the manufacturer's specifications. Each experimental set was performed in triplicate.

VRP production and purification

To produce high titer stocks for in vivo studies, BHK (VEErep/Pac-Ubi-C*) cells that express the WNV capsid protein from a Venezuelan encephalitis virus replicon were inoculated with Replivax WN [41] at an MOI of 0.05 and supernatants were harvested 96 hpi. Replivax WN contains a deletion of the capsid protein. The supernatants were clarified and then concentrated over a 100,000 kilodalton (Kda) cutoff centrifugal filter at 1500xg. The concentrate was applied to a 10-40% sucrose gradient and centrifuged at 35,000 rpm for 2.5hr at 4°C. The fractions containing infectious particles were collected and washed three times with L15 medium over a centrifugal filter to remove residual sucrose. The final concentrate was resuspended in L15 medium containing 10mM HEPES and 0.1% FBS. VRP titers were determined on Vero cells by immunostain against WNV-specific antigen and reported as infectious units (iu)/ml.

***In vivo* experiments**

C57BL/6 mice were obtained from Charles River laboratories and housed under specific-pathogen-free conditions in the Biological Resources Facility at NCSU. All procedures were conducted in accordance with the NCSU Institutional Animal Care and Use Committee. Eight to twelve week old female C57BL/6 mice were inoculated in the rear footpads with 3.36μg of purified NS1. After 6h, mice were challenged via footpad administration of 20μg poly(IC:LC) (Hiltonol, Oncovir), PBS control, or $2x10^5$ iu purified Replivax VRP. Draining lymph nodes were harvested at 16h post challenge and processed for RT-PCR analysis.

Reverse transcription PCR

Total RNA was extracted using a Qiagen RNeasy kit with DNase treatment according to the manufacturer's specifications. Reverse transcription (RT) was carried out with the ImProm II RT kit (Promega) using oligo dT or random hexamer as primer for NS5 experiments. Real-time PCR analysis was carried out using the SensiMix SYBR & Fluorescein kit (BioLine) and the following primers; Mouse: *GAPDH* 5'-TGCCCAGAACATCATCCCTG-3' and 5'-ATCCACGACGGACACATTGG-3', *IL6* 5'GACTTCACAGAGGATACCACTCC-3' and 5'- TTCTGCAAGTGCATCATCGGT-3', *IFNβ* 5'- GGAGATGACGGAGAAGATGC-3' and 5'- CCCAGTGCTGGAGAAATTGT-3'. Reactions were set up in 96-well PCR plates (Genesee). Amplifications were carried out for 50 cycles, followed by a melt curve analysis of resulting products to confirm the specificity of the reactions. To construct standard curves, total RNA was isolated from the cells, and 300- to 600-bp fragments of the gene(s) of interest were amplified by RT-PCR using the appropriate primer sets. PCR fragments were gel purified and quantitated, and the copy number was calculated. Serial 10-fold dilutions were prepared for use as templates to create standard curves in real-time PCRs. All data are expressed as the ratio of copy numbers of target gene per 10^3 copies of GAPDH for samples run in triplicate.

Flow cytometry

HeLa cells were incubated in the presence of NS1 for indicated times then the cells were washed with PBS before fixing and permeabilizing on ice for 20 minutes (BD Cytofix/Cytoperm kit) or left unpermeabilized and fixed with 4% paraformaldehyde for five

minutes on ice. Cells were incubated with a primary antibody generated against the NS1 protein of Japanese Encephalitis virus and then a FITC-tagged secondary antibody. For *in vivo* detection of NS1, purified protein was tagged with the alexa-488 fluorophore using an Alexa-Fluor 488 protein labeling kit (Invitrogen) and this was used for injection into the footpad. At indicated times after inoculation the popliteal lymph nodes were harvested and analysis was performed on live cells. Analysis was performed on the Accuri C6 flow cytometer according to standard protocol.

Statistical analysis

Statistical analysis was performed software using student t-test in PRISM. P values below 0.05 were considered significant.

Results

WNV replicon cells inhibit TLR3 signal transduction in uninfected neighbors

To address whether WNV infected cells are able to influence TLR3 signaling in uninfected neighboring cells, we initially performed co-culture experiments. Uninfected HeLa cells were transfected with a reporter construct in which luciferase (Luc) expression is controlled by the IFNβ promoter as described previously [22]. The transfected cells were incubated for 16h with either HeLa WNV replicon cells (HeLa 1.1.1) which will secrete NS1 [21] or regular HeLa cells and subsequently stimulated with pIC to trigger TLR3 signaling. Lysates were harvested and analyzed for Luc activity, which was only induced in the cells transfected with the reporter plasmids. Figure 1A shows that reporter-transfected cells co-

cultured with regular HeLa cells were able to strongly induce Luc expression from the IFNβ promoter, while HeLa cells cultured with HeLa 1.1.1 cells were significantly inhibited in their TLR3 response. Similar results were obtained by co-culture of HeLa 1.1.1 cells with HEK 293 cells overexpressing TLR3 (data not shown). Therefore, WNV-infected cells can modulate TLR3 responses in uninfected neighboring cells. To investigate whether this phenomenon was cell contact dependent or mediated by a soluble factor in the culture fluid, the experiments were repeated by incubating reporter-transfected naïve cells with supernatants harvested from WNV HeLa 1.1.1 cells. Induction of Luc expression was greatly reduced in HeLa cells incubated with HeLa1.1.1 supernatants while incubation of transfected cells with normal HeLa cell supernatants had no effect on TLR3 activation (Fig. 1B).

To directly address whether this inhibition is mediated by NS1 and not another viral factor expressed by the HeLa 1.1.1 cell line, NS1 was expressed in HEK 293T cells from a stably integrated lentivirus vector. The expression and secretion of NS1 from these cells were confirmed by immunoblot (data not shown). Supernatants from NS1-expressing cells and a lentivirus vector control cell line were harvested and incubated with HeLa cells transfected with the IFNβ reporter prior to TLR3 stimulation. IFNβ promoter activation was significantly inhibited in cells that were incubated with NS1 containing supernatants compared to cells exposed to the vector control supernatants (Fig. 1C). These results demonstrate that the inhibition of TLR3 signal transduction is dependent on NS1.

NS1 associates with naïve cells

Any scenario in which secreted NS1 might inhibit TLR signaling is likely to involve association of sNS1 with naïve cells. In fact, Alcon Le Poder et al. reported that purified dengue virus sNS1 was rapidly endocytosed by hepatocytes [30] and Avirutnan et al. demonstrated preferential dengue virus sNS1 binding to endothelial cells [31]. Secreted NS1-containing or vector control HEK 293T cell supernatants were added to HeLa cells and analyzed by immunofluorescence after 16 hours of incubation using an NS1-specific antibody. NS1-specific punctate staining was found only in cells incubated with NS1-containing supernatants (Fig. 2A). To further confirm association of NS1 with naïve cells, whole cell extracts were prepared after incubation of HeLa cells with control or sNS1-containing supernatants. Cells were thoroughly washed prior to lysis to remove any unbound sNS1. An NS1-specific band was detected by western blot in whole-cell extracts of cells incubated with sNS1-containing supernatants, confirming its association with naïve cells (Fig. 2B). To answer the question of whether NS1 simply binds to the cell surface or becomes internalized, flow cytometric analysis was performed on permeabilized and non-permeabilized cells, pre-incubated with either sNS1-containing or control supernatants. At both 6h and 24h after sNS1 addition, NS1 could be readily detected both on non-permeabilized cells as well as in permeabilized cells. In fact, NS1 staining was stronger in permeabilized cells than in nonpermeabilized cells even at 6 hours after addition, indicating that internalization occurred rapidly (Fig. 2C).

Purification of sNS1

The previous experiments demonstrate that supernatants from NS1-expressing cells are able to inhibit TLR3 signaling in naïve cells. To determine whether sNS1 was directly responsible for this effect, as opposed to a cellular factor potentially induced by NS1 expression, sNS1 was purified using an immunoaffinity approach. A Japanese encephalitis virus anti-NS1 monoclonal antibody [42] was used to purify sNS1 from supernatants of HEK 293T NS1 cells. Figure 3A shows the NS1 elution profile from the immunoaffinity column as detected by NS1-specific ELISA, and Figure 3B shows NS1 purity by SDS-PAGE silver stain. As a control for subsequent experiments, supernatants from vector control cells were treated identically to sNS1-containing supernatant and subjected to a mock purification process.

Purified NS1 inhibits TLR3 signaling

Purified secreted NS1 or control fraction (referred to as MCS) were added to HeLa cells and incubated for 16h followed by pIC treatment to trigger TLR3 signaling. MCS-treated cells responded to poly(I:C) with strong upregulation of IFNβ promoter activity, while cells incubated with sNS1 did not respond (Fig. 4A). To verify TLR3 inhibition outside the context of reporter assays, cytokine secretion in response to TLR3 stimulation was investigated. We previously reported that measurement of IL-6 secretion by ELISA is an appropriate readout for TLR3 signaling in HeLa cells, which do not produce appreciable amounts of IFNβ in response to TLR3 stimulation [22]. Preincubation of HeLa cells with sNS1 significantly inhibited TLR3-mediated IL-6 production in a dose-dependent manner as

compared to the MCS control (Figure 4B, plotted as fold-induction). The inhibition was lost when sNS1 was heated to 95°C, or upon antibody depletion of sNS1 from supernatants prior to addition to cells (Fig. 4C), confirming that the observed inhibitory effects were dependent on sNS1. The concentrations used are within the range reported for sNS1 circulating in the serum of dengue patients and the ranges reported for WNV NS1 circulation in the serum in a mouse model [28]. These experiments confirm that WNV sNS1 can inhibit cytokine production in response to stimulation of endogenously expressed TLR3.

TLR inhibition requires sNS1 association with naïve cells

Dengue virus NS1 has recently been shown to associate with cells via glycosaminoglycans (GAGs), such as heparan sulfate and chondroitin sulfate E [31]. Sodium chlorate treatment of cells can be used to inhibit the sulfation of GAGs and was shown to inhibit binding of dengue virus sNS1 to endothelial cells [31]. Supernatants containing sNS1 were collected from HEK 293T NS1 cells in a sulfate-free medium. HeLa cells were pretreated with increasing concentrations of sodium chlorate for 24h followed by incubation with sNS1-containing supernatants and subsequent TLR3 stimulation. Association of sNS1 with target cells was determined by flow cytometry. Sodium chlorate pretreatment inhibited NS1 binding to target cells in a concentration dependent manner (Fig. 5A). TLR3 stimulation in this context was assayed by IL-6 ELISA and showed that sodium chlorate pretreatment alleviated sNS1-mediated inhibition in a dose-dependent manner (Fig. 5B). Therefore, prevention of sNS1 binding to target cells prevented inhibition of TLR3 signaling.

Inhibition of TLR signaling in bone marrow derived immune cells

Up to this point, all experiments testing a potential role of sNS1 in TLR inhibition were conducted in HeLa cells. To investigate the effect of sNS1 on more immunologically relevant cell types, TLR3 and TLR4 signaling were investigated in bone marrow-derived mouse macrophages (BMDMs) (Fig. 6A) and bone marrow-derived myeloid dendritic cells (BMDCs) (Fig. 6B), both of which endogenously express these TLRs. As previous data for intracellularly-expressed NS1 indicated a range of NS1-mediated TLR signaling inhibition, LPS was included in these studies to determine if the same was true for sNS1. Both cell types were incubated with 5μg/ml of purified sNS1, equal amounts of MCS control, or heat-inactivated NS1 for 16h followed by stimulation with pIC or LPS. IL-6 secretion by BMDMs was assayed after 8h of stimulation and IL12p70 production by BMDCs was assayed by ELISA after 16h of TLR3 or TLR4 stimulation. Both MCS-treated cells and cells treated with heat inactivated sNS1 retained the ability to respond to pIC and LPS with significant secretion of IL-6 (Fig. 6A) or IL12p70 (Fig. 6B) while this response was severely impaired in cells pretreated with sNS1. These results demonstrate that impairment of TLR3 signaling by sNS1 is not restricted to HeLa cells but can also be observed in macrophages and dendritic cells of murine origin. Importantly, these results show that the inhibitory activity of sNS1 is not restricted to TLR3, but extends to TLR4 as well, which is in line with our previous observations made for intracellularly-expressed NS1. These observations should prove helpful in future evaluations of the effects of NS1 on WNV pathogenesis in the mouse model.

Secreted NS1 inhibits the *in vivo* response to poly(IC:LC)

With mounting evidence to support a TLR-inhibitory function of sNS1 *in vitro* we decided to test whether sNS1 could exert a similar function *in vivo*. A footpad injection model was chosen because it allows monitoring of innate responses in the popliteal lymph nodes (pLNs). Preliminary experiments using fluorescently-labeled sNS1 protein showed that sNS1 was readily detected in pLNs 6h after footpad injection (Figure 7A). Wild type C57BL/6 mice were injected via the footpad with 3.36μg of either purified or heat-inactivated NS1, or an equivalent volume of the MCS purification control. Six hours later mice were challenged with 20μg of poly(IC:LC) via the footpad. Poly(IC:LC) (Hiltonol) is a polylysine stabilized form of pIC that has been reported to induce potent innate immune responses similar to those of the YFV 17D vaccine in humans [43]. PLNs were harvested 16h after poly(IC:LC) administration and processed for total RNA extraction. Expression of IFNβ and IL-6 mRNA was monitored by real-time RT-PCR (Fig. 7B and C). MCS- and heat-inactivated sNS1-pretreated mice responded to poly(IC:LC) treatment with upregulation of IL-6 and IFNβ transcription. In contrast mice pre-injected with NS1 showed significantly reduced poly(IC:LC)-dependent induction of cytokine transcription. These data demonstrate that NS1 delivered cutaneously is able to inhibit innate responses in the draining lymph node.

NS1 inhibits the *in vivo* response to WNV VRP infection

The data presented thus far have focused on responses triggered by synthetic agonists of innate immune signaling pathways. Thus it was important to determine the effects of NS1 in the context of infection. In a previous study we demonstrated upregulation of IFNβ and

IL-6 transcription in the pLN at 24h post infection following footpad inoculation with WNV replicon particles (VRPs), demonstrating immune activation from a single round of viral replication. These data indicate that lymphoid tissues are a significant source of IFN production early in WNV infection [44] and that the footpad inoculation model is suitable to monitor early innate responses to infection with VRPs. Secreted NS1 or heat-inactivated sNS1 was delivered to mice via footpad injection as described above. Six hours later, mice were inoculated via footpad with WNV VRPs. PLNs were harvested 16h later and processed for RT-PCR analysis. Figure 8 shows a representative experiment out of three independent studies. Both IL-6 and IFNβ responses to VRP infection were significantly dampened in mice pretreated with sNS1 compared to mice receiving heat-inactivated sNS1 as a control (Fig. 8 A and B). Assaying for NS5 copies confirmed equal infection between the heat inactivated NS1 and NS1 treatment groups (Fig. 8C).

Discussion

Based on our previous studies regarding the ability of WNV NS1 protein to inhibit TLR signaling, we explored the possibility that the secreted form of NS1 might also play a role in this process. Our results show that NS1 secreted from either replicon-bearing cell lines or cell lines engineered to express NS1 is able to inhibit TLR3 signaling in naïve cells. These findings could be reproduced using sNS1 purified from cell culture supernatants. Importantly, purified sNS1 also inhibited immune responses *in vivo*.

For our studies, sNS1 was purified by immunoaffinity chromatography from the supernatants of cell lines engineered to secrete the protein. While we did not detect any co-

purified proteins in silver-stained electrophoresis gels, we cannot entirely rule out the possibility of sNS1 closely associating with another cellular factor(s), which is ultimately responsible for TLR inhibition. However, our data still demonstrate a dependence on sNS1 for TLR inhibition and based on the available evidence, the most likely explanation is that sNS1 is directly responsible for the TLR-inhibitory effects described.

Secreted NS1 was found to associate with naïve cells and could be detected both on the cell surface as well as intracellularly. While we have not formally demonstrated that sNS1 uptake occurs via endocytosis, this has been reported previously for dengue virus NS1 [30] and it is thus plausible that WNV NS1 enters cells in the same way. At this point it is not known whether sNS1 uptake into cells is required for its inhibitory function or if simply binding to target cells is sufficient; however, our data do demonstrate that the interaction of sNS1 with target cells is necessary for TLR inhibition. Avirutnan et al. demonstrated that sodium chlorate pretreatment of cells results in reduction of sulfation of cell surface-expressed glycosaminoglycans, which in turn prevents sNS1 binding to the cells [31]. In agreement with this report, our experiments have shown that sodium chlorate pretreatment of cells abolished sNS1 binding to the cells and prevented inhibition of TLR signaling in the presence of sNS1.

This report, to our knowledge, is the first study to describe TLR inhibition by a viral protein secreted during infection. Arjona et al. have reported that the WNV E protein is able to subvert TLR3 signaling, but this is only true for the E protein of mosquito cell origin [45]. Therefore, the mosquito-derived E protein could inhibit TLR3 during the very first infection cycle while E protein subsequently produced by mammalian cells would be unable to do so.

Our results argue that NS1 secreted from infected mammalian cells throughout the course of infection would inhibit TLR signaling by naïve cells.

At present the consequences of sNS1-mediated inhibition of TLR signaling for virus pathogenesis are not understood. Clearly the IFN system is very important in the control of flavivirus infections as illustrated by the enhanced susceptibility to flavivirus infection of mice deficient in either IFN receptor expression or IFN synthesis [46-49]. The importance of IFN is further illustrated by the many mechanisms evolved by flavivirus non-structural proteins to counteract either IFN induction or IFN signaling [50-57]. However, the role of individual PRRs, including TLRs, in flavivirus pathogenesis and their influence on IFN and proinflammatory cytokine production during flavivirus infections is not entirely clear.

Using MEFs from RIG-I and Mda-5 deficient mice it was demonstrated that RIG-I is important for the early type I IFN induction [58] while Mda-5 seems to play a role later in infection [6]. IPS-1 is an adapter protein common to both RIG-I and Mda-5 and analysis of IPS-1 knockout MEFs has shown that IFNβ induction in early infection is dependent on IPS-1 signaling [59]. These IPS-1 dependent pathways converge on activation of IRF3. Once type I IFN binds to the IFNβ receptor, an amplification loop for IFN synthesis is induced via expression and activation of IRF7 [60], a signaling intermediate also directly activated by TLR7, 8 and 9 via MyD88 [61]. Using MEFs derived from IRF3/7 double knockout (DKO) mice, IFNβ induction in response to early WNV infection was determined to be dependent on IRF3/7 [62]. However, differential effects of IRF3/7 were observed depending on the cell type as myeloid dendritic cells still showed significant IFN induction in the absence of IRF3 and IRF7. The contributions of IRF3/7 to the immune response to WNV infection *in vivo* are

significant as these DKO mice die within 6 dpi. However, the effect on mortality is not as substantial as is seen for IFNα/βR-/- mice, indicating a response to early WNV infection-mediated by signaling factors other than IRF3/7.

The effects of TLR deficiency on flavivirus pathogenesis are generally less dramatic than those seen in the absence of IPS-1-dependent pathways and several conflicting reports exist regarding the role of TLRs in flavivirus infection. Compared to WT mice, IP inoculation of TLR3-knockout mice with WNV resulted in a small enhancement of virus replication in the periphery [9]. However, this enhancement was not seen upon footpad inoculation [10]. In the IP inoculation study, TLR3 seemed to regulate the synthesis of IFNβ in the periphery while this was not found in the footpad inoculation model. In the latter context, TLR3 seemed to play a more prominent role in regulating virus replication and innate responses in the central nervous system [10]. A similar disparity is seen for the intraperitoneal, intradermal, and footpad inoculation models in TLR7-knockout mice. IP injection with WNV led to increased virus replication and higher mortality in TLR7 deficient mice [18], while TLR7 deficiency in an intradermal-inoculation model did not produce significant differences in mortality or viral burden [19]. Our own studies using footpad inoculations demonstrated increased susceptibility to WNV in the absence of TLR7, without significantly increased viral burden (Miller-Kittrell, M, unpublished data). In summary, TLRs have a role in flavivirus pathogenesis without having great influence on type I IFN production during flavivirus infection.

The majority of studies published on the role of PRRs in flavivirus infection have predominantly focused on the IFN system and therefore much less is known about the role of

other proinflammatory cytokines that are released in response to flavivirus infection in both TLR-dependent and independent manners. For example, it does not appear that MyD88-dependent signaling is required for IFNβ production early in infection [20], however, MyD88 signaling would likely induce IL-6, TNFα, and IL-12 production via IRF5 and contribute to immune cell migration in response to infection [63].

Our data show that sNS1 administered to mice via the footpad is able to modulate the *in vivo* response to stimulation with both poly(IC:LC) and WNV VRPs. Poly(IC:LC) has been reported to induce immune responses similar to those induced by the YFV17D vaccine [43], where TLR 4, 7 and 9 stimulation was identified [64]. Additionally, Bourne et al. showed IRF3-independent IFN production by poly(IC:LC) [44]. Therefore, poly(IC:LC) might not exclusively stimulate TLR3 but may also trigger other TLRs, RIG-I, Mda-5 and potentially other pathways, similar to what is expected from VRP infection. Our data indicate that at least TLR4-dependent signaling is inhibited by sNS1 in bone marrow derived immune cells, however additional studies are needed to determine the effect of sNS1 on signaling by other TLRs.

In summary our data demonstrates a novel role for a secreted nonstructural WNV protein in modulation of innate immune responses, which likely has important implications for WNV pathogenesis.

Ethics statement

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the NCSU Institutional Animal Care and Use Committee (Permit Number: 10-108B). All surgery was performed under isofluorane anesthesia, and every effort was made to minimize suffering.

Acknowledgements

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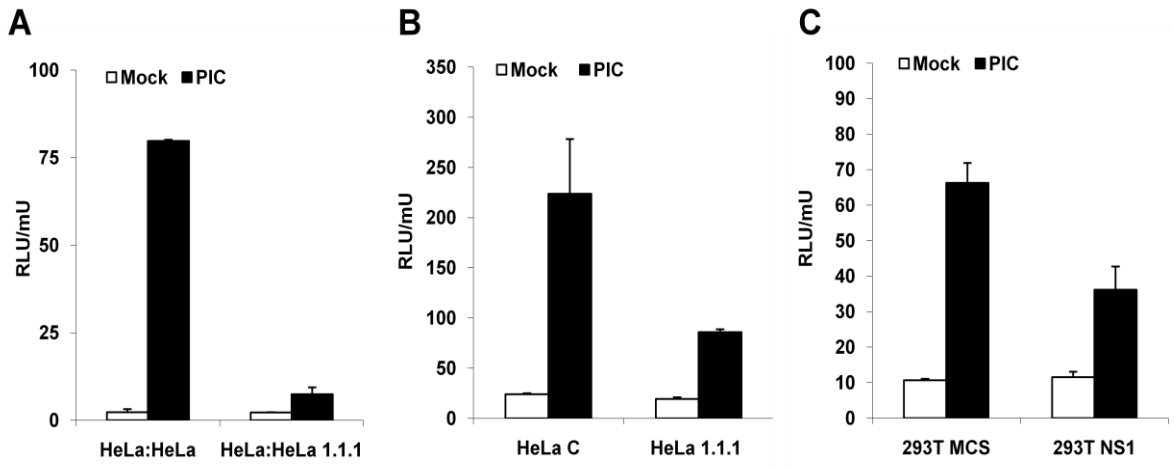

FIGURE 1: **WNV replicon cells inhibit TLR3 signal transduction in uninfected neighbors.** A. HeLa cells were transfected with an IFNβ-Luc reporter construct and a β-gal expressing plasmid and subsequently co-cultured with either regular HeLa cells or HeLa cells harboring a WNV replicon (HeLa 1.1.1). Luc activity was determined 4 hours after stimulation with pIC and normalized to β-gal activity. B. HeLa cells transfected with IFNβ reporter and β-gal as above were incubated for 16h with cell culture supernatants from either regular HeLa cells or HeLa 1.1.1 cells then stimulated with pIC before Luc activity was determined. C. HeLa cells transfected with IFNβ reporter construct and β-gal were incubated with supernatants from either control or NS1-expressing HEK 293T cells for 16h and stimulated as above.

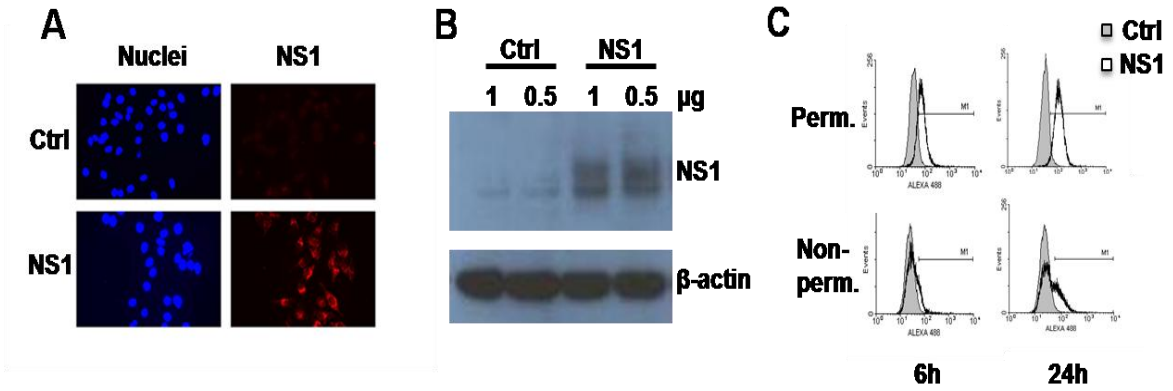

FIGURE 2: **NS1 associates with naïve cells.** A. HeLa cells were incubated with cell culture supernatants from either MCS control or NS1-expressing HEK 293T cells for 16h. NS1 association with cells was detected by immunofluorescence with an NS1-specific antibody. B. Whole-cell lysates of HeLa cells incubated with MCS control or NS1- containing supernatants were analyzed by immunoblot with WNV MHIAF (mouse hyperimmune ascitic fluid) as primary antibody. C. NS1 association with naïve cells determined by flow cytometry. HeLa cells were incubated with control or NS1-containing supernatants for 16h and left unpermeabilized to detect NS1 association with the cell surface or permeabilized prior to staining to detect NS1 taken up into the cells. NS1 was detected with an NS1-specific antibody.

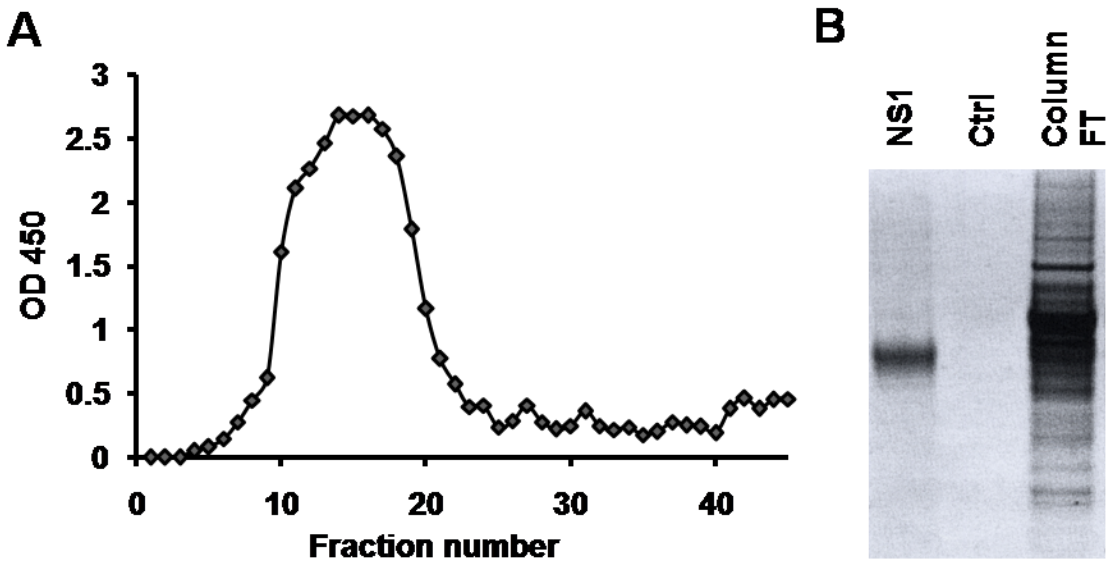

FIGURE 3: **Purification of secreted NS1.** NS1 was purified by immunoaffinity chromatography using an NS1-specific antibody. A. Elution fractions were analyzed for the presence of NS1 by NS1-specific ELISA. B. Silver stain of SDS-PAGE gel. Ctrl=MCS control supernatants undergoing identical purification conditions, FT= column flowthrough.

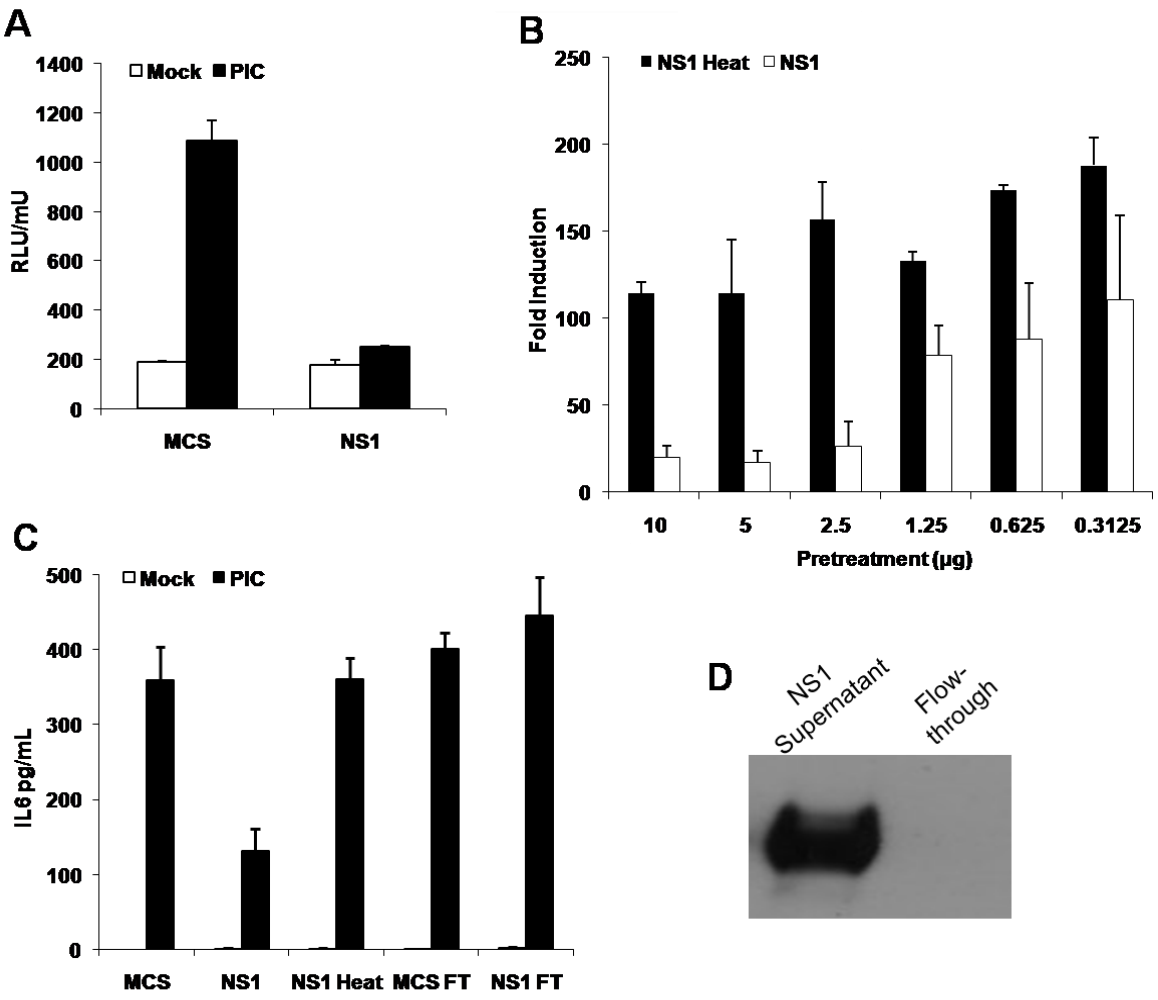

FIGURE 4: **Purified NS1 inhibits TLR3 signaling.** A. HeLa cells were transfected with IFNβ reporter construct and a β-gal expression construct and incubated with 4µg/mL purified NS1 or equal volume of MCS control for 16h prior to stimulation with pIC. B. IL6 secretion was measured from pIC treated HeLa cells following incubation with either MCS control or NS1. Results are the average fold-induction from two separate experiments. C. IL6 secretion was measured from PIC treated HeLa cells following incubation with MCS control, NS1, heat-inactivated NS1 or the flowthrough fraction from either MCS control supernatant or supernatant from NS1-expressing cells. D. Immunoblot analysis confirming depletion of NS1 from the flowthrough sample.

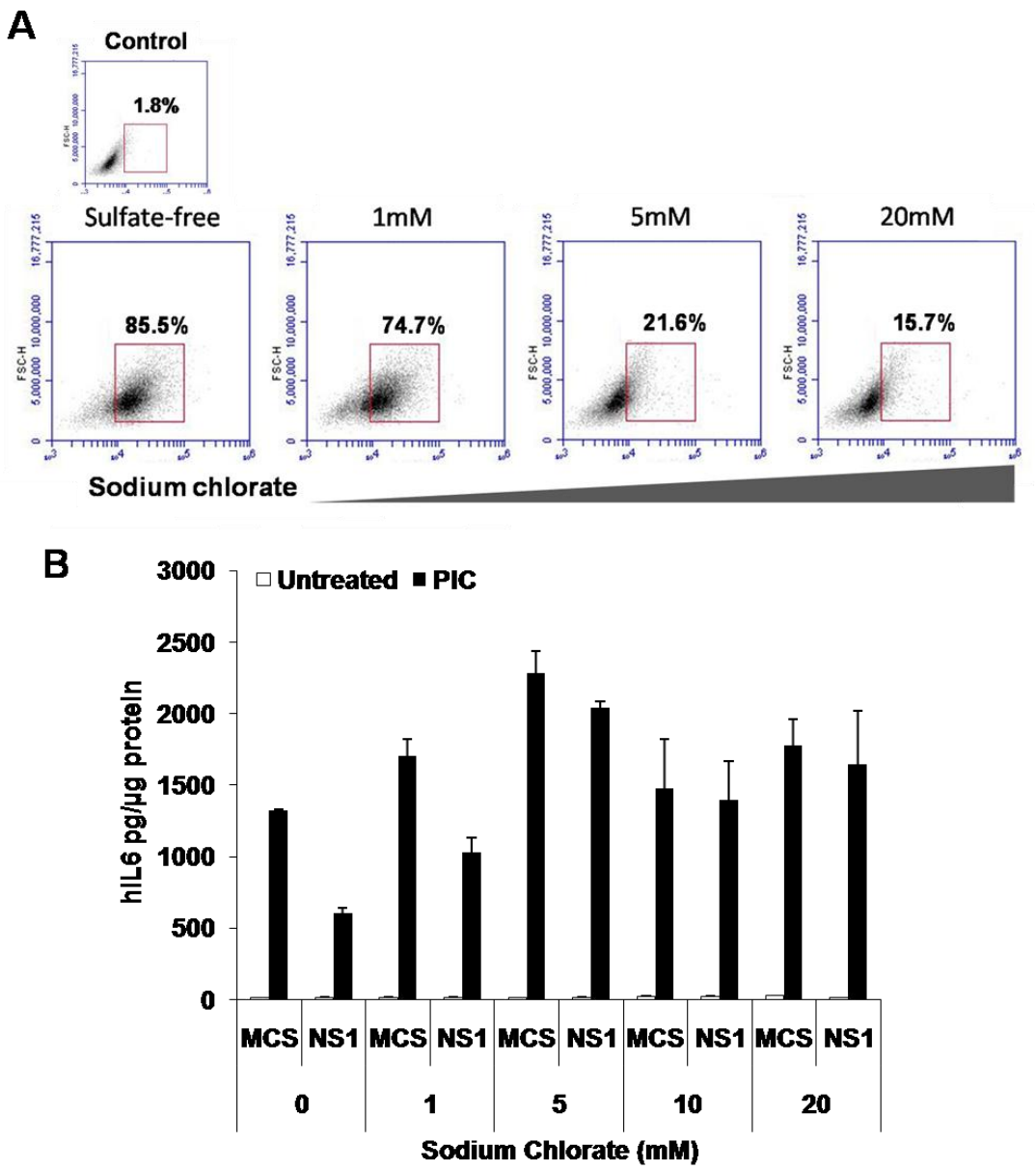

FIGURE 5: **TLR inhibition requires sNS1 association with naïve cells.** A. HeLa cells were incubated for 24h in sulfate-free medium alone or with increasing concentrations of sodium chlorate before addition of sNS1 or controls under the same conditions. Permeabilized cells were analyzed for NS1 association by flow cytometry using an NS1-specific antibody. B. Loss of NS1-cell interaction restores TLR3 signaling. IL-6 was measured in the supernatants from HeLa cells treated as in A and stimulated with 20µg/mL PIC for 8 hours.

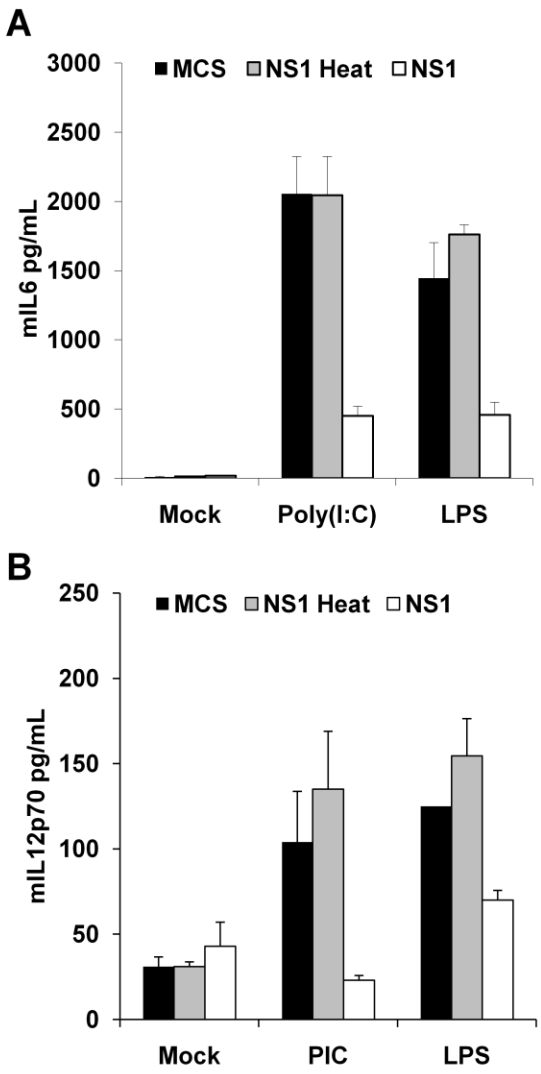

FIGURE 6: **Inhibition of TLR signaling in bone marrow derived immune cells.** A. BMDMs derived from C57BL/6 mice were incubated with 5μg/mL NS1 or control followed by 8 hour treatment with pIC at 5μg/mL or LPS at 1μg/mL. IL-6 release was measured by ELISA. B. Purified NS1 inhibits TLR3 signaling in BMDCs. Myeloid DCs derived from WT C57BL/6 mice were incubated with 5μg/mL NS1 or controls and stimulated with 10μg/mL PIC or 1μg/mL LPS for 12 hours. IL12p70 release into supernatants was determined by ELISA.

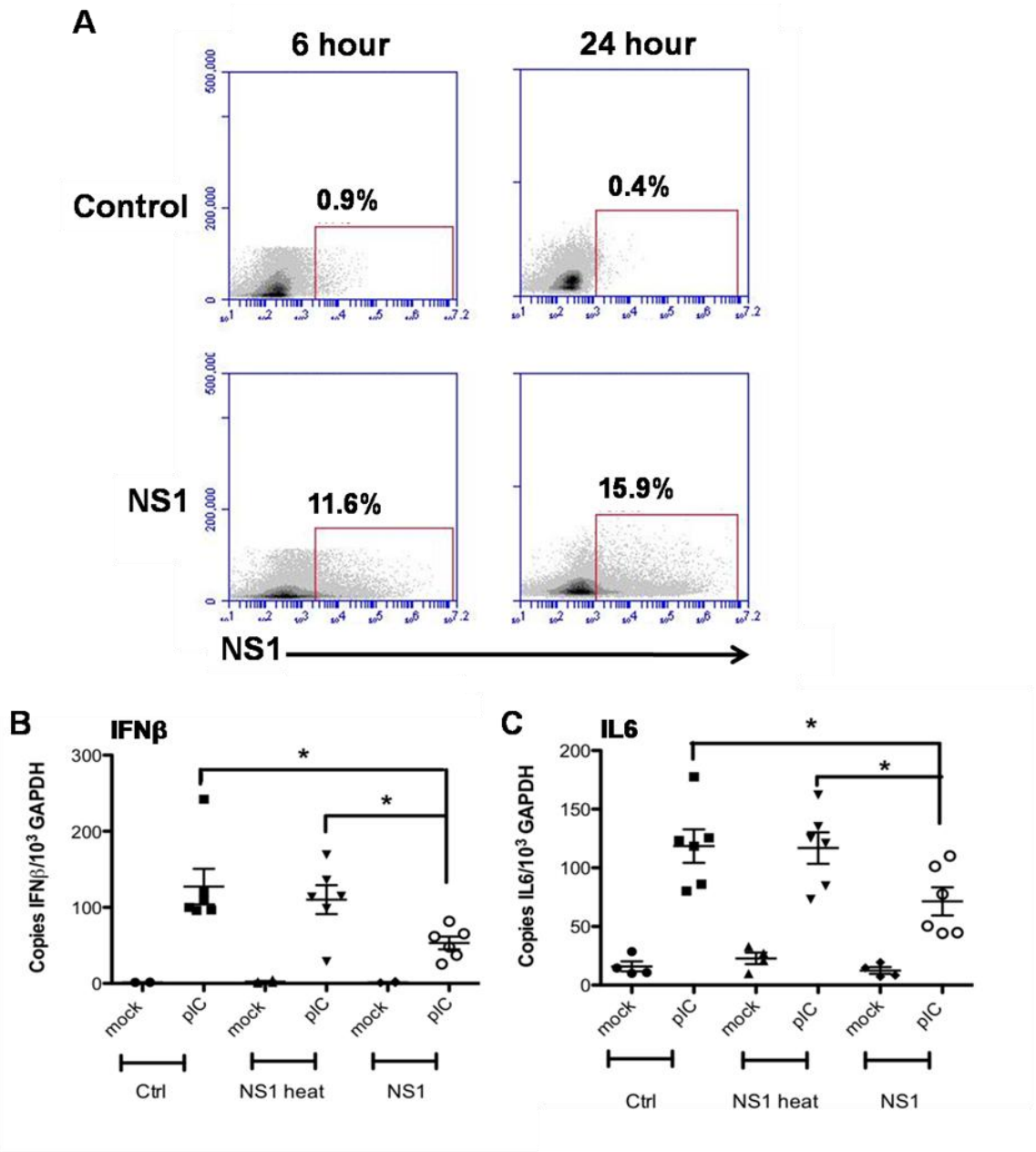

FIGURE 7: **Secreted NS1 inhibits the *in vivo* response to poly(IC:LC).** A. C57BL/6 mice were injected via each footpad with 5µg of purified NS1 labeled with Alexa-488 fluorophore and pLNs were harvested after 6 or 24 hours and analyzed for NS1 by flow cytometry. B, C. C57BL/6 mice were injected via each footpad with 3.36µg of purified NS1. After 6 hours the mice were stimulated by injection of 20µg of Poly(IC:LC) per footpad. 16 hours later popliteal lymph nodes were harvested and pooled per mouse before RNA extraction, cDNA preparation and subsequent qPCR analysis.

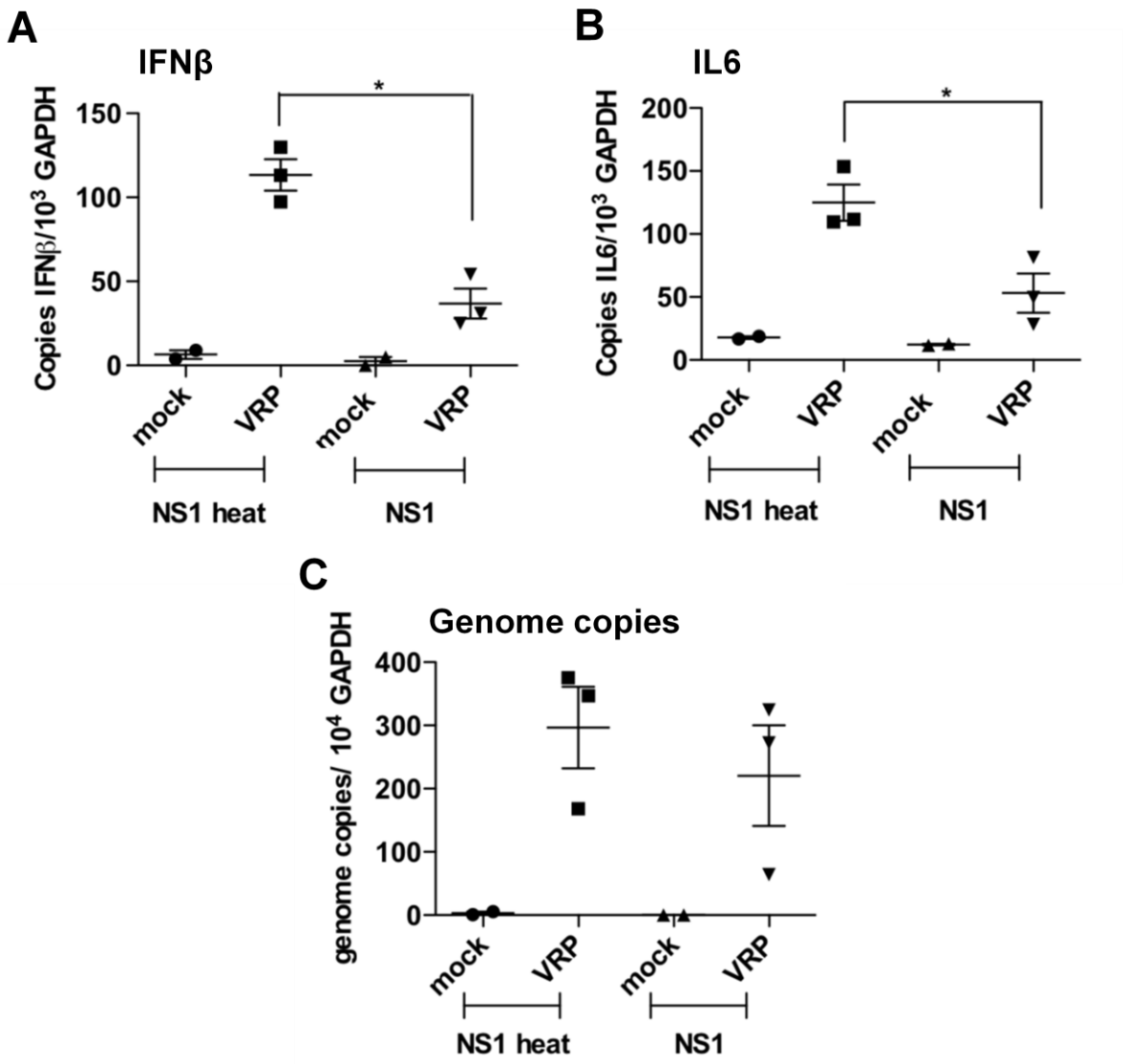

FIGURE 8: **Secreted NS1 inhibits the *in vivo* response to WNV VRP infection.** C57BL/6 mice were preinjected with NS1 as described above and stimulated by injection of $1x10^5$ pfu WNV VRPs per footpad. Popliteal lymph nodes were collected and processed as described in figure 7. IFNβ mRNA (A) or IL-6 mRNA (B) levels were analyzed by RT-PCR and (C) WNV VRP copy numbers were determined using primers specific for NS5.

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Chapter 4

Discussion

Discussion

The work described in this dissertation has helped to characterize the immunomodulatory activity of the WNV NS1 protein. It was originally demonstrated that either WNV infection or WNV stable replicon expression was sufficient to cause inhibition of IRF3 activation by TLR3 signal transduction [1]. NS1 was identified as the WNV non-structural protein capable of inhibiting TLR3 signal transduction after testing the inhibitory potential of each non-structural protein individually [2]. Further testing showed that intracellularly-expressed WNV NS1 is able to inhibit both IFNβ promoter activation and NFκB activation in response to TLR3 stimulation.

Our data generated using secreted NS1 has corroborated the findings for intracellularly-expressed NS1. Secreted NS1 was previously shown by others to associate with, and be endocytosed by, naïve cells [3]. Using this information, we purified NS1 from the supernatants of cells engineered to secrete this protein. Our data confirmed that sNS1 was able to inhibit IFNβ promoter induction and IL-6 secretion by HeLa cells in response to TLR3 stimulation. This finding also extended to the immune cell types; BMDMs and BMDCs. Finally, we were able to demonstrate an *in vivo* effect of sNS1 on stimulation induced by Poly(IC:LC). Stimulation by Poly (IC:LC) is believed to induce signaling through PRRs in addition to TLR3, suggesting an even broader function for NS1. Additionally, we were able to demonstrate that sNS1 can inhibit the innate immune response to VRP infection as well. IFNβ and IL-6 transcript levels were decreased in the lymph nodes of mice pretreated with sNS1 compared to those of mice that received the heat-inactivated protein.

Although there was clearly an effect of sNS1 on the immune response to VRPs, that did not translate to an increase in RNA replication as there was no significant difference in genome copies with NS1 pretreatment. As we used VRPs which are single-cycle infectious particles, we were unable to appreciate the effects of sNS1 on the spread of the virus, but it would appear that sNS1 does not provide a direct advantage for RNA replication. Previous data from the lab has demonstrated that intracellularly-expressed NS1 can function *in trans* to enhance WNV replication (Wilson JR, unpublished data). However, no enhancement of replication was seen when cells were pretreated with sNS1 (data not shown), suggesting that this form of NS1 cannot function *in trans* to rescue replication. Our previous and current experiments suggest that NS1 diminishes the antiviral effect produced in response to infection, and therefore an effect of NS1 pretreatment may be more evident in a spreading infection. Ultimately, *in vivo* experiments using live WNV for infection are needed to fully address these issues, but would present some difficulties. As sNS1 has other documented immunomodulatory effects, exclusive of the effects noted for TLR signaling, these effects would have to be accounted for and controlled for when analyzing data from live infections. Secreted NS1 has been shown to inhibit a variety of aspects of the complement system; therefore live WNV experiments would require the use of mouse models with deficiencies in complement pathway components. However, complement plays an important role in the immune response to WNV infection and therefore we would likely have to use a number of different mouse models that are deficient in different complement components. For example, research has shown that mice deficient in either C4 or in factor B are particularly susceptible to lethal WNV infection and are deficient in T cell responses [4]. We also know that sNS1

targets the complement component C4 [5, 6], therefore we would need to account for the potential effects sNS1 might indirectly have on WNV lethality and T cell responses. However, if we were to first determine the mechanism of NS1-mediated signaling inhibition we might be able to more easily separate the effects of sNS1 on complement from its effects on signaling.

To further define the inhibitory nature of WNV NS1 we determined the spectrum of TLR inhibition by the intracellularly-expressed form of this protein. In HEK 293 TLR-overexpressing cell lines we show inhibition of NFκB activation in response to TLR ligand stimulation. Additionally we were able to show inhibition of IL-8 production in response to TLR stimulation for many of the TLRs tested. Intracellularly-expressed NS1 was unable to inhibit signaling in the TLR7 and TLR8 cell lines, which may be due to the Bcl-xL gene included as part of the TLR expression construct. Nevertheless, NS1-mediated inhibition of signaling through these and the remaining TLRs was confirmed in the Raw 264.7 macrophage-like cell line which expresses these TLRs endogenously. This suggests that NS1 targets a pathway intermediate common to the TLRs for its inhibitory function.

Interestingly, NS1 was able to inhibit IL-6 production in response to stimulation by Sendai virus in the Raw 264.7 cell line. Previous data from our lab had indicated that NS1, expressed in the context of the replicon, is unable to inhibit Sendai virus-induced IRF3 activation in HeLa cells [1]. One possible explanation for this discrepancy is that Sendai virus is such a strong activator of immune signaling in HeLa cells that the effects would override possible NS1-mediated inhibition. Alternatively, this discrepancy may be explained by differences in the PRRs stimulated in these different cell lines. In HeLa cells, and other

non-immune cell types, Sendai virus is believed to signal solely through the RNA-helicase, RIG-I [7, 8]. In Raw 264.7 cells, however, there is evidence that Sendai virus will stimulate TLR7/8 in a replication-dependent manner [7]. There is also evidence that Sendai virus may trigger an as-of-yet unknown PRR as stimulation was detected that could not be explained by TLRs, PKR, or RNA-helicases [7]. This leaves an interesting possibility that NS1 may also be able to inhibit signaling through PRRs in addition to TLRs.

Our experiments infecting Raw 264.7 cells with Sendai virus also indicated that intracellularly-expressed NS1 was able to inhibit IFNβ promoter activation and production in response to infection. This inhibitory activity was only detected at early timepoints after infection. Though inhibition of IL-6 production in response to Sendai virus infection could be seen at 24 hours post-infection, IFNβ inhibition by NS1 was only seen at 12 hours post-infection. One possible explanation for these differences lies in the IFNβ positive feedback loop. After Sendai virus infection, even the smallest amount of IFNβ present would serve in an autocrine/paracrine fashion to amplify the IFNβ response. Here the IFNβ produced initially would bind to the IFNα/β receptor which signals through Jak/STAT and not the pathway intermediates common to the TLR signaling pathway. Therefore, our results show that NS1 is able to inhibit the early PRR-driven IFNβ response to Sendai virus infection, but also suggests that NS1 is not a global signaling inhibitor as IFNβ production, likely mediated via Jak/STAT dependent signaling, is not inhibited.

Experiments taking place concurrently with these studies confirmed that NS1 inhibition occurs after adaptor molecule recruitment, but takes place before the IKKs and MAPKs (Wilson JR, manuscript in review). By detailed biochemical pathway mapping these

studies identified the molecule TAK-1 as a potential candidate for NS1-mediated inhibition. TAK1 is a central component of TLR signaling that will directly activate NFκB, AP-1, and JNK. However, previous data indicate that NS1 is also able to inhibit IRF3 activation and translocation into the nucleus in response to TLR3 stimulation. Though a direct effect of TAK1 on IRF3 activation has not been identified, recent studies suggest that IRF3 activation may be controlled by JNK [9]. TAK1 undergoes autophosphorylation upon association with TAB1 and TAB2 and is constitutively phosphorylated when overexpressed with TAB1 or 2. Results from our lab have demonstrated that phosphorylation of TAK-1when TAB1 or TAB2 were overexpressed was inhibited in the presence of NS1. However, a direct interaction of NS1 with these molecules was not detected, suggesting that NS1 inhibits TLR signaling indirectly. The molecule functioning with NS1 to exert these inhibitory effects has yet to be identified.

TAK1 is involved in signaling that originates from a number of different receptors including; the IL-1 receptor, T and B-cell receptors, TGF-β receptor, TNF1 receptor, lymphotoxin-β (LTβ) receptor, and CD40. Preliminary data from VRP-infected mice pretreated with purified sNS1 has shown a decrease in CD40 expression on cells from the popliteal lymph node 16 hours after inoculation into the footpad (figure 1). CD40 is known to be a critical component in the development of adaptive responses as well as being a link between the innate and adaptive immune systems. Dendritic cells from $CD40^{-/-}$ and $CD40L^{-/-}$ mice were unable to elicit $CD4^{+}$ and $CD8^{+}$ T cell immunity, even though other activation markers were expressed and MHCII antigen presentation was intact [10]. Additionally, CD40 was found to be necessary for IL-12 production by DCs. These studies

suggested that the CD40/CD40L interaction generates a signal that functions together with antigen presentation and costimulation in the generation of functional $CD4^+$ and $CD8^+$ T cells [11].

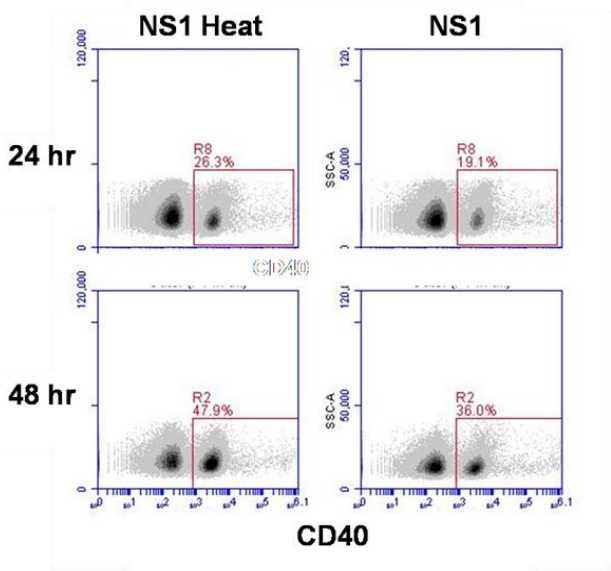

FIGURE 1: **NS1-mediated reduction in CD40 expression.** C57BL/6 mice were injected via the footpad with 5µg of NS1 or heat inactivated NS1 and infected with $1x10^5$ VRPs per footpad. Animals were sacrificed 24 or 48 hours later and flow cytometric analysis was performed on the popliteal lymph nodes using a fluorescently-labeled antibody specific for CD40. See Chapter 3 Materials and Methods.

A recent study has shown that CD40 expression on cells of the CNS is important in inflammation and recruitment of inflammatory cells into the CNS [12]. Other studies have established a protective role for T cells during primary WNV infection. Researchers showed that $CD8^+$ T-cell trafficking to the CNS was dramatically decreased in $CD40^{-/-}$ mice. This impairment of $CD8^+$ T cell recruitment correlated with elevated WNV titers in the CNS and increased death. The study demonstrated that in the brains of WT mice T cells readily

trafficked to the site of neuronal infection whereas in $CD40^{-/-}$ mice, T cells were retained in the perivascular space and were unable to migrate into the parenchyma [13].

CD40 is also critical for T cell-dependent immunoglobulin class switching. After infection of CD40 and CD40L-deficient mice with either lymphocytic choriomeningitis virus or vesicular stomatitis virus resulted in no lg isotype switching of virus-specific antibodies [10]. In another study, the murine gammaherpesvirus γMHV68 was able to efficiently establish a latent infection in B cells of $CD40^{-/-}$ mice, but not in B cells from WT mice [14].

A recent study showed that in the absence of CD40 expression, $CD8^+$ T cells were unable to differentiate into memory cells or receive help from CD4+ cells, suggesting that $CD8^+$ T cells receive help from CD4+ cells directly through CD40 and therefore CD40 is critical in generating memory $CD8^+$ T cells [15]. CD40 is known to be essential for germinal center formation [16] where the CD40-CD40L interaction is crucial in T-B interactions for T helper-dependent activation of B cells[10]. Interestingly, our recent *in vivo* studies examining popliteal lymph nodes by flow cytometry after VRP infection has also identified an NS1-dependent decrease in the expression of a number of prominent B-cell markers, including B220, CD19, CD23, and MHCII (figure 2). Future and ongoing experiments include further characterization of the cell types involved as well as ELISPOT assays to determine differences in the development of antigen-specific B-cell responses in the presence of NS1 after VRP infection. As TAK1 is also involved in signaling through the B-cell and T-cell receptors it is intriguing to speculate that the inhibitory function of NS1 may not be TLR-centered but rather centered in obstructing the adaptive immune response, where TLRs are simply casualties of NS1-mediated inhibition.

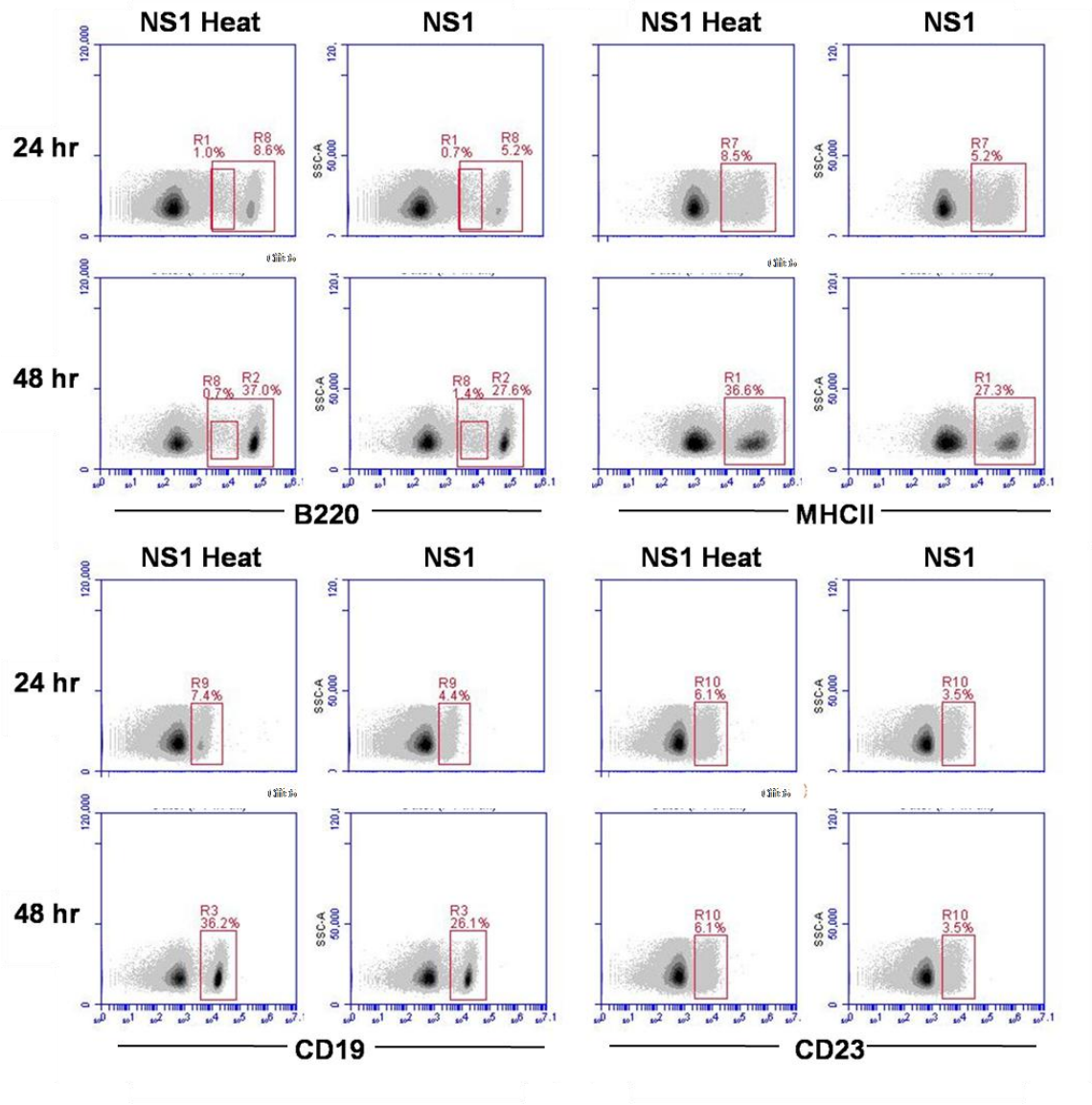

FIGURE 2: **NS1-mediated reduction in B-cell marker expression.** C57BL/6 mice were injected via the footpad with 5µg of NS1 or heat-inactivated NS1 and infected with $1x10^5$ VRPs per footpad. Animals were sacrificed 24 or 48 hours later and flow cytometric analysis was performed on the popliteal lymph nodes using fluorescently-labeled antibodies specific to the markers identified above. See Chapter 3 Materials and Methods.

Other studies from the lab have identified different mutations in the NS1 protein that can ablate its inhibitory function in signal transduction while maintaining its replicative function. These mutations are currently being introduced into the virus itself. In addition to mutations that ablate the inhibitory function of the intracellularly-expressed form of NS1, there are mutations which prevent NS1 migration to the plasma membrane as well as mutations that prevent secretion (Morrison CR, unpublished data). Importantly, recent data have shown that the NS1 P250L mutant, which exists only in monomeric form, is able to inhibit TLR signaling when NS1 is located intracellularly, but the secreted form is unable to inhibit signaling in naïve cells, despite the level of cell association being equal to that of the WT hexameric NS1 (Alayli FA, unpublished data). Transferring this mutation into the virus and subsequent *in vivo* studies will be very informative in determining the role of secreted NS1 in WNV infection. These studies would have to be conducted carefully as other immunomodulatory effects of NS1 involving the complement pathways are known. Studies using the other mutant viruses will be informative in resolving the main function of NS1 in immune inhibition and will be important in determining the contributions of NS1 to WNV pathogenesis.

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