

**INHIBITION OF TYPE I AND TYPE II INTERFERON SIGNALING BY
ALPHAVIRUS NONSTRUCTURAL PROTEINS**

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ABSTRACT

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Inhibition of type I and type II interferon signaling by alphavirus nonstructural proteins.
(Under the direction of Mark T. Heise)

Alphaviruses are mosquito-borne pathogens that represent an emerging threat due their capacity to cause large outbreaks of infectious disease ranging from severe arthritis, as observed with the current chikungunya virus outbreak in the Indian Ocean, to potentially fatal encephalitis, as witnessed by periodic outbreaks of Venezeulan equine encephalitis virus (VEEV). Type I interferon (IFN) plays a crucial role in limiting alphavirus replication and pathogenic strains must employ mechanisms to downregulate this response. In these studies, we have identified a previously unrecognized mechanism by which VEEV and Sindbis virus (SINV) inhibit the response to both type I and type II IFNs, which presumably would limit the antiviral effects of these cytokines within infected cells. Studies with VEEV, as well as propagation-defective replicon particles devoid of the viral structural genes, indicated that the viral nonstructural proteins (nsPs) disrupt critical signaling events downstream of type I and type II IFN receptor activation, as indicated by failed activation and nuclear localization of STAT1, a transcription factor central to multiple signaling pathways. Notably, these inhibitory events occurred upstream and independently of global transcriptional shutoff, which was previously proposed to be the mechanism by which alphaviruses downregulate IFN induction and signaling. Our subsequent studies with SINV demonstrated that other alphaviruses also antagonize Jak/STAT activation and that the efficiency of this inhibition correlated tightly with the relative virulence of SINV strains. Importantly, we were able to map this effect to a single amino acid determinant that was required for efficient STAT inhibition by AR86, an adult mouse neurovirulent SINV strain. This determinant, threonine at nsP1 538, was critical for adult mouse neurovirulence and could rescue efficient STAT inhibition when introduced into an avirulent virus. These studies

strongly suggest that STAT signaling inhibition by alphaviruses plays an important role in their capacity to cause disease, and they set the stage for future in vivo studies designed to assess the role that Jak/STAT signaling inhibition plays during alphavirus pathogenesis.

*To my parents, who have provided me with every opportunity,
And for whom I demand much from myself
Without any compulsion to fulfill expectations.*

*To my family, who model a balance of hard work
With an enjoyment of life and the company of each other.*

*And, to Kim, who keeps me grounded and happy,
Who motivated me to come home from lab,
Yet supported me when I couldn't.*

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LIST OF ABBREVIATIONS

CARD	Caspase recruitment domain
CBP	CREB [cAMP response element-binding] binding protein
CHIKV	Chikungunya virus
CIS	Cytokine-induced SH2 protein
CNS	Central nervous system
CPE	Cytopathic effect
DC	Dendritic cell
DLN	Draining lymph node
dsRNA	Double-stranded RNA
EM	Electron microscopy
GAF	Interferon- γ activated factor
GAS	Interferon- γ activated site
HAT	Histone acetyltransferase
HCV	Hepatitis C virus
HDAC	Histone deacetylase
HS	Heparan sulfate
IFN	Interferon
IFNAR	Interferon alpha [α/β] receptor
I κ B	Inhibitor of NF- κ B
IKK	I κ B kinase
IPS1	Interferon promoter stimulator-1
IRF	Interferon regulatory factor
ISG	Interferon stimulated gene
ISGF3	Interferon-stimulated gene factor 3

ISRE	Interferon stimulated response element
Jak	Janus kinase/Janus activated kinase/Just another kinase
JEV	Japanese encephalitis virus
MAPK	Mitogen-activated protein kinase
MAVS	Mitochondrial antiviral-signaling protein
MDA5	Melanoma differentiation-associated gene 5
MyD88	Myeloid differentiation primary response gene 88
NF- κ B	Nuclear factor kappa B
NLS	Nuclear localization signal
NSV	Neuroadapted Sindbis virus
NTPase	Nucleoside triphosphatase
PAMP	Pathogen-associated molecular pattern
pDC	Plasmacytoid dendritic cell
PI3K	Phosphoinositide 3-kinase
PIAS	Proteins that inhibit activated STATs
PKC	Protein kinase C
PKR	Protein kinase R/dsRNA-dependent protein kinase R
PRR	Pattern recognition receptor
PTP	Protein tyrosine phosphatase
RC	Replication complex
RdRp	RNA-dependent RNA polymerase
RLR	RIG-I-like receptor
RRV	Ross River virus
SAM	S-adenosyl-L-methionine
SH2	Src-homology 2
SHP1/2	SH2 domain-containing phosphatase 1/2

SINV	Sindbis virus
SIRS	Systemic inflammatory response syndrome
SOCS	Suppressor of cytokine signaling
ssRNA	Single-stranded RNA
STAT	Signal transducer and activator of transcription
TBEV	Tick-borne encephalitis virus
TBK1	TANK [TRAF family member-associated NF- κ B activator] binding kinase 1
TICAM1	TIR-domain-containing adaptor molecule
TIRAP	TIR-domain-containing adaptor protein
TLR	Toll-like receptor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adaptor inducing interferon- β
Tyk2	Tyrosine kinase 2
UPR	Unfolded protein response
VEEV	Venezuelan equine encephalitis virus
VISA	Virus-induced signaling adaptor
WNV	West Nile virus

CHAPTER ONE:

INTRODUCTION

1.1 Overview of alphaviruses

Alphavirus classification and general epidemiology

The family *Togaviridae* consists of two genera, the rubiviruses (Rubella virus) and alphaviruses, and derived its name from electron micrographs that revealed their enveloped structure that resembled a cloak (*Toga* derived from the Latin “cloak”). While the rubiviruses and alphaviruses share similar genome organization, their genomic homologies and replication strategy separates them into distinct genera (155); furthermore, unlike alphaviruses, rubiviruses are restricted to growth within humans (155). Arthropod-borne viruses, or arboviruses, were originally serologically classified into groups A, B and C where members of group A became the *alphaviruses*, and group B are current *Flaviviridae* members (248). The alphaviruses have a nearly global distribution and are maintained through transmission between arthropod (mostly mosquito) and vertebrate (commonly avian and rodent) hosts. However, many alphaviruses have emerged to cause periodic, yet sometimes explosive epidemics of human disease (339).

Members of the alphavirus genus can be classified into seven antigenic complexes (250, 305), which for convenience can be generically grouped according to their geographic distribution as Old World or New World viruses. Several Old World viruses are discussed in this work including the Sindbis-like viruses, which are members of the WEE complex, and members of the Semliki Forest antigenic complex including Ross River Virus (RRV), Chikungunya virus (CHIKV), and Semliki Forest virus (SFV). The New World viruses that will be discussed are from the eastern equine encephalitis (EEE) antigenic complex, the Venezuelan equine encephalitis (VEE) antigenic complex, and the western equine encephalitis virus (WEEV) antigenic complex, which includes WEEV, but not Sindbis-like viruses (SINV). The viruses discussed in this work are listed in Table 1.1, but a complete table of the seven antigenic complexes with their members is reported by Powers and colleagues (250).

Virion structure and genome organization

Structural information on the alphaviruses has been gathered for many years using a variety of methods including cryo-electron microscopy (cryo-EM) of whole virion particles and x-ray crystallography of individual viral components (122, 123, 202, 267, 330, 331). Recent image reconstruction technology has allowed 9 Å resolution in cryo-EM structures for SINV, RRV, and SFV (46, 205, 227). The virion is 700 Å in diameter and consists of a single stranded RNA genome of ~11.5 kilobases, which is encapsidated by the viral capsid proteins to form an icosahedral nucleocapsid that, itself, is contained within a lipid bilayer derived from the host cell plasma membrane. The surface of the membrane is coated with an icosahedral lattice (T = 4 symmetry) of the viral glycoproteins, 240 molecules each of E1 and E2, which form heterodimers and assemble into 80 trimeric “spikes” that protrude from the lattice (140).

The alphavirus genome is itself infectious when introduced into the cytoplasm of a susceptible cell since it mimics host messenger RNA containing a 5' methylguanosine cap and a 3' polyadenylated tail (Figure 1.1). Two open reading frames, which encode the viral nonstructural proteins and the viral structural proteins, are separated by a subgenomic promoter that functions from the minus strand. The nonstructural proteins form the viral replicase and function in RNA synthesis while the structural genes encode the capsid and glycoproteins required for virion assembly. The function of each viral protein is listed in Figure 1.1, and will be discussed in the context of its role in viral replication or the alphavirus lifecycle.

Alphaviruses grow well in a variety of cell cultures (112, 155), particularly those with defects in type I IFN pathways (discussed later), and are readily isolated/amplified from natural specimens by intracerebral inoculation of neonatal mouse brain, reasons for which they have been studied for many years. Additionally, the alphavirus genome is relatively simple and is highly amenable to genetic modification using reverse genetics, which is the rescue of infectious virus from a fixed clone in the form of cDNA. This technology is crucial for the types of studies that will be discussed in this work since it allows facile construction of virus mutants and ensures that virus preparations are genetically

homogenous allowing clear interpretation of experimental results and phenotypes. The infectious clones used are plasmids (propagated in *E. coli*) that contain the entire viral genome (cDNA) downstream of a bacteriophage promoter (T7 or SP6). To generate virus, viral transcripts are generated in vitro and are then electroporated into susceptible cells (usually BHK-21). As mentioned, the viral genome alone is infectious once introduced into the host cell cytoplasm, and thus virus can be harvested from the producer cell supernatants. Finally, since alphaviruses induce a cytopathic effect (CPE), the titer of viral stocks is easily determined using a standard plaque assay.

Lifecycle of alphaviruses

Although some evidence of direct viral fusion with the plasma membrane has been proposed (42, 69), alphaviruses generally are considered to infect the host cell through receptor-mediated endocytosis (305), which in most cases requires a specific receptor as well as attachment factors. Since the tropism of alphaviruses is broad, both in terms of their ability to infect multiple cell types within a single host and their wide host range encompassing vertebrates and invertebrates, two possible mechanisms have been proposed to explain the nature of the alphavirus cellular receptor. First, alphaviruses are able to use more than one protein as a receptor, which could potentially bind to multiple receptor binding domains within E2 (155). Alternatively, these viruses use a single cellular receptor, but this cellular factor is highly conserved across the animal kingdom (306). In the case of SINV, multiple cellular receptors and/or attachment factors have been proposed, depending on the host species and cell type, including the high-affinity laminin receptor (335), DC-SIGN and L-SIGN (147), heparan sulfate (HS) (149), and others (reviewed in (155)). For VEEV, the laminin receptor also serves as a cellular receptor in mosquito and vertebrate cells (195, 204).

Receptor engagement induces a conformational change within E2 and E1 as the virus is endocytosed via a clathrin-coated pit-dependent mechanism (Step 1, Figure 1.2) (65). With a drop in endosomal pH, the E2/E1 heterodimers dissociate and the fusogenic peptide within E1 is exposed, which inserts into and trimerizes within the endosomal membrane allowing the formation of a pore

and the coalescence of viral and endosomal membranes (Step 2, Figure 1.2) (155). Once delivered into the cytoplasm, the viral nucleocapsid disassembles and the viral genome associates with ribosomes for translational initiation (Step 3, Figure 1.2). The nonstructural genes are translated as a single polyprotein, which, in the case of alphaviruses that encode an Opal termination codon at the 3' end of the nsP3 gene, generates the nsP123 polyprotein as well as nsP1234 due to translational readthrough occurring at a frequency predicted to be ~10-20% (305) (Step 4, Figure 1.2).

An elegant model of viral RNA synthesis has been proposed where different conformations of the nonstructural replicase proteins mediate minus-sense and plus-sense (both genomic and sub-genomic length) RNAs (292). These nsP conformations are regulated by the degree of polyprotein processing (171, 281), which is mediated by a papain-like cysteine protease domain within nsP2 (Step 5 and 6, Figure 1.2). Synthesis of minus-sense viral RNA is mediated initially by the primary replication complex (RC) consisting of nsP123 and the viral RNA-dependent RNA polymerase (RdRp) nsP4 (168), which is cleaved rapidly from the nsP1234 precursor by either a *cis*-(59) or *trans*-(326) acting nsP2. While positive strand synthesis occurs at some level at all times post infection, the nsP123-containing RC synthesizes positive strands very inefficiently. As nsP123 levels accumulate, cleavage between nsP1 and nsP2, which is mediated by nsP2 in *trans*, occurs resulting in an intermediate RC consisting of nsP1, nsP23, and nsP4 (291), which is capable of full-length minus-strand and plus-strand synthesis, but is less active in synthesis of 26S subgenomic RNA. Minus-strand RNA ceases by ~4 hours post infection in BHK-21, HeLa, and Vero cells (282). Minus-strand synthesis requires continuous protein synthesis (282), and failure to shutoff minus-strand synthesis is associated with mutations within nsP2 (280). The nsP2/3 site is somewhat resistant to cleavage, which requires the prior release of nsP1 that exposes a cofactor within the nsP2 N-terminal domain (326). Cleavage of nsP23 provides the fully mature RC consisting of nsP1, nsP2, nsP3, and nsP4 that no longer recognize the promoter to synthesize minus-strand RNA, but efficiently synthesizes plus-strands favoring subgenomic 26S RNA synthesis over full-length genomes (170).

All nonstructural proteins are required for RNA synthesis, and their functions (Figure 1.1) have been elucidated through biochemical and structural studies. Nonstructural protein 1 anchors RCs to the plasma membrane and cytoplasmic membrane vesicles through cysteine-palmitoylated residues (159) as well as an amphipathic peptide (302). Membrane association through nsP1 interactions was not a strict requirement for replication of SFV and SINV, but disruption of these interactions resulted in delayed replication and attenuation of SFV *in vivo* (7, 362). In addition to its role in associating RCs to membranes, nsP1 encodes guanine-7-methyltransferase and guanylyltransferase enzymatic activities, which are required for the sequential guanosine cap methylation and capping of genome-length (42S) and subgenomic (26S) positive-strand RNAs (54, 160, 164). Mutations within nsP1, including the temperature-sensitive (*ts*) lesion *ts11* (A348T), result in defective minus-strand synthesis (197, 283).

As mentioned, nsP2 encodes the virus-specific papain-like cysteine protease that cleaves at the nsP1/2, nsP2/3, and nsP3/4 junctions, which share similar, but not identical, sequences(305). The crystal structure of the VEEV nsP2 C-terminus has been solved (267), and in addition to the centrally located protease domain, nsP2 also encodes a putative S-adenosyl-L-methionine (SAM)-dependent RNA methyltransferase at its C-terminus. The nsP2 methyltransferase has no known enzymatic function; however, mutations within this region have been shown to regulate minus-strand synthesis, as well as the ability of SINV to downregulate host transcription and translation (67, 84, 92, 103, 213), which is likely involved with the development of cytopathic effect (CPE) and inhibition of the host response (discussed later). The N-terminus of nsP2 encodes a helicase domain and nucleoside triphosphatase (NTPase) activity that are involved with RNA duplex unwinding during replication/transcription (102) and also encodes RNA 5'-triphosphatase activity that is required to prime the viral RNA 5'-most nucleoside for the subsequent nsP1-mediated capping reaction (325). Mutations within promoter elements of the virus genome (e.g. the 5' conserved sequence element within the nsP1 gene) resulted in adaptive mutations within the amino terminus of nsP2 suggesting this protein is directly involved with viral RNA binding (77, 218). Coincident with having multiple

functions within the cell, the alphavirus nsP2 has multiple subcellular distributions with approximately half of nsP2 present within the nucleus (239) and cytoplasmic nsP2 associating both with membrane-bound replication complexes and with ribosomes (220, 254). Nuclear localization signals have been mapped within SFV (259) and VEEV (221); disruption of NLS sequences revealed that nuclear functioning of nsP2 is not absolutely required for alphavirus replication, but could disrupt other functional properties including nsP2-mediated block of type I IFN induction (35).

Recently, the first crystal structures of the VEEV and CHIKV N-terminal nsP3 domains became available (202), which significantly enhanced our limited understanding of this protein. This region of nsP3 encodes a so-called macro domain (X domain) named after its homology to a region within histone macroH2A, which are highly conserved domains across all kingdoms of life yet their physiologic role is not understood. The CHIKV and VEEV macro domains were shown bind RNA, ADP-ribose and poly-ADP-ribose (PAR), and exhibited ADP-ribose phosphatase activity (202). Macro domains of SFV and SINV have also been characterized biochemically (70, 231, 234, 235), and while these activities have no defined role to date, they have been proposed to downregulate apoptosis (202). Alphavirus infection triggers PAR polymerase 1 (PARP-1) activation and PAR synthesis (230), which depletes cellular ATP and NAD⁺ stores and ultimately results in apoptosis-inducing factor (AIF) apoptosis. PAR binding by the viral macrodomain may somehow disrupt this response. While the N-terminal region of nsP3 is well conserved across all alphavirus complexes, the C-terminal region is hypervariable (305). This region is highly phosphorylated on serine and threonine residues by host kinases, modifications that play some role in minus-strand RNA synthesis (61) and pathogenesis (86, 319, 328). Finally, as mentioned above, translation of the alphavirus nsP1234 is altered by the presence of an Opal termination codon near the nsP3/4 cleavage site, which is common in all alphaviruses except SFV, Onyong 'nyong virus (ONNV), and SINV strain AR86, which all encode a sense codon at this position (305). Interestingly, a sense codon at this position does not result in greater accumulation of nsP4 (181) due to this protein's lability (60), however, replacement of a sense codon with Opal attenuates neurovirulence of both SFV and AR86 (307, 320).

While the role that greater translational readthrough plays in pathogenesis is unclear, the region between the Opal/sense codon and the nsP3/4 was recently shown to contain a degradation signal that reduced the half-life of nsP3 or other proteins to which the signal was introduced (324).

Complementation of *ts* mutants and sequence homology originally identified nsP4 as the catalytic subunit of the RC encoding the viral polymerase (119). The C-terminus shares homology with other viral RdRps (151), the catalytic core of which is not only required for RNA elongation but also possesses terminal adenylyltransferase activity believed to play some role in maintenance of the viral poly-adenylated tails (316). The N-terminal ~100 amino acids, on the other hand, share no homology with RdRps of other virus families (140), and in addition to the conserved N-terminal tyrosine that destabilizes the protein (60), this region is thought to mediate interactions with nsP1 and possibly unidentified host proteins (76, 140, 169).

The virus assembly process initiates with the translation of subgenomic RNAs (Step7, Figure 1.2) into a polyprotein from which the capsid protein is released from the nascent polypeptide by its own serine protease activity. Once cleaved, a C-terminal tryptophan residue of capsid sits within the protein's substrate binding pocket preventing further capsid-mediated proteolysis. Nucleocapsid assembly has been modeled in vitro where capsid dimers recognize viral RNA (226, 312), and subsequently autoassemble in a multi-step process (reviewed in (140)). Nucleation of this reaction requires the recognition of an encapsidation signal within the nsP1-coding sequence of the viral RNA by a specific capsid sequence (343, 344). Capsid autoproteolysis from the structural polyprotein reveals a signal sequence within the N-terminus of pE2, which is the E2 glycoprotein precursor with E3 still fused. Proper folding, glycosylation, and processing of the viral glycoproteins is achieved within the ER (Step 9, Figure 1.2) (reviewed in (140)). One function of the 6k protein is it contains the signal peptide for E1 translocation across the ER membrane, but 6k is then cleaved from E1 by host signalase. E1 and pE2 oligimerization occurs within the ER, which is required for transport to the plasma membrane (192). Within the *trans* Golgi, pE2 is cleaved by the host furin, an event important for infectivity of the newly synthesized virus (125). In addition to its role in mediating

proper E1 and E2 dimerization, E3 is also thought to prevent pre-mature E1 conformational changes during Golgi transport where it is exposed to low pH (155, 192).

To complete the virus lifecycle, nucleocapsids either diffuse or transit to the plasma membrane where they interact with the viral glycoproteins to initiate budding (Step 10, Figure 1.2). A specific motif within the cytoplasmic domain of E2 is proposed to interact with a pocket within the viral capsid to promote the budding process, which is aided by lateral interactions between the viral glycoproteins. Cholesterol content within the plasma membrane has been shown to aid budding, but this requirement was lost when mutations within E1 were introduced (194). Finally, the presence of 6k, which is introduced into budding viral particles at low levels, seems to be important for the budding process (155).

1.2 Human disease and animal models

A description of epidemiology and human disease associated with both Old World alphaviruses and New World alphaviruses will be given in this section. Since the agents investigated in this work are implicated in alphavirus-induced neurological disease, we will focus mostly on models of encephalomyelitis. While Sindbis-like viruses are technically Old World viruses, they have been used to model neurological disease, and therefore will be discussed in the latter section along with New World viruses.

Epidemiology and human disease associated with Old World alphaviruses

Chikungunya virus (CHIKV) was first isolated in 1953 as the causative agent of an epidemic of arthritic disease in southern Tanzania (260, 262), and has since been recognized to cause similar outbreaks of severe arthralgia in Africa, India, Southeast Asia and the Philippines. The capacity of arthritogenic alphaviruses to cause explosive epidemics is exemplified by the current CHIKV outbreak. In 2005, CHIKV emerged on island nations in the Indian Ocean including La Réunion

where one-third of the population became infected (72), then spread to India infecting 1.4 million people (212), and by 2007 caused isolated outbreaks in northern Italy (23, 337). Transmission of most alphaviruses involves the emergence from sylvatic cycles where humans are dead-end hosts; however, CHIKV can be maintained in the urban cycle being transmitted from human-mosquito-human (reviewed in (339)). A second factor in the massive CHIKV emergence was its adaptation to infect *Aedes albopictus* (Asian tiger mosquito), which enhanced human transmission and demonstrates the capacity of CHIKV to emerge globally since this mosquito populates every continent except Antarctica (45).

Chikungunya's name (Makonde for "that which bends up"), which was given during the 1952 outbreak in Tanzania, is a description of the debilitating symptoms this virus causes in humans (52). Patients suffer from a sudden onset disease that includes fever, rigor, headache, rash, and as the virus name refers, debilitating arthralgia and myalgia. Patients are infectious during the febrile period and usually a few days prior to its onset. While joint pain subsides, it can persist in a milder form for months after the onset of disease (112). Patients are treated with general anti-inflammatory drugs, but no specific therapy or antiviral is currently available.

Ross River virus (RRV) is another Old World alphavirus that has caused explosive outbreaks of polyarthritis in Fiji, Samoa and the Cook Islands. RRV is the most common cause of mosquito-transmitted disease in Australia, where it is endemic. The reservoir host for RRV in Australia is unclear although it is believed to be marsupials; however, like CHIKV, human-mosquito-human transmission is also likely with RRV during epidemics (266). As with CHIKV-induced disease, RRV causes an acute febrile illness in humans, which is rapid in onset and accompanied with rash, myalgia and arthralgia that can persist for up to 6 months.

Sindbis virus (SINV) is widely distributed, having been isolated from sources in Europe, the Middle East, Africa, India, Asia, Australia and the Philippines, and it is suspected to have existed in the Western hemisphere (112). The prototype SINV, strain AR339, was isolated from a pool of mosquitoes near Sindbis, Egypt in 1952 (311) and represents the most well-studied strain having

given rise to numerous laboratory derivatives. Although this strain caused no apparent human disease, several strains were isolated thereafter that were associated with a febrile illness. Strain AR86, which was isolated in South Africa in 1954 from a pool of *Culex spp.* mosquitos, was associated serologically with human disease (342). Another South African strain, Girdwood, was isolated from a skin lesion of an infected patient and represents one of very few SINVs isolated from a human (203). In addition to these African strains, moderate human disease is seen in northern Europe where related SINV strains have circulated (112), including Ockelbo82, which was isolated in Sweden in 1982 and was also associated serologically with human disease (233, 300). Unlike CHIKV and RRV, outbreaks of SINV disease occur when enzootic viruses that normally circulate between *Culex spp.* or *Culiseta spp.* mosquitoes and birds are able to establish infection in bridge vectors (*Aedes spp.*) that feed on humans (112). While many cases of SINV infection are asymptomatic or subclinical, mild to moderate disease has been reported that consists of fever, arthralgia, myalgia, and a maculo-papular rash with or without pruritis (73).

Mouse models of arthritogenic alphaviruses

The best developed animal models for alphavirus infection associated with arthritis are mouse models of RRV-induced arthritis/myositis using the T48 mouse-adapted strain (184, 224). In one model, subcutaneous infection of 24-day-old C57/B6J with RR64 (T48 infectious clone) results in the development of moderate to severe hind limb weakness, which persists for 2 to 3 weeks (224) and can be attributed to a dysregulated innate inflammatory response that depends on the complement system (222). Macrophage infiltration into muscle and joint-associated tissues (184) and complement-mediated activation of macrophages were linked to the tissue damage characteristic of this model (223), which is a useful tool in gaining an understanding of alphavirus pathogenesis as well as general mechanisms regulating the inflammatory response.

Animal models for CHIKV-induced arthritis are now being developed to better understand the underlying pathogenesis of the current epidemic. As seen with the RRV mouse model,

susceptibility of mice to CHIKV is strictly age dependent since 14-day-old mice develop arthritis and myositis similar to that seen in humans (361) and neonatal mice develop a severe multisystem disease (51). Type I IFN, as expected from work with other alphaviruses, is critical to CHIKV control since mice incapable of a normal response to type I IFN (IFN- α/β receptor +/- or IFN- α/β receptor -/-) are more susceptible (51). In adult mice, fibroblasts were the major IFN producers and CHIKV did not appear to infect or replicate within hematopoietic cells efficiently (285), however, in a recently published macaque arthritis model that more closely recapitulated human disease, macrophages supported persistent CHIKV infection in vivo (161).

While some studies have investigated SINV infection of mouse joint-associated tissues [(126), M. Suthar unpublished], infection of mice has primarily been used to model neurological disease as SINV causes encephalomyelitis in mice, but disease is dependent on mouse age and strain. For this reason, mouse models of SINV-induced encephalitis will be discussed along with those of New World virus-induced neurological disease.

Epidemiology and human disease associated with New World alphaviruses

Venezuelan equine encephalomyelitis/encephalitis (VEE) was recognized in the 1930s as a potentially fatal disease of horses, donkeys and mules (99, 339), the causative etiologic agent (VEEV) of which was first isolated in 1938 from the brain of a horse that had succumbed to infection (154). Although two human VEE cases were suspected in the 1940s, this was not demonstrated until the 1950s when an investigator contracted mild disease after voluntarily inoculating himself with a patient's viremic serum, after which VEEV was isolated from both the patient's and investigator's sera (263, 279). In the 1950s, VEEV was developed by the United States and the Soviet Union as a biological weapon due to its capacity for mass production and aerosolization (53), and today concerns of dual-use investigation persist since the virus can be easily manipulated genetically to enhance virulence. Regional outbreaks of VEE have occurred periodically in rural areas about every 10 years, the most expansive of which originated in Guatemala and/or El Salvador in 1969 but spread

throughout Central America and into Texas in 1971 (339). The largest outbreak occurred in Colombia and affected over 200,000 humans and resulted in more than 100,000 equine deaths (340).

The periodicity of VEE outbreaks is explained by its transmission, which involves two distinct cycles, each of which are dependent on ecologic factors, viral factors, and pre-existing immunity within human and equine populations. VEEV is endemic to swamp or sylvatic habitats covering an expansive geographic range where a particular virus strain infects a particular rodent species with spiny rats and cotton rats being the most common reservoir. The virus is maintained through transmission between rodents by *Culex spp.* mosquitoes in what is known as the enzootic cycle (340). Enzootic VEEV strains are not normally associated with human disease, both because these habitats are sparsely populated with human/equine hosts and because these strains are not as pathogenic and achieve significantly lower viremia during experimental infections (339). However, more recent data suggests that enzootic VEEV results in greatly underreported VEE cases in humans that live near swamp habitats (3, 252).

Due to ecologic changes—especially rainy summers that increase populations of additional mosquito species that can serve as bridge vectors—as well as mutations within the enzootic VEEV genome that favor infection/replication within bridge vectors and equids, a second transmission cycle is initiated. These “novel” VEEV strains achieve serum viremia of sufficient titer to allow horse-mosquito-horse transmission in what is known as the epizootic cycle. While humans do not directly participate in this cycle, they do become infected as dead-end hosts. In this way, horses serve as secondary amplification hosts within the epizootic cycle, which can result in massive epidemics of equine and human disease since these VEEV strains are more pathogenic and because equine and human populations overlap significantly in agricultural regions.

The determinants of epizootic emergence are not fully understood. Several studies proposed that relative to enzootic strains, epizootic VEEV strains were more resistant to the antiviral effects of type I IFN (136, 250, 301), suggesting that emergence required mutations to arise within the enzootic reservoir that were involved with suppression of the human/equine type I IFN response. However, at

least in the case of the 1992-1993 epizootic in Venezuela, this association was called into question since the epizootic strain (VEEV subtype IC) and its presumed progenitor enzootic strain (subtype ID) did not differ in their sensitivity to murine IFN- α/β (13); rather, efficient equine amplification by the epizootic strain involved a single mutation within the E2 glycoprotein, which is likely to arise frequently within the enzootic reservoir so the relatively rare emergence is more likely constrained by ecological factors rather than viral genetics (12, 108). Nonetheless, VEEV strains must downregulate type I IFNs to cause disease (discussed later), and this ability could play a role in the emergence of other epizootics.

After a mosquito bite, the incubation period for VEEV in humans is between two and five days (139) after which patients develop a flu-like illness that is rapid in onset and characterized by malaise, fever and chills, retro-orbital pain or headache, and myalgia (340). Signs of encephalitis are most commonly headache and vomiting, which usually begin 4 to 10 days after the onset of illness (112), but can also include somnolence, photophobia, confusion, paresis, seizures and occasionally coma. Laboratory studies reveal lymphopenia, and fatal cases can show myocarditis, hepatic necrosis, and hemorrhage in the brain, lungs, and/or gastrointestinal tract. Children and congenitally-infected neonates are especially susceptible to severe neurological damage and hemorrhagic manifestations (112). In addition to natural infection via a mosquito bite, VEEV can be transmitted through inhalation as occurred when a single broken vial of VEEV resulted in 20 Soviet laboratory workers contracting the illness through aerosol exposure in 1959 (53). Inhalation leads to an even more rapid onset of the disease signs mentioned above and can include pharyngitis (112).

Unlike the transmission associated with VEEV outbreaks, humans and horses are true dead-end hosts for EEEV, which is maintained within enzootic cycles involving passerine birds and ornithophilic mosquitoes and only results in equine (and human) disease due to spillover from this cycle. However, EEEV is significantly more virulent with case-fatality rates ranging from 30-40% in humans, children being especially susceptible, and rates reaching 80-90% in horses (63, 112). The geographic distribution of EEEV ranges from the eastern United States, through the Caribbean, to the

north and east coasts of South America and into the Amazon basin. Summer outbreaks of North American (NA) EEEV strains occur near swamps and result with about seven cases of EEE diagnosed each year in the United States (112), whereas outbreaks of South American (SA) EEEV strains occur year-round but result in mild or subclinical disease (5). Attenuation of SA relative to NA EEEV strains correlated with increased induction of and/or sensitivity to type I IFN in cell culture and a mouse model (2, 90). Unlike VEEV that induces high type I IFN levels in horses and infected mice, wild-type EEEV is a poor IFN inducer in mice, which has been attributed to its low infectivity of myeloid lineage cells and lymphoid tissues (89, 90, 178, 329). Serum viremia appears to be seeded by EEEV amplification within cells of mesenchymal lineage including fibroblasts, osteoblasts and skeletal muscle myocytes (329), which may allow the virus to avoid the massive type I IFN induction seen during VEE (89). Disease signs in human cases of EEE are similar to those described for VEE, however, they are more severe and death is a more common outcome, occurring within 2 to 10 days after the onset of encephalitis. In surviving patients, permanent neurologic sequelae including paralysis, mental retardation and seizures are common (35%-80%) after cases of EEE, but are less frequent in patients recovering from VEE.

Mouse models of alphavirus-associated neurological disease

After subcutaneous inoculation of mice with wild-type virulent VEEV strains, virus initially infects skin resident dendritic cells (Langerhans cells) that traffic to the draining lymph node (DLN) (199) where virus replication is detected within four hours (16). The virus then spreads via efferent lymphatics to other secondary lymphoid tissues including lymph nodes, spleen and thymus, where infection of reticuloendothelial cells seeds a significant serum viremia by 12 hours post infection. It is believed that a certain threshold of serum viremia must be surpassed to allow neuroinvasion, which is supported by the finding that avirulent enzootic VEEV strains that do not replicate to high titers after peripheral inoculation of horses can cause lethal encephalitis when inoculated intracerebrally (340). Thus, it seems the major restriction of epizootic emergence is the capacity of a particular

VEEV strain to seed high levels of serum viremia within horses. Unfortunately, no small animal models have been identified that consistently predict equine virulence and viremia (12).

The precise mechanisms of VEEV neuroinvasion are not completely understood, but the virus gains access to the olfactory and trigeminal nerves from which the infection spreads into the brain via cranial nerves I and V, respectively (44, 110). In addition to hematogenous spread of VEEV that seeds viral replication of dental and neuroepithelial tissues, access via the olfactory tract and bulb is also important for VEEV neuroinvasion after intranasal inoculation, whereby the virus can access the brain even in the face of pre-existing immunity (274). Sometime after these peripheral nervous tissues become infected, the initial phase of disease resolves with virus clearance from the serum and peripheral organs by 3 to 4 days post infection (110). Replication within the brain is first detected by 2 to 3 days post infection, and histopathologic changes of increasing severity are seen between days 4 to 6, which are comprised of perivascular cuffing due to lymphocytic infiltrates, neuronal degeneration, and gliosis. By day 6 post infection, virus has spread throughout all areas of the cerebrum and cerebellum, at which point disease signs in the mouse are severe and they ultimately succumb to meningoencephalitis (110).

A distinction between the mouse model of neurovirulent New World alphaviruses (VEEV and EEEV) and neurovirulent SINV is the relative capacity of these viruses for neuroinvasion. Neurovirulent SINV strains, which include AR86 and neuroadapted SINV (NSV), are able to cause lethal disease after peripheral inoculation, but only in young animals since these viruses are controlled effectively in the periphery of adult (6-week-old) mice (115) through type I IFN-dependent mechanisms (269). In neonatal mice, many wild-type SINV strains including TR339 cause lethal disease after subcutaneous inoculation, but the disease course of these animals is distinct from that seen with fatal outcomes of VEEV-induced encephalomyelitis. Neonatal mice fail to control SINV replication in peripheral tissues, and infection of dendritic cells, macrophages, bone, cartilage and skeletal muscle results in hyper-induction of pro-inflammatory cytokines (148, 149). Although neuroinvasion occurs at late stages of disease in these neonatal mice, this is not accompanied by overt

histopathological evidence of encephalitis (317), rather, the mice succumb to infection due to the dysregulated innate immune response that resembles systemic inflammatory response syndrome (SIRS) (268, 318), which involves destruction of hematopoietic cells within the liver and apoptosis of cells within the spleen and bone marrow (148, 317). Thus, it is speculated that newborn mice are immunosuppressed, and rather than mounting an overactive immune response that results in the encephalitis, it is an insufficient immune response in young animals that results in multisystemic virus dissemination, virus-induced neuronal apoptosis, hyper-active pro-inflammatory cytokine induction, and death (268).

Inoculation of mice intracerebrally (i.c.) bypasses the complex host-virus interactions resulting in neuroinvasion and allows the investigation of the inherent neurovirulence of SINV. There is also a clear age-dependent susceptibility of mice to SINV after i.c. inoculation that is partially explained by increasing neuronal resistance to apoptosis during development (113); however, several neurovirulent strains remain lethal in adult mice via this route. NSV was selected from an AR339 parent strain by successive intracranial inoculations alternately through neonatal and weanling mice (115), which selected for a highly virulent virus in 4-6 week-old mice. Another virus, AR86, was originally isolated after successive amplification in weanling (not neonatal) mouse brain (342), which likely resulted in selection of this strain's capacity to cause nearly 100% lethality in 6-week old animals. An infectious clone of AR86 was constructed, designated either ps55 or ps300, to identify possible viral genetic determinants of this strain's enhanced virulence profile (299). A unique feature of AR86 was that it encoded a threonine at nsP1 position 538, whereas other viruses of this group encode isoleucine at this position. The amino acid at nsP1 538 resides at the P3 position of the nsP1/2 cleavage domain (i.e. third amino acid from the nsP1 terminus), its role in neurovirulence was first evaluated by comparing wild-type AR86 (S300, nsP1 538 threonine) with a virus encoding the consensus isoleucine at that position (S340, nsP1 538 isoleucine). Intracranial inoculation of 6-week-old mice with wild-type S300 results in clinical signs of increasing severity including hind limb weakness, paresis, and finally paralysis by day 5 to 6 post infection, and ultimately death. Paralysis

of distal appendages likely reflects viral replication within and necrosis of anterior horn motor neurons within the spinal cord. In contrast, infection with S340 encoding the single isoleucine mutation at nsP1 538 results in significant attenuation causing fewer clinical signs and no mortality. Moreover, when the reciprocal mutation was made in the TR339 non-neurovirulent background (39ns1 = isoleucine→threonine at nsP1 538), this mutant caused more severe disease signs and mortality than wild-type TR339 strongly implicating threonine at nsP1 538 as a neurovirulence determinant. Surprisingly, both wild-type AR86 (S300) and mutant (S340) viruses established infection within similar regions of the brain and grew to equivalent titers at early times (12 to 72 hours post infection), but by day 6 post infection, foci of virus replication continue to expand in brains of wild-type (S300) virus-infected animals while these foci were considerably smaller in S340-infected mice, as determined by in situ hybridization (127). Thus, it appears that enhanced neurovirulence of SINV encoding threonine at nsP1 538 results from an ability to spread within CNS neurons more efficiently, or that this determinant confers resistance to host mechanisms of virus clearance from the CNS.

To further investigate the mechanisms of SINV neurovirulence, the adult mouse virulent AR86 strain (S300) and the avirulent Girdwood strain (infectious clone G100) were compared since these strains are highly homologous yet result in vastly different virulence profiles (307). Using a genetic approach, chimeric S300/G100 viruses were constructed and assessed for virulence using the adult mouse i.c. model. In addition to threonine at nsP1 538, this approach identified three additional S300-encoded determinants of adult mouse neurovirulence—a deletion within the hypervariable C-terminal domain of nsP3 (positions 386-403 of G100), the presence of cysteine at nsP3 position 537 rather than the Opal termination codon encoded by G100, and a single structural gene determinant at position 243 of E2 (serine). When introduced collectively into the avirulent G100 background, these four determinants rescue nearly full virulence of the wild-type S300 in terms of disease signs and mortality. Interestingly, comparison of the wild-type and mutant viruses that encode the four virulence determinants indicated that these viruses established infection and grew at early times post

infection within the brain and spinal cord to similar levels as the reciprocal viruses encoding the four attenuating mutations. However, at day 4 post infection, wild-type S300 grew to significantly higher titers than S363, which encodes the four attenuating mutations (307). Taken together, determinants of enhanced neurovirulence by AR86 (S300), particularly threonine at nsP1 538, appear to enable more efficient spread or resistance to immune clearance from CNS tissues.

1.3 The type I and type II IFN response

Historical perspective and current IFN family members

Interferon (IFN) was first recognized in 1957 as a component of supernatants from chick chorio-allantoic membrane cultures that had been infected with heat-inactivated influenza virus. When these supernatants were added to naïve cultures, this treatment was found to “interfere” with subsequent influenza infection (133). Interestingly, early studies on the antiviral properties of IFN involved alphaviruses, specifically Chikungunya virus (97, 130). In the following years, an IFN-like substance was found to be secreted by leukocytes upon stimulation with phytohemagglutinin (348) and was initially termed immune IFN (74), but is now known as IFN- γ . As individual IFN types were purified and cDNAs were cloned, it became apparent that these cytokines had not just antiviral properties, but demonstrated other biologic activities including antiproliferative and immunomodulatory effects (241), but by definition all IFNs are antiviral. The IFNs are comprised of three family members with distinct structures: type I IFNs, to which IFN- α , IFN- β , IFN- δ , IFN- ϵ , IFN- κ , and IFN- ω belong; type II IFN which is comprised of single member, IFN- γ ; and the more recently identified type III IFNs, which include various IFN- λ s (or IL-28A/B and IL-29). While the IFN- λ s are important to the antiviral response and mediate signaling events similar to type I and type II IFNs (for review see (14)), they were not specifically investigated in this work, and will not be discussed further. The genes that encode all type I IFNs, which include IFN- β and multiple IFN- α subtypes ($-\alpha 1$, $-\alpha 2$, $-\alpha 4$, $-\alpha 5$, $-\alpha 6$, $-\alpha 7$, $-\alpha 8$, $-\alpha 10$, $-\alpha 13$, $-\alpha 14$, $-\alpha 16$, $-\alpha 17$ and $-\alpha 21$), are located on

human chromosome 9 (chromosome 4 in mice) (246), and as the best characterized types, the terms IFN- α/β and type I IFN are used interchangeably. The gene encoding IFN- γ is found on human chromosome 12 (chromosome 10 in mice) (246). Historically, IFN signaling served a crucial role as a model to understand the specifics of cytokine/hormone signal transduction mechanisms.

Type I IFN induction

Induction of type I IFN is one of the earliest host responses to viral infection. While type I IFNs are detected after infection of many cell types (both immune and non-immune cells), the major producers in vivo are cells involved with the innate immune response, including macrophages and dendritic cells (DCs), which have a specialized pathogen surveillance role. However, unlike IFN- γ and other cytokines secreted specifically by immune cells, the ability of non-immune cells to respond to infection with local type I IFN production serves an important protective role. Both infected and uninfected bystander cells respond to local type I IFN stimulation with the subsequent induction of hundreds of genes, many of which have antiviral or immunomodulatory functions. Type I IFNs stimulate cytotoxic activities of immune cells (NK cells, T cells and macrophages) (241), induce chemokine secretion that promotes inflammatory cell recruitment to the site of inflammation (143), and regulate gene expression to favor pro-apoptotic functions (48). Moreover, type I IFN greatly modulates the activities of DCs, a cell type that bridges early innate immune signals with specific downstream adaptive antiviral immune responses (134). Maturation of DCs is induced by type I IFN by stimulating surface presentation of co-stimulatory molecules (315) as well as MHC class I antigens, either through classical presentation of endogenous viral antigen, or through cross-presentation of exogenous antigen (143, 210). Finally, basal type I IFN production is required for an optimal cellular response to additional viral or cytokine stimuli since many IFN stimulated genes (ISGs) encode proteins which themselves participate in immune signaling such as IFN regulatory factors (IRFs) (310), receptor adaptor molecules (e.g. MyD88), signal transducer and activator of

transcription (STAT) proteins, protein kinase R (PKR, discussed later)(31), and the type I IFN receptors themselves (1, 56).

Primary induction of type I IFN involves three steps: virus or pathogen recognition by various pattern recognition receptors (PRRs), activation of latent cytoplasmic transcription factors by protein kinases, and nuclear trafficking of these transcription factors which bind to promoter elements of the IFN- β and other early cytokine genes. Thus, IFN- β transcription does not require de novo protein synthesis and can be induced extremely rapidly upon infection. Transcription of IFN- β requires the coordinated assembly of several transcription factors within a compact region of its promoter forming an “enhanceosome”(75). These transcription factors include IFN regulatory factors 3 and 7 (IRF3 and IRF7), nuclear factor kappa B (NF- κ B) and ATF2/c-Jun, which bind different positive regulatory domains within the IFN- β enhancer. The enhanceosome is further modified through binding of CREB-binding protein (CBP) and p300, which are co-activators that have histone acetyltransferase (HAT) activities that maintain open chromatin structure to facilitate transcription (217). While IRF3/7 become activated in response to viral infection specifically and are essential to subsequent IFN- β transcription, cooperative binding of NF- κ B and ATF2/c-Jun enhance IFN- α/β induction (308).

A variety of stimuli, in addition to viral infection, activate NF- κ B, either through the canonical or non-canonical pathway. Although the latter pathway has only recently been identified, it appears that NF- κ B is more commonly activated by viral infection to induce type I IFN through the canonical pathway, which will be described briefly here. NF- κ B is a family of proteins consisting of at least five members, which hetero- and homo-dimerize to form functional transcription factors (33). These dimers are maintained in a latent form in the cytoplasm of an unstimulated cell through interactions with I κ B proteins. Upon stimulation, either through proinflammatory cytokine or toll-like receptor (TLR) signaling, a second complex composed of heterotrimeric I κ B kinases (IKKs) becomes activated and phosphorylates the I κ B inhibitory factor on specific serine residues, which leads to the polyubiquitination and degradation of the I κ B protein through the 26S proteasome.

Degradation of I κ B liberates the NF- κ B dimer (classically consisting of p50 and p65/RelA proteins) and exposes a nuclear localization signal (NLS) allowing its nuclear trafficking and binding to the IFN- β and other inflammatory cytokine gene promoters (33).

Unlike NF- κ B and ATF2/c-Jun, which are activated by many stimuli to induce numerous genes relating to inflammation, activation of IRF-3 and IRF-7 is achieved specifically in response to viral infection (143), and is required for IFN- β induction (310). Activation of IRF-3, in particular, is important for the initial response to infection since this factor is constitutively expressed in a latent cytoplasmic form while IRF-7 requires prior type I IFN signaling-dependent induction in most cell types (207, 310). IRF-3 activation is achieved through phosphorylation at Ser 396 (and other serine/threonine residues) by the IKK-related kinases IKK ϵ and TBK-1 after PRR stimulation, and once activated, forms homodimers and translocates to the nucleus to bind the IFN- β promoter (79, 290, 338, 357).

Numerous pattern recognition receptors that initiate innate immune and inflammatory responses have now been described, but recognition of RNA virus infection involves two main classes, toll-like receptors (TLRs) and RIG-I-like receptors (RLRs), which will each be briefly described. The TLRs are a large family of PRRs, which are important for recognition of pathogen-associated molecular patterns (PAMPs) on invading bacteria, fungi, protozoa and viruses. The PAMPs associated with viral infection include single-stranded RNA (ssRNA) (recognized by TLR7 and TLR8 within endosomes), double-stranded RNA (dsRNA) produced during replication of both dsDNA and ssRNA viruses (recognized by TLR3 within endosomes), unmethylated CpG DNA from dsDNA viruses (recognized by TLR9 within the endosome), and various viral envelope proteins (recognized by TLR2, TLR4 and TLR6 at the plasma membrane) (158). While all these TLRs are capable of inducing inflammatory cytokine secretion, only TLR2,3,4,7,8 and 9 are involved with induction of type I IFN (20, 21). TLR signaling is particularly important to immune cells, which show elevated, but cell-type-dependent, expression of these receptors. Plasmacytoid DCs (pDCs), which serve a specialized role for secreting abundant type I IFN, can respond to genomic DNA and

RNA in the absence of viral replication through TLR7 and 9 to induce IFN- α/β . Unlike RLR activation (discussed below), which requires active viral replication, phagocytic cells are thought to uptake apoptotic bodies of virus-infected cells where viral genomic or dsRNA intermediates can be recognized within endosomal vesicles by TLR3, which is preferentially expressed by phagocytic cells like CD8 α^+ DCs (143). Ligation of TLRs leads ultimately to activation of IKKs that in turn phosphorylate NF κ B and IRF3/7, but also results in the activation of MAP kinases that signal through ATF2/c-Jun. The signaling events between TLR ligation and IKK activation are complex, and will not be discussed here, but require adapter molecules (MyD88, TIRAP, TRIF/TICAM1, and TRAM) that associate with each TLR upon ligation and enable subsequent signaling involving both E3 ubiquitin ligase and protein kinase activities (143, 158). Since TLR3, and to some extent TLR4, use the adapter molecule TRIF/TICAM1 while all other TLRs signal through the MyD88 adapter, TLR signaling is generally divided into MyD88-dependent and MyD88-independent pathways.

Viral detection and IFN production by cells deficient in the expression of various TLRs suggested the existence of cytoplasmic viral nucleic acid sensors, a speculation that was recently confirmed with the demonstration that retinoic acid-inducible gene I (RIG-I) functions as a cytoplasmic dsRNA PRR (356). Soon thereafter, melanoma differentiation-associated gene 5 (MDA5) and LGP2, which were identified through sequence homologies to RIG-I, were added to the group of RIG-I-like receptors (RLRs) (355). These receptors are all DExD/H-box-containing helicases that bind dsRNA through a C-terminal helicase domain, and mediate signaling through an N-terminal caspase recruitment domain (CARD). The absence of a CARD domain in LGP2 explains this protein's biologic activity as a "dominant negative" regulator of RLR signaling (264, 355). RLR signaling requires active viral replication to produce dsRNA ligand and the CARD-containing adaptor molecule IPS-1 (a.k.a. MAVS/Cardif/VISA), which must localize to the mitochondrial outer membrane to mediate signaling. Reciprocal CARD interactions between the RLR and IPS-1 results in downstream activation of TBK1 and IKK ϵ protein kinases for IRF-3/7-dependent type I IFN induction, and/or activation of canonical IKK complexes to mediate NF κ B-dependent type I IFN

induction (143, 144), as described above. A final class of cytosolic PRR is PKR, which mediates both IFN induction and host translational shutoff. Since PKR effects have been well studied during alphavirus infection, they will be described later.

Type I and type II IFN signaling

Once secreted, IFN- β initiates a second cascade of signaling events through the type I IFN receptor (IFNAR), which we will refer to as the “IFN signaling pathway” to differentiate it from the “IFN induction pathway”. This subsequent signaling pathway is both critical for upregulation of IFN-stimulated genes (ISGs), but also for optimal type I IFN induction since (i) all IFN- α subtypes [except IFN- α 1 (human) and IFN- α 4 (mouse)] are induced in an IFN-signaling-dependent manner (185) and (ii) in most cell types, IRF-7 expression must first be upregulated to mediate the maximal late phase of IFN- β induction (207). For these reasons, the IFN signaling pathway is also referred to as the IFN amplification pathway. For simplicity, type I IFN signaling will be described in detail first, followed by a brief description of type II IFN signaling, which is highly related. The type I and type II IFN signaling pathways are illustrated in Figure 1.3.

The IFNAR (IFN- α/β R) is a heterodimeric receptor containing two subunits, IFNAR1 and IFNAR2 (IFNAR2c/IFNAR2.2/ β_L subunit). Receptor assembly is ligand dependent (49, 163), and since IFNAR2 has a higher affinity for type I IFN, it is more common for this binding event to occur first, followed by recruitment of the IFNAR1 subunit to assemble the ternary complex (95, 96). Differential binding affinities between IFN- β and the various IFN- α subtypes with the IFNAR subunits is thought, in part, to explain differential downstream signaling (322). The IFN receptors contain no catalytic function themselves, but rely on the activities of protein tyrosine kinases of the Janus kinase family [Jak/Janus activated kinase/“Just another kinase ”(350)], that constitutively associate with each subunit’s cytoplasmic tail proximal to the plasma membrane. This family of kinases has a tandem arrangement of a pseudo-kinase and a kinase domain at the C-terminus resembling the “two-headed” Roman god Janus. Once the ternary IFNAR1/2-IFN complex forms,

these tyrosine kinases are brought into favorable proximity/orientation to allow their trans- and/or auto-phosphorylation (50, 94, 228), resulting in their full activation. Tyrosine kinase 2 (Tyk2), which is associated with IFNAR1, and Janus kinase 1 which is pre-associated with IFNAR2, undergo phosphorylation in an interdependent manner on tyrosines within each kinase's activation loop at positions 1054/1055 (Tyk2) and 1022/1023 (Jak1) (94), which promotes kinase function directly or by improving substrate access to the active site (175, 322). Once activated, Tyk2 and Jak1 phosphorylate tyrosine residues present on the receptor tails, which serve as docking sites to allow the recruitment of STAT proteins through Src-homology-2 (SH2) domain-phosphotyrosine interactions. There is evidence that STAT2 is found pre-associated with the IFNAR2 subunit through a non-SH2 domain, but upon ligation of the receptor, STAT2 may relocate via its SH2 domain to the phosphorylated tyrosine 466 on the IFNAR1 subunit (182, 232, 278, 352), which is a required step for subsequent STAT1 recruitment and activation.

Once the IFNAR complex is activated and STAT proteins are recruited, STAT1 and STAT2 are in turn phosphorylated at tyrosine 701 and 690, respectively, by Jak1 and/or Tyk2. This co-localization at the receptor complex allows the phosphorylated STAT1 and STAT2 to heterodimerize through reciprocal phosphotyrosine-SH2 domain interactions, which concurrently releases the complex from the receptor. IFN regulatory factor 9 (IRF9/p48), which is retained within the cytoplasm through constitutive association with STAT2 (165, 209), additionally interacts with pY-STAT1 and pY-STAT2 to form the trimeric IFN-stimulated gene factor 3 (ISGF3) complex, which is the active transcription factor capable of promoter binding (85, 179). Recent work suggests that ISG induction requires acetylation of each component of ISGF3 by CBP, which is recruited to IFNAR2 upon IFN- α stimulation (309). Dimerization of phosphorylated STAT1 and STAT2 results in a conformational change that exposes a nuclear localization signal (NLS) within each of these factors. Although nuclear accumulation of tyrosine-phosphorylated STAT2 requires STAT1 (18), the NLS within STAT2 is functional since, in the context of a phosphorylated heterodimer, STAT2 can rescue a mutant STAT1 deficient for nuclear import (18, 255). The ISGF3 complex is shuttled to the nuclear

pore complex (NPC) by the carrier protein importin- α 5 (karyopherin- α 1) (214, 216), which recognizes the NLS within STAT1, where it engages importin- β 1 that mediates passage of the complex through the nuclear pore. The ISGF3, or other STAT1-containing complex, is then released after importin- β 1 binds a Ran-GTPase within the nucleus. Once liberated within the nucleus, ISGF3 is able to bind IFN-stimulated response elements (ISREs) present within the promoters of many ISGs. While multiple STAT complexes can bind IFN- γ -activated site (GAS) promoter elements (discussed below), including STAT1-STAT2 heterodimers, ISREs are only engaged by ISGF3 (246).

The response to IFN- γ is identical to the above sequence, with a few notable exceptions. The IFNGR is composed of two subunits, IFNGR1 and IFNGR2, to which the Jak1 and Jak2 tyrosine kinases are associated, respectively. Bioactive IFN- γ consists of a homodimer, and unlike the type I IFN receptor complex, ligand-induced activation of the IFNGR complex involves initial binding of two IFNGR1 chains to each IFN- γ monomer, which in turn creates binding sites for two IFNGR2 chains that are subsequently recruited (208, 303, 332). The active receptor complex thus consists of two receptor subunit heterodimers, which allows trans-phosphorylation of JAK1 and JAK2, and phosphorylation of tyrosine 440 on each IFNGR1 cytoplasmic tail where STAT1 binds. Unlike type I IFN signaling, IFN- γ stimulation predominately results in the formation of STAT1 homodimers (IFN- γ activated factors/GAFs). Nuclear localization of STAT1 dimers proceeds as described above, which bind to GAS promoter elements. Unlike the ISRE element which can only be bound by the ISGF3 complex, multiple combinations of STAT dimers are able to bind GAS elements such as homodimers of STAT1,3,4,5, or 6, and STAT1/2, STAT1/3, STAT1/4, STAT1/5, STAT2/3 and STAT5/6 heterodimers (246). The presence of ISRE, GAS, or both elements within the promoters of a given ISG partially explains the overlap of type I and type II IFN signaling pathways, and for maximal transcriptional activation of certain genes, cooperative signaling through both pathways may be required.

The above sequence of events represents the canonical JAK/STAT signaling pathway, but several other parallel signal transduction cascades are known to be intimately associated with this

classical pathway and/or mediate distinct signaling events in response to type I and type II IFNs. Activation of p38 MAPKs by type I IFN is well documented and ISRE/GAS-dependent transcription has been shown to require p38 kinase activity (101, 183). Moreover, activation of the Rac1/p38 MAP kinase pathways by type I IFN could be important for growth inhibitory activities of IFN (321). In addition to tyrosine phosphorylation at position 701, STAT1 becomes phosphorylated at serine 727, which is not required for dimerization or nuclear localization, but is necessary for full transcriptional activity (346). Enhanced transcriptional activity by serine-phosphorylated STAT1 dimers is due, at least in part, to the requirement for this modification for the recruitment of transcriptional co-activators in the nucleus, including CBP/p300 (discussed earlier) (246, 359) and GCN5 (236). Serine 727 phosphorylation is achieved by protein kinase C- δ (PKC- δ), which itself is activated by type I and type II IFN through a PI3K-dependent mechanism. A detailed discussion of the signaling pathways that cooperate with JAK/STAT is beyond the scope of these studies (for a detailed review, see (246)), but it will be important to consider the numerous and diverse consequences that inhibition of type I and type II receptor signaling has on downstream biologic activities.

Cellular Regulation of Jak/STAT signaling

Although a rapid cellular response to IFN is essential, the potency of this response can be detrimental to the host if not tightly controlled. Negative regulation of JAK/STAT signaling is mediated through downregulation of IFN receptor surface expression, dephosphorylation by protein tyrosine phosphatases, acetylation of STATs, induction of suppressors of cytokine signaling (SOCS) proteins, and disruption of STAT DNA binding by nuclear proteins that inhibit activated STATs (PIAS). Physiologic expression of truncated STATs (splice variants) also regulates STAT activity through dominant negative competition. While these cellular mechanisms are crucial to dampen overactive immune and growth inhibitory activities, several virus families have successfully usurped these regulatory mechanisms to their advantage, which will be discussed later.

Perhaps the most direct means of suppressing the IFN signaling pathways is to reverse the activation of various JAK/STAT factors, which is achieved through dephosphorylation by various protein tyrosine phosphatases (PTPs) including SH2-containing phosphatase 1 (SHP1), SHP2, and protein-tyrosine-phosphatase 1B (PTP1B). These are cytoplasmic phosphatases that are constitutively expressed in an inactive state (193), but are capable of dephosphorylating residues on IFN receptors, JAKs, or STATs themselves (15, 57, 137, 180, 358). Moreover, nuclear PTPs like T-cell protein tyrosine phosphatase (TCP45) dephosphorylate nuclear STAT1, which is shuttled back into the cytoplasm and remains non-responsive to subsequent activation for a period of latency (277, 313). Interestingly, CBP was recently found to directly acetylate STAT1 in the nucleus to counteract IFN signaling by inducing TCP45 binding, STAT1 dephosphorylation and nuclear export, and preventing subsequent STAT1 activation until the acetyl group is removed by HDAC3 (152). Thus, HATs/HDACs modulate IFN signaling in cooperation with PTP activities.

Identification of the SOCS family of proteins (71, 229, 304), which share a common central SH2 domain (10), initially implicated these proteins as negative regulators of JAK/STAT signaling, but they are now recognized as regulators of many other signaling pathways. SOCS proteins regulate IFN signaling through classical negative feedback since their expression is induced by cytokine stimulation, which is an important distinction from the constitutively expressed PTPs. SOCS proteins inhibit cytokine receptor complexes by binding phosphorylated JAKs to inhibit their kinase activity (SOCS1) (353), or by binding the receptor itself (cytokine-induced SH2 protein/CIS and SOCS3) which could either prevent STAT recruitment (10, 211) or interfere with JAK kinase activity (10). SOCS proteins also share a conserved C-terminal “SOCS box” domain that may be involved with ubiquitination of activated signaling molecules to target them toward proteasomal degradation (reviewed in (41, 153)).

A third group of proteins that negatively regulate STAT signaling are the PIAS, which principally act on tyrosine-phosphorylated STATs within the nucleus. PIAS act by binding transcription factors, including STAT1 and NF κ B (188, 189), and preventing their association with

DNA promoters, and through recruitment of negative transcriptional co-regulators (e.g. HDACs) or sequestration of positive regulators (reviewed in (293)). Finally, PIAS proteins can sumoylate target proteins and transcription factors, a mechanism through which they regulate multiple cellular functions in addition to cytokine signaling (273).

A final mechanism by which IFN signaling may be regulated is through expression of truncated signaling factors. Two isoforms of STAT1 are expressed from splice-variant transcripts—STAT1 α , the 91 kDa protein with full transcriptional activities, and STAT1 β , which is incapable of mediating IFN- γ -stimulated gene induction but participates in the IFN- α response as a component of a functional ISGF3 complex (56). Since STAT1 β can be activated on tyrosine 701 and can bind DNA, but it lacks the C-terminal transactivation domain responsible for transcriptional co-activator recruitment, it is thought to act as a physiologic dominant negative inhibitor of STAT1 α signaling (19). Interestingly, pathogen-induced STAT1 β induction has been shown to downregulate the IFN- γ response (11, 27). Similarly, overexpression of IFNAR2.1 (IFNAR2b/ β_s subunit), which is one of three IFNAR2 isoforms expressed from alternatively spliced mRNAs, was shown to counteract IFNAR signaling and antiviral responses (243).

Viral disruption of IFN signaling

An ever-growing list of viral evasion strategies designed to defeat the IFN response demonstrates that viruses, pathogenic viruses in particular, may antagonize virtually every signaling step previously described. Due to limits on genome size, virally-encoded proteins normally target a few central components of IFN induction, IFN signaling, and/or both pathways. Since the focus of this dissertation is on disruption of IFN signaling by alphaviruses, examples of antagonism of this pathway by other viruses will be discussed briefly; however, numerous strategies targeting IFN induction have been described (for a detailed review see (34)).

The earliest event in IFN signaling, ligation of the IFNAR with type I IFN, can be disrupted by two mechanisms. Poxviruses express soluble IFN decoy receptors that bind type I IFN (9) and

type II IFN (8) to competitively inhibit cellular IFN receptor activation. Alternatively, reduced IFN receptor ligation can be achieved through downregulation of receptor expression at the cell surface. Ligand-independent IFNAR downregulation occurs through the physiologic unfolded protein response (UPR) (also known as the ER stress response) by serine phosphorylation of IFNAR1, which targets it for ubiquitination and proteasomal degradation (28, 191). Rapid synthesis of viral proteins during VSV and HCV infection activates UPR, which inhibits antiviral defenses through IFNAR1 degradation (190).

Following receptor ligation, receptor assembly and Jak activation must occur for subsequent signaling, and these processes are inhibited by many viral families. The Marburg virus protein VP40 blocks IFN- α and IFN- γ -induced Jak tyrosine phosphorylation and downstream signaling (323). The flaviviruses, including West Nile virus (WNV), Japanese encephalitis virus (JEV), and tick-borne encephalitis virus (TBEV), similarly inhibit Jak activation by IFN- γ and/or IFN- α/β through expression of NS5 (26, 116, 187), which in the case of JEV was achieved through activation of a protein tyrosine phosphatase (186).

The STAT molecules are often inhibited during viral infection, either through affecting rates of phosphorylation and dephosphorylation, or by specifically targeting STAT toward degradation. The V, W, and C proteins of paramyxoviruses have been shown to modulate all of these processes to downregulate IFN signaling (reviewed in (341)). Several viruses usurp cellular negative regulatory mechanisms of STAT signaling such as Hepatitis C virus (HCV), another flavivirus, which induces cellular SOCS-3 expression to suppress STAT1 phosphorylation (32). Even if activated normally, STATs may be prevented from localizing to the nucleus during viral infection as in the case of Ebola virus infection where VP24 directly competes with STAT1 for importin- $\alpha 5$ binding, thus disrupting ISGF3 and GAF nuclear accumulation (256, 257).

Finally, even when upstream signaling events are functioning, viral infection may disrupt nuclear functions of transcription factors. Virus-induced shutoff of host transcription, which will be described later in the context of alphavirus infection, is an example of a non-specific mechanism to

inhibit IFN and ISG transcription. Poliovirus, through expression of its 3C^{pro} protease, achieves transcriptional shutoff by directly cleaving the transcriptional co-activator TATA-binding protein (TBP) (47, 351). Interestingly, expression of ISG54, which requires recruitment of GCN5 but not TBP, persisted in the presence of poliovirus-mediated transcriptional shutoff. Since GCN5 is inhibited by another viral protein, adenovirus E1A, and GCN5 is required for STAT2 function, it follows that transcriptional shutoff during adenovirus infection (but not poliovirus infection) effectively downregulates ISG54 expression (236). Thus, the multitude of virus-encoded mechanisms to inhibit the transcriptional apparatus can result in quite disparate effects on the IFN response, even in the face of perceived host transcriptional shutoff.

1.4 Host response to alphavirus infection

Type I IFN is essential for control of alphavirus replication

The host response to alphavirus infection involves both the innate and adaptive arms of the immune system. The type I IFN response is essential for early control of virus replication and for an effective specific adaptive response. After subcutaneous infection, SINV TR339 replication is detectable within the DLN, but the virus fails to disseminate and mice do not develop disease (272). In contrast, subcutaneous infection of adult mice incapable of responding to type I IFN (IFNAR^{-/-}) results in a course of infection similar to that seen in neonatal mice where TR339, and other avirulent SINV strains, replicates at the inoculation site and within the DLN within the first 24 hours, which seeds significant serum viremia and allows the virus to rapidly disseminate (40, 269). Importantly, unlike wild-type DCs that are somewhat resistant to SINV infection and replication (147, 268), SINV replicates efficiently in DCs of IFNAR^{-/-} mice in vitro and within the DLN in vivo, and the virus is able to spread to other myeloid-lineage cells in the spleen and liver (269, 270). The greater replication of SINV within myeloid-lineage cells of IFNAR^{-/-} mice could be responsible for the massive levels of proinflammatory cytokines measured in these animals (269). After subcutaneous

SINV inoculation, a combined deficiency of the type I and type II IFN response (IFNAR^{-/-} x IFNGR^{-/-}) results in even shorter survival times and more severe disease including hemorrhagic manifestations relative to IFNAR^{-/-}, indicating that IFN- γ signaling confers some protection in the absence of a functional type I IFN response (270). Finally, the finding that fatal disease is prevented if IFNAR^{-/-} mice are first immunized with an attenuated SINV strain prior to challenge with a virulent strain (40) indicates that adaptive responses remain functional in IFNAR^{-/-} animals, but these responses alone are not sufficient to control primary infection in the absence of competent IFN signaling.

Unlike subcutaneous infection of immunocompetent mice with SINV, peripheral inoculation with wild-type VEEV is able to replicate within myeloid lineage cells and lymphoid tissues (44, 110), sites in which SINV could only replicate in IFNAR^{-/-} animals (269, 270). VEEV infection results in greater type I IFN induction than SINV infection in immunocompetent mice, presumably because there is more RNA ligand present within IFN-producing cells. Despite this IFN induction, significant VEEV viremia ensues and allows neuroinvasion, which indicates VEEV is less sensitive to the antiviral state than SINV (discussed later). However, in the absence of a competent IFN response, mice succumb more rapidly to infection with wild-type VEEV, and strains that are normally avirulent become lethal in IFNAR^{-/-} animals (111, 349) indicating IFN- α/β signaling is important for control of VEEV replication. Accordingly, treatment of mice with pegylated IFN- α after either subcutaneous inoculation or aerosolized VEEV resulted in greatly improved survival (196).

The specific identification of alphavirus PAMPs and their corresponding PRRs that mediate downstream type I IFN induction is an active area of research. Different alphaviruses and different strains are known to induce varying amounts of type I IFN in vivo and in vitro. High levels of type I IFN induction is seen after infection of adult mice with VEEV and of neonatal mice with SINV, which likely reflects widespread, systemic viral replication in lymphoid tissues. Since expression of PRRs is cell-type specific, with TLRs being more highly expressed by subsets of DCs and

macrophages, the pathways leading to type I IFN induction by alphaviruses in vivo are complex, and depend not only on the nature of the viral PAMPs, but also the particular strain's cellular tropism.

Infection of cultured fibroblasts was first recognized to activate IRF-3, but only in the context of active viral replication (22). In myeloid DC cultures infected with SFV, it was later demonstrated that while type I IFN induction required IRF-3, it did not depend on either active viral replication or MyD88 (131) indicating that recognition of alphavirus PAMPs was indeed cell type-dependent, but that downstream signaling required IRF-3 activation. Further complexity arises with the nature of the input virions and whether they were derived from cells of mosquito or mammalian origin. RRV derived from mosquito cells efficiently infects myeloid DCs but induces type I IFN very poorly relative to mammalian-derived RRV, which is due to differential glycosylation of the envelope glycoproteins (288, 289) suggesting the involvement of carbohydrate recognition receptors (i.e. TLR2 and C-type lectins) as important factors in modulating type I IFN induction by alphavirus-infected myeloid cells. Moreover, UV inactivation of RRV resulted in little reduction in type I IFN induction by myeloid DCs suggesting that differential carbohydrate recognition was independent of virus replication (288).

In contrast to DCs and macrophages, which can express abundant endosomal PRRs that do not require viral replication for PAMP recognition, the RLRs are expressed ubiquitously and require viral replication to trigger type I IFN induction. In addition to dsRNA, which is recognized by both RIG-I and MDA5 in a length-dependent manner (141), RIG-I is known to recognize uncapped, single-stranded RNA containing exposed 5' triphosphate moieties (132, 244). For alphaviruses, type I IFN induction is likely to involve recognition of dsRNA replication intermediates since *ts* mutants that do not synthesize positive-sense RNA fail to induce detectable IFN (206). However, RIG-I-mediated recognition of free 5' triphosphates, which form during normal alphavirus replication and could be abundantly produced in strains with mutations within nsP1 that disrupt capping, is an interesting possibility (55). Infection of fibroblast cultures with SINV leads to little type I IFN production, which has been proposed to be a result of virus-induced shutoff of host macromolecular

synthesis (discussed later) (84). When primary mouse embryonic fibroblasts (MEFs) are infected with a non-cytopathic mutant SINV, however, induction of type I IFN depended on IRF-3 activation by TBK1 and the presence of MDA5 and PKR, but not RIG-I (39). In contrast, primary DC and macrophage cultures did not depend on MDA5 for type I IFN induction after SINV infection (100). The above studies indicate complex virus-host interactions that trigger type I IFN induction in manners that are cell type-dependent, alphavirus type/strain-dependent, and may or may not require active virus replication. Further elucidation of these interactions will enable a more complete understanding of pathogenesis since elimination of the IFN response dramatically alters disease profiles and is uniformly detrimental to the host when infected with virulent and avirulent alphaviruses.

Interferon-stimulated genes with anti-alphavirus activities

Protein kinase R (PKR/double-stranded RNA-dependent protein kinase) is an additional type of cytoplasmic PRR, which will be discussed in this section because its expression is increased in response to type I IFN and a potential inhibitory role during alphavirus replication has been studied for several years. PKR is constitutively expressed at a basal level in unstimulated cells, but it is maintained in an inactive form. Upon dsRNA binding (>30 nt), PKR dimerizes allowing activation of each monomer through auto-phosphorylation on serine or threonine residues in an ATP-dependent manner (142), which potentiates the enzyme's kinase activity. Although traditionally known for its role in translational shutoff, an antiviral mechanism to which SINV is partially resistant (discussed in the next section) (105, 327), PKR activation results in downstream induction of type I IFN through different pathways. The primary mechanism is through PKR-mediated IKK activation, which in turn phosphorylates I κ B resulting in NF κ B nuclear translocation and transcription of IFN- β and other cytokine genes (157); however, PKR also participates in diverse signaling cascades that interface with TLR signaling, that induce apoptosis through IRF1 activation, and that indirectly potentiate STAT1 serine phosphorylation to enhance STAT transcriptional activity (88). Type I IFN induction by SINV

is partially dependent on PKR expression in MEFs (39) and myeloid DCs (272) indicating that PKR may play a PRR role during alphavirus infection.

Mice with deletions in PKR, RNaseL, and Mx-1 (triple deficient, TD) do not develop detectable disease after subcutaneous inoculation with SINV TR339, however, virus replication within the draining lymph node was increased relative to that seen in wild-type animals (272). This finding was supported by infection of DC cultures, which were much more permissive to SINV replication in the absence of PKR (272). Despite this role for PKR in limiting early SINV protein synthesis, type I IFN signaling remains fully functional and limits virus spread beyond the DLN within SINV-infected PKR/RNaseL $-/-$ mice, and pre-treatment of PKR $-/-$ DC cultures with type I IFN prevents translation of SINV proteins (271). Thus, ISGs in addition to PKR and RNaseL (and Mx-1) prevent SINV dissemination within wild-type animals. In the absence of PKR, type I IFN stimulated factors appear to inhibit initial translation of SINV input genomes. Pre-treatment of PKR $-/-$ MEFs with type I IFN was shown to inhibit cap-dependent translation of SINV or other transcripts introduced into the cytoplasm by electroporation, but not translation of transcripts without cap structures (i.e. EMCV IRES-containing transcripts) or cellular mRNAs exported from the nucleus (314). The type I IFN-induced factors involved with the specific translational inhibition have not been identified.

Several additional ISGs that encode proteins with anti-alphavirus activities have been identified. Zinc-finger antiviral protein (ZAP) was shown to block translation of input genomes of several alphaviruses including VEEV, SFV and SINV (29, 200). The antiviral activity of ZAP, which appears to function as a homodimer to restrict SINV replication (167), is linked to its capacity to bind viral RNA and possibly target it to exosomal degradation (117). Transgenic mice expressing human MxA, even in the background of IFNAR $-/-$, limits SFV replication and reduces mortality (112, 124). Other ISGs were suggested to have anti-SINV activities when expressed from a second subgenomic promoter of the virus itself. After subcutaneous inoculation of adult IFNAR $-/-$ mice, SINV strain dsTE12Q expressing the ubiquitin homologue ISG15 resulted in reduced mortality (172, 173),

however, expression by a TR339-based double promoter virus had no effect on disease outcome (360). Similarly, recombinant SINV expressing mouse ISG12 from a second subgenomic promoter increased average survival time in neonatal mice (162). Through overexpression as well as siRNA-mediated knockdown, ISG20, ISG56, Viperin, as well as ZAP and ISG15 were shown to negatively affect SINV TR339 replication in vitro (360) where ZAP and ISG20 were the most potent and, when combined with expression or knockdown of the other ISGs, yielded an additive antiviral effect. Mechanistically, the actions of these ISG products toward SINV replication are not understood, and whether they are involved in the type I IFN-dependent antiviral response in vivo will require further study.

Type II IFN and noncytolytic viral clearance from the CNS

Intracranial inoculation of avirulent SINV has been studied for many years to model immune-mediated virus clearance from the CNS, which requires non-cytolytic mechanisms in order to preserve neuronal function since these cells have very limited capacity for regeneration. Intracranial infection of nonpathogenic SINV (AR339) into adult SCID mice, which have no functional B and T cells, results in a life-long persistent infection whereas wild-type mice rapidly clear infectious virus and viral RNA within 8 days (177). Passive transfer of anti-SINV hyperimmune sera or monoclonal anti-E2 antibodies results in clearance of infectious virus and reduced intracellular viral replication through non-cytolytic mechanisms, however, since viral RNA persists within neurons, removal of antibody results in virus reactivation (176). Clearance of viral RNA is thought to involve CD4⁺ and CD8⁺ T cells, but not through cytotoxic mechanisms since neurons are restricted in MHC class I and II antigen presentation. In the absence of an antibody response, μ MT mice that were persistently infected with SINV (TE strain) were able to clear infectious virus from the spinal cord, which required CD4⁺ or CD8⁺ T cell activities as determined by depletion studies or passive transfer of SINV-specific T cells into persistently infected RAG mice (30). Finally, IFN- γ production by CNS-infiltrating T cells was suggested to be important for clearance from CNS neurons since recombinant

SINV expressing IFN- γ from a second subgenomic promoter resulted in virus clearance from the spinal cord of SCID mice, which was presumed to functionally replace the activities of T cells at that site (30). Despite the suggested involvement of IFN- γ in clearance of SINV from the CNS, intracranial inoculation of IFN- γ deficient or IFNGR-/- mice resulted in virus clearance similar to that seen in wild-type (38, 270); however, the combined deficiency of antibody and IFN- γ (μ MT/GKO) resulted in a failure to clear virus from sites that μ MT animals cleared efficiently (i.e. the spinal cord and brainstem) suggesting IFN- γ acts in concert with antiviral antibody to mediate virus clearance from specific CNS sites (38).

Neuronal culture systems have been used to investigate the mechanisms of antibody- and IFN- γ -mediated SINV clearance (177). A rat neuronal cell line that can be differentiated in vitro (CSM14.1) is permissive to SINV infection regardless of its differentiation state, but SINV can establish persistent infection only in fully differentiated cells. Treatment of these persistently SINV-infected differentiated neurons with exogenous IFN- γ resulted in reduced virus growth, a reduction in SINV-induced cell death, inhibition of virus gene expression, and partial restoration of cellular protein synthesis (37). Thus, despite virus-induced shutoff of host gene expression in persistently infected neurons, these cells remained capable of responding to exogenous IFN- γ treatment, which could initiate an antiviral state. Responsiveness to IFN- γ was later shown to require the canonical Jak/STAT signaling pathway since inhibition of Jak1 kinase activity abrogated the IFN- γ -mediated protective effect (36). Therefore, it is plausible that IFN- γ acts in concert with antiviral antibody to mediate clearance of infectious virus and viral RNA from specific sites within the CNS, actions which require functional Jak/STAT signaling.

While the above studies implicate a protective role of IFN- γ and T cells during infection with nonfatal SINV, this assertion is misleading in the context of fatal SINV infection. Intranasal inoculation of wild-type mice with NSV results in 100% mortality, but mice lacking either CD4+ or CD8+ T cells were partially protected and IFN- γ -deficient mice were completely protected from mortality (265). Pathologic analysis of the hippocampus suggested that infiltration of CD4+ T cells

and macrophages contributed to immunopathogenesis (145). Balance of an immune response favoring virus clearance but avoiding immune-mediated damage is critical to survival and prevention of neurologic sequelae, and it is clear that these processes are modulated differently by different strains of alphaviruses.

1.5 Downregulation of the host response by alphaviruses

Enhanced disease seen in IFNAR^{-/-} mice after infection with virulent and avirulent strains of alphaviruses indicates that type I IFN is critical to the control of alphaviruses, but it also suggests that virulent strains must downregulate this response in order to cause disease. The major proposed mechanism by which alphaviruses downregulate the host response is through nonspecific shutoff of host gene expression, which will be discussed in detail. However, this nonspecific mechanism is inadequate to fully explain the complexity of virus-host interactions relating to the IFN response that result in pathogenesis, several examples of which will be given. Finally, the type I IFN system is important in the control of all alphaviruses, but different alphaviruses appear to employ different strategies to avoid this response as indicated by differing levels of IFN induction in vivo and differential sensitivity to IFN in vitro, which makes it difficult to generalize these strategies/mechanisms across species and strains.

Alphavirus-induced shutoff of host macromolecular synthesis

Alphavirus-induced shutoff of host transcription and translation has been studied for many years and is correlated with the development of cytopathic effect (CPE) (305). Identification of viral determinants of host shutoff has indirectly been accomplished through isolation of viruses capable of persistent infection that encode mutations either in the N-terminus, or more frequently, the C-terminus of nsP2 (240). In three independent studies, a single amino acid substitution for proline at SINV nsP2 position 726 was identified as a determinant of persistent infection in IFN-deficient BHK

cells (82, 240, 345); these substitutions also resulted in decreased viral RNA synthesis and less efficient transcriptional and translational shutoff by SINV (67, 82). The development of CPE after infection with virus or replicons encoding mutations at nsP2 726 was tightly correlated to the level of virus replication, however, the nsP2 C-terminal mutations were additionally associated with defective growth within, and clearance from IFN-competent cells (82, 84). Infection with SINV/G (nsP2 726 P→G) resulted in significant upregulation of many cellular genes and type I IFN secretion by NIH 3T3 cells while infection with the wild-type SINV downregulated most cellular genes significantly, failed to induce type I IFN secretion, and resulted in rapid CPE (84). SINV nsP2 was shown to mediate shutoff of RNA polymerase I- and II-dependent transcription (92) and two lines of evidence suggested that transcriptional, but not translational, shutoff plays the dominant role in downregulating the antiviral response. First, both SINV/G and a second noncytopathic mutant, SINV/2V (containing defective nsP2/3 cleavage), failed to inhibit host transcription and type I IFN production, but SINV/2V shutoff host translation very similarly to the Wild type (103). Second, when expressed alone, wild-type nsP2 was cytotoxic and could inhibit both host transcription and type I IFN production, but failed to have a major effect on host translation (92). This conclusion, however, was called into question since an additional mutation between the nsP2 protease and methyltransferase domains (R615A) conferred noncytopathic replication and reduced translational shutoff, but this virus (SINV 615A) demonstrated wild-type levels of transcriptional shutoff although direct effects on type I IFN production were not investigated (213). Thus, SINV nsP2 is proposed to suppress the host response, and in particular type I IFN production, through its role in transcriptional (92, 103) and/or translational shutoff (213). The functions of nsP2 from another Old World alphavirus, SFV, have also been intensively studied, but since these activities seem similar to those from SINV nsP2, they will not be discussed here.

Further analyses of SINV nsP2 functions indicated that this protein mediated shutoff of macromolecular synthesis and CPE development only when expressed in its fully processed form (92, 104), but if expressed autonomously its protease activity is not required (92). Ablating nonstructural

polyprotein cleavage sites in SINV or replicon allowed persistent infection in BHK-21 cells, but replication resulted with type I IFN production and virus clearance from IFN-competent cells (82, 92, 104). Additional mapping revealed that expression of the individual nsP2 domains alone (amino terminus, helicase, protease or SAM-dependent methyltransferase) could not mediate CPE or transcriptional shutoff, even if they were targeted to the nucleus by an NLS tag (83). This indicated that in order to mediate transcriptional shutoff, multiple nsP2 domains are interdependently required, which was confirmed using a non-biased mutagenesis strategy where short peptides were randomly inserted throughout all functional domains of nsP2. Expression of mutants with insertions in each nsP2 domain could prevent nsP2-dependent CPE development and alter the protein's subcellular localization, but only two mutations were not lethal to virus replication. These viable mutants persistently infected BHK-21 cells and IFNAR^{-/-} MEFs, but were cleared from IFN-competent cells (83). Thus, the mechanisms by which nsP2 mediates transcriptional shutoff (or the development of CPE) are not fully understood, but seem to require the functions of multiple domains.

Downregulation of the host response by New World alphaviruses is similar to that described above with the exception that transcriptional/translational shutoff by VEEV and EEEV is mediated by viral capsid and not nsP2. Accordingly, replicon RNA encoding the New World alphavirus nonstructural genes was found to be much less cytopathic than corresponding Old World replicons (93, 242), as was a chimeric virus containing EEEV-derived nsP and SINV-derived structural protein genes, which established persistent infection in BHK-21 cells but was cleared from NIH 3T3 cells (93). Expression of New World capsid protein rescued Newcastle disease virus replication (presumably due to antagonism of type I IFN production), inhibited RNA polymerase I- and II-dependent transcription, induced eIF2- α phosphorylation and possibly reduced host translation, and resulted in notable cytotoxicity (6, 93). These functions of VEEV and EEEV capsid proteins require an N-terminal peptide (4, 91), but neither the RNA-binding domain nor an active protease was required (93). As with Old World nsP2, a precise mechanism of New World capsid-mediated transcriptional shutoff has not been described, but the VEEV N-terminal capsid peptide (positions 33

to 68) localizes the capsid protein to the nuclear membrane and is proposed to disrupt the nuclear trafficking of cellular cargo required for transcription and the development of an antiviral response (17, 91). However, the EEEV N-terminal peptide did not have an effect on the subcellular distribution of the EEEV capsid, which localized to both the cytoplasm and nucleus, but was not found to accumulate at the nuclear envelope (4).

Resistance of SINV to mechanisms of protein synthesis shutoff

As mentioned previously, the role of PKR in promoting shutoff of both host and viral protein translation is well established as a means to downregulate virus replication. Once activated by dsRNA, PKR is one of four cellular kinases that phosphorylates the alpha subunit of eukaryotic initiation factor 2 (eIF2 α) on Ser-51, which renders its ternary complex (eIF2 α -GTP-tRNA_i) incapable of transporting initiator tRNAs to the 40S ribosome (237). Through the irreversible binding of phosphorylated eIF2 α -GDP with eIF2B, the activation of PKR ultimately results in reduced host and viral protein translation. Many virus families suppress PKR activation in order to both prevent translational shutoff and to limit PKR-mediated induction of type I IFN and proinflammatory cytokines (303). However, infection of immortalized fibroblast cultures with SINV has for many years been known to activate PKR (275, 276). Despite what appears to be nearly complete phosphorylation of eIF2 α by 3 to 4 hours post infection (327), translation of SINV structural proteins is resistant to this PKR-mediated translational block by alternatively recruiting eIF2A- tRNA_i, which does not require GTP, to the initiation codon of the subgenomic 26S RNA. Additionally, the presence of a stable RNA hairpin structure within the capsid coding sequence allows the 40S ribosomal subunit to stall while scanning the 26S RNA, which enables efficient initiation through eIF2A- tRNA_i binding (327). In this manner, translation of cellular mRNAs that require eIF2 α is blocked after PKR activation, which could suppress expression of antiviral effector molecules, but translation of SINV 26S RNA proceeds through the alternative recruitment of eIF2A.

Evidence for shutoff-independent mechanisms of type I IFN antagonism

Alphavirus mutants that cause little CPE within infected fibroblasts are associated with higher levels of virus-induced IFN- α/β production from these cells, which has led to the hypothesis that alphaviruses downregulate the type I IFN response through transcriptional shutoff. Host macromolecular synthesis shutoff is clearly important in the redirection of cellular resources toward viral functions and is likely a requirement for virulence, but its association with IFN antagonism has not been clearly demonstrated in vivo. Most importantly, mutations that limit cytopathogenicity in vitro also result in slower synthesis of viral RNA and reduced virus growth, which makes it difficult to ascribe their attenuation in vivo solely to an inability to shutoff gene expression efficiently. In this section, a few examples will be given of alphavirus disruption of the host IFN response that cannot be explained by generalized host shutoff.

Host transcriptional shutoff mediated by nsP2 of Old World alphaviruses is proposed to involve nuclear functions of this protein, which indeed is found at varying levels within cell nuclei. Determinants of nuclear localization and transcriptional shutoff by SINV nsP2 could not be assigned to any particular domain (83), but the NLS sequences within SFV have been definitively described (239, 259). Mutation of an arginine triplet (RRR) within this NLS to RDR renders the localization of nsP2 exclusively cytoplasmic within cells infected with this mutant (SFV-RDR) (258). Importantly, the SFV-RDR mutant is attenuated for neurovirulence (78), which is associated with higher in vitro induction of type I IFN (35), yet both wild-type SFV4 and the SFV-RDR mutant shutoff host transcription and translation equivalently (258). These findings call into question the hypotheses that (i) nsP2 nuclear targeting is required for transcriptional downregulation and (ii) that wild-type viruses suppress type I IFN induction through global shutoff of gene expression since, in this case, the mutant SFV-RDR shuts off macromolecular synthesis as efficiently as the Wild type. Our laboratory has reported analogous findings during infection with SINV AR86 infection where attenuation of S340,

which encodes a point mutation at nsP1 538, is associated with higher type I IFN production in vitro and in vivo, but both the wild-type S300 and S340 shutoff transcription and translation similarly (55). Thus, alphavirus mutants that induce elevated type I IFN production may or may not be associated with a deficient capacity to downregulate host macromolecular synthesis. Moreover, it is only recently that investigations have been made to identify the alphavirus ligands and PRRs mediating type I IFN induction (39, 100, 131). Mutations that reduce cytopathogenicity of alphaviruses and result in greater virus-induced type I IFN induction are known to quantitatively affect viral RNA accumulation, but whether these RNAs are qualitatively better PRR ligands for type I IFN induction has not been investigated.

Most work correlating type I IFN induction with deficient virus-induced host shutoff has been conducted using fibroblasts, either primary cells or cell lines. Infection of cultured embryonic fibroblasts with wild-type SINV does not result in IFN production (39, 84), however, type I IFN is induced significantly in neonatal mice (148) and to lower levels in adult mice (270). Type I IFN is likely produced by infected DCs in vivo, and infection of cultured DCs with wild-type SINV results in the expression of hundreds of cellular genes including IFN- α/β and is not associated with overt CPE in this cell type. While host shutoff is likely an important factor in type I IFN antagonism, its contribution toward pathogenesis must be considered in the context of the particular cell types involved with the antiviral response.

Neuronal cells are an important target for the encephalitic alphaviruses. As neurons mature in culture they become refractory to apoptotic signals explaining, at least in part, why SINV infection results with a much slower development of cytotoxic effects in this cell type. However, SINV does induce dramatic shutoff of host protein synthesis in these cells (37, 354). Treatment of SINV-infected rat neuronal cultures with IFN- γ resulted with restoration of cellular protein synthesis and clearance of infectious virus (37), presumably a result of the induction of ISGs with antiviral activities, which suggested that the infected cells were still responsive to cytokine stimuli even in the face of perceived shutoff of de novo gene expression. This “breakthrough” ISG induction may not be a unique feature

of neurons. Primary MEF cultures infected with SINV undergo shutoff of host protein synthesis within 4 to 6 hours post infection, which as mentioned, contributes to failed type I IFN induction (84). However, these infected cells remain at least partially responsive to additional stimuli including polyI:C and Sendai virus infection at times of perceived shutoff. Parallel experiments with the noncytopathic mutant SINV/G (nsP2 726 P→G) demonstrated that even in the absence of host shutoff, type I IFN induction was only detected at very late time points (>32 h.p.i.) (39). Thus, it seems evident that type I IFN antagonism in SINV-infected MEFs requires undescribed mechanisms that play a critical role at times prior to the initiation of macromolecular synthesis shutoff, and at later times these mechanisms act in concert with host shutoff to downregulate the host response.

1.6 Dissertation objectives

The above discussion indicates several gaps in our current understanding of how alphaviruses either suppress the induction of type I IFN or avoid the well-established antiviral effects of these cytokines in order to cause disease in mice, and presumably, humans. In particular, we have little understanding of the precise mechanism by which Old World virus nsP2 or New World virus capsid proteins mediate transcriptional or translational shutoff. Without mechanistic knowledge, it is difficult to thoroughly evaluate this hypothesis in vivo where the regulation of these processes in multiple tissues and cell types may be different. Secondly, the number of genes upregulated by IFN stimulation is numerous but relatively few have precisely described antiviral functions and even fewer are known to have anti-alphavirus activities. This is of particular importance in the context of VEEV infection where very high serum type I IFN levels are measured prior to neuroinvasion, which presumably indicates VEEV is capable of dismantling a pre-existing antiviral state. Finally, at the outset of these investigations, no additional mechanisms in addition to macromolecular synthesis shutoff were proposed for alphaviruses, but as was discussed in the previous section, they are very likely to exist. Several virulence determinants have been identified, which when disrupted, attenuate

virulence and cause elevated type I IFN induction without affecting the virus' ability to shutoff macromolecular synthesis (35, 78, 307). Moreover, many SINV strains that effectively shutoff gene expression in permissive cell lines fail to spread effectively beyond the DLN in mice with an intact type I IFN system (268). Interferon- γ is proposed to cooperate with antiviral antibody to mediate clearance of nonpathogenic SINV from the CNS (114), but whether virulent and avirulent viruses specifically disrupt IFN- γ -mediated signaling had not been studied.

Our major goal in these studies was to gain understanding of alphavirus pathogenesis by focusing on molecular mechanisms of VEEV and SINV IFN antagonism. In particular, we aimed to formally evaluate whether signaling events were disrupted at points upstream of gene induction, which together with transcriptional shutoff, could downregulate the host response. We initially tested whether signaling through the Jak/STAT pathway was affected by VEEV infection after Montgomery, et al. reported a specific interaction between VEEV nsP2 and importin- α 5 (221) that conceivably could disrupt STAT1 nuclear transport as is seen during Ebola virus infection (256, 257). Surprisingly, we found that VEEV nsPs robustly inhibited not only STAT1 nuclear import, but also the activation of several factors upstream in the type I and type II IFN signaling pathways.

A second major aim in these studies was to directly relate IFN signaling antagonism to alphavirus pathogenesis. To this end, we compared several alphaviruses as well as various strains of SINV with differing virulence profiles. These comparisons proved very informative in that they identified a single SINV determinant of STAT1 inhibition at nsP1 538, which suggests that a polyprotein precursor may be mediating Jak/STAT signaling inhibition rather than a single nsP. Additionally, this approach directly implicates Jak/STAT signaling inhibition as a key component of SINV neuropathogenesis since the nsP1 538 determinant is a well-established virulence determinant that is required for AR86 to cause mortality in adult mice. These findings form a solid basis for detailed *in vivo* analyses of the role for AR86-mediated Jak/STAT signaling inhibition during SINV-induced neurological disease, studies that are ongoing in our laboratory. The aims addressed herein are:

Aim 1: To determine whether the inhibition of ISG induction by alphaviruses is associated with dysfunctional Jak/STAT signaling.

Aim 2: To determine whether Jak/STAT signaling antagonism is a common feature of alphaviruses and whether this inhibition correlates with virulence potential.

Table 1.1: Alphaviruses discussed and their features.

Virus (clone), strain	Antigenic Complex	Origin, Source	Disease	Ref
NEW WORLD				
VEEV (V3000), Trinidad donkey	VEE	Trinidad, 1943, Donkey	Encephalitis (human,mouse, horse)	(58, 146)
VEEV (TC-83), Vaccine strain	VEE	Attenuated derivative of Trinidad Donkey	Mild neurologic	(24)
EEEV	EEE	North/South America	Encephalitis	
WEEV	WEEV	North/South America, SINV/EEEV recombinant	Encephalitis	
OLD WORLD				
SINV (S300/S55), S.A.AR86	WEE	South Africa, 1956, Mosquito	Arthritis (human), Encephalitis (adult mouse)	(299, 342)
SINV (G100), GirdwoodS.A.	WEE	South Africa, 1963, Human	Arthritis (human) Encephalitis (neontl. mouse)	(203, 307)
SINV (TR339), AR339	WEE	Egypt, 1953, Mosquito	Arthritis (human) Encephalitis (neontl. mouse)	(149, 215, 311)
NSV	WEE	AR339 derivative, Weanling/ neontl. mouse alt. passage	Encephalitis (adult mouse)	(115)
RRV (RR64), T48	SF	Australia, 1959 Mosquito	Fever, rash, polyarthritis (humans); Arthritis (mouse)	(156)
CHIKV	SF	Africa, Asia, Europe	Fever, arthritis, rash	
SFV	SF	Africa, Asia, Europe	Fever, encephalitis	

Figure 1.1

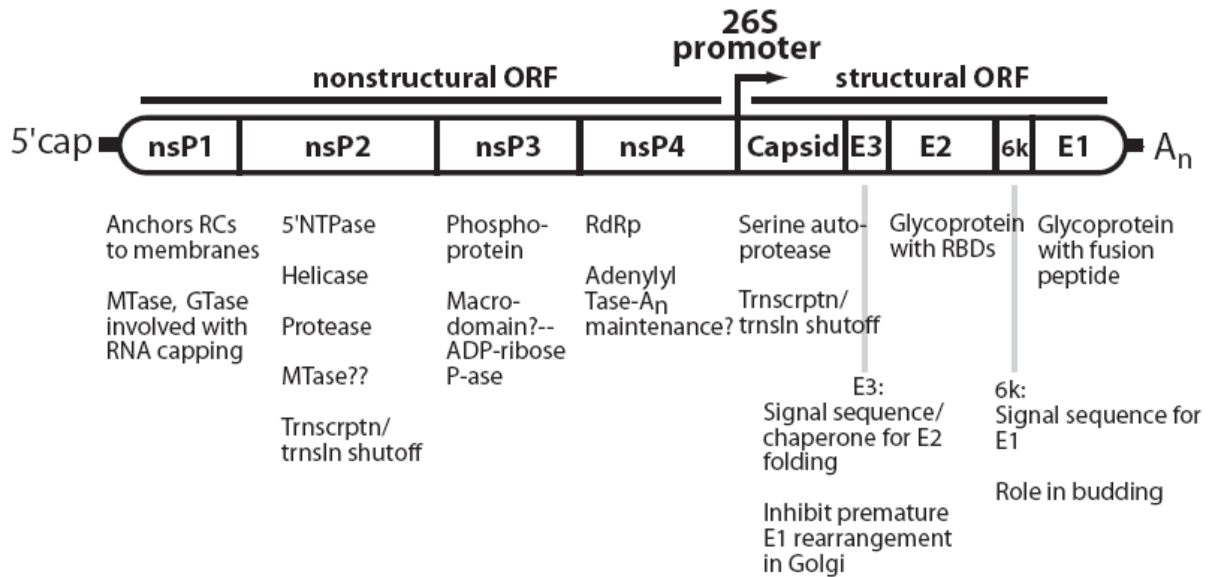


Figure 1.1: Alphavirus genome structure and protein functions

The alphavirus genome is polyadenylated (A_n), contains a 5'-methylguanosine cap, and encodes two open reading frames (ORF) separated by a subgenomic promoter that drives 26S RNA synthesis. Viral proteins encode multiple enzymatic and structural functions, which are indicated. MTase = methyltransferase; GTase = guanylttransferase; 5'NTPase = 5' nucleoside triphosphatase; P-ase = phosphorylase; RdRp = RNA-dependent RNA polymerase; "?" indicates an unclear functional role; Tase = transferase; Transcriptional (trnscrptn) and translational (trnsln) shutoff is mediated by nsP2 (Old World alphaviruses) and Capsid (New World alphaviruses).

Figure 1.2

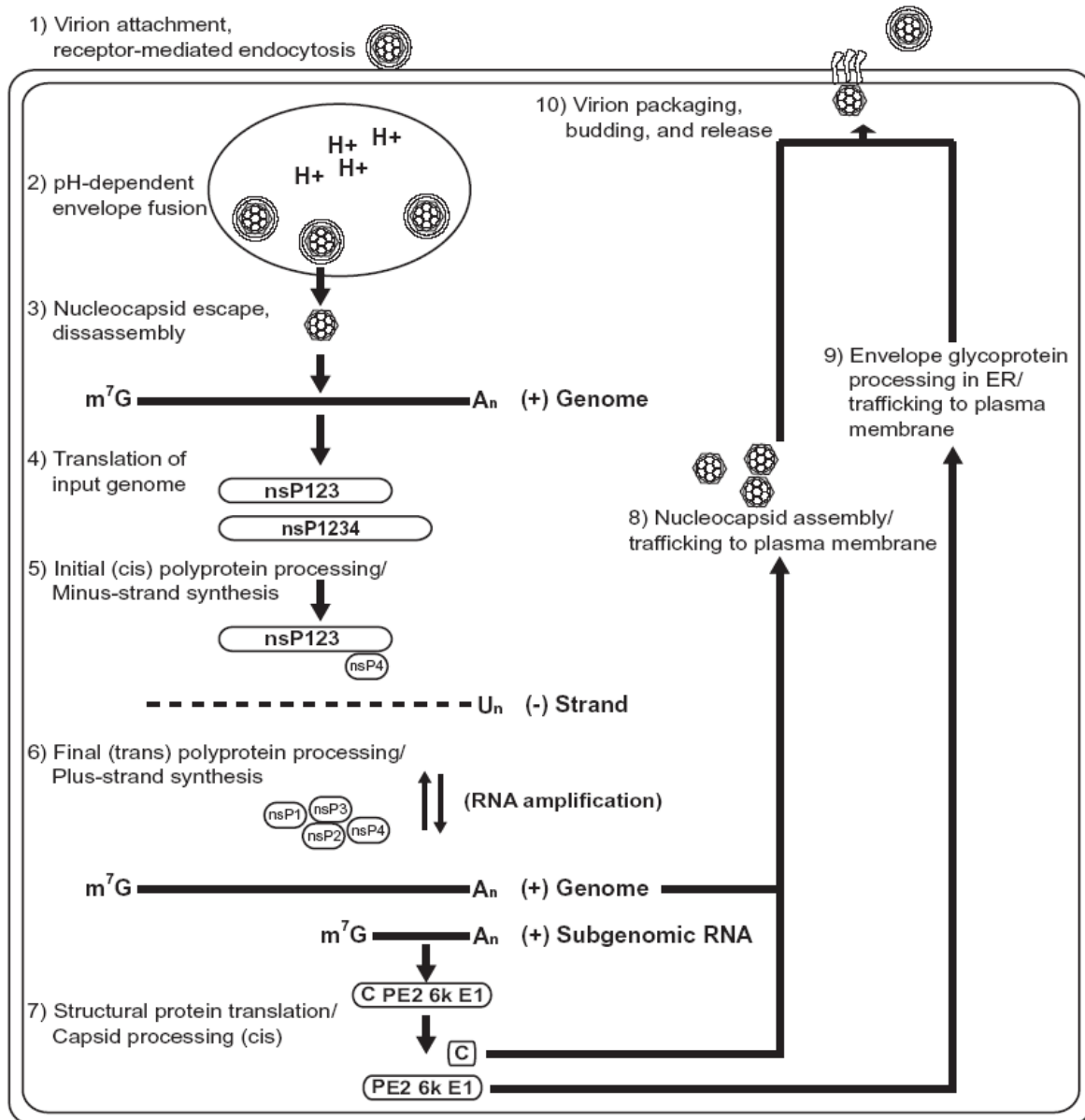


Figure 1.2: Alphavirus replication and lifecycle

The lifecycle of alphaviruses within a single cell is outlined. See text for detailed descriptions of Step1-Step10. Figure adapted from Jose, et al. (140).

Figure 1.3

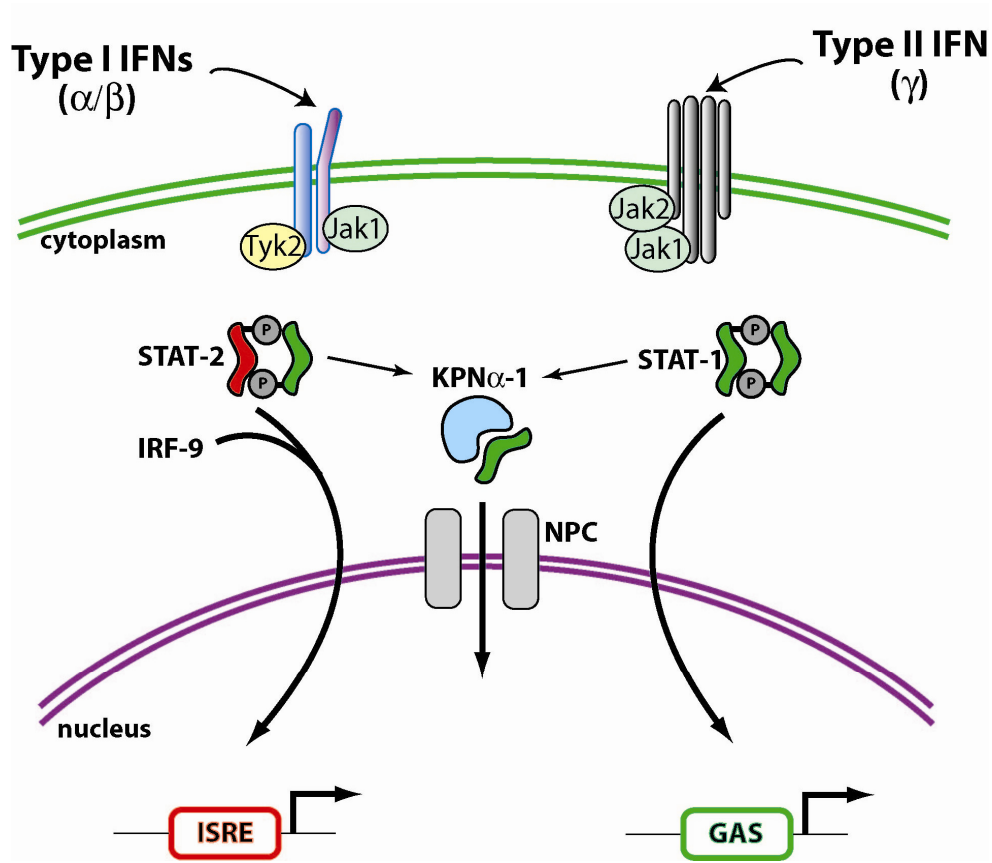


Figure 1.3: Type I and type II interferon signaling pathways

Signaling events downstream of the type I and type II IFN receptor complexes are depicted. A detailed description of each signaling event is included within the text.

CHAPTER TWO:

VENEZUELAN EQUINE ENCEPHALITIS VIRUS DISRUPTS STAT1 SIGNALING BY DISTINCT MECHANISMS INDEPENDENT OF HOST SHUTOFF

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2.1 Abstract

Venezuelan equine encephalitis virus (VEEV) is an important human and veterinary pathogen causing sporadic epizootic outbreaks of potentially fatal encephalitis. The type I interferon (IFN) system plays a central role in controlling VEEV and other alphavirus infections and IFN evasion is likely an important determinant of whether these viruses disseminate and cause disease within their hosts. Alphaviruses are thought to limit the induction of type I IFNs and IFN-stimulated genes (ISGs) by shutting off host cell macromolecular synthesis, which in the case of VEEV is partially mediated by the viral capsid protein. However, more specific strategies by which alphaviruses inhibit type I IFN signaling have not been characterized. Analyses of cells infected with VEEV and VEEV replicon particles (VRP) demonstrate that viral infection rapidly disrupts tyrosine phosphorylation and nuclear translocation of the transcription factor STAT1 in response to both IFN- β and IFN- γ . This effect was independent of host shutoff and expression of viral capsid, suggesting that VEEV employs novel mechanisms to interfere with type I and type II IFN signaling. Furthermore, at times when STAT1 activation was efficiently inhibited, VRP infection did not limit tyrosine phosphorylation of Jak1, Tyk2, or STAT2 following IFN- β treatment, but did inhibit Jak1 and Jak2 activation in response to IFN- γ , suggesting that VEEV interferes with STAT1 activation by the type I and II receptor complexes through distinct mechanisms. Identification of the viral requirements for this novel STAT1 inhibition will further our understanding of alphavirus molecular pathogenesis and may provide insights into effective alphavirus-based vaccine design.

2.2 Introduction

Venezuelan equine encephalitis virus (VEEV) is a mosquito-borne alphavirus in the family *Togaviridae* that is responsible for sporadic epidemics of encephalitis in equines and humans. Most cases of human and equine disease have been associated with epizootic VEEV strains (subtypes IAB

and IC) that undergo efficient amplification within horses, but recent studies indicate that endemic transmission of equine avirulent strains (subtype ID) is responsible for many unreported cases in humans that live near habitats where enzootic transmission occurs (3, 252, 340). When infected via the mosquito vector, patients may present with malaise, fever, and headache (340). While fatalities are rare (<1%), patients that recover from encephalitis may suffer from permanent neurological sequelae (174).

The type I interferons (IFNs) α and β represent a crucial innate defense system against most viral pathogens, including alphaviruses. These cytokines act in autocrine and paracrine pathways to induce the expression of numerous IFN-stimulated genes (ISGs), such as 2',5'-OAS, PKR, and Mx family members that are important for the control of viral infection (reviewed in (87)). The signaling events that follow IFN stimulation have been well described (reviewed in (180, 246)). In brief, when the type I IFNs bind the interferon α/β receptor subunits IFNAR1 and IFNAR2, these subunits dimerize at the cell surface allowing the apposition of two protein tyrosine kinases (PTKs), Janus activated kinase 1 (Jak1) and tyrosine kinase 2 (Tyk2), that are associated with the receptor's cytoplasmic tails. Juxtaposed Jak1 and Tyk2 are then activated through auto- and/or trans-phosphorylation (50, 94, 228), and they in turn phosphorylate tyrosine residues present on the receptor tails, which serve as docking sites for the recruitment of various signal transducers and activators of transcription (STAT) factors. Jak1 and Tyk2 subsequently phosphorylate STAT1 and STAT2, which form heterodimers, and in association with interferon regulatory factor 9 (IRF9), the trimeric complex localizes to the nucleus where it binds promoters containing IFN-stimulated response elements (ISREs) to drive expression of ISGs. This sequence of events is mirrored when type II IFN (IFN- γ) binds its cell surface receptor subunits (IFNGR1 and IFNGR2). Jak1 and Jak2 are activated at the IFNGR cytoplasmic tails, which in turn activate STAT1 by tyrosine phosphorylation. Unlike the response to type I IFN, IFN- γ stimulation results in the homodimerization of STAT1 molecules that translocate to the nucleus to bind ISG promoters containing IFN- γ activated sites (GASs).

Because the expression of ISGs is critical to limiting viral replication, viruses employ numerous strategies to antagonize the IFN response. Control of alphavirus infection relies on an intact type I IFN system since various attenuated strains of VEEV, Sindbis virus (SINV), and Semliki Forest Virus (SFV) become fully virulent in mice with disrupted IFN α / β receptors (78, 111, 349). Despite its crucial role in protection, treatment of mice with type I IFN or Poly I:C, an IFN inducer, failed to protect animals from a subsequent challenge with virulent VEEV suggesting the virus is partially resistant to these cytokines (111, 136), although administration of the more stable pegylated-IFN- α was successful (196). Previous studies indicate that sensitivity of different VEEV and Eastern Equine Encephalitis virus (EEEV) strains to IFN- α / β correlates with virulence potential (2, 5, 136, 301). Certain virulent epizootic strains of VEEV have been shown to be less sensitive to the effects of type I IFN than their less virulent enzootic progenitors (249, 301) suggesting IFN sensitivity is a potential marker for epizootic potential. However, more recent studies challenge this association (13) and implicate mutations within the E2 glycoprotein that allow equine-avirulent enzootic strains (subtype ID) to emerge as subtype IC strains that achieve efficient equine amplification (12, 13, 108, 333). In general, while the role for IFN resistance in VEEV pathogenesis/emergence remains unclear, all alphaviruses demonstrate some sensitivity to type I IFNs, and therefore must employ mechanisms to limit either the induction or cellular responses to these cytokines.

The major mechanism by which alphaviruses are believed to evade the antiviral effects of IFNs is through a global shutoff of host gene expression (84). Viruses containing mutations in the SINV nonstructural protein 2 (nsP2) carboxy-terminus possess limited ability to shutoff RNA polymerase II-dependent cellular transcription and induce higher levels of IFN- α / β relative to wild-type viruses, suggesting that generalized inhibition of host macromolecular synthesis contributes to viral blockade of the type I IFN response (84, 92, 103). Expression of nsP2 from the Old World alphaviruses SINV and SFV is linked to host cell cytotoxicity; however, studies with VEEV and Eastern Equine Encephalitis virus (EEEV) suggest that the New World alphaviruses mediate shutoff by viral capsid-dependent mechanisms (6, 91, 93, 242), although host shutoff also occurs in cells

infected with VEEV replicon particles (VRP) that do not express the viral capsid protein (S. A. Montgomery, unpublished data). An amino-terminal portion of VEEV capsid is required for transcriptional shutoff and for the generation of host cell cytopathic effect (CPE) through a mechanism proposed to involve the disruption of the nuclear import of cellular factors (17, 91). A similar region within EEEV capsid inhibits RNA polymerase II transcription and the antiviral effects of IFNs (4, 6, 17).

Although it is likely that host transcriptional and translational shutoff dampens the cellular antiviral response, these non-specific mechanisms are not solely responsible for the inhibition of the IFN response by alphaviruses. A mutation within the nuclear localization signal of SFV nsP2 leads to significantly higher levels of type I IFN induction with no apparent effect on host cell transcriptional or translational shutoff (35). Since this mutant is significantly attenuated in vivo (78), alphaviruses likely employ shutoff-independent mechanisms to antagonize the antiviral IFN response and these mechanisms are important determinants of virulence.

In this study, we tested whether infection with VEEV or VEEV replicon particles (VRP) specifically inhibits IFN signaling through the Jak-STAT pathway. Recent findings demonstrate that nsP2 of VEEV specifically interacts with the nuclear importin molecule karyopherin α -1 (KPNA1) (221). Since KPNA1 is known to bind and shuttle STAT1 to the nuclear pore complex to direct its nuclear import (287), we investigated whether VEEV inhibits STAT1-dependent IFN signaling. Our results indicate that cells infected with VEEV or VRP, which lack the genes encoding the viral structural proteins, fail to respond to type I and II IFN as demonstrated by decreased STAT1 tyrosine phosphorylation and nuclear localization. Since activation of the Jak-STAT pathway does not require de novo cellular gene transcription and translation, these findings provide additional evidence that alphaviruses are able to antagonize the IFN response by mechanisms independent of host transcriptional and translational shutoff.

2.3 Materials and Methods

Cell culture and reagents. Vero-81, BHK-21, HEK-293, and HeLa cells were grown under 5% CO₂ at 37 °C. Vero-81 cells (designated “Vero,” ATCC No. CCL-81) were obtained from the ATCC and maintained in DMEM/F12 media (Gibco) supplemented with 10% fetal bovine serum (FBS; Hyclone), L-glutamine (0.29 mg/mL; Gibco), nonessential amino acids (Gibco), penicillin-streptomycin (Gibco), and sodium bicarbonate adjusted to contain a final of 1.2 g/L (Gibco). HeLa and 293 cells were maintained in DMEM (Gibco) supplemented with 10% FBS, L-glutamine and penicillin/streptomycin. BHK-21 cells were maintained in alpha-MEM (Gibco) containing either 10% donor calf serum (Hyclone) or 10% FBS (Lonza), 10% tryptose phosphate broth (Sigma) and supplemented with L-glutamine and penicillin-streptomycin. Actinomycin D (ActD) and cycloheximide (CHx) were purchased from Sigma. Recombinant human IFN- β was obtained from Calbiochem and resuspended according to manufacturer’s instructions. The biological activity was determined by using a type I IFN bioassay previously described using A549 cells, and relative protection from EMCV-induced cytopathic effect was compared to the NIH human IFN- β reference standard (Gb23-902-531) (288). Recombinant human IFN- γ was used according to the concentration given by the manufacturer (R&D Systems).

VEEV and VEEV replicon particle (VRP) production. The generation of a full-length cDNA clone derived from the wild-type Trinidad donkey VEEV isolate (pV3000) has been described previously (58). A cell culture-adapted mutant encoding two attenuating amino acid changes in the E1 and E2 glycoprotein, pV3014, was used in these studies due to its greater specific infectivity relative to the parental pV3000 (25, 110). The mechanism of attenuation of V3014 may involve its efficient binding of heparan sulfate, which enhances cell culture infectivity but could increase viral clearance *in vivo* (25). In vitro-transcribed RNA from this pV3014 cDNA was used to electroporate BHK-21 cells, and after 24 h, supernatants were harvested, aliquoted, and stored at -80 °C. Viral

titers were quantified by plaque assay on BHK-21 cells. A split-helper system previously described (251) was used to generate VRP that express GFP or no transgene (VRP-empty). Supernatants of electroporated BHK-21 cells containing VRP were harvested and concentrated through 20% sucrose (wt/vol) at 72,000 x g for 4 h. Replicon titers were quantified as infectious units (IU) per mL by an indirect immunofluorescence staining assay of infected BHK-21 cells using antiserum raised against the viral nonstructural proteins (for VRP-empty titer) or by counting GFP positive cells (for VRP-GFP titer).

Immunoblot analysis. For most experiments, Vero-81 cells were lysed in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Igepal CA-630, 0.1% SDS, 0.5% deoxycholate, supplemented with Complete mini protease inhibitors (Roche) and phosphatase inhibitors (Sigma-P2850)) on ice for >5 min and scraped. Lysates were clarified by centrifugation for 5 to 10 min at 4°C and total protein was quantified by Coomassie Plus protein assay (Thermo). Equal amounts of total protein from each sample were denatured in SDS sample buffer and resolved by 8% SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane (Bio-Rad) in transfer buffer (48 mM Tris, 39 mM glycine, 10% methanol), blocked in 3-5% dry nonfat milk (or 5% bovine serum albumin) in PBST (137 mM NaCl, 2.7 mM KCl, 0.88 mM KH₂PO₄, 6.4 mM Na₂HPO₄, 0.1% Tween® 20), and then incubated with the indicated primary antibody overnight at 4°C. Membranes were exposed to horseradish peroxidase (HRP)-conjugated secondary antibody and then developed using ECL-Plus (Amersham) and exposed to film. The following primary antibodies were purchased from the indicated manufacturers: STAT1 (total), phospho-STAT1 (Tyr701), STAT3 (total), phospho-STAT3 (Tyr705), Tyk2 (total), phospho-Tyk2 (Tyr1054/1055), Jak2 (total) and phospho-Jak2 (Tyr1007/1008) from Cell Signaling; STAT2 and phospho-STAT2 (Tyr689 of mouse STAT2) from Upstate; Actin and GRP 78 from Santa Cruz; Jak1 (total) from BD transduction laboratories; and phospho-Jak1 (Tyr1022/1023) from Biosource. Goat anti-VEEV nsP2 was a gift from AlphaVax, Inc. Anti-mouse and anti-rabbit ECL HRP-conjugated

immunoglobulin G (IgG) secondary antibodies were purchased from Amersham, and HRP-conjugated anti-goat IgG was obtained from Sigma. For detection of immunoprecipitated total Jak protein by Western, light-chain-specific HRP-conjugated anti-rabbit and anti-mouse IgG was used.

Immunoprecipitations. For detection of phosphorylated Jak tyrosine kinases, total Jak protein was immunoprecipitated and then probed by Western blot with the appropriate phospho-specific antibody. Vero-81 cells were infected with VRP-GFP for 6 h and then stimulated with IFN for 20 min. Lysates were prepared as described above in phosphorylation lysis buffer (247) (0.5% Triton X-100, 150 mM NaCl, 10% glycerol, 1mM EDTA, 50 mM HEPES [pH 7.4], 1.5 mM MgCl₂, 200 μ M sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM NaF, Complete mini protease inhibitors). Lysates containing equal amounts of total protein (2.0-2.5 mg) were pre-cleared with protein G-agarose (Sigma) and preimmune normal serum for 2 to 5 h at 4°C, and then immunoprecipitated with protein G-agarose in the presence of the indicated antibody overnight at 4°C, washed 4 to 5 times with phosphorylation lysis buffer, and eluted in 2X SDS sample buffer for Western analysis. The anti-Jak kinase antibodies indicated above were used, except Tyk2 (total) (BD transduction laboratories) was used for immunoprecipitation. Isotype-matched normal IgG was purchased from Santa Cruz (rabbit) or eBiosciences (mouse).

Subcellular Fractionation. Nuclear purification was performed as previously described (220), except the crude nuclear fraction was not banded, but rather was washed 5 consecutive times in 0.5 to 1.0 mL homogenization medium prior to lysis in nuclear extraction buffer. Equal total protein from cytoplasmic and nuclear extracts were analyzed by Western blot as described above, and the purity of nuclear extracts was determined by Western blot to detect GRP 78, which localizes to the endoplasmic reticulum and should not be found within nuclear fractions.

Immunofluorescence microscopy. To assess subcellular localization of STAT1 by indirect immunofluorescence staining, Vero-81 cells were seeded on glass coverslips and infected with VRP-GFP for 6 h, treated with IFN- γ (200 U/mL for 40 min, rinsed in 1X DPBS, fixed for 30 min in 4% paraformaldehyde, incubated for 15 min in PBS-glycine (100 mM, [pH7.2]), permeabilized with 0.2% Triton X-100, blocked 1 hour with 10% normal goat serum in 1X immunofluorescence assay wash (3% bovine serum albumin, 0.05% Tween-20), stained for total STAT1 (Santa Cruz), and then stained with Alexa-Fluor 594-conjugated goat anti-rabbit IgG (Invitrogen) and DAPI (4',6'-diamidino-2-phenylindole [Roche]; 10ug/mL). Images were acquired with a Carl Zeiss LSM5 Pa confocal laser scanning microscope equipped with a 40X, 1.3 numerical aperture oil objective lens, using 505-530 nm band pass (green fluorescence) or 560 nm long pass (red fluorescence) filters. Pinhole diameter was set to 0.9 μ m (equivalent to one area unit). Confocal images were averaged 4 times with imaging software (Zeiss) and formatted using Adobe Photoshop.

Real-time PCR. Total cellular RNA was harvested using Trizol and PurLink™ purification system (Invitrogen), and equivalent amounts of total RNA was reverse transcribed using SuperScript III and random primers according to manufacturer's instructions (Invitrogen). cDNA was then quantified by TaqMan real-time PCR using the 7300 Real Time PCR System (Applied Biosystems). For each sample, 18s RNA and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) levels were quantified to calculate relative expression of the target genes using the $2^{\Delta\Delta CT}$ method, and similar results were obtained with each reporter gene. The following primer-probe sets were used (Applied Biosystems): 18S (Hs_99999901_s1), GAPDH (Rh02621745_g1), guanylate binding protein 2 (Hs00894842_g1), Trim 21(Hs00172616_m1).

Flow cytometry. To determine relative IFNGR1 surface expression, Vero-81 cells were seeded in 6-well plates and infected with VRP or mock-infected with diluent alone for 6 h. Cells were rinsed, and

then incubated on ice in enzyme-free Cell Dissociation Buffer (Gibco) for 10 min. Mock and VRP-infected cells were scraped and aliquoted into parallel groups for staining (2.5×10^6 to 5×10^6 cells/group). Each aliquot was then blocked with FACS Staining Buffer (FSB) (1% donor calf serum, 1% normal rabbit serum (Sigma), 0.1% NaN₃ in 1X DPBS), stained according to the manufacturer's instructions with anti-human CD119 (IFNGR1) conjugated to phycoerythrin (eBiosciences). Parallel cell aliquots were left unstained. Cells were then washed and fixed in 1% formaldehyde. Samples were read with a CyAn cytometer and were analyzed using Summit software (Dako).

To verify infectivity, VRP-infected cells in parallel wells containing glass coverslips were fixed in methanol and stained by indirect immunofluorescence assay using anti-VEEV-nonstructural protein mouse antiserum. In addition, separate wells were treated with IFN- γ and cell lysates were harvested for analysis of STAT1 phosphorylation as described above.

Statistical analyses. To determine whether VRP infection and/or IFN treatment resulted in a significant alteration of GBP-2 or Trim21 expression, we performed an analysis of variance using R software (www.r-project.org), which demonstrated that IFN treatment and VRP infection both had significant effects on ISG expression. A post hoc comparison of the IFN-treated groups revealed that VRP infection resulted in a significant reduction ($P < 0.05$) in both GBP-2 and Trim21 expression after each treatment time.

2.4 Results

VRP infection decreases IFN-stimulated gene expression independently of the viral structural proteins.

The reported interaction between nsP2 of VEEV and karyopherin- α -1 (KPNA1) (221) suggested that VEEV infection may disrupt IFN signaling by inhibiting STAT1 nuclear trafficking. To test this hypothesis, we first examined whether infection with VRP, in which the genes encoding

the viral structural proteins have been replaced with a reporter gene (Figure 2.1A), would interfere with STAT1-dependent, IFN-induced gene expression. Vero-81 cells, which cannot secrete endogenous type I IFN (66, 225), were either mock infected or infected with VRP-GFP, and then treated with type I or type II IFN for either 3 or 6 h. Total RNA was harvested and cDNA was generated to measure the IFN-mediated induction of two ISGs—guanylate binding protein 2 (GBP-2), which is induced in a STAT1-dependent manner following type II IFN treatment (201, 253) and tripartite motif protein 21 (TRIM21), which can be induced by type I IFN treatment (62, 334). These studies demonstrated that VRP infection significantly decreased induction of both genes after either 3 or 6 h of IFN treatment assessed by real time RT-PCR (Figure 2.1B and 2.1C). While the levels of housekeeping genes (18s RNA and GAPDH) were unaffected by VRP infection at these times, these studies cannot differentiate specific effects on IFN signaling from global transcriptional shutoff. Therefore, we next examined whether VRP or VEEV infection interferes with STAT1-mediated signal transduction.

VRP and VEEV infection disrupts the nuclear localization of STAT1 in response to IFN.

The translocation of STAT1 into the nucleus is an event critical to both type I and type II IFN signaling and requires the specific recognition of STAT1 by the importin molecule KPNA1 (287). Ebola virus VP24 interacts with this subfamily (NPI-1) of importins, an event that results in decreased STAT1 nuclear accumulation and IFN-induced gene expression (256, 257). Since nsP2 of VEEV directly interacts with KPNA1(221), we tested whether STAT1 trafficking is affected by the expression of VEEV nonstructural proteins. Vero-81 cells were infected with VRP for 6 h and then treated for 20 min with 1000 U of IFN- β or IFN- γ /mL after which subcellular fractions were harvested and subjected to Western blot. As shown in Figure 2.2A, total STAT1 was detected in the nuclear fractions in response to both IFN treatments in mock-infected, but not VRP-infected, lysates. The failure to detect STAT1 within nuclear fractions was not accompanied by any difference in total STAT1 levels within the cytoplasmic fractions, which suggests that the VRP-mediated block of

nuclear import was not associated with a decrease in either the expression or stability of STAT1 protein. Similar results were found in cells infected with VRP for only 5 h prior to IFN stimulation (data not shown). To corroborate these findings in the context of VEEV infection, we repeated this assay in Vero-81 cells infected with a derivative of the wild-type Trinidad donkey (TRD) VEEV strain (VEEV 3014) encoding the full-length genome. At 5 h post infection, we were again unable to detect STAT1 in the nuclear fraction in response to 1000 IU IFN- γ /mL, a concentration that clearly yielded nuclear STAT1 in mock-infected cells (Figure 2.2B), demonstrating that the VRP-mediated phenotype is relevant to cells infected with virus encoding the full-length VEEV genome. The same result was seen in cells infected with the wild-type V3000 virus (data not shown).

To confirm a defect in STAT1 nuclear accumulation, we used indirect immunofluorescence staining for STAT1 and confocal microscopy to assess the subcellular localization of STAT1 in VRP-infected Vero-81 cells. At a low MOI (3 IU/cell), the major population of cells showed both diffuse STAT1 staining as well as GFP fluorescence, indicating these cells were infected with GFP-expressing VRP (Figure 2.2C). In contrast, cells with distinct nuclear STAT1 staining, indicating they were IFN responsive, were GFP negative (arrows in Figure 2.2C).

Taken together, these experiments indicate that infection with VRP and VEEV reduced the nuclear accumulation of a transcription factor central to the IFN response. Previous reports indicate that an N-terminal region within the capsid protein of the New World alphaviruses VEEV and Eastern equine encephalitis virus (EEEV) is important for shutoff of host gene expression (4, 6, 91, 93) and that this activity may involve the disruption of nuclear import of host factors (17, 91). It is possible that the viral capsid protein contributes to the disruption of STAT1 nuclear import in cells infected with viruses encoding the full-length VEEV genome (Figure 2.2B), but this mechanism cannot explain the results found in VRP-infected cells that do not express the capsid protein. Finally, diminished STAT1 nuclear accumulation was not associated with a decrease in total STAT1 levels. Thus, if shutoff of macromolecular synthesis is required for the disruption of STAT1 trafficking, it

must affect targets other than STAT1, such as components of the IFN receptor complexes required for STAT1 activation.

Tyrosine phosphorylation of STAT1 in response to IFN- β and IFN- γ is reduced by VRP and VEEV infection.

The nuclear translocation of transcriptionally active STAT1 dimers requires prior activation by phosphorylation at tyrosine 701 (294, 295, 347). Since the reduced nuclear trafficking of STAT1 in VRP and VEEV infected cells could be due to defective STAT1 activation, we next determined whether STAT1 is tyrosine phosphorylated normally in infected cells. Surprisingly, the accumulation of STAT1 phosphorylated at tyrosine 701 (p-STAT1) was appreciably reduced in VRP-infected Vero-81 cells stimulated with various doses of IFN- β and IFN- γ (Figure 2.3A). Very similar results were achieved in two interferon-competent human cell lines, HeLa and HEK-293 (data not shown). This inhibition was not accompanied by any detectable difference in total STAT1 levels (compare lanes 9-12 of Figure 2.3A). At large IFN doses, a doublet band is seen in total STAT1 blots from mock-infected cells (lanes 5, 7, 13, 15 of Figure 2.3A). This doublet corresponds to phosphorylated and non-phosphorylated STAT1 and does not indicate an increase in total STAT1 levels. The equivalent total STAT1 levels between mock and VRP-infected cells again indicated that the VRP-mediated blockade involved neither a failure to express de novo STAT1 nor its specific degradation. Interestingly, phosphorylation of STAT2 at tyrosine 690, which also occurs at cytoplasmic tails of the IFN α/β receptor complex, was unaffected at this time post infection (6 h) (Figure 2.3B), although its activation was reduced at later times (Figure 2.4). While the activation of STAT1 and STAT2 is thought to be crucial during IFN signaling, a third factor, STAT3, can also be activated by the same receptor complexes to initiate separate signaling events (246). As seen with STAT1, VRP-infected cells had less tyrosine-phosphorylated STAT3, but equivalent total STAT3 levels to mock-infected cells (Figure 2.3C).

To determine the kinetics by which VRP inhibits the activation of STATs, and whether infection with virus encoding the full-length VEEV genome also limits STAT activation, we treated VRP or VEEV-infected Vero-81 cells with IFN- β at various times post infection and found that STAT1 activation was reduced as early as 4 h post infection (Figure 2.4). Infection with VEEV also severely reduced STAT1 activation, but at later times relative to VRP infection. These kinetics correlate with greater and more rapid accumulation of viral nonstructural proteins in cells infected with VRP relative to levels seen in VEEV-infected cells (Figure 2.4 and data not shown), although it is clear that large amounts of fully-processed VEEV nsP2 accumulate by 4 h post infection prior to any detectable effect on STAT1 activation in VEEV-infected cells. While the VEEV nsP2-KPNA1 interaction may play a role in the disruption of STAT1 nuclear import (Figure 2.2), these results indicate that defects in upstream signaling events likely contribute to the reduced levels of nuclear STAT1 and the decreased accumulation of STAT1-driven gene transcripts in infected cells.

Viral replication, but not de novo host gene expression, is required for the VRP-mediated blockade of STAT1 activation.

At early times (3 h) post infection, we failed to detect a defect in STAT1 activation within VRP-infected cells (data not shown), but the inhibition was nearly maximal at later times (4 to 5 h post infection) (Figure 2.4). These kinetics suggested that viral replication is required for the blockade to occur, which was confirmed by demonstrating that cells infected with UV-inactivated VRP (Figure 2.5A) were able to activate STAT1 to the same degree as mock-infected cells in response to IFN.

Since STAT1 and STAT2 require the same upstream factors for activation, it is unlikely that nonspecific mechanisms such as generalized host macromolecular synthesis shutoff would prevent activation of STAT1 but not STAT2 at a given time (4 to 6 h post infection). Specific antagonism may involve a direct interaction between a viral protein and a component of the Jak/STAT apparatus, or it may indirectly involve negative regulation of Jak/STAT signaling by a host factor that is induced

or potentiated by VRP infection. Such negative regulators include cellular protein tyrosine phosphatases (PTPs) and suppressors of cytokine signaling (SOCS) proteins. To determine if de novo host transcription is required for VRP to mediate the blockade of STAT1 activation, we treated Vero-81 cells with ActD (1.0 $\mu\text{g/mL}$), which specifically inhibits host, but not viral, transcription. As expected, mock-infected cells were able to respond to IFN in the presence of ActD since de novo gene expression is not required for the activation of latent STAT factors (Figure 2.5B, lane 7). The inhibition of STAT1 activation by VRP was not affected in ActD-treated cells (Figure 2.5B, lanes 6 and 8), indicating that the mechanism does not require de novo expression of a host gene that may be induced upon infection.

To inhibit host and viral translation, we treated Vero-81 cells with CHx (0.5 $\mu\text{g/mL}$) and found that STAT1 was activated similarly in the presence and absence of the inhibitor in mock-infected cells (Figure 2.5C, lanes 5 and 7). Furthermore, when cells were infected with VRP in the presence of CHx, the VRP-mediated inhibition of STAT1 phosphorylation was lost (Figure 2.5C, lanes 7 and 8), again indicating that productive replication and translation of viral proteins is required for the inhibition of STAT1 activation. Moreover, proteins such as viral capsid, which are introduced into the cell in limited quantities by the incoming replicon particles, are not sufficient to mediate the Jak/STAT signaling blockade in the absence of productive replication. Taken together, these data indicate that viral replication results in decreased STAT1 activation, an effect that cannot be achieved in cells treated with inhibitors of host macromolecular synthesis.

VRP infection inhibits the activation of Janus kinases associated with the type II, but not type I IFN receptor.

Stimulation of either the type I or type II IFN receptor results in the phosphorylation of STAT1 at Tyr701. The decrease in STAT1 phosphorylation in VRP-infected cells may reflect a defect at the level of STAT1 itself, such as an interaction with a viral protein that prevents STAT1 from being phosphorylated, or the virus-induced activation of a host cell protein tyrosine phosphatase

that inactivates STAT1. Alternatively, the activation of the type I and type II IFN receptor complexes, which is essential for IFN-induced tyrosine phosphorylation of STAT1, may be disrupted in VRP-infected cells. After type I IFN stimulation, Jak1 and Tyk2 become tyrosine phosphorylated at the IFNAR cytoplasmic tails. Similarly, after type II IFN stimulation, Jak1 and Jak2 become phosphorylated at the IFNGR subunits. Therefore, inhibition of Jak1 could lead to signaling defects in response to both IFN classes and result in decreased STAT1 (and STAT3) phosphorylation.

We assessed the activation of Jak kinases in VRP-infected Vero-81 cells by immunoprecipitation of Jak1, Tyk2 and Jak2 followed by Western blot analysis using phospho-specific antibodies that specifically recognize their activated epitopes. As shown in Figure 2.6A, Jak1 became phosphorylated at tyrosines 1022 and 1023 in response to both IFN treatments. Interestingly, VRP infection had no effect on this activation after treatment with IFN- β (Figure 2.6A; IP lanes 8 and 9), but there was a notable decrease in tyrosine-phosphorylated Jak1 after treatment with IFN- γ (Figure 2.6A; IP lanes 12 and 13). While we have obtained very consistent results indicating that VRP infection disrupts Jak1 activation after treatment with IFN- γ , but not IFN- β , we do not detect any consistent difference in total Jak1 protein levels between mock and VRP-infected cells (Figure 2.6A; WCL lanes 2-12). To further examine whether VRP differentially modulates signaling at the type I versus type II IFN receptors, we assessed the activation of Tyk2 and Jak2 at each receptor complex, respectively. Again, activation of the type I IFN receptor components occurred normally in VRP infected cells as indicated by phospho-Tyk2 levels equivalent to those in mock-infected cells. These events appear to be disrupted at the type II IFN receptor, which showed decreased Jak2 tyrosine phosphorylation (Figure 2.6 B and C), but normal levels of total Jak2.

Surface expression of IFNGR1 subunits is moderately reduced in VRP-infected cells.

Given differential effects of VRP infection on type I and type II IFN receptor complexes (Figure 2.6), we hypothesized that VRP infection reduces the cell surface expression of the IFN- γ receptor (IFNGR). Accordingly, we measured the relative surface expression of the IFNGR1 subunit

between mock and VRP-infected Vero-81 cells by flow cytometry. Surface IFNGR1 expression was slightly, but consistently, lower in VRP-infected cells (82-90% of that in mock-infected cells) in three independent experiments (Figure 2.7A). Despite this minimal decrease in IFNGR1 surface expression, a large decrease in STAT1 phosphorylation was detected in parallel VRP-infected cells stimulated with IFN- γ (Figure 2.7B). Although it was consistent, the minimal decrease in IFNGR1 surface expression is unlikely to account for the dramatic defect of downstream STAT1, Jak1, and Jak2 phosphorylation seen in VRP-infected cells in response to IFN- γ suggesting that additional mechanisms are involved.

2.5 Discussion

Alphaviruses are proposed to inhibit the type I IFN system through global shutoff of host transcription and translation, which would prevent the induction of all type I IFN classes and ISGs. The New World alphaviruses EEEV and VEEV utilize their capsid proteins to shutoff host transcription, which requires an N-terminal region within this protein (4, 6, 17, 91, 93). While the mechanisms by which the viral capsid inhibits transcription are unclear, this activity is associated with defects in type I IFN and ISG induction. In this study, we have found that VEEV also disrupts the cellular response to type I and type II IFN by specific mechanisms that do not require expression of the viral capsid gene and most likely act distinctly from, but in concert with, generalized host shutoff to down-regulate the host antiviral response.

We report that cells infected with VRP fail to activate the transcription factor STAT1 normally, a blockade that correlates with failed STAT1 nuclear localization and decreased STAT1-dependent gene transcription. This blockade did not require viral capsid protein since (i) no structural genes are expressed from the replicon genome, and (ii) components of incoming particles were not sufficient for STAT1 inhibition in either CHx-treated cells or cells infected with UV-inactivated VRP (Figure 2.5). Thus, de novo viral gene expression is required for the inhibition of STAT1 activation,

which likely involves the expression of the viral nonstructural proteins or requires productive viral RNA synthesis. We cannot rule out the possibility that the small amount of capsid introduced into the cell by incoming virions and viral replication are both required for STAT1 inhibition, however, preliminary results suggest that electroporation of VEEV replicon RNA alone is sufficient to disrupt STAT1 nuclear localization (data not shown).

While the shutoff of host macromolecular synthesis plays an important role in alphavirus pathogenesis (4, 84), several lines of evidence suggest that this function is most likely not required for the VRP-mediated Jak/STAT signaling inhibition. First, the decrease in STAT1 phosphorylation was not associated with any effect on total STAT1 levels, which indicates the blockade was not due to decreased synthesis (or increased degradation) of this factor (Figure 2.3A, lane 9-12). Second, although the type I IFN receptor (IFNAR) complex fails to phosphorylate STAT1 after 5 to 6 h of VRP infection (Figures 2.3 and 2.4), the defect is specific for STAT1 since this complex activates Jak1, Tyk2, and STAT2 normally, indicating that the IFNAR complex is fully functional at these times (Figures 2.4 and 2.6). Finally, since it is possible an unidentified host factor with a short half-life is required for STAT1 activation by type I IFN, we pharmacologically induced host shutoff with CHx or ActD but failed to recapitulate the magnitude of STAT1 inhibition achieved by VRP (Figure 2.5). Thus, even if VRP induces host shutoff by these early times post infection, the VRP must employ an additional strategy to block STAT1 phosphorylation since STAT1 activation can occur in the absence of de novo cellular transcription and translation (80, 81, 238).

While our findings indicate that STAT1 activation is inhibited in the absence of de novo host gene expression or viral structural protein synthesis, the particular mechanisms and viral factors required for this inhibition remain to be identified. Accumulation of tyrosine-phosphorylated STATs is determined by rates of phosphorylation and dephosphorylation, and VEEV infection could affect either or both processes. One possibility is that a particular viral nonstructural protein binds STAT1 (and STAT3) or the receptor complexes preventing its phosphorylation by Jak kinases. Alternatively, VRP may activate latent host proteins that regulate these processes, such as host protein tyrosine

phosphatases (PTPs). If this latter explanation is correct and there is rapid STAT dephosphorylation following IFN stimulation, the data presented in Figure 2.5 demonstrate that such a host factor is not transcriptionally induced upon VRP infection, and therefore must be present in a latent form at sufficient levels to counteract the very rapid STAT phosphorylation.

In the case of the type I IFN receptor (IFNAR), we found that the receptor complex remains functional even at times when STAT1 inhibition is nearly maximal (Figure 2.6). One possible explanation is that the VEEV-mediated mechanism(s), such as the direct binding of a viral nonstructural protein, is acting at the level of specific STAT factors preventing their association with the IFNAR and/or their activation by Jak1/Tyk2. The relative resistance of STAT2 to the VEEV-mediated blockade, in this case, could be explained by a reduced affinity of this nonstructural protein for STAT2. However, it is clear that STAT2 is affected at later times (after 6 h of infection; Figure 2.4). STAT2 associates with the IFNAR2 cytoplasmic tail prior to its engagement with the phosphorylated IFNAR1 subunit upon receptor ligation (182, 232). The orientation and duration of this pre-association may allow STAT2 to become phosphorylated more efficiently than STAT1, and thus be less sensitive to the VEEV-mediated mechanism. However, a potential role for STAT2-IFNAR2 pre-association in this phenotype remains to be determined.

In contrast to the events at the IFNAR complex, we detected defects in the activation of IFNGR components in VRP-infected cells, namely reduced Jak1 and Jak2 activation (Figure 2.6). While IFNGR surface expression was modestly reduced (Figure 2.7), it is unlikely that reduced receptor surface expression alone fully explains the large decrease in Jak1, Jak2, and STAT1 activation. It is possible that VEE prevents the activation of STAT1 by both receptor complexes through common mechanisms, but in the case of the IFNGR, additional mechanisms contribute to the defect in Jak1 and Jak2 activation. Taken together, our data suggest that signaling steps downstream of receptor surface expression, such as the kinase activities of Jak proteins and the phosphorylation of the critical tyrosine residues present on the receptor cytoplasmic tails, are disrupted by VEEV infection, and further studies are underway to define the mechanism(s) behind this process.

Additionally, it will be important to further assess whether VEEV infection interferes with the STAT1 signaling cascade at steps downstream of STAT1 tyrosine phosphorylation, since the previously demonstrated nsP2-KPNA1 interaction may further contribute to the inhibition of STAT1-dependent signaling.

These studies indicate that, in addition to its global interference with host macromolecular synthesis, VEEV is able to antagonize the type I and type II IFN response by specific mechanisms. It remains to be determined whether the inhibition of Jak/STAT signaling is specific for VEEV, or whether other alphaviruses employ similar strategies. Recent work by Griffin et al. indicates that the non-cytolytic clearance of Sindbis virus from infected neuronal cells requires functional IFN- γ and Jak1 signaling (36), which would suggest that this activity is not shared by all alphaviruses or that it may be cell type-dependent. It is important to note that VEEV is sensitive to the effects of type I IFN. For example, IFNAR deficient mice succumb to infection with VEEV much earlier than wild-type mice. The disruption of IFN signaling identified in these studies, therefore, may simply dampen the magnitude of the IFN response, or may be relevant to particular cell types important for initiating the innate immune response in the animal. Identification of the viral determinants that mediate the inhibition of Jak/STAT signaling in VEEV will provide a more complete understanding of the determinants for IFN sensitivity, viral virulence and pathogenesis, and may ultimately enable better design of alphavirus-based vaccines.

2.6 Acknowledgements

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Figure 2.1: VRP infection decreases IFN-stimulated gene expression independently of the viral structural proteins.

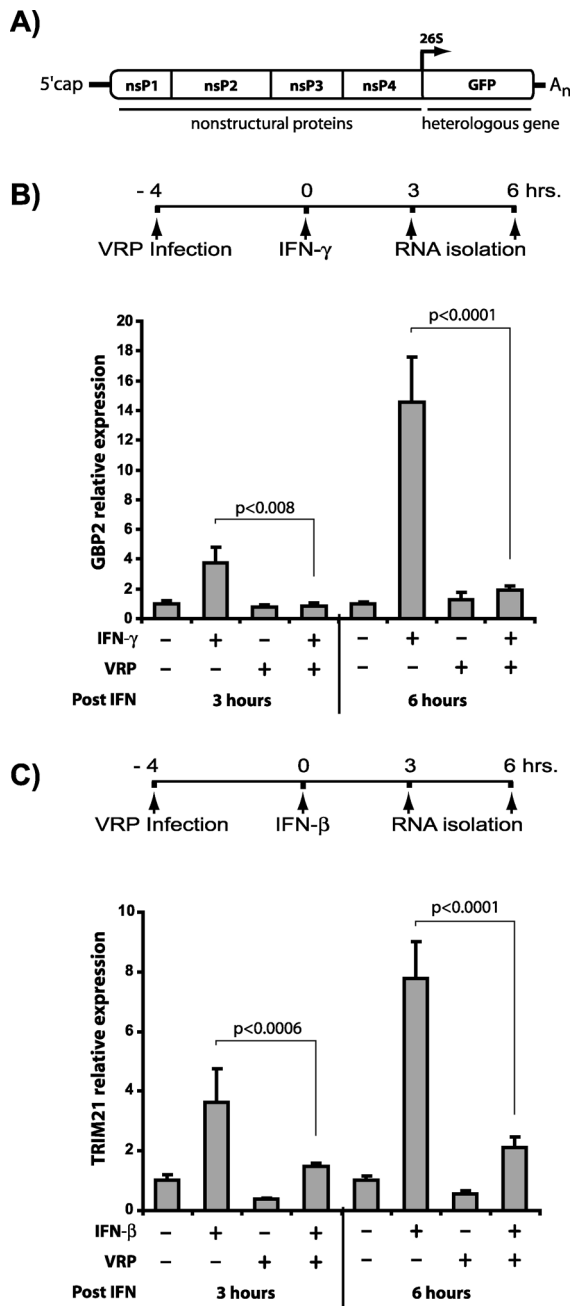


Figure 2.1: VRP infection decreases IFN-stimulated gene expression independently of the viral structural proteins.

Vero-81 cells were infected with VRP, which express GFP in place of viral structural proteins from the subgenomic 26S promoter (genome organization shown in panel A), at an MOI of 10 IU/cell for 4 h and then stimulated with 1,000 U of IFN- γ (B) or IFN- β (C)/mL. Total RNA was isolated after 3 or 6 h of IFN treatment, and relative guanylate binding protein 2 (GBP2) (B) or TRIM-21 (C) mRNA expression was determined by real-time PCR analysis of cDNA. Individual samples were normalized to GAPDH. The average of each group was calculated and is represented relative to mock-infected, untreated samples. Error bars indicate one standard deviation. To determine significance, an analysis of variance was performed. Post hoc comparisons of relevant groups at each time point are indicated by brackets along with associated *P* values.

Figure 2.2: VRP and VEEV infection disrupts the nuclear localization of STAT1 in response to IFN.

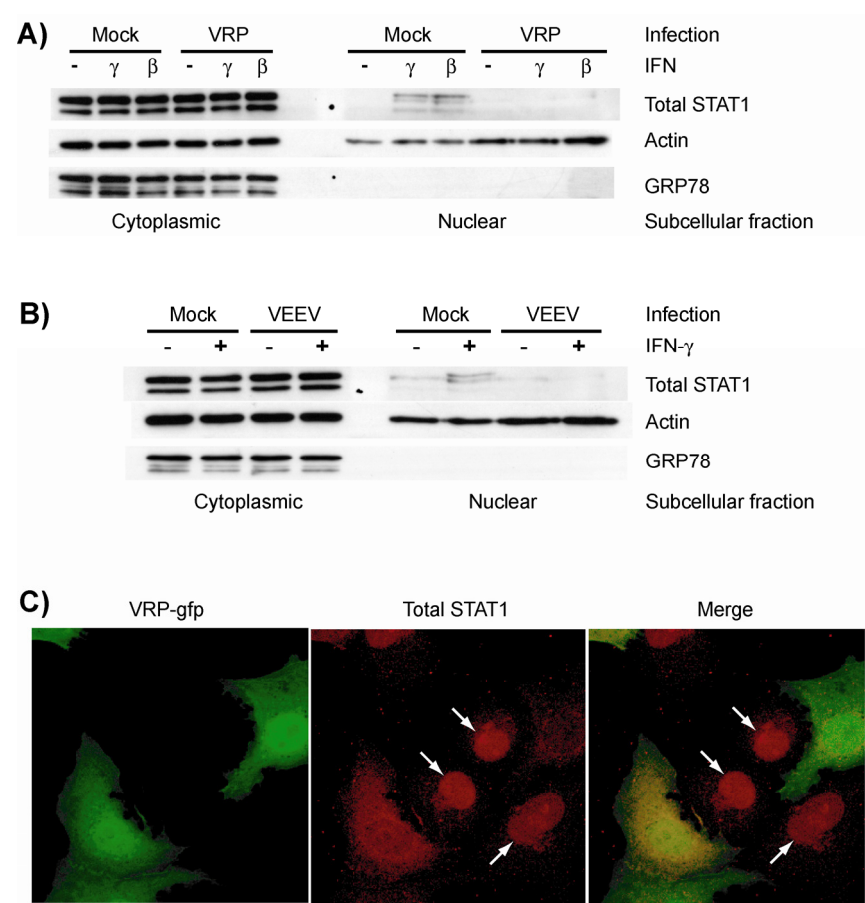


Figure 2.2: VRP and VEEV infection disrupts the nuclear localization of STAT1 in response to IFN.

Vero-81 cells were infected for 6 h with VRP at an MOI of 5 IU/cell (A and C) or for 5 h with VEEV 3014 at 10 PFU/cell (B) and then stimulated with IFN- γ or IFN- β for 20 to 40 min (200 to 1,000 U/mL). Subcellular extracts were prepared and analyzed by Western blotting to determine total STAT1 distribution (A and B). GRP 78, a protein found within the endoplasmic reticulum, verifies the purity of the nuclear fractions. In an indirect immunofluorescence staining assay (C), cells were fixed after IFN treatment and stained for total STAT1 protein. Infected cells expressing GFP and STAT1 subcellular distribution (red) were detected by confocal microscopy and demonstrate the nuclear redistribution of STAT1 in uninfected cells, but not VRP-infected cells.

Figure 2.3: Tyrosine phosphorylation of STAT1 and STAT3 in reponse to IFN-β and IFN-γ is reduced by VRP infection.

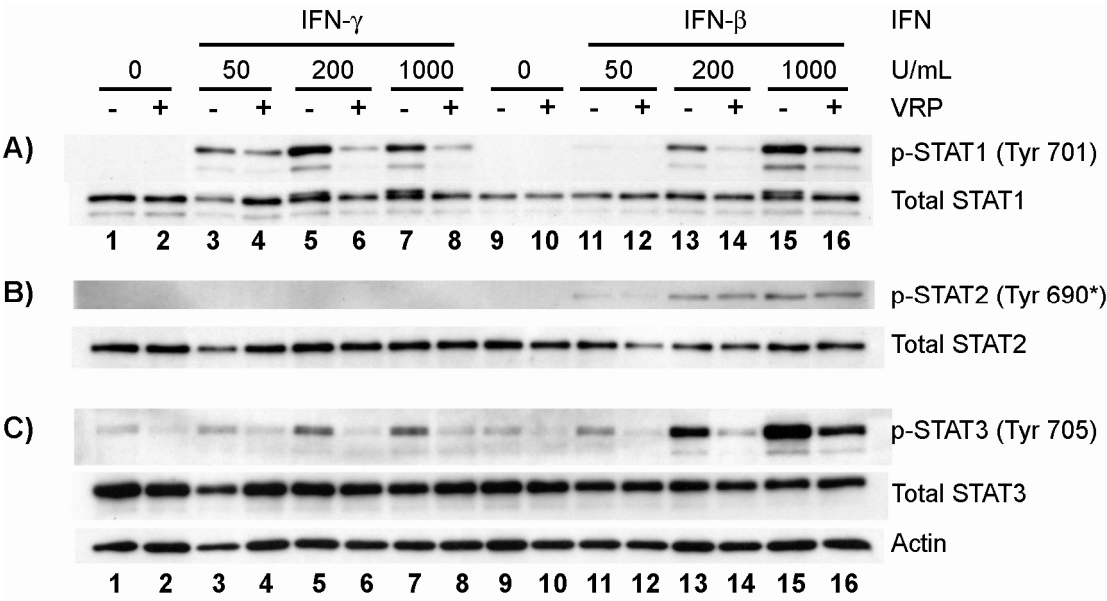


Figure 2.3: Tyrosine phosphorylation of STAT1 and STAT3 in response to IFN- β and IFN- γ is reduced by VRP infection.

Vero-81 cells were infected with VRP (MOI = 5 IU/cell) for 6 h and treated for 20 min with various concentrations of IFN- γ and IFN- β . Whole-cell extracts were harvested and 20 μ g of total protein from each lysate was resolved by SDS-polyacrylamide gel electrophoresis and analyzed in three separate blots to assess activation of STAT1 (A), STAT2 (B), and STAT3 (C) at the indicated phosphotyrosine residues. Blots were then stripped and reprobed using the respective total STAT antibody. All actin loading control blots were similar to that shown in panel C. *, The anti-mouse pTyr689-STAT2 antibody used (Upstate) cross-reacts with the corresponding human pTyr690-STAT2.

Figure 2.4: **Activation of STAT1, but not STAT2, is limited by VEEV and VRP at early times post infection.**

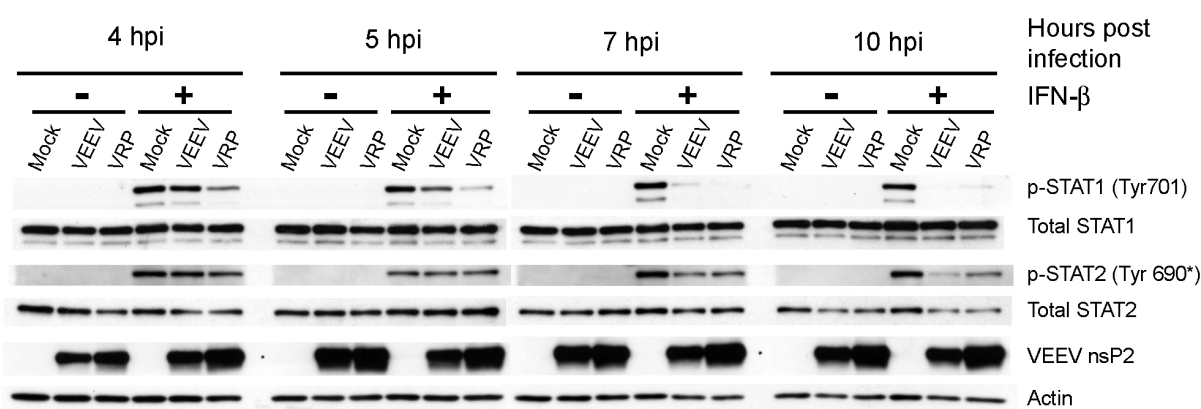


Figure 2.4: Activation of STAT1, but not STAT2, is limited by VEEV and VRP at early times post infection.

Vero-81 cells were infected for the indicated times with either VRP (MOI = 5 IU/cell) or VEEV 3014 (MOI = 20 PFU/cell) and then stimulated with IFN- β (500 U/mL) for 20 min. Samples were analyzed as described in Fig. 3. To compare the kinetics and amount of nonstructural protein accumulation, a separate Western immunoblot indicates fully-processed VEEV nonstructural protein 2 (VEEV nsP2).

Figure 2.5: Viral replication, but not de novo host gene expression, is required for the VRP-mediated STAT1 blockade.

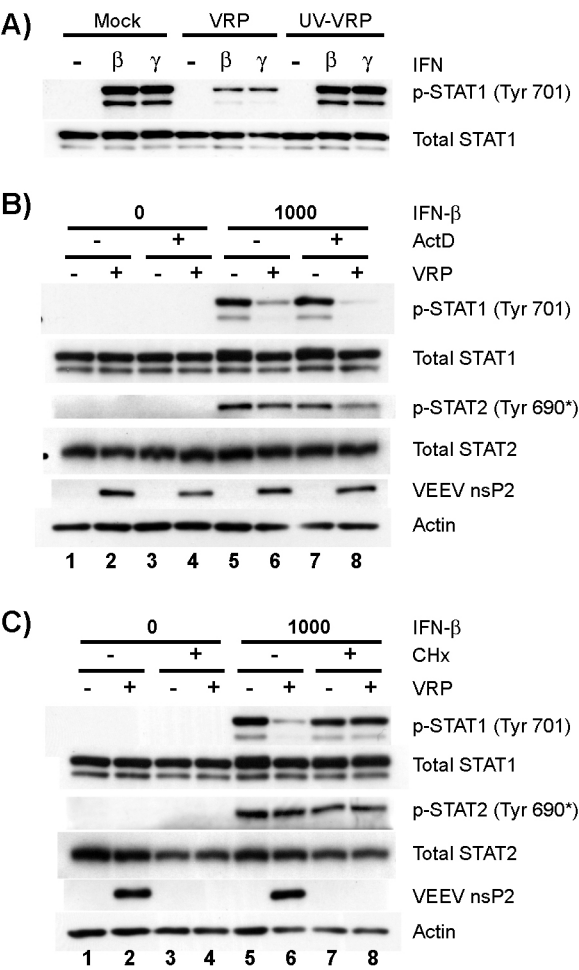


Figure 2.5: Viral replication, but not de novo host gene expression, is required for the VRP-mediated STAT1 blockade.

(A) Vero-81 cells were either mock infected, or infected with untreated VRP or VRP that had been exposed to ultraviolet light for 1 minute (UV-VRP). At 6 h post infection, cells were stimulated with 1,000 U of IFN/mL and analyzed as in Fig. 3. (B and C) Vero-81 cells were pre-treated for 1 h in the absence or presence of the inhibitors ActD or CHx prior to infection with VRP (MOI = 10 IU/cell) or diluent (mock). Virus was allowed to bind to cells in the indicated treatment groups in the presence of each inhibitor for 1 h, after which the inoculum was replaced with media containing the inhibitor. At 6 h post infection (7 h of inhibitor treatment), cells were stimulated with IFN- β (1,000 U/mL) for 20 min, and STAT phosphorylation was assessed as described in Fig. 3.

Figure 2.6: VRP infection inhibits the activation of Janus kinases associated with the type II, but not the type I, IFN receptor.

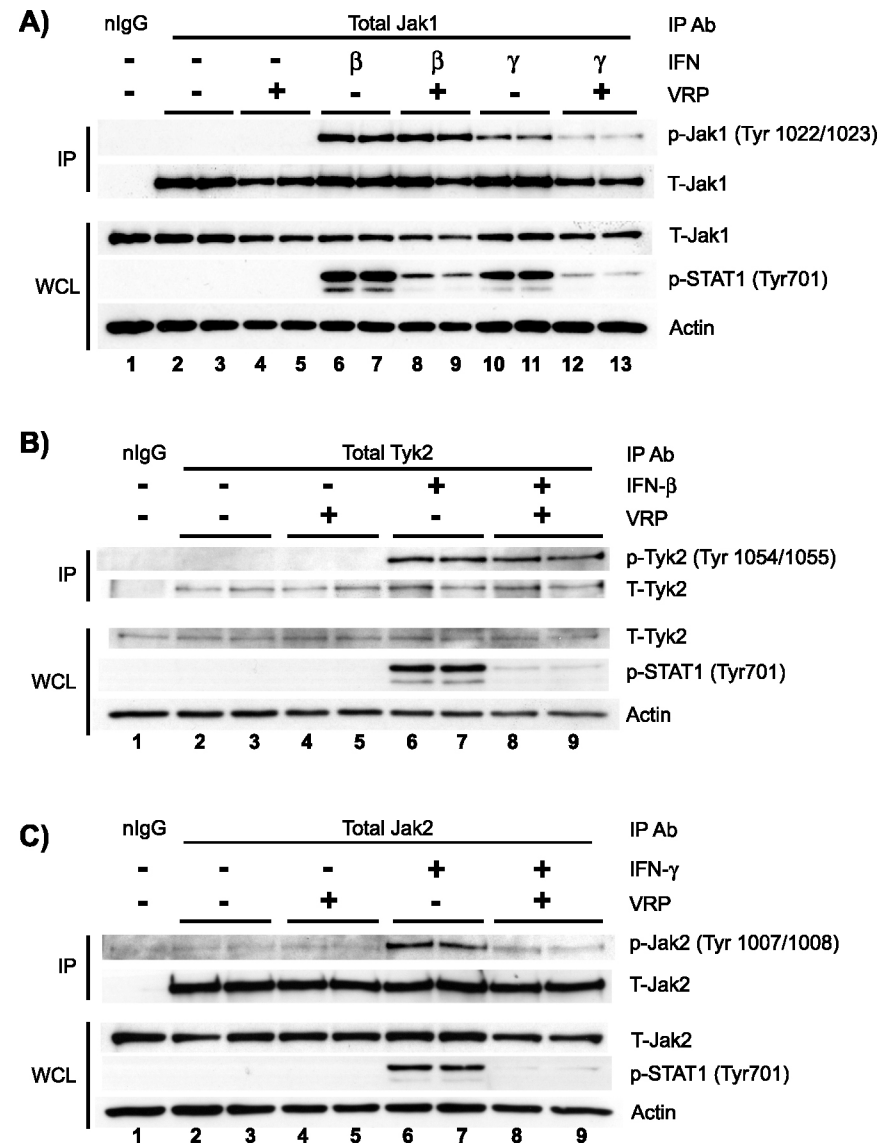


Figure 2.6: VRP infection inhibits the activation of Janus kinases associated with the type II, but not the type I, IFN receptor.

Vero-81 cells were mock infected or infected with VRP for 6 h and then stimulated for 20 min with IFN. Each infection and treatment was performed in duplicate. Whole-cell lysates (WCL) were prepared, and equivalent amounts of total protein were subjected to immunoprecipitation with antibodies recognizing total Jak1 (A), Tyk2 (B), and Jak2 (C), or with isotype-matched normal IgG (nIgG). To assess Jak protein activation, the immunoprecipitates were then analyzed by Western blotting using the indicated phospho-specific Jak antibodies (IP panels). These membranes were then stripped and reprobed with the corresponding antibody recognizing total Jak protein. A portion of the input whole-cell lysates (50 µg total protein) from each sample was also analyzed by Western blotting to detect effects that VRP has on total cellular Jak levels, as well as on STAT1 phosphorylation (WCL panels).

Figure 2.7: Surface expression of IFNGR1 subunits is moderately reduced in VRP-infected cells.

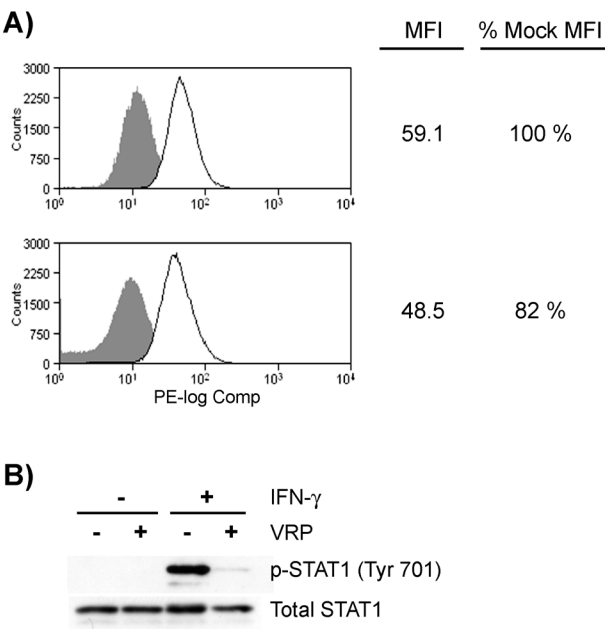


Figure 2.7: Surface expression of IFNGR1 subunits is moderately reduced in VRP-infected cells.

Vero-81 cells were infected with mock or VRP (MOI = 5 IU/cell) for 6 h, which resulted in the infection of 98.8% of cells as determined by an indirect immunofluorescence staining assay that detects the viral nonstructural proteins (data not shown). (A) Mock- or VRP-infected cells were pooled, counted, and then separated into replicate groups for staining. Cells were either stained with anti-CD119 (IFNGR1) directly conjugated to phycoerythrin (solid line) or left unstained (shaded histogram). Cells in each group were then washed, fixed, and analyzed by flow cytometry. Mean fluorescence intensities (MFI) of mock- and VRP-infected samples were compared, and the decrease in IFNGR1 surface expression was comparable between three independent experiments (10 to 18% decrease). (B) Mock- and VRP-infected cells in parallel cultures were stimulated with 1,000 U of IFN- γ /mL for 20 min, and harvested and analyzed as described in Fig. 3 to measure the inhibition of STAT1 phosphorylation.

CHAPTER THREE:

A DETERMINANT OF SINDBIS VIRUS NEUROVIRULENCE ENABLES EFFICIENT DISRUPTION OF JAK/STAT SIGNALING

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3.1 Abstract

Previous studies with Venezuelan equine encephalitis virus and Sindbis virus (SINV) indicate that alphaviruses are capable of suppressing the cellular response to type I and type II interferons (IFNs) by disrupting Jak/STAT signaling; however, the relevance of this signaling inhibition toward pathogenesis has not been investigated. The relative abilities of neurovirulent and non-neurovirulent SINV strains to downregulate Jak/STAT signaling were compared to determine whether the ability to inhibit IFN signaling correlated with virulence potential. The adult mouse neurovirulent strain, AR86, was found to rapidly and robustly inhibit tyrosine phosphorylation of STAT1 and STAT2 in response to IFN- γ and/or IFN- β . In contrast, the closely related SINV strains Girdwood and TR339, which do not cause detectable disease in adult mice, were relatively inefficient inhibitors of STAT1/2 activation. Decreased STAT activation in AR86-infected cells was associated with decreased activation of the IFN receptor-associated tyrosine kinases Tyk2, Jak1 and Jak2. To identify the viral factor(s) involved, we infected cells with several panels of AR86/Girdwood chimeric viruses. Surprisingly, we found that a single amino acid determinant, threonine at nsP1 538, which is required for AR86 virulence, was also required for efficient disruption of STAT1 activation, and that this determinant fully restored STAT1 inhibition when introduced into the avirulent Girdwood background. These data indicate that a key virulence determinant plays a critical role in downregulating the response to type I and type II IFNs, which suggests that the ability of alphaviruses to inhibit Jak/STAT signaling relates to their in vivo virulence potential.

3.2 Introduction

Members of the genus *Alphavirus* in the family *Togaviridae* include a variety of human pathogens with a nearly global distribution. These viruses are transmitted through mosquito vectors,

and are considered a threat due to their potential to cause large-scale epidemics. The Old World alphaviruses, such as chikungunya virus (CHIKV) and Ross River virus (RRV), have been linked to explosive epidemics of infectious arthritis, while the New World alphaviruses Venezuelan equine encephalitis virus (VEEV) and Eastern equine encephalitis virus (EEEV) have caused sporadic outbreaks of potentially fatal encephalitis. Sindbis virus (SINV), the prototype alphavirus, is an Old World alphavirus responsible for cases of self-limited arthralgia in humans. Infection of mice with SINV has also provided an excellent model of alphavirus-induced encephalomyelitis (68, 135, 148, 198).

Upon alphavirus infection, viral RNA is recognized by host pattern recognition receptors including the cytoplasmic RNA sensors PKR, MDA-5 and/or RIG-I, which can activate IRF-3/7-dependent signaling pathways to induce IFN- β and IFN- α 4 production (100, 131, 245, 272). Infection of mice with SINV results in detectable type I IFN (IFN- α/β) levels in the serum by 12 hours post infection (148). IFN- γ (type II IFN), which is secreted by specific immune effector cells, is detectable in the serum at slightly later times (24 hours) post SINV infection (148). The cellular response to secreted IFN- α/β and IFN- γ involves separate, but overlapping, signaling cascades that result in transcription of IFN-stimulated genes (ISGs), several of which have known antiviral functions. These signaling pathways have been well studied (for detailed reviews, see (246, 286)). In brief, IFN- α/β and IFN- γ bind distinct, ubiquitously expressed cell surface receptors, the IFN- α/β receptor (IFNAR) and the IFN- γ receptor (IFNGR), respectively. Ligation of the IFNAR results in the dimerization of the receptor subunits, IFNAR1 and IFNAR2, which allows apposition and auto-phosphorylation of Jak1/Tyk2 kinases that constitutively associate with each subunit. Once activated, these Jaks phosphorylate the receptor subunits allowing the recruitment and phosphorylation of STAT1 and STAT2, which dimerize and associate with IRF-9 to form the ISGF3 complex that binds IFN-stimulated response elements (ISREs) to drive transcription. In contrast, the activated type II IFN receptor complex is composed of IFNGR1/2 subunits and Jak1/Jak2 kinases that activate STAT1, which predominately forms homodimers to drive expression of IFN- γ stimulated genes

containing IFN- γ -activated sequence (GAS) elements in their promoters. The presence of both ISRE and GAS elements within a single ISG promoter partially explains overlap between the IFN- γ and IFN α/β response. Thus, STAT1 is a central component of the type I and type II IFN response.

The type I interferon (IFN) system is known to play a critical role in the control of most virus families including alphaviruses (for an excellent review, see (268)). Compared to immunocompetent adult mice that control most SINV strains, mice with targeted deletions of the IFNAR succumb rapidly to infection (269). The average survival time of SINV-infected mice is reduced further when they are doubly deficient in both the IFNAR and the IFNGR (270) suggesting that both type I and type II IFNs are important in limiting SINV replication and pathogenesis in adult mice. The IFN- γ response also has direct antiviral activity, but it is thought to play an important role at later times post SINV infection when CD8⁺ and CD4⁺ T cell-derived IFN- γ acts together with antiviral antibody to mediate non-cytolytic clearance of SINV from CNS neurons (for a detailed review, see (114)). As STAT1 is central to both type I and type II IFN responses, non-fatal SINV infection results in 100% lethality in STAT1^{-/-} mice (40, 270), although some STAT1-independent, IFN- α/β and IFN- γ responses with anti-SINV activities have been described (98).

Like most viruses, alphaviruses employ strategies to interfere with the host type I IFN response. Most work on these viruses has focused on virus-mediated nonspecific shutoff of cellular transcription and translation (4, 6, 84, 91-93). Recent studies with both SINV and VEEV suggest that both Old World and New World alphaviruses are also able to disrupt signaling events required for the cellular response to IFN- α/β and IFN- γ , namely, by inhibiting the accumulation of tyrosine-phosphorylated STAT1 and STAT2 (298, 354). This activity appeared to be mediated by the viral nonstructural proteins and was independent of shutoff of host macromolecular synthesis (298). However, the importance and relevance of the upstream Jak/STAT signaling inhibition in the face of potent and generalized shutoff of ISG transcription has not been demonstrated. In fact, the transcriptional induction of several ISGs did not correlate with the degree to which STAT1/2 activation was inhibited in mouse neuron cultures infected with VEEV replicon particles (354).

To further define the potential role that viral inhibition of Jak/STAT signaling plays in alphavirus pathogenesis, we evaluated whether viruses with differing virulence profiles exhibited the ability to inhibit Jak/STAT signaling. We focused our analysis on two SINV isolates, S.A.AR86 and a closely related strain, GirdwoodS.A. In adult mice, virus derived from the infectious clone of AR86 (S300) causes lethal disease following intracranial inoculation (127, 128, 299, 307) while the closely-related Girdwood virus (clone G100) is avirulent, even though both S300 and G100 replicate to similar levels within the CNS of mice at early times post infection (307). The difference in virulence is mediated by four genetic determinants within AR86, which when introduced into the avirulent G100 background results in a gain of full virulence. Herein, we report that the adult mouse virulence potential of SINV strains S300, G100 and TR339 directly correlates with the relative ability of these viruses to disrupt the activation of Jak proteins and/or STAT1/2 in response to both IFN- β and IFN- γ . Strikingly, we found that a single virulence determinant unique to AR86, a threonine encoded at nsP1 538, is both necessary and sufficient for rapid and efficient inhibition of STAT1 activation. The previously demonstrated importance of this determinant for adult mouse neurovirulence and for avoiding clearance from the CNS (127, 307) suggests that inhibition of Jak/STAT signaling contributes to alphavirus pathogenesis, perhaps through downregulation of the response to type I IFNs and/or through suppression of noncytolytic SINV clearance from CNS neurons, which involves IFN- γ signaling (30, 36-38).

3.3 Materials and Methods

Cell culture and reagents. Vero-81 cells (designated “Vero”, ATCC No. CCL-81) and BHK-21 cells were grown at 37°C under 5% CO₂. Vero cells were maintained in DMEM/F12 media (Gibco) supplemented with 10% fetal bovine serum (Hyclone), L-glutamine (0.29 mg/mL; Gibco), non-essential amino acids (1X; Gibco), penicillin/streptomycin (1X; Gibco), and sodium bicarbonate (final of 1.2 g/L; Gibco). BHK-21 were maintained in α -MEM (Gibco) containing 10% donor calf

serum (DCS; Hyclone), 10% tryptose phosphate broth (Sigma), and supplemented with L-glutamine and penicillin/streptomycin as above. For virus and replicon production in BHK-21 cells, fetal bovine serum (10%; Lonza) was used in place of DCS. Recombinant human IFN- β (Calbiochem) was resuspended in sterile DMEM containing 10% fetal bovine serum and aliquots were stored at -80°C. Biologic activity of each IFN- β preparation was determined by a type I IFN bioassay on A549 cells where protection from EMCV-induced cytopathic effect was scored relative to the NIH human IFN- β standard (Gb23-902-531), as previously described (288). Recombinant human IFN- γ was used at the activity reported by the manufacturer (R&D Systems).

Virus and replicon production. The infectious clone-derived viruses used are listed in Table 3.1, which annotates the mutations each clone encodes. Virus is generated from plasmid templates named with a “p” prefix (i.e. S300 virus is generated from plasmid pS300). S300, G100, S350, G106, S363, S340, S343 and S344 were all generated as previously described (307). Both pS300 and pS55, which differ only in the linearization site used, each encode wild-type AR86; thus, S300 and the previously designated S55 (127, 128) are synonymous. Similarly, S51 is synonymous with S340 (127, 307). Primer-directed mutagenesis of pG100 was used to construct clones pG119, pG121, and pG120, as described previously (307). pTR339, which encodes the consensus A.R.339 sequence, was generated previously by replacing cell-culture adaptive mutations in the E2 gene and is representative of the original virulent A.R.339 natural isolate (149, 215).

Infectious SINV clones contain the viral cDNA inserted between an SP6 promoter and a unique restriction site, either PmeI or XbaI, used for linearization. In brief, linearized cDNA templates were used for SP6 in vitro transcription reactions (Ambion). Capped, poly-adenylated transcripts were then electroporated into BHK-21 cells, and after 24 h, supernatants were harvested, clarified at 3,000 RPM for 15 min, and then virus was concentrated through 20% sucrose at 24,000 RPM for greater than 4 h at 4°C. Virus pellets were resuspended in virus diluent (Dulbecco’s Phosphate Buffered Saline [1X DPBS; Gibco] supplemented with 1% DCS, 0.122 mg/mL CaCl₂, and

0.10 mg/mL MgCl), aliquoted, and stored at -80°C. All concentrated full-length virus stocks were titrated by plaque assay on BHK-21 cells.

For replicon particle packaging, a tripartite helper system has been described previously (129). The replicon RNA genomes used in this study each encode green fluorescent protein (GFP) in place of the viral structural genes and are thus capable of GFP expression from the subgenomic 26S RNA promoter, but are propagation defective. Construction of the AR86-based pREP89 (126) and the Girdwood-based pRgird (129) replicon clones were previously described, and the GFP gene was inserted 3' of the 26S promoter 5'-UTR using the ClaI site as described previously for insertion of other foreign genes (129). Helper transcripts for the capsid helper construct, pCAP86 (126), and glycoprotein helper, pGIRDGLY (129), were generated as previously described. Replicon particles were packaged by co-electroporating BHK-21 cells with transcripts of replicon genomes and helper RNAs, and particles were harvested and concentrated as above. The resulting replicon particles designated S300 RP (REP89-gfp, AR86-based) and G100 RP (Rgird-gfp, Girdwood-based) contained equivalent coats and were titrated on Vero cells by counting GFP-positive cells. The packaging of VEE-based replicon particles (VRP), which encode the wild-type (V3000) nonstructural genes but contain adaptive mutations in the E2 glycoprotein (pV3014 coat) to maximize tissue culture infectivity, was described previously (298).

Metabolic labeling. To assess relative amounts of de novo protein synthesis, Vero cells were infected with S300 or G100 at a multiplicity of 20 PFU per cell, or with diluent alone (mock) for the indicated times. Prior to each indicated harvest time, cells were starved for 2 h in DMEM deficient in cysteine/methionine (Gibco) and then labeled for 1 h with media containing ³⁵S-labeled cysteine/methionine (33 µCi; Amersham Pro-mix). Cells were then rinsed in ice-cold 1X PBS and then lysed in NP-40 lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% Igepal CA-630 [Sigma], Complete mini protease inhibitors [Roche]). Lysates were clarified by centrifugation and equal volumes were resolved by 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel

electrophoresis. The gel was fixed in buffer containing 10% acetic acid and 40% methanol, dried, and exposed to a phosphorimager screen, which was scanned using a Personal Molecular Imager FX (Bio-Rad). To quantify relative de novo host protein synthesis, a host protein that resolved at the expected molecular weight of actin was quantified using Quantity One software (Bio-Rad), and each value was normalized to the average signal of this band from three mock-infected samples.

Virus infections and IFN treatment. For all experiments, Vero cells were infected for 1 h at 37°C with inocula containing the indicated viruses or replicon particles prepared in virus diluent. After virus binding, warm media was added to cells without removing the inocula. At the indicated number of hours post infection (hpi), cell supernatants were replaced with media containing IFN- β or IFN- γ prepared immediately prior to stimulation. Cells were treated for 20 min with IFN, washed once or twice with ice-cold 1X PBS, and extracts were prepared as described below.

Immunoblot analysis. For direct immunoblot, Vero cells were lysed in ice-cold radioimmunoprecipitation assay buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA, 1% Igepal CA-630, 0.1% sodium dodecyl sulfate [SDS], and 0.5% deoxycholate, Complete mini protease inhibitor cocktail tablets, phosphatase inhibitor cocktail [Sigma-P2850]) on ice for greater than 5 min, after which cells were scraped, lysates were clarified for 10 min at 4°C and total protein content was quantified using a Coomassie Plus protein assay (Thermo). Equal total protein from each sample was denatured for 5 min at 95°C in SDS sample buffer, resolved by SDS-polyacrylamide gel electrophoresis (8%), and transferred to polyvinylidene difluoride membrane (Bio-Rad). Membranes were blocked 1 h in 5% nonfat dry milk in PBS-T (137 mM NaCl, 2.7 mM KCl, 0.88 mM KH₂PO₄, pH 7.5, 0.1% Tween 20), exposed to primary antibody overnight at 4°C, washed five times in PBS-T and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody 1 h at room temperature, and finally developed with ECL-Plus (Amersham) and exposed to film. Dilutions of the following primary antibodies were prepared as recommended by the manufacturer: STAT1 (total),

phospho-STAT1 (Tyr701), Tyk2 (total), phospho-Tyk2 (Tyr1054/1055), Jak2 (Total), and phospho-Jak2 (Tyr1007/1008) were all purchased from Cell Signaling Technology; STAT2 (total) and phospho-STAT2 (Tyr689 of mouse STAT2) were purchased from Upstate; Jak1 (total) was from BD transduction laboratories and phospho-Jak1 (Tyr1022/1023) from Biosource; Actin was purchased from Santa Cruz. Goat anti-VEEV nsP2 was kindly provided by Alphavax, Inc., and mono-specific anti-SINV nsP2 rabbit polyclonal sera was a generous gift from Charles Rice. HRP-conjugated IgG secondary antibodies were purchased from Amersham (anti-rabbit and anti-mouse) and Sigma (anti-goat). For detection of immunoprecipitated protein, light-chain-specific HRP-conjugated IgG (anti-rabbit and anti-mouse) secondary antibodies were purchased from Jackson Immuno Research Laboratories, Inc.

Where indicated, membranes were stripped for 30 min at 50°C in stripping buffer (62.5 mM Tris-HCl pH 6.7, 2% SDS, 100 mM β -mercaptoethanol), washed at least 4 times with PBS-T, and then blocked and re-probed as described above.

Immunoprecipitations. To analyze activation of Jaks through tyrosine phosphorylation, Vero cells were seeded in 6-well plates, infected and treated with IFN- β as described above, and incubated on ice with 200 μ L of phosphorylation lysis buffer (247)(0.5% Triton X-100, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 50 mM HEPES [pH 7.4], 200 μ M sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM NaF, Complete protease inhibitor tablets [Roche]) per well for at least 10 min. Wells were scraped at 4°C and lysates were harvested, pooled (2 wells per group), clarified, and total protein content quantified as described above. Equivalent amounts of lysate (0.5-0.8 mg total protein) were pre-cleared with protein-G agarose (Sigma) plus pre-immune normal sera for 2 h at 4°C and then exposed to the indicated total Jak antibody overnight at 4°C. Pulldowns were performed at 4°C for two h with protein-G agarose, and the immunoprecipitates (IPs) were washed five times with phosphorylation lysis buffer prior to dissociation with 2X SDS sample buffer. IPs were subjected to immunoblot as described above with the indicated antibodies, with the exception that Tyk2 (total; BD

Transduction laboratories) was used for immunoprecipitation and Tyk2 (total; Cell Signaling Technology) was used for immunoblot. As a negative control, lysates from a mock-infected and IFN-treated group was subjected to immunoprecipitation with isotype-matched controls: normal mouse IgG2a, IgG2b (eBiosciences), or normal rabbit IgG (Sigma). A portion of the input lysates (5-7% of that used for immunoprecipitation) was subjected to direct immunoblot analysis to assess levels of total Jak, phospho-STAT1, and SINV nsP2, as described above.

Indirect immunofluorescence assay. To assess the percentage of infected cells in all STAT1 activation assays, parallel cultures in each experiment were stained at 10 h post infection in an indirect immunofluorescence assay. Infected cells were washed twice with ice-cold 1X PBS, fixed in ice-cold methanol for 10 min at -20°C, air dried and stored at 4°C until staining. Cells were incubated for 15 min in PBS-glycine (100 mM [pH7.2]) and then blocked 1 h at room temperature in blocking buffer (10% normal goat serum [Sigma], 3% bovine serum albumin, 0.05% Tween-20 prepared in 1X PBS). Cells were then stained with SINV hyperimmune sera for 1 h at room temperature, washed three times in IFA wash buffer (3% BSA, 0.05% Tween-20, prepared in 1X PBS), counterstained with Alexafluor-488 goat-anti-mouse IgG (Invitrogen) and DAPI (4',6'-diamidino-2-phenylindole [Roche]; 10ug/mL). The percentage of infected cells was calculated by acquiring fluorescent images and counting the number of positively-stained cells (green) relative to the total number of DAPI-positive nuclei. Since threonine at nsP1 538 is associated with delayed expression of 26S RNA (127), cells infected with viruses encoding this determinant (e.g. S300) gave lower fluorescent signal and their calculated infectivities are most likely underestimated.

3.4 Results

Differential regulation of STAT1 phosphorylation by strains of SINV.

The alphaviruses VEEV and SINV have previously been reported to antagonize the IFN- α/β and the IFN- γ response by limiting the accumulation of tyrosine phosphorylated STAT1/2 after stimulation with these cytokines (298, 354). To determine whether SINV strains that have different mouse neurovirulence profiles differ in their ability to disrupt type I and type II IFN-mediated STAT activation, we assessed STAT phosphorylation in cells infected with SINV strain AR86 (infectious clone S300), which is nearly 100% lethal in adult mice (127, 299), and two SINV strains that do not cause disease in adult mice—TR339 and Girdwood (clone G100). Since various alphaviruses induce quantitatively different amounts of type I IFN both in vivo and in vitro (43, 89, 148, 269), it was important to normalize the amount of IFN present in each culture. For this reason, Vero cells were infected since these cells are highly permissive for alphavirus infection, are unable to synthesize endogenous type I IFN due to a defective genetic locus (66, 225), and are therefore widely used to compare virus-mediated IFN antagonism specific to the IFN signaling pathway (26, 138, 256, 298, 354). After 6, 8 or 10 hours of infection with each virus, Vero cells were stimulated with recombinant IFN- β or IFN- γ to assess STAT1 activation as indicated by phosphorylation at tyrosine 701, which is required for STAT1 to form transcriptionally active dimers (180)(Figure 3.1). Strikingly, infection with the neurovirulent S300 resulted in a more rapid and more complete disruption of STAT1/2 activation in response to IFN- β , and of STAT1 activation in response to IFN- γ , when compared to infection with non-neurovirulent G100 or TR339 viruses (Figure 3.1). Total STAT levels were not affected by infection with any virus at any timepoint indicating the loss in STAT1 phosphorylation did not result from a decrease in STAT1 expression nor specific STAT1 degradation. Moreover, differential STAT1 phosphorylation between SINV-infected cultures cannot be explained by differences in infectivity since G100 and TR339 each infected greater than 95% of cells as indicated by indirect immunofluorescence stain of parallel cultures at 10 hpi using anti-SINV

hyperimmune sera (data not shown). These data corroborate those previously reported by Yin et al. (354) and indicate that SINV TR339 and G100 partially inhibit the activation of STAT1/2 in response to IFN- α/β . Thus, it appears that several SINV strains are capable of inhibiting Jak/STAT signaling, but importantly, a strain with enhanced virulence exhibits more potent activity.

S300 and G100 initiate shutoff of de novo protein synthesis with similar kinetics.

Activation of STAT1 by IFN occurs in the absence of de novo gene expression (80, 81, 238), and we previously demonstrated that the mechanism by which VRP disables Jak/STAT activation does not involve nonspecific shutoff of host transcription/translation, which occurs within several hours after VRP infection (298, 354). However, since maximal STAT1/2 inhibition occurs slightly later during S300 infection relative to infection with VRP (8 versus 6 hpi, respectively), it is possible that shutoff of host gene expression could impact STAT1/2 activation at these later times due to turnover of IFN receptor complex components. Therefore, to rule out the possibility that enhanced STAT1 inhibition reflects an ability of S300 to shutoff host protein synthesis more efficiently than a non-virulent SINV, we metabolically labeled Vero cells at various times after infection with S300 and G100. As shown in Figure 3.2A, S300 and G100 both inhibit de novo protein synthesis with similar kinetics. In fact, when a host protein that migrates at the expected molecular weight of actin was quantified, G100 shows slightly more rapid and complete shutoff (Figure 3.2B) indicating that nonspecific inhibition of gene expression is very unlikely to explain the enhanced ability of S300 to downregulate STAT1/2 tyrosine phosphorylation. This analysis, however, does not rule out the possibility that S300 specifically inhibits expression of a factor required for STAT1 activation, but otherwise, downregulates global gene expression similarly to G100.

Reduced STAT1 phosphorylation in S300-infected cells correlates with defects at the level of, or upstream of Jak activation.

IFN-mediated activation of STAT proteins requires upstream activation of the receptor-associated kinases Jak1/Tyk2 (IFN- α/β) or Jak1/Jak2 (IFN- γ) (50, 296, 297, 336). Therefore, we assessed whether S300 infection interfered with activation of these kinases following IFN treatment. Vero cells were infected with either S300 or G100 and stimulated with either IFN- β or IFN- γ after 8 hours of infection, a time that gave nearly maximal STAT1/2 inhibition by S300. After stimulation, total Jak protein was immunoprecipitated and subjected to immunoblot using phospho-tyrosine specific Jak antibodies. As shown in Figure 3.3, STAT1 inhibition (measured by immunoblot of input lysates) was directly correlated with reduced activation of Tyk2/Jak1 in response to IFN- β (Figure 3.3A and 3.3C) and reduced Jak1/Jak2 phosphorylation in response to IFN- γ (Figure 3.3B and 3.3D). Moreover, though G100 infection resulted in some inhibition of Tyk2/Jak1 or Jak1/Jak2 phosphorylation compared to mock-infected cells, inhibition of tyrosine phosphorylation of both STAT1 and Jak proteins was notably greater in S300- relative to G100-infected cells. Total Jak protein levels, which were assessed by both direct immunoblot of input lysates (Figure 3.3; IB panels) and by stripping/reprobing blots of immunoprecipitated total Jak protein (Figure 3.3; IP:IB panels) with antibodies recognizing both phosphorylated and unphosphorylated forms, were not consistently different between S300- and G100-infected cells. These data are consistent with the hypothesis that S300 infection antagonizes IFN signaling upstream of, or at the level of IFN receptor-associated Jak activation.

Efficient disruption of STAT1 tyrosine phosphorylation requires determinants within the nonstructural genes of S300.

In order to further define the mechanisms by which SINV strain AR86 (S300) efficiently inhibits STAT1 signaling and IFN receptor complex activation, we first focused on identifying the viral determinants of this activity. Initially, we evaluated infection with S300/G100 chimeric viruses, which encode heterologous nonstructural and structural genes. As shown in Figure 3.4, phosphorylation of STAT1 at Tyr-701 was again efficiently inhibited in response to IFN- β by 8 hours

in cells infected with wild-type S300 as well as the chimera encoding the S300 nonstructural genes (G106). Conversely, even though both G100 and the chimera encoding the G100 nonstructural proteins infected greater than 95% of the cells (data not shown), neither virus efficiently inhibited STAT1 activation. These data indicate that AR86 (S300) determinants that enable efficient Jak/STAT signaling inhibition are encoded by the viral nonstructural genes. To corroborate these data, we tested whether propagation-defective replicon particles, which encode S300 or G100 replicase genes but express GFP from their subgenomic promoter in place of the viral structural genes, were able to recapitulate the phenotype seen with the full length viruses. IFN- β -stimulated tyrosine phosphorylation of STAT1/2 was inhibited more efficiently by the S300-based replicon (S300 RP) relative to the replicon encoding the G100 replicase genes (G100 RP) after 6 hours of infection (Figure 3.5). However, while enhanced STAT2 inhibition was maintained in S300 RP-infected cells at 8 hpi, STAT1 activation at this time point was dramatically inhibited by both replicons. Thus, as reported previously (298), the kinetics of STAT1 inhibition during replicon versus full length virus infection differ; but, taken together, these data indicate that the enhanced inhibition of STAT1/2 activation by the adult mouse neurovirulent AR86 (S300) virus is mediated by determinants within the viral nonstructural genes.

The presence of threonine at S300 nsP1 538 is required for efficient inhibition of STAT1 activation.

Results from the chimeric virus and replicon studies indicated that determinants within the nonstructural protein coding region mediate the effects on STAT activation. To further define these determinants, we next evaluated whether any of the three previously defined AR86 determinants of adult mouse neurovirulence in this region (307) was required for STAT1 inhibition either individually, or in combination. Two of these virulence determinants reside within nsP3—an 18 amino acid deletion present in the C-terminal region of S300 nsP3 and a cysteine that replaces the G100-encoded opal termination codon just upstream of the nsP3/4 cleavage domain. A third virulence determinant, threonine at nsP1 538, is unique to the AR86 strain among Sindbis viruses. As

shown in Figure 3.6, introducing the four defined attenuating mutations into S300 (S363) abrogated enhanced STAT1 inhibition giving results equivalent to those seen during G100 infection. When we evaluated the individual contribution of the three nonstructural virulence determinants, we found that threonine at nsP1 538 was required, since S340 (encoding the consensus isoleucine at that position) also failed to inhibit STAT1 activation effectively. Conversely, introduction of either the G100-encoded nsP3 attenuating mutations (S343 and S344) did not result in any detectable loss of STAT1 inhibition relative to S300. We obtained similar results when measuring STAT1 activation in response to IFN- γ (data not shown). While we cannot rule out that other differences within S300 and G100 nonstructural genes are required for Jak/STAT signaling inhibition, these data clearly demonstrate that threonine at nsP1 538 is absolutely required for the enhanced inhibition seen in S300-infected cells.

Presence of threonine at nsP1 538 is sufficient for the inhibition of STAT1 activation during S300 infection.

Our loss-of-function analysis demonstrated that threonine at nsP1 538, a codon unique to the adult mouse neurovirulent AR86 strain, was required for the enhanced STAT1 inhibition by S300. We next evaluated whether replacement of the isoleucine at nsP1 538 in G100 with threonine also resulted in enhanced inhibition, or whether other determinants present within the nonstructural genes were also required for maximal STAT1 inhibition. Vero cells were infected with the wild-type G100 virus or mutants encoding AR86 virulence determinants at either nsP1 538 (isoleucine to threonine), the 18 amino acid deletion in nsP3, or the opal to cysteine change near the nsP3 C-terminus. Strikingly, introduction of threonine at nsP1 538 into the G100 background (G119) was sufficient to enhance STAT1 inhibition by G100 to the level seen during S300 infection (Figure 3.7), while introduction of either nsP3 virulence determinant had no effect. G119 and S300-mediated inhibition were also comparable when we measured STAT1 activation after IFN- γ treatment (data not shown).

Therefore, the virulence determinant at nsP1 538 is necessary and sufficient for enhanced STAT1 inhibition by the adult mouse neurovirulent S300 virus.

3.5 Discussion

The importance of the type I IFN system in the immediate control of virus replication is illustrated by the quantity and diversity of mechanisms encoded by most (if not all) virus families to antagonize the production of, and/or the cellular response to this family of cytokines. In the case of alphaviruses, this is further illustrated by the findings that avirulent SINV strains become fully virulent in mice deficient in the type I IFN response (IFNAR^{-/-}) (269, 270) suggesting that SINV must downregulate IFN production and/or signaling to some extent in order to cause disease in immunocompetent mice. Previous work has suggested that alphaviruses, including SINV, antagonize type I IFNs through global shutoff of host macromolecular synthesis (6, 84, 91, 93). Recent work from our group and others has demonstrated that VEEV and SINV infection results in a failure of cultured cells to respond to type I and II IFN as indicated by reduced STAT1 tyrosine phosphorylation (298, 354), however, in one study, inhibition of host macromolecular synthesis was proposed to play the dominant role in IFN antagonism since STAT1 inhibition did not result in reduced ISG transcription in cultured mouse neurons infected with VRP (354). In light of these previous results, we set out to test whether there was any correlation between the *in vivo* virulence profile of different SINVs and their ability to antagonize STAT activation. Importantly, we found that SINV strain AR86 (S300), which causes lethal disease in adult mice, demonstrated more complete and rapid inhibition of STAT1 activation than two avirulent SINV strains, Girdwood (G100) and TR339, which only partially inhibited STAT1 activation (as previously reported for TR339 (354)). AR86 and Girdwood each downregulated host protein synthesis with similar kinetics, and enhanced STAT1 inhibition mapped to a single determinant at nsP1 position 538 known to modulate AR86 neurovirulence (127, 307), but not shutoff of host transcription/translation (55).

These results suggest that Jak/STAT signaling inhibition, and not host cell shutoff, contribute to the enhanced virulence profile of AR86.

Though we do not yet understand the mechanism by which AR86 inhibits IFN- α/β and IFN- γ signaling, analysis of the type I and type II receptor complexes demonstrated clearly that defective STAT1 activation was associated with defects at the level of Jak protein activation at both receptor complexes. Decreased Jak activation could result from an inhibition of Jak kinase activity, from protein tyrosine phosphatase activity, or through a loss of receptor-ligand interaction (e.g. via decreased IFN receptor surface expression). Additional studies are required to determine whether the virus interferes with the ability of receptor complexes to associate and/or maintain expression at the cell surface. Previous studies with VRP infection indicated that Tyk2/Jak1 activation by IFN- β was normal at times of maximal STAT1 inhibition, but IFN- γ -mediated Jak1/Jak2 phosphorylation was reduced without a significant decrease in IFNGR2 surface expression (298). These results suggest that while both VEEV and AR86 inhibit STAT1 activation by IFN- β and IFN- γ , these viruses may act through distinct mechanisms, but this possibility requires further study.

Mapping studies (Figures 3.6 and 3.7) revealed that a single S300 determinant at nsP1 position 538 was necessary and sufficient for enhanced Jak/STAT inhibition. This determinant has previously been shown to play an essential role in adult mouse neurovirulence by the AR86 virus (127, 307). Recently, we demonstrated that SINV nsP1 538 modulates type I IFN induction independently of its effects on Jak/STAT signaling (55), which suggests this determinant regulates both the induction and signaling arms of the type I IFN response, each of which could be important for virulence. A possible link to type I IFN disruption is the ability of this determinant to modulate cleavage of the nonstructural polyprotein precursor. The nsP1 538 lies within the nsP1/2 cleavage domain, and relative to the consensus isoleucine at this position, the virulence-associated threonine codon delays the kinetics by which polyprotein intermediates are processed into mature nsPs (128). Interestingly, when we compare our various panels of mutant viruses, potent STAT1 inhibition correlates tightly with viruses that maintain expression of proteins corresponding to polyprotein

precursors (data not shown). Therefore, we are currently evaluating whether enhanced STAT1 inhibition is a direct result of delayed polyprotein processing, and if so, whether inhibition might be mediated by a P123 precursor or some other cleavage intermediate.

We cannot rule out the possibility that the threonine codon is modulating Jak/STAT inhibition independently of its effects on polyprotein processing. Furthermore, although the threonine codon is essential for efficient inhibition of STAT activation, the fact that viruses lacking threonine at this position retain partial inhibitory activity suggests that other determinants contribute to STAT inhibition. Interestingly, mutating TR339 nsP1 538 from an isoleucine to threonine (39ns1) resulted in enhanced inhibition of STAT1 phosphorylation (data not shown); however, this inhibition did not reach the magnitude seen when threonine at nsP1 538 was introduced into G100 (G119) suggesting that other differences between TR339 and Girdwood (G100), which is more closely related to AR86, also contribute to the inhibitory mechanisms. When combined with previous results with VEEV (298, 354), these data implicate the viral nonstructural proteins as mediators of Jak/STAT signaling inhibition. However, while we observed no role for the structural proteins in Jak/STAT inhibition, it is clear that determinants within alphavirus structural proteins (2, 301) and noncoding regions (349) also contribute to the resistance of alphaviruses to type I IFN.

Antagonism of Jak/STAT signaling is a feature of other encephalitic viruses including the flaviviruses Japanese encephalitis virus, tick-borne encephalitis virus, and West Nile virus (WNV), all of which have been shown to inhibit responses to IFN- α/β and/or IFN- γ (26, 116, 187). Recently, expression of the NS5 protein of the virulent NY99 WNV strain, but not NS5 from the attenuated Kunjin WNV, was shown to effectively disrupt STAT1 activation by IFN- α/β and IFN- γ . Effective STAT1 inhibition by the Kunjin WNV strain could be rescued by the introduction of a single amino acid encoded by the virulent NY99 (NS5 S653F) (166) in a manner analogous to our findings with SINV. At least in the case of SINV AR86 infection, the importance of the determinant at nsP1 position 538 both for adult mouse neurovirulence and for Jak/STAT inhibition suggests this inhibition contributes to pathogenesis. We have previously reported that S300 (WT, nsP1 538 Thr) and S340

(mutant, nsP1 538 Ile) establish infection and replicate within the CNS to similar levels at early times. However, by day 6 post infection, when S300-infected mice begin to succumb to infection, S300 continues to replicate within the CNS while the S340 virus is largely cleared (127) suggesting that the differences in virulence between these strains results in part from an ability of S300 to avoid viral clearance (127, 307). Antiviral antibody and IFN- γ mediate non-cytolytic clearance of non-pathogenic SINV from the CNS (30, 38, 176, 177), and IFN- γ treatment of SINV-infected rat neuronal cultures results in the clearance of infectious virus, a reduction in viral protein synthesis, and restored cellular protein synthesis within 24 hours (37), effects that were later determined to require Jak1-dependent signaling (36). Since AR86 was a potent inhibitor of IFN- γ -mediated STAT1 activation, it is possible that this virus is more resistant to mechanisms of IFN- γ -dependent viral clearance from the CNS, which ultimately leads to the induction of lethal neurologic disease. Studies are underway to directly assess this possibility.

In summary, we have demonstrated that an adult mouse neurovirulent strain of Sindbis virus exhibits an enhanced ability to interfere with Jak/STAT activation by either type I or type II IFNs and that this effect is mediated by a virulence determinant at nsP1 position 538. These studies strongly suggest that the ability to block Jak/STAT signaling contributes to alphavirus virulence. Therefore, additional studies are needed to both define the molecular mechanisms underlying Jak/STAT antagonism by alphaviruses and to investigate the relative role of inhibition of either type I or type II IFN receptor signaling in the pathogenesis of alphavirus-induced disease.

3.6 Acknowledgements

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Table 3.1: List of SINV infectious clones used.

Virus name	Background strain and mutations
S300	Wild-type S.A.AR86.
G100	Wild-type GirdwoodS.A.
TR339	Wild-type A.R.339.
G106	AR86 nt 43-6411 in Girdwood background.
S350	Girdwood nt 43-6465 in AR86 background.
S363	AR86 with nsP1 538 Thr→Ile; 18 aa insertion of G100 nsP3 aa 386-403 (un-Δ); Cys→Opal at [AR86] nsP3 537; Ser→Leu at E2 243.
S340	AR86 with nsP1 538 Thr→Ile.
S343	AR86 with 18 aa insertion of G100 nsP3 386-403.
S344	AR86 with Cys→Opal at nsP3 537.
G119	Girdwood with nsP1 538 Ile→Thr.
G120	Girdwood with Opal→Cys at nsP3 555.
G121	Girdwood with nsP3 aa 386-403 deletion.

Figure 3.1: SINV neurovirulent strain AR86 efficiently inhibits STAT1/2 activation in response to type I and type II IFNs.

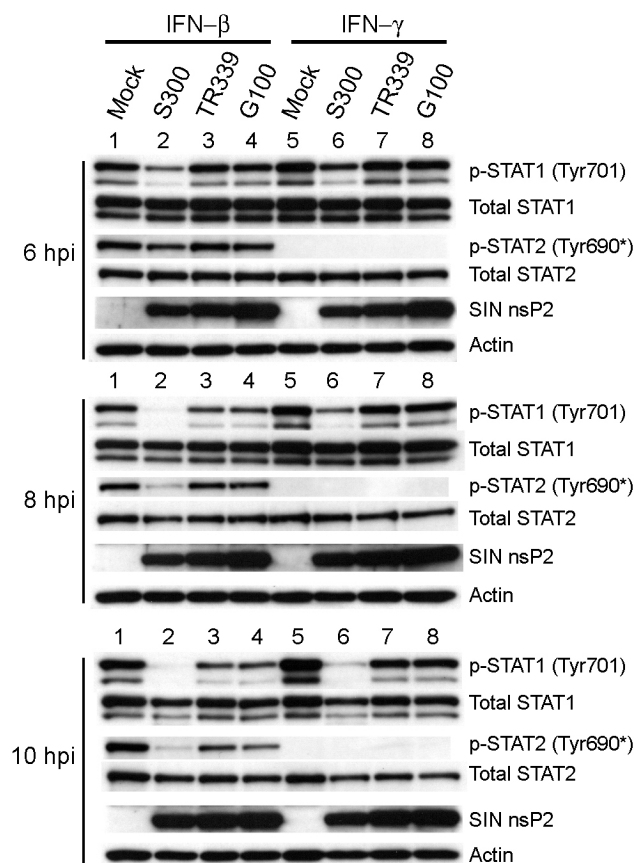


Figure 3.1: SINV neurovirulent strain AR86 efficiently inhibits STAT1/2 activation in response to type I and type II IFNs.

Vero cells, which respond to but do not secrete type I IFNs, were infected at an MOI of 10 PFU/cell with SINV strains AR86 (S300), TR339, and Girdwood (G100). At 6, 8, or 10 h post infection (hpi), the cells were treated with IFN- β or IFN- γ (1,000 U/mL) for 20 min after which whole cell lysates were prepared. Equal total protein from each sample was then subjected to immunoblot using the indicated antibodies. *, The phospho-specific STAT2 antibody used (Upstate) is directed against mouse STAT2 phosphorylated at tyrosine 689, but cross-reacts with the corresponding position of human (primate) STAT2 (pY-690).

Figure 3.2: S300 and G100 initiate shutoff of de novo protein synthesis with similar kinetics.

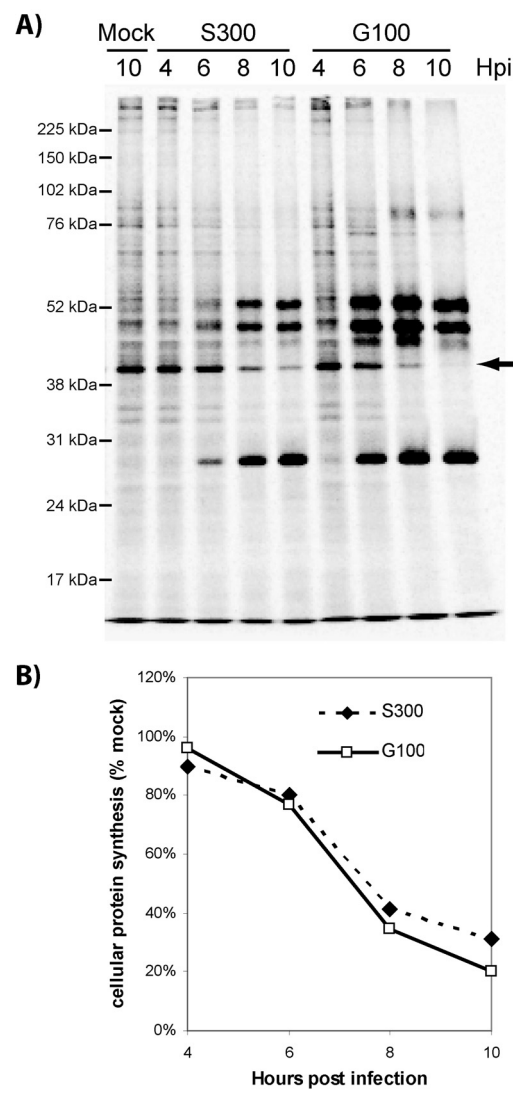


Figure 3.2: S300 and G100 initiate shutoff of de novo protein synthesis with similar kinetics.

To measure de novo protein synthesis, Vero cells were infected with diluent (Mock), S300 or G100 (MOI=20 PFU/cell) and then labeled with media containing 33 μCi ^{35}S -labeled cysteine/methionine per mL for 1 h prior to harvest at the indicated times post infection (hpi). Prior to each labeling period, cells were starved for 2 h with media deficient in cysteine/methionine. Extracts of the radiolabeled cells were prepared and resolved by SDS-polyacrylamide gel electrophoresis. The gel was dried, fixed, and exposed to phosphorimaging (Bio-Rad) as described in Material and Methods (A). A host protein band that resolved at the expected molecular weight of actin (arrow) was quantified using Quantity One software (Bio-Rad). (B) Densities of this band were normalized to Mock-infected samples and plotted to compare de novo host protein synthesis in S300- versus G100-infected cells.

Figure 3.3: Reduced STAT1 phosphorylation in S300-infected cells correlates with defects in Jak activation by type I and type II IFNs.

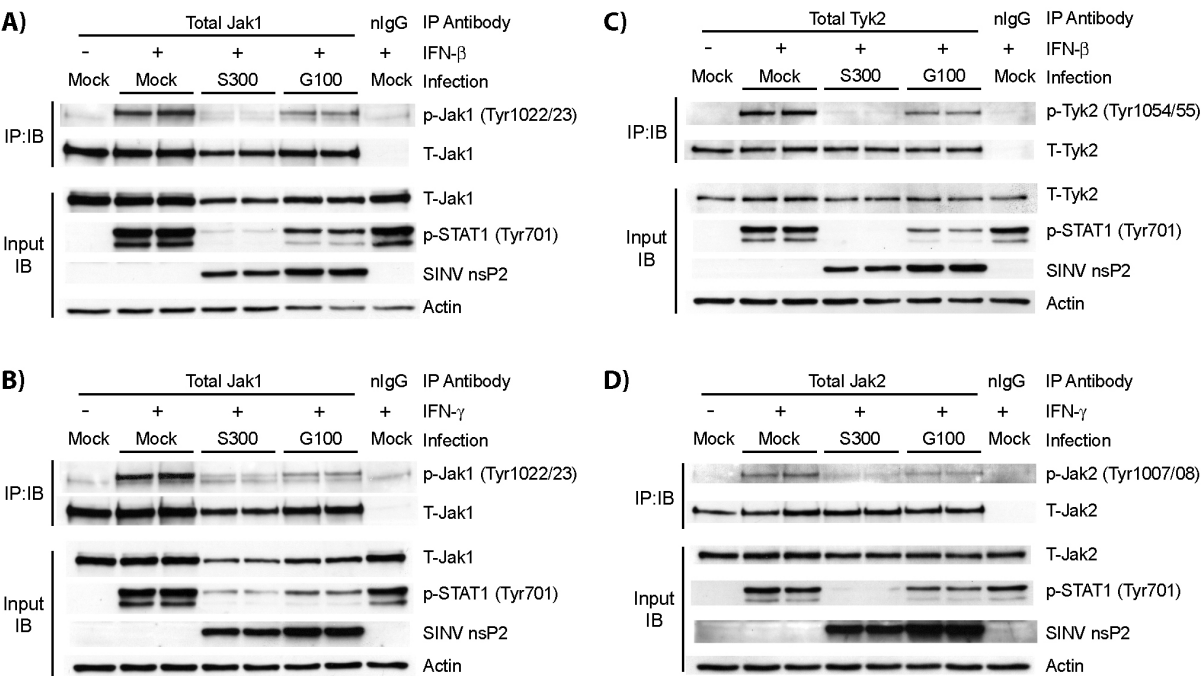


Figure 3.3: Reduced STAT1 phosphorylation in S300-infected cells correlates with defects in Jak activation by type I and type II IFNs.

Vero cells were infected (MOI = 10 PFU/cell) with S300, G100 or diluent alone (Mock) for, and then treated for 20 min with 1,000 U IFN- β (A and C) or IFN- γ (B and D) per mL. Cell extracts were then prepared as described in the Materials and Methods and total protein content was quantified. Equal amounts of total protein were then subjected to immunoprecipitation with antibodies directed against total Jak1 (A and B), total Tyk2 (C), or total Jak2 (D) protein, and to assess activation, the immunoprecipitates were resolved by SDS-PAGE and immunoblotted using the indicated phospho-specific Jak antibody (IB:IP panels). This blot (IP:IB) was then stripped and re-probed using an antibody recognizing the appropriate total Jak. A portion of the input lysates (5-7%) was subjected to direct immunoblot analysis to assess total Jak protein levels (Input IB panels). This blot was stripped and re-probed consecutive times with phospho-specific STAT1 (Tyr701) and anti-SINV nsP2 polyclonal sera.

Figure 3.4: Efficient disruption of STAT1 tyrosine phosphorylation requires determinants encoded by the S300 nonstructural genes.

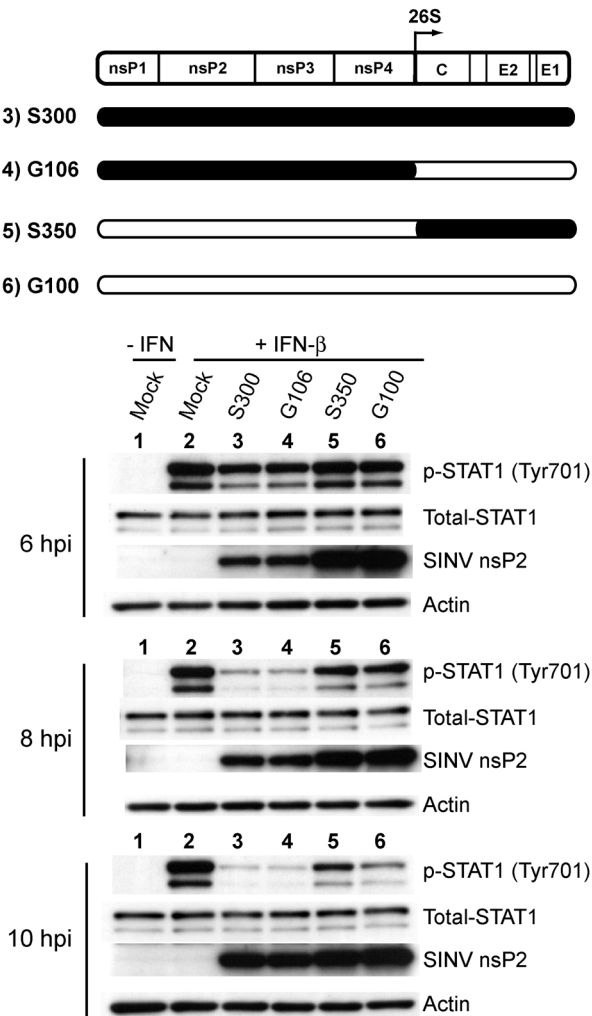


Figure 3.4: Efficient disruption of STAT1 tyrosine phosphorylation requires determinants encoded by the S300 nonstructural genes.

Vero cells were infected for 6, 8, or 10 h with the indicated viruses (MOI = 15 PFU/cell) prior to 20 minute stimulation with 1,000 U IFN- β /mL. Whole cell extracts were prepared and analyzed as described in Figure 1. A schematic of the SINV genome indicates the origin of the nonstructural/structural gene chimeras where black indicates genes of S300 origin and white indicates genes of G100 origin.

Figure 3.5: SINV replicon particle infection inhibits STAT1/2 activation with similar trends as full-length viruses, but with more rapid kinetics.

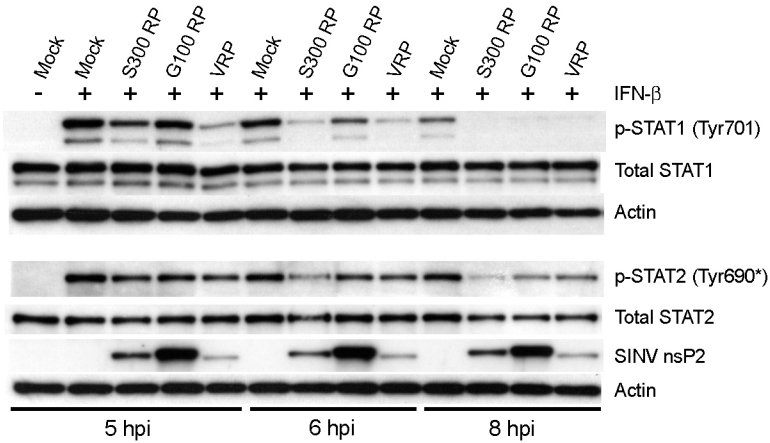


Figure 3.5: SINV replicon particle infection inhibits STAT1/2 activation with similar trends as full length viruses, but with more rapid kinetics.

Replicon genomes, which express green fluorescent protein (GFP) in place of the viral structural genes, encoding the replicase genes of either S300 or G100 origin were packaged with equivalent capsid and glycoprotein helper constructs and used to infect Vero cells (MOI = 5 IU/cell). At 5, 6, and 8 h post infection, cells were treated with 1,000 U IFN- β /mL for 20 min. Whole cell extracts were harvested and analyzed as described in Figure 1. VEEV replicon particles (VRP), which were previously shown to inhibit STAT1 activation, were included as a positive control. (S300 RP and G100 RP indicate S300-derived replicon particles and G100-derived replicon particles, respectively).

Figure 3.6: The presence of threonine at S300 nsP1 538 is required for efficient inhibition of STAT1 activation.

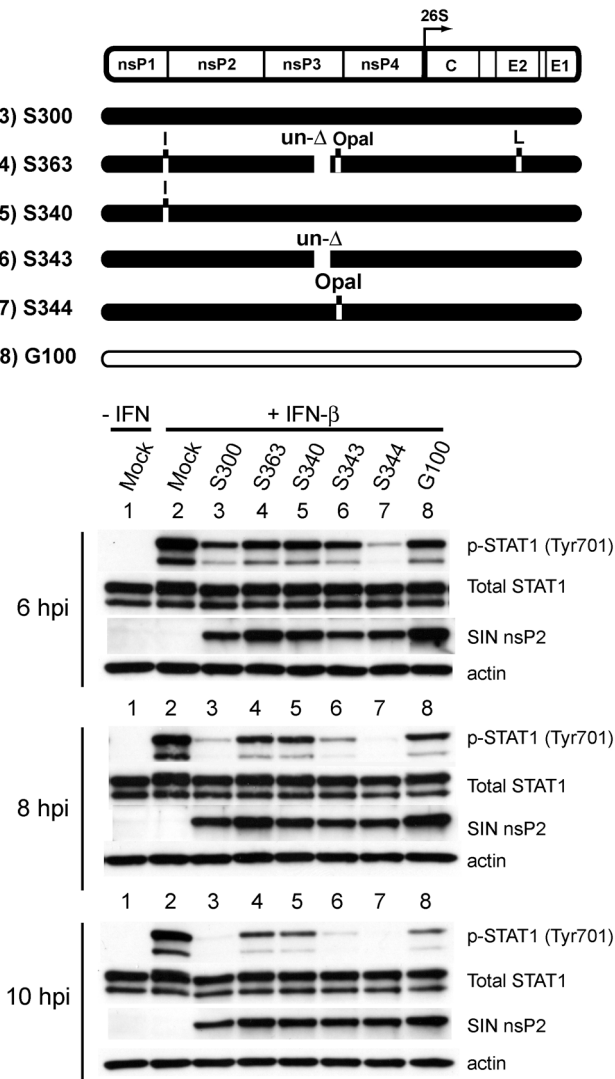


Figure 3.6: The presence of threonine at S300 nsP1 538 is required for efficient inhibition of STAT1 activation.

For a loss-of-function analysis, Vero cells were infected with the indicated viruses for 6, 8, or 10 h (MOI = 15 PFU/cell) and then stimulated for 20 min with 1,000 U IFN- β /mL. Whole cell lysates were harvested and then subjected to Western blotting analysis as described in Figure 1. A schematic of the SINV genome denotes the locations of four attenuating mutations of G100 origin (white) that are inserted individually, or in combination, into wild-type S300 background (black): Isoleucine (I) at nsP1 538 within the nsP1/2 cleavage domain replaces threonine encoded by wild-type S300, an 18 amino acid insertion (un- Δ) replaces a deletion present in the wild-type S300 at nsP3 position 386-403, an Opal termination codon replaces the wild-type S300-encoded cysteine at nsP3 position 537, and a leucine (L) replaces a serine encoded by wild-type S300 at position 243 of the E2 glycoprotein.

Figure 3.7: Presence of threonine at nsP1 538 is sufficient for the inhibition of STAT1 activation during S300 infection.

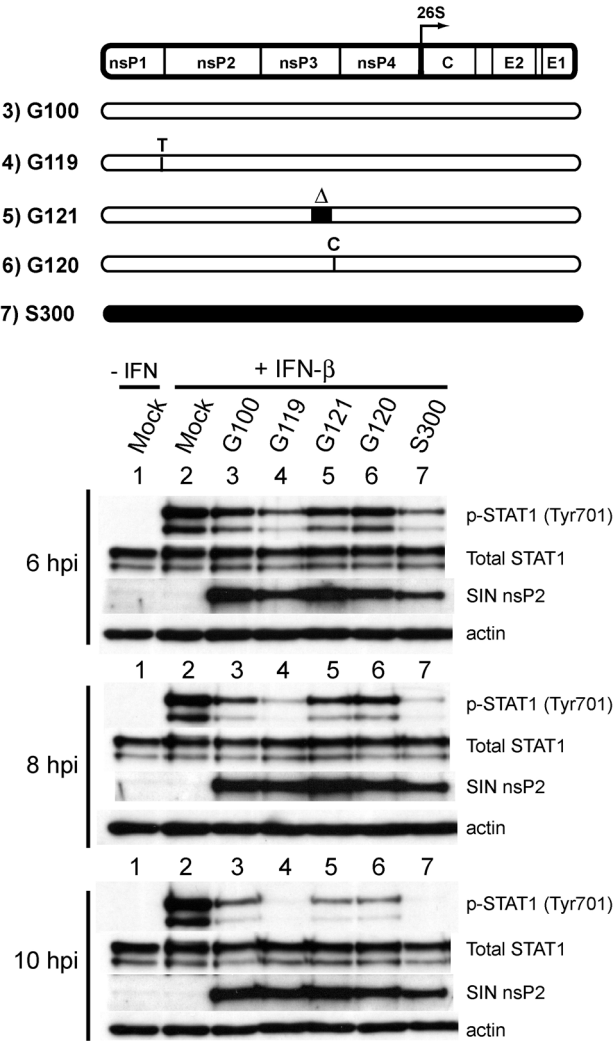


Figure 3.7: Presence of threonine at nsP1 538 is sufficient for the inhibition of STAT1 activation during S300 infection.

To assess for gain-of-function, Vero cells were infected for 6, 8, and 10 h with the indicated viruses (MOI = 15 PFU/cell) and then stimulated for 20 min with 1,000 U IFN- β /mL. Whole cell extracts were prepared and analyzed by Western blotting as described in Figure 1. The diagram indicates the location of four virulence determinants of S300 origin (black), which were individually introduced into the avirulent G100 background (white). Threonine (T) replaces the G100-encoded isoleucine at nsP1 538 within the nsP1/2 cleavage domain, nsP3 positions 386-403 are deleted from wild-type G100 (Δ), and the wild-type Opal termination codon at G100 nsP3 position 555 is replaced with cysteine (C).

CHAPTER FOUR:
DISCUSSION

4.1 VEEV inhibits type I and type II IFN signaling

Alphaviruses antagonize IFNs through mechanisms independent of host shutoff

Infection of mice subcutaneously with VEEV results in abundant type I IFN production detected within serum, which corresponds to the efficient replication of the virus within myeloid cells of lymphoid tissues (43, 110) where PRRs have ample opportunity to engage virus PAMP ligands. It is clear that responsiveness to IFN is required for mice to control virus replication since (i) avirulent VEEV strains cause uniform lethality in IFNAR^{-/-} mice (111) and (ii) virulent VEEV infection results in more rapid disease progression in mice either treated with anti-IFN- α/β antibody (109) or deficient in IFN signaling (IFNAR^{-/-}) (349) relative to untreated, wild-type mice. However, high levels of virus-induced endogenous type I IFN or exogenous treatment with IFN- β or IFN inducers (polyI:C) is not usually sufficient to protect wild-type mice from mortality with virulent VEEV (111). Thus, at the outset of these investigations, virulent VEEV was expected to employ mechanisms that limit its sensitivity to type I IFN.

In vitro studies in mouse fibroblasts and primary mouse cortical neurons suggest that VEEV is less sensitive than other alphaviruses to the antiviral effects of murine IFN- α/β treatment (268, 354), which presumably was associated with New World alphavirus capsid-dependent mechanisms of host shutoff. However, protein translation shutoff clearly occurs during infection with VEEV replicon particles (VRP) in the absence of capsid expression (S. Montgomery, unpublished) and evidence from our laboratories and others indicates that determinants that limit IFN sensitivity by VEEV and EEEV are encoded by the 5'-untranslated region (349) and nonstructural genes as well as the structural genes (2, 249) calling into question whether host shutoff was the only mechanism that limited VEEV sensitivity to type I IFN. Therefore, our initial investigations focused on identifying whether the signaling events upstream of ISG induction occurred normally within VEEV-infected cells, and since activation of these components does not require de novo gene expression, any phenotype we uncovered was likely independent of nonspecific effects on host shutoff.

We analyzed whether STAT1 trafficking was disrupted by VRP infection because nsP2 interacts specifically with importin- α 5, the nuclear importin- α molecule required for STAT1 nuclear trafficking (221), which ultimately lead to the identification of defective IFN signaling events at multiple levels. Although upstream activation of the type II IFN receptor complex and of STAT1 were found to be inhibited, it remains possible that the VEEV nsP2-importin- α 5 interaction further contributes to Jak/STAT signaling antagonism. Our findings clearly demonstrate for the first time that shutoff-independent mechanisms of IFN antagonism are employed by alphaviruses since (i) nsP but not capsid protein expression is required and (ii) STAT1 inhibition occurs more rapidly by the VEEV-mediated mechanism than could be explained by shutoff alone (i.e. Actinomycin D treatment, Figure 2.5).

Jak/STAT signaling inhibition could play an important role during several stages of VEEV pathogenesis, either through type I IFN signaling inhibition and/or by suppressing the response of infected cells to IFN- γ . Of particular interest is whether STAT1 signaling inhibition contributes to the ability of VEEV to resist high levels of serum IFN- α/β and continue to replicate efficiently during the critical initial lymphoid phase of infection. Although peripheral replication in mice is controlled and cleared within several days post infection, the early establishment of viremia determines neuroinvasion, which is controlled in a type I IFN-dependent manner during infection with avirulent VEEV (349). Moreover, significant viremia within equine hosts is a requirement for VEEV emergence and epidemic disease in humans. Thus, whether enzootic and epizootic strains differ in their capacity to inhibit Jak/STAT signaling will be an interesting avenue of investigation.

Taken together, it is clear that VEEV efficiently inhibits STAT1 activation, but the relevance of this inhibition to viral pathogenesis is not understood at this time. In the face of potent downstream virus-induced transcriptional (and translational) shutoff, the role for upstream signaling inhibition is unclear. Moreover, relative to other alphaviruses, VEEV appears to be much more resistant to a pre-existing antiviral state suggesting it encodes mechanisms to avoid or suppress the activities of ISG-encoded proteins (268, 354). This resistance would seemingly make upstream

STAT1 inactivation unnecessary. However, since STAT1 inhibition is a relatively conserved activity among other virus families and this molecule is central to multiple and diverse host responses, it is likely that VEEV has evolved to employ this activity for a reason, and it may reduce the overall IFN response to a threshold that can be tolerated by the virus.

Possible mechanisms of VEEV-mediated Jak/STAT signaling inhibition

At this point, we have not precisely defined the mechanism of virus-mediated disruption of Jak/STAT signaling, although our analyses have greatly narrowed the possibilities. Perhaps the most interesting and unusual findings were the specificities of the inhibition at both the level of STAT factors and of IFN receptor complexes, which gives some insight into potential mechanisms. First, we found that in response to type I IFN treatment in cells infected with VRP for 4 to 6 hours, STAT1 activation was dramatically reduced without any reduction in STAT2 activation (Figures 2.3 and 2.4). A reduction in STAT2 activation was seen at later times post infection, but it never reached the magnitude detected with STAT1. Secondly, after 6 hours of infection, VRP inhibited Jak1 activation at the IFNGR but its activation occurred normally in response to IFN- β (Figure 2.6). These findings make events upstream, such as receptor downregulation or disrupted receptor complex activation, unlikely mechanisms. Indeed, the mild reduction in IFNGR1 surface expression did not correlate with the significant reductions in Jak1, Jak2 and STAT1 phosphorylation (Figure 2.7); while we did not assess IFNAR surface expression, normal Jak1, Tyk2 and STAT2 activation at times of failed STAT1 activation indicate that in response to IFN- β , the IFNAR complex is activated and functions normally. It is certainly possible that VEEV employs multiple mechanisms that differentially inhibit type I and type II IFN receptor signaling. However, since Jak1 participates in signaling at each receptor complex, it is a likely target. In support of this, we found that STAT3 activation was dramatically reduced in VRP-infected cells in response to IFN- α/β , IFN- γ and IL-6, which signal through distinct receptor complexes all of which require Jak1 (118, 261) (Figure 2.6 and data not shown). If Jak1 is a common target either directly by a viral effector or by a virus-activated host

factor, it is unclear how STAT1 fails to become activated after IFN- β stimulation while Jak1, Tyk2 and STAT2 are phosphorylated normally. Since STAT2 activation eventually is inhibited, we expect that the activation of the type I IFN receptor complex will be inhibited at later times (>6 hpi), which would be consistent with Jak1-specific inhibition, but this possibility requires further study.

One limitation we have in trying to identify the VEEV factor(s) required for Jak/STAT signaling inhibition is that we do not have a mutant or strain deficient in this activity (one reason we focused on the SINV studies). The vaccine VEEV strain TC-83, which is known to be more sensitive to type I IFN, differs from the wild-type TRD strain at two positions within the replicon genome, but neither a TC-83-based VRP nor VRP encoding each of the TC-83 changes individually resulted in any decrease in STAT1 inhibition (data not shown). Moreover, a mutation at the site within the nsP1/2 cleavage domain within VEEV nsP1 (nsP1 A533V) that is analogous to RRV nsP1 A532V and SINV G100 I538T (55) did not affect STAT1 inhibition (data not shown), although this is not unexpected since this mutation may enhance STAT1 inhibition and the inhibition seen during wild-type VEEV is nearly complete (see RRV A532V discussion in Chapter 4.2).

Future directions

An important future study will be to screen a variety of additional VEEV strains, both virulent and avirulent, to determine whether a particular strain or VEEV mutant cannot efficiently inhibit STAT1 activation. In the absence of such a mutant, we attempted to evaluate whether expression of nsPs outside the context of replicating virus inhibited STAT1 activation. To do this, we treated cells with IFN- β after co-transfection of a STAT1-gfp construct along with an nsP expression construct of interest. Using this approach, STAT1-gfp can be resolved from endogenous STAT1 in an immunoblot analysis. This system should be a useful screening approach to identify the viral effector(s) involved since VRP infection inhibits STAT1-gfp tyrosine phosphorylation with similar kinetics and efficiency as endogenous STAT1 (data not shown). Overexpression of VEEV nsP2 dramatically inhibited STAT1-gfp activation, but this is likely explained by large decreases in total

STAT1-gfp expression (Figure 4.1). Most likely, nsP2 overexpression results in generalized downregulation of gene expression, as is known to occur during overexpression of SINV nsP2 (92). We cannot rule out, however, that in addition to affects on gene expression, nsP2 overexpression is also inhibiting STAT1-gfp phosphorylation. There are no known mutations that limit nsP2-mediated cytotoxicity in the context of virus-free overexpression. Therefore, future studies will rely on responsiveness of endogenous STAT1 (e.g. nuclear localization by IFA), or may require an inducible expression system where nsP2 expression can be initiated after sufficient STAT1-gfp accumulation (or induced in a stable nsP2-containing cell line). Alternatively, our data in Chapter 3 suggest a polyprotein could play a role in Jak/STAT inhibition, and therefore, we should also assess whether the expression of various VEEV nsP polyprotein precursors disrupt STAT1-gfp activation.

One limitation of the current studies is the use of Vero cell cultures. At the cost of being a somewhat artificial system, Veros were required to eliminate the confounding effects of endogenously produced type I IFN induced by VEEV/VRP replication. We did find that VRP suppressed STAT1 activation in two IFN-competent human cell lines, HeLa and 293 (data not shown), suggesting that we could likely measure VRP effects within infected primary cells even in the presence of virus-induced endogenous IFN. In particular, it will be interesting to evaluate VEEV or VRP effects on primary murine myeloid cells including macrophages and DCs, since these cells are targeted by VEEV *in vivo* and the virus replicates to high titers even in the presence of rapid and abundant induction of type I IFN in the serum. Infection of DC cultures with SINV resulted in much less cell death relative to fibroblast cell lines (271), which suggests we could assess virus clearance from VEEV- or VRP-infected DC cultures treated with exogenous type I IFN. However, these studies will likely require the prior identification of a VEEV strain deficient in STAT1 inhibition to compare its clearance with that of wild-type VEEV. Since emergence of VEEV involves efficient amplification within horses, it will also be important to evaluate whether STAT1 inhibition can be detected within VEEV-infected cells of equine origin. If so, follow-up studies comparing enzootic and epizootic strains in their relative capacity to inhibit Jak/STAT signaling may implicate this

activity as an additional determinant of VEEV emergence and could mechanistically explain the previously proposed association of epizootic strains and IFN resistance (249, 301).

Concurrent with our VEEV publication (Chapter 2), Yin et al. reported that both VEEV and SINV inhibited STAT1 activation in primary murine neuronal cultures (354), suggesting that Jak/STAT signaling inhibition is likely to occur in vivo. Despite partial inhibition of STAT1 and STAT2 activation, analyses of the induction of several downstream ISGs indicated that full-length VEEV reduced ISG expression, but VRP infection actually resulted in the upregulation of a few ISGs. The conclusion was made that STAT1/2 inhibition did not contribute significantly toward the downregulation of the antiviral state, but rather occurs primarily by transcriptional shutoff mediated by the viral structural genes (i.e. capsid). It is likely, however, that upregulation of ISGs in neurons infected with VRP results from virus-induced endogenous type I IFN production, which would allow upregulation of IFN signaling components that could amplify downstream ISG induction upon exogenous IFN stimulation. Moreover, several ISGs evaluated are thought to be additionally induced by IRF3/7-directed promoter activity (106, 107), and thus could be induced through STAT1-independent mechanisms (i.e. ISG56). Finally, these studies clearly demonstrate that despite its upregulation of a few ISGs, VRP clearly induces dramatic host translational shutoff within neurons by 12 h.p.i. Therefore, it will be of great interest to evaluate whether VRP replication within primary neurons is resistant to type I (or type II) IFN treatment, and whether any resistance requires the ability of VRP to inhibit Jak/STAT signaling.

4.2 Jak/STAT signaling inhibition and SINV neurovirulence

An adult mouse neurovirulence determinant allows SINV to suppress IFN signaling

Our studies with VEEV left us with several important questions. First, whether STAT inhibition is unique to VEEV infection or if other alphaviruses employ similar activities. Second,

which viral factors are involved in signaling inhibition and through what mechanisms? Finally, what is the relevance of Jak/STAT inhibition in the context of alphavirus pathogenesis? Our subsequent SINV studies answered several of these questions and advanced our understanding of alphavirus molecular pathogenesis. It was clear that SINV also employs mechanisms to inhibit STAT and Jak activation (Figure 3.1 and 3.3) while RRV fails to do so (data not shown). Secondly, identification of threonine at nsP1 538 either implicates nsP1 itself or a polyprotein precursor as the mediator of signaling inhibition. Finally, the importance of this determinant both for STAT1 inhibition and for virulence strongly suggests that Jak/STAT signaling plays a critical role during SINV neuropathogenesis.

Immunocompetent adult mice effectively control replication of most SINV strains after intracranial inoculation. This is dependent on a functional type I IFN response since the absence of the IFNAR results in rapid virus dissemination and death (40). Adult mouse neurovirulent SINV strains, therefore, must employ mechanisms to downregulate the type I IFN response to cause disease. This is accomplished, at least in part, through nsP2-mediated transcriptional shutoff that limits expression of IFN- α/β and ISGs (84). Attenuation of S300 by the nsP1 T538I mutation (S340) is associated with elevated type I IFN induction in vitro and in vivo (55). However, unlike the case of noncytopathic attenuated SINV mutants (84, 92), increased IFN induction by S340 is not a result of deficient host shutoff (55). The current investigation suggests that this single mutation, in addition to having drastic effects on the induction of IFN- α/β , also blocks the cellular response to these cytokines. At this time, it is unclear whether Jak/STAT signaling inhibition contributes to a suppression of type I IFN induction during S300 infection by inhibiting the amplification pathway. In vitro, it seems clear that the nsP1 538 determinant (or at least the corresponding position in RRV) modulates type I IFN induction independently of IFNAR-dependent amplification(55), but this does not exclude the possibility that Jak/STAT signaling inhibition further antagonizes type I IFN induction.

Interestingly, the wild-type S300 and attenuated mutant (S340; Ile nsP1 538) replicate within the brain very similarly at early times when abundant type I IFN is detected in the serum of S340-infected mice (127, 307). However, by day 6 post infection, foci of S340 virus replication in the brain are largely cleared while those of S300 persist, which suggests that S300 either spreads more efficiently or employs mechanisms to resist clearance (127). Previous studies indicate that noncytolytic SINV clearance is mediated through cooperation of antiviral antibody and IFN- γ -dependent mechanisms (30, 37, 38). Interferon- γ treatment of cultured rat neuronal cells infected with avirulent SINV resulted in partial restoration of host protein synthesis, downregulation of viral protein synthesis, and clearance of infectious virus through mechanisms that required Jak1 kinase activity (36, 37). By day 3 to 4 post intracranial SINV infection, an adaptive response is first detected involving anti-SINV antibody and the infiltration of T cells, which are involved with IFN- γ production (114, 219). Temporally, the appearance of this adaptive response corresponds to the divergence of S300 from S340 in terms of disease course and neuropathology, which is consistent with the hypothesis that S300 is more resistant to antibody- and/or IFN- γ -dependent mechanisms of virus clearance. Accordingly, infection of RAG-/- mice with S363, which encodes the nsP1 538 isoleucine mutation as well as the three other attenuating mutations (Figure 3.6), results in 100% mortality suggesting that the adaptive response is required for S340 clearance (M. Suthar, unpublished). The enhanced inhibition of IFN- γ -stimulated STAT1 activation provides a plausible mechanism to explain how S300 suppresses this adaptive response allowing it to persist within CNS neurons and ultimately cause fatal disease in adult mice.

Possible mechanisms of SINV-mediated Jak/STAT signaling inhibition

Infection with S300 resulted in dramatic defects in the activation of IFN receptor complexes as indicated by reduced Tyk2, Jak1 and Jak2 activation by either IFN- β or IFN- γ (Figure 3.3). While we cannot rule out additional downstream targets, the magnitude of the inhibition of Jak phosphorylation could fully explain failed STAT1 or STAT2 activation by both receptor complexes.

Therefore, it seems likely that the inhibitory mechanism occurs at the level of the type I and type II IFN receptor complex. One possibility is that SINV infection downregulates the surface expression of IFN receptor subunits. Although we have not evaluated surface expression of either type I IFN or type II IFN receptor subunits, our VEEV studies indicated that defective STAT1 activation by IFN- β treatment was not associated with a defect in the activation of the type I IFN receptor complex components. At this time we cannot exclude the possibility that VEEV and S300 act through different mechanisms.

Potential host factors directly involved with IFN receptor antagonism could involve decreased rates of phosphorylation or increased rates of dephosphorylation. Protein tyrosine phosphatases (PTPs) are a more likely candidate than SOCS proteins since PTPs are normally constitutively expressed and await activation while SOCS proteins are typically induced by type I IFN. In these studies, the 20 minute IFN stimulation times are not sufficient for significant accumulation of IFN-induced SOCS proteins. A more direct mechanism could involve interference with Jak activation through the binding of a viral factor. Alphavirus replication complexes are known to associate with the cytoplasmic membrane through direct nsP1-membrane lipid interactions, which require palmitoylation of cysteine residues and an amphipathic peptide within nsP1 (159, 302). This membrane association could be important for nsP1 or other viral nsPs to interact with and/or antagonize IFN receptor complexes, but has not yet been evaluated.

Since the release of nsP1 from the nsP123 precursor is thought to be required for subsequent nsP23 cleavage (326), it follows that the residue at nsP1 538, which resides at the P3 position within the nsP1/2 cleavage site, could dramatically affect the processing kinetics of the nonstructural polyprotein. Indeed, both wild-type AR86 and a mutant TR339 (39ns1) encoding threonine at nsP1 538 displayed delayed kinetics with which the nsP123 (or nsP1234) polyprotein is processed relative to viruses encoding the consensus isoleucine at that position (128). Similarly, a RRV mutation (A532V) at the corresponding position delays polyprotein cleavage in vitro (C. Cruz, unpublished). Preliminary results with the TR339 39ns1 and the RRV A532V mutants indicate that these viruses are

also more efficient inhibitors of IFN- β -mediated STAT1 activation relative to the wild-type strains (data not shown). These results clearly demonstrate the importance of this position for STAT1 inhibition in unrelated alphavirus strains, perhaps through an effect on polyprotein processing. Western blot analyses demonstrated that effective STAT1 inhibition occurred correlated with accumulation of nsP2-containing polyprotein precursors; however, these precursors accumulated prior to significant STAT1 inhibition indicating that their presence did not strictly determine STAT1 effects. It remains possible that polyprotein precursor accumulation must precede the development of a specific effector function. Preliminary studies of *ts* mutants that are incapable of polyprotein processing when infected cultures are incubated at the nonpermissive temperature suggested that polyprotein accumulation did not alone promote STAT1 inhibition (discussed below; Figure 4.2). Taken together, our data does not support a simple model where accumulation of nsP123 or other intermediates results in the inhibition of STAT1 activation, but a role for delayed polyprotein processing has not been ruled out.

Future directions

Identification of threonine at nsP1 538 as a determinant of efficient STAT1 inhibition and its previously demonstrated role in SINV neurovirulence warrants several important *in vivo* studies. Since S300 (virulent, nsP1 538 threonine) inhibits IFN- γ -mediated STAT1 activation more efficiently than S340 (attenuated, nsP1 538 isoleucine), we hypothesize that this mechanism renders S300 more resistant to IFN- γ -mediated clearance from infected neurons. To test this hypothesis, it will first be important to confirm our STAT inhibition findings in S300- and S340-infected cultures of primary mouse cortical neurons. In these primary cells, SINV (TR339) infection is known to induce expression of IFN- β message without detectable production of bioactive type I IFN protein (354); however, since S340 is known to induce elevated levels of endogenous type I IFN relative to S300 in mouse cell lines and *in vivo*, these studies must control for the relative contributions of exogenous recombinant IFN versus virus-induced endogenous type I IFN toward STAT1 activation. Using these

neuron cultures, we will also compare the relative abilities of S300 and S340 to replicate in the presence of exogenous IFN treatment. Treatment of in vitro-differentiated rat neuronal cultures with exogenous IFN- γ resulted in partial restoration of cellular protein synthesis and clearance of infectious SINV (strain 663) (37), but since this IFN- γ antiviral activity required functional Jak1 activity (36), we hypothesize that neurons infected with S300 will fail to clear infectious virus and be less sensitive to the IFN- γ antiviral effects when compared to S340-infected cells since S300 more efficiently inhibits Jak1/Jak2/STAT1 activation by IFN- γ . We will also be able to compare IFN- β -mediated clearance using this same culture system.

Concurrent with these in vitro experiments, we will directly assess the role of IFNGR signaling after intracranial inoculation of mice with S300 and S340. We expect that the inability of S340 to efficiently disrupt STAT1 activation will make this virus more sensitive to IFNGR-mediated clearance, and to test this, we will infect wild-type and IFNGR $^{-/-}$ mice with S300 and S340 to see whether each virus's disease course is affected. In the absence of IFNGR signaling, which would abrogate IFN- γ -mediated STAT1 activation, we hypothesize that S340 will cause more severe disease and will be able to persist within the brain similarly to S300 at later times (5 to 6 days post infection). We would like to evaluate the role for IFNAR signaling in mediating the difference in virulence between S300 and S340, but it is probable that after i.c. inoculation, S340 will efficiently spread and replicate within the periphery in the complete absence of a functional type I IFN response (IFNAR $^{-/-}$). To restrict differences between S300 and S340 pathogenesis to events within the CNS, we could employ a tissue-specific knockout approach where IFNAR can be targeted specifically in cells of neuroectodermal origin (64). Infection of these mice will determine whether the failure of S340 to spread within CNS neurons of wild-type mice requires functional IFNAR signaling, although it is possible that S300 will also spread more efficiently in these tissue-specific IFNAR knockout mice, complicating the interpretation.

By 4 days post infection, the disease courses of S300 and S340 begin to diverge, which could result from the successful control of S340 replication and spread by infiltrating inflammatory cells.

We will evaluate whether S300- and S340-infected cells within the brain are responding normally to this immune response using two separate assays. First, we will evaluate STAT1 phosphorylation and nuclear translocation within infected brain neurons using immunohistochemistry. By co-staining brain sections with a phospho-specific STAT1 antibody and with anti-SINV hyperimmune sera, we can (i) determine whether cells within a particular tissue section are responding to cytokines by activating STAT1 and whether infected cells within the same section are resistant to this response, and (ii) compare the relative ability of S300- and S340-infected brain neurons to inhibit STAT1 phosphorylation and nuclear translocation. In a second analysis, we will compare gene expression profiles within infected brains through an mRNP tagging ribonomics approach by infecting mice with viruses expressing an epitope-tagged poly-A binding protein (PABP) from a second subgenomic promoter(150, 284). Immunoprecipitation of epitope-tagged PABP from extracts of infected brains can differentiate transcripts present within virally-infected cells from transcripts that appear in flow-through fractions representing uninfected cells. Using this approach, we can compare expression profiles from brains of S300- and S340-infected mice and determine whether IFN- α/β - or IFN- γ -responsive genes are (i) downregulated within infected cells specifically, (ii) whether the magnitude of this downregulation differs between S300- and S340-infected cells since we hypothesize that STAT1 inhibition by S300 would result in greater inhibition of ISG transcription, and (iii) whether effects on gene expression are specific or if STAT-independent gene targets are equally reduced. The latter would suggest that downregulation of gene expression is due to virus-induced global transcriptional shutoff. Taken together, these studies directly evaluate whether disruption of Jak/STAT signaling in vivo can explain why S300 is resistant to immune clearance mechanisms allowing it to ultimately cause fatal disease.

Several in vitro studies are currently underway to identify the viral factors required for SINV-mediated Jak/STAT signaling inhibition. The first is designed to determine whether additional mutations that delay nonstructural polyprotein processing kinetics also enhance STAT1 inhibition. Several *ts* lesions that were previously described to abolish or diminish polyprotein processing have

been cloned into the G100 background. Three of these mutants, G100-*ts*17, G100-*ts*18 and G100-*ts*24, encode *ts* lesions mapping to nsP2 that were previously shown to result in failed RNA synthesis due to defective polyprotein processing (120, 121). Two additional *ts* mutants were cloned—G100-*ts*11 which encodes a mutation in nsP1 that gives its temperature-sensitive phenotype, but does not affect polyprotein processing, and G100-*ts*7 which encodes an nsP2 mutation, but has a modest polyprotein processing defect (121). We performed our STAT1 activation experiment described before, except Vero cells were infected with each *ts* mutant, wild-type G100, or wild-type S300 and the viruses were allowed to replicate at 30°C, the permissive temperature. After either 3, 4 or 5 hours of incubation, the cultures were shifted to 40°C to allow polyprotein accumulation. At 8 hours post infection, cells were treated with IFN- β and analyzed as before. As mentioned, studies with G100 mutants encoding *ts* lesions indicated that nonstructural polyprotein precursor accumulation did not result in enhanced STAT1 activation relative to wild-type G100; however, all viruses encoding *ts* lesions within nsP2 (*ts*7, *ts*17, *ts*18 and *ts*24) inhibited STAT1 activation much more efficiently when grown at the permissive temperature for at least 5 hours (Figure 4.2). In future studies, we will have an expanded panel of gain-of-function mutants useful for the identification of common features that result in effective blockade of Jak/STAT signaling.

A second strategy to identify the SINV factor(s) involved with Jak/STAT inhibition involves a comparison between G100 and TR339. The South African isolates AR86 (S300) and Girdwood (G100) share significant homology across the viral nsPs (99% amino acid identity) while the TR339 isolate is more divergent (G100 and TR339 share 97.5% identity). As mentioned, introduction of threonine at nsP1 538 into G100 and TR339 resulted in enhanced STAT1 inhibition, but the relative magnitude of this enhancement was much greater in the case of G100 (G119; Figure 3.7). Therefore, we will attempt to map additional determinants of maximal STAT1 inhibition by using TR339 39ns1 and G119 as parent viruses, and through construction of chimeras, identify whether any additional nsP changes confer the maximal STAT1 inhibition seen during S300 and G119 infection.

A third approach we have undertaken to identify nsP precursors involved with STAT1 inhibition is to express various intermediates outside the context of replicating virus. The G100 nsP123 coding sequence, with or without mutations that abrogate cleavage at the nsP1/2 and/or the nsP2/3 cleavage sites, was cloned downstream of the constitutive chicken β -actin promoter. Expression of these constructs yielded precursors of the expected molecular weight and did not result in decreased expression of co-transfected STAT1-gfp as was seen during expression of VEEnsP2; however, no construct appeared to affect IFN- β -induced STAT1-gfp tyrosine phosphorylation (Figure 4.3). As mentioned earlier in the case of VEEV, this system should be viable since S300 infection inhibits STAT1-gfp activation to a similar degree as it does endogenous STAT1. It is possible that the HA epitope tag fused to the C-terminus of each polyprotein precursor is disrupting its function. Alternatively, since constructs encoding G100 nsP3 were designed to terminate just prior to the Opal termination codon in order to allow readthrough of the HA tag, this may disrupt a putative degradation signal present at the SFV nsP3 C-terminus (324). The establishment of an in vitro system that recapitulates STAT1 inhibition by simply overexpressing viral nsPs warrants further study as it would enable detailed mutagenesis studies that are independent of virus replication and could precisely define required viral domains.

4.3 Conclusions

In summary, the IFN response has long been recognized as critical to the host anti-alphavirus response. The work described in this dissertation has identified a novel mechanism by which alphaviruses antagonize the type I and type II IFN response, advancing our understanding of alphavirus molecular pathogenesis. The mechanisms described are independent of the well-characterized global effects alphaviruses have on host gene expression, which was previously suggested to play the dominant role in type I IFN antagonism. Attenuating mutations within several alphaviruses are known to induce greater type I IFN levels without any reduction in the ability to

shutoff host transcription/translation. Therefore, it will be important to evaluate what effect these attenuating mutations have on Jak/STAT signaling since the SINV nsP1 538 determinant clearly plays a dramatic role. Furthermore, the differences in IFN sensitivities among alphavirus strains is a well-studied phenomenon that was previously proposed to play a role in VEEV emergence and is required for pathogenic VEEV strains to replicate to titers sufficient for mosquito transmission. It will be important to evaluate the relative abilities of these VEEV strains to inhibit Jak/STAT signaling since this mechanism could determine a strain's sensitivity to IFN.

The determinant at nsP1 538 was previously identified and well-studied in our laboratory due to its dramatic effect on adult mouse neurovirulence. In the *in vitro* studies reported here, we independently identified this determinant as a critical factor in efficient STAT1 inhibition. While studies to elucidate the precise mechanism by which VEEV and SINV inhibit Jak kinase and STAT activation are ongoing, this work provides a clear mechanistic picture of one role the nsP1 538 determinant may play in the infected mouse, tying together numerous previous studies. A working hypothesis that can be directly tested using our SINV model of adult mouse encephalomyelitis is that S300 is resistant to clearance from CNS tissues by inhibiting STAT1/2 activation by IFN- α/β and/or by IFN- γ produced by infiltrating T-cells. As a whole, Jak/STAT signaling inhibition could play a major role in alphavirus pathogenesis and emergence, and it will be important to consider during the development of specific therapies and alphavirus-based vaccine strategies.

Figure 4.1: VEEV nsP2 limits expression and activation of STAT1-gfp.

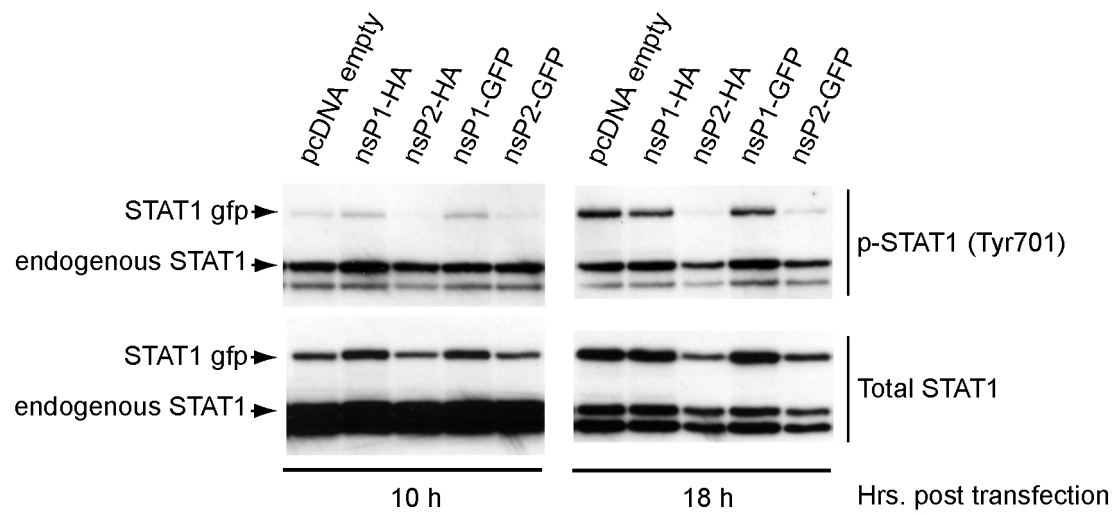


Figure 4.1: VEEV nsP2 limits expression and activation of STAT1-gfp.

pCAGGS-VnsP1 and pCAGGS-VnsP2 constructs expressing each viral protein from the constitutive chicken β -actin promoter and fused to either an HA epitope or green fluorescent protein C-terminal tag were co-transfected with a STAT1-gfp construct. At the given times post transfection, all groups were stimulated with 1,000 U IFN- β /mL for 20 min, at which point extracts were prepared and analyzed by Western blotting as described previously. Blots were cropped to display both STAT1-gfp as well as endogenous STAT1, which were well resolved. As a negative control, pcDNA3.1(+) (Invitrogen) was co-transfected with STAT1-gfp. Similar constructs expressing VEEV nsP3 and VEEV nsP4 reduced neither STAT1-gfp activation nor expression (data not shown).

Figure 4.2: Accumulation of nonstructural polyprotein precursors does not alone confer STAT1 antagonism.

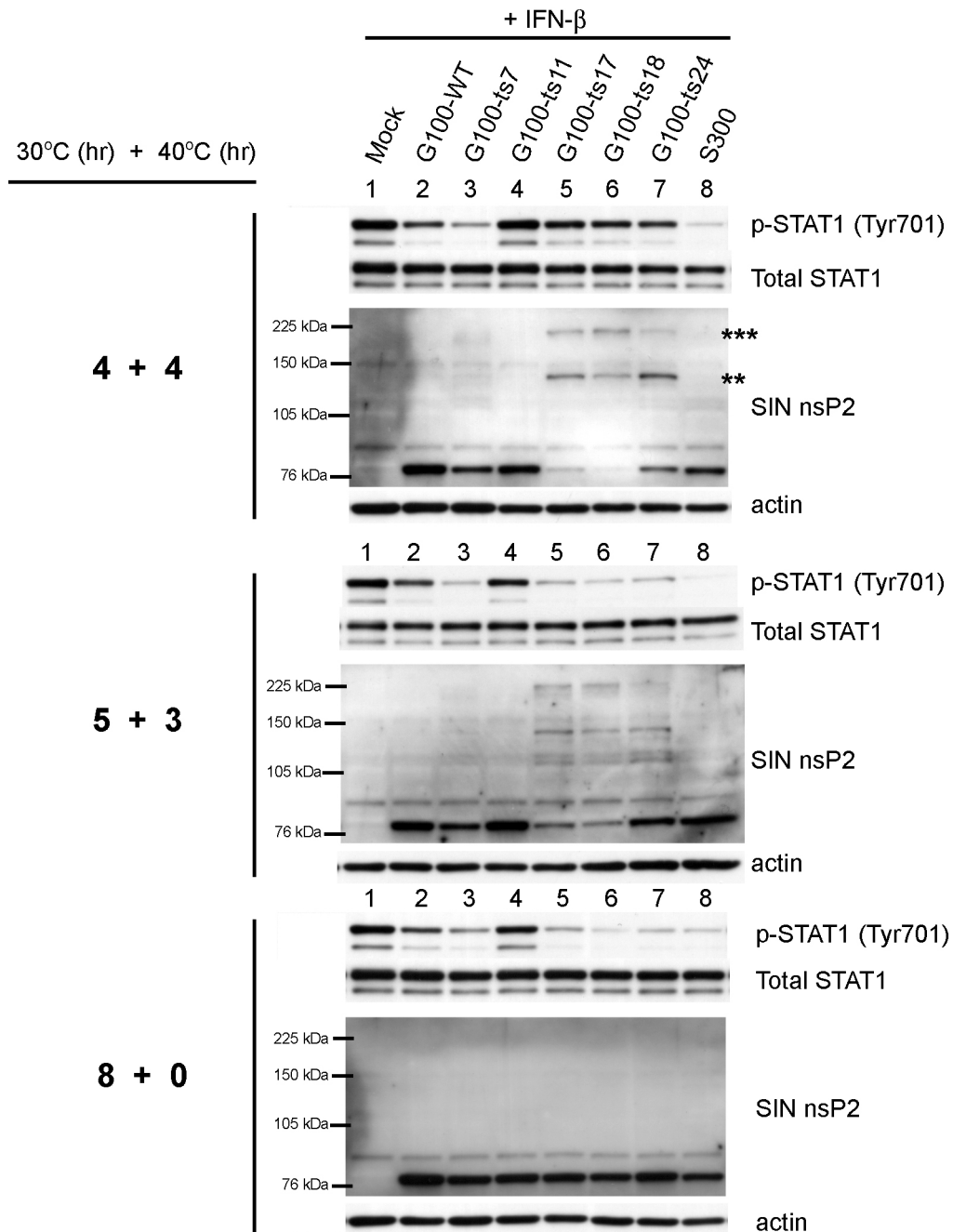


Figure 4.2: Accumulation of nonstructural polyprotein precursors does not alone confer STAT1 antagonism.

Several mutant G100 viruses were constructed to evaluate whether delayed polyprotein processing directly contributed to inhibition of STAT1 activation. Three mutants encoded *ts* lesions within nsP2 that were predicted to abrogate polyprotein processing: G100-ts17 (A517T), G100-ts18 (F509L) and G100-ts24 (G736S). As negative controls, two additional *ts* mutants were constructed. G100-ts7 also encoded an nsP2 mutation (D522N), but this was predicted to have a mild effect on polyprotein processing. Finally, the mutant G100-ts11 encodes a mutation in nsP1 (A348T) that results in a temperature-sensitive phenotype, but through mechanisms independent of polyprotein processing, which occurs normally. Replicate plates of Vero cells were infected with wild-type G100, each *ts* mutant, and wild-type S300 at the permissive temperature (30°C) for either 4 or 5 h, and then shifted to the non-permissive 40°C temperature for the remainder of the 8 h incubation, and then treated with IFN- β (1,000 U/mL) for 20 min. at 40°C. An additional plate was incubated and IFN-treated at 30°C for the entirety of the experiment. After IFN treatment, lysates were prepared and equal total protein from each sample was analyzed by Western blotting as previously described. Each blot was probed for p-STAT1(Tyr701) and actin, and then stripped and reprobed consecutive times with anti-SINV nsP2-specific polyclonal sera and then total STAT1. **, predicted nsP23 precursor. ***, predicted nsP123 precursor.

Figure 4.3: Overexpression of SINV nonstructural polyprotein precursor intermediates fails to inhibit STAT1 activation.

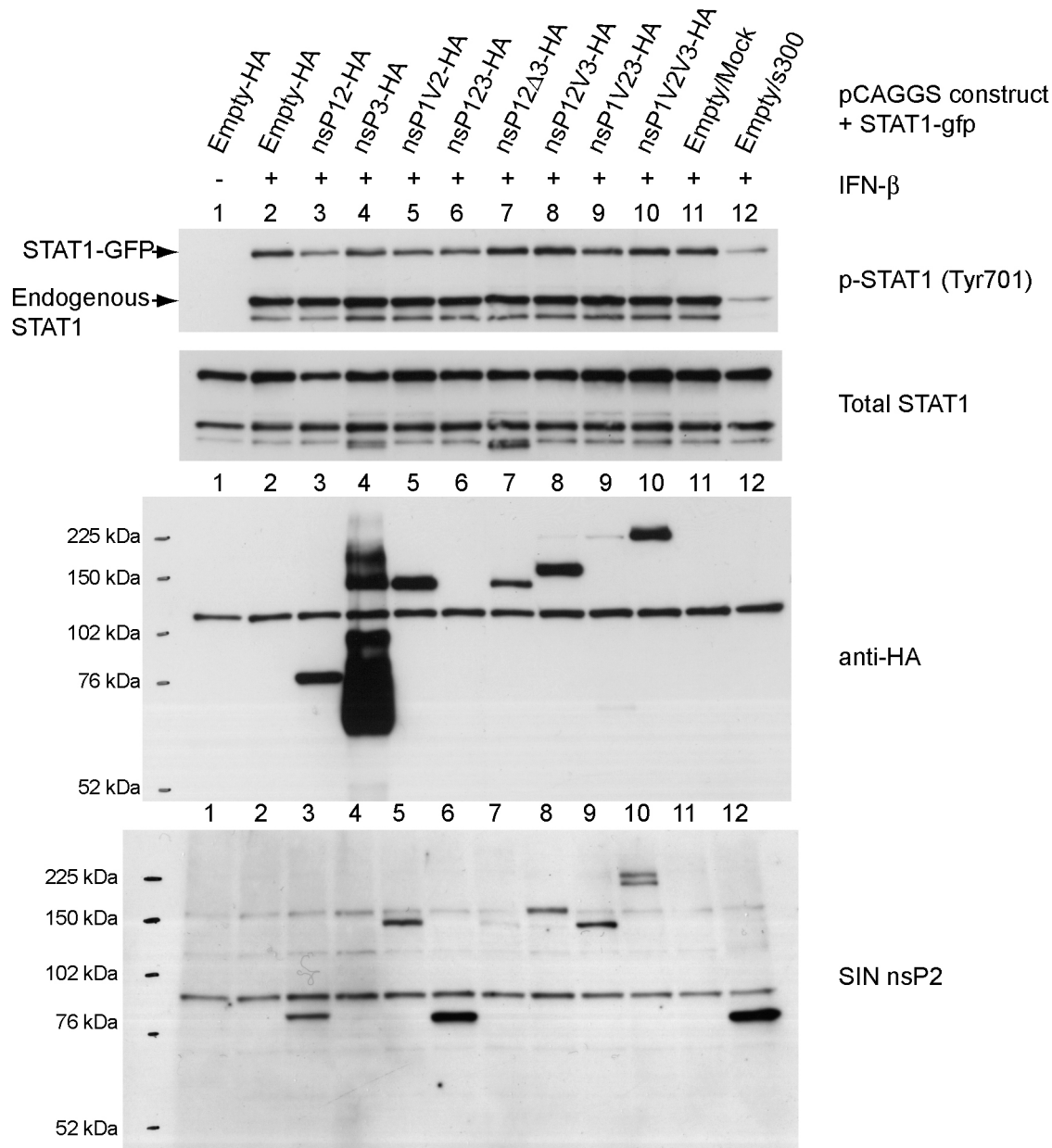


Figure 4.3: Overexpression of SINV nonstructural polyprotein precursor intermediates fails to inhibit STAT1 activation.

A panel of expression constructs were created by cloning G100 nonstructural polyprotein intermediates into the plasmid pCAGGS-HA (Empty-HA) containing a constitutive chicken β -actin promoter and a C-terminal HA epitope tag. Wild-type nsP12, nsP3 and nsP123 were constructed along with several mutants. To prevent nsP2-mediated cleavage at the natural nsP1/2 and nsP2/3 cleavage sites, a mutation of Gly \rightarrow Val was introduced into the P2 position of each site. The constructs nsP1V2-HA, nsP1V23-HA and nsP1V2V3 all had mutations abrogating cleavage at the nsP1/2 site while the constructs nsP12V3 and nsP1V2V3 were incapable of cleavage at the nsP2/3 site. A final construct, pCAGGS-G100nsP12 Δ 3-HA contained an in-frame deletion within nsP2 (amino acids 81 to 727). Each construct was co-transfected with STAT1-gfp and at 17 hours post transfection, cells were stimulated with 1,000 U IFN- β /mL for 20 minutes. Two additional pCAGGS- Empty-HA/STAT1-gfp transfection groups were infected with wild-type S300 (MOI=20 PFU/cell) or diluent (Mock) for 8 h prior to IFN- β stimulation as a positive control (lanes 11 and 12). After IFN- β treatment, cell lysates were prepared and analyzed by duplicate Western blots. The first membrane was probed for pSTAT1(Tyr701) and then stripped and reprobed with total STAT1. The second membrane was probed with anti-HA and then stripped and reprobed with anti-SINV nsP2 polyclonal sera to detect relative expression of expression constructs. All constructs expressed products of the predicted molecular weights.

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