# EVASION AND RESISTANCE OF HOST ANTIVIRAL DEFENSE PATHWAYS BY ALPHAVIRUSES

by

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**Evasion and Resistance of Host Antiviral Defense pathways by Alphaviruses**

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The *Alphavirus* genus consists of positive-sense single stranded RNA viruses which cause millions of annual infections, and are classified as arthritogenic (Sindbis (SINV) and Chikungunya virus (CHIKV)) or encephalitic (Venezuelan (VEEV) and eastern equine encephalitis viruses (EEEV)) based on disease phenotype. The nature, severity and outcome of disease following infection by a particular alphavirus are primarily dependent on evasion of or resistance to the Interferon-α/β (IFN-α/β) upregulated antiviral state. In mice, induced serum IFN-α/β levels are highest following VEEV infection, lower following SINV infection, and negligible after EEEV or CHIKV infection. SINV or CHIKV cause a self-limiting, non-fatal infection in mice with functional IFN α/β responses, suggesting limited antagonism of the IFN-α/β response, while infection with EEEV or VEEV is lethal. Whereas EEEV avoids both IFN-α/β and antiviral state induction via suppression of myeloid cell replication and lymphoid tissue dissemination, VEEV infection is associated with extensive systemic replication that occurs despite rapid induction of serum IFN-α/β, suggesting successful replication despite upregulation of antiviral effectors. However, viral determinants of this differential sensitivity of various alphaviruses to IFN-α/β are unknown.

In this study I have investigated the relative sensitivity of SINV, CHIKV, EEEV and VEEV to the IFN-α/β induced antiviral state, and found that VEEV is most resistant to the activities of antiviral effectors expressed either collectively or individually. I mapped this

activity to the nonstructural protein-expressing region of the genome, and determined that VEEV nonstructural protein 2 (nsP2) induced translation shutoff is critical for the antiviral state resistance of VEEV. I definitively separated virus induced transcription and translation shutoff as independent activities, and identified the viral proteins mediating these activities for multiple alphaviruses. Furthermore, I demonstrated that expression of nsP2 alone is sufficient to block Signal Transducer and Activator of Transcription 1 (STAT1) phosphorylation, and that viral infection induces proteasome-mediated degradation of Janus Kinase 1 (JAK1). Additionally, I demonstrate the role of IRF7 in host antiviral response induction following alphavirus infection. Overall, I have delineated the interactions of multiple alphaviruses with host antiviral mechanisms, and illustrated the different strategies that closely related alphaviruses can use to successfully overcome the IFN-α/β response.

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# PREFACE

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# 1.0 INTRODUCTION

## 1.1 ALPHAVIRUSES

### 1.1.1 Classification of alphaviruses

The *alphaviruses* are a member genus of the family Togaviridae, and consist of over 40 species of single-stranded positive-sense RNA viruses that are widely distributed in temperate and tropical areas of the world. Member species are found on all continents, including Antarctica, and have a wide range of vertebrate reservoir species including rodents, birds, primates, marine mammals and fish (1-4). Alphaviruses can be classified into seven antigenic complexes based on testing cross-reactivity of antibodies raised against different viruses in Hemagglutinin (HA) inhibition tests (2). Most alphavirus species are transmitted between vertebrate hosts by arthropod vectors (4, 5), primarily mosquitoes, but also mites and lice (6), and specificity between arthropod vector and virus likely limits the geographic dispersal of a particular alphavirus. Furthermore, barring mutations to change virulence or vector specificity (7-12), the virus-vector relationship in large part determines the frequency and type of infected vertebrate species and a particular virus' propensity to cause epidemics or spread out of established endemic areas.

Historically, alphaviruses have been separated into Old and New World groups based on geographic distribution, with Old world alphaviruses such as Sindbis virus (SINV), Chikungunya virus (CHIKV) and Semliki Forest virus (SFV) endemic to Asia and Africa, and New World alphaviruses such as Venezuelan equine encephalitis virus (VEEV), eastern equine encephalitis virus (EEEV), western equine encephalitis virus (WEEV) and Mayaro virus found in the Americas (2). It is likely that several genetic recombination events between Old and New World alphaviruses have occurred throughout the evolutionary history of alphaviruses. For example, WEEV nonstructural proteins and capsid are closely related to EEEV, whereas the structural glycoprotein regions appear to be derived from SINV and/or SFV (2, 4, 13). More recently, alphaviruses have been grouped by disease phenotype into arthritogenic and encephalitic disease causing alphaviruses (14-16). Infection with arthritogenic viruses such as SINV and CHIKV results in a fever and rash with mild to severe arthralgia and arthritis which may persist and periodically recur in infected patients for many years following infection. In contrast, encephalitic virus infection results in fever which can progress to lethal encephalitis, and frequently leaves neurologic sequelae and long-term damage in survivors. In general, lethality following alphavirus infection ranges from rare (<1%, generally associated with co-morbidities) in the case of arthritogenic alphaviruses (15, 17) to moderate (1-5%) and severe (30-70%) following encephalitic alphavirus infection (14, 18-21). However, humans and other large mammals are usually dead-end hosts and unimportant for sustenance of the sylvatic or epidemic infection cycle of most alphaviruses.

### 1.1.2 Virion Structure and genome organization

The alphavirus virion is a 60-70nm diameter particle exhibiting icosahedral (T=4) symmetry (22) and consists of a nucleocapsid surrounded by a lipid envelope derived from infected cells (4, 23). The nucleocapsid is comprised of 240 units of the capsid protein and encloses a single molecule of the viral genome (24). The glycoproteins E1 and E2 are embedded in the lipid bilayer in the form of 80 glycoprotein spikes consisting of trimers of an E1-E2 heterodimer (25, 26), with the E2 component of each heterodimer interacting with an underlying capsid unit (27, 28). The enclosed viral genome does not assume a structured configuration in the virion (29).

The alphavirus genome (Fig. 1, (30)) consists of a single positive-sense single-stranded RNA molecule approximately 12 Kb in size (4) that mimics messenger RNA (mRNA), bookended with a Type-0 7-methylguanalyte cap at the 5’ end (31, 32) and a poly (A) tail at the 3’ end (33). Replication is biphasic as a consequence of the structure of the genome, with the initial two-thirds encoding the nonstructural polyprotein open reading frame (ORF) and the latter third the structural polyprotein ORF which is expressed from a subgenomic promoter (4, 34). The polyproteins are cleaved into individual viral proteins over the course of the replication cycle by both viral and cellular proteases (Fig. 1).

The specificity of genomic RNA replication is achieved by recognition of four cis-acting conserved sequence elements (CSEs), which are highly conserved in their secondary structures across all alphaviruses (35). The 19-nucleotide CSE at the 3’ end of the genome immediately proximal to the poly (A) tail is the promoter for negative-strand synthesis (36). Similarly, the complement of the CSE at the 5’ end of the genome acts as the promoter for positive-strand synthesis in the negative strand (36, 37). There is a transcription enhancing CSE ~51-nucleotides long in the nonstructural protein 1 (nsP1) coding region of the genome (38, 39). Finally, a 24-

nucleotide CSE at the start of the subgenomic RNA region is the translation promoter and enhancer for expression of the structural polyprotein in the subgenomic RNA itself, while its complement in the negative strand is the promoter for synthesis of the subgenomic RNA (40-42).

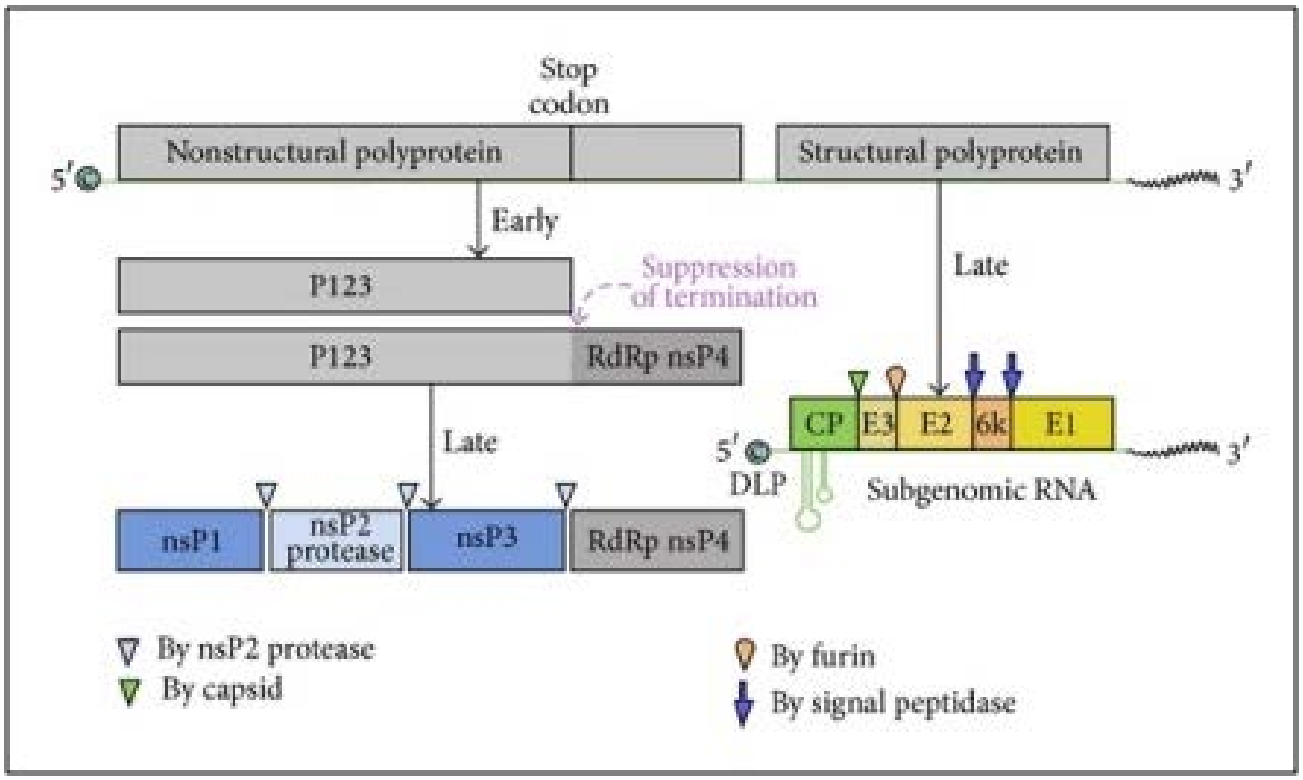



**Figure 1. Structure of the alphavirus genome (adapted from (30)).**

### 1.1.3 Viral Replication Cycle

The alphavirus replication cycle (Fig. 2, (35)) is a highly regulated and ordered process, and slight variations can lower the efficiency of replication as well as alter the efficacy of the host response to infection. As described in more detail below, replication commences with attachment and entry into the cell, followed by translation of the nonstructural polyprotein, the components of which synthesize viral RNA. The negative strand is initially synthesized, followed later in the

infection by the positive strand and the subgenomic mRNA which encodes the structural proteins. The cycle completes with packaging of the genome into the nucleocapsid and budding of new virions (4).

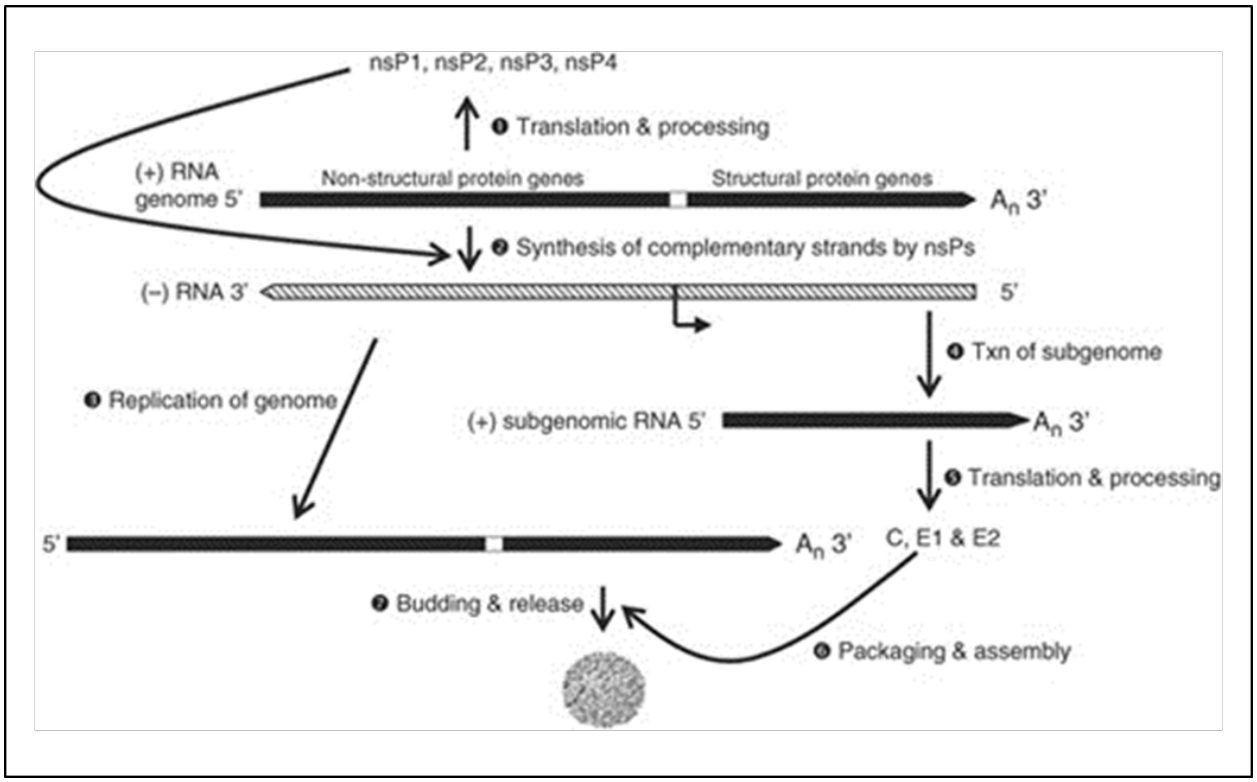



**Figure 2. The alphavirus replication cycle (adapted from (35)).**

### 1.1.3.1 Attachment and Entry

The alphaviruses have a broad host range and can infect a large number of cell types *in vitro*, necessitating the use of either different receptors to enter various cell types, the use of a receptor common to all cell types, or some combination of both strategies (4). The glycoproteins E2 and E1 are responsible for attachment and entry into the target cell. Following E2 binding with the target receptor, the glycoproteins undergo conformation changes and the virion undergoes clathrin-mediated endocytosis into the cell (43). Subsequent acidification of the endosome

disrupts the E2-E1 heterodimer, thereby exposing the fusion peptide in E1 which inserts into the endosomal membrane (44). Fusion of the viral and endosomal membranes is dependent on the presence of the sterol 3B hydroxyl group of cholesterol (45-47) in vertebrate, but not invertebrate, cells. Following fusion, the nucleocapsid is released into the cytoplasm, possibly destabilized by the endosomal acidic conditions and/or by competitive binding of ribosomal RNA at the genome binding site of capsid (48-50). Subsequently, the viral genome is released into the cell to begin the process of replication (43).

#### 1.1.3.2 Expression and function of nonstructural proteins

The alphavirus genome mimics host cell mRNA molecules and is translated upon entry into the cytoplasm. Translation of the viral genome nonstructural ORF produces two polyproteins, P123 and P1234, which are further cleaved into the individual nonstructural proteins 1-4 (nsP1, nsP2, nsP3 and nsP4) (4). An opal stop codon (UGA) between nsP3 and nsP4 (51) is read through at a frequency of 10-20% which balances the ratio of P123 and P1234 levels and is important for successful replication (4, 52). This opal stop codon is found in most, but not all alphaviruses (53, 54), and removal or mutation of this codon decreases viral replication and may affect viral fitness in mosquito vectors (55, 56). The nonstructural polyproteins are cleaved by the nsP2 viral protease in a strict order which controls the replication process (57, 58). All nonstructural proteins are involved in all stages of viral RNA synthesis.

The alphaviral nonstructural proteins are multifunctional, though all functions have not been elucidated thus far. NsP1 has been identified as the protein with Guanine-7-methyltransferase and guanylyl transferase activity that is critical for capping the 5’ ends of viral RNA molecules (59). The nsP1 mediated capping process differs from the mechanism used by host cells to cap host mRNA molecules (60). NsP1 is associated with membranes, mediated by

palmitoylation of the residue Cys420, although a secondary structure (amino acids 245-264) can also interact with the lipid bilayer (61-65). Membrane association anchors replication complexes to membranes and is likely required for efficient RNA synthesis.

The protein nsP2 exhibits NTPase, helicase and RNA triphosphatase activities associated with its N-terminal domain, each of which are required for RNA synthesis (66-69). The C-terminal domain contains a papain-like serine protease that cleaves the nonstructural polyprotein, the order of cleavages kinetically and temporally controlling viral replication (70-72). In addition, the nsP2 of Old world, but not New world, alphaviruses possess nuclear localization and export sequences which transfer ~50% of nsP2 into the nucleus (73, 74). Similarly, nsP2 has been implicated in the disruption of host transcription, translation and IFN signaling depending on the virus studied (74-77), and is speculated to interact with a variety of host proteins to modulate viral-host interactions.

The function of nsP3 is unknown outside of its role in viral RNA synthesis. The C-terminal domain has several serine and threonine residues that are phosphorylated during infection, the significance of which is unknown (78-80). NsP3 has recently been shown to interact with G3BP and inhibit stress granule assembly during infection (81, 82).

The alphaviral RNA-dependent RNA polymerase (RDRP) is nsP4, the C-terminal of which contains the replicase domain as well as terminal adenylyl transferase activity required for poly (A) tail addition to the ends of viral RNA molecules (83-85). The level of nsP4 is highly regulated during infection by several mechanisms (55). First, the ratio of P1234 to P123 is low, which has a negative impact on replication if increased (4). In addition, the N-terminus consists of a tyrosine residue, which facilitates degradation of free floating, but not replication complex

associated nsP4 via the N-end rule pathway, limiting nsP4 to exclusively viral replication complexes (86-89).

#### 1.1.3.3 Genome synthesis

The production of nsPs begins viral RNA synthesis. Three species of alphaviral RNA are produced; the positive strand, the negative strand and the subgenomic RNA strand, the production of each of which is regulated tightly by the levels and order of cleavages of the nonstructural polyproteins (72). The negative strand is produced first, followed by the positive strand and the subgenomic RNA (4). Replication occurs on the cytoplasmic surfaces of cellular endosomes and lysosomes, in specialized viral induced structures termed cytopathic vacuoles which contain the viral nonstructural proteins (90, 91). Functional replication complexes are formed shortly after infection, and their number stabilizes following the initial burst of negative and positive strand RNA synthesis (92-94).

Negative strand synthesis begins at the 3' CSE after initial production of the nonstructural polyproteins P1234 and P123, and strictly requires P123 and nsP4 (95). P1234 is cleaved in *cis*, or *trans* by other P1234 or P123 molecules to produce the negative strand replication complex P123-nsP4 (4, 95, 96). Negative strand synthesis also requires continuous viral protein production, likely due to the continuous cleavage of P123 into constituent nsPs with increasing levels of viral protein production (4). The level of negative strand is 2-5% of the amount of positive strand (97), and the CSE at the 5' end of the negative strand initiates positive strand and subgenomic RNA synthesis.

As the levels of nonstructural proteins increase, the *trans* cleavage of P123 or P1234 in a bimolar reaction produces nsP1, P23 and nsP4, but cannot cleave P23, which begins the switch from negative to positive strand and subgenomic RNA synthesis 3-4h following infection (95,

98). P23 is subsequently cleaved into nsP2 and nsP3 in *trans* by P23 or P234, producing a replication complex consisting of individual nsP1/2/3/4 proteins that exclusively synthesize the positive strand and subgenomic RNA (96). Finally, the level of nonstructural proteins increases to the point where P123 and P1234 cannot exist, with cleavage of the nsP2-nsP3 bond occurring concurrent with synthesis to produce P12, nsP3 and P34. P12 undergoes inefficient auto-proteolysis or *trans* cleavage by P12 to produce nsP1 and nsP2, and P34 plays no role in viral RNA synthesis, as its cleavage requires a nonstructural protein intermediate containing both nsP2 and nsP3, which are individual proteins by this stage of infection (4). The rate of viral RNA synthesis stabilizes at this stage of infection, and no new replication complexes are produced or assembled (4).

#### 1.1.3.4 Assembly and Budding and expression of structural proteins

The subgenomic RNA is synthesized from the CSE found at the termination of the nonstructural coding region in the negative strand (42). Levels of the subgenomic RNA are 5-20 fold higher than the genomic RNA (35), and encode the structural polyprotein which consists of capsid, PE2, 6K and E1 proteins. Additionally, expression of the structural polyprotein is enhanced by the presence of a secondary structure between nucleotides 77-139 (99). The secondary structure of the translation initiation site in the subgenomic RNA has a reduced requirement for eukaryotic initiation factors (eIFs), notably eIF4G and eIF4A (100-102), believed to help the subgenomic RNA resist the effects of alphavirus induced transcription and translation shutoff (103). Assembly and budding begin following expression of structural proteins.

The alphavirus structural proteins are primarily involved in the assembly and budding of new virions, although capsid of New World alphaviruses (e.g. VEEV, EEEV) can inhibit host transcription (104-106). The capsid protein is the sole constituent of the alphavirus nucleocapsid.

The C-terminal contains a chymotrypsin-like serine protease that cleaves capsid from the structural polyprotein immediately following translation. The terminal tyrosine residue of capsid remains in the cleavage site and prevents further trans-cleavage activity (107-109). The packaging signal in most alphaviral genomes is located in the nsP1 coding region, and is recognized by the N-terminal domain of capsid. Packaging of viral genomic RNA is specific and efficient, dependent on the secondary structure of the packaging signal, although mutants exist which can target heterologous RNA for packaging (110-115). Alphaviruses exclusively package genomic RNA with rare exceptions (Aura virus) that can also package subgenomic RNA (116-118) . Assembly of nucleocapsid is catalyzed by RNA-capsid binding, and assembled cores locate to the plasma membrane by binding to the cytoplasmic tail of E2 (4, 119, 120).

Following proteolysis of capsid, the glycoproteins translocate into the endoplasmic reticulum (ER), mediated by signal sequences at the N and C-terminals of PE2 and the C-terminal of 6K (4, 27, 121). 6K is cleaved from PE2 and E1 in the ER by signal peptidase (27), and PE2-E1 heterodimerize and traverse the trans-Golgi network (25), where E3, the leader peptide of PE2 is cleaved by furin and discarded in most alphaviruses (26, 122, 123). The E2-E1 heterodimer is transported to the plasma membrane following post-translation modifications (124, 125), where the cytoplasmic domain of E2 interacts with and binds to the nucleocapsid to initiate virion budding.

Viral budding occurs at the plasma membrane and requires interaction between the cytoplasmic domain of E2 and hydrophobic pockets in individual capsid proteins in the assembled nucleocapsid, although most of the budding process itself is driven by lateral interactions between neighboring E2-E1 heterodimers (126-129). Glycoproteins expressed alone do not form virus-like particles (VLP) efficiently, demonstrating a need for capsid-E2 interaction

during budding (130). Budding requires cholesterol (131, 132) and may be enhanced by the activity of 6K, though few copies of this protein are integrated into the virion (124, 133). Released particles are fully mature and capable of immediately infecting cells.

#### 1.1.3.5 Non-replicating Replicons

The genome structure and replication cycle of alphaviruses has been exploited for use as expression vectors (Fig. 3) in which the structural protein coding region of the genome can be replaced with non-viral genes of choice (134-136). Replicons lack viral structural proteins and are incapable of propagation following the initial round of infection, and are packaged with helper RNAs expressing structural proteins which can be manipulated to target specific cell types (42, 137). Expression of encoded genes of interest is high as the number of subgenomic RNA molecules produced is 5-20 fold higher than the genomic RNA (35), and multiple subgenomic promoters can be added in series to express several genes of interest from the same replicon. In this study, we have used replicons to map different viral activities, such as transcription and translation shutoff and STAT1 signaling inhibition to different regions of the genome.

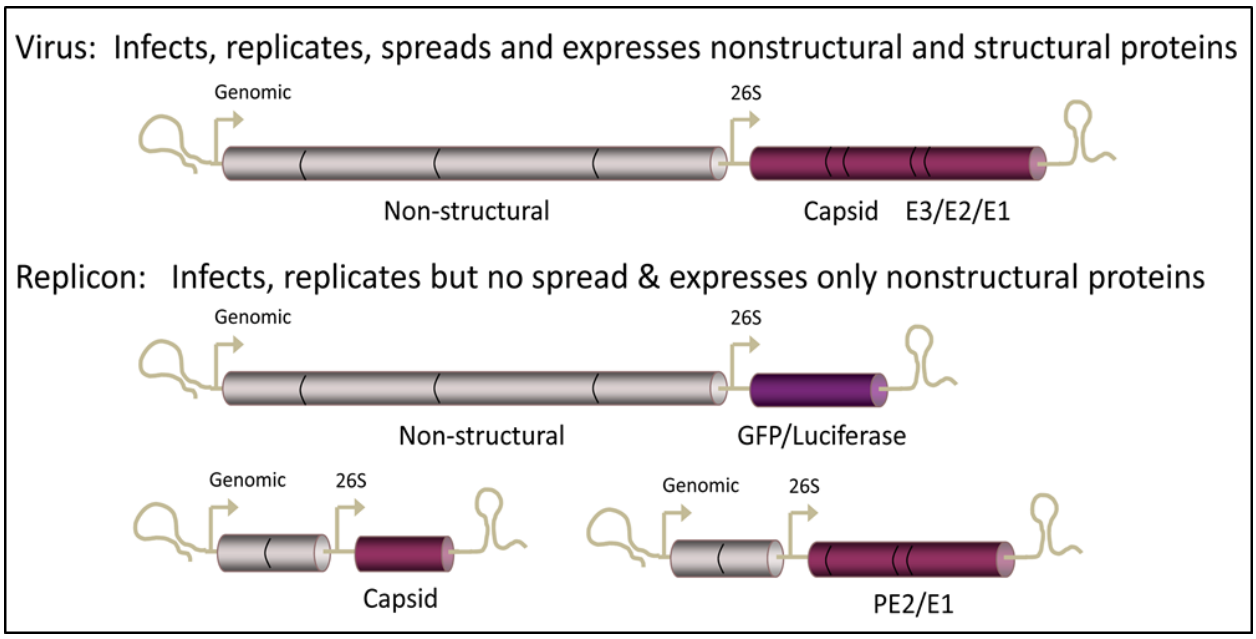



**Figure 3. Alphavirus replicon genome structure.**

## 1.2 ALPHAVIRUS PATHOGENESIS

### 1.2.1 Cellular Receptors

The receptor or receptors mediating alphavirus entry into target cells have not been definitively identified. Alphaviruses have a broad host range and can infect multiple cell-types *in vitro*, suggesting the use of a receptor common to all infectable cell types or a different receptor on each cell type, binding to which may be mediated by conformation changes in E2. Several receptors promoting entry have been identified for different alphaviruses, including the high affinity laminin receptor on baby hamster kidney (BHK) cells (SINV) and mosquito cells (SINV and VEEV (138, 139)) and DC-SIGN and L-SIGN on dendritic cells (SINV (140)), although these candidates are not required for entry in all cell types.

The role of heparan sulfate (HS), a highly negatively-charged glycosaminoglycan constitutively expressed on most cell types, in alphavirus entry and pathogenesis has been explored recently. Laboratory passaged/adapted strains of most alphaviruses (SINV, CHIKV, VEEV etc.) acquire positive-charge mutations in E2 that efficiently bind HS and increase infectivity *in vitro* compared to wild-type strains which do not bind HS or bind very weakly (141-145). In marked contrast, infection of mice with HS binding strains attenuates virulence, whereas wild-type strains remain virulent, although HS binding can promote viral spread in specific tissues such as the brain (145-147). Notably among alphaviruses, neurovirulent naturally circulating EEEV strains use HS as an attachment receptor, and amelioration of HS binding leads to induction of a prodromal disease, cytokine production, decreased brain replication and increase in average survival time (AST) of infected mice (148).

### 1.2.2 Infection of Vertebrate cells

Alphaviruses can successfully replicate in most vertebrate cells, with virion release occurring 4-6h post-infection (p.i.). Infected cells display evidence of cytopathic effect (CPE), with extensive observed blebbing, shrinkage and rounding (149, 150). Alphavirus infection leads to extensive modulation of the intracellular environment, highlighted by establishment of host transcription and translation shutoff (76), loss of membrane potential (151) and depletion of nicotinamide adenine dinucleotide (NAD) (152). Cell death in most cell types occurs by viral induced apoptosis between 24-48h p.i., with viral budding continuing from apoptotic blebs (153). However, certain cell types such as mature neurons can undergo necrotic cell death (154, 155). Apoptosis may enhance replication, as overexpression of members of the B-cell lymphoma 2 (BcL-2) family of anti-apoptotic proteins or pharmacologic inhibition of caspases reduces viral

yield from infected cells (156-159). Super infection of cells with multiple alphaviruses is unsuccessful due to nsP2 mediated cleavage of P123 and P1234 translated from the genome of the super-infecting virus, which prevents negative strand synthesis replication initiation.

### 1.2.3 Infection of Invertebrate cells

Alphavirus infection has primarily been studied in mosquito cell lines derived from *Aedes albopictus* and *Aedes aegypti*. Unlike infection in vertebrate cell lines, SINV or SFV establish a persistent, noncytopathic infection in mosquito cells, characterized by eventual nonapoptotic pathway mediated cell death (160-162). The mechanism of persistence is unknown, though linked to intracellular factors in mosquito cells that restrict viral replication (163).

### 1.2.4 Pathogenesis *in vivo*

Alphavirus pathogenesis in vertebrates begins with infection of a vertebrate host by a virus-carrying vector, most commonly mosquitoes. Subcutaneous virus extravascular deposition leads to uptake by sentinel immune cells (dendritic cells, Langerhans' cells etc.) and transport to the nearest draining lymph node (164, 165), where infection of cells produces a serum viremia which systemically seeds viral replication sites distant from the site of inoculation (164, 166-169). Replication in peripheral tissues seeds a high-titer viremia which may be picked up by uninfected vectors in a blood meal to continue the infection cycle (147), although the reasons why some species can infect new mosquitoes and others cannot is unclear. Many alphaviruses infect the brain and replicate in neurons (164, 170, 171), the severity encephalitic disease being greatly dependent on sensitivity of the virus to innate antiviral mechanisms. Infection of myeloid tissues

upregulates antiviral cytokines and induces an antiviral state in sites distant from the initial infection site, resistance to which greatly affects the extent of peripheral and brain replication, and ultimately determines the outcome of infection (35).

### 1.2.5 Pathology and Virulence in murine models

Mouse models for both arthritogenic and encephalitic alphaviruses exist and are extensively used to study alphavirus pathogenesis *in vivo*. A primary determinant of virulence in murine models is the ability of alphaviruses to resist or avoid antiviral effects of induced IFN-α/β (35).

Infection of adult mice competent at induction of IFN-α/β responses with SINV is avirulent, whereas neonatal infection is fatal, characterized by disseminated replication in lymphoid tissues, muscles and skin, with induction of high levels of inflammatory cytokines and death caused by systemic inflammatory response syndrome (SIRS) like disease (172-174). Avirulence in adult mice has been ascribed to restriction of replication in peripheral tissues, in particular myeloid lineage cells that are initially infected in the draining lymph node, and dependent on functional IFN-α/β responses (172, 173, 175).

The induction and severity of disease after infection of adult mice with CHIKV is age-dependent, with fatal infection of neonates and mild musculoskeletal inflammatory disease observed in adults with competent IFN-α/β responses (176-178). Additionally, severity of disease is greatly exacerbated by IFN-α/β or STAT1 deficiency. Infection of adult mice results in replication in muscle, joint and skin causing self-limiting arthritis, myositis and rheumatic disease associated with macrophage and natural killer cell infiltration into sites of replication, which are important for control and clearance of virus (179, 180), while infection of neonates is

more severe at these sites and proceeds to neuroinvasion, with fatality dependent on age and presence of IFN-α/β responses (177, 180)

Regardless of age, infection of mice with VEEV causes a fatal biphasic febrile and encephalitic disease, which mimics observations made in severe human cases of VEEV infection (181, 182). Infected dendritic cells travel to lymph nodes where robust replication seeds a serum viremia that infects both lymphoid and non-lymphoid tissues in the periphery, causing widespread cell death and lymphocyte destruction (165, 183, 184). VEEV subsequently crosses the blood brain barrier either through infection of the olfactory bulb, or directly via cytokine mediated permeabilization (for example, by tumor-necrosis factor-α) of the barrier (185, 186). Neurons are the primary cell type infected in the brain leading to widespread destruction, encephalitis and death (184, 187).

EEEV infection of mice is fatal, although few signs of a prodromal disease are observed following infection (148). Reflecting restriction due to HS binding and microRNA suppression of translation in hematopoietic cells, infection of myeloid cells in the draining lymph node is largely avoided, and the virus instead replicates in muscle and bone endosteum and periosteum osteoblast-lineage cells, which seeds a low-titer viremia leading to neuroinvasion, potentially via direct crossing of the blood-brain barrier (188, 189). Replication in the brain is extremely efficient and leads to widespread destruction of neurons and eventual death (188, 189). As with the effects of VEEV infection, the lack of prodrome and onset of severe encephalitis in mice is reflective of human disease caused by EEEV.

## 1.3 IFN-A/B INDUCTION AND SIGNALING

The IFN-α/β system is an innate immune mechanism important for resistance, control and clearance following infection with a wide range of pathogens. Control and clearance of viral infections by IFN-α/β is well documented (190-194). Specifically, induction of IFN-α/β responses, and relative resistance of alphaviruses to the IFN-α/β system largely determines the outcome of infection in both *in vitro* and *in vivo* model systems (35, 173, 195-197). The IFN-α/β induction and signaling pathway components are targeted by various pathogens to suppress IFN-α/β signaling and upregulation of an IFN induced antiviral state (198-203). Here, IFN-α/β induction and signaling following RNA virus infection is briefly reviewed.

### 1.3.1 IFN-α/β induction

IFN-α/β can be induced by multiple cellular pathways (Fig. 4, (204)). Pattern Recognition Receptors (PRRs) found in the cytoplasm and the endosome can detect invading viral RNA and trigger signaling cascades to upregulate IFN-α/β production. Cytoplasmic RNA-dependent PRRs include Protein Kinase R (PKR), Melanoma Differentiation-Associated protein 5 (MDA5), and Retinoic acid-inducible gene 1 (RIG-I) which are constitutively expressed in most cell types (205-207), whereas endosomal PRRs (Toll like receptors 3, 7, 8, C-type lectins etc.) are only expressed by specific subsets of immune cells (208, 209). TLR7 is expressed primarily and constitutively in plasmacytoid dendritic cells (210, 211), whereas certain C-type lectins are expressed in dendritic cells and macrophages (212).

Detection of double stranded viral RNA species by PKR, TLR3, RIG-I or MDA5, or lipopolysaccharide by TLR4, or single stranded RNA species by TLR7/8 activates IRF3,

mediated by TBK1 and IKKε dependent phosphorylation (213-215). Activated IRF3 translocates to the nucleus and triggers transcription of multiple genes, including IFN-β, IFN-α4 and IRF7 (216, 217). Alternatively, IRF7, which is constitutively expressed in macrophages and dendritic cells, can also be phosphorylated and activated by PRRs upon ligand binding, leading to IFN-α/β induction from myeloid cells (218-221). PRR genes are also targets of IRF3 and IRF7 and their levels are further upregulated by activated IRF3 and IRF7, leading to a second wave of IFN production consisting primarily of various IFN-α subtypes (222). Secreted IFN-α/β can further signal in an autocrine or paracrine manner to induce antiviral effector molecules.

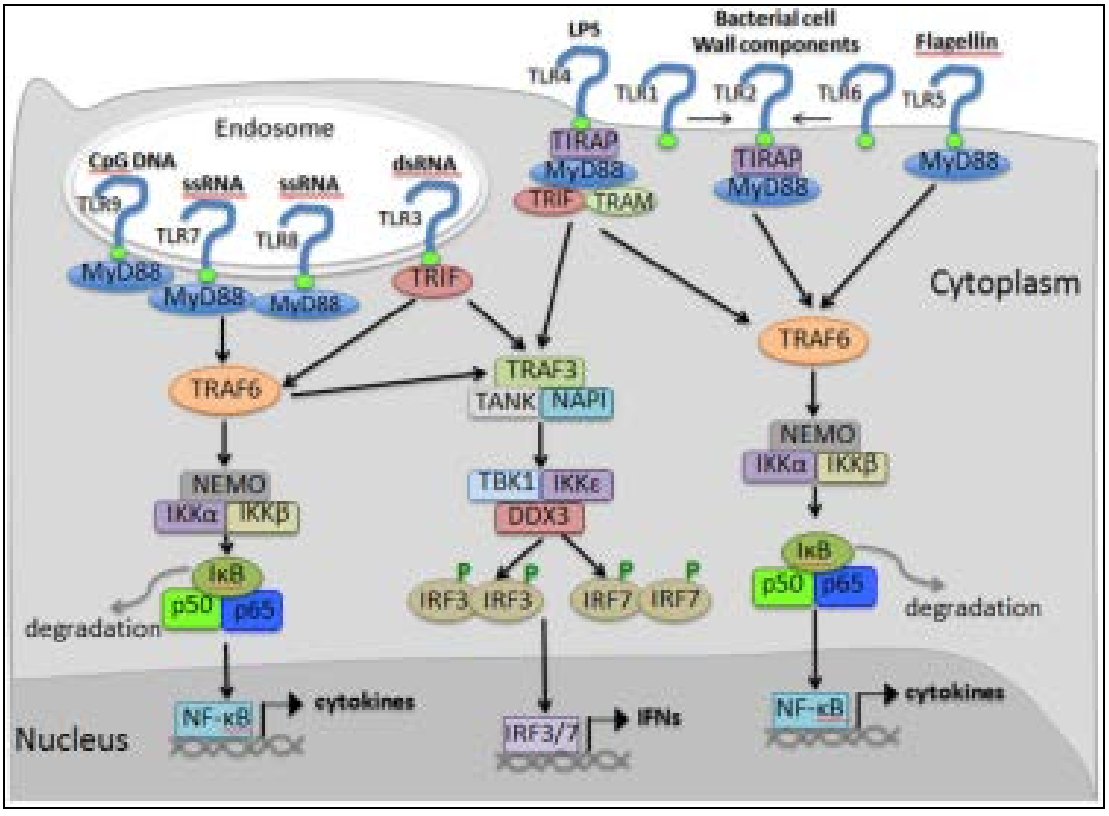



**Figure 4. Type-1 IFN induction pathways (adapted from (204)).**

### 1.3.2 IFN-α/β signaling cascade

The Interferon-α/β receptor (IFNAR) is a heterodimer consisting of two subunits, IFNAR1 and IFNAR2, and is localized at the cell surface (223). The proteins JAK1 and Tyk2 bind to the IFNAR complex and facilitate signaling following IFN-α/β binding to the receptor (224). Signaling begins with high affinity ligand binding to IFNAR (Fig. 5, (225)) (226), which undergoes a structural reconfiguration to bring the two receptor chains into close proximity. Subsequently, JAK1 and Tyk2 undergo activation by transphosphorylation, and further phosphorylate IFNAR1 and IFNAR2 at conserved tyrosine residues. Phosphorylated IFNAR binds to STAT1 and STAT2 via Src homology domain 2 (SH2) binding of phosphorylated tyrosine, and JAK1/Tyk2 subsequently phosphorylate STAT1 and STAT2 on conserved tyrosine residues (227, 228). Phosphorylated STAT1 and STAT2 are released from the receptor complex and heterodimerize, exposing a nuclear localization signal (NLS), and bind to a single molecule of interferon regulatory factor 9 (IRF9) to form the ISG factor 3 (ISGF3) complex (229, 230). ISGF3 translocates to the nucleus where STAT1 is further phosphorylated on a conserved serine residue (231, 232), which promotes DNA binding and activates the transcription of several hundred Interferon stimulated genes (ISGs) via Interferon stimulated regulatory elements (ISREs) in the promoter regions of ISGs (233, 234). ISGs consist of a diverse range of gene products categorized into numerous groups based on potential functions, including immune modulators, adhesion molecules, RNA binding, translation inhibitors, protease inhibitors (235-237), which collectively can target all stages of the viral life cycle including entry, genome translation, replication, packaging and egress to prevent successful viral replication. Production of ISGs results in establishment of a potent antiviral state in cells.

In addition, an important mechanism by which Type-1 IFNs mediate antiviral activity is through the activation of natural killer (NK) cells. NK cells, a component of innate immunity, are important factors for control of infection and neoplasia through both secretion of cytokines (such as IFN-γ) and cytolysis of target cells (238, 239). Type-1 IFNs have been shown to regulate NK cell activation and enhance cytotoxicity in response to both tumor proliferation and viral infection (240-243). Several studies have been performed to explore the role of NK cells during infection with both arthritogenic and encephalitic alphaviruses. Infection of mice with Ross River virus (RRV) or CHIKV triggers virus-mediated inflammation of joint tissues with NK cells being a major component of the infiltrate, independent of adaptive immune system activity. NK cells have also been detected in the synovial exudates of human patients infected with RRV or CHIKV (179, 244-246), and NK cell mediated synovial tissue injury and cytotoxicity may play a role in the persistent inflammation and arthritis observed in RRV or CHIKV infected patients, although specific mechanisms have been poorly characterized.

Infection of mouse models with alphaviruses capable of causing encephalitic disease also suggests that NK cells are important mediators of observed neuronal immunopathology (247, 248). Encephalitic disease in C3H/HeN mice infected with TC-83, a vaccine strain of VEEV, was observed only in the presence of NK cells, suggesting the importance of these cells in inducing a pathogenic host response during infection. Similarly, wild-type B6 mice infected with virulent SFV strains displayed increased mean time to death after NK cell, but not Tc-cell, depletion when compared to mice with normal NK cell activity (248), suggesting a role for NK cell mediated immunopathology during lethal SFV infection in mice. Overall, data suggest NK cells may play a detrimental overall effect and contribute to pathology following alphavirus infection *in vivo*.

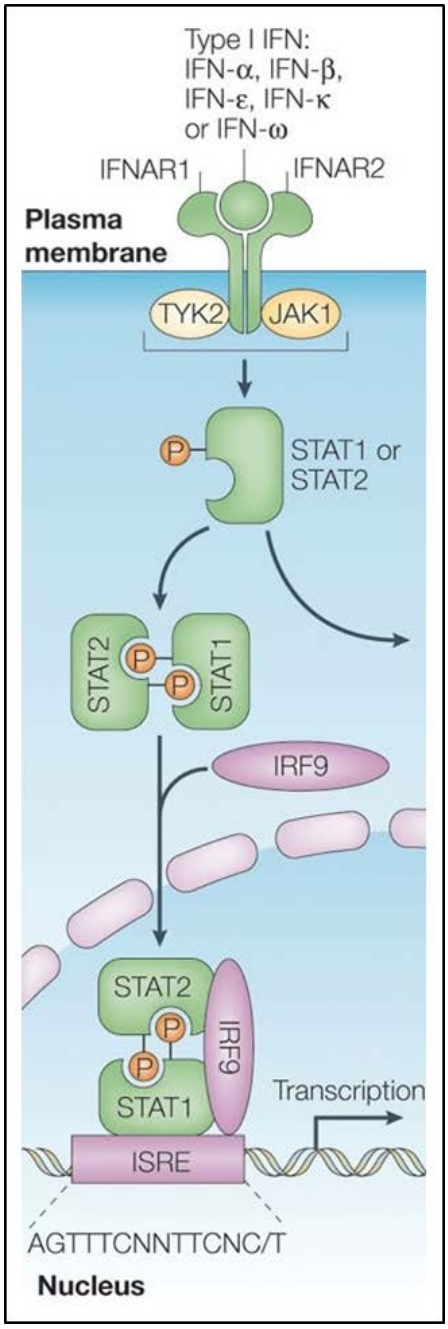



**Figure 5. The Type-1 IFN signaling pathway (adapted from (225)).**

## 1.4 HOST-VIRUS INTERACTIONS FOLLOWING ALPHAVIRUS INFECTION

Infection of permissive cells with alphaviruses can inhibit IFN-α/β signaling and global host macromolecular synthesis shutoff, including inhibition of both host transcription and translation. The role of these activities in the suppression of, and resistance to, induction and downstream effectors of host antiviral mechanisms during infection is currently an active area of study.

### 1.4.1 Alphavirus infection and host transcription

To potentially suppress host cell antiviral responses, alphaviruses can shutoff global host transcription during infection, dependent on virus and cell type. The viral proteins that mediate this activity differ between Old and New world alphaviruses. The nsP2 of SFV, SINV and CHIKV (75, 106, 249) can shut off host transcription, whereas capsid is responsible for this activity during VEEV and EEEV infection (104-106, 250).

Mutations in SINV and CHIKV nsP2 and VEEV capsid have been described that can abrogate this activity in infected cells or from plasmid expressed nsP2. A SINV nsP2 mutant at a conserved proline residue found in the nsP2 of most alphaviruses (P726G) (251-253) was isolated from replicon vectors selected to be non-cytopathic and found to inhibit transcription shutoff. Another mutation in SINV which abolished the cleavage site between nsP2 and nsP3, and prevented the nuclear localization of nsP2 (254) also inhibited this activity in infected cells. Similarly, a CHIKV nsP2 mutant at this position (P718S, P718G), or mutation of the NLS (KR649-650AA) was also deficient at inducing host macromolecular synthesis shutoff (74), though the distinction between transcription and translation shutoff was not made in this study. For VEEV capsid, mutation or deletion of a predicted NLS spanning amino acid residues 64-68,

or a nuclear export signal (NES) upstream of the NLS both blocked nuclear translocation of capsid and subsequently abrogated transcription shutoff (255, 256), whereas a mutation in the corresponding location in EEEV capsid appeared to do so without affecting nuclear transport (250).

A hypothesis for the mechanism of nsP2-mediated transcription shutoff was recently proposed (75), as the authors of that study found that expression of nsP2 induced ubiquitin mediated degradation of Rpb1, the catalytic core of RNA Pol II, in vertebrate but not mosquito cells and was independent of nsP2 protease activity. Rpb1 degradation catalyzed through the helicase and S-adenosylmethionine (SAM) like domains of nsP2, and a previously described mutation in SINV (P726G) also inhibited degradation of Rpb1.

In contrast, capsid mediated transcription shutoff has been proposed to occur through interference with nuclear transport via blockade of the nuclear pore (255, 256), although this mechanism implies an indirect mechanism of shutoff. VEEV capsid binds to importin α/β and the nuclear export receptor chromosomal maintenance 1 (CRM1), also known as Exportin-1, and accumulates in the nuclear pore channel, thereby blocking nuclear-cytoplasmic transport and inhibiting transcription. Similar to results observed with SINV nsP2, transcription was not shutoff in mosquito cells during infection (256).

### 1.4.2 Alphavirus infection and host translation

Infection of target cells with alphaviruses can result in inhibition of global host translation by 3-6h p.i. in permissive cells. Old world alphavirus nsP2 has been shown to inhibit host translation during infection, whereas the New world alphaviral protein mediating this activity is unknown (76, 254, 257). Transcription and translation shutoff are distinct activities induced through

independent mechanisms, although the downstream effect of transcription shutoff is likely observed, and additive, during measurement of translation levels in infected cells. VEEV replicons lacking structural proteins can induce translation shutoff but do not inhibit transcription of genes, whereas VEEV virus expressing capsid inhibits both, suggesting that the two activities are independent (76). A mutation in SINV nsP2 (P726G) has also been shown to reduce shutoff of host translation (251, 253). In contrast, removal of the nsP2-nsP3 cleavage site in a SINV mutant abrogated shutoff of transcription, but efficiently induced translation shutoff in infected cells (254). A mutant in VEEV nsP2 (Q739L), selected from a replicon non-cytopathic in BHK cells, possibly reflects inability to inhibit host macromolecular synthesis (258). Additionally, mutations in CHIKV nsP2 (P718S, P718G, KR649-650AA) can also inhibit host macromolecular synthesis shutoff when expressed from a plasmid independent of other viral proteins (74, 259), although the distinction between transcription and translation shutoff is not clear in these studies, and P718S and P718G, when added to full-length virus, did not appear to affect host translation (unpublished).

Virus-induced shutoff (here representing the sum of transcription and translation shutoff) may have evolved, in part, to coordinate translation of the viral genomic and subgenomic RNAs. Specifically, five major questions concerning the effect of viral induced translation shutoff on both virus and cell are important for furthering our understanding of alphavirus-host cell interactions: 1) how does translation of the viral genomic and subgenomic RNAs occur and how is it linked to viral RNA synthesis during the infection cycle; 2) as structural proteins are produced later in infection, how does subgenomic RNA translation resist the effect of virus induced translation shutoff; 3) what is the molecular mechanism of alphavirus induced translation shutoff; 4) are there differences in the mechanism of translation shutoff between

viruses and; 5) what is the effect of IFN on translation shutoff induction. These questions are intimately linked to understanding the ability of alphaviruses to both successfully initiate replication and antagonize host antiviral defense mechanisms in a variety of cell types, and the differential ability of various alphaviruses to induce translation shutoff may help explain their relative resistance to antiviral pathways.

Several studies have demonstrated the differential translation of alphaviral genomic and subgenomic RNAs, specifically the reduced requirement of eIFs for subgenomic RNA translation as well as its resistance to eIF2α phosphorylation. The subgenomic RNA can translate efficiently via a ribosome scanning mechanism in the absence of eIF4G or eIF4A activity, or eIF2α phosphorylation in infected cells (100-103, 260); cleavage of eIF4G by expression of poliovirus 2A protease, inhibition of eIF4A association with eIF4F by Pateamine A, or eIF2α phosphorylation by arsenite treatment did not inhibit expression of reporter genes from subgenomic RNA molecules. In contrast, the translation of viral genomic RNA was inhibited in these circumstances, suggesting that genomic RNA canonically initiates translation. Subgenomic RNA resistance to eIF2α phosphorylation, but not eIF4A inhibition, was mapped to the presence of a stable hairpin loop downstream of the AUG initiation codon, which stalled ribosomes onto the correct initiation site and bypassed the requirement for functional eIF2 during initiation. The requirement for AUG was only partial, as RNAs with mutated start codons were still able to initiate translation, albeit with diminished efficiency (41, 101, 103). However, these results were not true in uninfected cells transfected with subgenomic RNA reporters or in a cell-free system, or when virally synthesized subgenomic RNA was transfected into infected cells. It has been proposed for a number of viruses including SINV that efficient viral RNA translation is linked to *de* novo RNA synthesis (261-263), and this may explain the differential phenotype of translation

initiation and eIF requirement observed in infected versus uninfected cells. During infection, only RNA molecules synthesized by the replication machinery are translated, whereas exogenous RNAs with equivalent structure are excluded from translation initiation complexes. Cytoplasmic vacuoles, or spherules (91), the sites of viral replication are also sites where translation occurs, and ribosomes and eIF3, but not eIF4G or eIF2 localize to these vacuoles (103), suggesting that RNAs produced during replication are spatially connected to translation initiation factors, whereas incoming exogenous RNAs cannot initiate via the same pathway due to competition of a particular factor or factors involved in translation initiation In addition, the relocation of eIFs to vacuoles may also play a role in translation shutoff induction. However, both of these assertions are controversial in the field, as no further evidence beyond these initial studies has been provided for this hypothesis. A study using SINV subgenomic RNA mutants concluded that efficient subgenomic RNA translation was correlated with the extent and efficiency of translation shutoff (264). Similarly, efficient viral replication was correlated with host translation shutoff (265) in SINV infected cells. These results suggest that viral replication and subgenomic RNA translation can compete with cellular RNAs for eIFs which may affect host RNA translation efficiency and contribute to virus mediated translation shutoff, as well as enhancing expression of viral proteins during infection.

It has also been suggested that activation of PKR and subsequent phosphorylation of eIF2α during alphavirus infection contributes to induction of host translation shutoff. However, the efficiency and extent of translation shutoff induction is similar in both PKR expressing and $PKR^{-/-}$ cells (257, 266), and translation shutoff is actually more rapid in cells expressing a non-phosphorylatable form of eIF2α (William Klimstra, unpublished results). In addition, translation shutoff occurs significantly before detection of phosphorylated eIF2α suggesting that this activity

is induced by a mechanism independent of PKR (257). Another possible mechanism may be viral mediated degradation of eIFs following infection. Data from our lab (William Klimstra, unpublished) show levels of various eIFs to be constant throughout the infection cycle, with partial degradation of eIF4G observed at late times (18-24h) post infection, making it unlikely that this mechanism is used by alphaviruses to induce translation shutoff.

The capsid of Rubella virus, a member of the *Rubivirus* genus of the *Togaviridae* and close relative of alphaviruses, has been shown to bind with and sequester the poly (A) binding protein (PABP) and inhibit cellular translation (267), though this study was performed in a cell-free system and it remains to be determined whether the alphavirus capsid can perform a similar function. Similarly, VEEV nsP2 can potentially bind to the ribosome protein S6 (RpS6) (268), a key component of the ribosome 40S subunit, though the effect of this interaction on translation shutoff remains unknown. Phosphorylation of RpS6 is an important factor in the formation of polysomes and efficient mRNA translation (269, 270). A major reduction in RpS6 phosphorylation levels was observed following infection, which correlated with a reduction in cellular translation in alphavirus infected cells. Interestingly, knockdown of this protein diminished translation of viral but not cellular RNAs (268) in infected cells. However, the major RNA species translated in alphavirus infected cells is the subgenomic RNA, suggesting that the interaction of nsP2 with RpS6 may selectively promote viral RNA translation, and indirectly inhibit host translation. Overall, shutoff of cellular translation may be linked at least in part to successful replication and translation of viral genomic and subgenomic RNA, and is potentially mediated by the viral nsP2 in both Old and New world alphaviruses.

### 1.4.3 Alphavirus infection and IFN signaling

Alphavirus infection inhibits both IFN-α/β and IFN-γ signaling in multiple cell-types and our group provided the first evidence of STAT1 phosphorylation blockade by SINV and VEEV (76, 77, 253, 271, 272). Subsequently, the nsP2 protein of Old world alphaviruses was shown to be responsible for this activity (77), whereas the New world alphaviral protein mediating this activity is unknown but speculated to be nsP2 (76, 272). CHIKV nsP2 expressed independently from other viral proteins blocked STAT1 phosphorylation and subsequent nuclear translocation (74, 77).

Several studies have described mutations in SINV and CHIKV nsP2 that can reduce or abrogate this activity during infection. The SINV mutant P726G was defective in inhibiting STAT1 signaling (253, 271), and CHIKV nsP2 mutant KR645-650AA (74), when expressed independently from other viral proteins, prevented phosphorylation of STAT1. Infection with VEEV replicons demonstrated the independence of this activity from induction of host transcription and translation shutoff (76, 272). Inhibition of transcription by Actinomycin D treatment, or protein synthesis by cyclohexamide during infection did not affect virus mediated STAT1 signaling disruption. However, the lack of mutants which disrupt STAT1 signaling but not host transcription or translation prevents definitive resolution of this question.

Infection of Vero cells with a VEEV replicon resulted in phosphorylation of JAK1 and Tyrosine kinase 2 (Tyk2) following IFN-α/β signaling, but inhibition of JAK1 and JAK2 phosphorylation after treatment with IFN-γ (272), suggesting use of distinct mechanisms to inhibit STAT1 signaling following IFN-α/β and IFN-γ treatment. Subsequently, a moderate reduction in the levels of Interferon-γ receptor (IFNGR) at the cell surface was observed; however, the reduction was insufficient to fully explain loss of STAT1 phosphorylation

following IFN-γ treatment in infected cells. The precise mechanisms by which alphaviruses inhibit STAT1 following Type-1 and Type-2 IFN signaling are not known, but are proposed to target a step or steps in the IFN signaling cascade between receptor-ligand binding and STAT1 phosphorylation.

### 1.4.4 Alphavirus infection and the IFN upregulated antiviral state

The multifunctional alphavirus nonstructural proteins are necessary for successful viral replication and more recently have begun to be implicated as critical role players in antagonizing the induction of, and mediating resistance to, cellular antiviral mechanisms. The biphasic IFN-α/β pathway is the most important of these systems; IFN-α/β induction and signaling lead to the establishment of a potent antiviral state which limits the replication and spread of viral infections, and resistance to the inducible IFN-α/β response has been proposed as a major determinant of alphavirus infection and pathogenesis in cultured cells and murine models (35).

In animal models of alphavirus pathogenesis, infection with VEEV results in induction of very high levels of serum IFN-α/β (183) within 12h p.i., which peak around 24h p.i. and diminish thereafter. In contrast, infection with SINV results in lower levels of IFN-α/β induction, while CHIKV or EEEV infection induces barely detectable levels of serum IFN-α/β (188, 273). Infected myeloid lineage cells (dendritic cells and macrophages) are the likely source of serum IFN-α/β (148, 165, 173, 274-276) *in vivo*, and the capacity and extent of myeloid cell infection by alphaviruses, with the exception of EEEV and WEEV, is dependent on resistance to IFN-α/β responses. *In vivo*, VEEV infection is fatal with the virus efficiently infecting immune tissues such as spleen and lymph nodes, resulting in widespread destruction and observation of pathological changes in those tissues despite upregulation and secretion of antiviral cytokines

(183, 277). The effects of SINV (173) or CHIKV (145, 177) infection of immune tissues are mostly undetectable in adult mice competent for induction of IFN-α/β responses, whereas infection of Type-1 IFN deficient mice is fatal and exhibits widespread lymphoid tissue infection with these viruses. Infection of mice with EEEV is also uniformly fatal despite a lack of tropism for myeloid cells and tissues, recently discovered to be due to miR142-3p mediated suppression of replication in macrophages and dendritic cells (188, 276, 278), which in turn prevents induction of IFN-α/β and other antiviral cytokines. The disparity in serum IFN-α/β levels observed between SINV and VEEV infected mice is most likely due to differing sensitivities of these viruses to IFN-α/β; the more sensitive virus (SINV) is rapidly controlled and cleared by induced IFN-α/β whereas the ability of VEEV to replicate successfully in the face of the IFN-α/β response (76) potentially leads to an even greater induction of IFN-α/β in infected mice. Thus, outcome of infection *in vivo* mouse models, and possibly human cases, is likely correlated with resistance to or avoidance of IFN-α/β responses.

Successful induction of IFN-α/β is dependent on cell type during *in vitro* infection with alphaviruses. Infection of various non-myeloid lineage and mesynchemal like cells does not induce IFN-α/β production. MEF cells, primary neurons and human foreskin fibroblasts all fail to secrete IFN-α/β following SINV, VEEV, CHIKV or EEEV infection (76, 251, 253, 257). Upon SINV infection of MEF cells, IRF3 dimerizes, translocates to the nucleus and binds to target DNA sequences, but transcription of target genes, or protein synthesis, does not occur (253). Activation of IRF3 is dependent on MDA5 activity, but not that of RIG-I (253). Similarly, CHIKV infection of human foreskin fibroblasts also leads to IRF3 dimerization and subsequent transcription of target genes, but no production of effector antiviral proteins or IFN-α/β (257). Infection of primary neuron cells with VEEV results in some transcription of IFN-α/β and ISG

mRNA but no detectable synthesis or secretion of IFN-α/β (76), whereas transcription of IFN-α/β and ISG mRNA is blocked more efficiently in SINV infected primary neurons.

Alphavirus induced transcription and translation shutoff can efficiently inhibit induction of IFN-α/β responses in unprimed cells. Further, upregulation of ISGs following IFN-α/β treatment is efficiently blocked by alphavirus induced STAT1 signaling antagonism. For SINV, transcription shutoff appears to be more complete and important for control of IFN-α/β response induction (254), whereas VEEV induces this activity less efficiently (76), possibly due to the temporally deferred synthesis of capsid during infection. For VEEV, translation shutoff appears to be more important in restricting IFN-α/β response induction (76). Inhibition of STAT1 signaling possibly also prevents further sustenance of the antiviral state (35, 76). In MEF cells, alphaviruses induce efficient transcription and translation shutoff to inhibit IFN-α/β production, with mutants defective at shutoff capable of eliciting IFN-α/β from infected cells. However, failure to transcribe IFN-α/β mRNA or IRF3 target genes occurs before establishment of virus induced transcription and translation shutoff, which suggests avoidance of PRR mediated detection by alphaviruses prior to establishment of transcription and translation shutoff in non-myeloid cell types.

Several upregulated ISGs have been examined for anti-alphaviral activity, and overexpression of multiple antiviral effector molecules has been shown to inhibit alphavirus replication (32, 196, 279-281). Interferon-inducible protein with tetratricopeptide repeat 1 (IFIT1) can distinguish between self and non-self mRNA molecules by selectively binding to a Type-0 cap (found on some viral RNAs, including alphaviruses), but not Type-1 or Type-2 caps (found on most host cell mRNAs) (282, 283) and inhibiting translation initiation. Alphaviruses evade this restriction through the presence of a secondary structure formed by the genome

sequence directly distal to the 5' cap structure, which prevents IFIT1 from binding to the cap. Overexpression of IFIT1 only modestly inhibits alphaviral replication but highly represses mutants with a disrupted 5' secondary structure. The activity of PKR appears to suppress alphavirus replication, as replication levels in $PKR^{-/-}$ dendritic cells and in lymph nodes of $PKR^{-/-}$ mice are 10-fold higher compared to WT controls (172, 281). However, IFN-α/β in $PKR^{-/-}$ mice or in dendritic cell or MEF cultures derived from $PKR^{-/-}$ mice inhibits SINV infection as efficiently as in PKR+/+ cells. The Zinc-finger antiviral protein (ZAP) and Interferon-stimulated gene 20 (ISG20) can potently inhibit replication of SINV (196, 280, 284), whereas expression of viperin and Interferon-stimulated gene 15 (ISG15) did so only modestly (196). In contrast, expression of RNase L may have a beneficial effect on SINV replication (285) (Kate Ryman, unpublished data), as this protein appears to play an indirect role in the formation of stable replication complexes during SINV infection.

In contrast to the lack of observed IFN-α/β induction in non-myeloid lineage cells, SFV and SINV (172, 286) infected myeloid dendritic cells, and SINV infected macrophages (206) secrete IFN-α/β, dependent upon IRF7 but not IRF3 (unpublished). Similarly, infection of $PKR^{-/-}$ myeloid dendritic cells with SINV delayed but did not reduce production of IFN-α/β (172). Infection of bone-marrow derived dendritic cells with SINV resulted in IFN-α/β dependent upregulation of ~400 potentially antiviral genes, with no observed cytopathic effect, and the infection was self-limiting and cleared from culture (172). However, the ability of alphaviruses to shutoff cellular transcription and/or translation in myeloid cells is unknown, as are the mechanisms by which myeloid cells successfully secrete IFN-α/β during infection by alphaviruses. Overall, alphaviruses use shutoff of host macromolecular synthesis to prevent IFN-α/β induction in infected cells in a cell-type dependent manner, but can also avoid detection by

PRRs early after infection. In addition, shutoff-independent mechanisms to inhibit IFN-α/β induction may exist.

## 1.5 HYPOTHESES

Most previous studies exploring the inhibition of IFN-α/β responses by alphaviruses have focused on the ability of alphaviruses to block IFN-α/β induction and/or IFN receptor signaling in unprimed cells. While relevant, these studies primarily describe the small subset of cells infected shortly after inoculation in vivo and in most cases utilize fibroblast cultures when the initially infected cells in vivo are frequently of myeloid lineage (35). During VEEV and SINV infection, induced serum IFN-α/β upregulates an antiviral state in the many uninfected cells, including fibroblasts and neurons, and, thus, infection of IFN-primed cells represents the dominant event during VEEV and SINV infection in vivo. Other than a single study performed by our group (76), this aspect of infection has not been studied and, in our opinion, is critical to understanding the major differences in virulence observed between VEEV, SINV and other alphaviruses in vivo. The IFN-α/β induced antiviral state can efficiently control SINV but not VEEV infection, and viral determinants of this differential sensitivity are unknown.

Alphaviruses can induce transcription and translation shutoff, and inhibit STAT1 signaling in infected cells. Successful induction of transcription and translation shutoff and STAT1 signaling inhibition in IFN-α/β primed cells is virus dependent (76) with translation shutoff observed following VEEV, but not SINV, infection of IFN-α/β primed cells. The roles of this activity and others (transcription shutoff and STAT1 signaling inhibition) in determining outcomes of infection both in vivo and in vitro are unknown.

Furthermore, the majority of studies describing the role of capsid and nsP2 in inhibiting host translation, transcription and STAT1 signaling have been performed using full-length virus, or proteins expressed from a virus replicon, a context which does not preclude interference, dependence of protein expression upon replication competence, or the synergistic effects of other viral proteins on the actions of nsP2 or capsid. Relatively few studies have expressed nsP2 and capsid independent of other viral proteins, and those studies have either described a generalized "host shutoff" or examined only transcription shutoff. Indeed, some of these studies have discounted translation shutoff as playing a role in IFN antagonism (74, 271). The role of nsP2 and capsid of different alphaviruses in mediating these activities independent of other viral proteins is unresolved, and indeed for some alphaviruses is unknown.

In addition, the interaction of alphaviruses with myeloid cells that are infected following inoculation has only recently begun to be explored (276). Although myeloid cells infected with VEEV and SINV can secrete IFN-α/β, the characteristics of this induction are not known. Infection of non-myeloid primary cells and cell lines abrogates IFN-α/β induction through host macromolecular synthesis shutoff. Presumably, alphaviruses can shutoff host transcription and translation in myeloid cells, although this aspect of alphavirus infection has not been previously explored. The ability of myeloid cells to avoid or resist alphavirus induced macromolecular synthesis shutoff and secrete IFN-α/β, and the host factors involved in this process are unknown.

To explore the aforementioned questions and further understand how the interplay between host antiviral mechanisms and alphavirus replication determines disease progression and infection outcomes, I have proposed the following hypotheses in this study:

- In Chapter 2, I hypothesize that the ability of VEEV to induce host transcription and translation shutoff is critical for resisting the IFN-α/β upregulated antiviral state, and explains the differential virulence observed following murine infection with SINV and VEEV.
- In Chapter 3, I hypothesize that the viral nsP2 inhibits STAT1 signaling by disrupting the IFNAR signaling cascade at a step prior to STAT1 phosphorylation.
- In Chapter 4, I hypothesize that IRF7 mediated IFN-α/β induction is important for control of VEEV infection in mice, and that the IFN-α/β induction observed following VEEV infection of myeloid, but not non-myeloid, cells occurs despite establishment of transcription and translation shutoff.

The results obtained from this study will advance our understanding of alphavirus pathogenesis and reveal molecular mechanisms used by these viruses to evade or resist host immune defenses and cause disease.

# 2.0 HOST TRANSLATION SHUTOFF MEDIATED BY NON-STRUCTURAL PROTEIN 2 IS A CRITICAL FACTOR IN THE ANTIVIRAL STATE RESISTANCE OF VENEZEULAN EQUINE ENCEPHALITIS VIRUS

## 2.1 INTRODUCTION

The *Alphavirus* genus of the *Togaviridae* family of viruses consists of positive-sense single-stranded RNA viruses broadly classified into arthritogenic (e.g. SINV and CHIKV) and encephalitic (e.g. VEEV, EEEV) disease-causing groups. Members of this genus are responsible for millions of annual infections and ongoing epidemic outbreaks in several parts of the world, such as the current CHIKV epidemic in the Indian Ocean region (287) which has recently spread to the Caribbean, United States and Central and South America (288-290). Infection with arthritogenic alphaviruses causes a febrile illness, which can lead to arthralgia/arthritis lasing for months or years after infection (176). In contrast, encephalitic alphavirus infection results in prodromal disease of varying duration and severity which can progress to fatal encephalitis in a significant number of cases depending upon the virus (35).

Alphavirus replication and disease severity in mouse models is dependent on their resistance to the antiviral state generated following IFN-α/β induction, and it has been proposed that human disease severity is also associated with resistance to or avoidance of the antiviral effects of IFN (15, 35, 76). Infection of mice with VEEV elicits the highest levels of induced

systemic IFN-α/β, which rapidly primes uninfected sites *in* vivo, while significantly lower levels are observed following SINV infection (35), and little to no IFN is induced by EEEV infection (188). For CHIKV, robust IFN induction is observed in the serum of infected patients (291, 292), and infected non-human primates (293), whereas little IFN is detected in the serum of infected mice (35). However, non-hematopoietic cells are the primary source of IFN during CHIKV infection (273). Mice with functional IFN-α/β responses efficiently control SINV (172, 173, 294) and CHIKV infection (176, 287). In contrast infection with VEEV (181, 182) or EEEV (278) is usually fatal. While the severity of EEEV infection is linked to its inability to replicate in myeloid lineage cells and consequent suppression of IFN and other innate immune responses (148, 276), mortality and disease progression observed following VEEV infection is due, at least in part, to greater resistance to the antiviral state induced by IFN (76). However, viral determinants and mechanisms mediating this resistance are unknown.

IFN signaling results in upregulation of hundreds of ISGs many of which possess antiviral activities (235, 295), of which several have been shown to inhibit alphavirus replication (32, 279, 280). Notably, in conditions where replication of other alphaviruses is highly restricted by IFN priming, successful replication of VEEV can be observed (76, 296). The resistance of VEEV to multiple antiviral effectors in IFN-primed cells suggests the use of a global mechanism that overcomes their inhibitory activities, rather than resistance to the activity of each ISG individually. To suppress the induction of cell stress responses, alphaviruses have been shown to block host cell transcription (104, 251) and translation (76, 254), and it is possible that the induction of one or more such processes during infection of IFN-primed cells by VEEV is able to suppress the pre-existing antiviral state. The Old world alphaviruses that have been studied mediate host transcription and translation shutoff through an activity of the nonstructural protein

nsP2, while the capsid protein of New world alphaviruses has been implicated in the shut off host cell transcription (104, 106). The viral protein involved in host translation arrest during New World alphavirus infection has not been determined.

Most previous studies exploring the mechanisms of alphavirus mediated IFN-α/β antagonism were performed in unprimed cells, cells treated with IFN-α/β post infection, or cells over-expressing individual ISGs such as Interferon-inducible protein with tetratricopeptide repeat 1 (IFIT1) (32, 271, 283, 297). However, rapid induction of serum IFN-α/β in mice after VEEV and SINV infection upregulates an antiviral state in most cells at sites where the infection has not progressed, causing the of majority cells infected by these viruses *in vivo* to be primed to resist infection. Thus, previous *in vitro* work in unprimed cells primarily represents the few cells initially infected after inoculation of mice. The interaction of VEEV and SINV with a pre-established antiviral state was explored in recent studies (35, 76), which demonstrated that VEEV was far more resistant to a pre-existing antiviral state than SINV.

Previous studies have also focused on the effect of a generalized shutoff, or when specific, virus-induced transcription shutoff on induction of IFN-α/β responses (74, 271), while the role of translation shutoff in antiviral state antagonism has not been emphasized. For SINV, both transcription and translation shutoff are induced by the same protein (254), and the relative contribution of these functions in resisting the antiviral state is difficult to explore. Similarly, most previous work with VEEV or EEEV has implicated capsid induced transcription shutoff to play a major role in suppression of IFN-α/β induction, despite the temporally deferred synthesis of this viral protein during infection (297, 298). Induction of host translation shutoff by VEEV has been localized to the nonstructural protein region of the genome, which is translated before

the capsid region during infection (76), suggesting a role for this activity in the antiviral state resistance of VEEV.

Here we have examined possible mechanisms underlying resistance of VEEV to an IFN-α/β induced, pre-established antiviral state and identified/confirmed the proteins that mediate host transcription and translation shutoff with CHIKV, SINV, VEEV and EEEV through individual protein expression. In *in vitro* testing, VEEV was more resistant than SINV, CHIKV and EEEV to the global antiviral state in mouse and human cells, and this resistance became evident at a point after initial translation of the incoming virus genome. Furthermore, a panel of mutant viruses deficient in host macromolecular synthesis shutoff demonstrated that sensitivity to the antiviral state was correlated with slower rates this activity. Using a plasmid expression system to study host macromolecular synthesis shutoff independent of virus replication rates, we found that expression of VEEV, CHIKV or SINV nsP2, or VEEV or EEEV capsid expression, but not control nsPs or GFP was sufficient to block host translation, with VEEV and EEEV capsid translation blockade likely secondary to transcription shutoff. VEEV and EEEV nsP2 did not inhibit transcription. VEEV or EEEV capsid and CHIKV or SINV nsP2 expression directly inhibited host transcription. EEEV nsP2 also failed to block host translation revealing a stark difference between VEEV and EEEV. Importantly, in the absence of transcription shutoff, host translation in IFN-primed cells was inhibited more efficiently by VEEV nsP2 than that of SINV nsP2. Furthermore, when VEEV nsP2 was expressed in IFN-primed cells, levels of ISG's were lower, and replication of an unrelated IFN-sensitive virus (yellow fever virus 17-D) was enhanced over IFN-primed control cells. Overall, we conclude that VEEV nsP2-induced host translation shutoff early after infection downregulates the antiviral state by decreasing levels of ISG's and creating an environment more permissive for viral replication.

## 2.2 RESULTS

### 2.2.1 VEEV is more resistant to the IFN-induced antiviral state than other alphaviruses.

Previously, we compared the relative resistance of SINV and VEEV to an IFN-induced anti-viral state in primary mouse neurons (76). Treatment of primary neuron cultures with 1000 international units (IU) IFN-α/β post-infection had limited effect on viral growth; however, pre-treatment with 1000 IU IFN-α/β for 24h prior to infection substantially inhibited growth of SINV but not VEEV (76). Here we determined the relative resistance of multiple Old world (SINV and CHIKV) and New world (VEEV and EEEV) alphaviruses to a pre-existing antiviral state in both mouse and primate cells. The dose of IFN used for priming was selected such that replication of all tested alphaviruses except VEEV was significantly inhibited.

Treatment of Vero cells with 5000 IU human leukocyte IFN for 24h prior to infection significantly ($P<0.0005$) reduced the growth of all tested alphaviruses at 6h post-infection (p.i.) (Fig. 6A). By 24h p.i., this early blockade on virus growth was subsequently overcome by VEEV and replication in IFN-pretreated cells was comparable to untreated cells (Fig. 6B). In contrast, the antiviral effects of IFN significantly ($P<0.0005$) virus growth of all other tested alphaviruses. Similar results were obtained in mouse embryonic fibroblast (MEF) cells (data not shown), indicating that the resistance phenotype of VEEV is not mouse- or primate-specific. These results indicate that VEEV is resistant to a global IFN-induced anti-viral state.

We next tested the ability of alphaviruses to replicate in the presence of individually overexpressed IFN effectors that had been previously shown to possess anti-alphaviral activity (196). Tet-inducible MEF cells over-expressing IFIT1 and ISG20 were infected with VEEV, EEEV, SINV and CHIKV and viral replication was measured using qRT-PCR at 24h p.i. Similar

to pre-treatment experiments, VEEV replicated successfully; in contrast, EEEV, CHIKV and SINV were significantly inhibited ($P<0.001$, Fig. 6C). Taken together, these results indicate that VEEV is resistant to both a global anti-viral state and individual IFN induced effector proteins.

During alphavirus infection, nonstructural proteins are produced first, and are required for production of structural proteins (4). To identify the role of nonstructural proteins in the resistance phenotype of VEEV, we used VEEV, SINV, CHIKV and EEEV replicons lacking structural proteins and expressing fLuc. Luciferase activity in MEF cells pre-treated with IFN was reduced during infection of all replicons when compared to untreated cells. However, luciferase activity in IFN primed cells infected with Vrep Luc was significantly higher ($P<0.0003$) than those infected with other replicons (Fig. 6D). We concluded that the resistance phenotype of VEEV is localized, at least in part, to the nonstructural protein region of the genome.

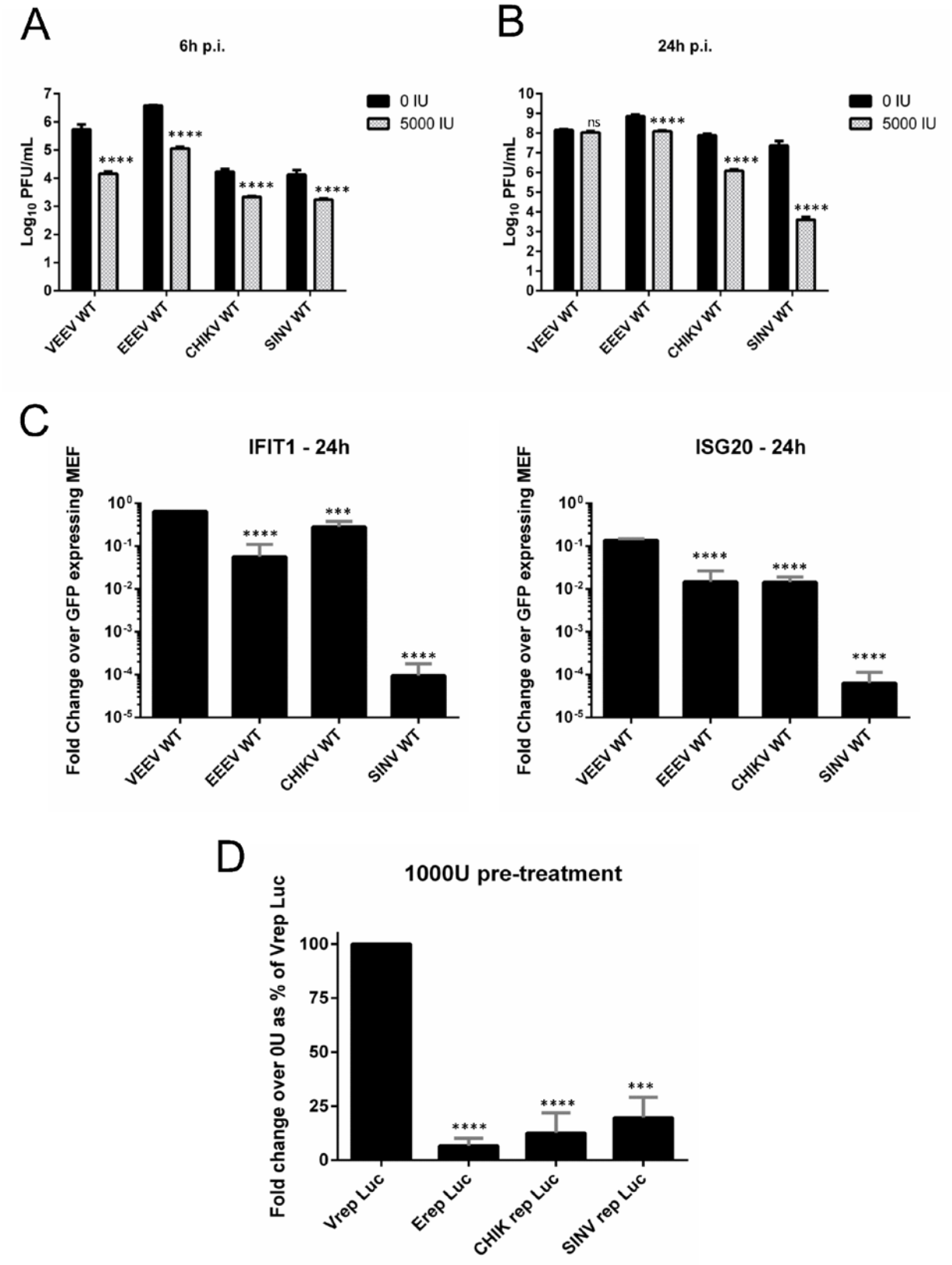

A
6h p.i.
Log10 PFU/mL
0 IU
5000 IU
****
VEEV WT
EEEV WT
CHIKV WT
SINV WT
B
24h p.i.
ns
C
IFIT1 - 24h
Fold Change over GFP expressing MEF
***
ISG20 - 24h
D
1000U pre-treatment
Fold change over 0U as % of Vrep Luc
Vrep Luc
Erep Luc
CHIK rep Luc
SINV rep Luc

**Figure 6. VEEV is resistant to a pre-established anti-viral state and to individual overexpressed IFN effectors.** (A and B) Vero cells mock-treated or pre-treated with 5000 IU human leukocyte IFN for 24h were infected in triplicate with indicated viruses (M.O.I. = 2.5). Supernatants were collected at 6h (A) and 24h (B) p.i. and virus replication was quantified using plaque assays. For each virus, data represents the fold decrease in viral replication versus no IFN, expressed as a fold change over replication of VEEV. ****, P<0.0005 using t-test. Data is representative of two independent experiments. (C) Tet-inducible MEFs stably expressing IFIT1, ISG20 and GFP (control) were infected in triplicate with indicated viruses (M.O.I. = 1). Cell lysates were collected at 24h p.i and viral RNA levels were measured using RT PCR as described in Materials and Methods. Data is viral RNA levels from IFIT1 or ISG20 expressing cells as a fold change of viral RNA levels from GFP expressing cells. ****, P<0.0001; ***, P<0.001 using One-way ANOVA. All error bars are standard deviations. (D) MEF cells were mock-treated or pre-treated with 1000 IU mouse IFN for 24h and infected with indicated replicons (equal dilution). Lysates were collected in passive lysis buffer at 16h p.i. and luciferase activity was measured. Data is relative light units (RLUs) per μg total protein expressed as a fold change over no IFN. Infection was performed in triplicate. ****, P<0.0001; ***, P<0.0003 using t-test.

### 2.2.2 Translation of incoming genomes of all tested alphaviruses is similarly affected by IFN.

The previous experiments demonstrated the ability of VEEV to more efficiently initiate replication in the presence of an antiviral state compared to other tested alphaviruses and suggested a role for noncoding regions or nonstructural proteins in the antiviral state resistance of VEEV. Subsequently we sought to determine step(s) in the replication cycle where VEEV escaped suppressing effects of the antiviral state. Possible points where upregulated antiviral effectors could potentially block alphavirus replication initiation are virus entry, virion envelope fusion with the endosomal membrane, sequestration/degradation of incoming viral RNA and suppression of initial translation of the incoming viral genome. We previously observed that translation of mRNA messages entering an IFN-primed cell across the cytoplasm was substantially reduced, while that of nuclear originating mRNA was not (299). Additionally, this block was at the step of initial translation of the mRNA. Furthermore, recent work using chimeric alphaviruses has shown that, in general, attachment, entry and nucleocapsid dissociation are not affected by IFN (283). Therefore, we speculated that the IFN induced

antiviral state might block virus replication at the point of initial genome translation. We used capped and poly-adenylated reporter RNA molecules in which the Firefly Luciferase (fLuc) gene was flanked by authentic 5' and 3' non-translated regions (NTR) and fused to truncated nonstructural protein 1 (nsP1) of VEEV, EEEV, CHIKV and SINV (Fig. 7A) to measure the effect of antiviral activity on translation of incoming virus genomes as previously described (299). Translation of reporter RNA mimics initial translation of an incoming viral genome as the reporter is incapable of replication. In addition, electroporation of reporter RNA's bypasses the entry and fusion steps of the viral infection cycle by delivering RNA directly into the cytoplasm.

The activity of fLuc in electroporated MEF cells pre-treated with IFN revealed a dose dependent decrease in translation of incoming RNA independent of the origin of the reporter (Fig. 7B), similar to published observations using chimeric viruses (283). Activity was significantly lower (reduced >100 fold; $P<0.0015$) in cells pre-treated with the highest dose of IFN (1000 IU) compared to untreated cells for all viral reporter RNA's. We conclude that replication of alphaviruses is significantly diminished in IFN-primed cells after entry and at the point of initial translation of the genome and production of nsPs. Notably, translation of all reporter RNA's, while being heavily suppressed was not completely ablated even in cells receiving the highest dose of IFN. While, recently, IFIT-1 activity was demonstrated to inhibit translation differentially between alphaviruses (283), VEEV does not appear more resistant to the overall effect of IFN priming in this assay. This suggests that low-level translation of viral nonstructural proteins occurs following infection of IFN-primed cells. Based upon this result, and consistent with replicon data (Fig. 6D), we hypothesized that an activity or activities of one or more VEEV nsPs may contribute to the antiviral state resistance of VEEV.

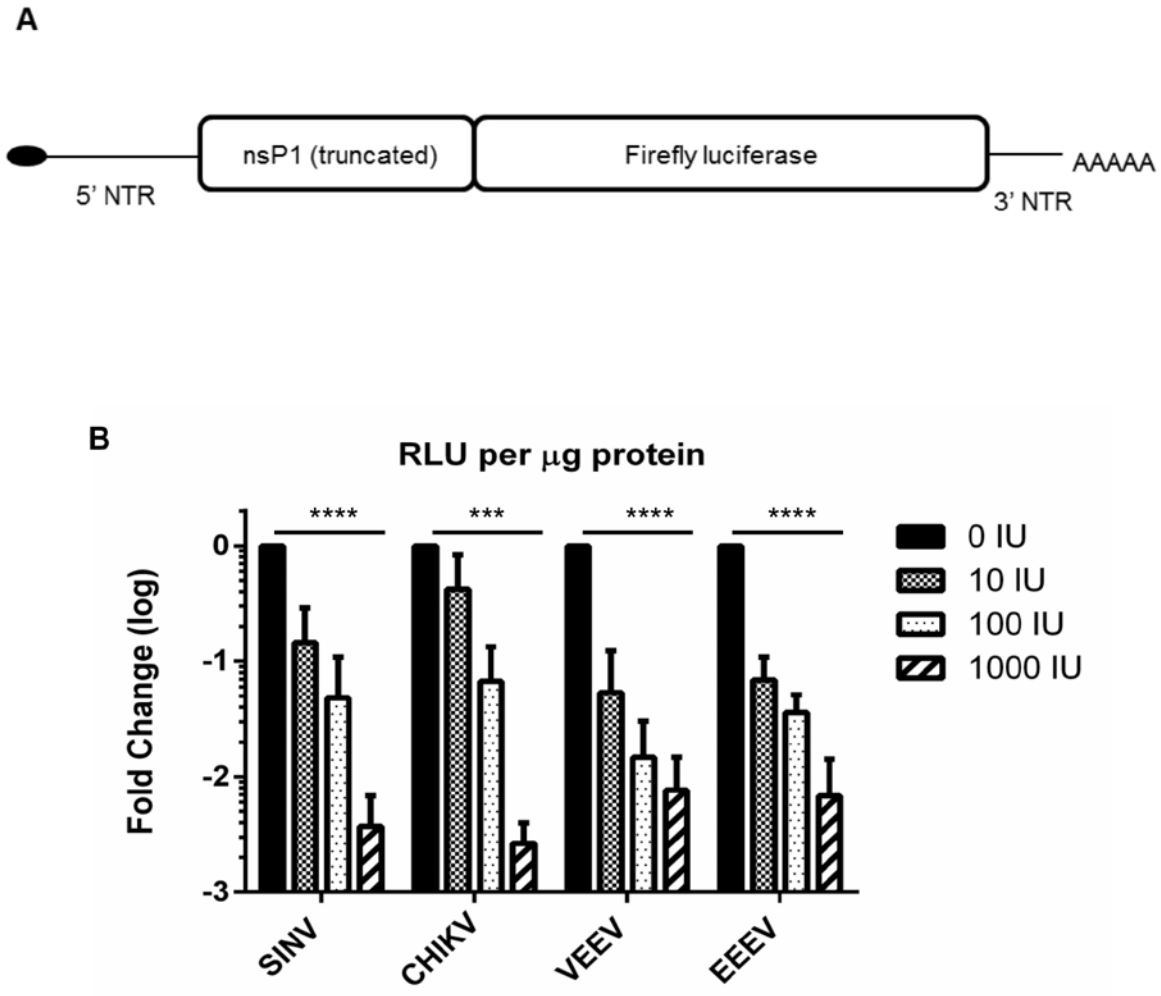



**Figure 7. Translation of incoming genomes is attenuated in IFN pre-treated cells.** (A) Schematic of translation reporter RNA with wild-type virus 5' and 3' NTRs. (B). MEF cells were untreated or pre-treated with 0, 10, 100 or 1000 IU mouse IFN for 16h and electroporated with reporter RNAs of indicated viruses as described in Materials and Methods. Cells were lysed 2h post-electroporation and luciferase activity was measured. Data is RLUs per μg total protein expressed as a fold change over no IFN; average of three experiments for per treatment. ***, $P<0.0015$; ****, $P<0.0001$ using One way ANOVA. All error bars are standard deviations.

### 2.2.3 Phenotypes of mutant VEE viruses suggest a role for host macromolecular synthesis shutoff in antiviral state antagonism.

Alphaviruses can inhibit host macromolecular synthesis by causing transcription and translation shutoff in many replication-permissive cells (76, 106, 266, 300). We hypothesized that VEEV induces host macromolecular synthesis shutoff in IFN-primed cells to inhibit the antiviral state during infection. To test this hypothesis, we created a panel of VEEV viruses incorporating published mutations in the nsP2 or capsid of VEEV and other alphaviruses known to reduce host macromolecular synthesis shutoff (Table 1). The wild-type nsP2 sequences of most alphaviruses contain a conserved proline residue at amino acid position 713 in VEEV, 726 in SINV and 718 in CHIKV, with the notable exception of EEEV which possesses a lysine at the analogous position. Published data with mutations at or near this position (SINV P726G, CHIKV P718S, VEEV Q739L) have been reported to decrease host macromolecular synthesis shutoff or, with Q739L, cytopathic activity, which may reflect shutoff efficiency (74, 253, 258, 301). In addition, a five amino acid deletion in VEEV capsid greatly reduces its ability to shut off host cell transcription (105, 106).

**Table 1. Summary of published and generated mutants.**

| Virus | Wild Type | Phenotype |
|---|---|---|
| VEEV WT | HLN**P**GGTCVSIGYGYADRASESIIGAIARQFKF | Wild type |
| SINV WT | CLN**P**GGTLVVKSYGYADRNSEDVVTALARKFVR | Wild type |
| CHIK WT | LLK**P**GGSLLIRAYGYADRTSERVICVLGRKFRS | Wild type |
| | **Published mutations in nsP2 and Capsid** | |

| | | |
|---|---|---|
| SINV P726G | CLN**G**GGTLVVKSYGYADRNSEDVVTALARKFVR | Non-cytopathic in cells (253, 301) |
| CHIKV P718S | LLK**S**GGSLLIRAYGYADRTSERVICVLGRKFRS | Does not antagonize macromolecular synthesis shutoff (74) |
| VEEV Q739L | HLNPGGTCVSIGYGYADRASESIIGAIAR**L**FKF | Non-cytopathic in BHK cells (258) |
| VEEV CD | Deletion of Amino acids 64-68 in Capsid | No transcription shutoff (105, 255) |
| | **Generated mutations in nsP2 and Capsid** | |
| VEEV CD | Deletion of Amino acids 64-68 in Capsid | No transcription shutoff (105) |
| VEEV CD/739L | Deletion in capsid + nsP2 739L | Unknown |
| VEEV P713G | HLN**G**GGTCVSIGYGYADRASESIIGAIARQFKF | Unknown |
| VEEV P713S | HLN**S**GGTCVSIGYGYADRASESIIGAIARQFKF | Unknown |
| VEEV Q739L | HLNPGGTCVSIGYGYADRASESIIGAIAR**L**FKF | Non-cytopathic in BHK cells (258) |

We measured the following phenotypes of the panel of mutant viruses: 1.) replication in unprimed and IFN-primed cells; 2.) the ability to shutoff host macromolecular synthesis by measuring translation shutoff and IFN induction; and 3.) replication efficiency in IFN-primed cells compared to wild-type (WT) VEEV. We reasoned that a correlation of one or more mutant phenotypes with the ability of mutants to replicate in the presence of an antiviral state would implicate those activities in the antiviral state resistance of WT VEEV.

We introduced these mutations into an enzootic wild-type VEEV virus (Table 1) and tested their effects on viral replication by performing a one-step growth curve in untreated and IFN pre-treated Vero cells (Fig. 8A and B). The rate and efficiency of infection was similar between WT and mutant viruses. In untreated cells the mutants divided into two groups (Fig. 8A); slower growing mutants (Q739L, CD/739L) which significantly ($P<0.0001$) lagged WT

VEEV replication at early times p.i., and mutants which grew to levels and at rates not significantly different from WT VEEV (CD, P713G, P713S). Most mutants achieved levels of replication similar to WT VEEV by 24h p.i. Substitution of the conserved proline at position 713 in VEEV nsP2 with glycine had no impact on replication kinetics, in stark contrast to the substantial effect of the analogous mutation in SINV (253). Additionally, the effect of the capsid deletion mutation on viral replication was observed only in conjunction with Q739L. The replication of mutants in IFN-primed cells (Fig. 8B) was similar to that observed in unprimed cells. 713G and 713S replication was not significantly different from VEEV WT. The mutant CD was slower significantly only at 6h and 24h p.i. ($P<0.0001$) compared to VEEV WT. In contrast, growth of 739L and CD/739L was significantly ($P<0.0001$) inhibited compared to VEEV WT at all times p.i., and similar to the effect observed in unprimed cells, the combined effect of two mutations significantly inhibited viral replication. Finally, we titered all viruses on multiple cell types (BHK, Vero and Huh7, data not shown). We observed different titers between cell types, but crucially, the difference between WT and mutant titers was similar in each cell type, suggesting that the viral infectivity was similar between WT and mutant viruses.

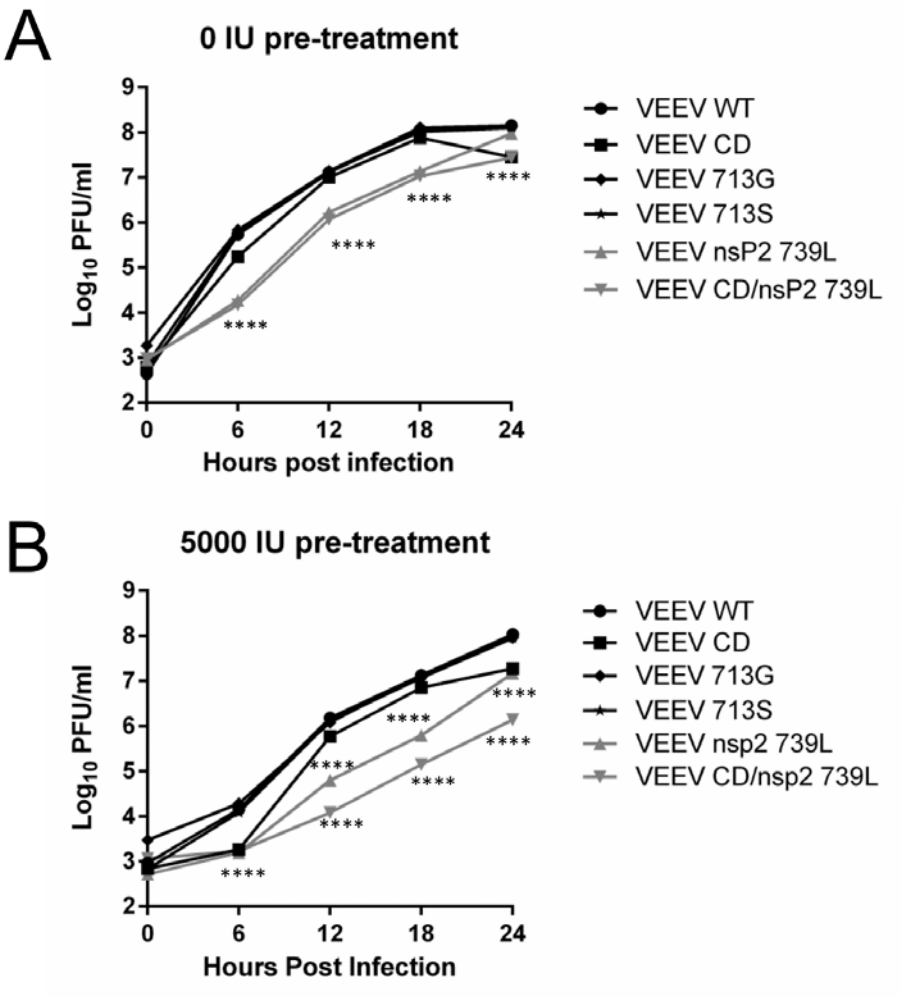



**Figure 8. Growth characteristics of VEEV mutants.** Vero cells untreated (A) or pre-treated with 5000 IU IFN (B) were infected with indicated viruses (M.O.I. = 2.5) and supernatants were collected at 6, 12, 18 and 24h p.i. Virus replication was quantified using plaque assays. ****, P<0.0001 using Two-way ANOVA. All error bars are standard deviations. Data is representative of two independent experiments. (C) Data from (A) and (B) was used to quantify fold change over no IFN. ****, P<0.0001; ***, P<0.001; **, P<0.01 using One-way ANOVA. Ns = not significant. All error bars are standard deviations.

We next tested the ability of mutant viruses to shut off host macromolecular synthesis, measured in this case as translation shutoff which represents cumulative inhibition of transcription and translation activities. Neuro 2a cells were used as a substitute for primary neurons, which were previously used to study the resistance of VEEV to the antiviral state (76). Infected Neuro 2a cells were labeled with [$^{35}$S] Cys/Met for 2h at 6h and 18h p.i and lysates were

resolved on polyacrylamide gels to measure total protein synthesis (Fig. 9A and B). As previously reported, WT VEEV efficiently shut off host translation by 6h. Shutoff induced by mutants 713G and 713S was not significantly different from WT VEEV early or late after infection. The mutants 739L, CD and CD/739L were significantly ($P<0.01$) impaired at inducing shutoff early after infection, and while the extent of shutoff achieved by 739L was not significantly different from WT VEEV by 18h, CD and CD/739L were unable to induce a complete shutoff even by 18h p.i. ($P<0.0001$). Shutoff induced by CD/739L was significantly lower than CD alone both early ($P<0.05$) and late ($P<0.0001$) p.i., reflecting the combined effects of the two mutations present in the virus.

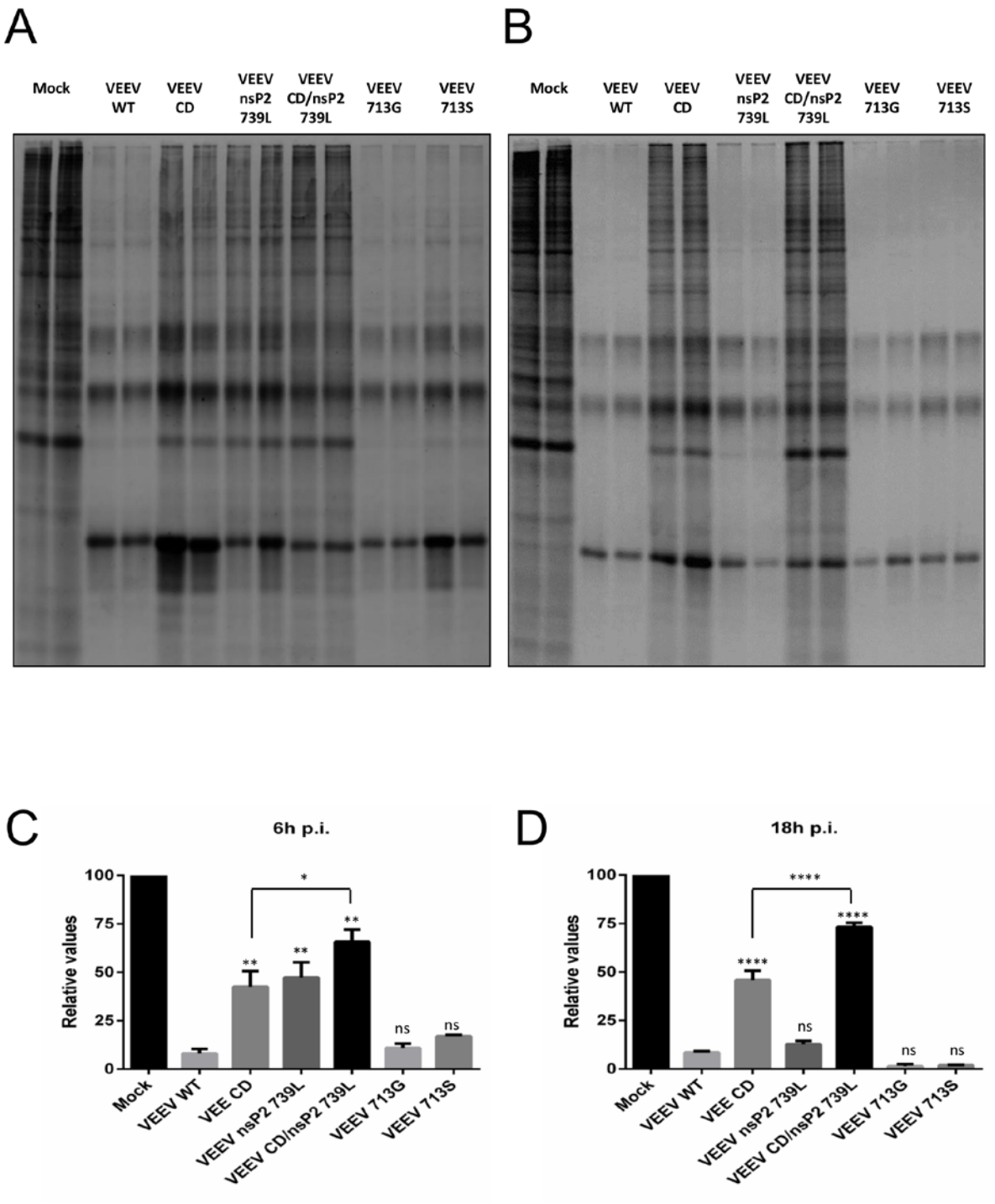



**Figure 9. Host macromolecular synthesis shutoff by VEEV mutants.** (A-D) Neuro 2a cells were infected with indicated viruses (M.O.I. = 2.5) and labeled with 100μCi/ml of [$^{35}$S] Cys/Met for 2h at 6h (A) and 18h (B) p.i. Lysates were collected and resolved on SDS-PAGE gels and visualized as described in Materials and Methods. (C and D) Densitometry performed on gels from (A) and (B) respectively. ****, P<0.0001; **, P<0.01 using One-way ANOVA, compared to VEEV WT. Ns = not significant. Data is representative of two independent experiments. All error bars are standard deviations.

IFN induction following infection can result in upregulation of an antiviral state via autocrine or paracrine signaling (224). The ability of VEEV to shutoff host macromolecular synthesis would also prevent IFN production and subsequent establishment and sustenance of an antiviral state. We explored the role of host macromolecular synthesis shutoff in antagonism of IFN induction by measuring secreted IFN levels following infection of MEF cells with WT and mutant VEEV viruses as this might affect rates of virus replication in cell types competent for IFN production. MEF cells were chosen as they are capable of secreting IFN following treatment with appropriate stimuli. VEEV WT infection did not result in IFN secretion at early or late times p.i. (Fig. 10A and B). IFN was only detected in CD and CD/739L supernatants at both early and late times p.i. (Fig. 10A and B). CD/739L induced slightly, but not significantly, greater amounts of IFN than CD early (3h; 830IU vs 330IU), and significantly ($P<0.0001$) greater amounts late during infection (24h; 8000IU vs 4000IU). Notably, while 739L infected cells did not secrete IFN, CD/739L infected cells secreted the greatest amount of IFN at all measured times p.i, suggesting that different components of the host macromolecular synthesis shutoff mechanism have a combined effect on antiviral state antagonism. Taken together, these results suggest, consistent with previous reports with SINV (271) that host macromolecular synthesis shutoff plays a role in suppression of IFN induction in fibroblast type cells by VEEV. These results also demonstrate that both VEEV nsP2 and capsid contribute to this suppression during infection

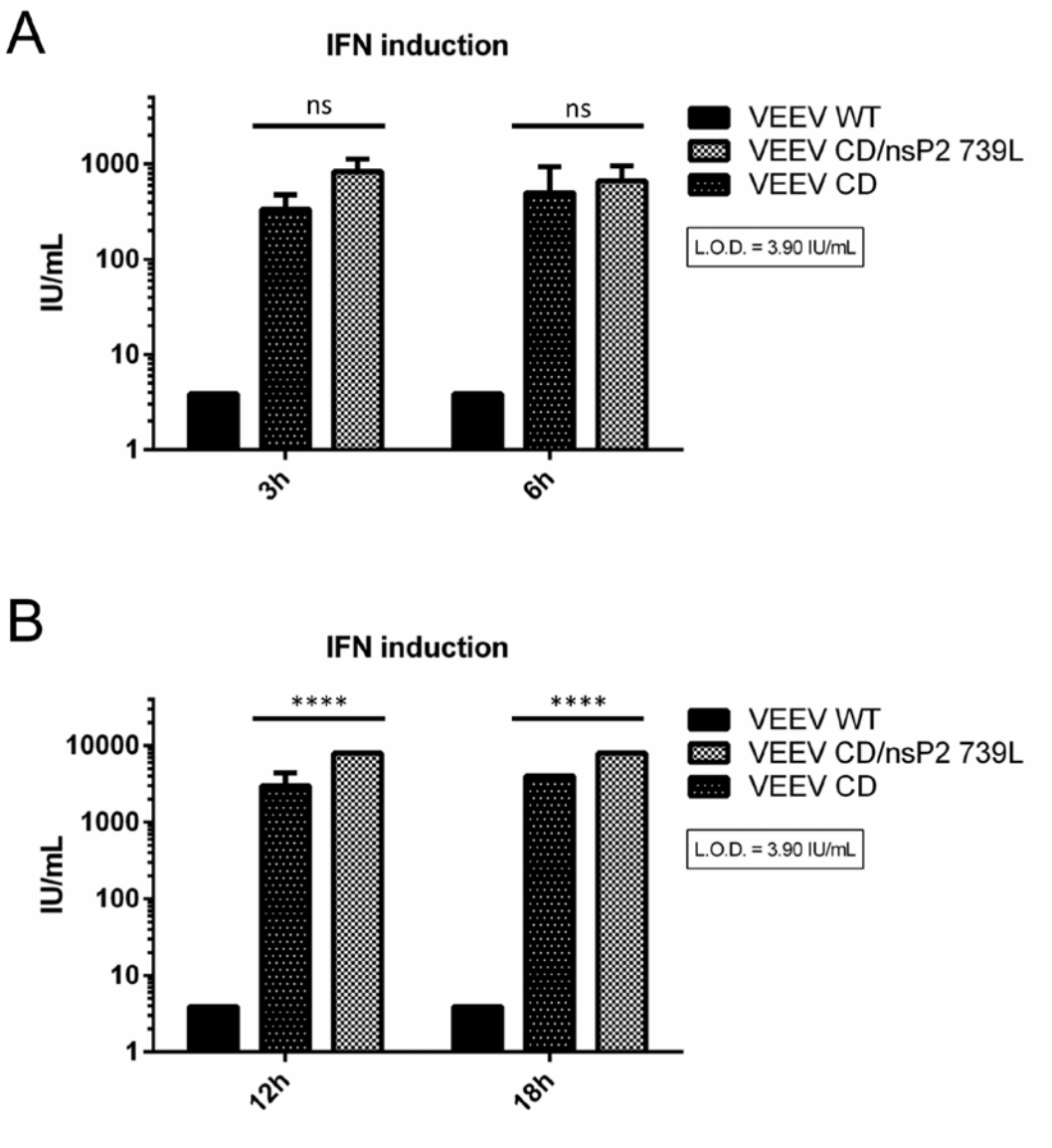



**Figure 10. IFN induction by VEEV mutants.** MEF cells were infected with indicated viruses (M.O.I = 5) and supernatants were collected at early (A) or late (B) times p.i. IFN bioassays were performed as described in Materials and Methods to determine secreted IFN levels. ***, P<0.0005; **, P<0.006 using Two way ANOVA. Ns = not significant. Data is the representative of at least two independent experiments for each virus. All error bars are standard deviations.

We tested the ability of VEEV mutant viruses to replicate in the presence of a pre-established antiviral state in order to identify whether macromolecular synthesis shutoff played a role in antiviral state resistance. Vero cells were selected as they are incapable of producing IFN upon stimulus (302, 303); thus the antiviral state generated by IFN pre-treatment would be

equivalent for all VEEV mutants and would not be affected by virus induced IFN. Vero cells were pre-treated with 5000 IU human leukocyte IFN for 24h and infected with VEEV WT and mutants at equal M.O.I. The fold reduction in replication in IFN-treated cells versus replication in untreated cells was used to determine the sensitivity of VEEV mutants to IFN-priming (Fig. 11A). The mutants CD, 713G and 713S were not significantly different from WT VEEV in resisting the effects of the anti-viral state, whereas 739L and CD/739L were significantly ($P<0.0001$) more sensitive than VEEV WT at late times p.i. Additionally, CD/739L was significantly ($P<0.0001$) more sensitive to the antiviral state than 739L, reflecting the presence of mutations affecting both components of macromolecular synthesis shutoff. Growth of 739L and CD/739L was also significantly inhibited ($P<0.05$) in cells over-expressing single antiviral effector proteins IFIT1 or ISG20 (Fig. 11B) when compared to VEEV WT.

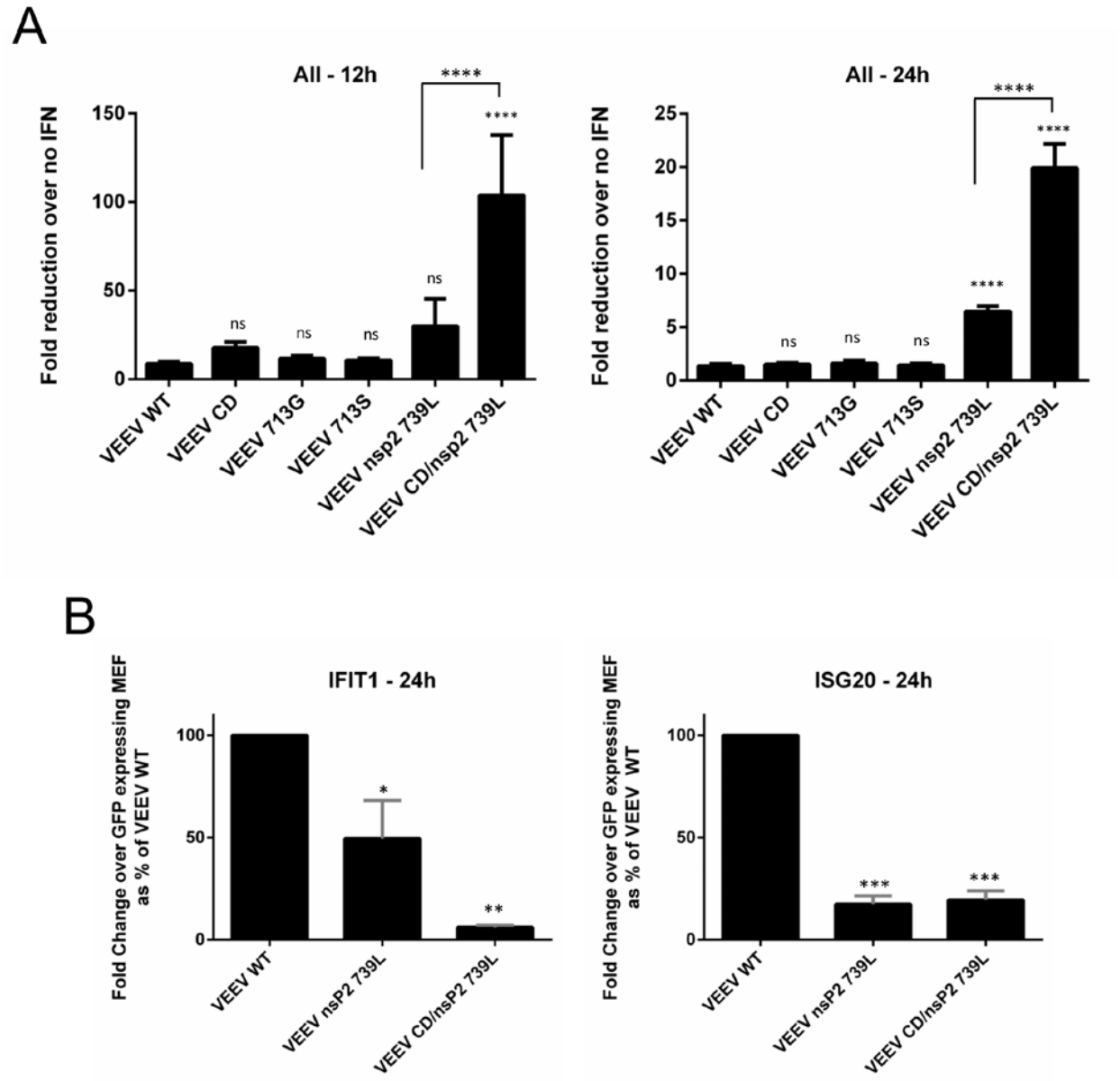



**Figure 11. Sensitivity of VEEV mutants to a pre-established anti-viral state.** Vero cells were mock-treated or treated with 5000 IU human leukocyte IFN for 24h before infection in triplicate with indicated viruses (M.O.I. = 2.5). Supernatants were collected at 12h (A) and 24h (B) p.i. and virus replication was quantified using plaque assays. For each virus, data represents the fold decrease in viral replication versus no IFN, expressed as a fold change over replication of VEEV WT. ****, P<0.0001; ***, P<0.0007; **, P < 0.008 using t-test. Ns = not significant. Data is representative of two independent experiments. All error bars are standard deviations. (C) Tet-inducible mouse embryonic fibroblasts (MEFs) stably expressing IFIT1, ISG20 and GFP (control) were infected in triplicate with indicated viruses (M.O.I. = 1). Cell lysates were collected at 24h p.i and viral RNA levels were measured using RT PCR as described in Materials and Methods. Data represent viral RNA levels from IFIT1 or ISG20 expressing cells as a fold change of viral RNA levels from GFP expressing cells. ***, P<0.0002; **, P<0.006; *, P<0.05 using One-way ANOVA. All error bars are standard deviations.

Summarizing the results of *in vitro* experiments with the VEEV mutants (Table 2), we found that the efficiency of replication early during infection was the viral phenotype most closely associated with resistance to the antiviral state during late stages of infection, and was positively associated with host shutoff. Lesser shutoff of host macromolecular synthesis likely contributed to the reduced growth of 739L and CD/739L observed in IFN-primed cells. Viruses containing mutations negatively affecting host macromolecular synthesis shutoff (739L and CD/739L) were thus more sensitive to the antiviral state, while mutations that had a negligible to slightly positive, albeit non-significant, effect on these viral activities (713G, 713S) were similarly resistant as VEEV WT to the antiviral state.

**Table 2. Summary of VEEV mutant phenotypes at early and late times p.i.**

| | **Early times p.i. (6h)** | | | | **Late times p.i. (18-24h)** | | | |
|---|---|---|---|---|---|---|---|---|
| | **No IFN** | | | **Priming** | **No IFN** | | | **Priming** |
| **Virus** | **Replication efficiency** | **Macromolecular synthesis shutoff** | **IFN induction** | **Resistance to anti-viral state** | **Replication efficiency** | **Macromolecular synthesis shutoff** | **IFN induction** | **Resistance to anti-viral state** |
| **WT** | +++ | +++ | None | + | +++ | +++ | None | +++ |
| **nsP2 713G** | +++ | +++ | None | + | +++ | +++ | None | +++ |
| **nsP2 713S** | +++ | +++ | None | + | +++ | +++ | None | +++ |
| **nsP2 739L** | + | + | None | + | +++ | +++ | None | ++ |
| **CD** | +++ | + | High | + | +++ | + | High | +++ |
| **CD/739L** | + | + | High | + | ++ | + | High | ++ |

### 2.2.4 VEEV nsP2 promotes host translation shutoff.

Due to the potential for pleiotropic effects of nsP2 and/or capsid mutations on multiple viral activities confounding interpretation of virus infection experiments, we developed a plasmid expression system similar to that previously described (304) to assess the role individual VEEV proteins in host macromolecular synthesis shutoff and their involvement in antiviral state resistance. To ensure the observed effects of mutations in viral proteins were not due to differential expression levels in this system, we measured expression levels of WT and mutant nsP2 and capsid and VEEV nsP1 and nsP3 following transfection of plasmids (Fig. 12A). There were no apparent differences observed between WT and mutant VEEV, SINV and CHIKV or WT EEEV nsP2. The expression level of EEEV capsid appeared lower than that of VEEV capsid, while VEEV nsP1 and nonstructural protein 3 (nsP3) expression levels appeared similar to each other.

We had previously observed shutoff of host translation, but not transcription, in cells infected with a VEEV replicon expressing only nsPs (76), and hypothesized that one or more VEEV nsPs induced this activity during infection. We transfected Huh7 cells with plasmids encoding capsid and WT/mutant nsP2 proteins from different alphaviruses, and measured their ability to repress host translation using [$^{35}$S] pulse-labeling to detect steady-state translation. Expression of VEEV/EEEV capsid and VEEV/SINV/CHIKV WT nsP2 was sufficient to significantly ($P<0.0001$) diminish host translation (by >50%) when compared to GFP expressing control cells (Fig. 12B and C). The extent of translation shutoff is lower than that observed during replicating virus infections (Fig. 9), likely due to lower efficiency of the transfection process and/or additional effects of infection on host cell viability. Additionally, the viral proteins expressed from plasmids likely arrest their own transcription/translation. VEEV nsP2

739L delayed induction of host translation shutoff during viral infection, but exhibited only a consistent, but non-significant decrease in translation shutoff compared to VEEV nsP2 WT when expressed from plasmid. SINV nsP2 726G did not shutoff host cell translation. VEEV nsP1 and VEEV nsP3 did not affect host translation (Fig. 12B). Notably, expression of WT EEEV nsP2 was also unable to shutoff host translation, in contrast to WT VEEV nsP2.

We also observed ablation of host translation in cells expressing VEEV capsid. In order to determine if translation shutoff by viral proteins was an independent activity, or potentially caused by shutoff of host transcription, we tested the ability of individually expressed viral proteins to induce host transcription shutoff. We used a quantitative RT-PCR (qRT-PCR) assay to measure levels of an intron from a highly expressed, constitutively active gene whose half-life in cells is thought to be in the order of minutes. This allows use of intron levels as a measure of pol II transcription levels in a cell (305) and this type of assay has previously been used to analyze transcription of the human insulin gene (305, 306) and to measure transcription shutoff during La Crosse virus infection (307, 308). We transfected Huh7 cells with plasmids expressing SINV, CHIKV, EEEV and VEEV nsP2 and VEEV and EEEV capsid proteins and used qRT-PCR to measure levels of gamma actin intron #3 (307) at 12h post-transfection (Fig. 12D). Levels of this intron were reduced 10-fold ($P<0.0001$) with 6h of Actinomycin D treatment as a control for pol II transcription inhibition. SINV and CHIKV nsP2 and EEEV and VEEV capsid significantly inhibited transcription (by >50%; $P<0.02$) when compared to a GFP control. In contrast, EEEV and VEEV nsP2 did not inhibit transcription in transfected cells. These observations with individually expressed proteins confirm and extend published observations using viruses (74, 251, 252, 254, 300). From our results we concluded that VEEV nsP2 was the viral nsP responsible for host translation, but not transcription shutoff.

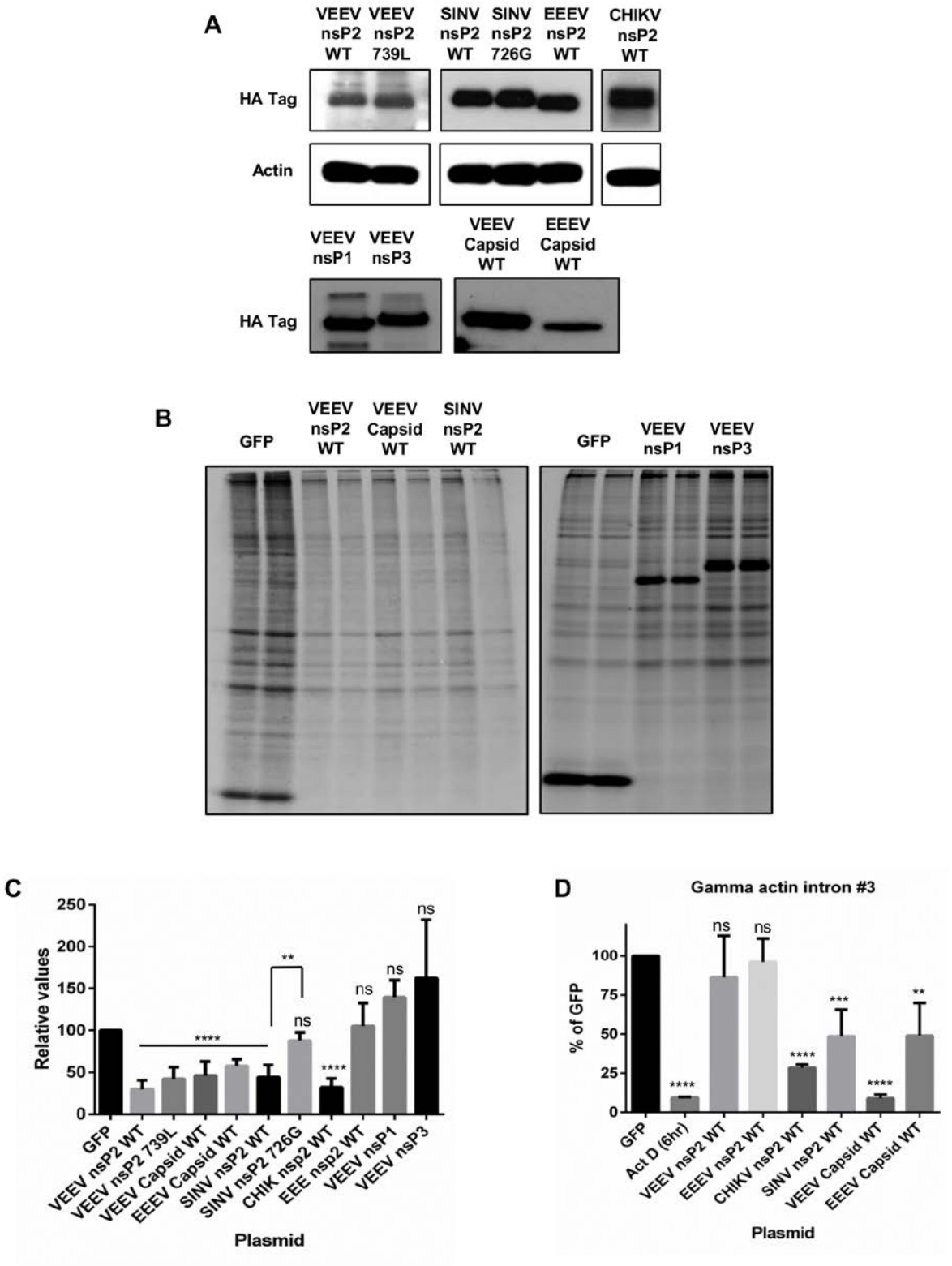



**Figure 12. Individually expressed viral proteins block transcription and translation.** (A) Huh7 cells were transfected with indicated plasmids and lysates were collected at 18h post transfection. Western blots for HA-tag were performed as described in Materials and Methods. (B and C) Huh7 cells were transfected with plasmids coding

for indicated viral proteins and labeled with 100μCi/ml of [$^{35}$S] Cys/Met for 2h at 8-24h post transfection. Lysates were collected and resolved on SDS-PAGE gels and visualized as described in Materials and Methods. (B) Representative image of nsP and capsid induced shutoff compared to GFP control. (C) Densitometry was performed to quantify the extent of shutoff following transfection of indicated plasmids. ****, P<0.0001; **, P<0.01 using t-test and One-way ANOVA. Ns = not significant . Data is the average of four independent experiments. All error bars are standard deviations. (D) Huh7 cells were transfected with plasmids coding for indicated viral proteins and lysates were collected in triplicate at 12h post transfection. RT PCR for human gamma actin intron #3 was performed as described in Materials and Methods. ****, P<0.0001; ***, P<0.01; **, P<0.02 using t-test. Ns = not significant. Data is representative of four independent experiments. All error bars are standard deviations.

### 2.2.5 Induction of host translation shutoff by VEEV nsP2 in IFN pre-treated cells contributes to its resistance to the antiviral state.

Our previous experiments using propagation-competent viruses indicated a role for both components of host macromolecular synthesis shutoff in the antiviral state resistance of VEEV, mediated by nsP2 (translation shutoff) and capsid (transcription shutoff). As the nonstructural proteins are produced earlier than structural proteins during viral infection (4), we addressed the role of translation shutoff in the antiviral state resistance of VEEV in a context independent of replication rates and absent other viral proteins using the nsP overexpression system. MEF cells were used in these experiments as they establish a potent antiviral state in response to IFN treatment in contrast to Huh7 cells. MEF cells were pre-treated with mouse IFN for 16h and transfected with plasmids encoding GFP (control) or VEEV/SINV WT nsP2. Cells were labeled with [$^{35}$S] Cys/Met at 24h post transfection. Host translation in untreated cells was significantly inhibited by the expression of either VEEV or SINV nsP2 WT when compared to GFP expressing control cells (data not shown). However, a significant reduction in host translation in IFN-primed cells (>50%; P<0.0004) was only observed in VEEV nsP2 WT transfected cells (Fig. 13A and B), indicating that VEEV was capable of inhibiting translation despite the presence of an antiviral state, whereas SINV nsP2 was not. This is consistent with the published

observation that VEEV successfully induced global translation shutoff during infection of IFN-primed cells (76). This data, in conjunction with the observation that VEEV successfully replicates in the presence of an antiviral state (Fig. 6), and our mutant virus studies (Table 2) supports the conclusion that the ability of VEEV nsP2 to induce translation shutoff in IFN-primed cells contributes to a host cell environment permissive to virus replication. We also tested the ability of VEEV nsP2 739L to shutoff translation in IFN-primed cells. Similar to observations made in untreated cells, VEEV nsP2 739L expressed from plasmid was successful at inducing translation shutoff (Fig. 13A and B).

We speculated that if this model were correct, expression of VEEV nsP2 in IFN-primed cells should result in modulation of the cellular environment to favor viral replication by decreasing protein levels of antiviral ISG's. Several ISG's have been shown to have short half-lives (309-312) between 6-18h, which makes the antiviral state potentially vulnerable to a sustained translation shutoff. In order to test this hypothesis, we quantified protein levels of two ISG's in IFN-primed MEF cells transfected with plasmids expressing VEEV nsP2 and SINV nsP2 (Fig. 13C and D). IFIT1 levels in VEEV nsP2 expressing MEF's were significantly (by 40-50%; $P<0.05$) lower than levels in GFP expressing control cells, while levels in SINV nsP2 expressing cells did not significantly differ from those of GFP expressing cells. Similarly, levels of T-cell specific GTPase (TGTP) were significantly lower (by 25%; $P<0.001$) in VEEV nsP2 expressing MEF's when compared to GFP and SINV nsP2 expressing cells (Fig 16C and D). This data taken together with the resistance of VEEV to individual overexpressed antiviral proteins (Fig. 6C) suggests the resistance phenotype of VEEV is at least partially mediated through a global effect such as host macromolecular synthesis shutoff rather than the presence of multiple separate viral resistance factors.

To demonstrate the functional significance of host translation shutoff in diminishing the antiviral state, we measured the replication of an IFN sensitive virus, YFV 17-D strain, engineered to express nano-Luciferase (nLuc) as a cleavable fusion with the YFV capsid protein (described in Watson et.al., manuscript in preparation) in IFN-primed Huh7 cells transfected with plasmids expressing GFP (control) or virus nsP2 proteins. Replication of 17-D virus was inhibited 50% in Huh7 cells primed with only 5IU IFN when compared to replication in untreated cells (data not shown). Huh7 cells were treated with 2000 IU IFN for 16h and infected with 17-D nLuc 8h post transfection. The replication level of 17-D in VEEV nsP2 expressing IFN-primed cells was significantly ($P<0.002$) higher than that observed in GFP or VEEV nsP1 expressing control cells (Fig. 13E). In contrast, 17-D replication in SINV nsP2 expressing cells was not significantly different from replication in controls. Similar results were observed in Vero cells (data not shown). We conclude that VEEV nsP2 induced translation shutoff in primed cells reduces the efficacy of the antiviral state, supporting the replication of an IFN-sensitive virus.

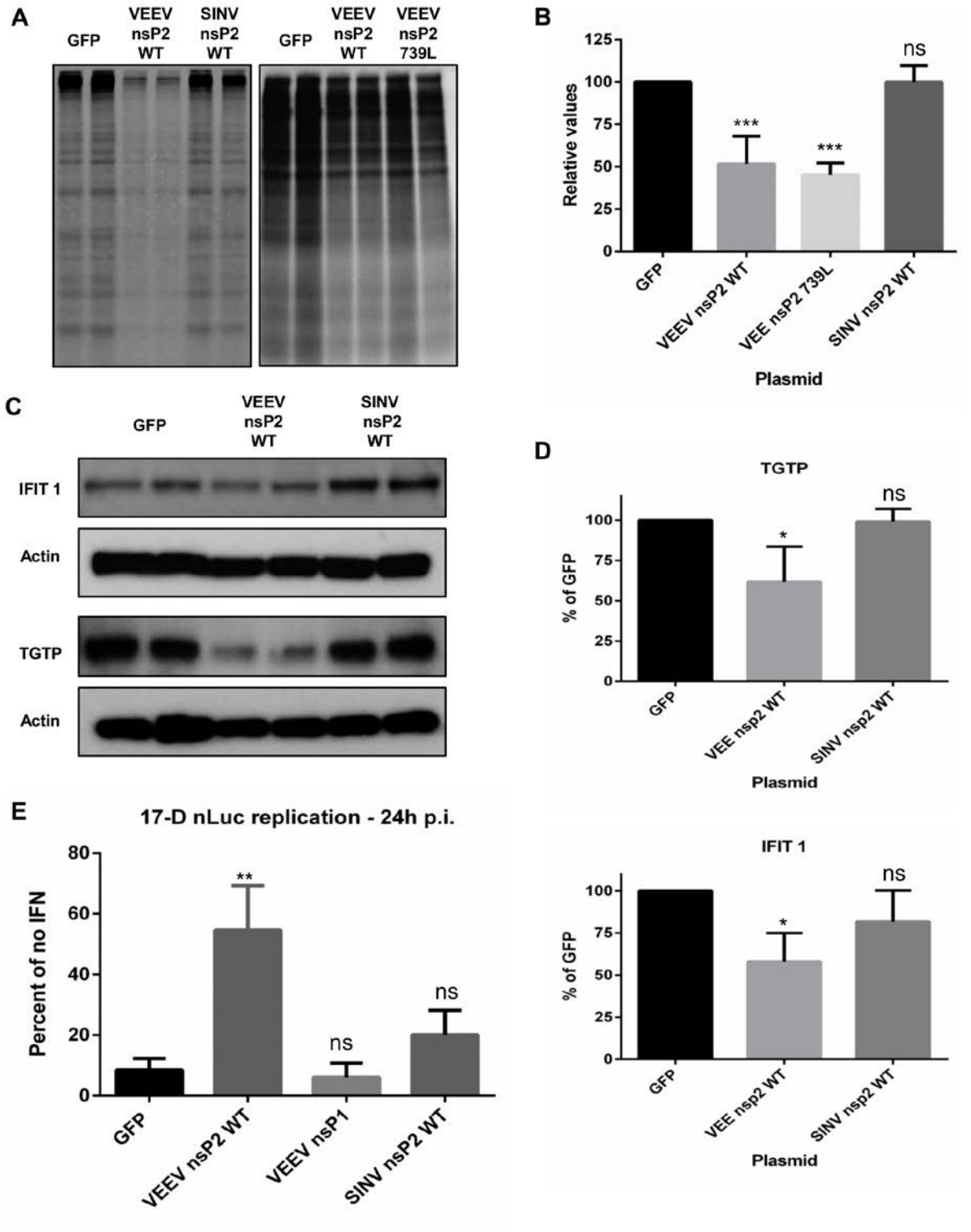



**Figure 13. VEEV nsP2 can shut off global host macromolecular synthesis and downregulate the antiviral state.** (A and B) MEF cells were treated with 100-150 IU/mL (10mL total) mouse IFN in a 60mm dish or 400 IU/mL (10mL total) mouse IFN in a 100 mm dish for 16h and transfected with indicated plasmids. Cells were labeled with 100µCi/ml of [$^{35}$S] Cys/Met for 2h at 24h post transfection. Lysates were collected and resolved on

SDS-PAGE gels and visualized as described in Materials and Methods. (A) Representative images of nsP2 induced shutoff in IFN primed cells compared to Mock or GFP control. (B) Densitometry was performed to quantify the extent of shutoff. ***, P<0.0004 using t-test. Ns = not significant. Data is the average of four independent experiments. (C and D) MEF cells were treated with 125-150 IU/mL mouse IFN in a 60mm dish for 16h and transfected with indicated plasmids. Lysates were collected at 24h post transfection and western blots for ISGs were performed as described in Materials and Methods. (C) Representative blot of IFIT1 and TGTP levels in VEEV and SINV nsP2 transfected cells. (D) Densitometry was performed to normalize IFIT1 and TGTP levels to actin and compared to GFP transfected control. *, P<0.04 using t-test. (E) Huh7 cells were treated with 2000 IU/mL for 16h and transfected with plasmids expressing indicated proteins. Cells were infected with 17-D nLuc at 8h following transfection (M.O.I. = 1.5) and lysates were collected for luciferase assay at 24h p.i. Data is expressed as RLU/μg in IFN-primed cells as a percentage of untreated cells for each plasmid. Results are average of three independent experiments. **, P<0.002 using t-test. Ns = not significant. All error bars are standard deviations.

To determine the if difference in functionality between these proteins was due to altered localization or activity in IFN pre-treated cells, we investigated whether a change in localization of VEEV/SINV nsP2 occurred in response to IFN pre-treatment (Fig. 14A and B). In unprimed cells, SINV nsP2 was present in the nucleus and cytoplasm, which is consistent with published observations (75). Similarly, VEEV nsP2 was present mainly in the cytoplasm in most transfected cells (256) (Fig. 14A). Pre-treatment with IFN did not noticeably alter the distribution of SINV nsP2 (Fig. 14B). Surprisingly, VEEV nsP2 localized to the nucleus in nearly all primed and transfected cells (Fig. 14B). This raised the possibility that nucleus-localized VEEV nsP2 induced transcription shutoff in IFN-primed cells which indirectly ablated host translation (Fig. 13A and B). However, expression of SINV nsP2 significantly (P<0.003) inhibited host transcription compared to GFP expressing control in unprimed cells, while VEEV nsP2 did not (Fig. 14C). However, no inhibition of host transcription was observed upon expression of VEEV or SINV nsP2 in primed cells (Fig. 14D). Therefore, the movement of VEEV nsP2 into the nucleus in IFN-primed cells does not induce host transcription shutoff.

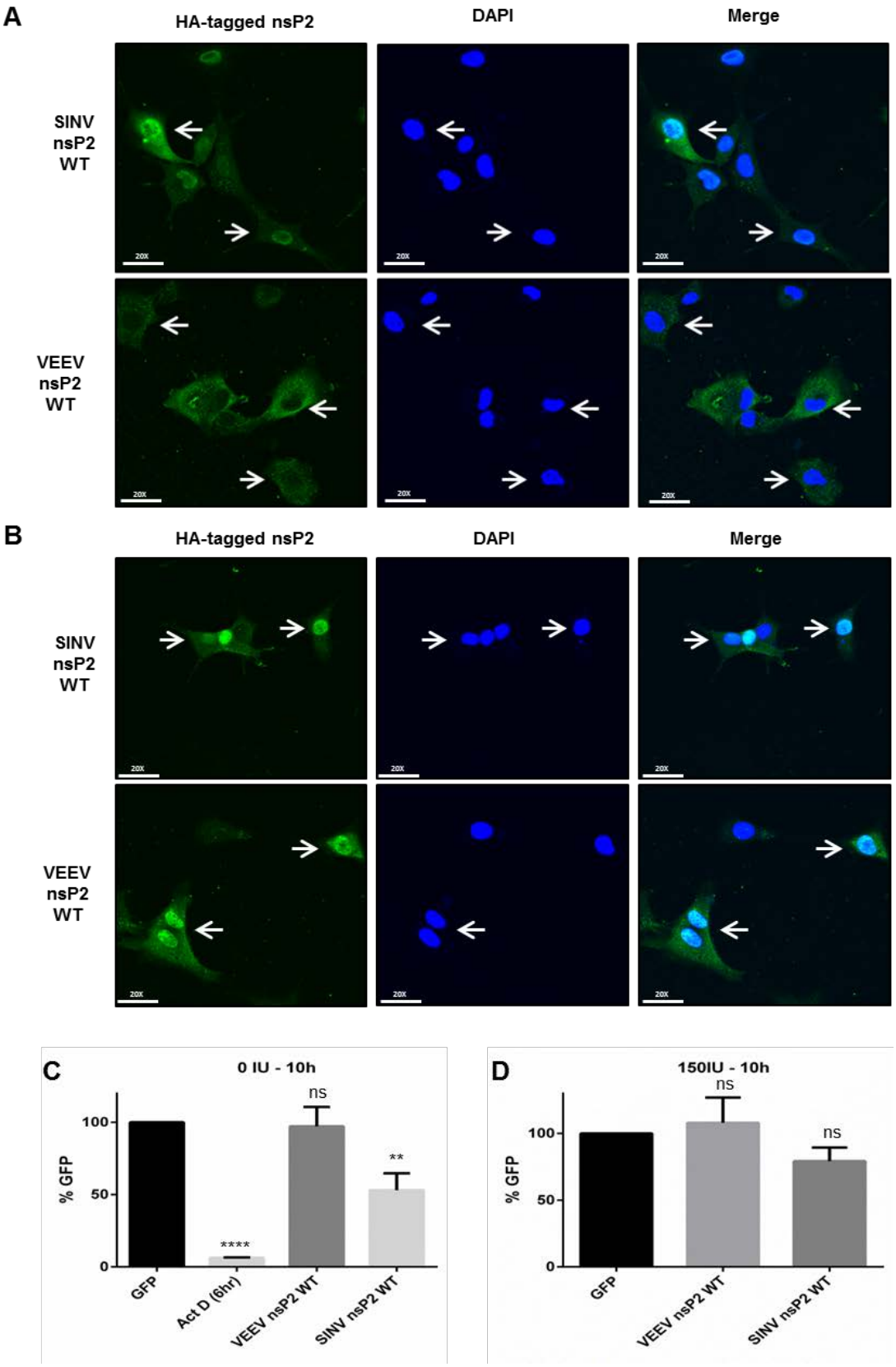

A
HA-tagged nsP2
DAPI
Merge
SINV nsP2 WT
VEEV nsP2 WT
B
HA-tagged nsP2
DAPI
Merge
SINV nsP2 WT
VEEV nsP2 WT
C
0 IU - 10h
% GFP
100
50
0
ns
**
****
GFP
Act D (6hr)
VEEV nsP2 WT
SINV nsP2 WT
D
150IU - 10h
% GFP
100
50
0
ns
ns
GFP
VEEV nsP2 WT
SINV nsP2 WT

**Figure 14. Localization of VEEV nsP2 is altered in IFN pre-treated MEF cells.** (A and B) MEF cells were untreated (A) or pre-treated (B) with 150 IU/mL mouse IFN in a 60mm dish for 16h and transfected with plasmids expressing indicated viral proteins. Cells were fixed at 24h post transfection and stained for hemagglutinin (HA) tag as described in Materials and Methods. (C and D) MEF cells were untreated (C) or pre-treated (D) with 150 IU/mL mouse IFN in a 60mm dish for 16h and transfected with plasmids expressing indicated proteins. Lysates were collected at indicated times post transfection. RT PCR for human gamma actin intron #3 was performed as described in Materials and Methods. Results are average of three independent experiments. ****, P<0.0001; **, P<0.003 using t-test. Ns = not significant. All error bars are standard deviations.

### 2.2.6 VEEV nsP2 or capsid mutants are attenuated in mice but do not increase systemic IFN.

To determine if alterations in translation shutoff were attenuating *in vivo*, we infected mice with VEEV WT, CD, 739L and CD/739L viruses. Morbidity and mortality profiles and disease phenotypes demonstrated that attenuation co-varied with replication rates and IFN sensitivity measured *in vitro* (Fig. 6 and 11; Fig. 15A and B). IFN-α induction in the serum of infected mice at 12h p.i. (peak IFN is typically 12-18h p.i. (35)) as measured by enzyme-linked immunosorbent assay (ELISA) was similar between viruses, while IFN-α/β measured by bioassay showed the mutant viruses to induce generally lower levels than the WT (Fig. 15C), likely reflecting more limited replication. Therefore, systemic IFN induction appears to be independent of differential macromolecular synthesis shutoff characteristics *in vivo* and unlikely to determine virulence between viruses.

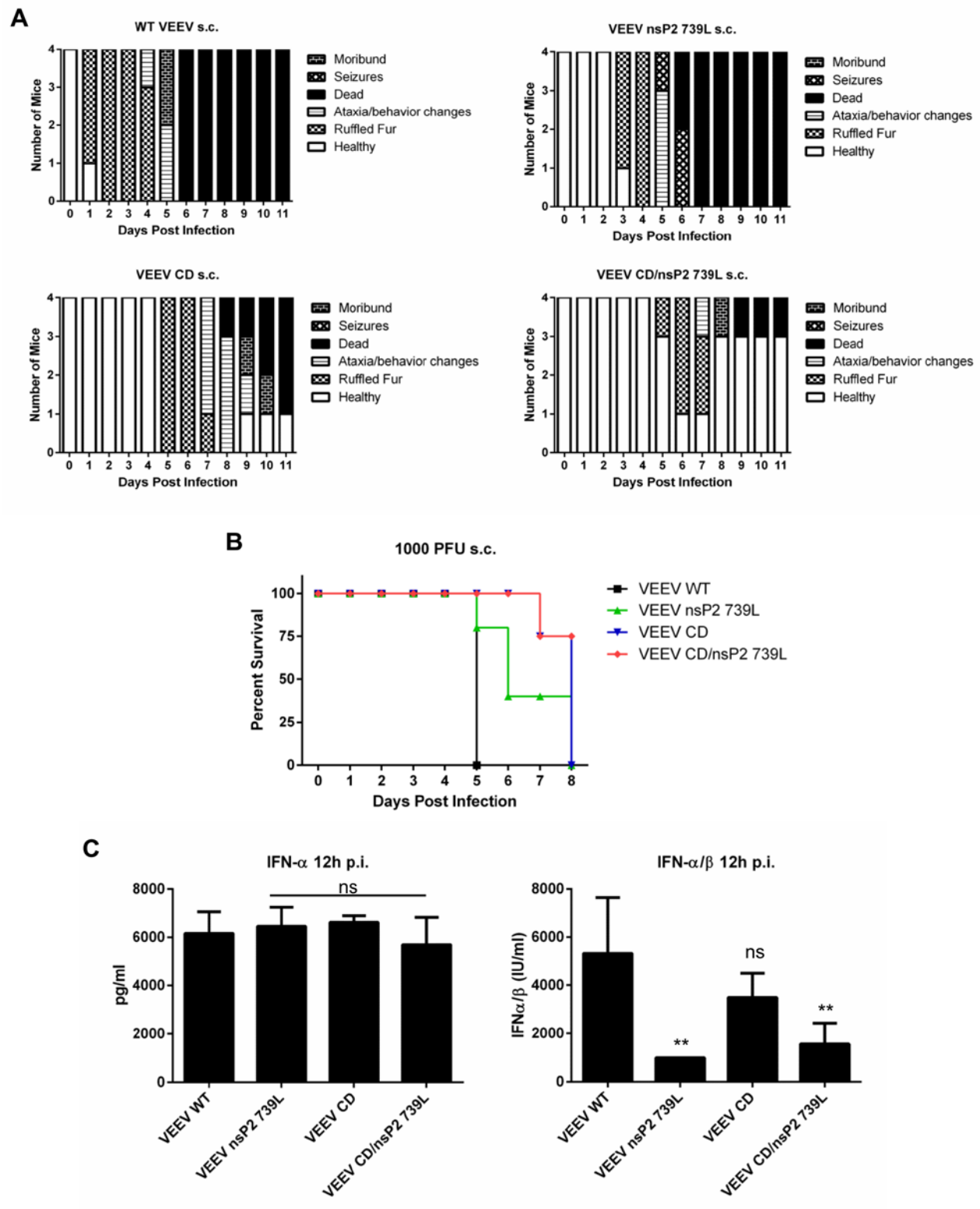



**Figure 15. VEEV mutants are attenuated in vivo.** (A) CD-1 mice were infected subcutaneously in the hind leg footpad with 100 PFU of indicated viruses and scored for degree of sickness at 24h intervals. (B) CD-1 mice were infected subcutaneously in the hind leg footpad with 1000 PFU of indicated viruses and average survival time (AST)

was determined. 4 mice per group. (C and D) Serum from mice infected with indicated viruses was collected at 12h p.i. and used to measure IFN-α using ELISA (C) or used to measure IFN-α/β using a bioassay (D) as described in Materials and Methods. **, P<0.006 using One-way ANOVA. Ns = not significant. All error bars are standard deviations.

## 2.3 DISCUSSION

### 2.3.1 Interaction of VEEV with the IFN-induced antiviral state.

During infection of mice, and presumably humans, most alphaviruses initially interact with myeloid cells which are likely the major source of systemic serum IFN (165, 173, 183, 188, 313). Notably, VEEV infection induces the highest levels of serum IFN of all alphaviruses tested (35, 314), with lower levels observed during SINV infection (35), while CHIKV and EEEV infection results in little to no IFN production (188, 273). IFN induction during CHIKV infection occurs from non-hematopoietic cells (273). This induction of large amounts of IFN during VEEV infection, and lesser amounts during SINV infection, occurs within 12h of inoculation *in vivo* (35, 313), upregulates ISG's and establishes an antiviral state at uninfected sites distal to the initial infection site, including the central nervous system (315-317) an important site of replication during VEEV infection. Thus, it is likely that most, if not all, cells infected by VEEV (and possibly SINV) following the initial round of infection *in vivo* have already been exposed to IFN and are primed to resist infection.

The ability of the IFN induced antiviral state to limit virus infection is believed to be closely associated with severity of alphavirus induced disease and mortality. Alphaviruses overcome the antiviral state by either preventing its activation (EEEV; (188, 276)) or by resisting its inhibitory effects on viral replication (VEEV, SINV; (35)). Previous reports have suggested that the balance between virus replication and IFN secretion/response early during infection determines the severity of disease (271). However, those studies were performed using an *in vitro* model of SINV infection and did not take into account the induction of large amounts of IFN from myeloid cells following *in vivo* inoculation, which rapidly primes uninfected cells *in vivo*. Alphaviruses resistant to this priming successfully replicate, infect the brain and cause disease (VEEV), while the replication of sensitive alphaviruses is controlled (e.g., SINV). It is notable that SINV infection in normal adult mice is completely controlled with no visible signs of disease observed during the course of infection (173), while VEEV infection leads to essentially uniform mortality (181).

Our data show that VEEV replication is most resistant to the effects of IFN priming among all alphaviruses tested, and that this resistance localizes at least in part to the nonstructural protein/non-translated regions of the genome. VEEV replication in IFN-primed cells approached levels observed in untreated cells, while other alphaviruses were 10-4000 times more sensitive to the antiviral state. VEEV replication in IFN-primed cells lags by ~6h compared to replication in unprimed cells, which suggests VEEV modulates the cellular environment to favor viral replication during this phase. Using reporter RNA's that mimicked initial translation of the incoming viral genome in an infected cell, we found that the antiviral state suppressed VEEV, EEEV, SINV and CHIKV replication at the point of initial translation, in agreement with and extending published observations (281, 283, 299). Nevertheless, low-level translation of

reporters in primed cells indicates that the nsPs of alphaviruses that infect IFN-primed cells are indeed produced. This suggests that an activity or activities of one or more nsPs of VEEV would be present to successfully antagonize, in whole or in part, the antiviral state, while the activities of other tested alphavirus nsPs do so to a lesser degree.

### 2.3.2 Nsp2 is a suppressor of multiple host responses but activity varies between alphaviruses.

Previous studies, primarily with SINV, have demonstrated that mutations in the C-terminal region of nsP2 can impact shutoff of transcription and translation (106, 253, 254). With VEEV and EEEV New World viruses, the capsid protein affects host transcription (104, 105), which we confirm but our data suggest that this activity is delayed *versus* the translation inhibiting activity of nsP2. Additionally, data from CHIKV and SINV (254, 257) indicates that translation shutoff occurs before shutoff of transcription during infection, and the activities are mediated through distinct mechanisms (i.e. transcription shutoff does not directly mediate translation shutoff).

With SINV, a single mutation (726G) can render the virus defective in abrogation of each of these activities (253, 271). A widely conserved "PGG" domain in which the 726G (SINV) or 718S (CHIKV) mutation substitutes the proline has been suggested to be the critical site for these activities of nsP2 (74, 253, 271). We confirm that the "P-G" mutation at aa 726 affects each activity of the SINV TR339 consensus strain using both viruses and individually expressed nsP2 (nsP2 726G transcription shutoff data not shown). For CHIKV, in contrast to published data with P718S which suggests this mutation abrogates nsP2 mediated host macromolecular synthesis shutoff (74), our data indicates P718S has no effect on this activity of CHIKV nsP2 (data not shown). The substitution of "G" or "S" for "P" in the VEEV "PGG" domain (713G), analogous

to the SINV/CHIKV mutations had no effect on these activities. Indeed it had a slight enhancing effect on translation shutoff (current studies and data not shown) as well as virulence in mice (not shown). EEEV does not have the canonical “PGG” domain; rather its sequence is “KGG.” It is tempting to speculate that the sequence differences result in the failure of EEEV nsP2 to inhibit translation. However, substitution of the “K” residue for “P” in the “PPG” site of VEEV had no effect on transcription or translation shutoff (data not shown). This along with the 713 “G” and “S” mutant data, suggest that this is not a critical residue with New World viruses and, perhaps, Old World viruses other than SINV. The 739L mutation of VEEV, selected for limited cytopathogenicity in BHK cells (258), lessened translation shutoff in the context of virus infection but the individually expressed protein affected translation shutoff in a reproducible but ultimately non-significant manner. Studies with WT and 739L VEEV replicons were inconclusive due to low infectivity of Vrep 739L (data not shown) for multiple cell types. It is possible that the effects of 739L on translation shutoff only manifest in the context of virus infection, and not when VEEV nsP2 is expressed alone. While VEEV nsP2 739L shut off host translation when expressed from a plasmid, the delayed induction of translation shutoff in VEEV 739L virus infected cells compared to VEEV WT implies that this mutation may be at least partially deficient at this activity independent of its effect on viral replication. While VEEV 739L virus replicated more slowly than WT, replication in 739L infected cells suggests that nsP2 levels were presumably sufficient to induce translation shutoff during infection. We conclude that the C-terminal region of New world alphavirus nsP2 but not the “PGG” domain is directly involved in translational shutoff, in contrast to the critical requirement of the “P” residue for efficient function of SINV nsP2. Further, the importance of the “PGG” domain appears to be

limited to SINV nsP2, suggesting that activities attributed to nsP2 may be localized to unique regions of the protein in different alphaviruses.

### 2.3.3 Effect of VEEV induced translation shutoff on the antiviral state.

Our virus mutagenesis data suggest that replication efficiency of VEEV is most closely associated with antiviral state resistance *in vitro*, which itself positively associates with macromolecular synthesis shutoff. The sensitivity of mutants to IFN priming correlates with their growth rates *in vitro*, suggesting that an inability to efficiently shutoff host macromolecular synthesis reduces the growth of mutant viruses and renders mutants more sensitive to cell stress responses. The viruses grow more slowly in cells that do not make IFN but other stress responses could be active as well as constitutive ISG induction. Our data show host macromolecular synthesis shutoff through its ability to prevent synthesis of new ISG's, control IFN induction and enhance viral growth in infected cells plays a role in resistance to the anti-viral state, as defective mutants (739L and CD/739L) are more sensitive to the antiviral state when compared to mutants (713G, 713S) having no effect on this process.

During alphavirus infection, the nonstructural proteins including nsP2 are produced first, and are required for production of structural proteins including capsid from the subgenomic promoter (4). Thus it is likely that virus induced *translation shutoff* in IFN-primed cells is important early during resistance of VEEV to the antiviral state, and the effect of capsid mediated transcription shutoff on the antiviral state is additive to, and very likely delayed *versus*, the activity of nsP2 due to temporally deferred synthesis of capsid during infection. We demonstrate that VEEV nsP2 induces translation shutoff in IFN-primed cells while SINV nsP2 is unable to shutoff host translation or transcription. As expected, we observed lower levels of

measured ISG's in IFN-primed cells expressing VEEV nsP2 when compared to SINV nsP2. Translation shutoff also supports the replication of Yellow Fever vaccine strain 17-D, a virus highly susceptible to IFN priming, as replication of 17-D virus in IFN-primed cells expressing VEEV nsP2 approaches replication levels in unprimed cells. Our data suggest VEEV nsP2 induced translation shutoff in IFN-primed cells early after infection is an important factor in the resistance phenotype of VEEV by decreasing levels of pre-existing ISG's and reducing production of new ISG molecules, which engenders a cellular environment permissive to viral replication. In contrast, the inability of SINV nsP2 to shutoff transcription or translation in IFN pre-treated cells likely explains in part its sensitivity to IFN priming *in vitro*, and lack of disease in immunocompetent adult mouse models where SINV induces significant serum IFN. It is unclear whether one or both these activities contributes to antiviral state resistance, and whether or not one predominates, as both are localized to a single domain of nsP2 in SINV. Nonetheless, the inhibition of nsP2 activity by IFN likely results in the sensitivity of SINV to the antiviral state. Similarly, the sensitivity of other alphaviruses to the antiviral state may in part result from their inefficient induction of translation shutoff early after infection. Interestingly, although EEEV nsP2 is unable to induce host translation shutoff, EEEV demonstrates mortality similar to VEEV in mice (148, 188), and is far more virulent in humans than VEEV (318). While EEEV capsid can shutoff transcription in cells (104), EEEV mediated suppression of systemic innate immune responses *in vivo* involves avoidance of lymphoid tissue targeting through heparan sulfate binding (148) as well as suppression of replication in myeloid cells via virus genome binding to a hematopoietic cell-specific microRNA, miR142-3p (276). Thus, very closely related alphaviruses utilize very different strategies to overcome innate immunity. Furthermore, our current and previous results suggest the efficacy of innate immunity suppression as major factors

in the relative virulence of arthritogenic alphaviruses (rarely fatal), VEEV (occasionally fatal) and EEEV (frequently fatal).

In addition to translation shutoff, the effect of capsid mediated transcription shutoff on the antiviral state is additive to, and delayed *versus*, the activity of nsP2, due to temporally deferred synthesis of capsid during infection. The extent of shutoff observed during infection of unprimed cells with CD/739L at 18h p.i. is roughly equal to that of CD and 739L combined, demonstrating the additive effect of both activities on macromolecular synthesis shutoff. Similarly, IFN induction following CD/739L infection was significantly greater that CD, reflecting the deficiency of that mutant in the inhibition of both host transcription and translation, verses transcription only for CD. Additionally, the effect of transcription shutoff by capsid was observed during [$^{35}$S] labeling of new translation products, suggesting that while nsP2 and capsid can both induce eventual translation shutoff, their effects are additive during infection.

We propose three possible explanations for the ability of VEEV, and the lack thereof of SINV, to induce translation shutoff in IFN-primed cells: 1), that the mechanism of translation shutoff used by VEEV is different from that of SINV; 2), that VEEV shuts off translation in IFN-primed cells using a mechanism different from that used by alphaviruses in unprimed cells; or 3), that the induction of translation shutoff during VEEV infection in both primed and unprimed cells is more efficient and substantive than that induced by SINV. Future studies aimed at identifying the molecular pathways underlying alphavirus induced translation shutoff will distinguish between these possibilities.

Data from our lab (unpublished) using translation reporters containing internal ribosome entry sites (IRES) from cricket paralysis virus (CrPV), Hepatitis C virus (HCV) and Encephalomyocarditis virus (EMCV) suggest that there may exist two possible mechanisms of

alphavirus induced translation shutoff (Fig. 16A and B). Different IRES sequences require different combinations of eIFs to initiate translation (319): CrPV IRES binds directly to the 40S ribosomal subunit to initiate translation (320, 321); in contrast, the HCV IRES binds to eIF3 and the 40S subunit of the 43S complex and initiates translation (322, 323), whereas the EMCV IRES requires all initiation factors except eIF4E (324, 325). Efficient translation of reporter RNAs expressing luciferase under the CrPV and HCV IRES sequences, but not the EMCV IRES sequence, and without addition of a poly (A) tail, in VEEV infected cells suggested that the virus is targeting the eIF4F (consisting of eIF4E, eIF4G and eIF4A) complex to induce translation shutoff (Fig. 16A). Conversely, addition of a poly (A) tail to reporter molecules completely ablated translation of all reporters regardless of IRES sequence, which suggested that a poly (A) tail related mechanism is also involved in translation shutoff, and is likely exerting a dominant effect on translation (Fig 16B). The relationship between the two mechanisms is unknown, and it is possible that they are mediated through a common pathway. Future studies will explore the molecular basis underlying these identified mechanisms.

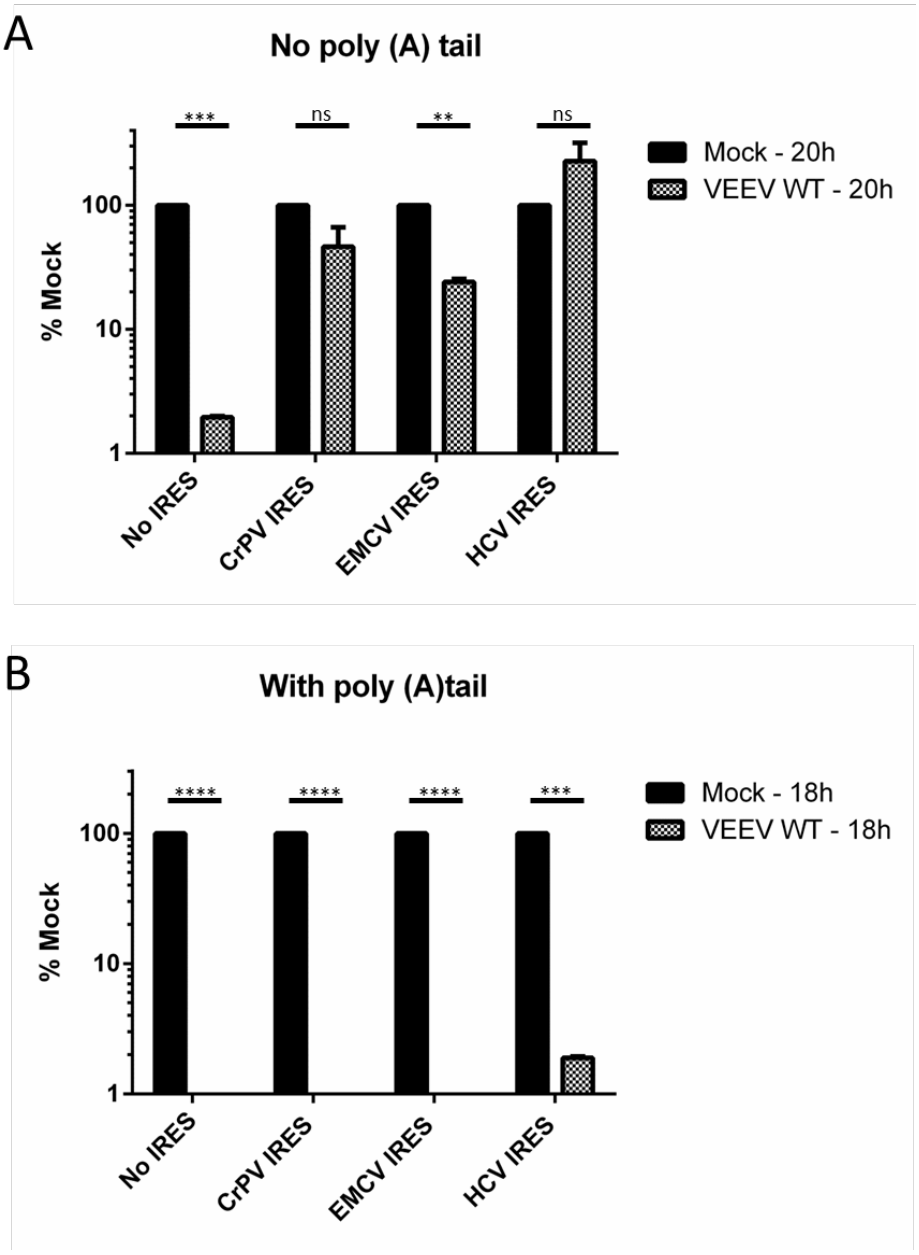



**Figure 16. Translation of IRES reporters in VEEV infected cells.** MEF cells were infected with VEEV WT (M.O.I. = 5) and electroporated with indicated reporters as described in Materials and methods. Cells were lysed 1.5h post-electroporation and luciferase activity was measured. Data is RLUs per µg total protein expressed as % of Mock. Data is representative of three independent experiments. ****, P<0.0003; ***, P<0.0004, **, P<0.009 using T-test. All error bars are standard deviations.

# 3.0 JAK1 DEGRADATION BY THE ALPHAVIRUS NON-STRUCTURAL PROTEIN-2 CONTRIBUTES TO IFN A/B SIGNALING INHIBITION DURING INFECTION

## 3.1 INTRODUCTION

The type-1 IFN system is a critical innate immune mechanism used to control and clear pathogens following infection. IFN-α/β has been shown to be important for the control of a variety of viruses (190-194), including alphaviruses (35, 173, 195-197), and viruses have evolved various strategies to inhibit the effects of IFN, such as inhibition of STAT1 phosphorylation, degradation of STAT1 and inhibition of JAK1 activation (198-203). Inhibition of STAT1 phosphorylation following IFN-α/β and IFN-γ treatment has also been observed during alphavirus infection of multiple cell-types (76, 77, 253, 271, 272). Additionally, CHIKV nsP2 expressed individually has been shown to block STAT1 nuclear translocation following IFN treatment (77), and mutations in the C-terminal region of SINV and CHIKV nsP2 appear to reduce this activity. The SINV 726G virus (253, 271) and the individually expressed CHIKV nsP2 mutant KR649-650AA, but not D711G-P718S, (74) have been described as defective in blocking STAT1 signaling. However, the New world alphavirus protein involved in STAT1 phosphorylation inhibition is unknown. We previously observed STAT1 signaling inhibition in

cells infected with a VEEV replicon lacking structural proteins (76), and hypothesize that one or more nsPs induce this activity during viral infection.

Inhibition of STAT1 signaling is believed to be induced independently of host transcription and translation shutoff, as it does not appear to require *de novo* gene or protein synthesis (272). Moderate reduction in the level of IFNGR observed during infection (272) are insufficient to fully account for the diminution of STAT1 phosphorylation observed in infected cells. However, the precise mechanism(s) by which alphaviruses inhibit STAT1 phosphorylation are not known. Therefore, we propose that the step(s) in the IFN signaling cascade that appear to be targeted by nsPs lie between receptor-ligand binding and STAT1 phosphorylation.

## 3.2 RESULTS

### 3.2.1 NsP2 is the New world alphavirus protein responsible for STAT1 signaling inhibition.

To determine the alphavirus protein(s) responsible for STAT1 signaling inhibition, we transfected Huh7 cells with plasmids encoding WT and mutant SINV, CHIKV, EEEV or VEEV nsP2 proteins and measured their ability to suppress STAT1 signaling. Huh7 cells were used as they are very efficiently transfected, and robustly phosphorylate STAT1 in response to IFN treatment. Transfected cells were treated for 30 min with 1000 IU/mL human leukocyte IFN at 24h post transfection and stained for STAT1 and HA-tagged nsP2 (Fig. 17). We used the presence or absence of STAT1 in the nucleus as a measure of successful IFN signaling inhibition by plasmids expressing viral nsP2 (Fig. 17) as this step of the IFN signaling cascade is

downstream of STAT1 Y701 phosphorylation. Expression of WT nsP2 proteins from SINV, CHIKV, EEEV and VEEV significantly ($P<0.0001$) abrogated STAT1 nuclear translocation in transfected cells (Fig. 17B) when compared to a GFP-expressing control. The mutants SINV nsP2 726G, VEEV 739L and CHIKV nsP2 711G/718S significantly ($P<0.0007$) reduced this activity when compared to SINV, VEEV and CHIKV nsP2 WT, respectively, highlighting the multifunctional nature of the C-terminal region of nsP2, which is also involved in induction of transcription and translation shutoff during infection. In addition, STAT1 nuclear localization was not prevented by expression of VEEV nsP1 or nsP3 (Fig. 17B). Further, levels of STAT1 did not appear to decrease by nsP2 expression (also shown in Fig. 21C). Taken together, these results suggest nsP2 expressed independently of other viral proteins possesses STAT1 signaling inhibition activity for arthritogenic and encephalitic alphaviruses and mutations that reduce this activity during virus infection also reduce the activity of nsP2 alone.

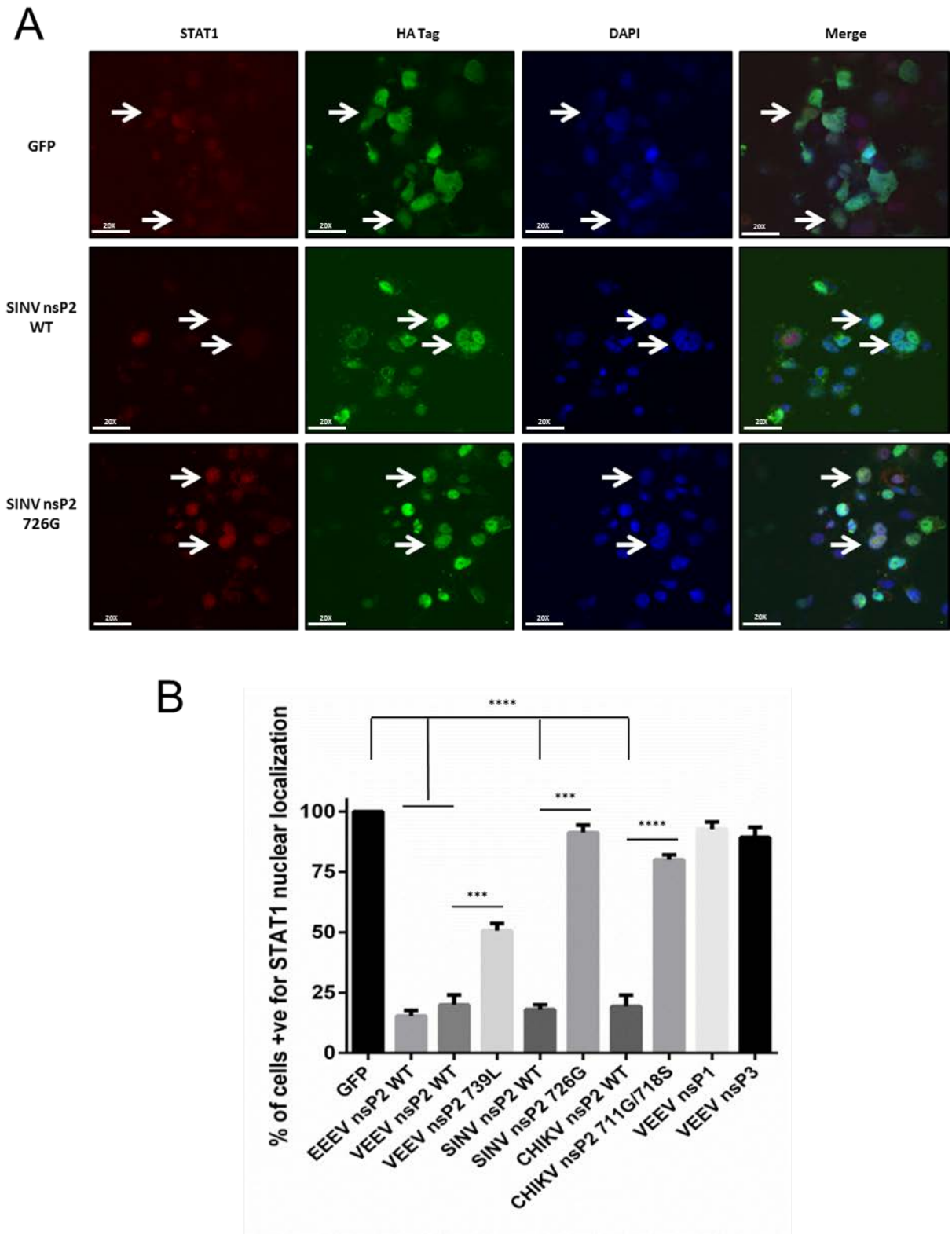



**Figure 17. Individually expressed viral proteins block nuclear translocation of STAT1 following IFN treatment.** (A and B) Huh7 cells were transfected with indicated plasmids and treated with 1000 IU human leukocyte IFN at 24h post transfection for 30 min. Cells were fixed and stained for STAT1 and Ha Tag as described

in Materials and Methods. (A) Immunofluorescence images of Huh7 cells co-stained for STAT1 and viral nsp2 (Ha Tag). (B) Huh7 cells transfected with indicated plasmids that successfully expressed viral proteins were counted for the presence of STAT1 in the nucleus in triplicate. Fifty cells were counted for each transfection in triplicate. ****, $P < 0.0001$; ***, $P<0.0007$ using t-test. Data are representative of two independent experiments. All error bars are standard deviations.

### 3.2.2 Proteasome-mediated degradation of JAK1 is associated with STAT1 signaling inhibition during alphavirus infection.

Based on data from previous experiments, we reasoned that nsP2 inhibited STAT1 signaling by blocking a step between receptor binding and STAT1 phosphorylation in the IFN-α/β signaling cascade. Possible steps which nsP2 could interdict in the process were JAK1/Tyk2 phosphorylation, receptor phosphorylation and STAT1 binding to phosphorylated receptors. Alternatively, nsp2 could upregulate negative regulators of the JAK-STAT signaling pathway such as the suppressors of cytokine signaling (SOCS) proteins (326). Huh7 cells were infected with WT VEEV and western blots were performed to examine the levels of phosphorylated and total JAK1 (Fig. 18). Levels of JAK1 in infected cells were markedly reduced when compared to mock infected cells, and correlated with the abrogation of STAT1 phosphorylation. We concluded that VEEV induced degradation of JAK1 during infection which suppressed STAT1 signaling and inhibited the IFN response.

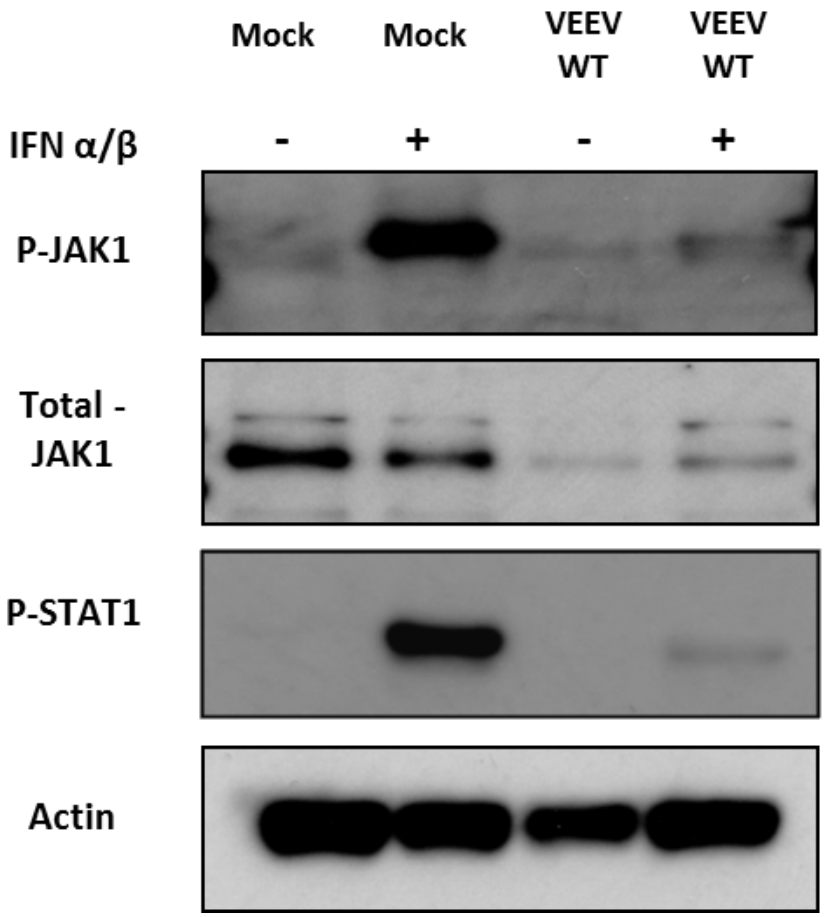



**Figure 18. JAK1 levels are reduced following infection VEEV infection.** Huh7 cells were infected with VEEV WT (M.O.I. = 5) and treated with 1000 IU IFN α/β for 30 min at 18h p.i. Lysates were collected and Western blots performed as described in Materials and Methods.

We next studied the degradation of JAK1 and its effect on STAT1 phosphorylation. JAK1 degradation can occur via the proteasome following disruption of the JAK1-Hsp90-CDC37 complex (327-329). We hypothesized that inhibition of the proteasome would restore JAK1 levels and rescue STAT1 phosphorylation in infected cells. Huh7 cells were infected with WT VEEV and concurrently treated with MG132, a known proteasome inhibitor. Degradation of JAK1 was observed in untreated cells infected with WT VEEV (Fig. 19A and B). However, levels of JAK1 in MG132 treated infected cells were restored to those observed in mock infected cells ($P<0.04$) (Fig. 19A and B). In addition, restoration of JAK1 levels also resulted in an

increase of STAT1 phosphorylation (Fig. 19A). We observed similar results following infection with WT EEEV, CHIKV and SINV (Fig. 20), suggesting that the same degradation pathway results in JAK1 degradation after infection with Old World and New World alphaviruses.

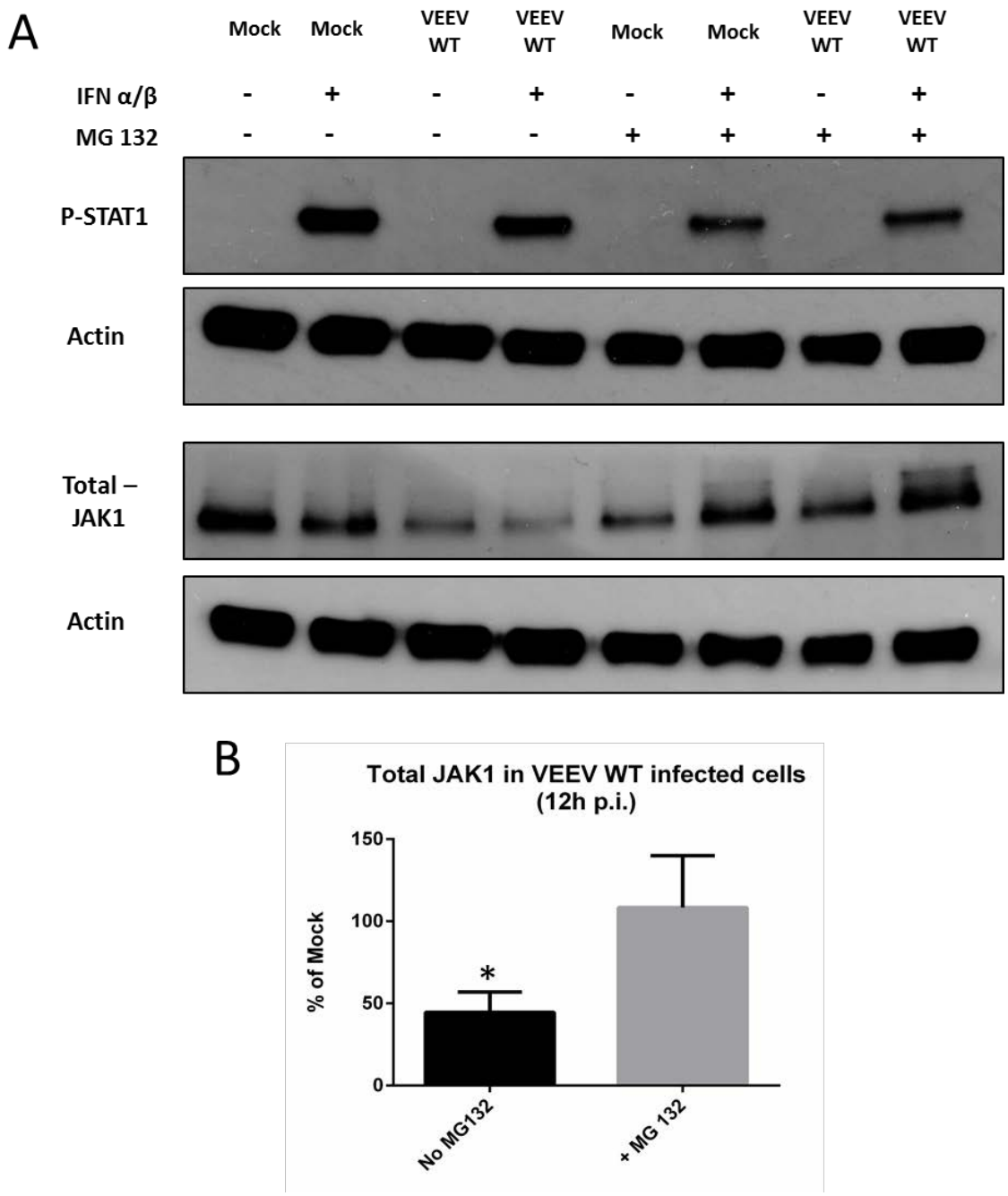



**Figure 19. Degradation of JAK1 is rescued by MG132 treatment in VEEV infected cells.** (A) Huh7 cells were treated with media containing MG132 (10μM) for 1h prior to infection. Cells were infected with VEEV WT (M.O.I. = 20) and media added after 1h infection contained MG132 (10μM). Cells were treated with media containing MG132 (10μM) and 1000 IU IFN α/β for 30 min at 12h p.i. Lysates were collected and Western blots performed as described in Materials and Methods. (B) Densitometry was performed on gels from (A). *, P<0.04 using T-test. All error bars are standard deviations. Data is representative of two independent experiments.

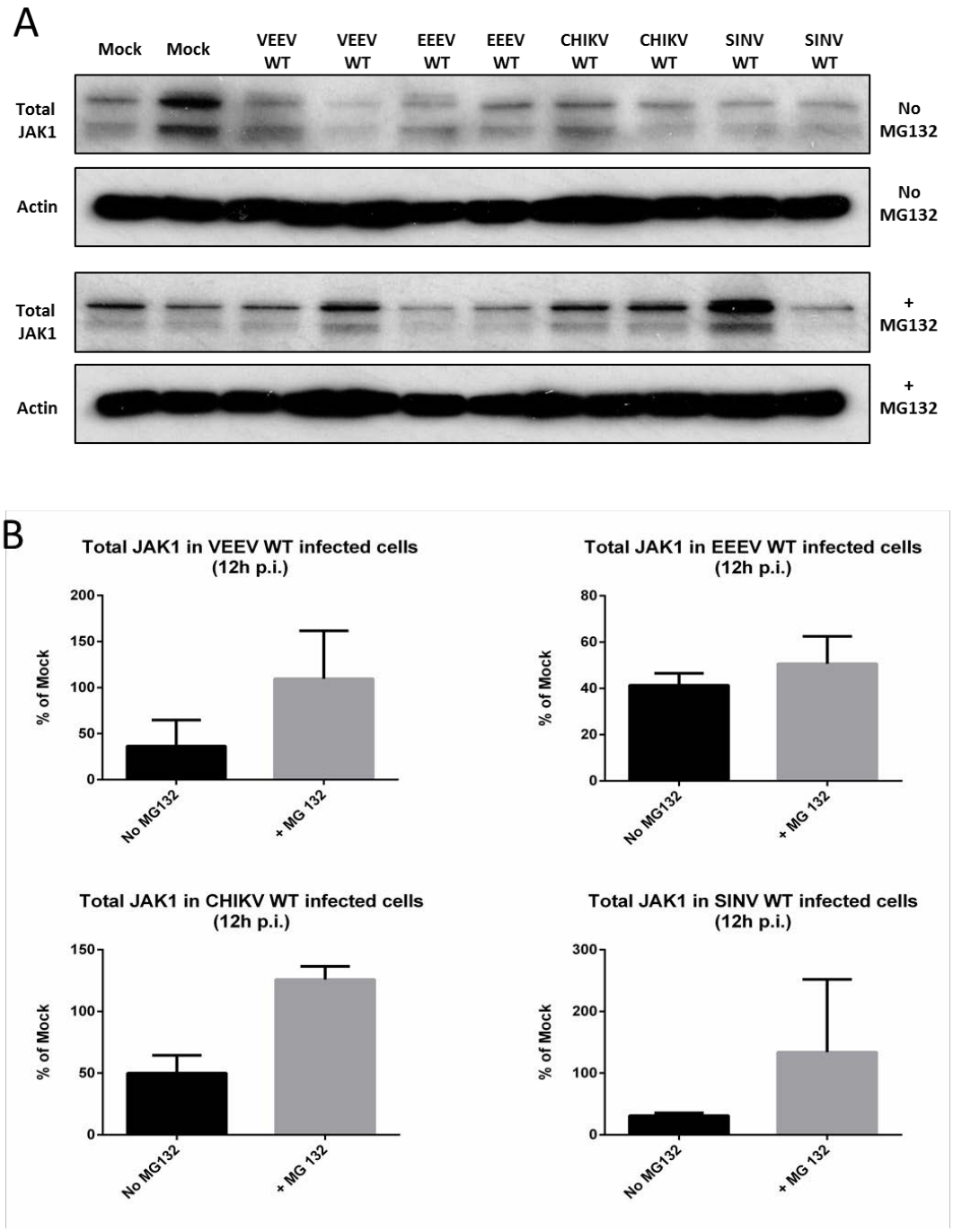



**Figure 20. All tested alphaviruses degrade JAK1 following infection.** (A) Huh7 cells were treated with media containing MG132 (10μM) for 1h prior to infection. Cells were infected in duplicate with indicated viruses (M.O.I. = 20) and lysates collected at 12h p.i. Western blots were performed as described in Materials and Methods. (B) Densitometry was performed on gels from (A). Data is representative of two independent experiments for each virus.

### 3.2.3 The role of IFN signaling inhibition in the antiviral state resistance of VEEV WT.

We analyzed the ability of mutant viruses to antagonize IFN signaling by blocking phosphorylation of STAT1. Phosphorylation at Y701 is required for successful nuclear translocation of STAT1 and subsequent ISG induction following IFN signaling (225). Huh7 cells were used as IFN elicits a large and consistent signaling response. Infected Huh7 cells were treated with 1000 IU of human leukocyte IFN for 30 min at 6 and 24h p.i. and western blots were examined for phospho-STAT1 and Actin (Fig. 21A and B). STAT1 phosphorylation inhibition was not observed in any infected samples at 6h, which is consistent with other reports (76, 271, 272). However, efficient inhibition of STAT1 phosphorylation was detected 24h p.i. in WT VEEEV infected cells. The 713G/S mutant viruses inhibited STAT1 phosphorylation to a similar or greater extent than VEEV WT, indicating that these mutations do not reduce this activity of nsP2, in stark contrast to the effect of 726G in SINV. In contrast, CD, 739L and CD/739L all showed reduction of this activity in infected cells, with an increased effect observed in the double mutant CD/739L. The blockade of STAT1 phosphorylation following virus infection was not due to virus mediated STAT1 degradation (Fig. 23C). This was also true after infection with WT SINV, CHIKV and EEEV viruses, each of which blocked STAT1 phosphorylation at late times p.i. (data not shown).

We analyzed the correlation between STAT1 signaling inhibition and antiviral state resistance (as measured by the ability of the virus to replicate successfully in IFN-primed cells in Fig. 11) using VEEV mutants (Table 3) and found no clear correlation between the two processes. Mutants found to be either resistant (CD) or sensitive (739L, CD/739L) to the effects of IFN-priming were impaired in their ability to inhibit STAT1 phosphorylation, suggesting that

this activity may not contribute directly to antiviral state resistance as evaluated in our experimental design.

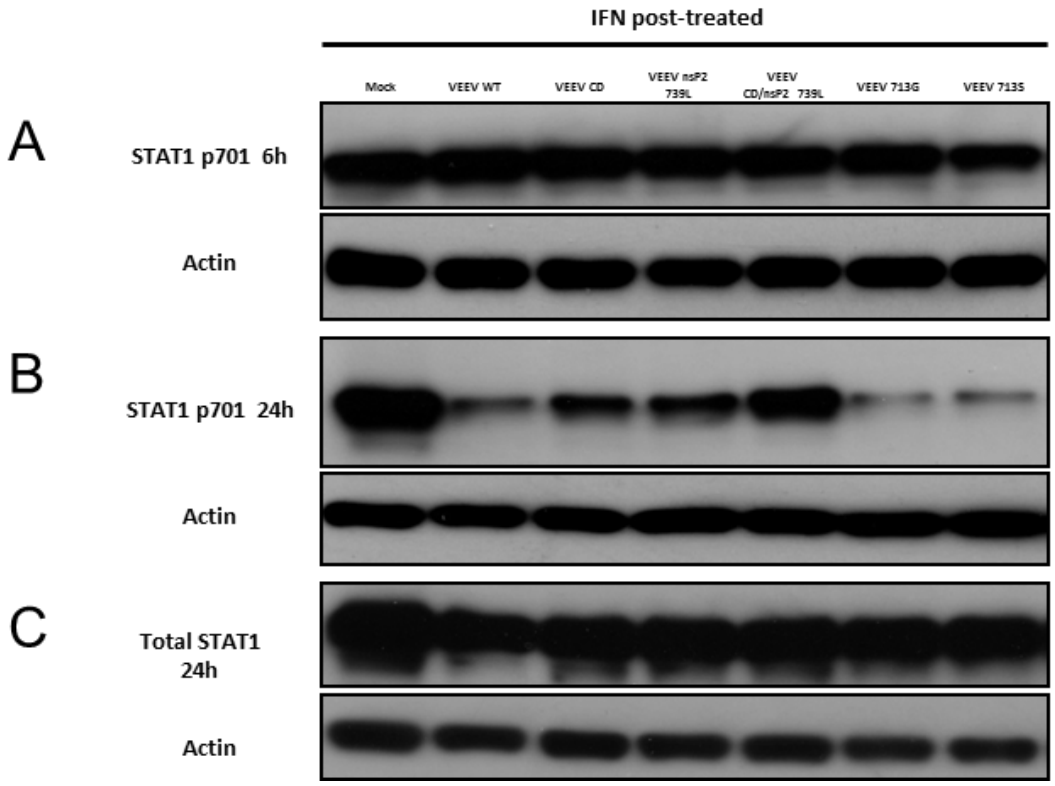



**Figure 21. IFN signaling antagonism by VEEV mutants.** (A-C) Huh7 cells were infected with indicated viruses (M.O.I. = 4) and treated with 1000 IU human leukocyte IFN at 6h (A) and 24h (B) p.i. for 30 min. Lysates were collected and western blots for pSTAT1 and Actin were performed as described in Materials and Methods. (C) Total STAT1 was measured from the same lysates at 24h p.i. Data is representative of two independent experiments.

**Table 3. STAT1 signaling antagonism and antiviral state resistance.**

| | Early times p.i. (6h) | | Late times p.i. (18-24h) | |
|---|---|---|---|---|
| **Virus** | **Resistance to anti-viral state** | **STAT1 phosphorylation inhibition** | **Resistance to anti-viral state** | **STAT1 phosphorylation inhibition** |
| **WT** | + | + | +++ | +++ |

| | | | | |
|---|---|---|---|---|
| **nsP2 713G** | + | + | +++ | +++ |
| **nsP2 713S** | + | + | +++ | +++ |
| **nsP2 739L** | + | + | ++ | ++ |
| **CD** | + | + | +++ | ++ |
| **CD/739L** | + | + | ++ | + |

## 3.3 DISCUSSION

### 3.3.1 STAT1 signaling inhibition during alphavirus infection.

During alphavirus infection, inhibition of STAT1 phosphorylation has been observed in multiple cell-types (77, 253, 271). In addition, mutations in the viral protease nsP2 of multiple alphaviruses have been shown to abrogate or reduce this activity virus (253, 271) (Fig. 13 and 14). Using a plasmid expression system to express individual WT and mutant viral proteins, our studies confirm the observation that individually expressed CHIKV nsP2 can abrogate IFN signaling (77), and extend this to VEEV, EEEV and SINV nsP2, thus conclusively identifying nsP2 to be the viral protein that induces this activity during infection for Old world and New world alphaviruses. We also identify/confirm mutations in SINV, VEEV and CHIKV nsP2 that abrogate this activity. The CHIKV nsP2 mutant D711G-P718S did not inhibit STAT1 signaling in our experiments, in contrast to published results (74), which may be due to differences in cell type or viral strain analyzed in the studies.

Additionally, levels of JAK1 decreased following infection with all tested alphaviruses when measured by western blot, but were restored by blocking the proteasome with MG132. Our studies identified the proteasome-mediated degradation of JAK1 to be critical for abrogation of STAT1 signaling during infection, as restoration of JAK1 levels upon restriction of proteasome activity also restored STAT1 phosphorylation when measured by a western blot. JAK1 is a target for binding and stabilization by the Hsp90-CDC37 chaperone complex (327), which appears to provide scaffolding between JAK1 and the IFNAR1 while simultaneously preventing JAK1 degradation. Disruption of Hsp90 with inhibitors, or siRNA-mediated knockdown of Hsp90 or CDC37, results in proteasome mediated degradation of JAK1, which to our knowledge is the only degradation pathway for JAK1 described to date (327-330). Interestingly, it has recently been shown that CHIKV nsP2, nsP3 and nsP4 all bind directly to Hsp90, and treatment of infected cells or mice with Hsp90 inhibitors reduces the level of viral replication and improves the outcome of *in vivo* infection (331, 332). Therefore, we propose a model in which nsPs produced following initial infection sequester Hsp90 to promote viral replication, competing directly with cellular proteins that are the binding partners of Hsp90 in unstressed cells, and leading to degradation of those cellular proteins. Since Hsp90 is thought to constitute 1-2% of total protein mass in unstressed cells (333-335), this presumably explains why JAK1 degradation and STAT1 signaling inhibition are observed only late during infection, as it is possible that sufficient amounts of Hsp90 have to be sequestered before the effect on their binding partners is observable. Future experiments aimed at understanding the role of Hsp90 in the degradation of JAK1 and inhibition of IFN signaling will explore these hypotheses.

### 3.3.2 STAT1 signaling inhibition and antiviral state resistance.

The role of STAT1 signaling inhibition in the resistance phenotype of VEEV is unclear from our studies. Consistent with other reports (76, 271, 272), STAT1 phosphorylation was not inhibited early after infection and levels of STAT1 protein were also not reduced, suggesting that VEEV does not target STAT1 for degradation. This suggests that in unprimed cells, STAT1 signaling is abrogated only after the efficient induction of translation shutoff and may act secondary to the latter in resisting the establishment of an induced antiviral state by blocking IFN-α/β or IFN-γ signaling or both. Similarly in IFN-primed cells, the activity of an existing pool of phosphorylated STAT1 is antagonized early by virus mediated translation shutoff, while antiviral state sustenance and reestablishment is prevented by STAT1 signaling inhibition late during infection, albeit complementary to the effect of host macromolecular synthesis shutoff. Data from primary neurons suggest that the level of phosphorylated STAT1 in IFN-α/β primed cells infected with VEEV or SINV stays constant or shows a slight increase (76), suggesting that STAT1 signaling inhibition acts to prevent a further increase of the pool of phosphorylated STAT1, though this may be difficult to detect in our current assays. Our mutagenesis data also point to the major role played by macromolecular synthesis shutoff in the resistance phenotype of VEEV, and show no clear correlation between abrogation of STAT1 signaling antagonism and increased sensitivity to the antiviral state. Taken together, the data suggest that STAT1 signaling antagonism is likely a secondary mechanism of antiviral state resistance for alphaviruses and complements the activity of host macromolecular synthesis shutoff. Nonetheless, this activity may delay IFN-mediated clearance from infected cells *in vivo* by preventing the continuation and sustenance of antiviral signaling, as is observed in IFN-γ mediated clearance (which also signals through STAT1 (336)) of SINV from infected neurons (337, 338).

# 4.0 SYSTEMIC IFN A/B SECRETION AFTER VENEZEULAN EQUINE ENCEPHALITIS VIRUS INFECTION IS PRIMARILY DEPENDENT ON IRF7 AND OCCURS DESPITE RAPID VIRUS-INDUCED TRANSLATION SHUTOFF IN INFECTED CELLS.

## 4.1 INTRODUCTION

Alphavirus infection of murine models and humans begins with subcutaneous deposition of virus in the skin leading to infection of dendritic cells, macrophages and Langerhans cells, which presumably facilitate virus spread to the regional draining nodes (165, 339). The interaction of alphaviruses and myeloid cells plays a significant role in shaping the course and outcome of infection, and the virulence of different alphaviruses can be partly explained by how they exploit this interaction to replicate successfully (35). For instance, EEEV avoids induction of host innate antiviral responses by suppressing genomic replication via myeloid cells specific micro-RNA mediated inhibition of initial genomic translation in myeloid cells (276), which allows the virus to seed distant sites of replication without activating systemic innate antiviral immunity. In contrast, replication of VEEV in the draining lymph node results in secretion of large amounts of serum IFN-α/β, which primes distant sites against viral infection by upregulation of antiviral proteins (183, 313). It has been proposed that the resistance of VEEV to IFN-α/β and other innate antiviral cytokines enables the virus to replicate successfully and cause disease despite

inducing robust IFN-α/β responses (35, 76). However, most studies that have examined the inductive phase of the IFN response to VEEV have used fibroblast models in which virus induction of host macromolecular shutoff precludes the production of detectable IFN-α/β. Responses of myeloid to virus infection cells in vitro and, presumably, in vivo, differ greatly as high levels of IFN are produced (76, 104, 251, 254). The molecular characteristics of VEEV infection of myeloid cells have not been extensively studied. In particular, host factors important for control of VEEV replication and production of IFN-α/β in myeloid cells have not been described. In addition, the ability of VEEV to inhibit host transcription and translation in myeloid cells, and the effect of this inhibition on production of IFN-α/β has also not been explored.

Besides IFN-α/β and some ISGs (ex. ISG20, unpublished data), few host factors exhibiting antiviral activity against VEEV have been identified. In one study, IRF2$^{-/-}$ mice were more susceptible to VEEV infection when compared to WT mice (340). Similarly, IRF1 and IRF2 were important for eliciting protective responses after inoculation with an attenuated strain of VEEV when challenged by WT VEEV (341). IRF3 signaling following VEEV infection fails to elicit transcription of IFN-α/β or antiviral effector genes in MEF cells, but its role in IFN-α/β induction from myeloid cells remains unknown (253). The role of IRF7 in induction of IFN-α/β following VEEV infection has not been explored. However, as IRF7 is constitutively expressed in myeloid lineage cells (218-221), we hypothesize this molecule plays a role in IFN-α/β induction from VEEV infected macrophages and dendritic cells.

Successful induction of host shutoff in non-myeloid cells can block IFN-α/β induction (253) at a step subsequent to IRF3 dimerization and nuclear localization. For myeloid cells, IFN-α/β may be secreted by both infected and uninfected cells in VEEV infected lymph nodes (342),

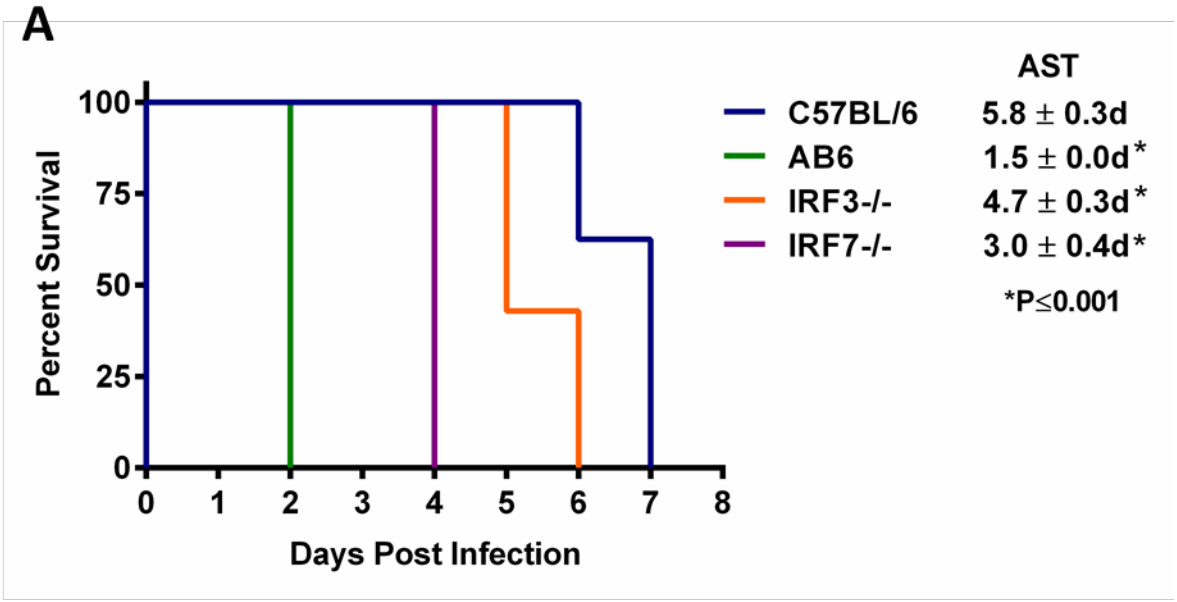
suggesting that myeloid cells can both avoid viral mechanisms of innate immune pathway disruption to secrete IFN-α/β, and release IFN-α/β upon detection of infection in neighboring cells. We hypothesize that VEEV can efficiently shutoff host transcription and/or translation in myeloid cells, yet infected cells will remain competent for production of IFN-α/β in an IRF7 dependent manner.

## 4.2 RESULTS

### 4.2.1 IRF7 is important for controlling VEEV replication in vivo.

In order to determine IFN system induction pathway factors with potential antiviral roles against VEEV infection, we infected mice lacking IFNAR1 (AB6), IRF3 or IRF7 with WT VEEV (Fig. 22A and B). As previously published (340, 341), IFNAR$^{-/-}$ mice succumbed fastest to VEEV infection (average survival time (AST)) 1.5 vs. 5.8 for WT, $P<0.001$), highlighting the critical role of the IFN-α/β system in controlling alphavirus infection. AST for IRF7$^{-/-}$ mice was decreased by ~3 days ($P\leq0.001$) compared to WT, whereas AST reduction (~1 day, $P\leq0.001$) in IRF3$^{-/-}$ mice was more modest, suggesting an important role for IRF7 in controlling VEEV infection. Furthermore, systemic IFN-α/β induction was heavily dependent on the presence of IRF7 but not IRF3 (Fig. 22B). Consistent with previous reports (35, 183), WT mice responded to VEEV infection with robust IFN-α/β induction (>10,000 IU/mL at 24h p.i.). In contrast, mice lacking IRF7 secreted little to no IFN-α/β in serum (50-200 IU/mL at 24h p.i.), whereas levels of serum IFN-α/β in IRF3$^{-/-}$ mice were not significantly different from levels observed in WT mice until 48h p.i. ($P<0.001$), suggesting IRF7 plays a critical role in IFN-α/β induction during VEEV

infection in vivo, whereas IRF3 may be important during the clearance phase of infection. The role of IRF7 in controlling VEEV was further highlighted when viral titers from various tissues were measured (Fig. 23). Titers in tissues of IRF7$^{-/-}$ mice were universally and significantly higher ($P<0.001$) when compared to WT mice. In contrast, replication of VEEV in IRF3$^{-/-}$ mice was not significantly different in the majority of tissues when compared to replication in WT mice. Taken together, these results suggest an important role of IRF7 in controlling VEEV replication in vivo, potentially through IRF7 mediated induction of IFN-α/β.



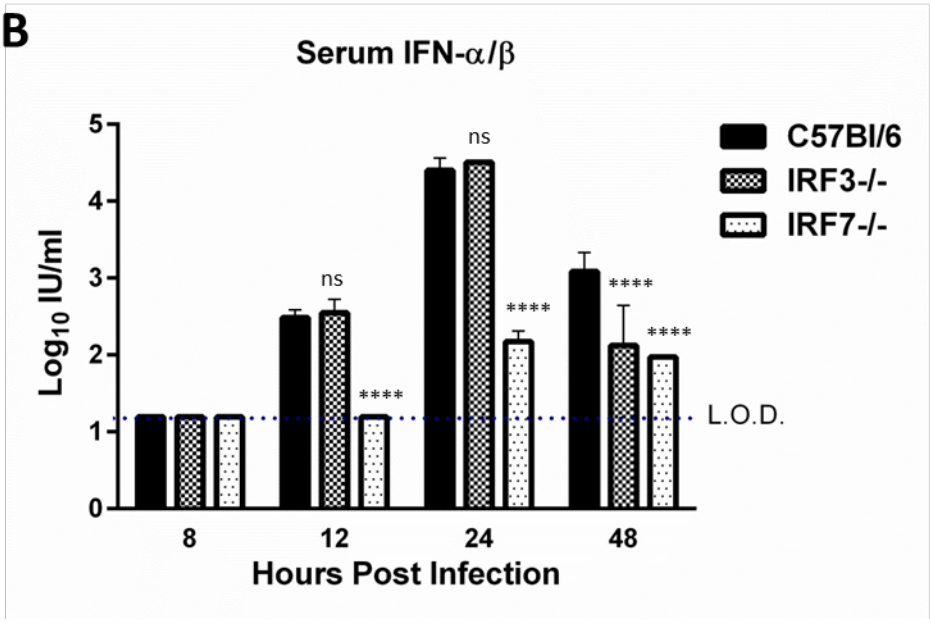




**Figure 22. The role of IRF7 in protection against VEEV infection.** (A) BL6, AB6, IRF3$^{-/-}$ and IRF7$^{-/-}$ mice were infected subcutaneously in the hind leg footpad with 1000 PFU of VEEV WT and AST was determined. 4 mice per group. (B) Serum from indicated mice was used to measure IFN-α/β using IFN bioassay as described in Materials and Methods. ****, P<0.001 using Two-way ANOVA. *, P<0.001 using Mantel-Cox Log-Rank test. All error bars are standard deviations. Data is representative of two independent experiments.

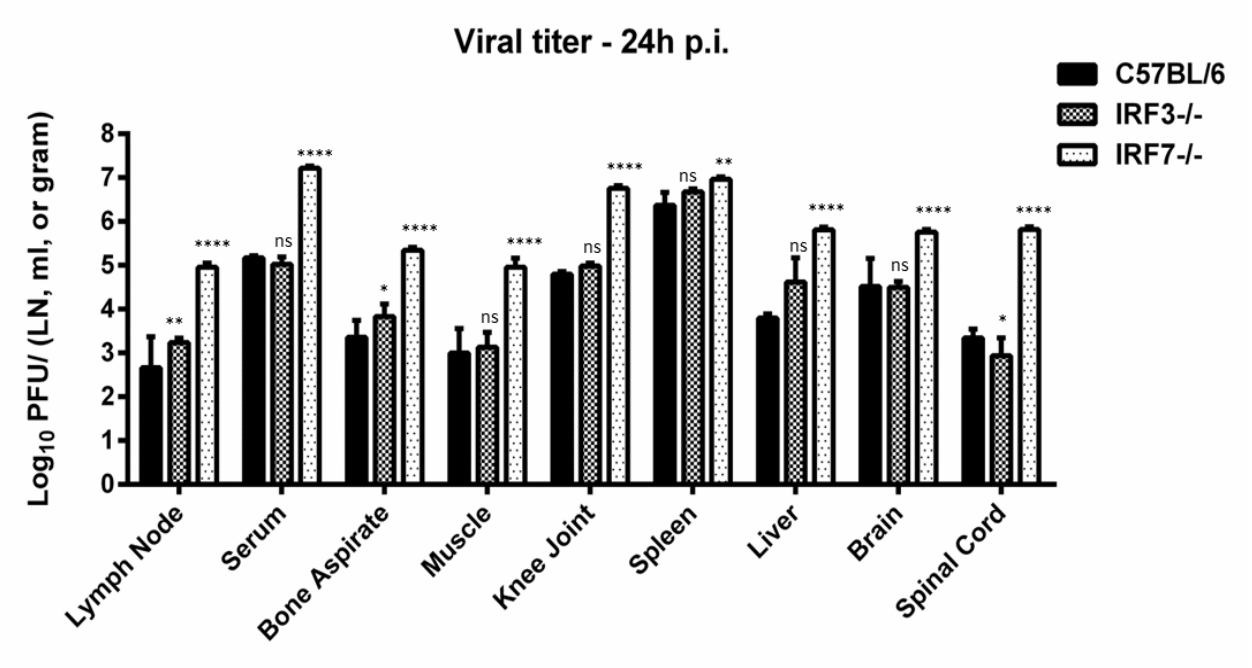



**Figure 23. Titers of VEEV in infected tissues.** BL6, IRF3-/- and IRF7-/- mice were infected subcutaneously in the hind leg footpad with 1000 PFU of VEEV WT and indicated tissues were harvested at 24h p.i..6 mice per group. Viral titers were determined using plaque assays as described in Materials and Methods. ****, P<0.0001; **, P<0.001; *, P<0.01 using Two-way ANOVA. All error bars are standard deviations.

### 4.2.2 IRF7 is important for IFN-α/β induction in macrophages and dendritic cells.

Previously published data suggest that serum IFN-α/β observed during VEEV infection is produced primarily by myeloid cells (dendritic cells and macrophages) in infected lymph nodes (183, 313), whereas IFN-α/β induction is suppressed during non-myeloid cell infection (253). To test these findings in vitro, we cultured primary macrophages and dendritic cells from WT, IRF3$^{-/-}$ or IRF7$^{-/-}$ mice and infected them with VEEV WT (Fig. 24). Cultured primary macrophages and dendritic cells were used to test the IFN-α/β induction response in cells similar to those initially infected by VEEV following in vivo infection. Induced IFN-α/β levels from cells lacking IRF3 were higher than that observed from cells cultured from WT mice. In contrast, levels were equivalent or lower from cells lacking IRF7, suggesting that IRF7 was important for

IFN-α/β induction (Fig. 24). We speculate that the IFN levels were higher in IRF3$^{-/-}$ cultures due to enhanced virus replication, possibly an effect of limited IRF3-mediated gene induction and/or greater IRF7 mediated gene induction. In contrast, levels in IRF7$^{-/-}$ cultures were lower or equivalent to WT cultures, suggesting inability of other IFN gene induction factors to compensate for the deletion of IRF7. This data suggests myeloid cells can successfully secrete IFN-α/β during infection, and that IRF7 is an important contributor towards IFN-α/β induction following VEEV infection of primary myeloid cells.

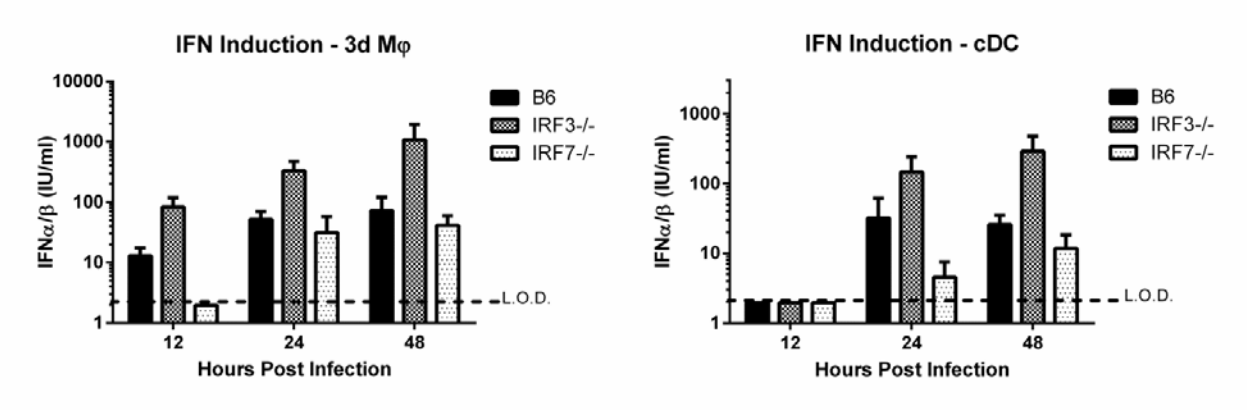



**Figure 24. IFN induction and IFN mRNA levels in primary macrophages and dendritic cells following VEEV infection.** Primary 3day macrophages and conventional dendritic cells were infected with VEEV WT (M.O.I. = 1) and supernatants were collected for IFN bioassay at indicated times p.i, as described in Materials and methods. Data is the representative of at least two independent experiments. All error bars are standard deviations.

### 4.2.3 Induction of IFN-α/β occurs despite rapid translation shutoff induction by VEEV.

Alphaviruses can inhibit host transcription and translation during infection (76). However, all previous studies examining the characteristics of host shutoff have used non-myeloid cell lines (such as MEF, BHK etc.) that may not accurately represent myeloid cell infection in vivo. We used Raw 264.7 cells (a mouse macrophage cell line) as they are more susceptible to infection than primary macrophages (~95% as measured by flow cytometry, data not shown) which helps

avoid obscuring of shutoff in infected cells by positive signal from uninfected cells in the cultures. In addition, and similar to infection of primary myeloid cells, infected Raw 264.7 cells are capable of producing large amounts of IFN-α/β following VEEV infection (Fig. 25). We tested the ability of VEEV to shut off host translation in myeloid cells by labeling infected Raw 264.7 cells with $^{35}S$ in a pulse-chase analysis to measure levels of newly synthesized peptides (Fig. 26A and B). In addition, we tested the ability of VEEV to inhibit host transcription (Fig. 26C) by measuring levels of mouse gamma actin intron #3 by RT-PCR. Surprisingly, VEEV achieved maximum shutoff rapidly in infected cells as early as 6h p.i. (Fig. 26A and B). However, this shutoff is not complete (~70-80%, Fig. 26B) and lower than that observed in non-myeloid cells (Fig. 9, VEEV WT ~90% in Huh7 cells), which suggests low level production of host proteins in infected cells, or protein production in uninfected cells may still occur during infection. In contrast, host transcription was inhibited late (15h p.i., $P<0.0001$) but not early after infection (Fig. 26C) similar to the effect observed in non-myeloid cells (76). Interestingly, secretion of IFN-α/β by Raw 264.7 cells continues and increases throughout the course of infection despite early establishment of host translation shutoff (Fig. 25), suggesting induction by either the few uninfected cells in culture, induction occurring in infected cells due to incomplete shutoff established by VEEV, or induction by cells in which translation is efficiently shutoff by mechanisms that upregulate antiviral cytokine production in the face of virus induced transcription and translation shutoff.

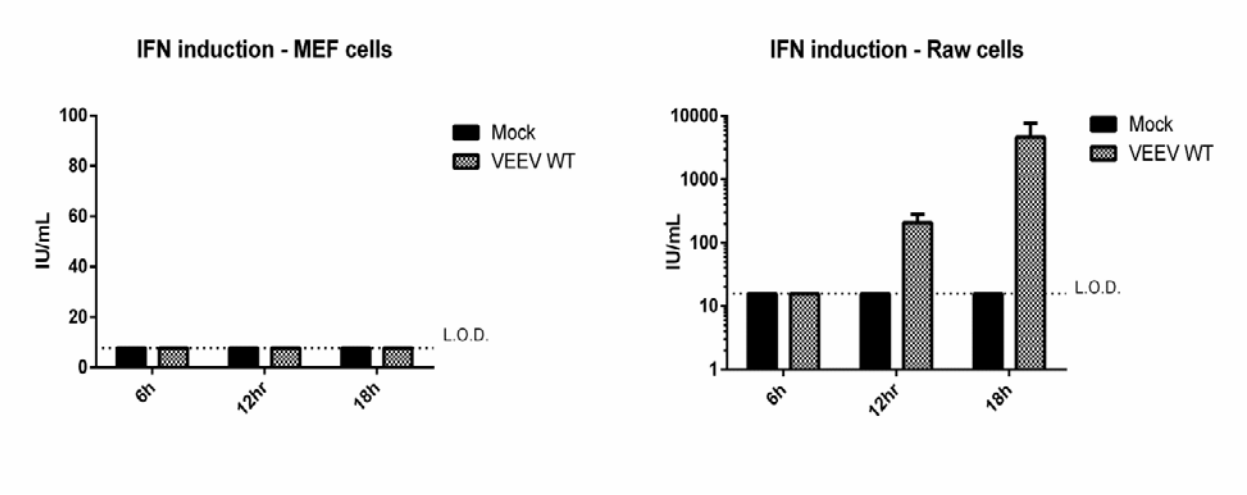



**Figure 25. IFN induction following infection of Raw 264.7 cells by VEEV WT.** MEF and Raw 264.7 cells were infected with VEEV WT (M.O.I. = 10) and 100μL supernatant was collected at indicated times p.i. (supernatant was replaced with 100μL fresh media). IFN bioassays were performed as described in Materials and Methods to determine secreted IFN levels. Infection at each time point was performed in duplicate. Data is representative of three independent experiments.

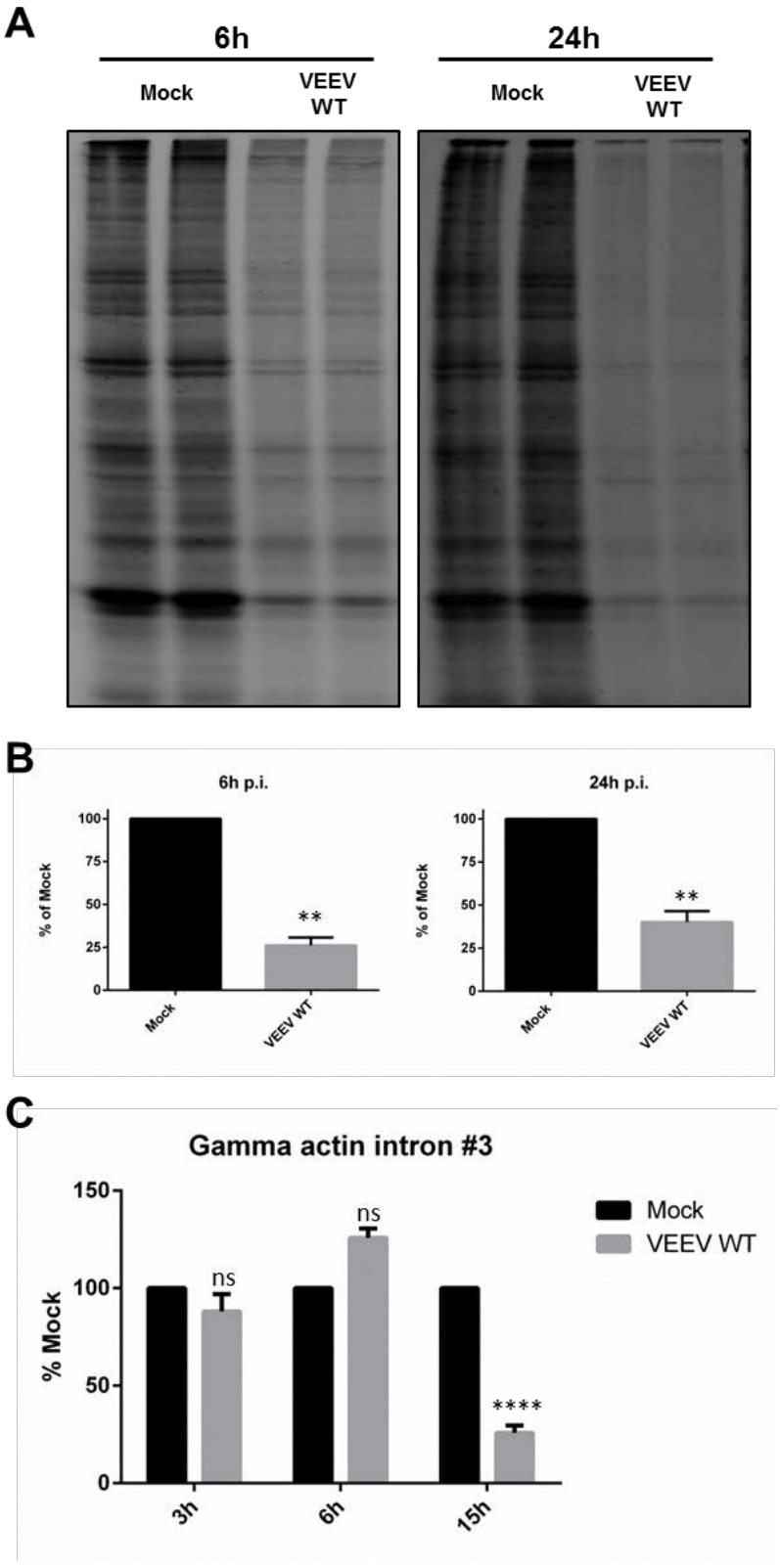



**Figure 26. Translation and transcription shutoff in Raw 264.7 cells following VEEV WT infection.** (A) and (B) Raw 264.7 cells were infected with VEEV WT (M.O.I. = 10) and labeled with 100μCi/ml of [$^{35}$S] Cys/Met for 2h at

6h and 24h p.i. Lysates were collected and resolved on SDS-PAGE gels and visualized as described in Materials and Methods. (B) Densitometry performed on gels from (A). (C) Raw 264.7 cells were infected with VEEV WT (M.O.I. = 5) and lysates were collected at indicated times p.i. RT PCR for mouse gamma actin intron #3 was performed as described in Materials and Methods ****, P<0.0001; **, P<0.006 using T-test. Data is average of two replicates (A) and three replicates (C). All error bars are standard deviations.

## 4.3 DISCUSSION

### 4.3.1 The role of IRF7 during VEEV infection.

The importance of IRF7 in the control of alphavirus infection has been previously illustrated, as IRF7$^{-/-}$ mice are more susceptible to SINV and CHIKV infection (343, 344). In addition, IRF7, but not IRF3, has been implicated in systemic IFN-α/β induction during West Nile virus infection mice (345, 346). Our data suggest that IRF7 is required for protection of mice from VEEV. There is a significant decrease in AST of IRF7$^{-/-}$ mice infected with VEEV when compared to WT mice, with higher viral loads in all examined tissues. A likely mechanism of IRF7 control of VEEV replication is its role in the efficient induction of IFN-α/β from myeloid cells in vivo. We observed a dramatic reduction in induced serum IFN-α/β in VEEV infected IRF7$^{-/-}$ mice. As IFN-α/β induction from non-myeloid cells is blocked during VEEV infection (76, 253), we surmise this activity of IRF7 is specific to myeloid cells, which is supported by the observation that IRF7 is constitutively expressed only in myeloid lineage cells such as plasmacytoid dendritic cells and monocytes (220), and thus provides an alternate signaling pathway following virus sensing by pattern recognition receptors that is not present in non-myeloid cells. Similarly, IFN-α/β induction from primary macrophages and dendritic cells lacking IRF7 was equivalent to or lower than cells cultured from WT mice. After alphavirus infection of non-myeloid cells, IRF3 is phosphorylated, dimerizes, and translocates to the

nucleus but no ISG or IFN-α/β mRNA is transcribed or IFN-α/β secreted (253). IRF3 translocation occurs before virus induced shutoff of transcription and translation, although these activities play a major role in subsequently preventing IFN-α/β secretion. It is possible that IRF7 mediated IFN-α/β and ISG induction is resistant to shutoff, and/or takes place faster than IRF3 mediated induction. Macrophages have pre-loaded RNA Pol II complexes on the promoters of many rapid-response genes, including ISG's, NFκB and IFNβ (347), which may help explain the IRF7 dependent disparity observed when measuring IFN-α/β secretion from myeloid and non-myeloid cells. Future experiments will explore the molecular underpinnings of IRF7 mediated IFN-α/β induction following VEEV infection of myeloid cells.

### 4.3.2 IFN-α/β induction from infected myeloid cells.

Infection of both primary myeloid-lineage cells and the Raw 264.7 macrophage cell line with VEEV resulted in IFN-α/β induction. Our data suggest that IFN-α/β secretion from infected Raw 264.7 cells continues throughout the course of infection, and occurs despite rapid and efficient, but not complete, induction of host translation shutoff in the entire cell culture early after infection and transcription inhibition late during infection. A combination of uninfected cells, infected cells with incomplete virus induced shutoff, and infected cells resistant to virus-induced translation shutoff, are likely responsible for the IFN-α/β observed in cell cultures and possibly in serum of infected animals. Infected cells may resist establishment of virus induced shutoff or be inducing IFN-α/β in a manner that can evade virus induced translation shutoff. Alternatively, the few uninfected cells in the culture may be responsible for observed IFN-α/β levels in the culture. When measured by flow cytometry, VEEV infection levels in Raw cells were ~95%

(data not shown), which suggests that some cells were resistant to infection. A previous study has demonstrated secretion of IFN-α/β from infected and uninfected myeloid cells both from VEEV infected lymph nodes and in vitro (342). Future experiments will elucidate how myeloid cells can evade viral mechanisms targeting antiviral cytokine induction following infection.

Our data is also in contrast to an *in vitro* derived model (using MEFs) explaining the induction of serum IFN-α/β following alphavirus infection in mice (271), where small amounts of IFN-α/β secreted by cells infected in the first round of infection prime uninfected cells, which subsequently secrete large amounts of IFN-α/β upon eventual infection. Levels of serum IFN-α/β peak 12-24h p.i. while mortality *in vivo* occurs 4-6 days p.i. (35), suggesting that primed non-myeloid cells are not major producers of IFN-α/β *in vivo*, though localized secretion in infected non-myeloid tissues may occur, and that IFN-α/β induction *in vivo* diminishes substantially as the infection progresses from targeting myeloid cells to non-myeloid cells (313). This differential ability of tested alphaviruses to prevent secretion of IFN-α/β (and possibly other antiviral cytokines) upon infection of various cell types likely determines the capacity of different tissues of the host to effectively respond to infection once the initial wave of myeloid cell induced IFN subsides.

# 5.0 CONCLUSIONS

The member viruses of the *Alphavirus* genus are endemic globally, and in recent years have been estimated to cause millions of infections in humans and domesticated livestock annually in ongoing epidemics. Alphaviruses can cause arthritogenic (SINV and CHIKV) or encephalitic (VEEV and EEEV) disease, with individual infection and disease profiles depending to a large extent on the ability of individual viruses to manipulate the IFN-α/β induced antiviral state either by evading or resisting the effects of downstream antiviral effector proteins on viral replication. In these studies we have assessed the relative resistance of various arthritogenic and encephalitic alphaviruses to the antiviral state and identified one of possibly multiple mechanisms that explain the differential sensitivity of those alphaviruses to IFN. We have also identified the viral proteins responsible for mediating transcription and translation shutoff and STAT1 signaling inhibition during infection, and begun to explore the role of IRF7 in inducing host responses against alphavirus infection. This work furthers our understanding of the success and failure of the mechanisms used by different alphaviruses to combat host antiviral pathways and their impact on virulence *in vivo*, and illustrates the markedly different strategies that can be used by closely related viruses to overcome host antiviral defenses and replicate successfully.

## 5.1 TRANSLATION SHUTOFF AND ANTIVIRAL STATE RESISTANCE

In Chapter 2, our studies examine the mechanism(s) underlying the relative resistance of SINV, CHIKV, EEEV and VEEV to both the IFN-α/β induced global antiviral state, and to individually over-expressed antiviral effector proteins. We conclude that VEEV is most resistant in either circumstance. IFN dramatically, but incompletely, suppresses initial translation of infecting viral genomes of all tested viruses, but VEEV is able to escape this blockade at a step subsequent to the first round of translation in the viral replication cycle through the activity of a viral protein localized to the non-structural region of the genome, an activity the viral proteins of SINV, CHIKV or EEEV are able to induce much less effectively. Additionally, we demonstrate that VEEV, SINV or CHIKV nsP2 and VEEV or EEEV capsid, but not EEEV nsP2 inhibit cellular translation when expressed independent of other viral proteins. Similarly, SINV and CHIKV nsP2 and VEEV or EEEV capsid, but not VEEV or EEEV nsP2, abrogate cellular transcription. We show that a conserved "PGG" motif in the C-terminus of nsP2 in many alphaviruses is not essential for host interaction in all alphaviruses, as a mutation (P726G) previously identified in SINV abrogates transcription and translation shutoff in SINV, but not CHIKV or translation shutoff in VEEV. Our data unequivocally separates virus induced transcription and translation shutoff as two distinct activities, each of which is mediated by different viral proteins in different viruses. Further, we provide evidence to demonstrate that induction of nsP2 mediated translation shutoff in IFN-α/β pre-treated cells is critical for antiviral state resistance following infection with VEEV, and that a mutation previously identified in the C-terminal region of nsP2 (Q739L) reduces translation shutoff and increases sensitivity to IFN-α/β. In contrast, the nsP2 of SINV, an IFN-α/β sensitive virus, is unable to shutoff transcription or translation in IFN-α/β primed cells. Functionally, translation shutoff in IFN-α/β primed cells reduces levels of ISG's and dismantles

the antiviral state, as evidenced by the successful replication of an IFN-α/β sensitive virus, YFV 17-D, in primed cells expressing VEEV nsP2. *In vivo,* infection with VEEV mutants deficient in translation shutoff elicits similar levels of IFN-α/β as WT VEEV, suggesting that an activity of the virus downstream of IFN induction is crucial for the resistance phenotype of VEEV. Our studies identify antiviral state resistance as a mechanism of virulence used by VEEV to overcome the high level of serum IFN-α/β induced during infection *in vivo,* highlight how this mechanism is in marked contrast to the miRNA mediated evasion of the antiviral state by EEEV (a very closely related virus), and help explain mechanistically the different outcomes of infection observed *in vivo* for various alphaviruses.

## 5.2 STAT1 SIGNALING INHIBITION AND ALPHAVIRUS INFECTION

Our studies in Chapter 3 advance our understanding of the mechanism underlying inhibition of IFN-α/β signaling during alphavirus infection. We confirm that STAT1 phosphorylation is inhibited by SINV, CHIKV, EEEV and VEEV, and the viral protein nsP2 is responsible for this activity. Furthermore, this inhibition does not occur due to degradation of STAT1. Rather the step targeted in the IFN-α/β signaling cascade is at the level of JAK1 and Tyk2 signaling. We demonstrate that STAT1 phosphorylation is reduced due to proteasome mediated degradation of JAK1 during infection, and inhibition of proteasome activity by MG132 treatment restores levels of JAK1, and phosphorylated STAT1, in infected cells. We conclude that alphaviruses likely use the inhibition of IFN-α/β signaling to prevent both upregulation of ISG's in unprimed cells as well as sustenance of the antiviral state when infecting cells are already primed by IFN-α/β.

## 5.3 CHARACTERISTICS OF ALPHAVIRUS INFECTION OF MYELOID CELLS

In Chapter 4, our work examines the role of IRF7 in inducing host antiviral responses against alphavirus infection, in particular IFN-α/β. Infection of mice deficient in expressing IRF7 decreased average survival time, suggesting a protective role for IRF7 during alphavirus infection. We observed a decrease in serum IFN levels, and a dramatic increase in viral titers in multiple tissues in IRF7$^{-/-}$, but not IRF3$^{-/-}$ infected mice, suggesting an important role for IRF7 in controlling viral replication and induction of IFN-α/β. *In vitro,* IFN-α/β induction in primary macrophages and dendritic cells was similar or lower in the absence of IRF7 when compared to wild-type mice, whereas the absence of IRF3 increased IFN-α/β levels, possibly through greater viral replication due to lower IRF3-mediated gene induction, and/or the compensatory effect of increased IRF7 expression. In contrast to observations in fibroblast lineage cells, IFN-α/β secretion from myeloid lineage cells continued throughout the course of infection, despite successful establishment of virus-mediated host translation shutoff early after infection, which suggests myeloid cells differ markedly from fibroblast cells at upregulating antiviral responses and can escape viral activities targeting induced immune responses. We conclude that IRF7 is important for successful and robust induction of host antiviral responses following alphavirus infection, and that IFN-α/β induction in myeloid cells is resistant to virus induced host macromolecular synthesis shutoff, possibly through an IRF7 mediated mechanism.

## 5.4 CONCLUDING REMARKS

In conclusion, we identify a new antiviral state resistance mechanism and elucidate the molecular underpinnings of the relative resistance of alphaviruses to the IFN-α/β induced antiviral state. We identify the viral proteins mediating transcription and translation shutoff and STAT1 signaling antagonism, and demonstrate the functional effect of translation shutoff on the antiviral state. Mutagenesis data from our studies can be used to inform alphavirus vaccine design by using nsP2 and capsid mutants that attenuate critical viral activities. Additionally, nsP2 mediated translation shutoff and STAT1 signaling inhibition may be targeted for future therapies against alphavirus infection. Overall, our studies contribute to understanding the roles played by various virus induced activities and host responses in determining the outcomes following *in vitro* and *in vivo* infection by different alphaviruses.

# 6.0 MATERIALS AND METHODS

**Cell culture.**

Neuro 2a, Raw 264.7, Vero and Huh7 cells (acquired from American Type Culture Collection (ATCC)) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 200mM L-glutamine (L-glut; Sigma, 10,000 units/mL penicillin (Sigma), and 10 mg/mL streptomycin (Sigma). Tetracycline-inducible MEF cells (Clontech) were maintained in the above medium supplemented additionally with 50 mg/mL G418. The generation of, and target gene induction from, tetracycline-inducible MEF cells overexpressing GFP, IFIT1 and ISG20 has been previously described (196). BHK-21 cells (ATCC) were maintained in RPMI supplemented with 10% donor bovine serum (DBS), 10% tryptose phosphate broth (TPB), and supplements as above. Conventional dendritic cells (cDCs) and 3day macrophages were grown and maintained as previously described (188). All cells were grown at 37°C with 5% $CO_2$.

**Viruses and replicons.**

Construction of cDNA clones for VEEV ZPC738 (182), EEEV FL93-939 (300), SINV TR339 (141) and CHIK-LR (348) has been previously described. Mutant VEEV viruses P713G, P713S, P713K and Q739L were generated by site-directed mutagenesis using appropriate overlapping primers and Quikchange kit and according to manufacturer's guidelines (Agilent). VEEV

mutants CD and CD/nsP2 739L were generated similarly by deleting amino acids 64-68 from capsid using site-directed mutagenesis. YFV vaccine strain 17-D was used to construct a reporter virus expressing Nano-luciferase (nLuc) (349) by inserting the nLuc gene followed by the *Thosea asigna* virus (Tav) 2A-like protease in frame between Capsid and prM, as previously described for alphaviruses (350). Viruses were generated by electroporation of capped *in vitro*-transcribed RNA (mMessage mMachine; Ambion) into BHK-21 cells (277). Titer was determined using a BHK-21 plaque assay. Construction and packaging of VEEV, EEEV and SINV replicons expressing fLuc has previously been described (188). A CHIKV replicon expressing fLuc was constructed using methods similar to those previously described. Nano luciferase activity on lysates from 17-D nLuc infected cells was measured using the Nano-Glo Luciferase Assay kit (Promega). Protein levels were quantified using a BCA assay (Pierce) and data expressed as RLU/μg of cell protein.

**Protein expression plasmids.**

Individual virus proteins were cloned into the pCAGGS vector containing a HA tag at the N-terminus proximal to the insertion site (gift from Chris Basler) (351). The nsP and capsid proteolytic cleavage sites were chosen as the boundaries of the protein genes and a stop codon was added at the end of each gene. Viral genes were PCR-amplified from full-length cDNA clones using suitable primers with Not1 and Nhe1/Xma1 restriction sites added at the 5' and 3' ends respectively. Additionally, GFP was PCR-amplified from a previously constructed plasmid using suitable primers with Not1 and Nhe1 restriction sites added at the 5' and 3' ends respectively. PCR products and pCAGGS vector were digested and ligated, and individual clones were selected and confirmed by restriction digestion and sequencing. Huh7 and MEF cells were

transiently transfected with plasmids (10 μg per plasmid) using the Nucleofector II machine and manufacturer's protocols (Amaxa). Huh7 cells ($1X10^6$ per reaction) were nucleofected using Kit V and protocol T-022. MEF cells ($1X10^6$ per reaction) were nucleofected using Kit V and protocol T-020. Each transfection reaction was divided into two or three wells. A GFP-expressing plasmid was used as transfection control and reactions were used for experiments if GFP positive cells numbered >90%.

**Translation reporters.**

Reporters were constructed as described (280, 281, 299). Briefly, the fLuc gene was fused in frame with 5' and 3' NTR and poly (A) tail from SINV, CHIKV, VEEV or EEEV, such that reporter RNA would initiate translation of fLuc from authentic virus translation start sites. RNA was synthesized using *in vitro* transcription kits (mMessage, mMachine; Ambion). IRES translation reporters expressing Renilla luciferase were a gift from Martin Bushell (352). Cells were electroporated with virus derived reporter RNA's (5μg per reaction) using a BioRad Gene Pulser II machine (for each reaction, two pulses at 220 Volts and 1 mFarad). Each electroporation reaction was divided into three wells. Lysates were collected in passive lysis buffer (Promega). Firefly or Renilla luciferase activity was measured using the Dual Luciferase Reporter Assay kit (Promega). Protein levels were quantified by bicinchoninic acid assay (BCA; Pierce) and data expressed as RLU/μg.

**Virus infections.**

Cells were seeded in plates overnight before infections. Viruses were diluted to the M.O.I. indicated in figure legends in virus diluent phosphate-buffered saline (PBS with Calcium and

Magnesium, supplemented with 1% DBS). Cells were infected for one hour at 37°C, following which medium was added to cells. Supernatants were collected at indicated times and viral titers were determined as described above. For semi-quantitative PCR measuring viral replication, lysates were collected using Trizol reagent and total cellular RNA was extracted using manufacturer's protocols (Ambion). 100ng total RNA was reverse-transcribed into cDNA using a virus specific primer for VEEV (5'-GCGTAATACGACTCACTATACTGGTACTAGATTTATGCGC -3'), EEEV (5'-GCGTAATACGACTCACTATATGACAACCAACGAGTGTGGG-3'), SINV (5'-GCGTAATACGACTCACTATACGTGAGGAAGATTGCGGTTC-3') and CHIKV (5'-GCGTAATACGACTCACTATAGCTGTCTAGATCCACCCCATACATG-3'). For the semi-quantitative step, a primer for T7 (5'-GCGTAATACGACTCACTATA-3') and virus specific primers for VEEV (5'-TCCGTCAGCTCTCCCGCAGG-3'), EEEV (5'-AGAGTGGCTGACGTTCGCAC-3'), SINV (5'-CTGGGAAGGGCACACAACTT-3') and CHIKV (5'-GGCAGTGGTCTCAGATAATTCAAG-3') were used. Additionally, 100ng RNA was reverse-transcribed using random hexamers, and 18S primers were used as loading control (sense, 5'-CGCCGCTAGAGGTGAATTTCT-3'; antisense, 5'-CGAACCTCCGACTTTCGTTCT-3'). Values obtained were normalized to 18S levels using the ddCT method (353). DNA contamination was ruled out by performing reactions excluding reverse transcriptase.

**IFN bioassay and ELISA.**

The concentration of biologically active mouse IFN was measured using a bioassay as previously described (354). Briefly, $3X10^4$ L929 cells were seeded per well in a 96-well plate. Samples (200

μL) were acidified to pH 2.0 using 1M HCl for 24h at 4°C. Samples were then neutralized to pH 7.0 using 2M NaOH and 100 μL was added to the first well in duplicate. Samples were diluted 2-fold across the plate and incubated at 37°C for 24h. EMCV ($4X10^3$ PFU/well) was added to each well and incubated 24h at 37°C and then stained with crystal violet. The IFN concentration in samples (cell supernatants or mouse sera) was set as the dilution of sample required for 50% protection from CPE, compared with protection conferred by an IFN standard. The IFN-α ELISA was performed on mouse sera as per manufacturer's instructions.

**Western blotting.**

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 1 mM EGTA) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1μg/ml leupeptin, and 1 μg/ml pepstatin), and a phosphatase inhibitor cocktail (Sigma). Protein concentrations were determined as above. Equal amounts of protein (25 μg) from each lysate was resolved on an 8% SDS-polyacrylamide gel (SDS-PAGE) and transferred to a PVDF membrane (BioRad). Membranes were blocked for 1h in 5% milk in Tris-buffered saline (TBS) with 0.1% Tween 20 (TBST) and incubated overnight with primary antibody at 4°C. Primary antibodies were diluted in 3% BSA-TBST. Membranes were washed in TBST four times (15min each) and incubated for 1h in appropriate horseradish peroxidase-conjugated secondary antibody (Thermo Fisher) diluted in 2% milk-TBST. Membranes were washed in TBST four times and probed with an ECL chemiluminescence kit (Pierce). Densitometry was performed using Image J software. Membranes were probed with following antibodies: rabbit polyclonal against p-Tyr701 STAT1 (1:3000, Cell Signaling), STAT1 (1:3000, Santa Cruz), p-JAK1 (1:3000, Santa Cruz),

JAK1 (1:3000, Santa Cruz) and IFIT1 (1:1000, Sigma), goat polyclonal against TGTP (1:250, Santa Cruz) and mouse monoclonal against actin (1:5000, Millipore) and HA-tag (1:1000, Thermo Scientific).

**Metabolic labeling.**

Cells were infected with viruses or transfected with plasmids for the times indicated in figure legends. Thirty minutes before labeling, growth medium was replaced with starvation medium (Cys/Met free DMEM (Cellgro) supplemented with 1% FBS, L-glut, PS). Next, cells were incubated in starvation medium complemented with 100μCi/mL [$^{35}$S] Cys/Met (MP Biomedicals) for 2h at 37°C. Cells were washed with PBS and lysed in RIPA buffer. An equal volume of sample was resolved on an 8% SDS-PAGE gel. Gels were fixed and dried and exposed to photographic film (GE Healthcare) for 7 days at -80°C for visualizing labeled proteins. Densitometry was performed as described above. As a loading control, equal volumes of lysate were resolved and stained for levels of actin using western blot.

**Immunofluorescence.**

Huh7 cells were transfected with expression plasmids as described above and treated 24h later with 1000 IU/mL human leukocyte IFN for 30 min. Cells were fixed in 4% paraformaldehyde (PFA) overnight at 4°C and permeabilized with ice-cold methanol or 0.1% Triton X-100 at room temperature for 15 min. Cells were blocked with blocking buffer (BB, PBS supplemented with 1% bovine serum albumin and 0.3% Triton X-100) mixed with 10% donkey serum for 1h at room temperature. Primary antibody was diluted in BB and incubated overnight at 4°C. Next, cells were washed in PBS and incubated in fluorophore-conjugated secondary

antibody in the dark for 1h at room temp. DAPI (10μL per well) was added before cells were observed with a confocal fluorescence microscope. For each well, 50 HA-positive (virus protein-expressing) cells were counted for the presence of STAT1 in the nucleus, in triplicate. Cells transfected with a GFP expressing plasmid was used as a positive control for the assay as STAT1 translocation into the nucleus is not inhibited by GFP. Similar experiments were performed with MEF cells pre-treated with 150IU/mL mouse IFN for 16h and transfected as described above. The following primary antibodies were used: rabbit polyclonal anti-STAT1 (1:200, Santa Cruz), mouse monoclonal anti-HA tag (1:200, Thermo Fisher). Secondary antibodies used were Alexa 488 donkey anti-mouse and Alexa 594 donkey anti-rabbit (1:1000, Jackson Immunological). Images were acquired using Olympus Fluoview 1000 microscope at a magnification of 60X using Fluoview software version 3.1.

**Real-Time PCR.**

Lysates were collected from infected cells using Trizol reagent and total cellular RNA was extracted using manufacturer's protocols (Ambion). 100 ng total RNA was reverse-transcribed into cDNA using random hexamers. Primers were designed to detect levels of mouse IFN-α1, IFN-α4 and IFN-β. 18S primers were used as loading control (sense, 5'-CGCCGCTAGAGGTGAATTTCT-3'; antisense, 5'-CGAACCTCCGACTTTCGTTCT-3').

**Transcription shutoff analysis.**

Lysates were collected from transfected cells using Trizol reagent and total cellular RNA was extracted using manufacturer's protocols (Ambion). 100 ng total RNA was reverse-transcribed into cDNA using random hexamers. Primers were designed to detect levels of human gamma

actin intron #3 (sense, 5'-TTCTTTCGCTGTTCCAGGCT-3'; antisense, 5'-AGGCTTCAGGGAGGAAATGC-3') or mouse gamma actin intron #3 (sense, 5'-ACAGAACGCAAGCAGAAACG-3'; antisense, 5'-TGGCATTTCCTCCCTGAAGC-3'). 18S primers were used as loading control (sense, 5'-CGCCGCTAGAGGTGAATTTCT-3'; antisense, 5'-CGAACCTCCGACTTTCGTTCT-3'). Actinomycin D treatment (15 μg/mL treatment for 6h) was used as a positive control for transcription shutoff.

**Murine experiments.**

Inocula (10μL) containing 100 or 1000 PFU virus or PBS was administered subcutaneously to the hind leg footpad of CD-1, AB6, IRF3$^{-/-}$ or IRF7$^{-/-}$ mice using a 27 gauge needle and a gastight Hamilton syringe. For morbidity and mortality studies, mice were observed and scored for degree of sickness at 24h intervals and AST and percent mortality were calculated. For viral titers in tissues, indicated tissues were harvested 24h p.i. and stored at -80°C, and plaque assays were performed as described. For measuring serum IFN levels, serum was harvested at indicated times p.i. and IFN bioassays were performed as described.

**Statistics.**

Statistical tests used were Student's t-test, one-way ANOVA, two-way ANOVA and Mantel-cox Log-Rank Test, as indicated in figure legends. Graph Pad Prism software was used for all statistical analyses.

# APPENDIX A

## TABLE OF ABBREVIATIONS

**AST –** average survival time

**ATCC -** American Type Culture Collection

**Bcl-2 –** B-cell lymphoma 2

**BHK –** baby hamster kidney

**CHIKV –** Chikungunya virus

**CPE -** cytopathic effect

**CRM1 –** chromosomal maintenance 1

**CrPV –** Cricket paralysis virus

**CSE –** Conserved sequence element

**DBS –** Donor bovine serum

**DMEM -** Dulbecco's modified Eagle's medium

**EEEV –** eastern equine encephalitis virus

**eIF -** eukaryotic initiation factor

**eIF2α -** eukaryotic initiation factor 2α

**eIF3 -** eukaryotic initiation factor 3

**eIF4A -** eukaryotic initiation factor 4A

**eIF4G -** eukaryotic initiation factor 4 gamma

**ELISA -** enzyme-linked immunosorbent assay

**EMCV –** Encephalomyocarditis virus

**ER -** endoplasmic reticulum

**FBS –** Fetal bovine serum

**fLuc –** Firefly Luciferase

**GFP –** green fluorescent protein

**HA –** hemagglutinin

**HCV -** Hepatitis C virus

**HS –** heparan sulfate

**IFIT1 -** Interferon-inducible protein with tetratricopeptide repeat 1

**IFN –** Interferon

**IFNAR –** Interferon α/β receptor

**IFNGR –** Interferon gamma receptor

**IKKε –** Inhibitor of κB kinase ε

**IRES –** internal ribosome entry site

**IRF3 –** Interferon Regulatory Factor 3

**IRF7 -** Interferon Regulatory Factor 7

**IRF9 -** Interferon Regulatory Factor 9

**ISG –** Interferon-stimulated gene

**ISG15 -** Interferon-stimulated gene 15

**ISG20 -** Interferon-stimulated gene 20

**ISRE -** Interferon stimulated regulatory elements

**IU –** international unit

**JAK1 –** Janus Kinase 1

**MDA5 -** Melanoma Differentiation-Associated protein 5

**MEF –** murine Embryonic Fibroblasts

**MHC -** Major Histocompatibility Complex

**M.O.I. -** multiplicity of infection

**mRNA –** Messenger RNA

**MyD88 -** Myeloid differentiation primary response gene 88

**NAD –** nicotinamide adenine dinucleotide

**NES –** nuclear export signal

**nLuc –** nano Luciferase

**NLS –** nuclear localization signal

**nsP– n**onstructural protein

**nsP1–** nonstructural protein 1

**nsP2 –** nonstructural protein 2

**nsP3 –** nonstructural protein 3

**nsP4 –** nonstructural protein 4

**NTR –** non-translated region

**ORF –** open reading frame

**PABP –** poly (A) binding protein

**PBS -** phosphate-buffered saline

**PFA –** paraformaldehyde

**PKR –** protein kinase R

**PRR –** Pattern Recognition Receptor

**qRT-PCR -** quantitative RT-PCR

**RDRP –** RNA dependent RNA polymerase

**RIG-I –** Retinoic acid-inducible gene 1

**RIPA -** radioimmunoprecipitation assay

**RLU –** relative light units

**RpS6 –** ribosomal protein S6

**RRV –** Ross river virus

**SAM -** S-adenosylmethionine

**SH2 –** Src homology domain 2

**SINV** – Sindbis virus

**siRNA –** short-interfering RNA

**SIRS -** systemic inflammatory response syndrome

**SFV –** Semliki Forest virus

**SOCS –** Suppressors of cytokine signaling

**STAT1 –** Signal Transducer and Activator of Transcription, 1

**TBK1 –** TANK binding kinase 1

**TBS –** Tris-buffered saline

**TGTP -** T-cell specific GTPase

**TLR –** Toll like receptor

**TPB –** Tryptose phosphate broth

**TTC7B –** tetratricopeptide repeat domain 7B

**Tyk2 -** Tyrosine kinase 2

**VEEV** – Venezuelan equine encephalitis virus

**VLP –** Virus like particle

**WEEV –** western equine encephalitis virus

**WT –** wild-type

**YFV –** Yellow fever virus

**ZAP –** Zinc-finger antiviral protein

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