THE ROLE OF SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION (STAT) PROTEIN IN RIFT VALLEY FEVER VIRUS INFECTION

by

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DEDICATION

This is dedicated to my family and to Kyle for all their help and support throughout my academic career. Without their love and support, I would not be where I am today.
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LIST OF ABBREVIATIONS

Dulbecco’s Modified Eagle Medium ................................................................. DMEM
Dimethyl sulfoxide ........................................................................ DMSO
Fetal Bovine Serum ........................................................................ FBS
Signal Transducer and Activator of Transcription ............................. STAT
Multiplicity of Infection ................................................................ MOI
Phosphate Buffered Saline ................................................................ PBS
Polymerase Chain Reaction .............................................................. PCR
Quantitative Reverse Polymerase Chain Reaction .......................... qRT-PCR
Rift Valley Fever Virus ..................................................................... RVFV
Ribonucleic Acid ................................................................................ RNA
Interferon ............................................................................................ IFN
Human small airway epithelial cells ............................................. HSAECs
Ethylenediaminetetraacetic acid ................................................ EDTA
Glyceraldehyde 3-phosphate dehydrogenase ................................. GAPDH
Bovine serum albumin ...................................................................... BSA
4’,6-diamidino-2-phenylindole ....................................................... DAPI
Immunoprecipitation ........................................................................ IP
Wild type ............................................................................................... WT
THE ROLE OF SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION (STAT) PROTEIN IN RIFT VALLEY FEVER VIRUS INFECTION

Chelsea Pinkham, M.S.
George Mason University, 2014
Thesis Director: Dr. Kylene Kehn-Hall

First identified in the Rift Valley of Kenya, Rift Valley Fever Virus (RVFV) is a mosquito-borne zoonotic disease that can cause severe illness in humans and livestock. RVFV outbreaks can have remarkably devastating effects on livestock, in which RVFV causes spontaneous abortion in almost 100% of pregnant ruminants, referred to as an “abortion storm,” and a high mortality in young animals. These outbreaks can become a major burden for countries in which RVFV is endemic. Humans can also become infected with RVFV through a bite from an infected mosquito, which in many cases causes an acute febrile illness with symptoms including chills, malaise, dizziness, fever, and headache. In a small percentage of cases, the disease progresses to severe symptoms including blindness, hepatitis, encephalitis, and hemorrhagic fever. With the increased risk of spread, competent species in many areas of the world and the ease of international trade and travel, RVFV is considered an overlap select agent by the Centers of Disease
Control and Prevention and the U.S. Department of Agriculture. There are currently no approved vaccines or therapeutics available to the public in order to combat RVFV infection. Current research has focused more on host factors that aid in viral replication, unveiling a new collection of targets for treatments. Signal transducer and activator of transcription (STAT) proteins are a family of transcription factors that regulate many aspects of the host cell, such as immunity, cell growth, survival, and proliferation. The JAK-STAT protein cascade is initiated when a receptor receives signals from external factors, such as cytokines and growth factors, which in turn activate receptor-associated tyrosine kinases. Our research shows that both STAT1 and STAT3 are phosphorylated on their conserved tyrosine residue following RVFV infection. These phosphorylation events are dependent on the viral protein NSs, as the MP12-ΔNSs virus, which has a complete deletion of the viral protein NSs, does not induce STAT1 and STAT3 phosphorylation. Our data also indicates that two specific mutations in NSs impair STAT3 tyrosine phosphorylation. Treatment of cells with a STAT3 small molecular inhibitor, Stattic, reduces MP12 viral titers at 8, 16, and 24 hours post infection. Finally, STAT3 -/- MEFs are more susceptible to RVFV induced cell death than WT MEFs, indicating that STAT3 functions as a pro-survival factor.
INTRODUCTION

Rift Valley Fever Virus

*Background and Significance*

First identified in the Rift Valley of Kenya, Rift Valley Fever Virus (RVFV) is a mosquito-borne zoonotic disease that can cause severe illness in humans and livestock (1). Since its discovery in 1931, it has become endemic to sub-Saharan Africa and has dispersed to the Arabian Peninsula (2, 3). One of the largest outbreaks occurred in Egypt in 1977, in which an estimated 200,000 people were infected with the virus (4). More recently, an outbreak occurred in 2000 where the virus spread to Saudi Arabia and Yemen through the transport of infected animals and mosquitoes (4). While RVFV can spread through various routes, the virus is transmitted primarily through mosquitoes, particularly species of the *Aedes* and *Culex* genera (5). These two genera are present within developed areas, including Europe and the United States, indicating that there are competent vectors for viral spread (5). With the increased risk of spread, competent species in many areas of the world, and the ease of international trade and travel, RVFV is considered an overlap select agent by the Centers of Disease Control and Prevention and the U.S. Department of Agriculture (6).

RVFV outbreaks can have remarkably devastating effects on livestock, in which RVFV causes spontaneous abortion in almost 100% of pregnant ruminants, referred to as
an “abortion storm,” and a high mortality in young animals (5). Sheep are markedly susceptible to the virus, where newborns and young suffer from hepatitis while adult sheep exhibit hemorrhagic signs often seen in severe human cases (4). These outbreaks can become a major burden for countries in which RVFV is endemic. In these particular regions, much of the population is dependent on the livestock industry, posing an economic challenge to nations that are often in the infancy of their development.

Humans can also become infected with RVFV through various routes. One of the most common routes of infection is a bite from an infected mosquito, which in many cases causes an acute febrile illness with symptoms including chills, malaise, dizziness, fever, and headache (1). Generally, these viral infections result in an acute illness but in a small percentage of cases the disease can progress to severe symptoms including blindness, hepatitis, encephalitis, and hemorrhagic fever (1). Other common routes include close proximity to the slaughtering of infected animals or the handling of aborted fetus materials, in which direct contact with the infectious material, as well as the inhalation of aerosols produced during these processes, can both cause disease (7). Likewise, because of the ease of aerosolization, there is also a large concern for its use as a bioweapon since an aerosol would be the most probable route for an attack on a human or livestock population (8).

**Molecular Biology**

RVFV is a negative sense, single-stranded RNA virus of the family *Bunyaviridae*, genus *Phlebovirus*. The enveloped virus enters via heparin sulfate, which is present on the surface of most animal cells (9). It encodes three RNA segments termed the S-, M-,
and L-segments (1, 5). The S-segment codes for the nucleoprotein, as well as the nonstructural protein NSs in an ambisense fashion (1, 5). The M-segment is of negative polarity, in which the M-segment encodes NSm, 78-kDa protein, and the precursor to the glycoproteins (1, 5). The L-segment, which is also of negative polarity, encodes the RNA-dependent RNA polymerase (1, 5). After cellular entry, the virus is released into the cytoplasm where all replication steps occur (5). Once replication is complete, the fully mature virions are formed by budding from the Golgi.

Of particular interest is the 31-kDa nonstructural protein NSs, which is a major virulence factor of RVFV. RVFV strains that contain an in-frame deletion or a complete deletion of the NSs protein, such as the C13 strain or the MP12-ΔNSs strain, are highly attenuated (10). While RVFV replicates in the cytoplasm, NSs localizes to the nucleus where it forms its characteristic filamentous structures (11). NSs has various roles during infection, primarily acting as an interferon antagonist and blocking IFN-β gene expression at the transcriptional level (10). More specifically, it binds with Sin3A-associated protein (SAP30), Yin Yang 1 (YY1), Histone deacetylase 3 (HDAC3) and Sin3A-associated corepressor factors in order to be recruited to the IFN-β promoter (12). This blocks the coactivator, CREB binding protein (CBP), and facilitates transcriptional repression. Additionally, NSs interacts with the p44 subunit of TFIIH, which is sequestered by the characteristic filaments and prevents the assembly of transcription factor II (TFIIH) (13, 14). This results in a general repression of transcriptional activity in host cells. NSs also causes degradation of dsRNA-dependent protein kinase (PKR) via the proteasome, which in turn inhibits phosphorylation of eukaryotic translation initiation
factor 2α (eIF2α), preventing overall host translational suppression (15, 16). Overall, NSs has numerous roles in combating the host cell immune response at multiple levels, creating an environment where the virus can thrive and replicate efficiently.

**Signal Transducer and Activators of Transcription (STAT) Proteins**

Signal transducer and activator of transcription (STAT) proteins are a family of transcription factors that regulate many aspects of the host cell, such as immunity, cell growth, survival, and proliferation (17). The JAK-STAT protein cascade is initiated when a receptor receives signals from external factors, such as cytokines and growth factors, which in turn activates receptor-associated tyrosine kinases (17). These kinases, such as Janus kinase 2 (JAK2) or tyrosine kinase 2 (TYK2), proceed in phosphorylating STAT proteins causing them to assemble into homo- or hetero-dimers (17). This modification initiates a change in conformation which allows entry into the nucleus, association with co-activators and other transcription factors, and binding to specific DNA sites (18).

STATs are grouped based on the arrangement of their functional motifs. There is an amino terminus, a coiled-coil domain, DNA-binding domain, a SRC homology 2 (SH2) domain, a single conserved tyrosine residue, and a carboxy terminus (18). First, the coiled-coil domain consists of four helical coils and is responsible for interactions with other proteins (17). The DNA binding domain contains a series of beta-sheets and allows binding to specific DNA sites, which is followed by a linker domain containing alpha helices (17). Next is the SH2 domain, where the phosphorylated tyrosine residues
allow the reciprocal interaction with other STAT proteins, forming dimers (17). STATs interact with each other following tyrosine phosphorylation on the conserved residue, and subsequent dimerization through the SH2 domain interactions (18). STATs can also bind to each other in their unphosphorylated forms, but it is the dimerization of the tyrosine-phosphorylated STATs that allow specific binding to DNA in the nucleus (18). Finally, the carboxy terminus is responsible for transcriptional activation (17). This structure and function is consistent throughout the STAT family, but for the purposes of this research we chose to focus on STAT1 and STAT3.

**STAT1**

STAT1 was the first member of the STAT family to be identified, and plays a critical role in type I and II interferon responses (19). STAT1 was initially found to be a factor in the complex that binds to DNA following the response to type I IFNs (18). This complex comprises of dimers of STAT1 and STAT2, as well as IFN-regulatory factor 9 (IRF9), which initiate binding to the IFN-stimulated response element (ISRE), present in the promoter of specific genes (18). The conserved tyrosine phosphorylated residue (Y701) is also activated in response to type II IFNs, which results in the binding of the dimers to a gamma activated sequence (GAS) located in the promoter region of responsive genes (20). STAT1-targeted genes include pro-inflammatory and anti-proliferative genes, which contrasts greatly with the target genes of STAT3 (19). In addition, even though the generic model of signaling for STAT1 is through type I and II IFNs, STAT1 can also be stimulated by growth factors such as epidermal growth factor (EGF) (20). Furthermore, studies have determined that STAT1-deficient mice were
highly susceptible to multiple viruses, including influenza A and herpes simplex virus (21). Likewise, a recent study demonstrated the critical role that STAT1 plays in the immune response in humans. This study revealed that people with different STAT1 mutations were more susceptible to viral and bacterial infections (22).

In addition to its transcriptional roles, it has been established that STAT1 also plays a role in the cell cycle. It interacts directly with cyclin D1 and cyclin dependent kinase 4 (CDK4), proposing a role in G1 cell cycle progression (23). Moreover, STAT1 is required to induce apoptosis via the DNA-damage pathway, acting as a co-activator of the tumor suppressor protein p53 (24). The same study showed that STAT1 was a negative regulator of mouse double minute 2 homolog (MDM2), which is upstream of p53, due to increased levels in STAT1-deficient cells. These are only a few of the roles that have been recently uncovered and seem to be adding on to the ever growing list of STAT1 non-transcriptional functions.

STAT3

STAT3 performs in a similar way to STAT1, in that it becomes phosphorylated by an upstream kinase, forming dimers and translocating into the nucleus to induce transcription. After receptor stimulation from cytokines, such as IL-6 or IL-10, tyrosine kinases phosphorylate STAT3 on the conserved tyrosine residue (Y705). STAT3 also contains a serine phosphorylation site (S727) within the transactivation domain. Unlike STAT1, a conventional knockout of STAT3 results in embryonic lethality, which emphasizes its role in growth and development (25). STAT3 is also widely studied in the cancer field because of its constitutive activation in many cancers, specifically of the liver.
Although transcriptional activation is the classical function of STAT3, other cytoplasmic roles have been identified. Recently, STAT3 has been shown to be required for the stabilization of microtubules by inhibiting the action of the microtubule destabilizing protein, stathmin (26). STAT3 has also been identified as a regulator of autophagy by interaction with PKR (27). In addition to these roles, it was also demonstrated that STAT3 is a negative regulator of the type I IFN response (28). These examples are just a few roles that have been recently uncovered. Viral manipulation of STAT3 has also been established, and is discussed below.

**Stattic**

As previously mentioned, STAT3 is constitutively activated by upstream kinases in many cancer cell lines and human tumors. Consequently, there has been great interest in producing STAT3 inhibitors as therapeutics, particularly inhibiting the SH2 domain which is necessary for phosphorylation and dimerization. A recent study identified Stattic, a nonpeptidic small molecule inhibitor of STAT3, through a screening of chemical libraries (29). Stattic inhibits STAT3 by impairing the function of its SH2 domain in vitro, with selectivity over STAT1. Inhibition of phosphorylation on the SH2 domain subsequently prevents the activation, dimerization, and translocation to the nucleus of cells. In STAT3-dependent cancer cell lines, Stattic also increases the rate of apoptosis. This study shows that this inhibitor can be used as an interesting tool to explore the role of STAT3 in cancer, and in our case, microbial infections.
Viral Manipulation of STAT Pathways

Viruses have evolved to regulate STAT proteins in order to facilitate replication and evade the host immune response. Measles virus, a negative-sense single-stranded RNA virus, causes inhibition of IFN-α/β but not IFN-γ signaling (30). In addition, they revealed that STAT1 and STAT2 tyrosine phosphorylation is inhibited. Hepatitis C virus (HCV) and West Nile Virus (WNV), both Flaviviruses, evade the immune response by reducing accumulation of phosphorylated STAT1 in the nucleus (31, 32). Nipah virus, in the Paramyxoviridae family, has viral proteins that block both STAT1 and STAT2 activation and subsequent translocation to the nucleus. Ebola virus VP24 protein blocks production of interferon α/β by interfering with karyopherin α1 therefore preventing STAT1 translocation to the nucleus (33). These examples are some among many that illustrate virus’ abilities to evade host responses to incoming infections by modifying STAT1 activity.

Similarly, viruses can also manipulate the activity of STAT3 in order to avoid host cell mechanisms. Hepatitis B virus (HBV) enhances STAT3 and STAT5 phosphorylation, as well as STAT binding and transcriptional activity through the X-gene product (HBx) (34). The same study also noted that there was increased activity of JAK1 kinase, not JAK2 or TYK2. STAT3 activation is widely examined in HCV infections, due to its role in cell growth. One study indicated that HCV core protein activates STAT3 on its tyrosine residue, which results in rapid proliferation and cellular transformation (35). Conversely, another study demonstrated that STAT3 is activated by the complex formed by viral protein NS5A and JAK1, resulting in translocation to the
nucleus (36). A few years afterward, Machida et al. published work that showed HCV induces production of reactive oxygen species, consequently leading to DNA damage and activation of STAT3 (37). Although there have been differences in opinion of what is causing STAT3 activation in HCV infection, it is undeniable that STAT3 plays a major role in HCV.

**Rationale**

Current research has focused more on host factors that aid in viral replication, unveiling a new collection of targets for treatments. Popova et al. used reverse-phase protein microarray technology to identify signaling pathways that are induced during RVFV infection (38). Among those pathways, levels of both phosphorylated STAT1 and STAT3 increased following RVFV infection. Although these proteins undergo increased phosphorylation after RVFV infection, the mechanism by which this happens remains unknown. Thus, our project focuses on identifying the key roles that host proteins STAT1 and STAT3 play in RVFV infection. Identifying the mechanism by which RVFV exploits host proteins can provide additional targets for antivirals and therapeutics, which are not currently available to the public.
MATERIALS AND METHODS

Cell Culture

Vero cells (ATCC Cat # CCL-81) as well as wild-type mouse embryonic fibroblasts (MEFs) and STAT3 null derivatives (a kind gift from Dr. Levy and Dr. Watowich) were all maintained in Dulbecco’s modified minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin/streptomycin. Human small airway epithelial cells (HSAECs) were acquired from Cambrex Inc., Walkersville, MD and maintained in Ham’s F12 medium supplemented with 10% Fetal Bovine Serum (FBS), 1% L-glutamine, 1% penicillin/streptomycin, 1% nonessential amino acids, 1% sodium pyruvate, 0.001% of 55 mM β-mercaptoethanol (Gibco Cat # 2195-023). Human liver fibroblast cells (HepG2s) were maintained in 50% DMEM and 50% Ham’s F12 medium supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin. All cell lines were maintained at 37°C with 5% CO₂.

Viruses

Recombinant (r)MP12 virus was rescued using the following plasmids: pProT7-M(+), pProT7-L(+), pProT7-S(+), pT7-IRES-vN, pT7-IRES-vL, and pCAGGS-vG (1, 2). To generate an initial seed stock, BSR-T7/5 cells (seeded at 3 x 10⁶ cells per 75cm² flask) were transfected with 4μg each of pProT7-M(+), pProT7-L(+), pProT7-S(+), pT7-IRES-
vN and 2μg each of pT7-IREs-vL, and pCAGGS-vG using TransIT-LT1 (Mirus, Madison, WI). Ratio of total plasmid DNA amount to TransIT-LT1 was kept at 1:3. During rescue of recombinant MP12 virus, complete MEM media without Geneticin was used. At 24 hours (h) post transfection, transfection media was removed, cells washed once, and complete media added back. After an additional 72h, media supernatants were collected, clarified by centrifugation (5 min, 3000rpm, 4°C), aliquoted, and stored at -80°C. Infectious viral titers were determined by plaque assay on Vero cells. To generate a P1 viral stock, subconfluent monolayers of Veros were infected at an MOI 0.1 for 1h. Inoculum was then removed, cells washed once, and complete media added. Two days later when cytopathic effect was starting to be observed within the culture, media supernatants were collected twice with a 4h duration in-between harvests and stored at 4°C. After the last collection, supernatants were then pooled together, filtered (0.2μM), and stored at -80°C in aliquots. Viral titers were determined by plaque assay on Vero cells.

Other recombinant viruses used included the live-attenuated MP12 strain with a complete deletion of the NSs ORF (39), as well as a strain where a flag tag was added to the C-terminus of NSs (15). For viral infections, cells were cultured at 5x10^5 cells per well in 6-well plates. The next day, cells were infected with the appropriate virus at the specified multiplicity of infection (MOI) by adding 400 μL of the infectious media. Cells were incubated for 1 hour at 37°C and 5% CO₂. Infectious media was removed, washed once with phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+}, and 2 mL of complete media was added. The cells were left to incubate at 37°C and 5% CO₂ and collected using
the appropriate method at the specified time points. Particular experiments were done in 96-well and 12-well formats and the same protocol was followed with the volumes adjusted accordingly.

Viruses containing mutations in the NSs protein were obtained from two different sources. The V5-NSs MP12 virus encoding the wild type NSs protein and the V5-NSs R173A was acquired from Dr. Sina Bavari’s lab (USAMRIID), and previously described by Kalveram et al. (40). The rMP12 NSs-3xFLAG was generated in house (many thanks to Dr. Cynthia de la Fuente), and contains a NSs protein fused with three tandem repeats of the FLAG epitope. Its corresponding mutant rMP12 NSs F261P, which has exchanged a phenylalanine for a proline at residue 261 was generated by standard cloning techniques. All recombinant viruses were then generated through reverse genetics as described above.

**Treatments**

Statcic STAT3 Inhibitor V (573099) was purchased from EMD Millipore and dissolved in sterile DMSO. Cells were treated with 5 μM of Statcic or 0.1% DMSO as a control. All treatments were done in complete media 2 hours prior to viral infection, and then the same treated media was replaced after viral infection unless otherwise noted. For experiments involving interferon treatment, cells were plated at 5x10^5 cells per well in a 6-well plate. The next day, cells were treated with 500 U/mL of human recombinant β-interferon (EMD Millipore, 407318) diluted in cell specific media for 1 hour. Following treatment, media was removed and cells were collected using the appropriate protocol.
Cell Viability Assays

Cells were plated in a white-walled 96-well plate and allowed to incubate at 37°C and 5% CO₂ overnight. The Stattic STAT3 inhibitor was serial diluted in cell specific media and added to the cells to determine a working concentration. Cells were incubated for 24 and 48 hours with the inhibitor present. At the appropriate time point, the cells were analyzed using CellTiter-Glo Cell Luminescent Viability Assay (Promega, G7570) according to the manufacturer’s protocol. The reagents and plates were brought to room temperature for 30 minutes, and then a volume of the CellTiter-Glo reagent that was equal to the amount of cell culture media present was added to the plate. Cell lysis was induced by shaking the plate for 2 minutes. The plate was incubated at room temperature for 10 minutes to stabilize the luminescence signal, and then the luminescence signal was read on a DTX 880 multimode detector (Beckman Coulter). A similar protocol was followed for cell survival assays following MP12 infection. During these assays, media was gently removed from cells then replaced with 100 µL of fresh media. An equal volume of CellTiter-Glo reagent was added, and protocol proceeds as described above.

Plaque Assay

Cells were plated in a 96-well plate at 10,000 cells per well and allowed to incubate overnight at 37°C and 5% CO₂. The next day, cells were treated as described above and infected for one hour. Supernatants were collected at the indicated time points and stored at -80°C until use. Vero cells were plated at 2.5x10⁵ cells per well in 12-well plates and allowed to incubate overnight at 37°C and 5% CO₂. The following day, Vero cells were infected with dilutions of the supernatants collected. Supernatants were serial diluted
1:10 in triplicates from $10^{-1}$ to $10^8$ in complete DMEM. Vero cells were infected with 200 uL of each serial dilution for 1 hour. After infection, a 1-mL overlay of a 1:1 solution of 0.6% agarose in diH2O and 2x EMEM supplemented with 5% FBS, 1% L-glutamine, 1% penicillin/streptomycin, 1% non-essential amino acids, and 1% sodium pyruvate was added to the cells. The cells were incubated at 37°C and 5% CO$_2$ for 72 hours. After 72 hours, the cells were fixed using 10% formaldehyde for 2 hours at room temperature. Following fixation, the formaldehyde and agar plugs were discarded. The cell monolayers were stained with a solution of 1% crystal violet and 20% methanol to visualize plaques. Plaques were counted and the averages from triplicates were taken. Dilutions with less than 10 or more than 100 plaques were discounted. The viral titer (pfu/mL) was calculated by the average of the triplicates x dilution factor (5) x dilution.

**Western Blot**

Lysates were separated on NuPAGE 4-12% Bis-Tris gels (Life Technologies, NP0321BOX) and transferred to a polyvinyl difluoride (PVDF) membrane overnight at 4°C. Membranes were blocked at room temperature for 1 hour in a solution of previously boiled 3% nonfat dry milk in 1X PBS with 0.02% Tween-20. Primary antibodies to p-STAT1 Y701 (Cell Signaling, 7649S), total STAT1 (Cell Signaling, 9172S), p-STAT3 Y705 (Cell Signaling, 9145S), total STAT3 (Cell Signaling, 12640S), cleaved Caspase-3 (Cell Signaling, 9661S), Flag (Sigma, F1804), GAPDH (Cell Signaling, 5174S), Lamin A/C (Cell Signaling, 4777S), PKR (Cell Signaling, 3072S), RVFV Nucleoprotein (kind gift from Dr. Connie Schmaljohn, USAMRIID), or HRP conjugated actin (Abcam, ab49900) were diluted in the same 3% milk solution according
to the manufacturer’s instructions. Membranes were gently rocked in primary antibody solution overnight at 4°C. The next day, membranes were rinsed quickly once with 1X PBS with 0.02% Tween followed by four 5-minute washes. Membranes were incubated in the appropriate HRP-conjugated secondary antibody for 2 hours at room temperature. The same wash procedure was repeated, and the membranes were visualized by chemiluminescence using the SuperSignal West Femto Maximum Sensitivity Substrate kit (Thermo Scientific, 34096) on a Bio Rad Molecular Imager ChemiDoc XRS system (Bio-Rad).

**Immunofluorescence**

Veros or HSAECs were grown on coverslips in a 6-well plate, infected with MP12 or Flag-MP12 as described, washed with PBS and then fixed for 10 minutes in a solution of 4% paraformaldehyde. Cells were permeabilized with 0.1% Triton X-100 in PBS for 10 minutes and then washed three times with PBS. The cells were blocked for 1 hour at room temperature in a blocking solution of 1% BSA, 0.3M glycine, and 0.025% Triton X-100 in PBS. The primary antibodies, diluted according to manufacturer’s recommendations, were incubated in a solution of 1% BSA and 0.025% Triton-X100 in PBS for 1 hour at room temperature. Incubation was followed by one quick wash and two 5 minute washes in PBS with 0.025% Triton-X100. Alexa Fluor secondary antibodies were added at a 1:500 dilution in fresh buffer and incubated in the dark for one hour at room temperature. Slides were washed three times in PBS with 0.025% Triton-X100. Following washes, cells were incubated for 10 minutes in DAPI (dilution 1:1000) in PBS. After a short PBS wash, cover slips were mounted to glass slides using Fluoromount G.
and left to dry overnight. Slides were imaged using an oil-immersion 60X objective lens on a Nikon Eclipse TE 2000-U microscope, with all samples subjected to four-line averaging. At least three images were taken of each sample, with one representative image shown. The resulting images were processed through Nikon NIS-Elements AR Analysis 3.2 software.

qPCR

HSAECs were plated in a 12-well plate at 5x10^5 cells per well. Cells were mock-infected or infected with RVFV MP12 or MP12-∆NSs for one hour, washed once with PBS, and then complete media was added back onto the cells. Cells were collected at the indicated time points in 350 µL of RLT Buffer (with 2-Mercaptoethanol added) from RNeasy Mini Kit (Qiagen, 74104). Lysates were homogenized in a QIAshredder spin column that was centrifuged for 2 minutes at full speed, and then transferred to the RNeasy spin columns. Next, the total cell RNA extraction was performed according to manufacturer’s protocol. Following RNA extraction, RNA was treated with DNase I (Life Technologies, AM2222) for 30 minutes at 37°C to remove any contaminating DNA. DNase I was then inactivated by heating at 75°C for 10 minutes. Following DNase I treatment, RNA was reverse transcribed into cDNA using 1-2 µg of RNA (Applied Biosystems, 4387406) in a 20 µL reaction. For qPCR, the template cDNA was added to a 20 µL reaction with SYBR® Green PCR Master Mix (Applied Biosystems, 4309155) and 0.2 µM of primer. cDNA was amplified (1 cycle −95°C for 10 min, 40 cycles- 95°C for 15 sec and 60°C for 1 min) using the ABI Prism 7000. Fold changes were calculated relative to 18S RNA using the ∆∆Ct method. Primers used (5’-3’): SOCS3 (Forward:
TGAGAACTGCCAGGGAATCT, Reverse: GAGGAGCATGTCACCAGGAT), SOCS1 (Forward: CACTTCCGCACATTCCGTTC, Reverse: CAGTAGAATCCGCAGCGTC), IRF-1 (Forward: GGCTGGGACATCAACAAGGA, Reverse: TGCTTTGTATCGGCCTGTGT), and JunB (Forward: CCACCTCCGTTTACACCAA, Reverse: GAGGAGCTGATGGTGGTCG).
RESULTS

STAT1 and STAT3 are activated following RVFV infection

A recent study used reverse-phase protein array technology to identify host signaling pathways that are induced during RVFV infection (38). These results showed that phosphorylation of both STAT1 (Tyr 701) and STAT3 (Tyr 705) were increased after RVFV infection. Although these proteins undergo enhanced phosphorylation after RVFV infection, the mechanism by which this happens and its importance to RVFV replication remains unknown. As described previously, NSs is a major virulence factor that affects multiple host functions. To confirm the previously published results and to determine if this activation is dependent on the viral nonstructural protein NSs, western blot analysis was performed. Because STAT1 and STAT3 have roles in the interferon response, Vero cells, an interferon incompetent cell line was used. Vero cells possess interferon receptors, but they do not produce interferon due to a mutation in the interferon beta gene. Vero cells were mock infected or infected with the wild type MP12 strain or the MP12-ΔNSs strain containing a complete deletion of the NSs protein. STAT1 and STAT3 phosphorylation was most prominent at 16 hours post infection in Vero cells, but was increased over the mock at all time points assessed (Figure 1A). In addition, there was no enhanced phosphorylation of STAT1 or STAT3 in cells infected with MP12-ΔNSs. No phosphorylation was observed at 4 hours post infection with either viral
infection (data not shown). Analysis of nucleoprotein levels shows similar replication rates for both MP12 and MP12-ΔNSs viruses. Since Veros do not produce interferon but still respond to stimulation, Vero cells were stimulated with 500 U/mL of IFN-β as a control to insure that the Vero cells were able to activate STAT1 and STAT3 in absence of viral infection. Lysates were collected at various time points to analyze the STAT signaling in response to interferon stimulation (Figure 1B). Activation of STAT1 was exceptionally quick, showing increased expression in as little as 15 minutes. STAT3 activation lagged some in comparison to STAT1, with increased activation starting at 30 minutes after treatment. Overall, there was strong expression of both phosphorylated proteins at 30 minutes and 1 hour. STAT1 phosphorylation began to decrease after 2 hours, but was still increased over the untreated sample at 2 and 4 hours. STAT3 phosphorylation was back at similar levels to the untreated sample after 2 hours, and continued that way into the 4 hour time point. This confirms that Veros can be stimulated by interferon, and that interferon-induced signaling happens at very early time points. These data demonstrate that 1) STAT1 and STAT3 phosphorylation can occur in RVFV infected cells independent of interferon production, 2) phosphorylation is dependent on the viral protein NSs, and 3) the RVFV induced signaling is a later event as compared to the classical interferon induction of STAT1 and STAT3.

Next, an interferon-competent cell line was employed to see if STAT1 and STAT3 phosphorylation patterns were similar after RVFV infection. HSAECs cells were infected with the MP12 strain or the mutant strain MP12-ΔNSs. Western blot analysis showed that there was an increase in phosphorylation of both proteins after MP12
infection at later time points, similar to what was observed in the Vero cells (Figure 2A). More importantly, there was no phosphorylation of either STAT1 or STAT3 with the ΔNSs strain at the 16 and 24 hour time point. STAT1 and STAT3 phosphorylation was seen as early as 8 hours post infection, with STAT1 and STAT3 activation most prominent at 16 hours post infection. There was some phosphorylation of both proteins in cells infected with the ΔNSs strain at 8 hours post-infection, which may be the lingering host interferon response. In order to see how STAT1 and STAT3 are activated at early time points after infection, which corresponds to the induction of interferon, HSAECs were mock-infected or infected with MP12 or MP12-ΔNSs and collected at various early time points (Figure 2B). As a control, HSAECs were also stimulated with IFN-β and collected at the same time points. At 1 and 2 hours post-infection, there is little to no STAT1 phosphorylation in cells infected with MP12 and with MP12-ΔNSs. There is a robust phosphorylation of STAT1 in IFN-β stimulated cells. STAT1 phosphorylation does not occur until 4 hours in MP12 infected cells. STAT1 phosphorylation is increased at 4 and 8 hours over the mock as well as over MP12-infected cells. By 8 hours, the IFN-β stimulated cells have decreased phosphorylation of STAT1. Similarly, STAT3 phosphorylation was seen early on in cells infected with MP12-ΔNSs, while cells infected with the wild type MP12 virus had delayed STAT3 phosphorylation, only detectable starting at 8 hours post-infection. The activation of both STAT1 and STAT3 was comparable in HepG2 cells (data not shown), despite the constitutive phosphorylation of both proteins which is common in liver cancer cell lines. These data demonstrate that the increased phosphorylation is consistent across different
cell lines, without changes in total STAT1 or STAT3 levels. Taken together, these results indicate that enhanced phosphorylation of STAT1 and STAT3 is linked to the nonstructural protein, NSs, and also occurs independently of interferon at later time points post infection.

**Mutations in NSs disrupt STAT3 activation**

Western blot analysis of activated STAT1 and STAT3 exhibited dependence on NSs, since little or no activation was seen in samples infected with the virus containing a deletion of NSs. We wanted to investigate activation of STAT1 and STAT3 in cells infected with RVFV containing mutations in NSs. One set, obtained from USAMRIID, contained a V5-tagged NSs protein. The wild type NSs protein (V5-NSs) has similar replication kinetics and was previously described (40). A mutation was introduced on residue 173, with the exchange of an arginine for an alanine (V5-NSs R173A). This mutant is unable to degrade PKR and forms irregular filaments in the nucleus. Another set, generated in-house, also has similar replication kinetics (data not shown). The wild type NSs protein is fused to three tandem repeats of the FLAG epitope (NSs-3xFLAG) and forms normal filaments following infection. Its corresponding mutant has a mutation in residue 261, with the exchange of a phenylalanine for a proline (NSs-FLAG F261P). This residue was shown to be important for the degradation and binding to p62 in vitro (data not shown). In infected cells, this mutant is unable to degrade both p62 and PKR, and does not form filaments in the nuclei of infected cells.

HSAECs were mock-infected or infected with MP12, a control MP12 NSs-tagged virus, or one of the viruses that contained NSs mutations for one hour. Cells were
washed, and then complete media was added back onto the cells. Lysates were collected at various time points, separated by SDS-PAGE, and analyzed by western blot. Membranes were probed for phospho-STAT3 (Y705), RVFV nucleoprotein, or β-actin as the loading control. Consistent with our previous data, there was an increase in phosphorylation of STAT3 after infection of MP12 and a similar increase was observed with the WT 3x-FLAG NSs virus at 8 hours post infection (Figure 3). The NSs mutant F261P exhibited reduced levels of phosphorylation when compared to the control. The MP12 V5-NSs did not display an increased activation as the wild type MP12, and had similar activation levels to the mock infected sample. However, the corresponding mutant MP12 V5-NSs R173A showed even less activation of STAT3, and had similar levels to the MP12-ΔNSs virus. At 16 hours post infection, there was significant STAT3 phosphorylation in all the controls (MP12, WT 3x-FLAG-NSs, V5-NSs). As expected, there was little to no phosphorylation in the mutant viruses, as well as the mock and MP12-ΔNSs sample. By 24 hours post infection, there are some levels of phosphorylation in the mock sample. The wild type MP12 virus is still showing increased phosphorylation, but the other control viruses are displaying similar levels to the mock sample. But as seen with the previous time points, there is no activation of STAT3 with the FLAG-NSs F261P mutant as well as the MP12-ΔNSs virus. These data illustrate that mutations in NSs will nonetheless alter how the virus manipulates the host cell. While the mutations in NSs may not be directly affecting STAT3, it is causing a change in the cell environment in which the virus is not able to fully manipulate the host cell.
STAT1 and STAT3 translocation after RVFV infection

Based on the previous results, we wanted to determine the location of activated STAT1 and STAT3. STAT1 and STAT3 are normally stimulated by growth factors or cytokines, where they translocate to the nucleus and turn on the respective genes (19). Therefore, we wanted to visualize the distribution of STAT1 and STAT3 after RVFV infection. HSAECs were seeded on microscope slides and mock infected or infected with RVFV MP12 FLAG-NSs (MOI 3). Sixteen hours post infection, slides were fixed and STAT1 or STAT3 localization was analyzed by confocal fluorescent microscopy. Cells were stained for total STAT1 or STAT3, as well as viral proteins NSs or N. Mock-infected cells showed an even distribution of STAT1 and STAT3 throughout the nucleus and cytoplasm. Following infection with MP12, STAT1 appeared to localize more in the nucleus of infected cells (Figure 4A). Not only is STAT1 localized more to the nucleus in infected cells, but located in the nucleus of the neighboring uninfected cells as well. Similarly, STAT3 also appeared to be more nuclear (Figure 3D), although not as dramatic as the STAT1 nuclear localization. STAT3 was similar to STAT1 in that the cells that lacked NSs still showed translocation of STAT3 in neighboring, uninfected cells. This could be the result of released cytokines in response to infection, or perhaps that viral proteins are just below the limit of detection in those particular cells. In summary, these data show that STAT1 undergoes a shift in localization, moving from the cytoplasm to the nucleus after RVFV infection. The translocation of STAT3 was not as prominent, suggesting that STAT3 may be residing in the cytoplasm of infected cells.
Effects of Stattic on RVFV replication

Since the previous results indicated an increase in STAT1 and STAT3 phosphorylation following RVFV infection, we wanted to investigate the inhibition of STAT3 phosphorylation and what consequences it would have on RVFV replication. The inhibitor chosen was Stattic, a small molecule inhibitor that has been shown to selectively inhibit the function of the STAT3 SH2 domain in which upstream kinases dock (29). This in turn inhibits the activation, dimerization, and translocation of STAT3 (29). In order to establish a working, non-toxic concentration of the inhibitor, a cell viability assay was done with serial diluted concentrations of the inhibitor. The inhibitor was added to HSAECs and left for 48 hours to determine the optimal concentration. The cells were lysed and analyzed using Promega’s CellTiter Glo Luminescent Cell Viability Assay, in which it measures viability by a luminescent signal that is generated proportional to the amount of ATP present. After analyzing the luminescent signals, it revealed that about 75% of the cells were viable following treatment with 10 \( \mu \text{M} \) of Stattic (Figure 5A). At 5 \( \mu \text{M} \), 100% viability was regained and therefore this was the concentration that was used for the remainder of this study.

Next, in order to analyze the effects of STAT3 inhibition on RVFV replication, a plaque assay was performed. HSAECs were pre-treated with 5 \( \mu \text{M} \) of Stattic or 0.1% DMSO for two hours, infected with MP12 (MOI 3), then post-treated with the same treated media until the time of collection. Supernatants were collected at 24 hours post infection and analyzed by plaque assay to determine viral titers. There was a one log reduction in viral titers in cells treated with Stattic when compared to the DMSO control.
(Figure 5B). Additional time points were looked at to see if Stattic had a greater effect at different time points other than 24 hours post-infection. The same experiment was done in HSAECs, and supernatants were collected at 8, 16, and 24 hours post-infection. At all time points tested, there was a reduction in viral titers (Figure 5C). Taken together, this data demonstrates that RVFV replication is reduced when cells are pre- and post-treated with 5 μM of Stattic in an interferon-competent cell line.

Next, we tested how Stattic would alter RVFV in interferon-incompetent Vero cells. In order to establish a working, non-toxic concentration of the inhibitor in Vero cells, a cell viability assay was once again. The inhibitor was added to Veros and left for 48 hours to determine the optimal concentration. The cell viability assay revealed that the optimal concentration was the same; therefore a concentration of 5 μM was used on Vero cells (Figure 6A). To analyze the effects of STAT3 inhibition on RVFV replication in Vero cells, a plaque assay was performed. Vero cells were pre-treated with 5 μM of Stattic or 0.1% DMSO for two hours, infected with MP12, then post-treated with the same treated media until the time of collection. Supernatants were collected at 24 hours post infection and analyzed by plaque assay to determine viral titers. Stattic decreased viral titers by two logs at 24 hours post-infection (Figure 6B). This decrease is more significant that the decrease seen with the HSAECs, suggesting another possible link to interferon. We also confirmed by western blot that the inhibitor was decreasing levels of phosphorylated STAT3 (Figure 6C). There was decreased level of STAT3 phosphorylation at 8, 16, and 24 hours post-infection when treated with Stattic. Interestingly, there was also a reduction in STAT1 phosphorylation at 16 and 24 hours.
post-infection. This decrease in phosphorylation could be due to a reduction in virus, since Stattic reduces viral titers, or because of an off-target effect that is common amongst molecular inhibitors.

**RVFV Replication and Expression in STAT3-deficient Cells**

In addition to molecular inhibition of STAT3, we also wanted to investigate if RVFV was altered in STAT3-deficient cells. Therefore, STAT3 -/- mouse embryonic fibroblasts (MEFs) were used alongside wild-type (WT) MEFs to compare replication kinetics. WT and STAT3 -/- MEFs were infected with MP12 at MOI of 0.3 and 3.0 for one hour, washed with PBS, and then complete media was added back on to the cells. Supernatants and protein lysates were collected at the indicated time points. First, the protein lysates were separated out by SDS-PAGE and analyzed by western blot. As a control, total STAT3 levels were analyzed in each cell type and indicated that there was no STAT3 present in the null cell line (Figure 7A, B). To analyze the replication rate in each cell type, we probed for RVFV nucleoprotein. RVFV nucleoprotein was barely detectable by western blot at 16 hours post infection in wild type cells, but started to increase by 24 hours post-infection. Similarly, the STAT3 -/- MEFs had low levels of nucleoprotein up until 16 hours post-infection, with an increase in nucleoprotein levels at 24 hours post-infection.

Next, the supernatants from wild type and STAT3-deficient cells were analyzed by plaque assay to assess the effect on RVFV replication. In respect to both MOIs, viral titers were similar between cell types at 4 hours. When cells were infected with a MOI of 0.3, viral replication was slightly increased in STAT3 -/- MEFs at 8 and 16 hours post
infection compared to the wild type cells (Figure 8A, B). The STAT3-deficient cells had a slightly decreased viral titer compared to the wild type at 24 hours post infection. Similarly at a MOI of 3.0, viral titers in STAT3 -/- MEFs were slightly increased at 16 and 24 hours post infection over the wild type. In conclusion, these data revealed that a lack of STAT3 did not have an inhibitory effect on RVFV growth. In contrast, a lack of STAT3 seemed to provide a partial benefit to the virus which may be due to interruptions in cytokine signaling via the JAK-STAT pathway.

Finally, we wanted to investigate STAT3 and its role in cell survival, particularly during RVFV infection. STAT3 target genes are involved in many processes including immunity, survival, and proliferation. In this experiment, wild type and STAT3 -/- MEFs were mock-infected or infected with wild type MP12 at MOI of 0.3 and 3.0. Cell survival was analyzed by CellTiter-Glo at 24, 48, and 72 hours post infection to see the effects of STAT3 on cell survival. At 24 hours post-infection, cells infected at MOI 0.3 had similar levels of cell survival (Figure 9A). When compared to the wild type at 48 and 72 hours post-infection, STAT3 -/- MEFs had an increase in RVFV-induced cell death. The same was seen with MOI 3.0, although there was already a difference in cell survival at 24 hours post-infection with the higher MOI (Figure 9B). These data indicate that STAT3 plays a pro-survival role during RVFV infection, and that a lack of STAT3 increases RVFV-induced cell death.
STAT1 and STAT3 activation in an interferon-incompetent cell line following RVFV infection. A) Vero cells were mock-infected or infected with MP12 or MP12-ΔNSs at MOI 3.0 for one hour. Cell lysates were collected at 8, 16, and 24 hours post-infection and analyzed by western blot. Membranes were probed for p-STAT1 (Y701), p-STAT3 (Y705), RVFV nucleoprotein, and β-actin as a loading control. B) Vero cells were mock-treated or treated with 500 U/mL of IFN-β. Cell lysates were collected at various time points and analyzed by western blot. Membranes were probed for p-STAT1 (Y701), p-STAT3 (Y705), and β-actin as a loading control.
Figure 2

**STAT1 and STAT3 activation in an interferon-competent cell line following RVFV infection.** A) HSAECs were mock-infected or infected with MP12 or MP12-ΔNSs at MOI 3.0 for one hour. Cell lysates were collected at 8, 16, and 24 hours post-infection and analyzed by western blot. Membranes were probed for p-STAT1 (Y701), p-STAT3 (Y705), RVFV nucleoprotein, and β-actin as a loading control. B) HSAECs were mock-infected, infected with MP12 or MP12-ΔNSs, or treated with 500 U/mL of IFN-β. Cell lysates were collected at early time points and analyzed by western blot. Membranes were probed for p-STAT1 (Y701), p-STAT3 (Y705), and β-actin as a loading control.
**Figure 3**

**Effects of NSs mutations on STAT3 activation.** HSAECs were mock-infected or infected with wild type MP12, MP12-NSs-3xFLAG, MP12-FLAG-NSs F261P, MP12-V5-NSs, MP12-V5-NSs R173A, or MP12-ΔNSs for one hour. Cell lysates were collected at 8, 16, and 24 hours post-infection and analyzed by western blot. Membranes were probed for p-STAT3 (Y705), RVFV nucleoprotein, or β-actin as a loading control.
Figure 4
STAT1 and STAT3 localization following RVFV infection. HSAECs were mock-infected or infected with MP12-FLAG-NSs for one hour. Cells were fixed at 16 hours post-infection and stained for total STAT1 (A) or total STAT3 (B). As a positive control, cells were also stained for RVFV nucleoprotein or NSs. DAPI stain was used to visualize nuclei. Representative pictures from each sample are shown.
Figure 5
Effects of Static on RVFV in interferon-incompetent cells. A) Serial diluted inhibitor was added to Vero cells and left for 48 hours. At 48 hours post-treatment, cells were lysed and analyzed for viability using Promega’s CellTiter Glo Assay. Viability was normalized to the DMSO solvent control. B) Vero cells were pre-treated with 0.1% DMSO or 5 µM of Static for two hours, and then infected with MP12 for one hour. Cells were washed with PBS, and then treated media was added back. Supernatants were collected 24 hours post-infection and analyzed by plaque assay. C) Same procedure was performed for panel B, but supernatants were collected 8, 16, and 24 hours post-infection. D) Vero cells were pre-treated with 0.1% DMSO or 5 µM of Static for two hours, and then infected with MP12 for one hour. Cells were washed with PBS, and then treated media was added back. Cell lysates were collected at 8, 16, and 24 hours post-infection and analyzed by western blot. Membranes were probed for p-STAT1 (Y701), p-STAT3 (Y705), and β-actin as a loading control.
Figure 6

Effects of Stattic on RVFV in interferon-competent cells. A) Serial diluted inhibitor was added to HSAECs and left for 48 hours. At 48 hours post-treatment, cells were lysed and analyzed for viability using Promega’s CellTiter Glo Assay. Viability was normalized to the DMSO solvent control. B) HSAECs were pre-treated with 0.1% DMSO or 5 µM of Stattic for two hours, and then infected with MP12 for one hour. Cells were washed with PBS, and then treated media was added back. Supernatants were collected 24 hours post-infection and analyzed by plaque assay. C) The same procedure was performed as in panel B, but supernatants were collected at 8, 16, and 24 hours post-infection.
Figure 7

**Effects of STAT3-deficient cells on RVFV.** WT and STAT3-/- MEFs were mock-infected or infected with MP12 for one hour at MOI 0.3 (A) or MOI 3.0 (B). Cell lysates were collected at 4, 8, 16, and 24 hours post-infection and analyzed by western blot. Membranes were probed for total STAT3, RVFV nucleoprotein, or β-actin as a loading control.
STAT3-deficient cells do not alter RVFV replication. WT and STAT3-/- MEFs were mock-infected or infected with MP12 for one hour at MOI 0.3 (A) or 3.0 (B). Supernatants were collected at 4, 8, 16, and 24 hours post-infection and analyzed by plaque assay.
Figure 9
Lack of STAT3 increases RVFV-induced cell death. WT and STAT3-/- MEFs were mock-infected or infected with MP12 for one hour at MOI 0.3 (A) or 3.0 (B). At the indicated time points, cells were analyzed for survival using Promega’s CellTiter-Glo Assay. Cells were normalized to their corresponding mock-infected sample. C) WT and STAT3-/- MEFs were mock-infected or infected in the same manner, lysates were collected and western blot analysis was performed. Membranes were probed for cleaved Caspase-3 and β-actin as a loading control.
DISCUSSION

RVFV and other viruses have elegantly evolved the ability to evade the host immune response, as well as employ host cellular machinery to carry out its life cycle. It is important to understand how RVFV exploits the host during infection in order to further understand the life cycle and develop appropriate therapeutics. STATs are involved in cellular responses such as immunity, survival, development, and proliferation. As described earlier, STAT1 and STAT3 both have numerous functions in the cell and could be exploited for any number of reasons in order to provide a more ideal environment for replication.

Popova et al. showed in their work that phosphorylation of STAT1 and STAT3 are increased following infection with RVFV, but the mechanism was never investigated further. In this study, it is confirmed that both STAT1 and STAT3 are increased after infecting with the live-attenuated MP12 strain. Not only is phosphorylation increased with both proteins but this activation does not occur with the RVFV strain containing a deletion of NSs (MP12-ΔNSs), suggesting that NSs is required for STAT1 and STAT3 activation. These results were also confirmed in a fully virulent strain of RVFV ZH548 (data not shown). The activation of STAT1 is interesting in that most viruses prevent the phosphorylation or promote the degradation of STAT1 in order to evade the host immune response (30, 32, 33). In contrast, most of the literature showed that STAT3 was
activated by particular viral infections in order to promote cell survival, and in the case of HBV and HCV, proliferation and transformation (34, 35). Although for HCV, this seems to be a controversial topic since it was reported that the core protein was the causative agent of STAT3 activation, while another demonstrated STAT3 activation as a result of DNA damage and reactive oxygen species (35, 37).

In the case of RVFV, STAT1 and STAT3 activation was most evident at 16 hours post-infection. After checking later time points, we wanted to demonstrate that the phosphorylation of either protein was not as a result of a general interferon response to a viral infection. Therefore, we stimulated cells with IFN-β to see how quickly the signaling cascade was activated. It showed that activation of STAT1 and STAT3 as a result of IFN-β stimulation occurred very quickly, with the amount of phosphorylation tapering off by 2 hours post-treatment. Interestingly, there was a modest increase in STAT3 phosphorylation at early time points with the MP12-ΔNSs virus, indicating an initial interferon response to viral infection. This initial phosphorylation fades by 4 hours post-infection. In order to see if this activation could occur independent of interferon, we employed Vero cells that are interferon-incompetent. STAT1 and STAT3 phosphorylation was also seen in Vero cells, indicating that this activation happens in both interferon-competent and interferon-incompetent cell lines.

Mutations in the NSs protein alter STAT1 and STAT3 phosphorylation, showing a decreased rate of activation with the viruses containing NSs mutations, particularly at 16 and 24 hours post-infection. NSs can localize to the nucleus with the FLAG-NSs F261P mutant, but the characteristic large filaments do not form. This mutant is also
unable to degrade PKR or p62, which occurs with wild type MP12 infection. This may indicate that proper filament formation may be necessary in order to induce STAT3 phosphorylation, or that PKR or p62 could be involved in the activation of STAT3. The same case could be stated for the V5-NSs R173A mutant, since it forms irregular filaments in the nucleus and it is unable to degrade PKR (40).

Given that proper filament formation may have an influence on STAT3 phosphorylation, we needed to see if STAT1 and STAT3 were translocating to the nucleus following RVFV infection. Confocal microscopy clearly demonstrates the nuclear localization of STAT1 following infection with MP12. This indicates that it could be dimerizing and making its way to the nucleus to induce transcription of response genes, including ones involved in cell survival. Following RVFV infection, there was only a modest localization of STAT3 in the nucleus of cells. This suggests a possible cytoplasmic role of phosphorylated STAT3. Consistent with only a partial nuclear localization of STAT3, our group has demonstrated that one target of STAT3, SOCS3, the negative regulator of STAT3, is down-regulated during RVFV infection (unpublished data). These data are consistent with the general transcription repression induced by NSs. We speculate that at earlier time points, the interferon response is responding as usual with the MP12-ΔNSs virus and STAT3 phosphorylation eventually is stopped by its regulator, SOCS3. In contrast in wild type MP12, STAT3 is not activated until later time points which is dependent on NSs, and the subsequent down-regulation of SOCS3 maintains the consistent phosphorylation of STAT3. Future experiments will determine
if STAT3 and STAT1 transcriptional targets are generally down-regulated or if only selective targets are being activated.

Stattic, a small molecular STAT3 inhibitor, has been investigated as potential therapeutics for cancer and human tumors. Here, we employ the inhibitor to analyze the effects on RVFV. Stattic reduced MP12 viral titers by about two logs by 24 hours post-infection. This decrease was not as drastic with HSAECs, only decreasing viral titers about one log, further suggesting a link to interferon. Even though Stattic reduced MP12 viral titers, these data were not mirrored in the STAT3 -/- MEFs which contain a complete knockout of STAT3. MP12 viral titers were compared in wild type and STAT3 -/- MEFs and demonstrated there was no difference in the viral kinetics, indicating that we may be seeing some off-target effects with Stattic. Alternatively, the differences observed may be due to species differences, as HSAEC and Vero cells are human and monkey respectively, as compared to mouse cells. However, a pro-survival role of STAT3 was evident by a decrease in cell survival following MP12 infection. Compared to the wild type MEFs, the STAT3-/- MEFs had an increase in RVFV-induced cell death. Studies have shown that STAT3 positively regulates expression of anti-apoptotic and anti-oxidant genes (41). There has been a recent emergence in the role of STAT3 in mitochondria, following the study that confirmed STAT3 localization to the mitochondria (42–44). Mitochondria from STAT3 -/- cells are more susceptible to elevated calcium levels, which causes opening of the mitochondria permeability transition pore. Therefore, our next step is to investigate STAT3 and its cell survival roles during RVFV infection, possibly stemming from the involvement in the mitochondria.
Assuming that STAT1 and STAT3 phosphorylation are dependent on NSs, interaction of NSs with STAT1 or STAT3 was investigated in cytoplasmic fractions (data not shown). Although there was no evidence of interaction, NSs could be interacting with STAT1 or STAT3 elsewhere. Further investigation will be needed in order to rule out any direct interaction with STAT1 or STAT3 in the nucleus or in enriched mitochondrial fractions. In the event that there is no interaction with NSs, mass spectrometry data has revealed a shift in binding partners following MP12 infection. Mock samples showed STAT3 interaction with multiple cytoskeletal proteins, while MP12-infected samples showed interaction with proteins involved in the mitochondria and endoplasmic reticulum (data not shown). These provide some leads to investigate possible STAT3 binding partners, and what role they could be playing during infection.

Overall, these conclusions point to a role in STAT1 and STAT3 during RVFV infection. Following RVFV infection, both STAT1 and STAT3 are activated on their conserved tyrosine residues. Mutations in NSs can impair STAT1 and STAT3 activation, indicating that proper filament formation may be required for activation. The lack of STAT3 results in an increase in RVFV-induced cell death, implying a pro-survival role during infection. Further research will need to be done to uncover the mechanism by which RVFV manipulates STAT1 and STAT3 in order to maintain an ideal environment for amplification.
REFERENCES


BIOGRAPHY

Chelsea Pinkham was born in Chesapeake, Virginia and graduated from First Colonial High School, Virginia Beach, Virginia, in 2008. She received her Bachelor of Science from George Mason University in 2012. After participating in the Aspiring Scientist Summer Internship Program, she pursued her Masters in Biology at George Mason University with a concentration in Microbiology and Infectious Diseases. Following graduation, she intends to stay at George Mason University to pursue her PhD in Biosciences.