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The Impact of the Non-Structural Small Segment Gene on Host Immune Responses Against Rift Valley Fever Virus

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IMACTS OF THE NON-STRUCTURAL SMALL SEGMENT GENE ON HOST IMMUNE RESPONSES AGAINST RIFT VALLEY FEVER VIRUS

By

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Dedication

To my parents, whose constant love and encouragement to help me achieve my goals has been unwavering every step of the way.

In loving memory of Sherelle Kawana Hill. We miss you, but I know my big sister is still inspiring me forward to be the best that I can

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IMACTS OF THE NON-STRUCTURAL SMALL SEGMENT GENE ON HOST IMMUNE RESPONSES AGAINST RIFT VALLEY FEVER VIRUS

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Rift Valley fever virus (RVFV) is responsible for large periodic outbreaks of enzootic hepatitis of ruminants. Inevitably, due to the close proximity and relationship between, the mosquito vector species, livestock, and humans, infection spreads to humans causing a range of mild to severe clinical conditions. The severe health and economic impacts RVFV exerts on endemic regions demands the urgent development of effective preventive and therapeutic interventions. To address the need for livestock and human vaccines, several live attenuated vaccine platforms have been proposed. Primarily the MP-12 strain which contains multiple amino acid mutations collectively contributing to attenuation, and Clone 13 which has a single large deletion in the S-segment non-structural gene (NSs). When used in vaccination of animals both vaccine constructs show promising results. However, extensive investigation on the fundamental differences in innate immune responses, which ultimately impact the quality, intensity and duration of host immune defenses has not been undertaken. Additionally, detailed analysis on the susceptibility and host responses of human innate immune cells, such as macrophages, has not been reported. To address the deficiencies in understanding host-virus interaction of RVFV vaccines and human innate immunity, I investigated the cellular responses of human primary macrophage cells to RVFV infections containing NSs-intact and NSs-deleted genotypes. The use of cDNA based transcriptional analysis allowed for detailed and specific identification of modulated gene expression induced by RVFV and the functions of NSs. Findings from these studies revealed that removal of NSs function lead to more dramatic host defense response that was reflected at the transcriptional and protein levels. Further bioinformatics analysis of the differentially expressed genes identified signaling pathways and biological functions which are relevant to antiviral processes, immune regulation, and pathogenesis. The experimental approach provided an excellent means of identifying host responses which were compromised by NSs activity. The major benefit is that these differences can be used for hypothesis generation to identify the critical host components modulated during RVFV infection which may assist or compromise the development of immunity. Specifically, the alteration of RIG-I signaling, cytokine production, and antigen presentation mechanisms by NSs may potentially be exploited as targets for rationally improve RVFV vaccines.

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List of Abbreviations

(r)	Recombinant
ANOVA	Analysis of Variance
CCL	Chemokine Ligand
CDC	Centers for Disease Control
cDNA	Complementary Deoxyribonucleic Acid
DC	Dentritic Cell
DIVA	Differentiation of Infected Versus Vaccinated
ELISA	Enzyme-linked Immunosorbency Assay
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
GM-CSF	Granulocyte Monocyte Colony Stimulating Factor
GO	Gene Ontology
hr	Hour
HPIV	Highly Pathogenic Influenza Virus
IFN	Interferon
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IPA	Ingenuity Pathway Analysis
IRES	Internal Ribosome Entry Sequence
IRF	Interferon Regulatory Factor
ISG	Interferon Stimulated Gene
LPS	Lipopolysaccharide
MDA-5	Melanoma Differentiation Antigen 5
MFI	Mean Fluorescent Intensity
MHC	Major Histocompatability Complex
ml	Milliliter
MOI	Multiplicity of Infection
MΦ	Macrophage
NSs	Non-Structural S Protein
PBMC	Peripheral Blood Monocytic Cell
PKR	Protein Kinase R
pi	Post Inoculation
PRR	Pattern Recognition Receptor
RIG-I	Retinoic Acid Inducible Gene I
RNA	Ribonucleic Acid
RVFV	Rift Valley fever virus
TCID50	50% Tissue Culture Infectious Dose
TF	Transcription Factor
TLR	Toll-like Receptor
TNFα	Tumor Necrosis Factor Alpha
ul	Microliter
USDA	United States Department of Agriculture
VSV	Vesicular Stomatitis Virus

Chapter I: Introduction

RIFT VALLEY FEVER VIRUS HISTORY

NATURAL HISTORY AND ECONOMIC SIGNIFICANCE

Zoonotic pathogens have caused more than 65% of infectious disease outbreaks in the past 60 years, and have caused more than $200 billion in economic losses worldwide over the past decade [1-2]. Furthermore, arboviral infections comprise an important segment of the emerging and reemerging diseases important to humans. These arboviral diseases may burden human health and economic activity with great severity. Furthermore, in the 21st century's fast paced and increasingly globalized world, infectious diseases do not recognize national boundaries, and have the potential to spread rapidly to previously unaffected areas, as demonstrated by the recent spread of West Nile, Chikungunya, Dengue, Yellow fever, and Rift Valley fever viruses [3-9].

Improving general public health and infection control capacities in tropical and developing countries is vital not only for the developing world, but also for the health and wellbeing of international travelers, deployed military personnel, and foreign aid and development workers. Additionally, the natural or intentional introduction of zoonotic agents onto virgin soil could have negative impacts on human health, economically important agricultural species, and would certainly complicate disease control efforts. It is therefore a necessity to strengthen diagnostic, vaccine, and therapeutic capacity against emerging diseases.

One of the major viral families contributing to potential emerging diseases of humans and economically important animals are members of the *Bunyaviridae* family. Together the bunyavirus members have a nearly worldwide distribution with over three hundred members divided into five genera, the Orthobunyavirus, Hantaviruses, Nairuvirus, Phlebovirus, and

Tospovirus. Among these virus family members are several pathogens with histories as important public health threats, which cause a variety of clinical conditions depending both on the viral and host species. This range of diseases includes acute febrile illness, hepatitis, encephalitis, respiratory disease, and hemorrhagic fever. In addition, the Tospovirus genus is exclusively a plant pathogen. The extensive number of viruses, unique life cycles, and diversity of the diseases caused within the bunyaviridae family makes understanding the mechanisms of pathogenesis a challenging but important endeavor for the scientific and medical communities.

One of the most important bunyavirus members in modern history to affect human and animal health is Rift Valley fever virus (RVFV). The acute enzootic hepatitis associated with RVFV causes a high rate of mortality in sheep and cattle resulting in millions in lost revenue [10-12]. Rift Valley fever virus is a member of the genus *Phlebovirus*, family *Bunyaviridae* [13-14]. The virus is composed of three genome segments; L (Large), M (Medium), and S (Small), of which the L and M segments are negative sense and the S segment is encoded in an ambisense orientation [15].

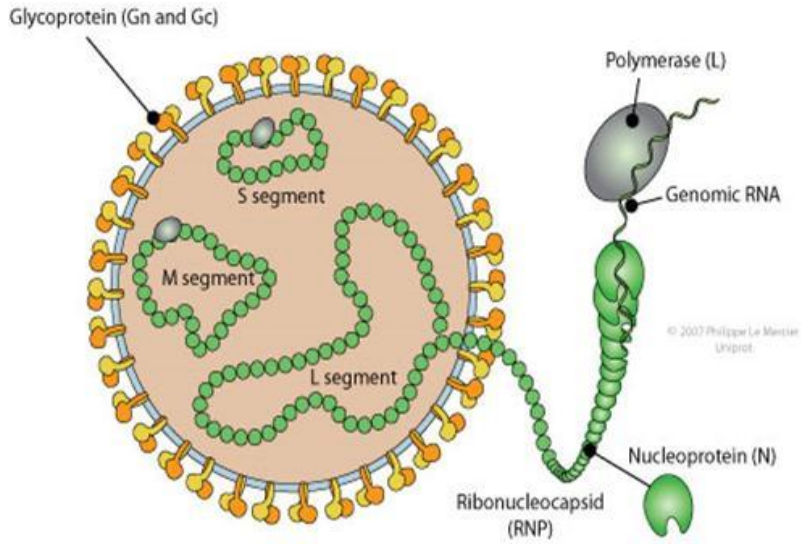


Figure 1.1: Genome structure of *Phleboviridae*. The characteristic tri-segmented genomic organization of Rift Valley fever virus. Viral RNA binding proteins (Polymerase and Nucleoprotein) in addition surface glycoproteins (Gn and Gc) are shown. [16-17]

Major outbreaks of Rift Valley fever have occurred beginning with the 1931 outbreak first identified by Daubney and Hudson as an outbreak of acute lethal hepatitis in lambs and ewes, accompanied by high abortion rates of pregnant ewes occurring within the Rift Valley region of Kenya, where the virus was first isolated and given the name Rift Valley fever [18-19]. Large outbreaks have occurred in Sub-Saharan Africa, Madagascar, and the Arabian Peninsula affecting both livestock and humans [2]. Outbreaks in Kenya from the early 1900's through the 1970's impacted thousands of sheep, with the largest outbreak occurring in 1950-1951 [20]. Major epizootic RVFV has subsequently been identified in the 1950's in South Africa and Egypt starting in the 1970's [21-26]. Areas around the Rift Valley region including Kenya, Tanzania, Uganda, and Sudan remain some of the primary areas for human and livestock exposure in recent history [27-32]. Rift Valley fever virus is now endemic throughout the majority of Africa and has most recently spread through most of the Arabian Peninsula after first introduced during outbreaks in Saudi Arabia and Yemen beginning in 2000 [9,32-35].

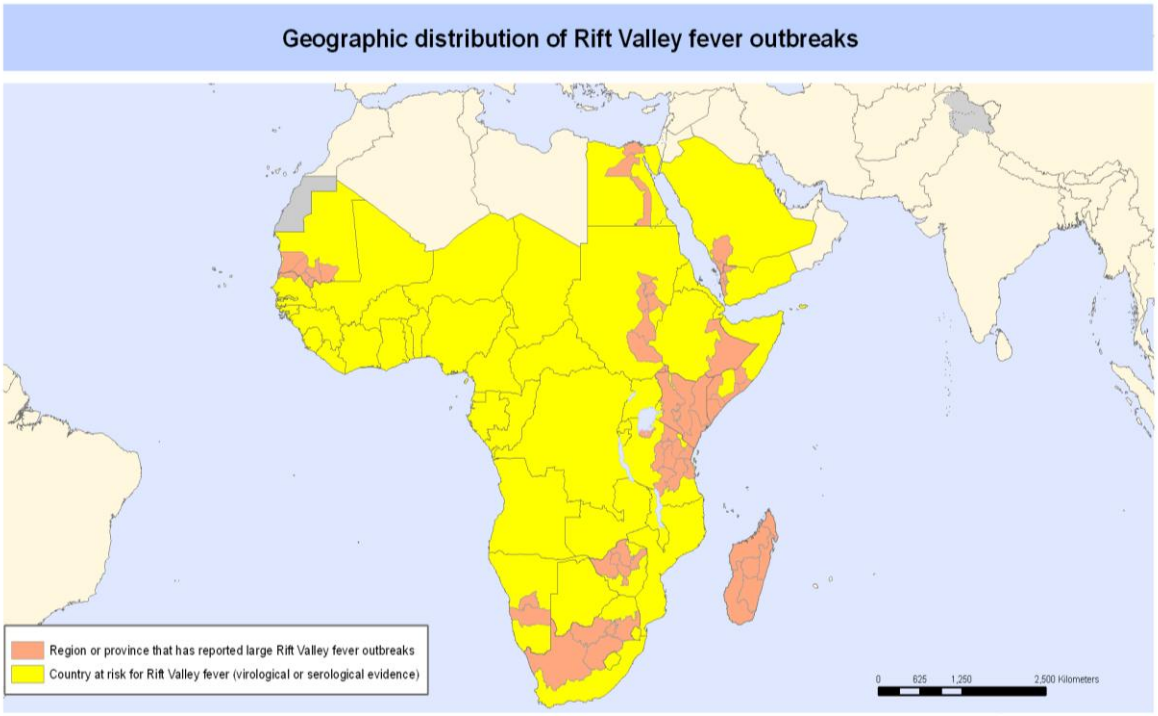


Figure 1.2: Geographic distribution of Rift Valley fever outbreaks. Distribution of RVFV on the African continent and Arabian Peninsula. Serological evidence of endemic (pink) and at risk (yellow) areas for RVFV circulation. [36]

Aedes and *Culex* mosquito *spp.* serve as the principle arthropod vectors during times of RVFV outbreaks [37-39]. During inter-epidemic periods, it is believed that infected female floodwater *Aedes* mosquitoes are capable of maintaining the virus population through trans-ovarial transmission into desiccation resistant eggs, which hatch during flooding to give rise to virus-infected larvae which start the next outbreak [40-42].

Humans may be infected with RVFV by either mosquito bite, contact with virus-laden body fluid during the slaughtering of infected animals, or aerosolized infection by intentional or accidental means [43-44]. Some of the earliest descriptions of Rift Valley fever in humans occurred as a result of accidental laboratory infection [45-46]. The majority of symptomatic human cases results in fever, myalgia, and malaise followed by recovery [47-48]. Of greater concern are the

severe symptoms characterized by encephalitis and hemorrhagic fever which occur in ~1% of confirmed cases [49-50]. Ocular complications are more common than encephalitis or hemorrhagic fever in humans, affecting approximately 20% of patients, and may lead to permanent vision impairment [51-54].

Infected sheep, goats, and cattle typically exhibit more severe clinical manifestations than their human counterparts [2]. High rates of severe and lethal hepatitis along with high abortion rates among pregnant livestock are characteristic of outbreaks [11-12,55]. It has been reported that zoonotic outbreaks of RVFV could cause up to 30% mortality rates in adult sheep, 90% mortality rates in lambs, and 100% abortion rates in infected pregnant ewes [56-57]. Therefore RVFV outbreaks are typically first identified in livestock due to the devastating disease burden to local sheep and cattle herds.

While RVFV outbreaks to date have only occurred in sub-Saharan Africa and the Arabian Peninsula, due to the wide-spread distribution of suitable vector mosquito species on many continents, there is the chance through accidental or intentional means that RVFV reach previously unexposed countries, including areas in the Americas and Europe [58-62].

The most devastating impacts on both public health and economy would occur from epidemics/epizootics of RVFV in previously unexposed (naïve) regions, and those developed and developing countries which rely heavily on intense ruminant based agricultural-industries. Due to the potential to cause severe disease in human and livestock and spread rapidly if introduced into new territories, RVFV is listed as a CDC and USDA Select Agent. Additionally RVFV is one of the "List A" pathogens, designated by the Office International des Epizooties (OIE) because of its capacity to negatively impact vital agricultural production and trade of animals or animal products at national and international levels. Based upon rates of West Nile

virus infection in humans during the initial outbreak, the estimated economic impact if RVFV spread to the United States could cost between one and two billion dollars [63].

VACCINE DEVELOPMENT EFFORTS

Because the high mortality rates in young animals and high abortion rates in RVFV infected sheep and cattle may strain already fragile food sources, protection of suceptable livestock is critical [64]. Vaccination of susceptible livestock species has been one of the primary means of preventing and limiting RVFV outbreaks; however, prior to widespread acceptance of any RVFV vaccination strategy, improvements in safety and efficacy beyond that of the current vaccination strategies used to protect susceptible animals during times of RVFV outbreaks are required. Additionally, improved understanding of the determining mechanisms guiding successful vaccination in humans, particularly the host-virus interaction among live attenuated RVFV vaccine candidates is necessary prior to routine administration to humans.

The Smithburn neurotropic strain is a live attenuated vaccine strain developed in South Africa for use as an agricultural vaccine for domestic ruminants [65]. Adverse complications as a result of vaccination with the Smithburn strain including unacceptable rates of abortion in cattle and goats, and less than optimal rates of successful vaccination, which stressed that alternative control measures are needed [66-67]. A formalin inactivated RVF vaccine (TSI-GSD-200) is available for use in laboratory workers and military personnel whom may be at particularly high risk for contracting RVFV. The high financial cost associated with the inactivated vaccine, and the requirement for booster administration of the vaccine before long term immunity is achieved [68] makes the formalin inactivated vaccine less desirable for widespread administration.

Vaccination studies aimed at protecting livestock against RVFV disease outbreaks have shown the Smithburn strain is relatively successful in generating IgG and protective responses in >60% of animals [66,69]. While the Smithburn strain has been used in Africa to vaccinate animals during times of RVFV outbreaks, currently there is neither a widely available vaccine for human use nor a standardized therapeutic regimen against RVFV available. These gaps in RVFV treatment and prevention capacity make the development of safe and effective vaccination strategies for humans and animals urgent priorities. Live, attenuated strains of RVFV (i.e., MP-12 and Clone 13) have been studied extensively as vaccine candidates. While these vaccines are capable of protecting animals from lethal challenge [70-72], thorough understanding of the exact molecular mechanics that governs the development of lasting protection as a result of vaccination with either strain of live attenuated RVFV is not yet fully understood.

The live attenuated RVFV strains were developed independently from two separate parental RVFV isolates collected during outbreaks in east Africa. Closely related parental strains ZH501 and ZH548 were obtained from patients treated at Zagazig hospital during the 1977 Egyptian outbreak[49,73]. Respectively, ZH501 was isolated from a patient displaying severe RVFV symptoms, while ZH548 was isolated from a patient with mild self-limiting symptoms.

The MP-12 vaccine candidate was established by serial passage of the ZH548 isolate in the presence of 5-fluro-uracil, which introduced multiple attenuating mutations in each of the three RVFV genome segments [74]. In total, 25 nucleotide changes were introduced, with at least one mutation on each of the three genome segments[75]. The nucleotide changes resulted in eleven total amino acid changes in MP-12 from ZH548 virus. The authentic Clone 13 virus was derived from the parental 74HB59 strain of RVFV which was isolated from a nonfatal human case in the Central African Republic, and contained a heterogeneous population of RVFV mutants[76]. Plaque

purification revealed that one of the mutants could not be detected by antibody against NSs. This mutant was found to have a 549 nucleotide deletion in the NSs gene [76].

The MP-12 and Clone 13 live attenuated RVFV strains have been studied as possible vaccines to protect livestock and potentially humans from RVFV infection. Both have been found to be avirulent in immune competent adult animals[77-78] . Additionally both strains stimulate the production of neutralizing antibody responses in livestock[72,79], and are protective against lethal RVFV challenge in sheep[71,80]. While MP-12 has been successfully demonstrated to generate antibody responses in human volunteers[81], Clone 13 has not yet been administered to human subjects.

While the safety and efficacy of both of these promising live attenuated vaccine strains has been studied in mice and large animal models for the production of neutralizing antibody and protection against lethal infection, there is still a limited understanding regarding the similarities and differences between how each strain interacts with the innate immune system and achieves immunogenicity. In particular, selection of the live attenuated vaccine candidate which delivers the most optimal safety and efficacy profile for humans is highly desired. As part of that selection process determining how the RVFV NSs protein impacts the education of innate immune responses required for producing antibody responses in humans is of great significance. Ideally, characterization of molecular signatures or "biomarkers" which are indicative to the context of the necessary signaling events required to generate successful immunization is desired. Similar characterization attempts have been undertaken for other pathogens, where early gene expression patterns were used to predict vaccination success [82-83]. Having an efficient and effective means of screening vaccine candidates based on in vitro and in silico data could greatly reduce the time and cost associated with multiple animal experiments.

Additionally it has been suggested that a doubly mutated live attenuated virus which contains both the attenuating amino acid changes of MP-12 and the Clone 13 like deletion of NSs might have improved safety and immunogenicity characteristics when compared to other possible vaccine candidates[84-85]. Comparing the host-virus interactions between human cells and the various live attenuated vaccine strategies is a critical step in advancing vaccine efforts aimed for human use.

Though comparisons between NSs intact and NSs impaired RVFV can be made using authentic MP-12 and Clone 13 strains, the naturally occurring differences in the L,M, and S segment genetic sequences limits the ability to indiscriminately conclude that all variations in the host-virus interaction are a result of NSs functions alone. Fortunately, through the use of reverse genetics it is possible to construct recombinant (r) NSs-intact, rMP-12 and NSs-deleted, rMP-12 C13-type viruses which share genetic identity at the L and M segment genomes, yet differ in sequence at the S segment (Figure 1.3) [86]. The ability to simultaneously utilize NSs-intact and NSs-deleted viruses of otherwise identical genetic background in comparison of macrophage host responses allows for specific detection of differential responses generated as a result of NSs mediated alteration of macrophage functions. These differential responses could not only provide a unique insight into the molecular properties modulated (i.e., activated or inhibited) by the presence or absence of RVFV NSs protein, but also provide novel cellular targets for countering the pathogenic effects of RVFV NSs.

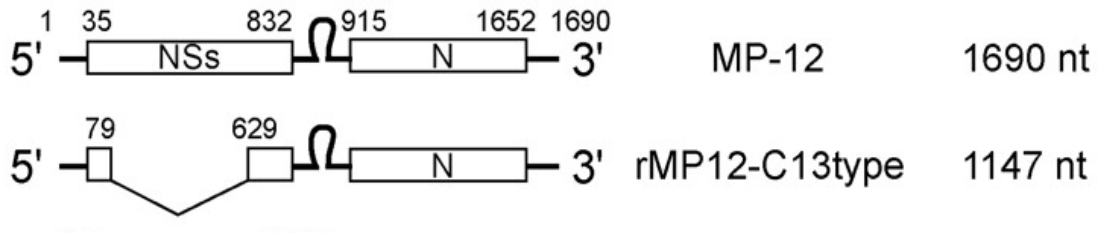


Figure 1.3: Diagram of NSs-intact and NSs-deletion S segment. Diagrams show MP-12 based RVFV S segment organization of nucleocapsid (N) and (NSs) genes. Lower diagram shows the large 69% truncation between nucleotides 78 and 629 of rMP12-C13type NSs that removes NSs activity [86].

GAPS IN KNOWLEDGE

It has been well-established that the nature of the host innate immune response helps shape the final outcome of viral infection or the success of vaccination [87-91]. Early host defenses contribute susceptibility or resistance to infection by directly restricting the ability of viruses to replicate within the permissive target cells, and in large part by regulating the onset of not only the innate immune responses but also the adaptive immune responses generated later by both T and B lymphocytes [82]. As such, the generation of host antiviral defenses mediated through the classical IFN-α/β-related signaling pathway has been recognized as a key step toward the generation of effective host antiviral responses [92].

Signaling through IFN-α/β also regulates many cellular activities relevant to innate and adaptive immunity [92-93]. This is highlighted by the implication of over 300 so called interferon stimulated genes (ISGs) whose expression is regulated by IFN-α/β [94]. Thus, to establish their infection, many viruses have evolved various strategies to evade IFN-α/β-related antiviral signaling pathways, including RVFV [95-97]. Additionally, the critical roles of several ISGs (e.g.,

Mx proteins and PKR) in the host defense against RVFV infection have been demonstrated [98-100]. While IFN-α/β signaling plays a key role in the host defense against RVFV [101-102], other possible early signaling molecules and cascades that might be involved to optimize the protection elicited by vaccination with live attenuated strains of RVFV have been largely neglected. Thus, it is critical to investigate how the vaccine strains of RVFVs interact with the host innate immune response as a first step towards better understanding and the protective immune responses elicited against either rMP-12 or Clone13-type attenuated RVFV.

Increased understanding of innate immunity's role in RVFV infection will support the scientific understanding of RVFV pathogenesis, and aid the eventual development of future therapeutic and vaccine strategies. Studies focused on delineating the impact of RVFV's major virulence factor, the s-segment nonstructural protein (NSs), on the innate immune response may further improve the rational design of preventive and therapeutic strategies in the future. Specifically, knowledge of the contribution NSs has on the functions of immune cells can potentially be exploited to direct improvements in RVFV vaccines to enhance immunogenicity, reduce pathological risks, and even promote the establishment of effective vaccines with the potential to allow for differentiation of infected versus vaccinated animals (DIVA). These improved vaccines will be particularly important for usage to vaccinate immunocompromised, pregnant, and elderly individuals who may otherwise be at higher risk of RVFV infection and vaccine complications as well. Thus, fully understanding the molecular impact of RVFV NSs protein on the induction and maintenance of the host innate antiviral responses may eventually lead to identifying better host factors for immune manipulation to thwart unfavorable host response against RVFV.

MACROPHAGE PARTICIPATION IN INFECTION AND IMMUNITY

MΦ are intrinsically dedicated immune cells responsible for producing a wide array of inflammatory mediators as part of the response to viral infections. MΦ carry out diverse and important roles in clearing infection, resolving inflammation, and tissue damage that may result from virus induced injury [103]. These processes may be beneficial or detrimental to the final outcome of infection or development of immunity depending on their context, intensity, and duration. Given the multifaceted activities of this cell population and their ability to reside within various organs including the initial and late stage sites of infection, it is highly important to understand the host-virus interaction dynamics between MΦ cells and RVFV.

The role of leukocytes, particularly macrophages (MΦ) and dendritic cells (DC), in contributing to the pathologic and/or protective mechanisms in response to RVFV infection has not been fully investigated. Their contribution as guardians against invading pathogens and potentially harmful foreign material is well established [104-107]. Though critical for host defense, macrophages may also be exploited by viruses for reproduction, concealment, or dissemination. This is well demonstrated by the ability of several viruses to infect macrophages, such as HIV, Dengue, Avian Influenza, and Punta Toro virus [108-112].

While a major site of RVFV infection in vivo occurs within the hepatocytes of the liver, RVFV displays a broad cellular tropism both in vivo and in vitro, including the ability to infect lymphoid cells [113-115]. Furthermore there is a high degree of variation in the clinical symptoms experienced by infected human patients. The multi-organ distribution of RVFV and variation in clinical symptoms experienced by different patients suggest that host innate immunity is an important determinant factor for susceptibility or resistance to RVFV. As resident and

circulating MΦ cells are present in nearly all organs which RVFV is present, it is possible that MΦ cells play a significant role in tissue pathology or protection.

NSs as the major RVFV virulence factor

The RVFV small-segment non-structural protein (NSs), is the major single virulence factor necessary for RVFV pathogenesis [78]. NSs is not an essential gene for viral replication [86,116]; however, NSs function has been shown to maximize the replication efficiency of RVFV. This is particularly the case in IFN-competent cell types, by acting as a potent antagonist of the interferon α/β host defense response pathway [76-77,86]. Additionally the removal of NSs completely abolishes virulence in young and adult animals [72]. However it is clear that mutations in other gene segments, i.e., the L and M segments of MP-12 also contribute to attenuation of RVFV [75,117], thereby demonstrating that virulence of pathogenic RVFV strains is under the control of multiple genomic segments [75,117].

Previously identified functions of NSs include obstruction of the interferon α/β system by binding the IFN-β promoter, promoting the post-translational degradation of protein kinase R (PKR), and inhibition of host transcriptional machinery by binding to the general transcription factor II H [77,118-121]. These functions of NSs are focused on preventing host cells from establishing strong host antiviral responses, thereby allowing RVFV to fully utilize host resources for establishing infection with enough vigor to cause pathology. Specifically, the inhibition of host type-I interferon and subsequent limitation of antiviral interferon stimulated gene production has been recognized as a major function of NSs contribution to RVFV virulence [77,119]. It remains possible however that NSs or other RVFV proteins may impact host factors important for RVFV virulence and immune responses.

The previous studies defining NSs function, while highly beneficial to understanding RVFV pathogenesis were investigated largely, if not exclusively, performed using parenchymal cells (i.e., hepatocytes, or cell lines i.e.,Vero, 293T cells, and MRC-5) cells which may not be as representative of the cell types which first encounter RVFV at the initial sites of infection when considering the various routes of natural infection. Though these cell types may mimic some aspects of in vivo immune responses, the majority of them are not specialized to act major players in antiviral defense or stimulators of adaptive immunity.

Dedicated classical immune cells such as MΦ and dendritic cells are abundant at the sites of natural RVFV infection (i.e., skin and airway) and highly specialized to utilize a highly diverse spectrum of host defense genes and signaling pathways. MΦ exert the intrinsic abilities to migrate within the circulatory and lymphatic system, target foreign pathogens for destruction, and activate T and B cells. As such, the analysis of MΦ responses to RVFV provides a unique opportunity to gain novel insight into the activity of previously unexplored innate immune mechanisms which occur after exposure to RVFV. Additionally while studies continue to improve scientific understanding on the mechanisms that NSs employs to achieve immune evasion, to date there has not been a comprehensive study to provide a global picture of molecular and cellular impacted by RVFV NSs protein. Both the mutagen attenuated strain of RVFV (MP-12) and the naturally attenuated RVFV, Clone 13 (C13) are highly attenuated in mice and sheep [71-72,76,122], however the L and M segments of these two viruses are not identical, as previously mentioned (figure 1.3) [77,117].

One of the most versatile and high throughput methods for identifying and quantifying the global molecular factors present during various physiologic states is cDNA microarray technology and use of associated bioinformatics analysis tools. By using cDNA microarray

technologies, examination of the complete transcriptional level responses to virus exposure can be achieved. Transcriptional profiling allows a contextually expansive identification and quantification of genes and signaling pathways relevant to the current and future biological activities of cells at the molecular level to be derived. From this transcriptional analysis, hypothesis generation of the protective and/or pathologic gene expression, commitment of molecular resources, and direction of cellular activities can be projected. This technique has been used considerably to fill gaps in knowledge on the mechanisms of viral pathogenesis for a number of pathogens and their associated diseases [123-126].

The scientific community has begun to address the gaps in knowledge surrounding the temporal and contextual disposition of the innate immune responses to many viruses, including arthropod borne viruses and specifically RVFV. To close these gaps, information surrounding the host-virus interaction's contribution to RVFV pathogenesis needs to be ascertained. A basic understanding of the permissiveness of key innate immune cells populations to RVFV, and a more complete understanding of the array of intracellular antiviral mechanisms whose expression or function is altered by NSs mediated antagonism is vital for understanding the factors required to limit replication of RVFV. Additionally, improved clarity of the influence NSs expression has on modulating the intrinsic host defenses and immunoregulatory signaling capabilities of innate immune cells, specifically production of surface expressed and secreted immune signaling proteins necessary for recruiting additional immune cells and directing adaptive immune responses, is needed to advance vaccine and therapeutic development efforts.

The overall purpose of the research is to comprehensively characterize a more complete profile of the consequences RVFV NSs has on altering the host immune system, and to do so with use of a pathologically and immunologically relevant but previously overlooked cell type

i.e., macrophages. This effort will increase scientific understanding of the host-virus interaction during RVFV infection, and ultimately contribute to the improved ability to rationally design novel therapeutic approaches against RVFV. **The central hypothesis is that human macrophage cells infected with NSs-deleted rMP-12 RVFV display a differential profile of innate immune responses compared to NSs-intact RVFV.** This hypothesis is based on the previously identified functions of NSs, most critically as a potent antagonist of host IFN-α/β-mediated antiviral responses, and inhibitor of general host transcription. Novel information pertaining to the role of NSs in altering diverse host defense mechanisms can be gained by observing differential responses to RVFV in macrophages. Macrophages and other "classical innate immune cells" may possess distinctive arrays of intrinsic innate immune functions (i.e., phagocytosis, antigen presentation, and the production of inflammatory mediators) which are not as directly discernable in epithelial cells. This study is of high importance for advancing RVFV research and vaccine development, as the ability of immune cells to call upon dynamic and unrestricted range of host defense factors is critical for developing quality protective responses.

Chapter II: Rift Valley fever virus Modulation of Macrophage Function

BACKGROUND AND RATIONALE

The severity of disease course in humans after exposure to RVFV may vary. Some individuals will remain asymptomatic while those patients that become symptomatic may experience a range of clinical manifestations, from flu-like self limited to severe or even fatal diseases. These various outcomes of infection are based upon both host and viral factors. These factors include the immune competency of the host, the virulence of the strain, and the dose.

As part of immunological basis for disease severity, the innate immune responses exerted by epithelial cells, and rapidly responding resident and infiltrating immune cells, have tremendous potential to impact the final outcome of disease severity. Resident and infiltrating immune cells, including MΦ and DC that respond to infection at the initial inoculation sites, may migrate into the lymphatic system to stimulate long term protective immune responses from acquired immunity. These migrating immune cells may also be used by viruses as vehicles to facilitate dissemination throughout the body. Because these early immune responses are critical to disease severity, understanding how RVFV virulence mechanisms may modulate human MΦ functions is an important aim of RVFV research and vaccine development.

Understanding how RVFV interacts with cellular constituents of the host innate immune response is key to understanding RVFV pathogenesis. This information is important to guide the development of safe and effective strategies against RVFV. Specifically, clarification of how the NSs protein of RVFV influences the ability of host cells to induce defense responses necessary to limit viral pathogenesis is an extremely important aspect of RVFV research.

As most RVFV-laden mosquito saliva will be deposited into the dermis while the female mosquito probes for a blood meal [127-129], innate immune cells (i.e., MΦ and DC), would then be among the first cells to encounter invading RVFV virions. Due to the rapid nature in which MΦ or DC may encounter invading pathogens they are uniquely situated to limit viral replication or to be used by virus as Trojan horses to further disseminate the virus to other tissues.

While the functions and contribution of MΦ and DC as key innate immune cells have been studied with many pathogens, including the closely related phlebovirus, Punta Toro virus [112], detailed study of MΦ functional modulation and susceptibility as a result of RVFV exposure has yet to be thoroughly investigated. Additionally, macrophages are dedicated immune cells likely more capable of exerting the full range of intrinsic antiviral and immunoregulatory innate immune functions. Thus, investigation on the susceptibility and impact of infection with attenuated rMP-12 versus NSs (70%)-deleted rMP-12 (designated Clone 13type) on the intrinsic biological functions of human MΦ cells is a vital step in extending understanding of possible critical immunomodulatory processes occurring during RVFV infection.

OBJECTIVE

Due to the limited information regarding antigen presenting cell involvement with respect to RVFV pathologic processes or host defense, analysis of MΦ cell functional aberrations in response to RVFV provides necessary novel insight on the cellular processes which determine the outcome of infection. Specifically differential responses induced by the major RVFV virulence factor NSs, are of particular importance for understand the cellular and molecular basis for success of RVFV vaccines. Examination of a broad array of possible secreted cytokines and phenotypic activation and maturation indicators, were investigated in order to more thoroughly

evaluate the ability of NSs to enact modulation of intrinsic host defense functions beyond the previously well describe restriction of Type-I interferon production and general host transcriptional activity.

SPECIFIC AIM

Characterize how NSs-intact versus NSs–deleted rMP-12 viral exposure impacts the biology of primary human macrophages (MΦs) with regards to their innate intrinsic functions, i.e., ability to secrete inflammatory mediators and uptake antigen/s.

EXPERIMENTAL DESIGN AND METHODS

Viruses

Recombinant (r) MP-12 viruses were generated by co-transfection of cDNA corresponding to the genomic sequences, pPro-T7-S(+), pPro-T7-M(+), and pPro-T7-L(+) or (pPro-T7-S(+)C13; Clone 13 like) and expressed under the T7 promoter into BHK/T7-9 cells. Additionally cDNA designed for rapid expression of the viral polymerase pT7-IRES-vL and structural proteins pT7-IRES-vN and pCAGGS-vG were included to enhance viral recovery [86]. These recombinant MP-12 and NSs deletion mutant viruses were obtained as generous gifts from Dr. Ikegami, UTMB, and subsequently rMP-12 and rMP-12 NSs-deletion (Clone 13 like) viruses were passaged three times to obtain a working stock used in all experiments**.**

Cells

Human peripheral blood monocytic cells (PBMC) were separated from healthy donors using histopaque 1077 gradient. All PBMCs were derive using UTMB approved IRB protocol #04-159. Included donors were between the ages of 20 and sixty, were not currently taking prescription medication, and were generally but not exclusively male donors. Donors were also

HCV and HIV negative without a current history of chronic illness. In total; six individual donors were used to complete the studies. After separation, isolation of monocytes from PBMCs through antibody enrichment and negative selection of CD14+ PBMCs using a magnetic separation system (EasySep® Human Monocyte Enrichment Kit, StemCell Technologies) to yield monocytes for subsequent differentiation into macrophages. Enrichment routinely produced CD14+ cells with ≥ 80% purity.

Maintenance of monocytes in RPMI-1640 with the presence of GM-CSF (500 U/ml) for 7 days was used to drive the differentiation of monocytes into macrophages prior to harvesting for experimental manipulation. Cytokines were replenished every 3-4 days by addition of 250u/ml of GM-CSF to maintain the concentration of GM-CSF in the medium. Cells were harvested at 7 days of culture to assess their morphologic and phenotypic characterization, and use in inoculation experiments. Following the EasySep® protocol and subsequent culture with GM-CSF for 7 days, highly adherent $CD14^{+}CD1a^{-}CD40^{low}CD86^{-}HLA\text{-}DR^{+}$ cells of MΦ phenotype were consistently derived as previously described by use of this method [130-131].

Cytokine Assays and Interferon Bioassay

Virus induced cytokine and chemokine molecule production was determined as previously performed in our laboratory using Bio-plex assay (Bio-Rad) and interleukin 29 ELISA (PBLinterferonsource). Supernatant samples harvested at indicated time-points post infection were gamma-irradiated (2 million rads) to inactivate virus particles. 50ul of irradiated supernatant sample was assayed following manufacturers protocol (Biorad, Hercules, CA) for human 27-plex panel. Observed concentrations of cytokines were then determined based on standard curve generated during each assay.

Significant differences in cytokine concentration were determined by statistical t-test. By this method the quantification of the concentrations of 27 distinct cytokines as they are secreted into the cell supernatant by RVFV infected MΦ was achieved.

Macrophage cell activation

Activation of MΦ antigen presenting cell functions was assessed by CD83, CD86, CD40, and HLA surface expression, determined by flow cytometry. Additionally, MΦ phagocytosis activity was determined by uptake of dextran-sulfate beads using flow cytometry analysis. PBMC derived macrophage cells were inoculated with RVFV for 12, 24, or 48hrs and then harvested by non-enzymatic cell disassociation buffer treatment for 5min and cell scraping to remove adherent cells. Surface expression of maturation markers CD86, CD40, and HLA-DR in response to RVFV (MP-12 and C13type virus) infection was determined by flow cytometry using specific antibodies and isotype controls purchased from (invitrogen). Briefly, $1x10^6$ RVFV exposed MΦ were incubated with FITC-conjugated antibody against target protein for 30 min in staining buffer (PBS; +2% fetal bovine serum; +0.1% sodium azide). After 30min incubation cells were washed three times in PBS and fixed in 500ul of 4% paraformaldehyde.

Phagocytic function as determined by uptake of FITC-dextran-sulfate beads was performed by incubating $1x10^6$ RVFV or mock inoculated MΦ for 1hr with 40ug/ml FITC-dextran beads (40,000MW) at 37^oC. The relative activity of phagocytosis was determined by the relative intensity of FITC detected during flow-cytometry analysis from dextran-sulfate exposed macrophages.

In addition to analysis of dextran-sulfate bead uptake, the surface expression of mannose receptor (CD206), which has been linked as a mechanism for mediating dextran endocytosis [132] was measured to further investigate the correlation of phagocytosis rate to a well known

mechanism for mediating dextran endocytosis. Additionally, to test the suggestion that the RVFV exposed MΦ may undergo a phenotypic modulation in surface protein expression resembling that proposed of classical (Th1) activated MΦs, the modulation of CD206 expression on the surface of RVFV exposed MΦ was investigated. Briefly, RVFV infected MΦs or LPS and mock treated controls were inoculated for 48hrs. After 48hrs of exposure to the respective stimulus, $1x10^6$ MΦ were incubated with FITC-conjugated goat-anti-CD206 antibody for 1hr in cell staining buffer. After 1hr incubation cells were washed three times with PBS and fixed with 4% paraformaldehyde.

To test that the modulation in RVFV exposed MΦ phagocytic activity was not restricted to uptake of inert dextran-sulfate, the gentamicin protection assay of live bacteria was performed. Stimulating exposure of $5x10^5$ MΦ with RVFV at MOI=0.1, LPS, or mock inoculation for 24hrs was performed to induce modulation of phagocytic activity. Subsequently, the treated MΦ were incubated with E.coli (XL-1 Blue) at MOI=10 for 60min at 37^oC to facilitate binding of bacteria to MΦ cells for endocytosis. After incubation of MΦ and bacteria, remaining unbound and extracellular bacteria are removed by washing 2X with PBS. To more completely clear the MΦ culture of extracellular E. coli, treatment with 40ug/ml gentamicin for 1hr was performed. After gentamicin treatment cells were washed 2X in PBS. The number of endocytosed bacteria was determined by lysing macrophage cells with 0.1% Triton-x-100 to release intracellular bacteria. 100ul of serially diluted MΦ cell lysate containing bacteria was then placed on agarose plates containg 10ug/ml ampicillin. Colony forming bacteria protected from gentamicin treatment by through MΦ engulfment were then counted to determine the rate of MΦ phagocytosis after 24hrs of RVFV exposure.

Completion of these experiments allowed us to more completely link the functional and phenotypic differences in macrophage maturation in response to NSs-intact and NSs-deleted RVFV.

Statistics

The results of cytokine and chemokine assays, IFN bioassays and ELISA, and titers measuring virus production are presented as mean ± standard deviation (SD) of three experimental replicates. Two-way ANOVA with Bonferroni's post test were used to establish statistical significance between groups. Asterisk indicated a significant difference ($p < 0.05$) was considered to exist. The comparison of fold expression between mRNAs and their corresponding proteins are presented by mean ± standard error of the mean (SEM).

RESULTS

Permissiveness of human MΦ Cells to RVFV

The first priority was to determine if rMP-12 and rMP-12/C13type could productively infect MΦ cells, and compare how the growth kinetics might be affected by NSs. Cells were inoculated with either rMP-12 or rMP-12/C13type at an MOI of 0.1 for determining their growth curves over indicated time periods post inoculation (pi). Inoculation of primary human MΦ with rMP-12 resulted in significantly higher viral titers 48 and 72 hrs pi ($p \leq 0.05$), compared to rMP-12/C13type (Figure 2.1). In contrast, inoculation of Vero E6 cells which are defective for the production of IFN-α/β, and therefore highly permissive to many viruses, including RVFV [133-134] allowed replication of rMP-12 and rMP-12/C13type to nearly identical levels. Furthermore growth of rMP-12 and rMP-12/C13type in primary human MΦ was reduced nearly 2-logs, when

compared to replication from IFN-defective Vero E6 cells, which resembled the kind of reduced viral yield reported for RVFV-infected immune competent cell cultures [86].

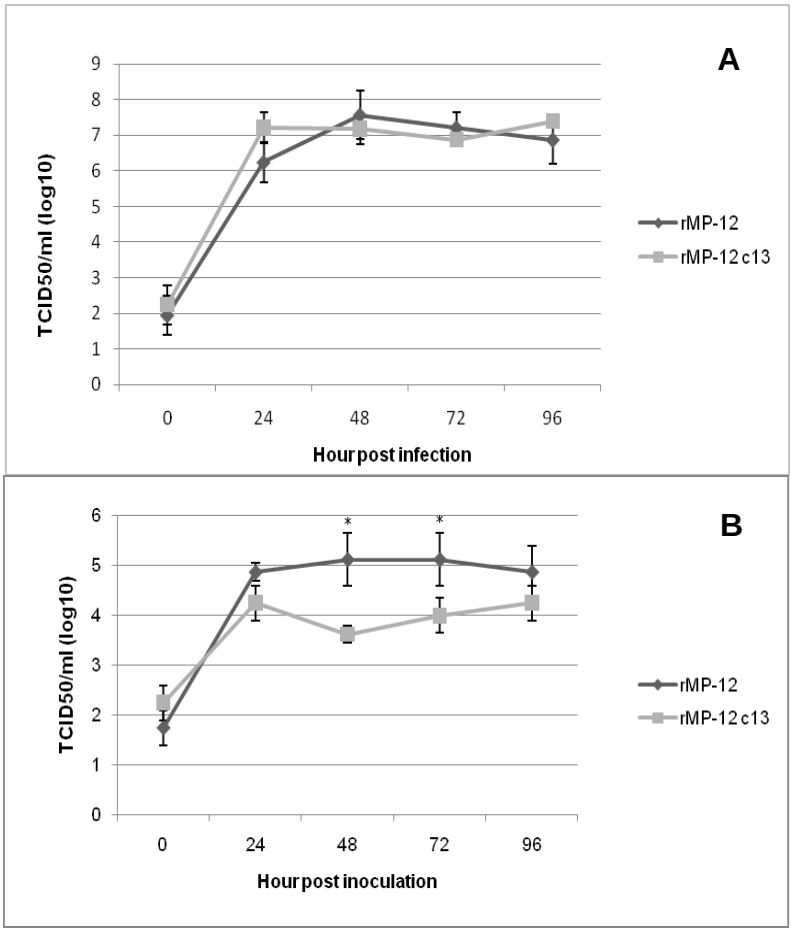


Figure 2.1: Replication efficiency of NSs-intact vs NSs-deleted RVFV. Vero E6 and primary human MΦ were infected with either rMP-12 (—◆—) or rMP12-C13type (—■—) with an MOI of 0.1. Their susceptibilities to either strain of RVFV was subsequently evaluated by assessing yields of infectious progeny viruses in the cultural supernatants harvested at indicated time points post inoculation (pi) by the standard Vero E6 cell-based infectivity assay. A) replication kinetics of rMP-12-versus-rMP12/C13type in Vero E6 cells. B) replication kinetics of rMP-12-versus-rMP12-C13type in primary human MΦ. Primary human MΦ titers are the results of a single donor and three replicates for each viral inoculation. * $p < 0.05$ (statistically different between rMP-12 and rMP12-C13type at indicated time points post inoculation).

In addition to heightened replication efficiency, the presence of a functional NSs in rMP-12 inoculated cells, appeared to confer an enhanced ability of RVFV to produce cytopathic effects in infected MΦ cells (Figure 2.2). While rMP-12 C13-type exposed MΦ also appeared to have some mild reduction in the attachment of cells, the general intensity of cytopathic production was greater when cells were exposed to NSs intact RVFV.

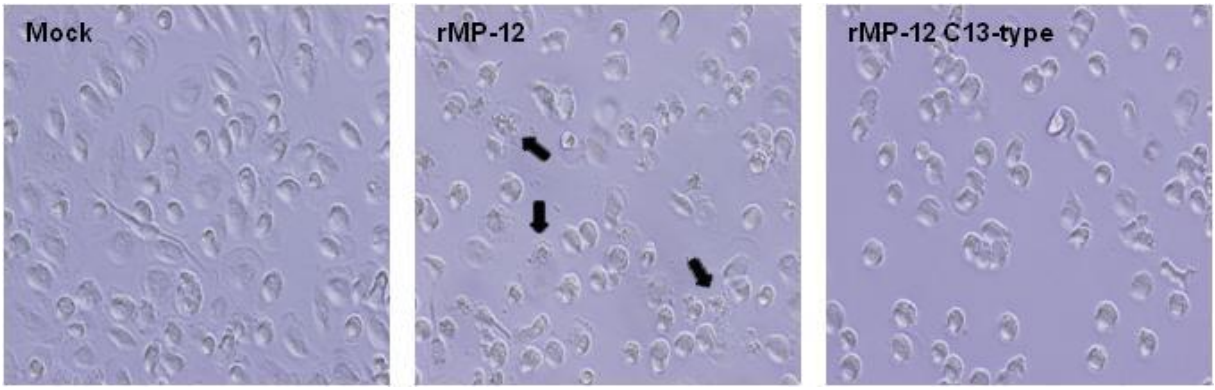


Figure 2.2: NSs protein enhances RVFV cytopathic effects. After 48hrs of infection MOI=0.1 rMP-12 is more potent than rMP12-C13type in causing CPE (arrows) of MΦ infected for 48 hrs. Magnification: 20X. Data are representative of three independently conducted experiments with similar results.

NSs Impact of Secretion of Proinflammatory Mediators

The NSs protein has previously been well characterized as an inhibitor of interferon beta production. The significant differences in RVFV replication kinetics as a result of the presence or absence of NSs expression could be due in large part to the intensity of proinflammatory cytokine induced antiviral activities. While the role of type I IFN has been extensively studied during RVFV infection [77,119,135], very little has been reported as to what extent NSs plays a role in altering the production of other immunomodulatory or antiviral molecules. To build upon the current understanding on the consequences of NSs function, a comprehensive look at the impact of the NSs protein on secretion of cytokines and chemokines was performed to see the extent of NSs impact on immune cell signaling capacity.

As the maximal viral yields for rMP-12 and rMP-12/C13type virus were reached by ~36-48hrs post infection, we choose to investigate the host inflammatory mediators produced at hours just prior to virus replication plateaus. Analysis of the host antiviral and immunomodulatory protein production was therefore assessed at 12 and 24hrs post infection. These time points allowed us to obtain information on the status of host innate antiviral and inflammatory signaling proteins produced which set the stage for the observed differences in replication capacities for NSs-intact and NSs-deleted RVFV in human MΦ.

We found that secretion of proinflammatory cytokines and chemokines from macrophages inoculated with NSs-intact RVFV was severely impaired in comparison to macrophages inoculated with NSs-deleted Clone 13-type RVFV. This drastic inhibition of cytokine and chemokine response from macrophages infected with NSs-intact RVFV further demonstrates how RVFV is able to restrict important aspects of the host innate immune response. While infection by either strain of RVFV resulted in the production of several innate inflammatory mediators (e.g., IL-1β, IL-6, IL-15, IFN-γ, TNF-α, MIP-1α, IP-10, and RANTES), rMP-12/C13type appeared to be much more potent than rMP-12 in promoting the inflammatory responses by infected MΦ (Figure 2.3), indicating that the NSs protein was inhibiting the ability to produce these and possibly other innate signaling molecules. The levels of secreted cytokines not reported in figure 2.3 but measured from bio-plex analysis of RVFV exposed MΦ is presented in Appendix 1.

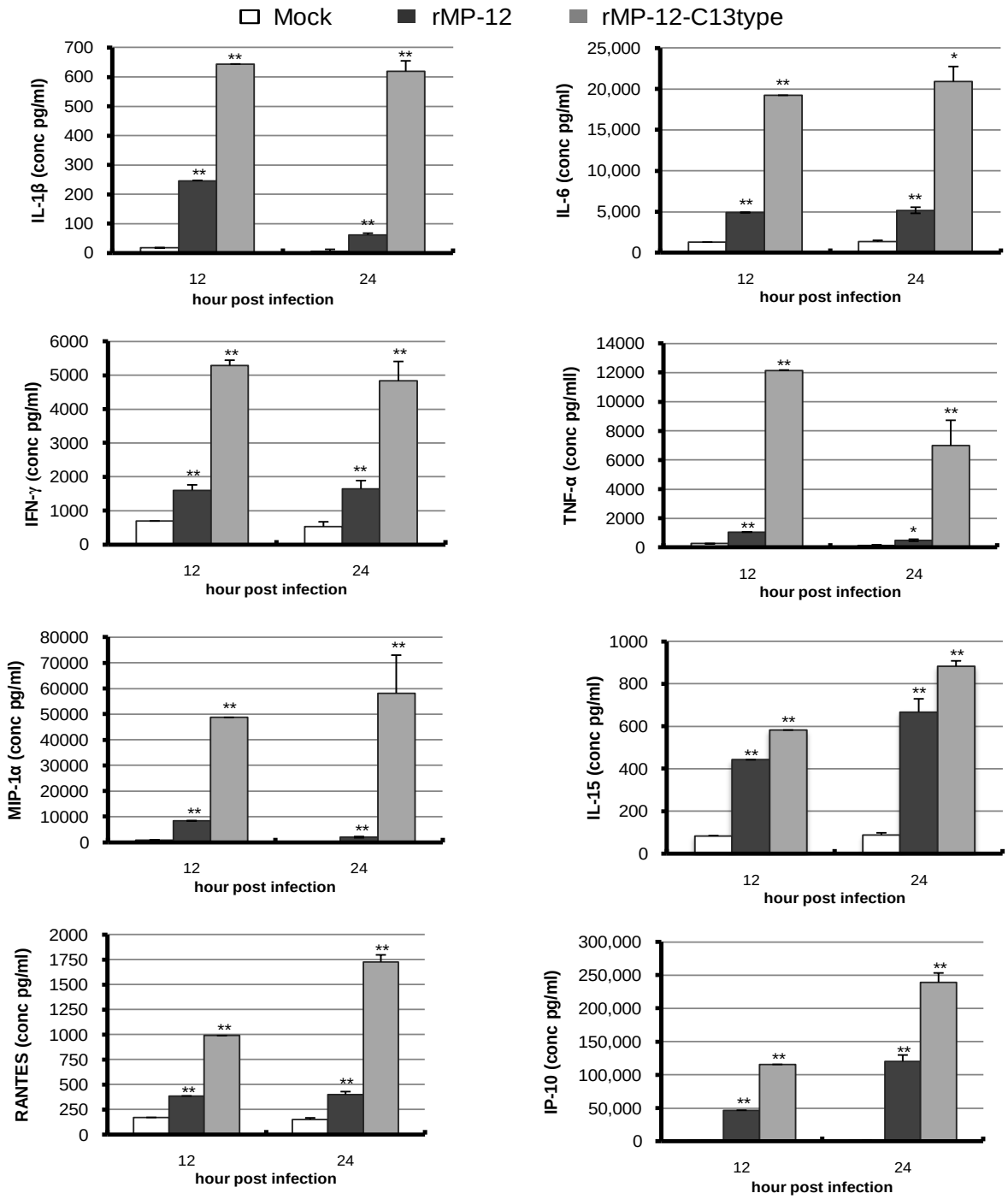


Figure 2.3: Expression of intact NSs reduces, but not prevents, the production of key inflammatory mediators by RVFV-infected MΦ. Aliquots of $7.5x10^5$ MΦ were mock-infected (□), or infected with rMP-12(■) or rMP12-C13type (▩) at an MOI of 0.1 for 12 and 24 hrs before harvesting cell-free supernatants for assessing the contents of key pro-inflammatory mediators by using Human 27-Plex Assay (Bio-Rad). Mean ± SD. * p <0.05; ** p < 0.01 (statistically different from mock-infected controls)

Interferon alpha/beta secretion was quantified by Vesicular stomatitis virus plaque reduction assay [136-138]. Interferon lambda1 was measured by ELISA (PBL, Piscataway, NJ). Unlike the rapid induction of the aforementioned inflammatory mediators (Figure 2.3 at 12 hrs pi), rMP-12-exposed MΦ did not elicit a significant IFN-α/β response until 24 hrs pi ($p < 0.05$) (Figure 2.4). Additionally, the ability to induce type III IFN (i.e., IFN-λ1) response was almost exclusively restricted to those cells infected by rMP-12/C13 virus (Figure 2.4). Taken together, these data demonstrates the RVFV NSs protein has a potent and previously unappreciated, role in restricting the intrinsic cytokine secretion responses of human MΦ. Though the detected amounts of cytokines produced from MΦ inoculated with RVFV containing intact NSs were significantly lower than rMP-12/C13type exposed MΦ, the cytokines IL-1β, IL-1ra, Il-6, IL-15, IP-10, MCP-1, MIP-1alpha, MIP-1β, RANTES, and TNF-α were still produced at appreciable levels.

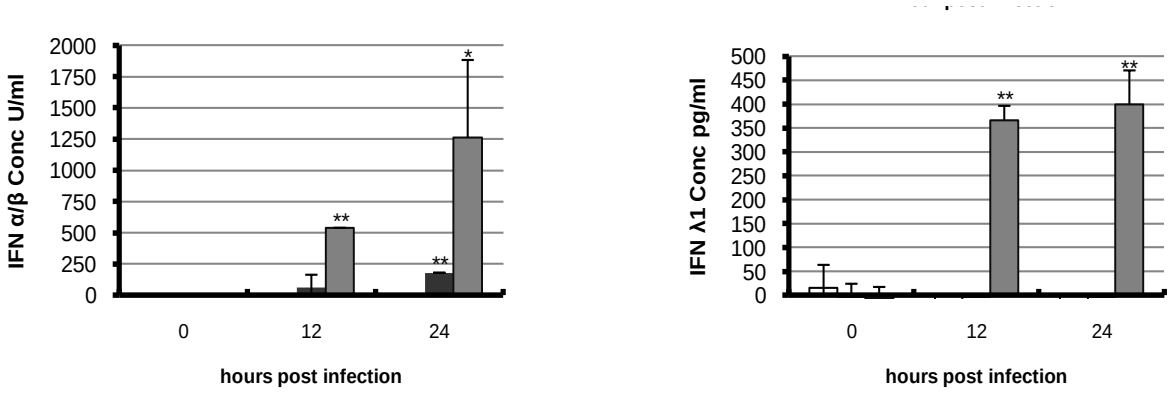


Figure 2.4: NSs restricts type I and type III IFN production. ELISA (IFN-λ1), and VSV-based plaque reduction assay (IFN-α/β) of aliquots of MΦ mock-infected (□), rMP-12(■) infected, or rMP12-C13type infected (▩) at an MOI of 0.1 12 and 24 hrs after inoculation. Data shown are representative of three independent experiments with similar results. Mean ± SD. * $p < 0.05$; ** $p < 0.01$ (statistically different from mock-infected controls)

While the cytokine levels produced may seem tremendously high in rMP-12 C13type infected macrophages, it should be carefully considered that the diversity of cells present within sites of infection (in vivo) may provide negative feedback regulatory mechanisms not represented by the single MΦ culture. It is also the case that under natural infection conditions the viral load encountered would be somewhat less than that used under experimental conditions and not in a homogeneous cell population, therefore such a concentrated level of cytokines and chemokine production may not be observed.

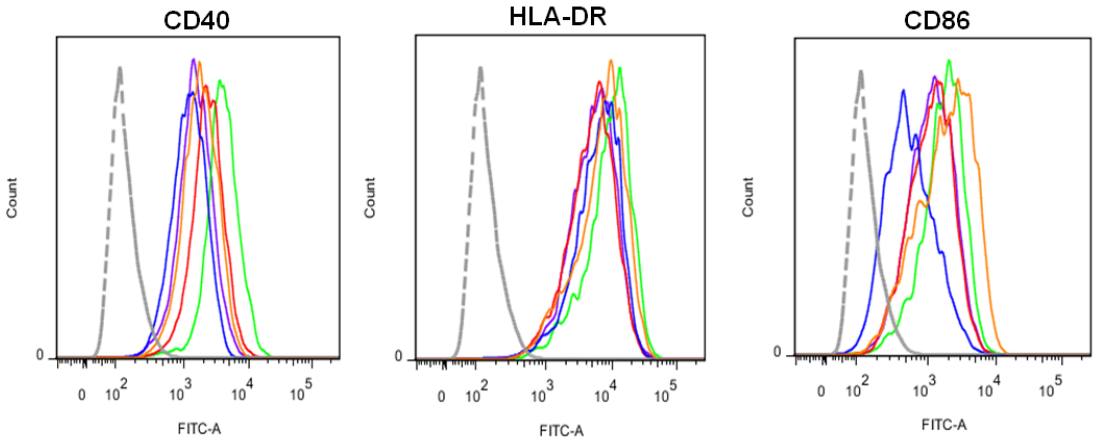
It is also important to note that while the overall secretion of proinflammatory mediators generated in response to rMP-12 is less than that of rMP-12-C13type, the balance between the virus antigen pool and proinflammatory conditions that limit virus replication and stimulate innate and adaptive immunity is an important aspect for proper education of T- and B-cell responses [139]. With this in consideration the production of innate signaling molecules at the lower levels observed during rMP-12 may be sufficient or even optimal for directing further steps in innate and adaptive immune protection. Ultimately carefully executed in vivo experiments are necessary to discern the benefit or detriment heightened levels of inflammatory mediators, such as those observed during rMP-12-C13type infection, have on the quality and kinetics of innate-adaptive immune cell interactions.

NSs Mediated Alteration of other MΦ Intrinsic Functions

In addition to compromising the inflammatory response, we investigated whether the expression of NSs could modulate MΦ's intrinsic ability to present processed antigens in the context of MHC-peptide complexes to antigen-specific T and/or B cells. Because the increased antigen presentation potential is often correlated with the elevated expression of co-stimulatory molecules (e.g., CD40, CD80, CD86, HLA-DR, and etc.) on the surface of professional antigen presenting cells, such as MΦ and dendritic cells (DC), it was important to examine the extent of surface expression for selected co-stimulatory molecules on the membrane of differentially infected or treated MΦ by flow cytometry.

As shown in (Figure 2.5), whereas the percentages of $CD40^+$ and $HLA\text{-}DR^+$ MΦ were not significantly altered upon infection with either strain of RVFV tested, the intensities of their increased expression, as judged by the mean fluorescent intensity (MFI), were more readily detected in rMP-12-C13type- than rMP-12-infecetd MΦ. Specifically, the MFIs of CD40 and HLA-DR expression increased from 2,113 (CD40) and 7,236 (HLA-DR) to 4,357 and 10,600 for rMP-12-C13type-infected cells, respectively, compared to 2,596 and 6,037 of rMP-12-infected ones. Furthermore, both the percentage and the intensity of CD86-expressiong MΦ were more significantly increased in cells infected with rMP-12-C13type-infeced MΦ (i.e., 94.3%-positive with an MFI of 1,997) than those infected with rMP-12-infecetd (i.e., 87.7%-positive with an MFI of 1,470), compared to those of mock-infected controls (i.e., 66.1%-positive with an MFI of 1,092). Taken together, these data suggest to us that the expression of RVFV NSs could dampen the expression of co-stimulatory molecules on the membrane of RVFV-infected MΦ. As an additional measure to demonstrate that NSs is responsible for the differential ability of RVFV to promote host defense responses, including phenotypic maturation. Inclusion of a rMP-

12 NSm-del virus, which contains a deletion in the non-structural M-segment protein further aided drawing the conclusion that NSs was responsible for mediating the modulation of MΦ phenotypic maturation. Infection of MΦ with NSm-del virus produced a pattern of phenotypic maturation which closely mimicked that of rMP-12, indicating that the enhanced phenotypic maturation observed in rMP-12 C13-type infected cells was due to deletion of NSs



	CD40		CD86		HLA-DR	
	% positive	MFI	% positive	MFI	% positive	MFI
Mock	96	2113	66.1	1092	99	7236
rMP-12	95.6	2596	87.7	1470	99.4	6037
C13type	99.3	4357	94.3	1997	99.8	10600
NSmDel	86.6	1842	88.1	1481	99.2	6363
LPS	91.1	2117	90.5	2600	99.6	8506

Figure 2.5: Augmentation of phenotypic maturation marker expression in response to virus is reduced by NSs. Upper panels display histogram of flow cytometry analysis for aliquots of $1x10^6$ RVFV inoculated MΦ. Bottom table indicates corresponding mean fluorescent intensity (MFI) of cell surface expressed protein. % positive cells represents those cells with MFI above isotype antibody control (dashed grey line). Results presented are from a single donor.

The increase in proinflammatory cytokine production but not anti-inflammatory cytokines detected during RVFV (Figure 2.3) was a strong indication that the MΦ were maturing in a classical Th1 phenotype, which typically function in processes of microbial killing and tissue destruction [140]. Alternatively or (Th2) activated macrophages are instead induced to

mature under an environment rich in IL-4 or TGF-β and typically associate with functions involving resolution of inflammation in addition to tissue repair.. The indication that cells progressed towards a Th1 phenotype was further indicated by the increase in surface expression of antigen presenting and costimulatory molecules (Figure 2.5) [103]. While it is commonly believed that macrophage activation should increase phagocytosis activity, reduced levels of phagocytosis and scavenger receptor molecule expression in response to macrophage activators has been reported coinciding with increased proinflammatory cytokine secretion [103]. Thus, to further identify the extent of Th1 type MΦ activation, investigation of RVFV NSs protein impact on MΦ ability to uptake FITC-conjugated dextran was measured to determine NSs modulation of the intrinsic phagocytosis function of MΦ. After infection of MΦ by rMP-12, and especially, rMP-12-C13type, MΦ rapidly and drastically reduced their intrinsic ability to engulf exogenous antigens (Figure 2.6). These results further emphasize that RVFV NSs protein has a great propensity to regulate the kinetics of many intrinsic MΦ functions. Ultimately there are great implications for how the modulation of MΦ or other immune cell functions by NSs might critically impact the host ability to mount protective defense mechanisms especially at the site/s of infection and/or their draining lymph nodes.

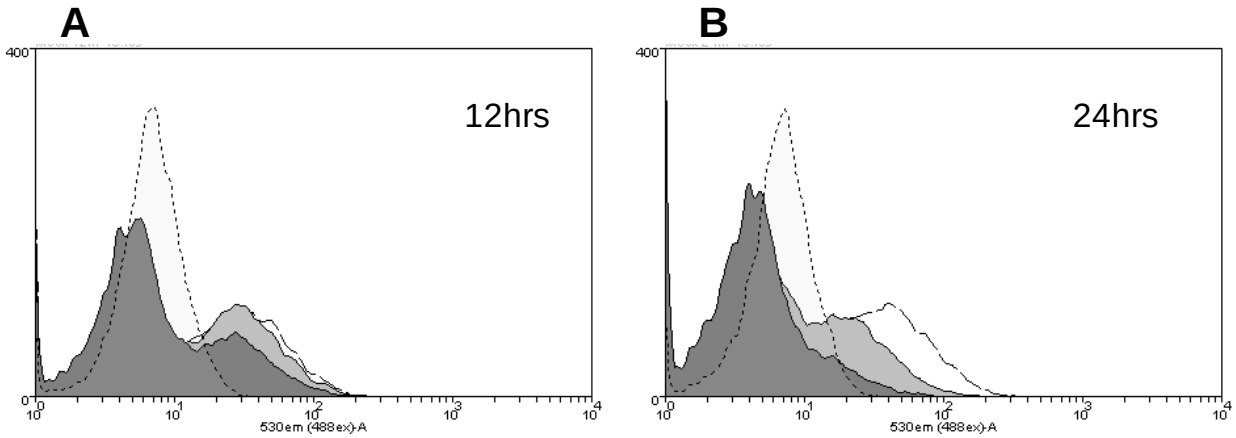
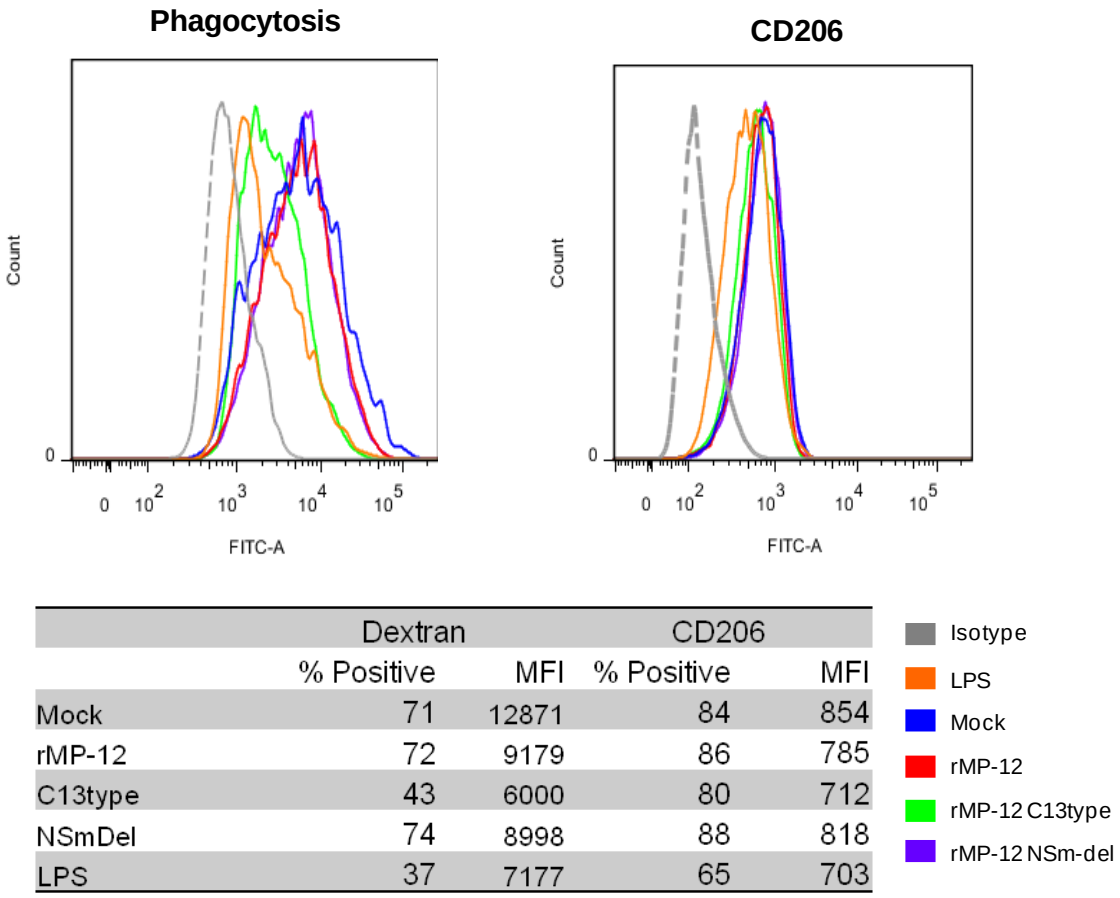


Figure 2.6: Down-regulation of exogenous antigen uptake by infected primary human MΦ is delayed by NSs. The uptake of FITC-conjugated dextran sulfate by primary human MΦ infected with rMP-12 (light grey) or rMP12-C13type (dark grey) at an MOI of 0.1 for 12 hrs (A) and 24 hrs (B). Their capacity to uptake dextran sulfate assessed by flow cytometery. Mock-infected MΦ incubated with dextran sulfate at 4^{o} C (broken line) or 37^{o} C (white shade) were included as negative and positive controls, respectively. Representative data from three independent experiments using MΦ derived from different individuals are shown.

While phagocytosis mediating CD206 is constitutively expressed on MΦ and DC, the intensity of their expression is highly sensitive to manipulation by inflammatory cytokines and toll like receptor ligands [103]. Proinflammatory stimulus such as TNF-α or IFN-γ induce classical-activation of macrophages, resulting in reduced CD206 surface expression and phagocytosis, while alternatively-activated MΦ responding to anti-inflammatory IL-4 increase CD206 surface expression and phagocytic rate [103,141]. CD206 is known to mediate the endocytosis of dextran-sulfate and therefore may be mechanistically responsible for the observed phenotypic alterations in phagocytosis activity [132]. We therefore sought to examine NSs ability to alter levels of CD206 surface-expression as a further indication of NSs impact on macrophage activation. As expected CD206 expression was reduced in response to rMP-12-C13-type infection and LPS (Figure 2.7). The reduced intensity of CD206 surface expression was relatively mild compared to both the

more drastic reductions in phagocytic activity and the increases in costimulatory molecule expression respectively observed from similarly infected MΦ cells. For instance after 48 hours of RVFV exposure rMP12-C13 type infected cells decreased phagocytosis to 43% at an MFI=6000 compared to mock treated cells of which 71% were highly phagocytic indicated by the intense MFI=12871 (Figure 2.7), indicating that the overall propensity for phagocytosis was reduced by roughly half. By comparison CD206 expression only decreased in intensity ~17% by rMP-12-C13type infection when compared to mock.



	Dextran		CD206	
	% Positive	MFI	% Positive	MFI
Mock	71	12871	84	854
rMP-12	72	9179	86	785
C13type	43	6000	80	712
NSmDel	74	8998	88	818
LPS	37	7177	65	703

Figure 2.7: Mannose receptor expression is moderately reduced during RVFV infection. Phagocytosis of FITC-dextran and expression of the mannose receptor (CD206) was assessed from aliquots of $1x10^6$ RVFV exposed MΦ cells after 48 hours of virus exposure at MOI=0.1. Lines representative of histogram readings of fluorescent intensity values of internalized FITC-dextran or surface expressed CD206 respectively. Line colors correspond to respective RVFV strain or control treatment type. Results presented are from a single donor.

Rapid live bacteria ingestion rate is sustained by NSs expression in RVFV infected MΦ

To further explore the negative impact of RVFV NSs protein on the phagocytic activity of MΦ beyond the uptake of dextran sulfate, and to verify that the observed reduction in phagocytosis was not specific or unique to dextran-sulfate antigen, the gentamicin protection assay to determine whether the expression of NSs also modulates MΦ's capacity to uptake and/or kill intracellular bacteria. Briefly MΦ which were inoculated at a MOI=0.1 with rMP-12 or rMP-12-C13type RVFV for 24hrs were exposed to bacteria at a MOI of 10. After incubation, any remaining non-phagocytosed extracellular bacteria were removed by washing with PBS and treatment with 40ug/ml gentamicin. The quantity of E. coli taken up by MΦ was then determined by lysing the MΦ cells with 0.1% 0.1% Triton-X-100 at the indicated times after infection. Phagocytosis activity of live bacteria was the highest among mock and rMP-12 infected MΦ, compared to those elicited by either rMP-12-C13type-infecetd or LPS-stimulated MΦ (Figure 2.8). In fact, the capacities of MΦ to ingest extracellular bacteria were greatly reduced in response to LPS or rMP-12-C13type infection. We also noticed that the total numbers of intracellular bacteria recovered from differentially treated MΦ (i.e., Mock-versus-LPS-versus-rMP-12-versus-rMP-12-C13type at 3 and 6 hrs were similar to those recovered at 0 hrs post-treatment. Thus, these data indicate that differentially treated MΦ populations, displayed a uniformly weak ability to kill the ingested bacteria, at least within the time frame tested. the 6 hrs post-bacterial exposure.

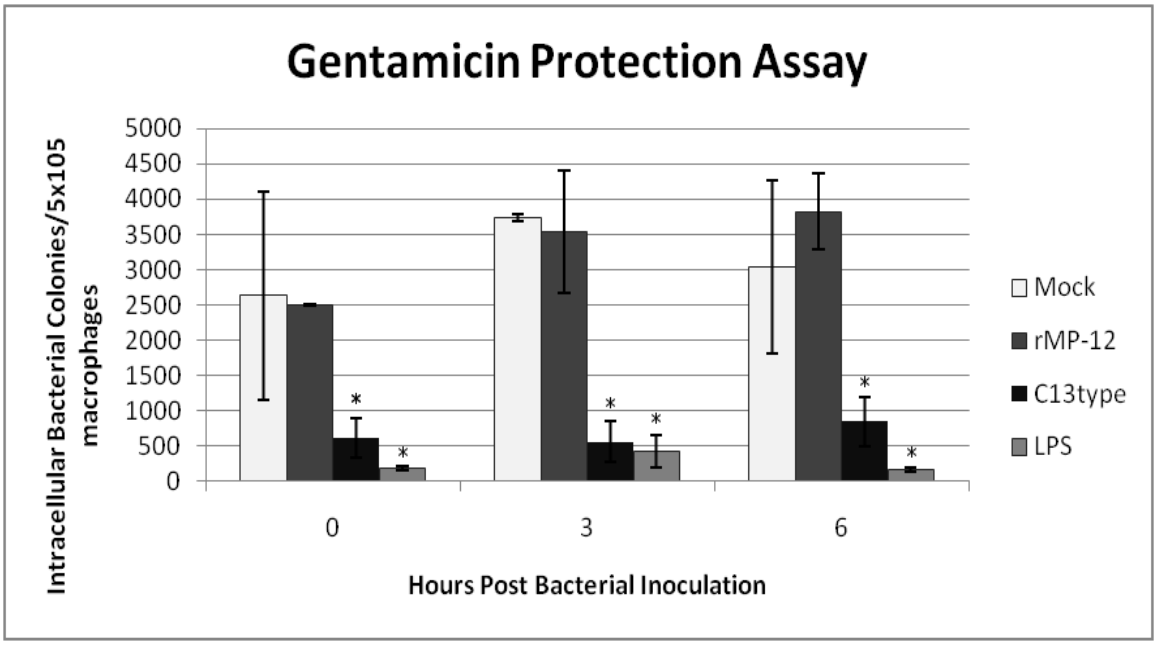


Figure 2.8: NSs maintains the high MΦ phagocytic function rate for live bacteria in response to RVFV infection. MΦs either mock-infected (white bars) or infected with rMP-12 (dark grey), C13-type (black), or treated with LPS (light grey) for 24hrs, were cultured with live E. coli for indicated time points after infection. Colonies formed from remaining live bacteria which were engulfed into MΦs were counted and are represented by bar height means ± SD. Data shown are representative of three independent experiments with similar results. * p <0.05; (statistically different from mock-infected controls)

DISCUSSION

While information on the immunogenicity of MP-12 and Clone 13 RVFV, as live vaccine candidates, and their respective regulation of type I interferon production has been studied, never before has a comprehensive study of RVFV NSs-mediated impact on the response of any of the classic innate immune cells, like MΦ, has not been performed. Furthermore, the correlation between the extent of host immune response and the severity of diseases or the efficacy of live attenuated MP-12 or Clone 13 against RVFV infection in the setting of human infection has not been established. In particular, a large gap exists with regard to how classic innate immune cells

respond to RVFV infection, and their subsequent impact on either the pathogenesis of or the protection against RVFV infection. These results revealed previously unexplored consequences of NSs modulation of innate immune cell host defense functions. The usage of recombinant generated RVFV strains allowed for the specific comparison of NSs function by modulation of the NSs gene independent of differences in the genetic backgrounds of the original MP-12 and Clone 13 strains. Assessment of MΦ phenotypic responses to RVFV found that the intrinsic functions of MΦ, e.g., the ability to secrete inflammatory mediators and function as professional antigen presenting cells, in response to RVFV infection were greatly repressed, but not totally eliminated, by the expression of NSs protein.

Conclusively this study revealed for the first time that not only were human primary MΦ permissive to productive RVFV infection, but their subsequent responses to infection also differentially regulated by the presence or absence of RVFV NSs protein. Specifically, the secretion of proinflammatory cytokines, including type I IFNs, and the expression of co-stimulatory molecules, accompanied by the dampened antigen uptake all occurred in a more dynamic fashion in NSs-defective rMP-12-C13type-infected than NSs-intact rMP-12-infected MΦ. Specifically, enhanced expression of co-stimulatory molecules (e.g., CD40, CD86, and HLA-DR), reduced CD206 expression, and significantly altered phagocytic activity of MΦ, as the results of RVFV infection, have never been previously reported. In addition, the ability RVFV-infected MΦ to increase the production of IL-1, IL-6, TNF-α, IFN-γ, MIP-1α, and IP-10, the hallmark proinflammatory cytokines of Th1-type activation, and to enhance the expression of key co-stimulatory molecules (i.e., CD40, CD86, and HLA-DR), along with a reduced CD206 expression is suggestive that MΦ infected by NSs-intact rMP-12 and, especially, NSs-defective

rMP-12-C13type seemed to preferentially differentiate toward classically activated phenotype, and thereby might favor the induction of Th1- over Th2-type of adaptive immune responses [103].

Intriguingly, given the recently described role of endosome functions in facilitating bunyavirus entry[142], the observed drastic decrease in CD206 expression provides a strong biological basis for further investigation on the role of phagocytosis and endosome processing associated molecules and pathways on the pathologic or protective processes during RVFV infection. Even though the nonpathogenic rMP-12 was inferior to rMP-12-C13type in activating MΦ, it remained fully capable of stimulating MΦ to a lesser, and perhaps sufficient or even optimal extent to promote protective host immune responses against RVFV infection. Additional studies of cellular interplays between MΦ and/or DC and T and/or B cells at the sites of peripheral infections and their draining LNs in the context of rMP-12-versus-rMP-12-C13type infection are needed to fully compare the impact of NSs-mediated alteration in innate immune responses on the nature, intensity, and duration of RVFV-specific adaptive immune responses. Successful completion of such a study would greatly expand not only the knowledge of RVFV pathogenesis but also benefit the development of therapeutic and preventive strategies against RVFV.

The extent to which NSs reduced the secretion of a broad array proinflammatory mediators, while not totally unanticipated, provided insight that the depth to which host innate responses against RVFV were modulated was not limited to type I IFN. It should also be carefully noted that while the intensity of rMP-12 induced cytokines is reduced (Figure 2.3), the diversity of cytokines induced under rMP-12 infection greatly overlaps that of rMP-12-C13ype with the exception of type III interferon, which was not detectable after rMP-12 infection but was readily secreted in response to rMP-12-C13type RVFV (Figure 2.6). Interestingly strong

IL-29 production was detected from rMP-12-C13type infected MΦ; however, rMP-12 inoculated cells failed to produce detectable levels of IL-29. While it has previously been reported that NSs medicates the specific inhibition of IFN-β production [120], ELISA detection of the abundant IL-29 produced from rMP-12-C13type infected MΦ but not rMP-12 infected cells indicates that IL-29 may also be targeted by NSs. The type III interferon system, which includes IL-29 and IL-28, is a recently identified antiviral and immunomodulatory signaling systems with many overlapping effects to that of the type I interferon α/β signaling [143].

IL-29 signaling is effective in a more select tissue distribution compared to type I interferon due to the limited expression of the unique type III interferon receptor [144-145]. While epithelial cells are highly responsive to IL-29, previously the effectiveness of type III IFN during RVFV infection suggested that diseases due to hepatotrophic viruses, such as RVFV, in mice were not significantly alleviated by type III IFN [145-146]. Contrastingly, the effectiveness of IL-29 against hepatotropic viruses for humans has been investigated with some signs of success [147-148], as type III interferon treatment has been suggested to combat the hepatitis C virus [148-150]. The advantage being believed that fewer unwanted side effects due to systemic immunomodulation would occur [151]. Being that a major site of viral replication and tissue injury during RVFV infection occurs within the liver, the beneficial effects of IL-29 against hepatotropic viruses in humans indicate that RVFV inhibition of IL-29 may play a previously underappreciated role during RVFV infection. Mice maintain type III interferon signaling through IL-28A and IL-28B, similar to humans, but the IL-29 protein which is highly active in humans is non-functional in mice [152]. Discrepancies between human and mouse genetics and physiology may be a sign that the mouse model system is less than ideal for studying type III interferon interactions with hepatotrophic viruses.

The goal of identifying how the intrinsic functions of MΦ cells change in response to RVFV infection, and the influence of the NSs protein on regulating the intensity to which MΦ generated host defenses was achieved. Though these results greatly extend understanding of the alteration of innate immune responses, there still exist a plethora of other host factors beyond those which can be readily examined by multiplex cytokine analysis or flow cytometry which may also be altered by NSs during RVFV infection.

Given the chance that an exceedingly varied number of intrinsic host defense mechanisms that previously have been unidentified may be altered directly or indirectly by NSs function during RVFV infection, complementary high-throughput transcription level and complementary proteomic approaches would provide a more exhaustive ability to identify the ramifications RVFV infection exerts on MΦ cell functions.

Chapter III: Macrophage Global Transcriptional Responses

BACKGROUND AND RATIONALE

A robust and delicate balance of the timing, intensity, and duration of the innate immune response is required not only for eliciting acute innate antiviral response but also for instructing the development of adaptive immune response generated later by antigen-specific T and B lymphocytes. Thus, efforts to delineate the mechanics of host innate immune response to alleviate or exacerbate clinical symptoms, as the consequences of viral infections, have come to the forefront of an intensive scientific and medical investigation. The innate response to a given pathogen must be of the proper kinetics and content to inhibit viral replication while not causing excess inflammation and damage to the host. Because innate immune responses, especially those mediated by type I IFNs, serves as the first line of host defense against viral infections, virtually all viruses, including RVFV, have evolved strategies to evade host innate immune responses for their survival in the infected hosts [77,153-154]. It is likely that effective viral suppression of innate immune response initiated at the site/s of infection would facilitate efficient viral replication during the early phase of infection, allowing systemic viral dissemination to other organs, where the overwhelming viral burden often out competes the ineffectual host immune response, which may then collaborate to cause tissue injury and diseases [155-157].

RVFV NSs protein, the major virulence gene of RVFV, has been well-demonstrated to contribute to RVFV pathogenesis by interfering with host general transcriptional activation, suppressing the activation of IFN-β-encoding gene, and degrading the protein of PKR [118-121] .

Together, these functions of NSs enable RVFV to establish an intracellular environment particularly favorable for an early and efficient viral replication and their subsequent dissemination to its prime target organ, the liver the liver. Recent efforts to develop live attenuated vaccines for humans and animals would be benefited by a more thorough understanding of the innate immune responses elicited by RVFV vaccine candidates. Thus, comparing NSs-intact and NSs-deleted RVFV transcriptional responses provides a means to further scientific understanding of the full pathogenic functions of NSs and a means of forwarding RVFV vaccine candidates. Specifically, investigating the global transcriptional responses towards NSs-intact and NSs-deleted RVFV are provided to add additional comprehensive information regarding the composition and kinetics of the genes, signaling cascades, and biological functions most relevant of the host pathogen interaction during RVFV infection

Because the ability to promptly elicit innate cytokine and chemokine responses that eventually regulate the development of adaptive immune response to the infection is a prominent feature of MΦ functions, particular interest in delineating the likely inducing factors driving innate inflammatory responses to RVFV infection and their subsequent inferred biological functions. Microarray gene expression technology is a powerful tool with the potential capacity of examining, at the mRNA level, the full diversity of up- or down-regulation of gene expression. Analysis of the global gene responses of primary human MFs to RVFV provides an efficient means of gathering vast information pertaining to the status of a cell at the transcriptional level during RVFV infection. This information can be further mined to compare similarities and differences between NSs-intact and NSs-deleted RVFV induction or restriction of signaling cascades vital for generation of protective immunity.

OBJECTIVE

The NSs protein has previously been well characterized as an inhibitor of IFN-β expression [119]. In chapter II, it was demonstrated that many of the intrinsic functions of MΦ (i.e., expression of co-stimulatory molecules, production of inflammatory mediators, and phagocytosis/endocytosis) could be greatly modulated by the presence of RVFV NSs protein. While these findings were highly significant, they were rather limited in the capacity to fully identify the mechanism and cascades of host innate immune response which were differentially affected by NSs-intact-versus-NSs-defective RVFV. To build upon the current knowledge of RVFV NSs protein as the major virulence factor and the expanded array of impacted host factors (Chapter II), a comprehensive look at the impact of the NSs protein on the global gene expression of MΦ in response to rMP-12-versus-rMP-12-C13type over time by using microarray-based functional genomics analyses was sought. Through such high throughput methodologies and bioinformatrics tolls, novel and useful information with regard to the molecular basis of innate signaling cascades will be generated, allowing identification of potential cellular targets for future rational design of preventive and therapeutic interventions against RVFV.

SPECIFIC AIM

Determine the global and temporal gene expressions of primary human MΦ in response to NSs-intact versus –deleted rMP-12 infection.

EXPERIMENTAL DESIGN AND METHODS

Specimen collection and Affymetrix GeneChip Array Analysis

Human primary MΦ, differentiated as previously described in chapter II materials and methods, were infected with one of two attenuated strains of the Rift Valley fever virus (rMP-12 or rMP-12 C13). All MΦ cells used in microarray experiments were from a single donor whose cells were not used in cell surface phenotype studies, chapter II (Figures 2.5 through 2.8) The RNA was collected at 12 and 24 hrs post infection and further processed, converted to cDNA, labeled and hybridized to (Affymetrix; Human Genome U133 Plus 2.0) Genechip arrays. Experiments were performed in triplicate, but RNA was lost for one of the samples which was infected with the rMP-12 strain for 12hrs (Table 3.1), therefore only a total of 17 arrays, each with 54,675 probe set identifiers representing more than ~47,400 transcripts, including 38,500 well-characterized genes, and various internal controls were completed. We were interested in comparing the transcriptional responses that differed between NSs-intact (rMP-12) and NSs-deleted (rMP-12 C13) at each time point.

Tube Number	Treatment	Time
1	Mock	12 hr
2	Mock	12hr
3	Mock	12 hr
4	rMP-12	12 hr
5	rMP-12	12 hr
6	rMP-12	12 hr*
7	rMP-12-C13Type	12 hr
8	rMP-12-C13Type	12 hr
9	rMP-12-C13Type	12 hr
10	Mock	24 hr
11	Mock	24 hr
12	Mock	24 hr
13	rMP-12	24 hr
14	rMP-12	24 hr
15	rMP-12	24 hr
16	rMP-12-C13Type	24 hr
17	rMP-12-C13Type	24 hr
18	rMP-12-C13Type	24 hr

Table 3.1: Groupings of virus treatments and harvest times used to generate samples for cDNA microarray analysis. *RNA sample was lost and not included in microarray data

DNA hybridization of microarray experiments were performed in the Molecular Genomics (MG) Core Facilities at UTMB. Detailed information about the procedures used can be found on the MG Core Facilities website (genomics@scms.utmb.edu). **Affymetrix Human Genome U133 Plus 2.0** "Gene Chips" able to distinguish ~38,500 well-characterized genes using 54,675 probe set identifiers to accurately identify more than ~47,400 transcripts, including various internal controls (Affymetrix, Santa Clara, CA).

Normalization and quality analysis of microarray sample data was RMA (Robust Multi-chip Average) normalized [158] using GeneSpring GX software (Agilent Technologies, Santa

Clara, CA) and GeneSifter (VizX Labs, Seattle, WA), and probe sets with expression values below the level of background noise (as determined by detection p value using GeneSifter and filtered on expression level, 20Th of 100th percentile, using GeneSpring) were disregarded in further analyses. As shown by Box Whisker (Figure 3.1) and Profile plots (Figure 3.2), RMA normalization successfully resulted in comparable hybridization signals across arrays.

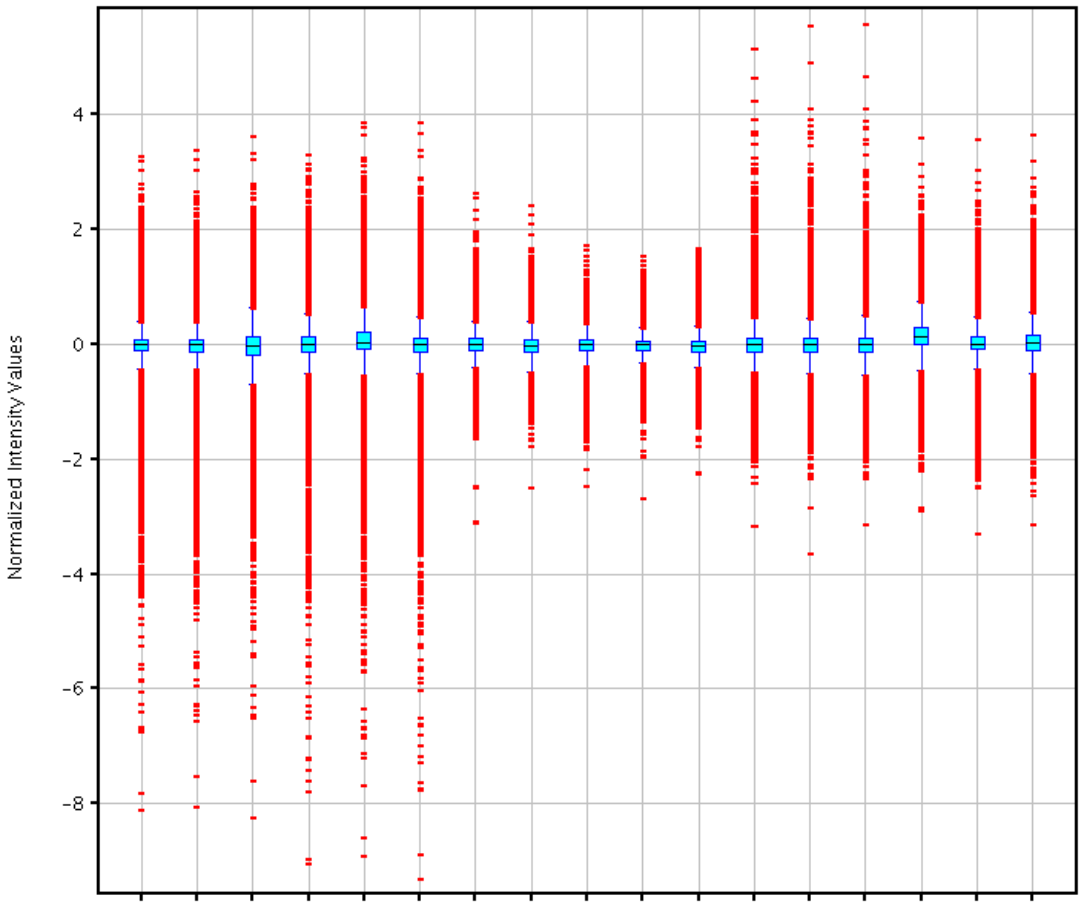


Figure 3.1: Box Wisker plot of expression distribution generated after RMA normalization by GeneSpring microarray expression analysis software. As shown, the RMA normalization procedure resulted in low inter-array variability that could be attributed to normal, technical considerations that might otherwise lead to identification of differences between samples that are not biological in nature. Box plots indicate that distributions of the logged signal values after summarization are well aligned at their centers and quartiles.

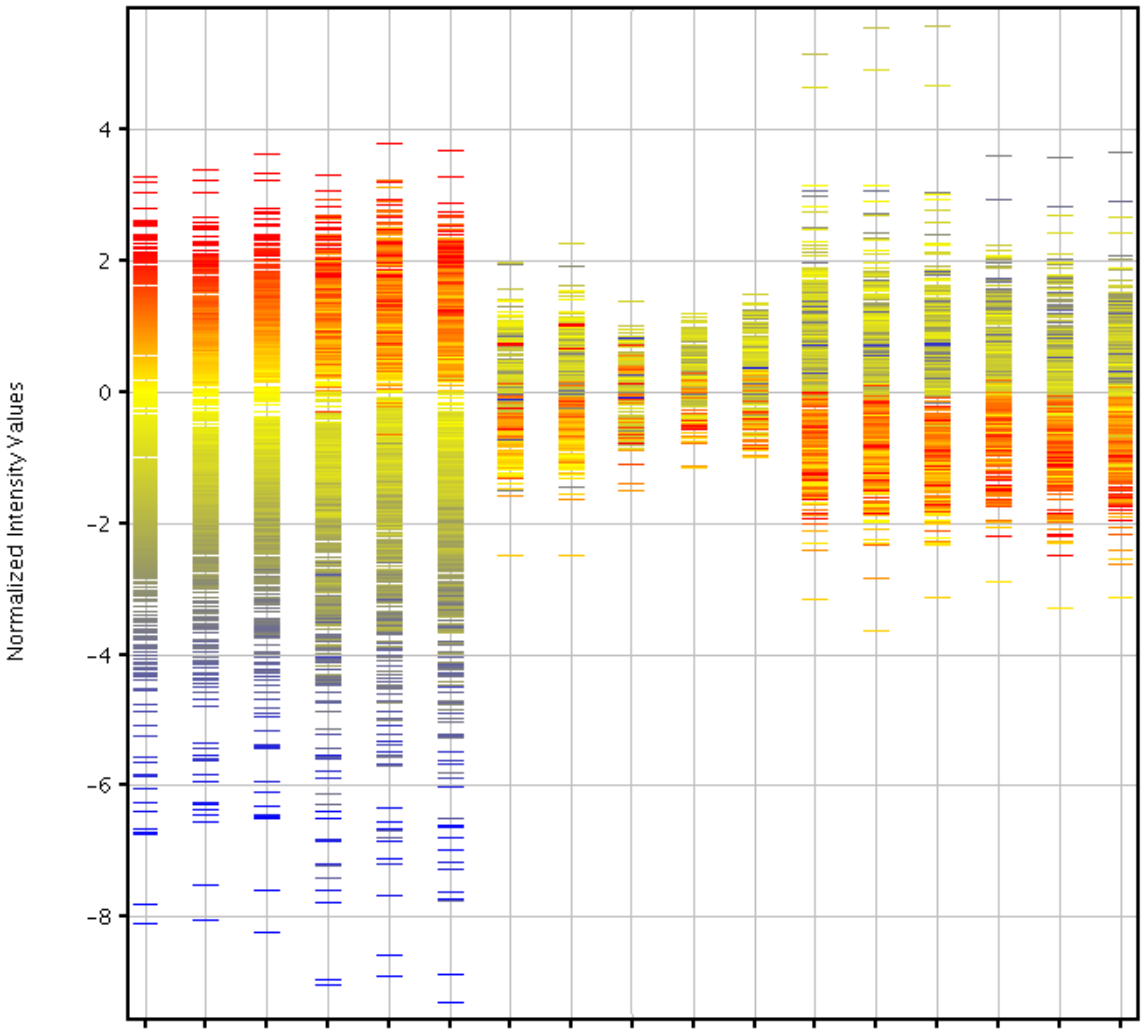


Figure 3.2: Profile Plot of normalized expression generated by GeneSpring after RMA normalization. The colors represent normalized intensity value ranges, with blue at the lower end of the scale, yellow the median, and red at the upper end to represent the highest signal values. Samples, numbered 1 -17 represent the following samples: mock infection at 12 hr (1-3) and 24hr (4-6), rMP-12 infection at 12 hr (7 and 8) and 24hr (9-11), and rMP-12 C13 infection at 12 hr (12-14) and 24hr (15-17). As can be seen from this plot, replicates are consistent within each group and also differ from other groups.

For statistical purposes, all 17 arrays were analyzed as six separate groups (mock-, rMP-12-, and rMP-12 C13--infected cells at 12 and 24 hr). GeneSpring was used to perform RMA normalization, followed by pairwise comparisons of average group values and Student's t test with Benjamini and Hochberg correction. Only probe sets that resulted in a fold-change of at least 1.5 and adjusted p value of less than 0.05 were considered as significantly altered. Data

was further filtered using GeneSifter, after RMA normalization, by performing all relevant individual pairwise comparisons (57 comparisons in total). Criteria for selection of genes were as follows: All 9 possible pairwise comparisons (or 6 for rMP-12) for one group versus another (e.g., mock- versus rMP-12-infected cells at 12 hr) were expected to be at least 1.5-fold and at least 50% greater than the fold-change observed between any two controls (e.g., mock-infected replicate 1 versus mock-infected replicate 2 at 12 hr). Exceptions were made for those probe sets that were significantly altered (fold change ≥ 1.5, p value ≤ 0.05) at the earlier or later time point, with a magnitude that exceeded observed fold-change between replicate controls by at least 50%. This allowed for more accurate identification of those genes that increased or decreased in expression levels over time but that fell below the stringent statistical criteria at the lower transcriptional levels. In order to ensure that results obtained from comparison of rMP-12- and rMP-12 C13-infected cells was specific to infection, any gene with an expression level that differed between cells infected with the two different viruses was considered as irrelevant if not altered by either virus, compared to mock-infected control cells at that same time point.

RESULTS

RVFV induces relative impairment of macrophage functions through the activity of NSs protein. This ability of the RVFV NSs protein to restrict such a broad array of innate immune functions likely leads to an poor performing host defense response, resulting in less than optimal recruitment and activation of immune cells capable of controlling virus proliferation. Such effects on classical innate immune cells would have drastic consequences on the ultimate outcome of infection. In order to further understand the pathogenesic mechanisms of RVFV and

characterize the molecular mechanisms directing the observed host responses elicited in the presence and absence of the RVFV, NSs virulence gene. We employed cDNA microarray method as a means to discovering the host transcriptional aberrations induced by RVFV.

A total list of 3,007 different genes that were statistically significantly altered for at least one comparison (mock-infected versus rMP-12-infected or mock-infected versus rMP-12 C13-infected at either 12hr or 24 hr) could be discerned. Roughly twice as many probe sets differentially detected in response to rMP12 C13 infection (compared to mock-infected controls) at both time points, compared to what was observed for macrophages infected with the rMP-12 strain (Figure 3.3). Specifically, as a result of RVFV infection at 12hrs post inoculation NSs-intact RVFV induced the expression of 614 genes, 20 of which were only expressed by rMP-12. Infection by rMP-12 C13-type induced 1159 genes total, 565 of which were up-regulated at 12hr, s only as part of the response to NSs-deletion. At 24hrs after infection a sustained pattern of more diverse transcriptional responses by rMP-12 C13-type was observed, where rMP-12 produced only 519 up-regulated genes compared to 1165 in response to rMP-12 C13 type. At this time period 676 altered genes were up-regulated only in response to NSs-deleted RFVF while an alternative 30 genes were expressed specific to responses against NSs-intact RVFV.

Interestingly, though it has been reported that NSs causes a generalized suppression of host transcriptional activity [118-119,121], the exposure of primary human MΦ to rMP-12 RVFV did not completely agree with the previously described negative effects of NSs on host gene transcription. Microarray analysis identified that more genes were significantly down-regulated compared to mock infected cells during rMP-12 C13-type infection than with rMP-12 (Figure 3.3). In total 1580 and 1569 genes were down-regulated at 12 and 24hrs respectively in response to rMP-12 C13 infection, while the NSs-intact rMP-12 virus infection only caused the reduced

expression of 584 and 510 genes 12 and 24hrs after infection (Figure 3.3). Because defense responses involve many complex interactions that may cause the encouragement or restriction of biological functions in order to make the host cell an unfavorable environment for propagation of the virus, the observation of many down-regulated genes in response to RVFV infection is not totally unexpected. These observations indicate that during RVFV infection of MΦ cells, the absence of NSs permits the induction of tremendous up- and down-regulation of gene expression. Conversely NSs both prohibits the ability of host cells to enhance the expression of genes most likely required to facilitate conduction of host defenses, and also sustains the expression of genes that may be discouraging to the production of quality immune functions. Host defense processes that ultimately cause the down-regulation of gene transcription may first arrive from signaling components such as IFN α/β, TNF, or apoptosis related caspase signaling [159]. Thus, while NSs does induce the down-regulation of some host transcriptional activity compared to mock infected cells, contrary to the assumed wide-ranging negative impact on host gene transcription, the ability to decrease the expression of host genes as part of the innate immune responses is further enhanced by the removal of NSs. This observation further indicates that the more robust immune signaling occurring during infection with NSs-deleted virus also encourages the reduction of genes whose maintenance may be counterproductive to promoting quality protective immune responses.

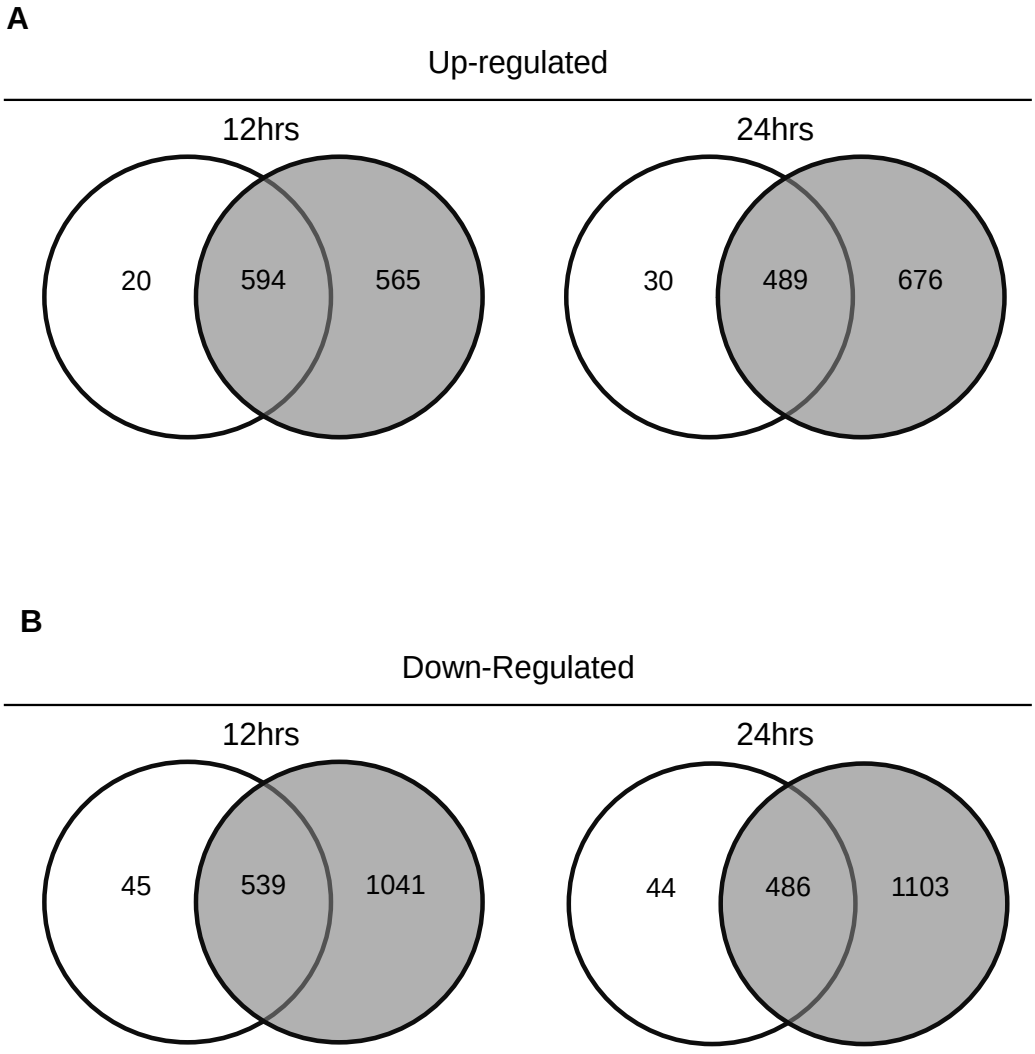


Figure 3.3: Venn diagrams reveal rMP-12 vs rMP-12-C13-type induced differential and overlapping gene expression. Aliquots of MΦ cultured in 6-well plates were infected with rMP-12 or rMP-12 C13-type at MOI=0.1 or remained uninfected (as controls) for indicate time points. Cells were lysed for extracting total RNAs for the subsequent microarray analysis by using Affymetrix Genechips. After the stringent pair-wise comparisons and the statistical analysis, numbers of genes whose expressions were significantly altered (e.g., fold-change ≥1.5 and at least 50% greater in magnitude than any change observed between control samples, $p<0.05$) in rMP-12-infected (white circles), rMP-12 C13-type (grey circles), or both (overlapping region) versus uninfected MΦ are represented. Two Venn diagrams were created to reflect accumulatively up-regulated (A) and down-regulated (B) genes, respectively.

Of the 3007 total genes detected as significantly altered across 12 and 24hrs, only 4 genes (IL-8Rβ, IL21R, and P311, which were all up-regulated, and CRIM1 that was down-regulated) demonstrated selective ability to have their expression altered in response to rMP-12 infection, but not infection with the rMP-12 C13 strain (Table 3.2). Furthermore the mathematical significance identified these genes as statistically significant when expression levels were directly compared between rMP-12-infected versus rMP-12 C13-infected cells (B-H adj. p value ≤ 0.05, fold-change ≥ 2.0).

Table 3.2 Unique rMP-12 Gene Expression Alterations

Gene ID	Fold Change
IL-8Rβ	2.6
IL-21R	2.2
P311/C5orf13	2.1
CRIM1	-2.1

Table 3.3 Top 10 Genes up- and down-regulated genes unique to rMP-12 C13-type infection

Up-Regulated Genes	
Gene ID	Fold Change
CCR7	12
IL29	10.5
ITGB8	7.9
IFNA4	7.4
IFNA7	6.7
IFNA10	6.4
IFNW1	5.3
CKB	5
RXFP1	4.7
IFNA21	4.6
Down-Regulated Genes	
Gene ID	Fold Change
CLEC5A	-5.1
OLFML2B	-3.1
SERPINE1	-3
HHEX	-2.9
LONRF3	-2.8
SLC36A1	-2.7
TRPS1	-2.5
ID2	-2.5
ATP8A1	-2.5
NPL	-2.4

Alternatively there were 255 genes which demonstrated altered expression only as a result of rMP-12 C13type infection, indicating that in addition to a generally less intense fold-change induction of host defense responses, the diversity of genes altered under RVFV infection is reduced in variety by NSs. (Appendix B, Table 1). The 10 genes displaying the greatest fold changes intensities either up- or down-regulated respectively, are displayed in (Table 3.3).

The large number of altered genes induced by RVFV infection prevented the displaying of the fold change intensities along with gene symbols for all of the transcriptional response. Categorization of the altered genes into functional groups made the visualization of genes with well established relevance to processes of infection and immunity more convenient. Heat maps were generated of the genes with association to "antiviral response" or "inflammatory response" functions (Figure 3.4A), and "cytokines and chemokines" (Figure 3.4B). Interestingly, despite the negligible expression of genes encoding for IFN-α and only minimal IFN-β expression or protein secretion in response to infection NSs-intact virus, rMP-12-infected MΦ were capable of activating many well known interferon-stimulated genes (ISGs), e.g., ISG15, ISG20, MX1/2, and OASL, were expressed at levels comparable to those elicited by rMP-12 C13-type infected cells, which produced copious amounts of IFN (Figure 3.4A). Additionally, the pattern recognition receptors (PRRs) and also ISGs, RIG-I, MDA5, TLR-3, and TLR-7, which are responsible for detecting RNA viruses, were also up-regulated during RVFV infection. Genes encoding for RIG-I and TLR-3 displayed exceptionally enhanced expression in response to RVFV during both NSs-intact and NSs-deleted virus with fold changes of ~10 and ~20 fold increase respectively at 12- and 24hrs time points and with both viruses. MDA5 and TLR-7 expression while up-regulated were not as intense, only expressing ~2 fold increases in response to both viruses at 12- and 24hrs. The ssRNA detection molecule TLR-8 was not detected as significantly expressed. Also observed was the differential activation of genes encoding for various key inflammatory mediators or their receptors. Specifically, the expressions of CCL5/RANTES, CXCL9/MIG, CCL4/MIP-1β, CXCL13/BLC (B lymphocyte chemoattractant), CCL14, CCR7, and IL-15RA were preferentially induced in response to rMP-12/C13type, whereas those of CXCL11/IP-9, CXCL10/IP-10, CCL8/MCP-2, and CCL7 were equally induced by either strain, i.e., rMP-12 or

rMP-12/C13type (Figure 3.4B). In contrast, the expressions of CXCR4, CXCL3/GRO-γ, CXCL5/ENA-78, CCL22, and several members of the SOCS (suppressor of cytokine signaling) family (e.g., SOCS-3, -4, -5, -6, and -7), and PRC1 (Protein regulator of cytokinesis 1) were significantly down-regulated upon infection with either strain of RVFV.

Additionally, the expression of several members belonging to the tumor necrosis factor superfamily (TNFSF), were differentially regulated in MΦ inoculated with rMP-12-versus-rMP-12/C13type. Specifically, MP-12 and, especially, rMP-12/C13type were capable of inducing the expression of TNF and/or TNFSF10/TRAIL. Whereas rMP-12/C13type is unique in its ability to down-regulate the expression of TNFSF14/CD258. Alternatively, expression of TRIM5 (tripartite motif-containing protein 5), an intrinsic immune factor relevant to the innate immune defense especially against retroviruses [160-161] was more intensely expressed in MΦ over time periods examined by rMP-12, than rMP-12/C13type.

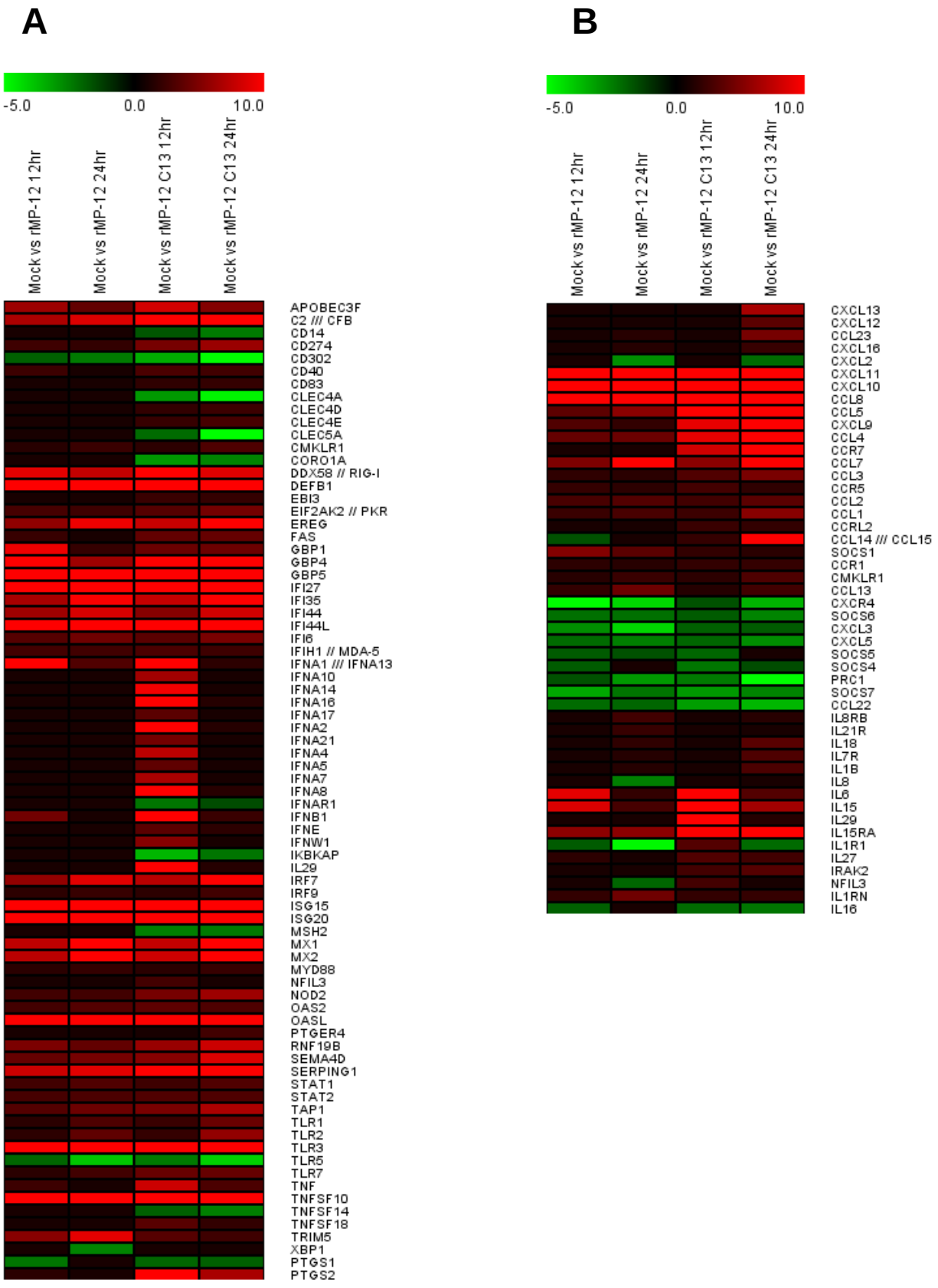

A
-5.0
0.0
10.0
Mock vs rMP-12 12hr
Mock vs rMP-12 24hr
Mock vs rMP-12 C13 12hr
Mock vs rMP-12 C13 24hr
APOBEC3F
C2 /// CFB
CD14
CD274
CD302
CD40
CD83
CLEC4A
CLEC4D
CLEC4E
CLEC5A
CMKLR1
CORO1A
DDX58 // RIG-I
DEFB1
EBI3
EIF2AK2 // PKR
EREG
FAS
GBP1
GBP4
GBP5
IFI27
IFI35
IFI44
IFI44L
IFI6
IFIH1 // MDA-5
IFNA1 /// IFNA13
IFNA10
IFNA14
IFNA16
IFNA17
IFNA2
IFNA21
IFNA4
IFNA5
IFNA7
IFNA8
IFNAR1
IFNB1
IFNE
IFNW1
IKBKAP
IL29
IRF7
IRF9
ISG15
ISG20
MSH2
MX1
MX2
MYD88
NFIL3
NOD2
OAS2
OASL
PTGER4
RNF19B
SEMA4D
SERPING1
STAT1
STAT2
TAP1
TLR1
TLR2
TLR3
TLR5
TLR7
TNF
TNFSF10
TNFSF14
TNFSF18
TRIM5
XBP1
PTGS1
PTGS2
B
-5.0
0.0
10.0
Mock vs rMP-12 12hr
Mock vs rMP-12 24hr
Mock vs rMP-12 C13 12hr
Mock vs rMP-12 C13 24hr
CXCL13
CXCL12
CCL23
CXCL16
CXCL2
CXCL11
CXCL10
CCL8
CCL5
CXCL9
CCL4
CCR7
CCL7
CCL3
CCR5
CCL2
CCL1
CCRL2
CCL14 /// CCL15
SOCS1
CCR1
CMKLR1
CCL13
CXCR4
SOCS6
CXCL3
CXCL5
SOCS5
SOCS4
PRC1
SOCS7
CCL22
IL8RB
IL21R
IL18
IL7R
IL1B
IL8
IL6
IL15
IL29
IL15RA
IL1R1
IL27
IRAK2
NFIL3
IL1RN
IL16

Figure 3.4: Expression of RVFV NSs dictates the temporal expressions of genes relevant to innate immune responses. Genes relevant to host antiviral response and the inflammatory response (A), and genes relevant to cytokine and chemokine signaling (B) whose expressions were significantly modulated in MΦ following rMP-12-versus-rMP-12 C13-typerMP12-C13type infection, compared to uninfected controls, were hierarchically grouped along with time points after infection to more comprehensively reveal the nature of NSs-mediated alteration of host antiviral and inflammatory responses to RVFV infection.

DISCUSSION

Even though it encodes a functional NSs, RVFV MP-12 strain is generally non-pathogenic in humans and generates noticeable adaptive immune responses, including specific neutralizing antibody responses, in human MP-12 vaccinated volunteers [81], the expression of NSs within MP-12 vaccine preparations may reduce the innate immune response and possibly compromise vaccine safety and efficacy. This may be particularly true for special patient populations including the young, elderly, pregnant women, or immunocompromised. Understanding how such a reduced capacity of MP-12 virus to activate host innate immune response, relative to that of NSs-defective live attenuated virus (e.g., Clone 13 or rMP-12-C13type), might affect the vaccine efficacy and safety in humans is therefore highly desirable for selection of the most appropriate preventative measure.

The results elucidated through transcriptional analysis reveal a diverse array of transcriptional responses which could be commonly or differentially modulated in MΦ in response to NSs-intact and NSs-deleted RVFV. These results supported the notion that NSs restricts the production of many host antiviral factors. Unlike previous studies which suggested that NSs was capable of reducing general transcription of infected hosts to below the background levels of uninfected cells [118], microarray analysis of the global transcriptional responses showed that rMP-12 infection also led to the up-regulation in expression of a large variety of genes. In

fact, only 255 genes with altered expression across the 24 hours examined were never altered during infection with rMP-12 virus. Additionally, there were three genes whose expressions were exclusively up-regulated in rMP-12-infected, but not rMP-12-C13type-infecetd, MΦ. Even though it has previously been assumed that the expression of RVFV NSs protein resulted in a nearly complete shutdown of IFN-β expression [100,119], primary human MΦ infected with rMP-12 retained the ability to significantly activate the expression of IFN-β at both transcriptional and translational levels, though at levels greatly reduced compared to rMP-12-C13type infection.

Due to the relatively low MOI used, the expression of IFN-β and many other genes may be a result of a combination of primary and secondary responses to rMP-12 or rMP-12-C13type infections. The primary responses consist of those occurring in cells directly infected by virus, whereas secondary responses are largely a result of cells which have not been infected, but are reacting to the secreted immunomodulatory proteins produced from those cells which are in close proximity and infected directly by virus. Because the lower initial virus load more closely reflects the step-wise cascade of host responses during natural infection, the similarities and differences in gene expression observed as a result of NSs restriction of immune responses is highly representative of the complexity of host-virus interaction dynamics that occur during vaccination or natural infection with RVFV. Ultimately the total number of genes that underwent altered expression in response to NSs-intact versus NSs-deleted RVFV did not diverge tremendously when considering the complete 24 hour time course examined, as there were only 255 genes uniquely altered by infection with rMP-12-C13type virus. Some genes were most intensely expressed at 12 hrs and subsided at 24 hrs pi. Thus, it would be desirable to investigate the global host response to RVFV infection at time points earlier than 12 hrs to determine whether genes prominently expressed at 12 hrs were derived from infected cells or

indirectly from uninfected or "by-stander" cells. While fewer genes would be identified as significantly modulated in cells earlier than 12 hrs pi, these genes likely play a pivotal role in initiating, relaying, and amplifying innate signaling cascades and, thus, are ideal targets for future strategies against RVFV and/or other viral infections. We noticed that among genes whose expressions were differentially regulated in MΦ infected with NSs-intact-versus-defective RVFV, the expressions of those genes encoding for type I IFNs, especially IFN-αs, were consistently suppressed over times (i.e., 12 and 24 hrs), as a result of NSs expression (Figure 3.4). These data suggest to us that while RVFV NSs could inhibit the expression of many genes relevant to the host innate immune response through causing a generalized transcriptional suppression, it appears to be more persistently targeting IFN-related antiviral circuit by profoundly suppressing the induction of type I and type III IFNs, thereby evading hosts' innate antiviral responses.

Global transcriptional analysis also revealed that like those differentially up-regulated genes, NSs-defective rMP-12-C13type was able to more dynamically down-regulated the global gene expression than NSs-intact rMP-12 in infected MΦ (Figure 3.3). While it might be easier to dissect the function of up-regulated genes for extrapolating their likely role/s in the host innate antiviral response, the contribution of those down-regulated genes and their corresponding biological functions in the pathogenesis of or protection against RVFV infection should not be overlooked. Although the utility in down-regulating these genes on the host defense against RVFV remains unknown, it is possible that their down-regulated expression might serve as a mechanism of host innate antiviral defense to limit further replication and/or dissemination of viruses. Alternatively, the viruses may induce the down-regulation of some host genes in order to more efficiently complete their virus replication cycles. Additional studies are, thus,

warranted for better understanding the likely role of these down-regulated genes in the defense against or pathology of RVFV infection.

Though transcriptional profiling reveals how infected cells intend to dedicate cellular resources, it does not allow detection of any post-transcriptional or post-translational alterations, such as NSs mediated PKR degradation [100]. This disadvantage requires that at least some subset of the genes, usually those of most logical connection to mechanisms relevant to the pathogenic features of the disease be validated at the protein level. Earlier the pattern of cytokine secretion was reported that corroborated the trends in cytokine and chemokine gene expression observed from MΦ infection with RVFV (Figure 2.3). In addition to the validation through protein quantification, computational functional genomics tools may statistically identify the most likely biological processes to which the RVFV exposed cells are prioritizing the commitment of their resources.

The diversity and intensity of the pro-inflammatory profile generated against rMP-12-C13type virus is reminiscent of the suggested hypercytokine responses observed during viral infections such as SARS-CoV or highly pathogenic influenza viruses (HPIV) [111,123-124,162-165]. While unregulated and often excessive innate inflammatory responses, also known as "cytokine storms", might confer the onset of severe respiratory diseases, such a notion might not apply to RVFV infection as NSs-deleted RVFV strains are nonpathogenic in Type-I IFN competent animals. With SARS-CoV and HPIV infections much of the disease severity was attributed to immunopathology induced by the excessive, but also ineffectual innate proinflammatory response. Without prior understanding of the extremely limited virulence of NSs-deleted RVFV in immunologically intact mammals [69,76,78], it could be incorrectly assumed from the narrow in vitro analysis alone that the dramatic number and intensity of proinflammatory responses

generated in response to rMP-12-C13type infection would in fact be detrimental, similar to HPIV or SARS-CoV. However, contrary to the relative resistance of SARS-CoV and HPIV to type I IFN, RVFV replication and disease severity appear to be highly sensitive to the antiviral effects associated with IFN and possibly other cytokines. Thus, the observed tremendous diversity and intensity of proinflammatory cytokines produced during rMP-12-C13type infection are more than likely attributable to protective mechanisms for restricting the dissemination of RVFV infection, and not to any pathologic processes reminiscent of localized or systemic "cytokine storm". Interestingly while IFN-β expression has been suggested to prevent RVFV replication in some cell systems [86], and critical for protection in animal models [77,91], the fact that the intense production of IFN-β in rMP-12-C13type MΦ was only able to reduce, but not more thoroughly eliminate, the replication of rMP-12-C13type RVFV replication was somewhat surprising. While the lag time between antiviral gene expression relative to the establishment of infection could be partially responsible for the ability or rMP-12-C13type virus to sustain its replication, the failure of MΦ to eliminate rMP-12-C13type infection suggests that additional antiviral mechanisms beyond IFN might be required to fully protect the host against RVFV infection. By global transcriptional analysis a more complete picture of the host's concerted effort to establish an antiviral state in response to RVFV is obtained. Furthermore, insight on the cellular communication mechanisms which subsequently educate and activate defense mechanisms of recruited and resident immune cell types present at the local sites of RVFV infection can be better appreciated.

In summary the transcriptional profiling supports the hypothesis that RVFV NSs protein profoundly inhibits the transcription of a broad array of host genes relevant to the host innate antiviral response. This microarray-based high-throughput approach allowed the production of

highly valuable gene lists whose expressions were commonly, differentially, or exclusively regulated by NSs-intact-versus-NSs-defective RVFV. Specifically, among the various genes whose expressions could be significantly modulated by either strain of RVFV tested, there were only 4 genes (IL-8RB, IL-21R, P311/C5orf13, and CRIM1) were exclusively regulated by NSs-intact rMP-12, compared to a total of 255 genes by NSs-defective rMP-12-C13type. Importantly, the magnitude of expression for many of the activated genes, especially those encoding for IFN-α/β and other innate inflammatory mediators would be correlated at both the transcriptional and translational levels (Figures 2.3 and 3.4). The general mutual agreement between mRNA expression and protein levels for the subset of innate immune cytokines is useful as it allows us to more faithfully use high-throughput transcriptional level analysis in hypothesizing the probable totality of host defense response mechanism induced by RVFV infection and/or altered by NSs expression. Ultimately as it is desired to utilize this high-throughput information for understanding how individual and coordinated alterations in MΦ gene expression influence the complex nature of RVFV pathogenesis and vaccine efficacy. The profiles of global MΦ transcriptional responses against RVFV infection obtained in these studies are highly valuable in understanding how NSs impacts the immunogenicity of the MP-12 vaccine.

Chapter IV: Microarray Data Interpretation and Function Analysis

BACKGROUND AND RATIONALE

Though basic transcriptional level analysis is capable of detecting the RNA expression of thousands of genes from a single sample, ultimately the list of significantly altered genes, and their corresponding fold changes alone only provides a quantitative measure of the genes as independent factors.

Due to the complex nature of biological systems, a gene displaying altered expression intensity is likely to only be relevant to the physiologic status of the cell in concert with the altered expression of several other genes. Additionally, while one gene may have a high degree of mathematical significance in terms of fold change magnitude, the biological significance of that expression may not be of coinciding intensity. Additionally, because cell signaling molecules, such as transcription factors and their activating kinases, serve as broad regulators and enhancers of gene expression and protein activity respectively, the relative fold-changes of mRNA or protein abundance for these types of genes may be modified only minimally in proportion to much more augmented downstream biological effects they induce.

Another important aspect of transcriptional responses to infectious diseases is the assessment that the intended biological functions of the altered genes are relevant to the physiologic processes which make the host more susceptible or confer resistance to the disease. This is of particular importance when deciphering the significance of what could be hundreds or thousands of different genes. Use of functional knock-down (i.e., RNA interference) or gene knock-out models are well established means for validating the necessity of a target gene in exerting an essential function that makes the host more or less susceptible to the infectious agent. However, targeting a single gene from the list of hundreds or even thousands of selected genes

for manipulation is a highly impractical approach. Thus, it is necessary to partition the enormous quantity of genes into smaller clusters based on important biological functions and/or signaling pathways using various bioinformatics tools for further analysis. From these functional gene clusters a more rational selection of the key target genes for which validation and further investigation of their contribution to disease susceptibility or resistance can be performed.

Because such large numbers of significantly altered genes were detected in response to RVFV infections, categorization of the differentially expressed genes based on their relevant physiological functions was desired. Computationally based functional enrichment not only makes it possible to reduce the analysis of host responses from a large list of individual genes into more concise functional clusters, published information of the cell signaling network allows the altered transcriptional responses to be placed and visualized as upstream and downstream components within the signaling pathways organization.

One method to extract larger gene lists into more manageable groups is through the usage of Gene ontology (GO) term-based functional gene enrichment analysis. Essentially, the functional gene enrichment analysis enables sorting the genes displaying altered expression into functional groups, based on their associated GO term-based biological and molecular functions. In addition, this analysis likely will allow placing the selected genes into various biological pathways, signaling networks, as well as identifying transcription factors (TFs) inferred to be responsible for the altered genes.

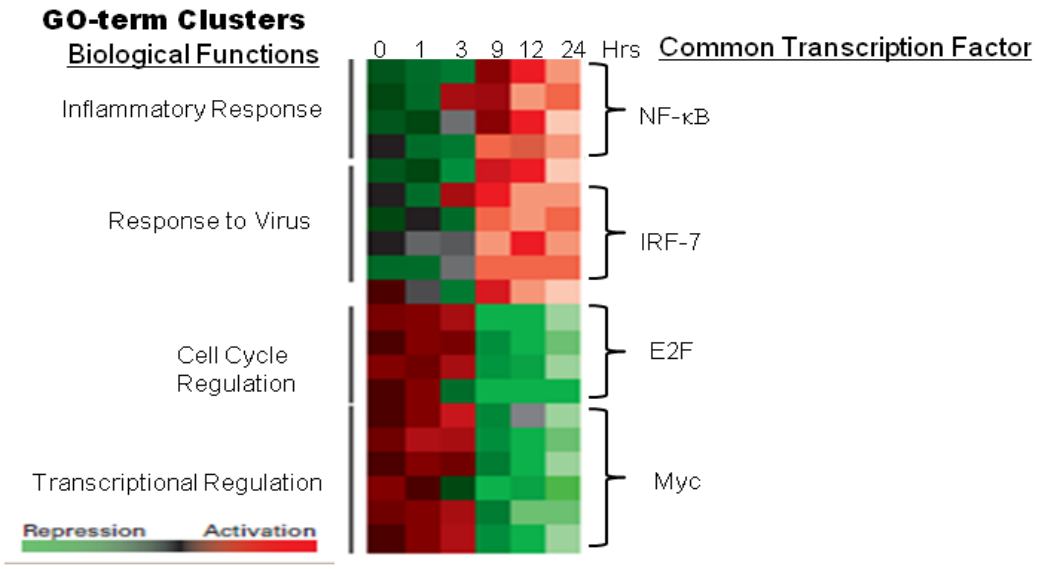


Figure 4.1: Conceptual diagram of gene expression profile clustered by GO-Term analysis to derive relevant biological functions and transcription factors involved expression of the significantly altered genes.

Priority ranking of the biological processes and signaling pathways can be achieved according to the abundance of the constituent molecules that a given pathway requires to faithfully execute its intended purpose being present in the input list of genes. Theoretically, the more molecules of inclusive of a pathway that exhibit altered gene expression indicates that the pathways final physiologic function has a high probability of being altered in the direction (up- or down-regulated) and intensity coinciding with the collaborative activity of the pathways individual constituent genes. Sorting, ranking, and classifying the significantly altered genes into a more limited number cellular biological processes, based on their published functional annotations, allows the logical focusing of a large dataset of individual genes into a manageable and prioritized list of select experimental targets.

Mechanistic validation, through methods such as functional knock-down or utilization of gene knock-out, are required for complete certainty of the biologic activity and relevance to

pathogenesis for genes or pathways implicated as important during infection based on transcriptional analysis. Computational bioinformatics tools are highly beneficial in focusing the workload of GO-terms and functions which should be prioritized. Particularly, any randomness in discerning the GO-terms projected to have biological activity and relevance during infection is minimized by virtue that statistical methods, such as Fishers exact test based analysis, rank most repetitive over-represented functions of a dataset compared with relatively less frequently occurring definitions [166]. Therefore, the biological activity and relevance during infection of implied GO-terms is more reasonably assumed due to the prioritization of those GO-terms with the highest number of supporting genes in the respective dataset lists of modulated genes. Based upon the statistical over-representation of functional annotations assigned to the significantly altered genes during RVFV infection, additional hypothesis generation regarding the importance of biological pathways such as RIG-I, IRF-7, and NF-κB signaling can be pursued. The use of IPA analysis has the additional benefit that the expression trends of molecules up-stream and/or down-stream of critical target molecules can be reasonably dissected for subsequent investigation. Upstream, molecules such as transcription factors or downstream molecules which may not have yet been generated due to the timing of the sample harvesting can be statistically hypothesized.

OBJECTIVE

Basic transcriptional analysis of host responses against viral exposure or infection provides an exhaustive list of genes whose relative mRNA concentrations are altered, either as a response against or consequence of the stimulus. While transcriptional analysis is highly powerful, this information can also be overwhelming, and does not provide an obvious means of

ranking the functional objectives of the genes identified as having altered expression intensities as a collective.

Because individual genes make up only pieces of larger cellular operational puzzle, it was desired to gain more meaningful insights into the biological and molecular mechanisms as well as signaling cascades that could be differentially regulated by rMP-12-versus-rMP-12-C13type infection in MΦ. This goal is achieved by using the relative abundance of genes directly connected to specific cellular operations which would allow for implication of the relative significance and intensity of the most relevant biological processes and signaling pathways triggered by RVFV infection. By using functional enrichment analysis, it was possible to identify biological and molecular functions that are most significantly up- or down-regulated in response to rMP-12-versus-and rMP-12-C13type infection. Of particular interest are pathways and functions unique to host responses against either rMP-12 or rMP-12-C13type infection. Ultimately, identifying these biological and molecular functions as protective or detrimental "biomarkers" would greatly enhance scientific knowledge of RVFV pathogenesis. In addition, they might serve as novel cellular targets for future preventive and therapeutic interventions against RVFV.

METHODS

FatiGO Gene Ontology Analysis

An initial functional profiling analysis of the significantly altered genes was achived by employment of FatiGO (http://www.babelomics.org/). This web-based fictional analysis tool determines whether a given list of genes has an enriched biologically relevant term, when compared to another gene list [167-168]. Typically a group of genes of interest is converted into a

list of functional terms and compared against the frequency of those functional terms in the rest of the genome. Specifically, all of the genes whose expressions were significantly altered (i.e., either up- and down-regulated genes) in infected MΦ cells at each time point, when compared to those of mock-infected cells, were analyzed against the rest of human genomes to assign the most characteristic gene ontology (GO) terms, such as the biological process and the molecular function, to each cluster of genes. In addition, the TRANSFAC database (http://www.gene-regulation.com/pub/databases.html) was employed to explore transcription factors (TFs) utilization based upon over-representation of corresponding DNA-bonding sites derived from the differentially regulated genes. For the statistical analysis, the GO terms identified as over-represented in a gene expression datasets i.e., input list vs the rest of the genome are compared by Fisher's exact test 2 x 2 contingency tables that compares the two groups and extracts the GO terms whose proportional frequency among the groups is significantly different. Multiple testing correction for all of the *p* values obtained for the selected GO terms were corrected by means of the false discovery rate (FDR) method of Benjamin and Hochberg correction to obtain an adjusted *p* value [168]. The adjusted p values < 0.05 were chosen to determine the level of statistical significance. Only probe sets resulting in a fold-change of at least 1.5 and p-value of less than 0.05 were considered significantly altered and explored by FatiGO analysis.

Ingenuity Pathways Analysis (IPA)

In addition to FatiGO based analysis, Ingenuity Pathway Analysis (IPA) was employed as an additional method of analysis to identify the involvement of relevant signaling networks and biological functions associated with the significantly altered genes were generated through the use of IPA (Ingenuity Systems, www.ingenuity.com). The IPA knowledge base is created

from millions of mapped relationships between genes, proteins, cells, tissues, drugs, and diseases, and uses a proprietary knowledge base, ontology, and algorithm to provide computational solutions for understanding biological systems (Ingenuity Systems). The relationships between components are supported by at least one published reference before inclusion as connected elements in the Ingenuity knowledge base.

Lists of altered gene identifiers and corresponding fold-change inductions from NSs-intact and NSs-deleted results were loaded into IPA software application. Molecules from the dataset that were associated with biological functions in the Ingenuity Knowledge Base were considered for the analysis. The functional analysis identified the biological functions that were most significant to the data set. A Right-tailed Fisher's exact test was used to calculate p values determining the probability that each biological function assigned derived as over-represented from data set is not due to chance alone.

In addition to evaluating the more downstream biologic functions being performed during RVFV infection. Construction of molecular pathways and signaling networks by IPA allows a comprehensive cascade of the regulatory events involved in the various host responses during RVFV infection to be depicted. Of particular interest was the implied connection of upstream signaling molecules and transcriptional regulators to the promotion of the array of host defense mediators and biological functions induced as a result of infection. To more completely investigate the signaling components and pathway activity involved in producing the differential responses to NSs-intact or NSs-deleted RVFV infections, genes significantly altered during infection with each virus were subjected to IPA analysis. Canonical pathways analysis by IPA identified the pathways constructed in the IPA library that were most significant to the signaling networks reflective of the gene expression data set. The significance of the association between

the data set and the canonical pathway was measured in 2 ways: 1) A ratio of the number of molecules from the data set that map to the pathway divided by the total number of molecules that map to the canonical pathway. 2) Fisher's exact test was used to calculate a p-value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone.

To generate signaling networks, after loading genes whose expression was significantly altered during infection into the IPA analysis software, the Network Eligible molecules from these input genes are mapped onto a global molecular network developed from information contained in the Ingenuity Knowledge Base. Network Eligible Molecules were then algorithmically placed into signaling networks based on their physical and functional connectivity to common molecules within defined signaling networks. Networks created with the IPA analysis tool and over-layed with transcriptional fold-changes representative of the input microarray data allowed for inferred visualization of potentially important upstream and downstream molecular events. These networks might also be useful for the identification of molecules that are not typically regulated via transcriptional alteration (e.g., kinases or transcription factors that are activated via phosphorylation events) and thus would not be directly detected by microarray analysis.

Results

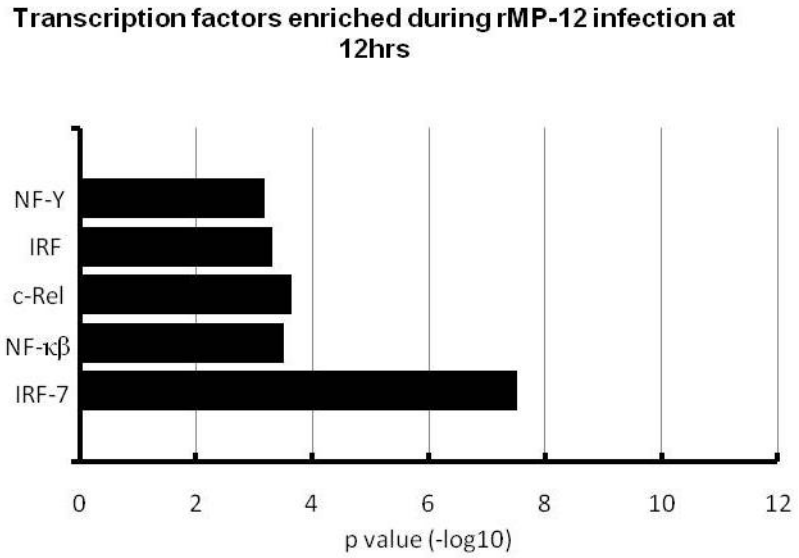
Genes altered in response to RVFV infections were subjected to statistical based gene-ontology analysis to anticipate the biological pathways both enhanced and limited as part of the innate responses to RVFV. Using the two recombinant virus strains allowed further clarification of how the diversity and intensity of biological pathway activities are altered by NSs function.

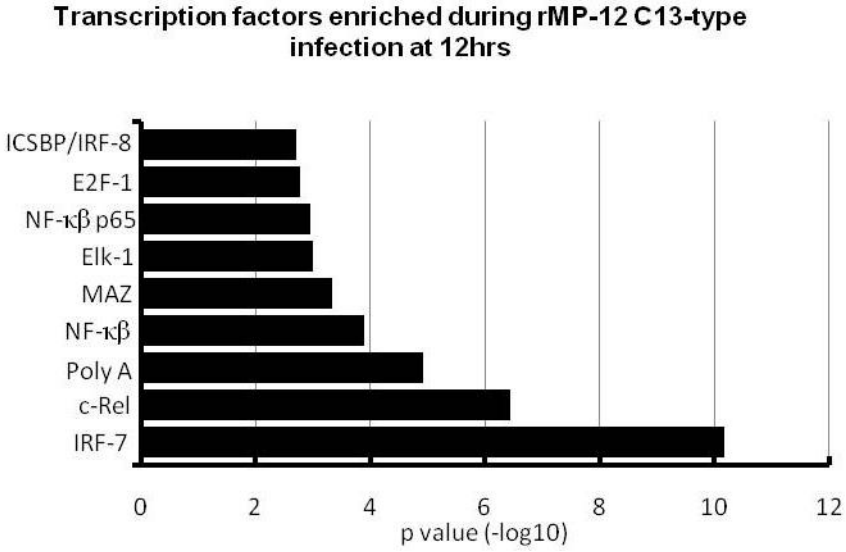
Elucidating how signaling pathways and transcription factor activity is altered during NSs-intact infection may stimulate studies focused on modulating the activity of NSs-targeted pathways as therapeutic interventions.

Coinciding with the generally reduced number and intensity of gene expression alterations during NSs-intact infection, the diversity and intensity of transcription factor utilization in response to RVFV was reduced in the presence of NSs. There were five transcription factors implicated as being responsible for generating the up-regulated genes expressed at 12hrs and 24hrs after rMP-12 infection. In contrast rMP-12 C13-type infected cells utilized 9 and 8 transcription factor elements at 12hrs and 24hrs after infection respectively (Figure 4.2). Specifically IRF-7 and NF-κB/c-Rel transcription factors were implicated during infection with either rMP-12 or rMP-12 C13-type infection. By comparison NF-Y was preferentially implicated with the up-regulated genes during rMP-12 infection, while ICSBP/IRF-8, NFκBp65, EZF-1, ELK-1, MAZ, and Poly A were uniquely implied from the list of up-regulated genes during rMP-13-C13type infection (Figure 4.2).

The NF-Y, TF is composed of NF-YA, NF-YB and NF-YC proteins in a heterotrimeric complex which regulates a large number of mammalian genes which contain the highly conserved *CCAAT* binding element within their promoters [169-170]. Because up to ~7-8% of human promoters contain functional *CCAAT* motifs, NF-Y has tremendous ability to regulating many genes, including housekeeping, cell cycle regulatory genes, and those involved in tissue development [169,171-172]. The implication that NF-Y activity is exclusively enhanced during MΦ responses to rMP-12, but not rMP-12/C13type, may reveal that NSs modulation of host gene expression promotes an altered cell cycle status that is favorable for RVFV replication.

A


Transcription factors enriched during rMP-12 infection at 12hrs
NF-Y
IRF
c-Rel
NF-κβ
IRF-7
0
2
4
6
8
10
12
p value (-log10)


Transcription factors enriched during rMP-12 C13-type infection at 12hrs
ICSBP/IRF-8
E2F-1
NF-κβ p65
Elk-1
MAZ
NF-κβ
Poly A
c-Rel
IRF-7
0
2
4
6
8
10
12
p value (-log10)

B

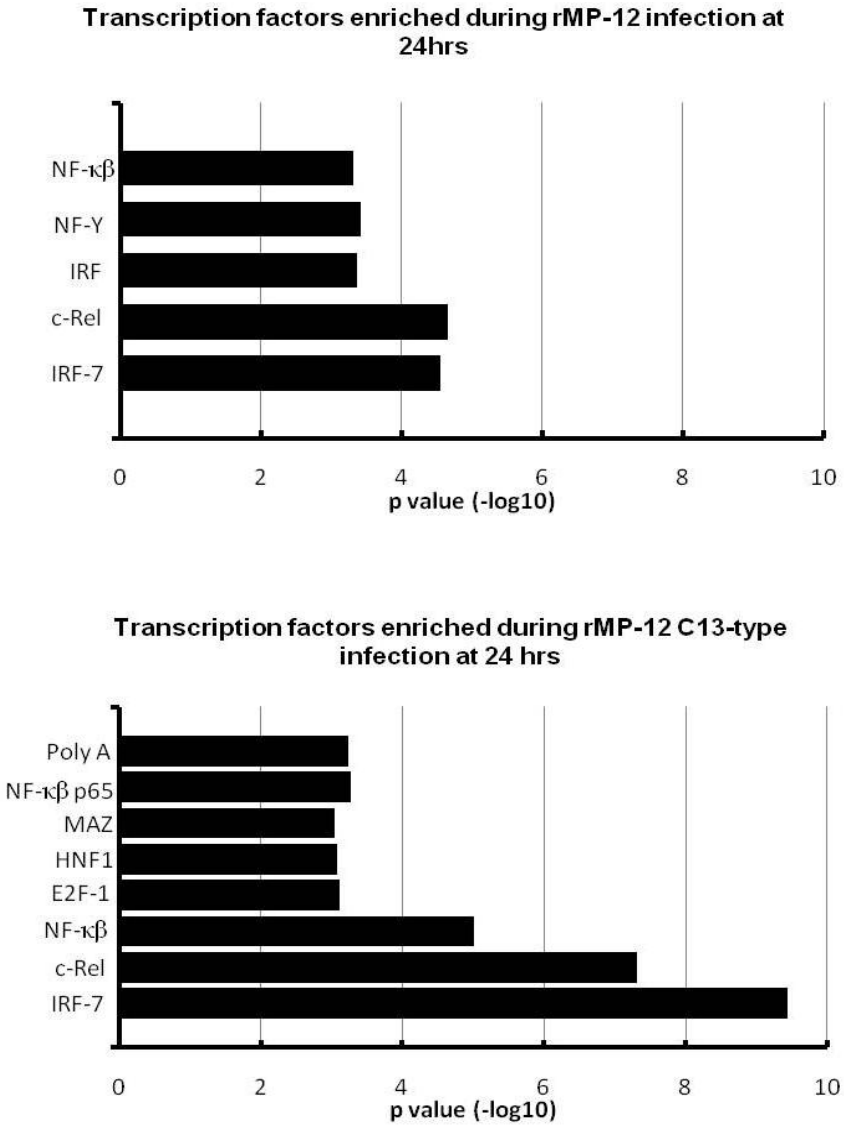


Figure 4.2: Implied transcription factor utilization in generating the up-regulated genes. Displayed are the temporal and differential usages of transcription factors (TFs) predicted to produce the up-regulated gene expression in RVFV-infected MΦ based on the over-representation of TF binding sites in within the promoter regions of up-regulated genes. Genes whose expressions were subjected to the TRANSFAC database-based analysis of TF activation, as described previously [124]. Inferred TF usages in response to rMP-12 and rMP12-C13type infection at 12 and 24 hrs pi are presented in A and B, respectively. Adjusted *p* values of < 0.05 among stringent pair-wise comparisons were used for selecting those TFs inferred to be used over time by differentially infected MΦ, compared to uninfected controls.

FatiGO analysis of the down-regulated genes identified two transcription factors assigned to those genes whose expressions were significantly down-regulated in rMP-12-infecetd MΦ at 12 hrs were those related to KROX (previously known as "early growth response") and Myc (Table 4.1). The inclusion of myc related transcription factor activity associated with down regulated genes during infection with rMP-12 is likely a result of NSs inhibitory activity on TFIIH during RVFV infection and the close association of myc with TFIIH [173-174].

Transcription factors of down-regulated genes 12hrs post rMP-12 infection

Term	Adjusted p value
KROX	5.18E-05
E2F-1	0.006127
Myc	0.01417
Rb:E2F-1:DP-1	0.036695
HNF-1	0.044404

Transcription factors of down-regulated genes 24hrs post rMP-12 infection

Term	Adjusted p value
KROX	2.34E-09
Myc	0.046303

Transcription factors of down-regulated genes 12hrs post C13type infection

Term	Adjusted p value
E2F-1	6.63E-08
KROX	8.24E-08
E2F	7.19E-07
LXR, PXR, CAR, COUP, RAR	0.000395
HNF-4	0.000439
E2F-1:DP-1	0.000952
CRE-BP1:c-Jun	0.002767
Pax-3	0.003205
c-Ets-1p54	0.003308
GABP	0.004024
Elk-1	0.004319
CREBATF	0.004473
HNF1	0.005799
Rb:E2F-1:DP-1	0.009265
HNF-1	0.009317
CDX	0.009689
E2F-1:DP-2	0.009689
E2F-4:DP-2	0.009689
USF	0.009689
CREB	0.009767
PITX2	0.010319
E2F-4:DP-1	0.012269
CRE-BP1	0.012644
Myc	0.012644
HLF	0.021921
MEF-2	0.023601
CCAAT box	0.025032
Staf	0.025032
ATF3	0.043621
AFP1	0.044
NF-Y	0.044

Transcription factors of down-regulated genes 24hrs post C13type infection

Term	Adjusted p value
E2F-1	1.37E-08
KROX	7.98E-08
E2F	2.79E-06
HNF-4	0.000372
LXR, PXR, CAR, COUP, RAR	0.000666
E2F-1:DP-1	0.003446
Pax-3	0.003528
Myc	0.003915
USF	0.00398
CRE-BP1:c-Jun	0.004683
HNF1	0.005503
Rb:E2F-1:DP-1	0.005503
E2F-4:DP-1	0.008877
HLF	0.009538
CREBATF	0.009721
CDX	0.010535
AFP1	0.014222
HNF-1	0.015524
GABP	0.01643
CRE-BP1	0.029642
CREB	0.044799
MEF-2	0.044799
CCAAT box	0.045582
E2F-1:DP-2	0.045582
E2F-4:DP-2	0.045582

Table 4.1: Trancription factors implied to control expression of down-regulated genes during RVFV infection.

Biological Processes down-regulated at 12hrs post rMP-12 infection

Term	p value
fatty acid metabolic process	0.048891
icosanoid metabolic process	0.048891
biopolymer biosynthetic process	0.048891
icosanoid biosynthetic process	0.048891

Biological Processes down-regulated at 24hrs post rMP-12 infection

Term	p value
translational elongation	0.022204

Biological Processes down-regulated at 12hrs post rMP-12-C13type infection

Term	p value
biosynthetic process	8.06E-09
cellular biosynthetic process	3.68E-06
cellular metabolic process	6.42E-05
M phase of mitotic cell cycle	0.000267
cell division	0.000267
mitosis	0.000373
translation	0.000684
mitotic cell cycle	0.000836
macromolecule biosynthetic process	0.000836
M phase	0.001093
organic acid metabolic process	0.001093
carboxylic acid metabolic process	0.001093
cell cycle phase	0.001093
primary metabolic process	0.001093
cell cycle	0.003241
cell cycle process	0.009805
cellular catabolic process	0.012048
alcohol metabolic process	0.029885

Biological Processes down-regulated at 24hrs post rMP-12-C13type infection

Term	p value
cell cycle	2.24E-05
cell division	0.000985
translation	0.000251
mitosis	0.002923

Table 4.2: The biological processes affiliated with the down-regulated genes during RVFV infection.

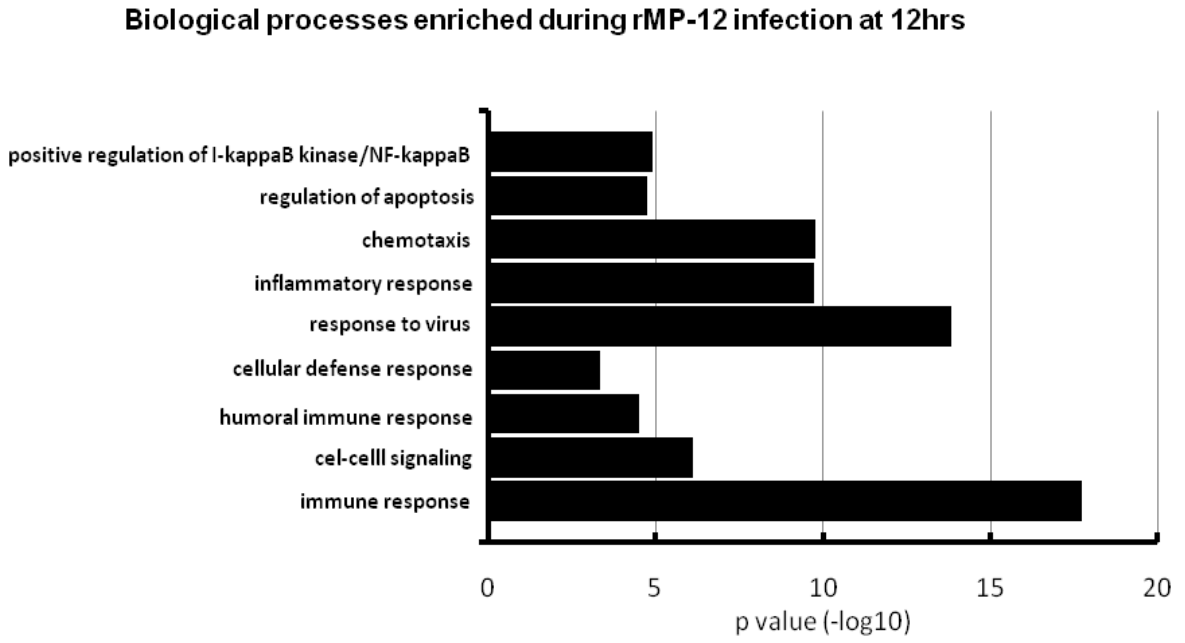
Analysis of the biological functions connected with the down-regulated genes suggested that the majority of the down-regulated genes were committed to biological processes involving

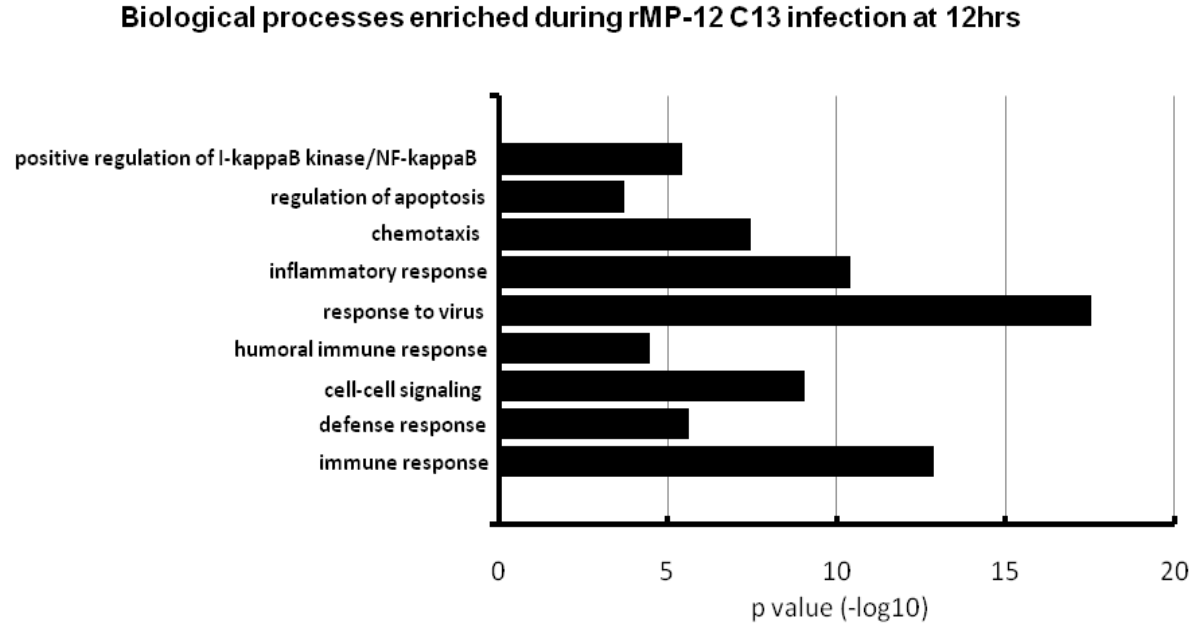
cellular biosynthetic and metabolic processes (Table 4.2). The decreased abundance of genes functioning to support cellular metabolism and biosynthesis during NSs-deleted RVFV infection suggests that part of the host defense response involves restricting access to host cellular machinery required for virus assembly. To combat this host defense NSs protein function may support cellular metabolism in order to sustain pools of raw materials necessary for virus assembly. This restriction in biosynthesis is similar to the finding that PKR activation during RVFV infection plays a vital role in limiting NSs-deleted virus replication, and that NSs promoted degradation of PKR is designed to support host and virus protein synthesis [99-100]. Alternatively, NSs-deleted RVFV was demonstrated to have a greatly reduced translation activity and virus protein accumulation as a result of unrestricted PKR activation [99-100].

While RVFV mediated PKR degradation impacts the translational steps to interrupt availability of host machinery, functional genomics analysis suggest that similar strategies in limiting the availability of critical host cell components required for RVFV infection also occurs at the transcriptional level. Interestingly, rMP-12-C13type virus infection also induced the most profound up-regulation of genes. Such divergent transcriptional alteration indicates that the protective innate host response dynamics includes a bi-directional regulation. The optimal ability to limit infection involves both the enhancement of genes related to antiviral responses and immune signaling, plus the reduction of non-immunologically related factors which may support virus replication. As the possible reduction in biosynthetic processes would influence the performance of potentially all subsequent cellular functions, including those required for antiviral responses, analysis of the biological processes of up-regulated genes able to persist through the implied reduction in cellular biosynthesis is essential.

With the goal in mind to better understand how the functions of the up-regulated genes expressed during RVFV infection limited virus replication and induced protective immune responses, Further GO-term analysis of the up-regulated transcriptional activities was performed. FatiGO analysis implied biological processes related to “immune response”, “response to virus”, “inflammatory response”, “chemotaxis”, and “positive regulation of I-κB kinase/NF-κB” were among the highest and most consistently activated functions common to infection with rMP-12 and rMP-12 C13-type infections at 12- and 24hrs. Interestingly, pronounced differences between the identified commitment to gene expression related to “humoral immune responses” were preferentially involved in response to rMP-12 C13-type infection. Specifically, “humoral immune response” was identifiable at 12- and 24hrs after infection with rMP-12 C13-type virus, but only 12hrs after infection with rMP-12. Alternatively, “cellular defense response” was exclusively involved at 12- and 24hrs during infection with rMP-12 but not from rMP-12 C13-type at any time point (Figure 4.3).

A


Biological processes enriched during rMP-12 infection at 12hrs
positive regulation of I-kappaB kinase/NF-kappaB
regulation of apoptosis
chemotaxis
inflammatory response
response to virus
cellular defense response
humoral immune response
cel-celll signaling
immune response
0
5
10
15
20
p value (-log10)


Biological processes enriched during rMP-12 C13 infection at 12hrs
positive regulation of I-kappaB kinase/NF-kappaB
regulation of apoptosis
chemotaxis
inflammatory response
response to virus
humoral immune response
cell-cell signaling
defense response
immune response
0
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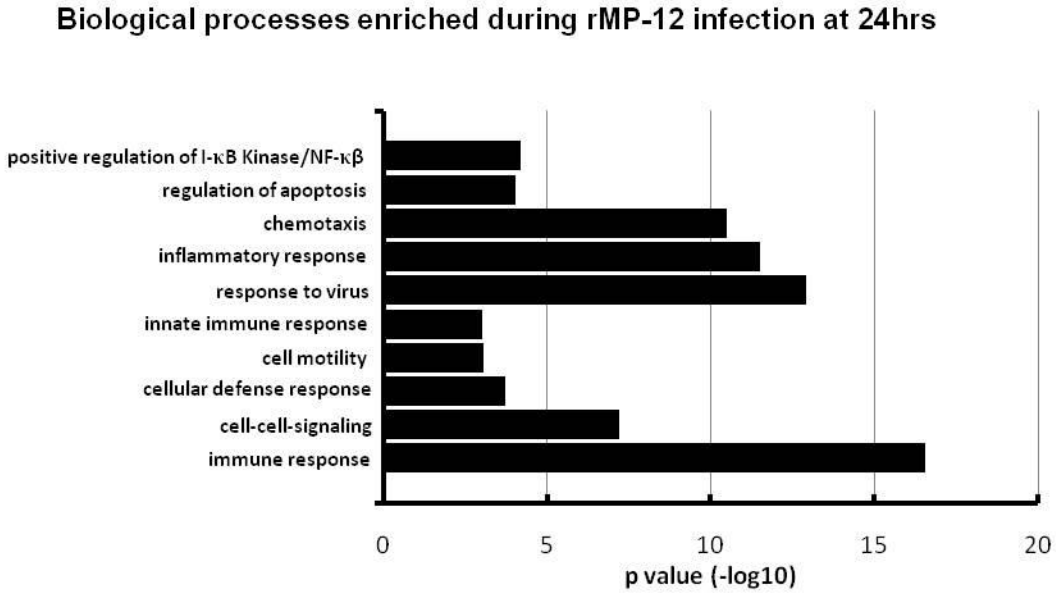
p value (-log10)

B



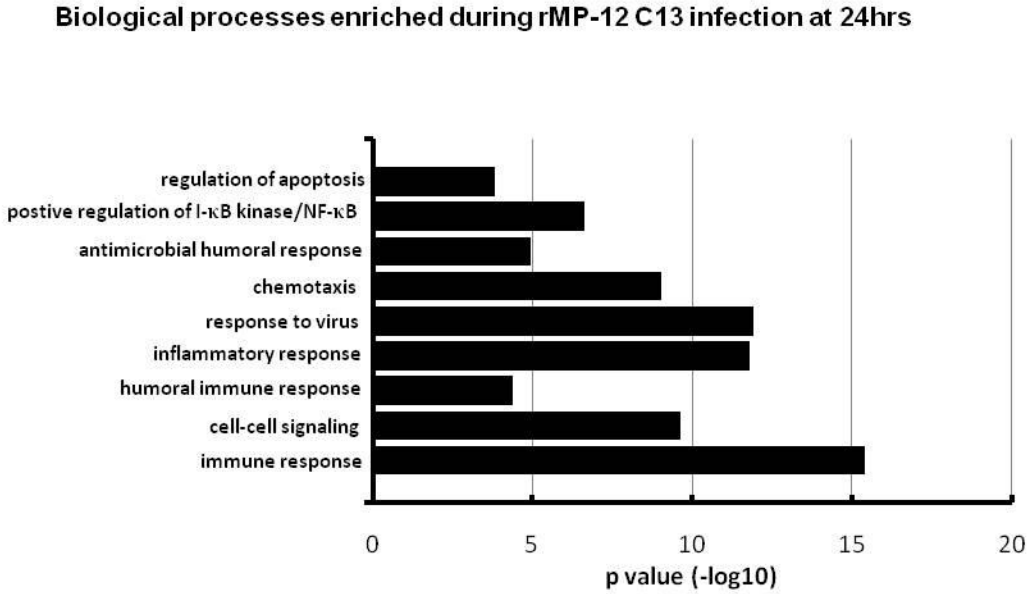


Figure 4.3: Implied biological processes of RVFV induced up-regulated genes. GO-based functional analysis reveals a similar subset of functional processes overrepresented by up-regulated genes elicited by rMP-12-versus-rMP12-C13type-infecetd MΦ. Up-regulated genes selected at 12 and 24 hrs after rMP-12-versus-rMP12-C13type infection were analyzed against the entire human genome gene set by the GO-based functional analysis. The enriched GO-annotated functional processes inferred by up-regulated genes triggered by rMP-12 and rMP12-C13type over time (i.e., 12 and 24 hrs pi) are presented in A and B, respectively. The height of an individual bar represents the level of the statistical significance of the enriched GO-annotated

term. An adjusted p value of < 0.05 was used as the minimal criterion for selecting enriched biological processes.

In addition to FatiGO analysis of functional associations relevant to the altered genes expressed during rMP-12 and rMP-12 C13-type infection, the use of Ingenuity Pathway Analysis (IPA) was employed to support and identify additional functional pathways that could be implied from the altered genes. While the exact nomenclature of the ontology's between different analysis software exists, IPA identified as "antimicrobial response" and "inflammatory response" highly relevant functions of the genes alternatively regulated by RVFV. The IPA analysis findings supported the observations found by using FatiGO analysis software. In addition many of the biological functions implicated were constructed with a greater number of molecules when the input gene list was from rMP-12 C13-type alternatively regulated genes when compared to rMP-12. The top functions associated with NSs-deletion responses compared to those of rMP-12 typically contained a greater number of genes expressed during infection that were associated with the biological function (Table 4.3).

		rMP-12		rMP-12 C13type	
12hrs	Biological Function	# of Molecules	p value	# of Molecules	p value
	Antimicrobioal response	45	4.11E-17	59	1.30E-15
	Inflammatory response	198	1.38E-17	336	1.30E-15
	Immune cell trafficking	123	1.74E-10	229	9.71E-09
	Cell-to-cell signaling	172	6.70E-10	245	9.71E-09
	Antigen presentation	85	6.51E-08	149	1.27E-08
24hrs					
	Antimicrobioal response	35	4.41E-16	56	1.72E-13
	Inflammatory response	193	3.29E-22	356	5.48E-16
	Immune cell trafficking	123	6.87E-13	247	2.02E-12
	Cell-to-cell signaling	183	1.04E-10	356	6.23E-08
	Antigen presentation	81	1.97E-09	119	7.37E-11

Table 4.3: Immunologically related biological processes associated with significantly altered genes.

The top canonical pathways associated with the altered genes of rMP-12 infection generated consisted of “interferon signaling”, “role of pattern recognition receptors in recognition to bacteria and viruses”, “Communication between innate and adaptive immune cells”, “activation of IRF by cytosolic pattern recognition receptors”, “TREM1 signaling” (Table 4.4). Again the number of genes involved in each signaling pathways were more abundantly expressed during rMP-12-C13type infection. By comparison the top canonical pathways associated with rMP-12 C13-type infection at 12hrs were “activation of IRF by cytosolic pattern recognition receptors”, “Role of RIG-I like receptors in antiviral innate immunity”, “role of pattern recognition receptors in recognition to bacteria and viruses”, “toll-like receptor signaling”, and “interferon signaling”. At 24hrs after infection many of these same pathways were still implicated (Figure 4.4)

Signaling Pathway	rMP-12 12hrs Genes expressed/Genes in pathway	rMP-12 12hrs p value	rMP-12 C13-type 12hrs Genes expressed/Genes in pathway	rMP-12 C13-type 12hrs p value
Interferon signaling	17/36	7.38E-12	21/36	1.80E-09
Role of RIG-I like receptors in antiviral innate immunity	10/51	5.42E-04	28/51	7.87E-13
Activation of IRF by cytosolic pattern recognition receptors	17/74	7.24E-07	37/74	2.16E-14
Role of pattern recognition receptors in recognition of bacteria and viruses	22/89	4.85E-09	37/89	4.64E-11
Communication between innate and adaptive immune cells	19/109	6.94E-07	27/109	8.09E-05
TREM1 signaling	16/25	7.88E-07	25/69	3.39E-07
Toll-like receptor signaling	9/55	7.88E-03	15/55	1.76E-02

Signaling Pathway	rMP-12 24hrs Genes expressed/Genes in pathway	rMP-12 24hrs p value	rMP-12 C13-type 24hrs Genes expressed/Genes in pathway	rMP-12 C13-type 24hrs p value
Interferon signaling	16/36	1.26E-11	19/36	4.57E-08
Role of RIG-I like receptors in antiviral innate immunity	8/51	3.60E-03	22/51	4.99E-08
Activation of IRF by cytosolic pattern recognition receptors	13/74	7.37E-05	31/74	3.25E-10
Role of pattern recognition receptors in recognition of bacteria and viruses	19/89	7.97E-08	36/89	6.97E-11
Communication between innate and adaptive immune cells	15/109	4.06E-05	27/109	1.76E-05
TREM1 signaling	15/69	7.46E-07	29/69	3.61E-10
Toll-like receptor signaling	8/55	1.09E-02	16/55	3.90E-03

Table 4.4: IPA analysis of signaling pathway activity implied from gene expression during rMP-12 and rMP-12-C13type infection.

Many of these implied pathways were indicated as part of the most highly relevant response pathways to either NSs-intact or NSs-deleted virus, however comparing the top 10 canonical pathways for rMP-12-C13 type to their corresponding significance during rMP-12 infection typically rMP-12 C13-type infection generated p-values which indicated a higher degree of pathway utilization than that generated by the expression of rMP-12 altered genes (Figure 4.4).

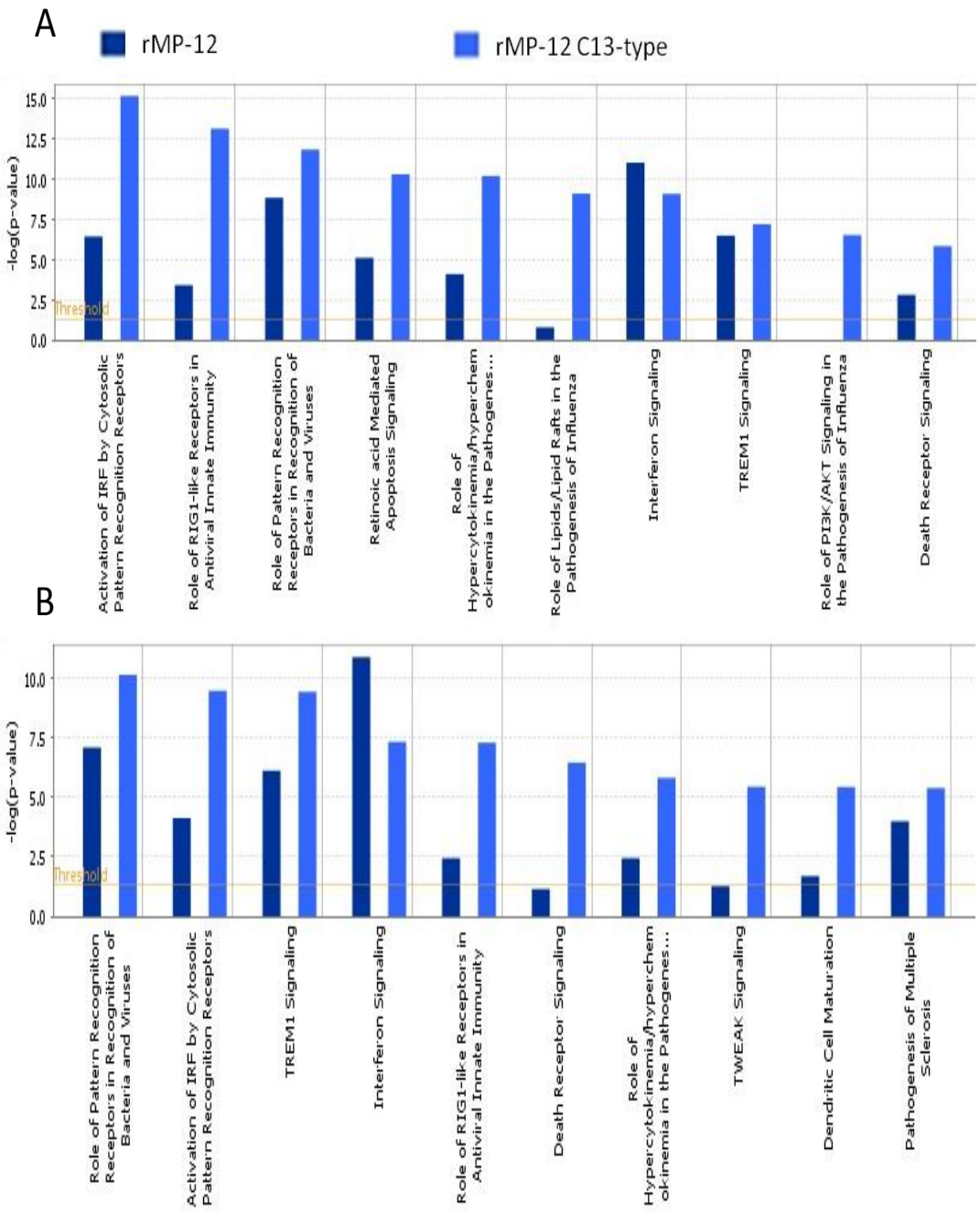


Figure 4.4: IPA analysis of the top-ten signaling pathways implied during rMP-12-C13type infection. Signaling pathway involvement of genes altered in response to rMP-12 or rMP-12-C13type infection, compared to mock-infected controls are shown,12 hours after infection (top panel) and 24 hours after infection (bottom panel). Statistical significance was determined using Ingenuity pathway Analysis after multiple hypothesis correction (B-H p value ≤ 0.05), converted to –log for graphing purposes. Bars represent p value converted to –log10 scale for graphical representation.

The desire to obtain information on the signaling cascades which involved the genes exclusively altered by either the presence or absence of NSs led to further IPA investigation of the genes uniquely expressed in response to rMP-12 or rMP-12 C13-type infection. Because only 4 genes were uniquely altered during rMP-12 infection it was impossible to gather statistically significant functional data from such little data. While only 4 genes could be detected as exclusively expressed in response to rMP-12, 255 uniquely altered gene responses could be detected across 24hrs of rMP-12 C13-type infection. Analysis of the 255 genes uniquely modulated during rMP-12-C13type infection by ingenuity pathway analysis (IPA) revealed significantly over-represented signaling pathways related to several antiviral immune system communication mechanisms. Specifically RIG-I signaling and IRF activation were significantly implicated in the signaling processes of genes altered during RVFV infection (Figure 4.5). The identification of these pathways as the top biological processes associated with the cluster of 255 genes exclusively altered during infection with NSs-deleted virus is also indicative that while RIG-I gene expression and IRF signaling are also detectable during responses to NSs-intact infection, the additional molecules altered by rMP-12 C13-type infection likely lead to a more robust and complete functional operation of the RIG-I and IRF signaling pathways for producing antiviral effects. In addition to RIG-I and IRF signaling functions, signaling pathways involved in cytokine signaling, TLR signaling, dendritic cell maturation, angiopoietin signaling, and MAPK signaling were some of the most highly indicated signaling pathways associated with genes differentially altered by rMP-12 C13-type but not rMP-12 infection (Figure 4.5).

RIG-I is one of the well known cytosolic receptors for viral RNA intermediates produced during the replication cycle [175-176]. As opposed to the RNA sensing Toll-like receptors (TLR-3, -7, and -8) which are membrane bound [177-180], RIG-I and MDA-5 reside within the cell cytoplasm [181], and ideal location as it is where the majority of negative sense RNA viruses replicate. The strong implication of RIG-I signaling during infection with RVFV was not surprising, as the RIG-I gene was one of the most consistent and highly up-regulated pattern recognition receptors (figure 3.4) during both rMP-12 and rMP-12-C13type infections. Furthermore, IRF signaling activation, which would occur as part of the downstream events directly related to RIG-I or other pattern recognition receptors signal transduction, was implicated by IPA analysis as the second most significantly functioning pathway associated with the genes expressed in response to rMP-12 C13-type infection. The combined presence of these intimately linked processes supports the probability that the RIG-I/IPS-1/IRF signaling pathway is one of the most critical factors for sensing RVFV infection and initiating antiviral responses. Furthermore, the top signaling pathways implied from the 255 rMP-12-C13type unique genes agreed with the much larger number of genes associated with RIG-I and IRF signaling in rMP-12-C13type infected cells vs rMP-12 at both 12- and 24hrs after infection. Taken together it is clear that NSs functions to prevent the early and sustained expression of a large number of genes with key functions of the RIG-I and IRF antiviral signal transduction pathways.

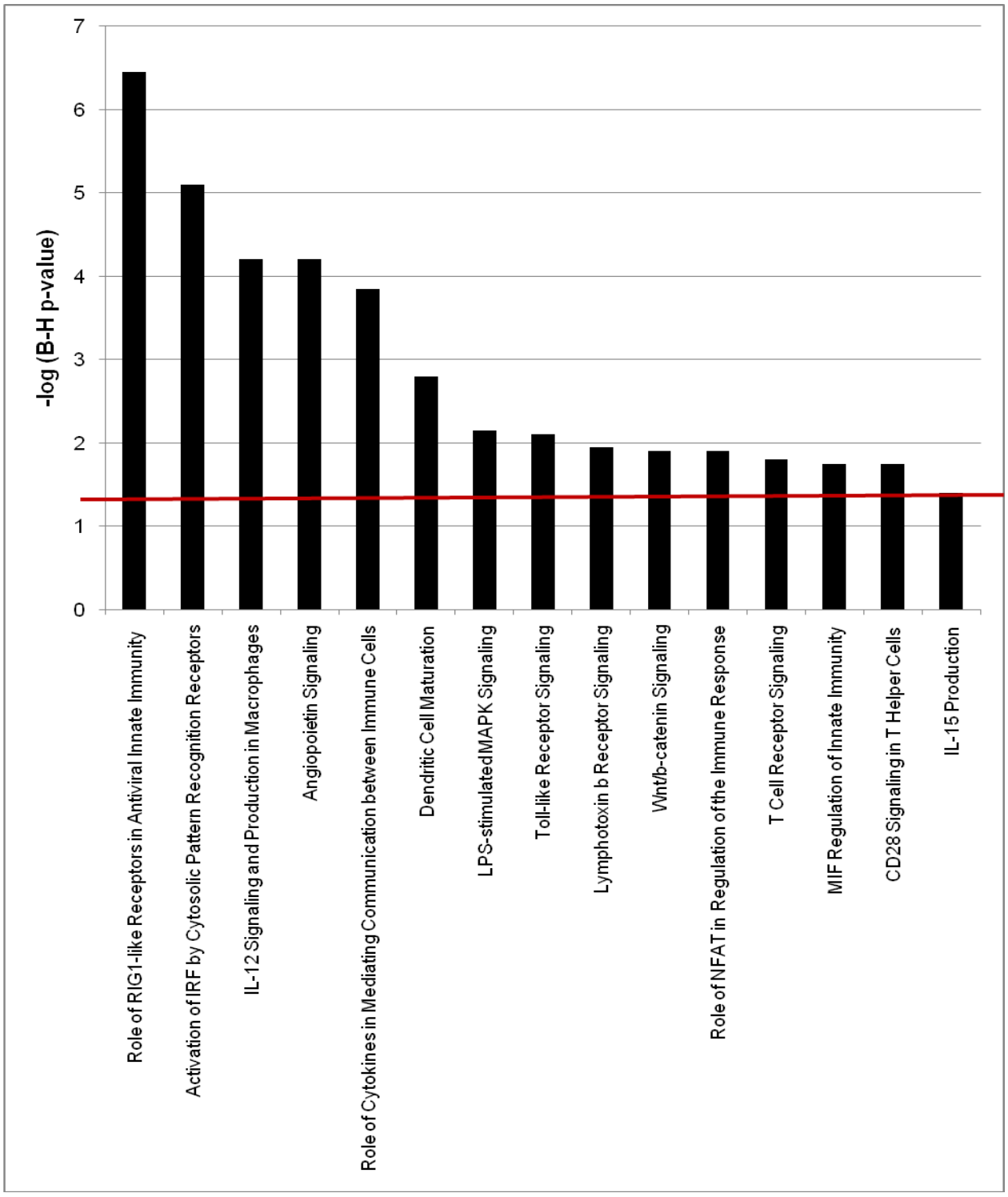


Figure 4.5: Signaling pathways relevant to genes exclusively altered in the absence of NSs. Immune-related signaling pathways over-represented by the 255 genes whose expressions were exclusively altered in rMP-12/C13type-infected MΦ. Statistical significance was determined using IPA after multiple hypothesis testing correction (B-H p value ≤ 0.05, converted to –log for graphing purposes).

Of the 255 genes showing exclusive altered gene expression responses during rMP-12-C13type infection, the most significant physiological process represented by genes uniquely altered in response to rMP-12 C13type infection, compared to mock controls, was cell-mediated immune response which was (43 genes, B-H adj. p values $1.0 \times 10^{-5} – 6.1 \times 10^{-2}$) according to IPA analysis.

The antigen presentation signaling network shown in (Figure 4.6) indicates that rMP-12-C13type inoculation results in the production of IgM and IgG antibodies. The genes overlaid onto the antigen presentation signaling network are derived from genes only altered during rMP-12-C13type infection. Interestingly while FatiGO analysis also identified a much stronger and more persistent humoral immune response, "cellular defense responses" were exclusive to MΦ responses against rMP-12 inoculation (Figure 4.3). IPA analysis however detected a cell-mediated immune response from the genes exclusive to rMP-12C13type infection. Importantly the cell-mediated physiological process category included several over-lapping signaling networks within this group that could be used to further describe the precise actions of the 255 genes. The exclusive up-regulation of a subset of genes involved in the antigen presentation signaling network is somewhat surprising and suggests that the timing or magnitude of antibody responses may differ between the two strains.

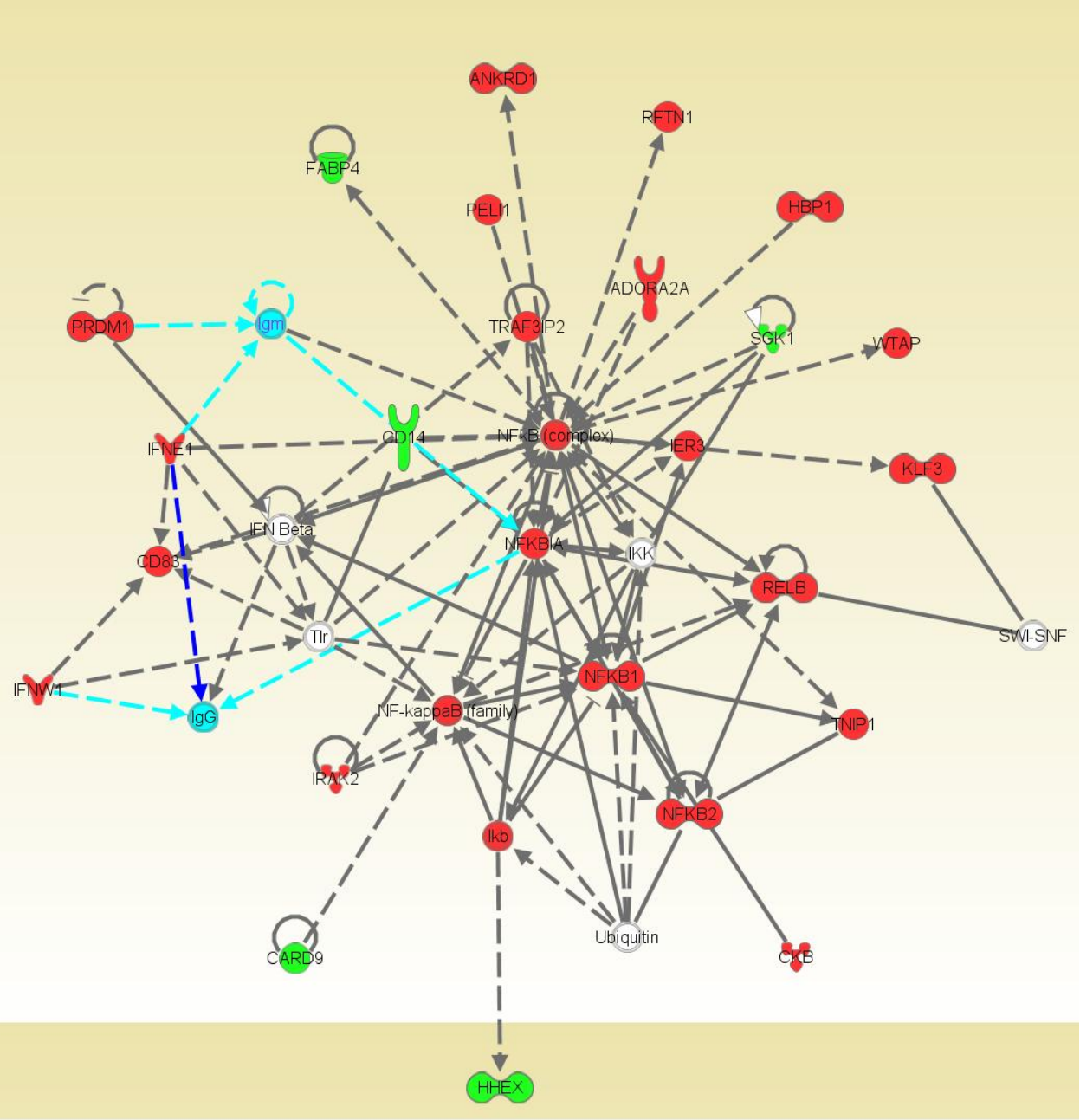


Figure 4.6: Ingenuity Pathway Analysis cell-mediated immunity signaling network. The 255 genes uniquely altered by rMP-12/C13type exposure, compared to unexposed controls, indicated the cell-mediated immunity signaling network involved in antigen presentation as one of the top functions. Expression data from rMP-12 C13-infected MΦ at 12 or 24 hr was overlaid onto the general network (red, indicates induction in rMP-12/C13type-exposed cells compared to mock controls, and green indicates down-regulation in response to viral exposure. Molecules shaded gray were not significantly altered at the level of transcription, based on microarray analysis. Antibodies that are most likely produced as a result of rMP-12/C13type-mediated gene activities involved with this signaling network are highlighted in bright blue.

Discussion

As expected the intensity and diversity of innate immune responses was greater when cells were inoculated with rMP-12-C13type virus than with rMP-12. Perhaps the most significant difference between the host responses was the more rapid kinetics of host transcriptional responses when reacting to rMP-12-C13type virus.

When the complete lists of altered transcriptional responses to rMP-12 and rMP-12-C13type viruses were analyzed by IPA software, immunologically important biological functions and signaling pathways were statistically implied as having a greater functionality when the NSs gene was removed from RVFV. This is in agreement with the overall greater numerical diversity and intensity of altered genes induced by rMP-12-C13type infection identified in (Chapter III). Specifically by analysis of genes only induced during rMP-12C13type a more robust construction of the RIG-I and IRF signaling pathways could be assumed. The greater significance associated with the implied functions of rMP-12-C13type responses is not totally surprising; as the number of genes from the list of rMP-12-C13type induced transcriptional responses was nearly double that of rMP-12. These results further support the hypothesis that MΦ cells infected by NSs-deleted RVFV more rapidly and intensely mount innate immune responses. Through computational gene-ontology analysis, in addition to the identification of a wide variety of individual genes affected by RVFV infection and NSs function, previously unnoticed differences in cellular processes of innate immunity such as humoral immune response, cell-to-cell signaling, and immune cell trafficking were extracted. Though most of these biological processes were similar between rMP-12 and rMP-12-C13type infection differential implication on the stimulation of innate immune responses leading to “humoral immune response” were detected as more intense and persistent during rMP-12-C13type

infection. Conversely though host responses against rMP-12 were not as capable of sustaining the necessary pattern of gene expression to imply "humoral immune response" through 24hrs, the gene expression pattern implicating "cellular defense responses" was generated occurred exclusively in response to rMP-12. While detailed in vivo experiments are needed to officially validate this implication, these data provides novel evidence that NSs function extends its influence on human innate immunity all the way to processes that will impact regulation of adaptive immunity.

While antibody production in response to the NSs-deleted RVFV is not unexpected, the exclusive up-regulation of the genes involved in this antigen presentation signaling network is somewhat surprising and suggests that the timing or quality of antibody responses may differ between the two strains as a result of vaccination. It is unclear whether humoral or cellular defense mechanisms are more critical for protection against RVFV. Both NSs-intact and NSs-deleted attenuated RVFV strains are capable of protecting animals from lethal challenge, suggesting that the safest and most highly effective vaccination strategy would be to combine the attenuating features of the authentic MP-12 and Clone 13 strains. However, the robustness of the early innate immune response mounted against NSs-deleted RVFVs, specifically the highly attenuated rMP-12-C13type virus, may replication and dissemination in vivo to such an extent that sufficient amounts of virus antigen are not available to produce long-term immunity [84].

Identification of the biological processes associated with gene expression provides insight as to what are the final cellular and molecular functional goals of the transcribed genes. While information on the end result functions is critical, the design of therapeutic strategies aimed at augmenting or reducing the activities of pathologically important biological processes require detailed understanding on the regulatory elements that comprise the signaling cascades necessary

to sustain the biological functions. The regulatory elements required to relay "danger" messages through antiviral and immunomodulatory signaling cascades often converge on only a few common critical signal transduction proteins or transcription factors. These convergence points may act as inhibitors or inducers for the expression of a wide range of genes involved in varying immunologically important biological processes. Viruses often encode for proteins that prevent the completion of one or more signaling cascades or transcription factor functions by targeting these convergence points. The mammalian innate immune system has evolved multiple systems of redundancy, which includes partially overlapping abilities to both initiate and respond to immune stimulants in order to overcome viral mechanisms of immune suppression. Some of the most well studied innate immune signaling pathways stem from the initial detection of virus RNA through TLR and/or RIG-I like receptors. Ultimately these signaling pathways may activate transcription factors of the IRF, NF-kB or AP-1/c-Jun systems in virus and cell type specific fashions. It has previously been shown that NSs works to block the latter stages of antiviral signaling by preventing the binding of transcription factors to the IFN-β promoter but not prevent their activation or migration to the nucleus [119]. Other than the negative interference with IFN-β expression, the impact of NSs activity on the ability of transcription factors to initiate the expression of their target genes is not well known. Part of the objective of transcriptional analysis was to find the most significant transcription factors utilized during RVFV host responses. Furthermore the identification of differentially utilized transcription factors as a result of NSs interference was important for gaining insight on previously unidentified targets of the RVFV NSs protein.

Interestingly by GO term analysis of the up-regulated genes expressed in rMP-12 and rMP-12-C13type infections, the IRF-7 TF was indicated as having a prominent role in the

regulation of host response genes (Figure 4.2). As it has been previously demonstrated that the NSs protein contributes to the virulence of RVFV by suppressing the ability of IRF-3 mediated IFN-β gene expression [119-120], the activity of IRF-7 could serve as a critical compensating means for mounting host defenses during inoculation with NSs-intact RVFV as the NSs protein will greatly inhibit the functions of IRF-3 mediated host responses. The exclusion of "ICSBP/IRF-8" and "NF-κβ p65" TF association GO terms during 12 and 24hr inoculation with NSs-intact RVFV highlights the potential of the NSs protein to restrict the full activation of proinflammatory mediators through NF-κβ and multiple interferon regulator factor dependent signaling pathways. Interestingly, the enrichment of genes associated with the NF-Y transcription factor occurred exclusively during inoculation with NSs-intact RVFV. Examining the contribution of RIG-I, NF-kB, IRF-7 genes through knock-out animal experiments or by functional knock-down with short-hairpin RNA is an important next step for determining what host transcriptional regulator or regulators controls the gene expression of the most important factors for conveying protection against RVFV. The improvement of vaccine or therapeutic strategies may depend on the ability to increase IRF-8, IRF-7 or NF-kb activation during rMP-12 infection or conversely decrease these transcription factors activities during rMP-12-C13type infection to bring the humoral defense responses and cellular defense response more in balance.

Farthest upstream of the signaling cascades that eventually result in immunologically relevant biological processes, are pattern recognition receptors responsible for detecting foreign invaders and initiating the antiviral signaling cascades which eventually lead to the activation of the previously mentioned transcription factors. While there are several pattern recognition receptors capable of detecting viral single-stranded or double-stranded RNA, transcriptional analysis found that the RIG-I and TLR-3 pattern recognition receptor signaling molecules had

increased expression in rMP-12 and rMP-12-C13type infection (Figure 3.4). This suggested that the signaling cascades induced by these two molecules may play more significant roles during the host responses towards RVFV than other possible viral RNA sensing proteins, such as TLR-7 or MDA-5, which were not expressed as vigorously. The use of IPA analysis determined that altered gene expression patterns implied that assembly of the RIG-I and IRF signaling pathways were highly active during infection with either RVFV strain, however the expression of genes related to these pathways was more than doubled in response to rMP-12-C13type infection when compared to the altered gene expression induced by rMP-12 virus (Table 4.3). This gross enhancement of genes related to RIG-I signaling during rMP-12-C13type infection implied that, while IRF-3, NF-κB, and AP-1 may still be activated during RVFV infection, the full performance of RIG-I mediated antiviral signaling was limited specifically at the step of IRF-3 binding to the IFN-β promoter as previously described [119], but also by the reduced abundance of signaling components further upstream. While microarray analysis supports data showing transcription factor access to the IFN-β promoter was blocked, a significant gap remained as to the consequences of NSs expression on the function of factors downstream of IFN-β expression. Transcriptional analysis also had the advantage of revealing the impact of NSs on upstream genes that may limit the full performance potential of the RIG-I/IRF signaling cascade.

To gain greater appreciation and visualization of the depth to which NSs modulates the expression of genes required for completion of antiviral signaling pathways analysis of the 255 unique genes produced during the response to NSs-deleted RVFV but not that of RVFV with functional NSs was performed. IPA analysis indicated that genes only expressed during rMP-12-C13type infection were most significantly dedicated to the operation of several signaling pathways. Interestingly the “RIG-I because these genes were restricted during the entire course

of rMP-12 infection it can be reasonably estimated that the signaling pathways that utilize their activity were not operating in their full capacity. The implied greater intensity of the intimately connected RIG-I and IRF signaling pathways during both rMP-12 and rMP-12C13type infections compared TLR signaling is an indication that even though strong TLR-3 signaling is identified at the transcriptional level (Figure 3.4), innate immune defenses against RVFV may depend more heavily on RIG-I mediated viral RNA detection.

Chapter V: Summary and Conclusions

SUMMARY

Should RVFV spread beyond the African continent to other geographic areas where ruminant species and humans are largely "naïve" to this mosquito-borne viral agent, the current preventive and therapeutic methods of treatment would be inadequate for controlling the devastating impact on public health and economic activity. Whereas the usage of live attenuated RVFV strains as vaccines is among the best preventive strategies, the variations in the genetic makeup of vaccine candidates, medical histories of human recipients, and the concerns over the potential reversion to wild type and/or undesirable safety and efficacy outcomes in farm animals and especially humans make accurate prediction of their prospective application challenging. Thus, advancing scientific knowledge of how permissive mammalian species respond to infection and/or vaccination against RVFV is an important endeavor to undertake this pivotal viral agent.

It has been well-established that NSs functions as a critical virulence factor for RVFV and other members of the bunyaviridae largely through its ability to interfere the host innate antiviral responses [78,182-185]. Specifically, the presence of RVFV NSs promoted a generalized transcriptional suppression of infected hosts and potently antagonized the expression of type I IFNs [77,118]. In addition, NSs has been demonstrated to initiate the proteasomal degradation of PKR [99-100]. However, whether other antiviral molecules and/or functions could also be affected by RVFV NSs protein have not been systematically investigated, making it an obstacle to fully understand RVFV pathogenesis and advance the countermeasures against it. Thus, the employment of cDNA-based microarray functional genomics analyses as a high throughput tool

to compare the impact of RVFV NSs protein on the global and temporal host gene response of human primary MΦ infected with NSs-intact-rMP-12-versus-NSs-defective rMP-12-C13type allowed the most expansive delineation of the impact of RVFV NSs on global host response to RVFV infection.

For the first time analysis of the intrinsic innate immune responses against RVRV were examined in primary classical innate immune cells of human origin. Not only was the model highly relevant to the initial stages of infection, it also expanded the scope of proinflammatory factors and cellular functions previously associated with the impacts of NSs activity. Here it was identified that RVFV infection causes the dramatic restriction of not only type I IFN, but also proinflamatory cytokines, chemokines, and the progression towards a phenotypic "Th1" activated MΦ.

Supporting the observed phenotypic changes to the intrinsic functions of MΦ infected by RVFV., global transcriptional analysis of the host responses occurring during RVFV infection of primary human cells revealed a large number of genes that were differentially expressed in response to NSs-intact vs NSs-deleted RVFV. The most significant differences in transcriptional responses, when comparing those induced by rMP-12 vs rMP-12C13type, was not the diversity of genes altered by either virus, but rather the kinetics of their modulated expression. NSs-intact RVFV delayed the induction of transcriptional responses relative to those generated against NSs-deleted RVFV. Additionally NSs functioned to reduce the intensity and sustainability of antiviral and immunomodulatory gene expression. The large overlap in overall modulation of transcriptional activity was demonstrated by the 2,748 transcriptional responses which were common to both NSs-intact and NSs-deleted RVFV inoculation across the 24hours of analysis. While microarray analysis supported the notion that NSs functions to reduce transcriptional

expression of a vast number of genes, the results slightly disagreed with the idea that NSs would near completely abolish de novo mRNA expression [118]. In fact, even the use of increased MOI which could theoretically negatively impact the primary antiviral response more thoroughly by infecting a greater percentage of cells, caused higher amounts of IFN-α/β secretion from MΦ cells than with relatively lower MOI (Figure 5.1).

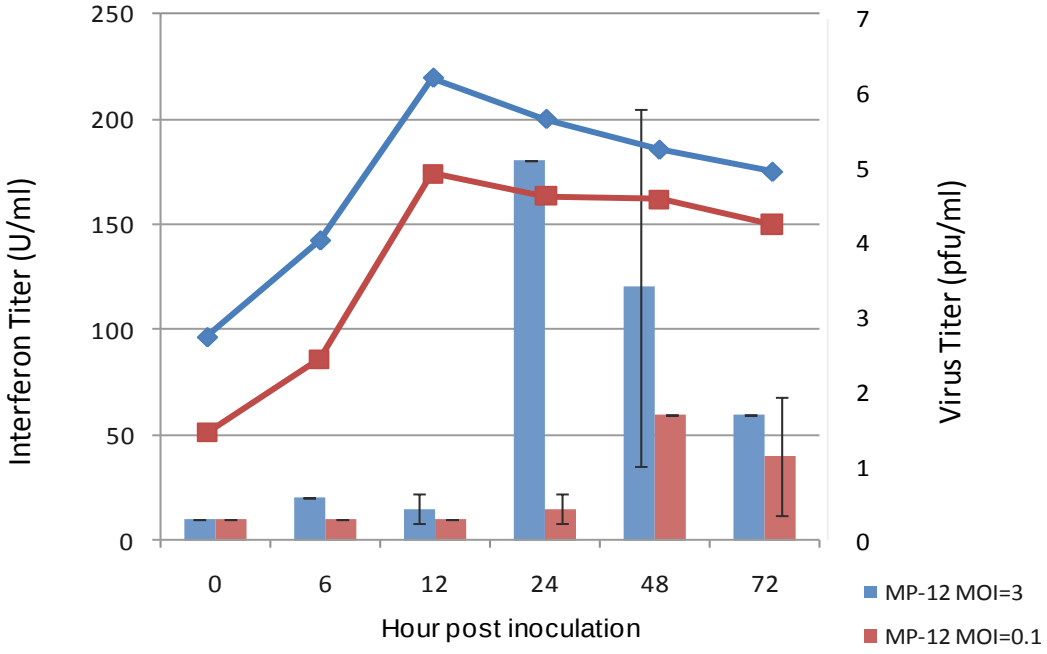


Figure 5.1: Higher multiplicity of infection (MOI) with RVFV results in more rapid and abundant type I IFN production. Cell-free supernatant aliquots from human MΦ cells inoculated with MP-12 virus secreted IFN-α/β as determined by VSV-based plaque reduction assay. Bar-graph of interferon titers overlaid with line-graph of virus titers of MP-12 recovered in supernatant aliquots during corresponding time point post inoculation. MOI-3 (blue line/bars) or MOI = 0.1 (red line/bars). Data shown are representative of three independent experiments with similar results. Mean ± SD.

Confirming the detected overlap but differential intensity in gene expression of cytokines and chemokines during rMP-12 and rMp-12-C13type infection, the protein secretion of proinflammatory cytokines and chemokines was confirmed from RVFV inoculated MΦ. As predicted, removal of NSs function during RVFV infection resulted in the dramatic enhancement

in capacity of MΦ cells to produce proinflammatory factors. Furthermore it was demonstrated that RVFV infected MΦ undergo a phenotypic maturation resembling that of "classic" Th1 type. The scale of phenotypic modulation towards this maturation profile was restricted by NSs function. The extensive proinflammaory cytokine profile generated including the expression of IFN-γ and TNF-α by MΦ likely contributed to the steering towards Th1 phenotype.

Interestingly interferon signaling was the most significant pathway derived from transcriptional responses to rMP-12 infection (Table 4.4). Here the more significant *p* value associated with IFN signaling of rMP-12 transcriptional responses compared to that of rMP-12 C13type (Figure 4.4 and Table 4.4) responses suggest that due to the relatively smaller list of input genes, (1029, for rMP-12) vs (2332, for rMP12-C13type), the over-representation of genes linked to "interferon signaling" out of the total number of genes from the rMP-12 altered gene list is proportionally greater than genes from the larger rMP-12-C13type transcriptional responses dataset. In this case even though relatively little interferon was detectable at the transcriptional or protein level compared to rMP-12-C13type, the attempt by host cells to focus a large fraction of host defense efforts to the production of elements involved in the interferon signaling pathway highlights the importance of this host defense mechanism during RVFV infection. However, the greater total number of genes expressed by rMP-12-C13type infected MΦ typically resulted in more constituent genes of the implied signaling pathways being expressed in rMP-12-C13type infected MΦ compared to rMP-12. For instance at 12hrs post rMP-12 infection 17/36 genes comprising the "interferon signaling" pathway were alternatively regulated giving a p value of 1.8E-09, however during rMP-12 C13-type infection 21/36 genes related to "interferon signaling" were alternatively regulated providing an observed p value of 7.38E-12. For many functions, other than interferon signaling, the more significant p-values

were associated with pathways associated with rMP-12 C13type infection (Table 4.4) Implicating that the over-representation of genes associated with rMP-12-C13type pathways is intense and likely correlates to more intense phenotypic activity relevant to host responses.

Among the possible viral detection mechanisms, RIG-I and TLR3 were the most prominently up-regulated pattern recognition receptor genes during both RVFV infections. The early induction, intensity, and persistence of the enhanced expression of these two genes suggested that they were highly involved in the onset of antiviral signaling cascades which would ultimately lead to the production of the observed IFN and other cytokine responses. Furthermore, functional analysis by IPA identified the commitment of a significant number of up-regulated genes were associated with the RIG-I signaling pathway. While TLR-3 gene expression was highly up-regulated during rMP-12 and rMP-12-C13type infection (Figure 3.4), the significance of TLR signaling was indicated to not be as robust during rMP-12 or rMP-12-C13type host defenses. The suggestion that RVFV relies on RIG-I mediated identification of the virus is reflective of the number of genes related to the RIG-I signaling and IRF signaling pathway compared to the lesser quantity of genes related to TLR signaling (Table 4.4). Perhaps more importantly, NSs function during rMP-12 infection appears to have restricted the expression of a significant number of RIG-I related genes (Table 4.4). The importance of the NSs restricted genes in RIG-I signaling is also supported by the finding that the most inferred signaling function of the 255 genes exclusively expressed during rMP-12-C13type infection are RIG-I and IRF signaling (Figure 4.5).

For the first time analysis of the host responses inspired by RVFV infection of human innate immune cells was examined in a highly detailed fashion. Largely confirming the hypothesis that NSs-deleted rMP-12 RVFV would induce a more rapid and intense profile of

innate immune responses than NSs-intact RVFV. The impact of NSs in dysregulating cellular host defense functions was obvious at the protein and transcriptional levels. While the global transcriptional profiling generated a wealth of information on previously unknown host responses modulated by NSs expression, a limitation of this study remains that transcriptional responses were against viruses of attenuated phenotypes, and therefore the differences between rMP-12 and rMP-12-C13type host responses observed in these studies cannot be fully regarded as patterns of gene expression totally identifiable with RVFV pathogenic mechanisms. Assessing the full scope of host factors modulated during pathogenic RVFV and their role in severe disease can only be achieved using non-attenuated strains such as ZH501.

The pathology observed during viral hemorrhagic fevers, such as Ebola and Lassa, has been suggested to stem from immunopathological based processes resulting in damage to endothelial cells [186-187]. While the results found through transcriptional and cytokine analysis indicate that the proinflammatory profile of innate immune system functions is activated during RVFV infection, the intense and excessive innate immune responses observed during other proposed virus induced immunopathological infections is not evident. Contrarily, NSs-intact RVFV greatly restricted innate immune responses. This kind of immune dysregulation while important for understanding the disease process and mechanisms for protection is not indicative of a disease with major proinflammatory basis. The results generated through analysis of human MΦ infection with RVFV therefore support the hypothesis that the majority of RVFV pathogenesis is a result of direct-tissue injury due to the innate immune systems inability to restrict replication and infection of multiple organ systems. Here we further identified that dedicated immune cells may also be important targets of RVFV infection, and may contribute to the local or systemic pathogenesis of RVFV infection.

Though exuberant production of proinflammatory mediators during RVFV infection is not a generalized feature of the host response, a limited number of cytokines were induced to a high degree even in the presence of NSs function. In the absence of a more diverse innate immune response, the more limited scope of secreted cytokines and chemokines in NSs-intact RVFV such as IP-10 and IL-15 may be significant factors determining pathogenesis in liver injury. For instance, the elevated expression of IL-15 has been found during infection with other hemorrhagic fever viruses [188-190]. Thus the unbalanced production of IL-15 during RVFV infection could be an important factor for inducing the activation of NK cells capable of limiting virus spread. It would be interesting to evaluate the role of NK cells during RVFV pathogenesis, and the capacity for IL-15 production during infection with virulent strain (ZH501) RVFV.

How the expression of NSs protein affects host mechanisms that link innate to adaptive immune responses were also observed for the first time, Chapter IV (Figures 4.3 and 4.6). The limitations of transcriptional level analysis restrict the bioinformatics based evaluations of the biological processes involving the innate and adaptive arms of immune immunity to being predictive in nature. While this is one drawback of transcriptional analysis, the implied propensities for stimulating signaling cascades leading to B cell activation and eventual immunoglobulin production were supported by protein level analysis showing that the enhanced surface expression of phenotypic markers on MΦ being induced more prominently by rMP-12-C13type infection than rMP-12 (Figure 2.5). In contrast, NSs-intact RVFV transcriptional responses suggested a unique reliance upon activation of cellular defense mechanisms, in addition to a less intense and persistent humoral immune responses.

Though antibody responses are often correlated with RVFV vaccine success [70-72], the contribution that cellular defense responses provide to either independently or in concert with

antibody mediated responses influence the quality of long-term protective immunity in animals or humans after RVFV vaccination is uncertain. It can therefore only be assumed that a balanced response sharing both features of both B cell and T cell mediated immunity is the most effective immune protection.

In addition, variations in genetic, cellular, or biochemical baselines among human individuals and various livestock species may influence the success or failure of particular vaccine platforms [66,191-193]. Ideally this consideration would be addressed through the availability of screening tools and availability of several modified vaccine preparations so that clinicians or veterinarians could select the optimal vaccine application. For most diseases however, multiple vaccine candidates and individualized screening methods are not available. Expanding analysis on global transcriptional responses against NSs-intact and NSs-deleted RVFV to multiple individuals of diverse genetic backgrounds or even various livestock species where necessary, would provide a better understanding of how the host genetics affect the outcome of infection or vaccination. This information would be highly beneficial to selection of the best vaccine candidates particularly when demographic information needs to be considered.

FUTURE EXTENSIONS OF RESEARCH

Improvements to the development of preventative and therapeutic strategies against RVFV will require better understanding of the host biomarkers of the initial stages of infection which indicate infected individuals will experience severe disease vs mild disease and recovery. Additionally, developing systems biology approaches capable of discerning host response profiles likely to result in the education of a well balanced adaptive immune response will aid the design and selection of vaccine candidates. Shown in this study, the utilization of transcriptional level analysis provides a powerful method for characterizing the potential beneficial or

determinantal factors involved in education of a quality immune response against RVFV. Further extending transcriptional analysis to include profiling of host responses induced by pathogenic vs attenuated vaccine strains will help clarify the molecular signatures or "danger signals" which can be used to focus rational design efforts for therapeutics targeted against differentially expressed genes and their corresponding biological functions.

Several signaling pathways and genes were differentially involved during rMP-12 vs rMP-12/C13type exposed human MΦ, including higher activity of NF-kB, IRF-7, and C-rel transcription factors in response to rMP-12/C13type exposure (Figure 4.2), the implication of RIG-I mediated signaling as the main pattern recognition receptor leading to the onset of antiviral signaling cascades during RVFV (Figures 4.3-4.5), and the more extensive expression of genes leading to stimulation of IgM and IgG production (Figure 4.6). The identification of these genes through transcriptional and statistical analysis warrants their priority as targets for analysis of their roles in shaping RVFV pathogenesis at more complete biological level. The evaluation of the biological impact of these targets by functional gene knockdown studies in vitro or in vivo should be an immediate experimental priority.

As pathogenic RVFV strains achieve a more absolute restriction of host antiviral mechanisms during establishment of infection, it should be taken into consideration that the attenuating mutations within the L and M segments of MP-12 and rMP-12 may also reveal virulence mechanisms complementary to NSs host virulence factor functions. While these hypothetical L and M segment factors may be difficult to validate considering they may only weakly modulate host defenses when functioning individually, there is the strong possibility that L or M segment functions have direct additive effects to the virulence activities NSs when expressed in conjunction. Taking into account the considerable reliance on NSs for RVFV

virulence, it is not surprising that the detection of other mechanisms which alter RVFV pathogenicity and host responses have remained elusive. Investigating the contribution of L and M segment virulence properties, which may more mildly fine tune the host response than NSs, would be beneficial for scientific inquiry and to improve vaccine design.

One way to analyze alterations of the host response as a consequence of L or M segment functions is to use mutant RVFV strains carrying substitutions of virulent strain L and M segments with those of MP-12 and perform global transcriptional analysis of infected cells. These mutant viruses would also need to have deletion of the NSs gene to mediate the discernment of the mild changes in host response characteristics relative to those induced by NSs containing viruses.

As methods for further functional analysis and systems biology investigation improve, the repositories of information generated through high-throughput microarray analysis can be reevaluated to allow a higher degree of detail, predictive power, and substantiation of the interpretations. The ability to re-analyze and cross compare datasets using meta-analysis approaches and multiple bioinformatics analysis tools will aid the finding of common genes, functions, or signaling events important during pathogenic strain infection or vaccination with attenuated virus.

Additionally, constant improvement and expansion of published literature sources from which the molecular connections between the genes and their attributed pathways and functions are derived will allow functional enrichment analysis to become more accurate in the future. The defining of host molecular biomarkers differentially regulated during pathogenic vs attenuated RVFV infection, and subsequent validation of their contribution to pathogenic or protective

processes is a key goal in the progression of improving therapeutic and preventative measures against RVFV.

The results presented herein provide useful data for rationally selecting immunologically relevant biomarkers impacted by the well known RVFV virulence gene, NSs. Future in vivo experimentation testing the significance of the common and differential transcriptional and phenotypic host responses of NSs-intact vs NSs-deleted RVFV have on disease severity will help provide beneficial information for advancing therapeutic and vaccine development.

Appendix

APPENDIX A:

Bio-plex results of RVFV exposed MΦ 12 and 24hrs after exposure. Data displayed as (mean ± standard deviation) of cell secreted cytokines from three replicates. Values for measurements below detection limit (BDL) or above detection limit (ADL) were not included.

	Conc (pg/ml)			
	rMP-12		rMP-12/C13type	
Cytokine	**12hrs**	**24hrs**	**12hrs**	**24hrs**
IL-1ra	26012 ± 2689	41678 ± 4893	40674 ± 7032	65835 ± 1918
IL-2	24 ± 4	29 ± 4	73 ± 10	88 ±15
IL-4	10 ± 3	15 ± 3	50 ± 3	46 ± 7
IL-5	BDL	BDL	BDL	BDL
IL-7	0	15 ± 9	10 ± 9	35 ± 8
IL-8	54019 ± 8758	54607 ± 6858	47255 ± 3567	58517 ± 2136
IL-9	862 ± 33	760 ± 44	1160 ± 131	1126 ± 70
IL-12(p70)	18 ± 1	30 ± 4	18 ± 1	30 ± 4
IL-17	455 ± 51	119 ± 97	900 ± 171	1111 ± 127
Eotaxin	794 ± 115	634 ± 43	1387 ± 212	1357 ± 131
FGF Basic	120 ± 8	51 ± 7	126 ± 8	128 ± 15
G-CSF	94 ± 11	90 ± 10	266 ± 31	300 ± 25
GM-CSF	106 ± 24	78 ± 19	262 ± 35	258 ± 11
MCP-1	52735 ± 3645	93070 ± 12032	84889 ± 24819	237944 ± 58800
MIP-1beta	131062 ± 41224	34731 ± 848	ADL	ADL
PDGF	66 ± 34	40 ± 13	226 ± 85	296 ± 14
VEGF	96 ± 38	110 ± 69	280 ± 71	267 ± 79

APPENDIX B

Gene ID	Gene Name	Gene Symbol	Function	Mock vs C13 12hr	Mock vs C13 24hr
				FC	
NM_001838	Chemokine (C-C motif) receptor 7	CCR7	Chemotaxis; inflammatory response	8.2	12.0
NM_172140	Interleukin 29 (interferon, lambda 1)	IL29	Negative regulation of cell proliferation; positive regulation of STAT; MHC class I biosynthesis	10.5	1.5
NM_002214	Integrin, beta 8	ITGB8	Cell-matrix adhesion; integrin-mediated signaling pathway	7.9	5.2
NM_021068	Interferon, alpha 4	IFNA4	Response to virus	7.4	-
NM_021057	Interferon, alpha 7	IFNA7	Response to virus	6.7	-
NM_006419	Chemokine (C-X-C motif) ligand 13 (B-cell chemoattractant)	CXCL13	Chemotaxis; inflammatory response; elevation of cytosolic calcium ion concentration	-	6.6
NM_002171	Interferon, alpha 10	IFNA10	Response to virus	6.4	-
NM_002177	Interferon, omega 1	IFNW1	Response to virus; cell cycle arrest	5.3	-
NM_001823	Creatine kinase, brain	CKB	Eenergy homeostasis; cellular chloride ion homeostasis	4.1	5.0
NM_021634	Relaxin/insulin-like family peptide receptor 1	RXFP1	Stimulates leukocyte adhesion and migration	4.7	3.3
NM_002175	Interferon, alpha 21	IFNA21	Response to virus	4.6	-
NR_015361	Hypothetical gene LOC440896	LOC440896	Unknown	4.6	2.6
NM_004267	Carbohydrate (N-acetylglucosamine-6-O) sulfotransferase 2	CHST2	Carbohydrate metabolism; inflammatory response	-	4.4
NM_003392	Wingless-type MMTV integration site family, member 5A	WNT5A	Cell growth; JNK cascade	-	4.3
NM_003410	Zinc finger protein, X-linked	ZFX	Transcription regulation	3.9	2.9
NM_006741	Protein phosphatase 1, regulatory (inhibitor) subunit 1A	PPP1R1A	Glycogen metabolism; hormone signaling	-	3.8
NM_002169	Interferon, alpha 5	IFNA5	Response to virus	3.8	-
NM_000609	Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	CXCL12	Chemotaxis; immune response; cell adhesion	-	3.6
NM_012082	Zinc finger protein, multitype 2	ZFPM2	Transcription regulation	3.6	2.0
NM_021268	Interferon, alpha 17	IFNA17	Response to virus	3.6	-
NM_176891	Interferon epsilon 1	IFNE1	Response to virus	3.6	1.8
NM_000857	Guanylate cyclase 1, soluble, beta 3	GUCY1B3	Nitric oxide mediated signal transduction	2.1	3.6
NM_015570	Autism susceptibility candidate 2	AUTS2	Precise function unknown	2.3	3.5

NM_004348	Runt-related transcription factor 2	RUNX2	Osteoblast differentiation	3.5	3.1
NM_024930	ELOVL family member 7, elongation of long chain fatty acids (yeast)	ELOVL7	Fatty acid biosynthesis	2.9	3.4
NM_001570	Interleukin-1 receptor-associated kinase 2	IRAK2	Inflammatory response; I-kappaB kinase/NF-kappaB cascade	2.7	3.4
NM_021202	Tumor protein p53 inducible nuclear protein 2	TP53INP2	Autophagy	1.6	3.3
NM_030766	BCL2-like 14 (apoptosis facilitator)	BCL2L14	Regulation of apoptosis	2.2	3.3
NM_001198	PR domain containing 1, with ZNF domain	PRDM1	Transcriptional repressor that binds specifically to the PRDI element in the promoter of the beta-interferon gene; drives the maturation of b-lymphocytes into Ig secreting cells	3.2	2.8
NM_194250	Zinc finger protein 804A	ZNF804A	Precise function unknown (possibly involved in cell transformation)	1.7	3.1
NM_020895	GRAM domain containing 1A	GRAMD1A	Unknown	2.0	3.1
NM_002448	Msh homeobox 1	MSX1	Regulation of cellular proliferation and differentiation	3.1	1.9
NM_020529	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	NFKBIA	Apoptosis; cytoplasmic sequestering of NF-kappaB; LPS-mediated signaling	1.6	3.0
NM_015150	Raftlin, lipid raft linker 1	RFTN1	B cell-specific major raft protein important for BCR signal transduction	1.9	3.0
NM_080625	Chromosome 20 open reading frame 160	C20orf160	Unknown	-	3.0
NM_005098	Musculin (activated B-cell factor-1)	MSC	Downstream target of the B-cell receptor signal transduction pathway; transcriptionally repressor that inhibits the transactivation capability of E47, an E2A protein	2.0	2.9
NM_003728	Unc-5 homolog C (C. elegans)	UNC5C	Apoptosis	2.8	2.6
NM_015549	Pleckstrin homology domain containing, family G (with RhoGef domain) member 3	PLEKHG3	Regulation of Rho protein signal transduction	1.6	2.8
NM_016032	Zinc finger, DHHC-type containing 9	ZDHHC9	Unknown	1.8	2.8
NM_000675	Adenosine A2a receptor	ADORA2A	Apoptosis; blood coagulation; cAMP biosynthesis; cellular defense response; inflammation	2.8	2.5
NM_002357	MAX dimerization protein 1	MXD1	Cell proliferation	2.8	2.5

NM_014358	C-type lectin domain family 4, member E	CLEC4E	Cell adhesion; downstream target of CCAAT/enhancer binding protein (C/EBP), beta; inflammation	2.2	2.8
NM_031272	Testis expressed 14	TEX14	Post-translational protein modification	2.8	-
NM_015257	Transmembrane protein 194	TMEM194	Unknown	2.8	2.5
NM_005723	Tetraspanin 5	TSPAN5	Unknown; belongs to family of preoteins that play roles in the regulation of cell development, activation, growth and motility	2.8	1.7
NM_021205	Ras homolog gene family, member U	RHOU	G1/S transition of mitotic cell cycle	2.7	-
NM_006813	Proline-rich nuclear receptor coactivator 1	PNRC1	Transcription regulation; interacts with many nuclear receptors including AR, ESR1, ESRRGA, ESRRG, NR3C1/GR, NR5A1/SF1, PGR, TR, RAR and RXR; interacts with GRB2	2.5	2.7
NM_015018	Dopey family member 1	DOPEY1	Establishment of protein localization	1.8	2.7
NM_006058	TNFAIP3 interacting protein 1	TNIP1	Negative regulation of viral genome replication; defense response	-	2.7
NM_173607	Chromosome 14 open reading frame 24	C14orf24	Unknown	2.7	2.5
NM_003151	Signal transducer and activator of transcription 4	STAT4	JAK-STAT cascade; cell proliferation; cytokine and chemokine mediated signaling	2.0	2.6
NM_175861	Transmembrane and tetratricopeptide repeat containing 1	TMTC1	Unknown	2.6	1.5
NM_012342	BMP and activin membrane-bound inhibitor homolog (Xenopus laevis)	BAMBI	Transmembrane glycoprotein related to the type I receptors of the TGF-beta family	2.6	-
NM_001025300	RAB12, member RAS oncogene family	RAB12	small GTPase mediated signal transduction	2.4	2.6
NM_000610	CD44 molecule (Indian blood group)	CD44	Cell adhesion	1.5	2.6
NM_002291	Laminin, beta 1	LAMB1	Cell adhesion; positive regulation of cell migration	1.8	2.6
NM_006806	BTG family, member 3	BTG3	Negative regulation of cell proliferation	1.6	2.6
NM_006779	CDC42 effector protein (Rho GTPase binding) 2	CDC42EP2	Actin filament assembly and cell shape control; formation fo pseudopodia	1.6	2.6

NM_000958	Prostaglandin E receptor 4 (subtype EP4)	PTGER4	One of four receptors identified for PGE2; activates T-cell factor signaling; mediates PGE2 induced expression of EGR1; regulates the level and stability of COX-2 mRNA, and leads to the phosphorylation of glycogen synthase kinase-3	-	2.6
NM_030647	Jumonji C domain containing histone demethylase 1 homolog D (S. cerevisiae)	JHDM1D	Transcription regulation	2.5	1.9
NM_003505	Frizzled homolog 1 (Drosophila)	FZD1	Wnt receptor signaling pathway; cell growth and differentiation	2.3	2.5
NM_014391	Ankyrin repeat domain 1 (cardiac muscle)	ANKRD1	defense response; localized to the nucleus of endothelial cells and is induced by IL-1 and TNF-alpha stimulation	2.5	-
NM_001497	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 1	B4GALT1	Acute inflammatory response	2.1	2.5
NM_030797	Family with sequence similarity 49, member A	FAM49A	Unknown	2.5	2.2
NM_005253	FOS-like antigen 2	FOSL2	One of 4 Fos gene family memebrs that can dimerize with proteins of the JUN family, thereby forming the transcription factor complex AP-1; FOS proteins have been implicated as regulators of cell proliferation, differentiation, and transformation	1.7	2.5
NM_015158	KN motif and ankyrin repeat domains 1	KANK1	Negative regulation of cell cycle	1.8	2.5
NM_032797	Apoptosis-inducing factor, mitochondrion-associated, 2	AIFM2	Induction of apoptosis	1.5	2.4
NM_024610	HSPB (heat shock 27kDa) associated protein 1	HSPBAP1	Stress response	1.9	2.4
NM_017940	Neuroblastoma breakpoint family, member 1 /// KIAA1245 /// CG10522-PA-like /// CLIP-190-like	NBPF1	Unknown	-	2.4
NM_182909	Filamin A interacting protein 1-like	FILIP1L	Unknown	-	2.4
NM_002101	Glycophorin C (Gerbich blood group)	GYPC	Minor sialoglycoprotein in human erythrocyte membranes; plays an important role in regulating the stability of red cells	-	2.4
NM_004184	Tryptophanyl-tRNA synthetase	WARS	Tryptophanyl-tRNA aminoacylation; negative regulation of cell proliferation	2.4	1.7

NM_032806	Chromosome 3 open reading frame 39	C3orf39	Unknown	2.4	1.8
NM_004906	Wilms tumor 1 associated protein	WTAP	mRNA processing; cell cycle	1.9	2.4
NM_080387	C-type lectin domain family 4, member D	CLEC4D	Cell adhesion; inflammation; immune response	2.0	2.4
NR_002814	CLR pseudogene	LOC374443	Unknown	2.4	-
NM_015091	KIAA0423	KIAA0423	Unknown	-	2.4
NM_015130	TBC1 domain family, member 9 (with GRAM domain)	TBC1D9	Regulation of Rab GTPase activity	1.9	2.4
NM_001010859	Hypothetical protein LOC150297	RP1-127L4.6	Unknown	2.3	1.9
NM_152574	Chromosome 9 open reading frame 52	C9orf52	Unknown	2.3	1.5
NM_002537	Ornithine decarboxylase antizyme 2	OAZ2	Polyamine biosynthesis	-	2.3
NM_020651	Pellino homolog 1 (Drosophila)	PELI1	Adaptor protein of IL-1R-associated kinase 1 (IRAK1) that promotes TGF-beta-mediated anti-inflammatory effects	2.3	-
NM_023038	ADAM metallopeptidase domain 19 (meltrin beta)	ADAM19	Cell adhesion; cells migration; cell-cell and cell-matrix interactions; signal transduction	2.1	2.3
NM_003176	Synaptonemal complex protein 1	SYCP1	Cell cycle	2.3	1.7
NM_006705	Growth arrest and DNA-damage-inducible, gamma	GADD45G	Activation of MAPKKK activity; DNA repair; response to stress	1.5	2.3
NM_014644	Phosphodiesterase 4D interacting protein (myomegalin) /// similar to phosphodiesterase 4D interacting protein isoform 2	PDE4DIP	Actin cytoskeleton organization	2.3	1.9
NM_005605	Protein phosphatase 3 (formerly 2B), catalytic subunit, gamma isoform	PPP3CC	Activation of pro-apoptotic gene products	2.1	2.3
NM_003965	Chemokine (C-C motif) receptor-like 2 /// similar to chemokine (C-C motif) receptor-like 2	CCRL2	Chemotaxis	2.3	2.0
NM_020231	KTEL (Lys-Tyr-Glu-Leu) containing 1	KTELC1	Unknown	2.0	2.3
NM_003897	Immediate early response 3	IER3	Functions in the protection of cells from Fas- or TNF alpha-induced apoptosis	-	2.3
NM_023929	Zinc finger and BTB domain containing 10	ZBTB10	Transcription regulation	2.2	2.3

NM_005755	Epstein-Barr virus induced gene 3	EBI3	Humoral immune response; positive regulation of alpha-beta T cell proliferation	2.3	2.1
NM_014828	TOX high mobility group box family member 4	TOX4	Transcription regulation	1.9	2.3
NM_145316	Chromosome 6 open reading frame 128	C6orf128	Unknown	2.3	1.5
NM_007195	Polymerase (DNA directed) iota	POLI	DNA replication: DNA repair; response to DNA damage	-	2.3
NM_024989	Post-GPI attachment to proteins 1	PGAP1	Attachment of GPI anchor to protein	1.9	2.3
NM_014479	ADAM-like, decysin 1	ADAMDEC1	Negative regulation of cell adhesion; integrin-mediated signaling pathway	2.0	2.3
NM_002041	GA binding protein transcription factor, beta subunit 2	GABPB2	Interacts with HCFC1, causing repression of transcriptional activity	2.2	1.7
NM_020240	CDC42 small effector 2	CDC42SE2	Phagocytosis; regulation of cell shape	2.0	2.2
NM_002502	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	NFKB2	Follicular dendritic cell differentiation; germinal center formation	-	2.2
NM_003998	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	NFKB1	Apoptosis	1.9	2.2
NM_004794	RAB33A, member RAS oncogene family	RAB33A	Small GTPase mediated signal transduction	2.2	1.8
NM_005780	Lipoma HMGIC fusion partner	LHFP	Member of the lipoma HMGIC fusion partner (LHFP) gene family; assocaited with deafness	-	2.2
NM_181900	StAR-related lipid transfer (START) domain containing 5	STARD5	Cholesterol homeostasis	-	2.2
NM_012257	HMG-box transcription factor 1	HBP1	Wnt receptor signaling pathway; cell cycle arrest	1.9	2.2
NM_013272	Solute carrier organic anion transporter family, member 3A1	SLCO3A1	Ion transport	1.6	2.2
NM_014572	LATS, large tumor suppressor, homolog 2 (Drosophila)	LATS2	G1/S transition of mitotic cell cycle	2.2	1.5
XM_931798	Hypothetical LOC643783	LOC643783	Unknown	1.5	2.2
NM_001017523	BTB (POZ) domain containing 11	BTBD11	Unknown	-	2.2
NM_005156	ROD1 regulator of differentiation 1 (S. pombe)	ROD1	mRNA processing; may play a role in regulation of differentiation	2.2	1.8
NM_002183	Interleukin 3 receptor, alpha (low affinity)	IL3RA	Cytokine signaling; apoptosis	1.7	2.2

NM_020185	Dual specificity phosphatase 22 /// similar to dual specificity phosphatase 22	DUSP22	Inactivation of MAPK activity; apoptosis	2.2	-
NM_006832	Fermitin family homolog 2 (Drosophila)	FERMT2	Cell adhesion; regulation of cell shape	1.9	2.2
NM_153607	Chromosome 5 open reading frame 41	C5orf41	Unknown	2.2	1.5
NM_016272	Transducer of ERBB2, 2	TOB2	Negative regulation of cell proliferation	2.2	1.5
NM_005637	Synovial sarcoma translocation, chromosome 18	SS18	Recruits beta-catenin to the nucleus and associates with it in an active complex; cell adhesion	1.7	2.2
NM_152992	POM (POM121 homolog, rat) and ZP3 fusion	POMZP3	Nuclear pore complex assembly and maintenance	-	2.2
NM_015900	Phospholipase A1 member A	PLA1A	Hydrolyzes phosphatidylserine (PS) exposed on the surface of cells such as apoptotic cells and activated platelets, and produces 2-acyl-lysophosphatidylserine (lysoPS), which is a lipid mediator for mast cells, T cells and neural cells.	1.9	2.2
NM_001271	Chromodomain helicase DNA binding protein 2	CHD2	Chromatin assembly or disassembly	2.2	-
NM_015017	Ubiquitin specific peptidase 33	USP33	Ubiquitin-dependent protein catabolism	1.9	2.1
NM_000390	Choroideremia (Rab escort protein 1)	CHM	blood vessel development	2.1	1.6
NM_020183	Aryl hydrocarbon receptor nuclear translocator-like 2	ARNTL2	Entrainment of circadian clock	2.1	1.9
NM_025147	Coenzyme Q10 homolog B (S. cerevisiae)	COQ10B	Unknown	1.9	2.1
NM_015945	Solute carrier family 35, member C2 /// hypothetical protein LOC100128167	SLC35C2	Response to oxygen tension	2.1	1.8
NM_015252	EH domain binding protein 1	EHBP1	Couples endocytosis to the actin cytoskeleton	2.1	1.7
NM_152618	Bardet-Biedl syndrome 12	BBS12	Protein folding	2.1	2.1
NM_016531	Kruppel-like factor 3 (basic)	KLF3	Transcription regualtor that Binds to the CACCC box; may play a role in hematopoiesis	2.1	-
NM_004482	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3 (GalNAc-T3)	GALNT3	Protein amino acid O-linked glycosylation via threonine	2.1	2.1

NM_014982	Pecanex homolog (Drosophila)	PCNX	Unknown	1.6	2.1
NM_019028	zinc finger, DHHC-type containing 13	ZDHHC13	Positive regulation of I-kappaB kinase/NF-kappaB cascade	1.6	2.1
NM_016141	Dynein, cytoplasmic 1, light intermediate chain 1	DYNC1LI1	Small GTPase mediated signal transduction; microtubule-based motor activity	1.5	2.1
NM_052818	NEDD4 binding protein 2-like 1	N4BP2L1	Unknown	1.7	2.1
NM_013396	Ubiquitin specific peptidase 25	USP25	Ubiquitin-dependent protein catabolism	2.1	-
NM_003414	Zinc finger protein 267	ZNF267	May be involved in transcriptional regulation	-	2.1
NM_001776	Ectonucleoside triphosphate diphosphohydrolase 1	ENTPD1	Cell adhesion; blood coagulation	-	2.1
NM_004233	CD83 molecule	CD83	Humoral immune response	1.9	2.1
XM_001714336	Hypothetical protein MGC39372	MGC39372	Unknown	-	2.1
NM_013336	Sec61 alpha 1 subunit (S. cerevisiae)	SEC61A1	Intracellular protein transmembrane transport	1.9	2.1
NM_003410	Zinc finger protein, X-linked	ZFX	Transcription regulation	2.1	1.5
NM_173475	DCN1, defective in cullin neddylation 1, domain containing 3 (S. cerevisiae)	DCUN1D3	Unknown	2.1	2.0
NM_006167	NK3 homeobox 1	NKX3-1	Transcription regulation; cell growth, transformation	2.1	1.7
NM_174908	Coiled-coil domain containing 50	CCDC50	May function as a negative regulator of NF-kB signaling and as an effector of epidermal growth factor (EGF)-mediated cell signaling; development	2.1	-
NM_181354	Oxidation resistance 1	OXR1	Response to oxidative stress; external encapsulating structure organization	2.1	-
NM_024586	Oxysterol binding protein-like 9	OSBPL9	Steroid metabolism; lipid transport	-	2.1
NM_004456	Enhancer of zeste homolog 2 (Drosophila)	EZH2	Transcription regulation; chromatin modification	1.7	2.1
NM_014330	Protein phosphatase 1, regulatory (inhibitor) subunit 15A	PPP1R15A	Regulation of translation; apoptosis; response to DNA damage stimulus; cell cycle arrest	2.1	-
NM_147200	TRAF3 interacting protein 2	TRAF3IP2	Positive regulation of I-kappaB kinase/NF-kappaB cascade	1.8	2.1
NM_017628	Tet oncogene family member 2	TET2	Unknown	2.1	-

NM_004414	Regulator of calcineurin 1	RCAN1	Interacts with calcineurin A and inhibits calcineurin-dependent signaling pathways; blood circulation; negative regulation of smooth muscle cell differentiation	1.5	2.1
NM_003379	Ezrin	EZR	Leukocyte adhesion; regulation of cell shape; membrane to membrane docking	2.1	-
NM_030940	Iron-sulfur cluster assembly 1 homolog (S. cerevisiae)	ISCA1	Mitochondrial protein involved in biogenesis and assembly of iron-sulfur clusters, functions in electron-transfer reactions	1.7	2.1
NM_000960	Prostaglandin I2 (prostacyclin) receptor (IP)	PTGIR	G-protein signaling, coupled to cyclic nucleotide second messenger; receptor for prostacyclin, major product of cyclooxygenase in macrovascular endothelium, elicits potent vasodilation and inhibition of platelet aggregation through binding to this receptor	-	2.0
NM_032164	Zinc finger protein 394	ZNF394	Transcription regulation	1.9	2.0
NM_022048	Casein kinase 1, gamma 1	CSNK1G1	Wnt receptor signaling pathway; involved in cell growth and morphogenesis	2.0	-
NM_003782	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 4	B3GALT4	Protein glycosylation	1.6	2.0
NM_002657	Pleiomorphic adenoma gene-like 2	PLAGL2	Transcription regulation; induction of apoptosis	1.5	2.0
NM_144593	Ras homolog enriched in brain like 1	RHEBL1	Small GTPase mediated signal transduction	2.0	1.8
NM_001531	Major histocompatibility complex, class I-related	MR1	Antigen processing and presentation; immune response	1.6	2.0
NM_031284	ADP-dependent glucokinase	ADPGK	Indicated role in glycolysis, possibly during ischemic conditions	1.7	2.0
NM_006509	v-Rel reticuloendotheliosis viral oncogene homolog B, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3 (avian)	RELB	T-helper 1 cell differentiation; myeloid dendritic cell differentiation; antigen processing and presentation	1.6	2.0
NM_000127	Exostoses (multiple) 1	EXT1	Endoplasmic reticulum-resident type II transmembrane glycosyltransferase involved in chain elongation step of heparan sulphate biosynthesis	2.0	-

NM_006153	NCK adaptor protein 1	NCK1	Regulation of translation; actin filament organization; signal complex assembly; cell migration	-	2.0
NM_022121	PERP, TP53 apoptosis effector	PERP	Induction of apoptosis; cell adhesion	1.6	2.0
NM_152905	Neural precursor cell expressed, developmentally down-regulated 1	NEDD1	Cell cycle	2.0	1.6
NM_000176	Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	NR3C1	Receptor for glucocorticoids acting as both a transcription factor and as a regulator of other transcription factors; also found in heteromeric cytoplasmic complexes with heat shock factors and immunophilins	2.0	1.5
NM_020117	Leucyl-tRNA synthetase	LARS	tRNA aminoacylation for protein translation	-1.5	-2.0
NM_177403	RAB7B, member RAS oncogene family	RAB7B	Small GTPase mediated signal transduction	-1.5	-2.0
NM_003255	TIMP metallopeptidase inhibitor 2	TIMP2	Natural inhibitor of matrix metalloproteinases, whicha re involved in degradation of the extracellular matrix; also directly suppress the proliferation of endothelial cells	-1.5	-2.1
NM_014911	AP2 associated kinase 1	AAK1	Functions during receptor-mediated endocytosis to trigger clathrin assembly, interact with membrane-bound receptors, and recruit encodytic accessory factors	-1.5	-2.2
NM_052813	Caspase recruitment domain family, member 9	CARD9	Positive regulation of stress-activated MAPK cascade; apoptosis	-2.0	-1.5
NM_020432	Putative homeodomain transcription factor 2	PHTF2	Transcription regulation	-2.1	-1.5
NM_003901	Sphingosine-1-phosphate lyase 1	SGPL1	Lipid metabolism; apoptosis	-2.1	-1.5
NM_002719	Protein phosphatase 2, regulatory subunit B', gamma isoform	PPP2R5C	Belongs to the phosphatase 2A regulatory subunit B family, implicated in the negative control of cell growth and division	-2.1	-1.5
NM_001010883	Family with sequence similarity 102, member B	FAM102B	Unknown	-2.5	-1.5
NM_020908	Transducin-like enhancer of split 3 (E(sp1) homolog, Drosophila)	TLE3	Wnt receptor signaling pathway; cell growth	-1.6	-2.0
NM_001769	CD9 molecule	CD9	Cell motion; cell adhesion; negative regulation of cell proliferation	-1.6	-2.1
NM_024996	G elongation factor, mitochondrial 1	GFM1	Translational elongation	-1.6	-2.1

NM_012305	Adaptor-related protein complex 2, alpha 2 subunit	AP2A2	Vesicle-mediated transport; regulation of defense response to virus by virus	-1.6	-2.2
NM_016199	LSM7 homolog, U6 small nuclear RNA associated (S. cerevisiae)	LSM7	Nuclear mRNA splicing, via spliceosome	-2.0	-1.6
NM_024654	Nucleolar protein 9	NOL9	Unknown	-2.0	-1.6
NM_001666	Rho GTPase activating protein 4	ARHGAP4	Cytoskeleton organization; Rho cell motility signaling pathway	-2.1	-1.6
NM_000358	Transforming growth factor, beta-induced, 68kDa	TGFBI	Negative regulation of cell adhesion; cell proliferation	-1.7	-2.0
NM_015224	Chromosome 3 open reading frame 63	C3orf63	Unknown	-1.7	-2.0
NM_006060	IKAROS family zinc finger 1 (Ikaros)	IKZF1	Transcription regulation; hemopoiesis	-1.7	-2.2
NM_001892	Casein kinase 1, alpha 1	CSNK1A1	Wnt receptor signaling pathway; regulation of apoptosis	-1.7	-2.3
NM_000591	CD14 molecule	CD14	Phagocytosis; apoptosis; inflammatory response	-1.7	-2.4
NM_006862	Tudor and KH domain containing	TDRKH	Unknown	-2.0	-1.7
NM_004290	Ring finger protein 14	RNF14	Ttrnascription regulation; ubiquitin-dependent protein catabolism	-2.2	-1.7
NM_016551	Transmembrane 7 superfamily member 3	TM7SF3	Unknown	-2.3	-1.7
NM_174896	Chromosome 1 open reading frame 162	C1orf162	Unknown	-1.8	-2.3
NM_005026	Phosphoinositide-3-kinase, catalytic, delta polypeptide	PIK3CD	B cell homeostasis	-1.8	-2.2
NM_006443	Chromosome 6 open reading frame 108	C6orf108	Cell proliferation	-1.8	-2.1
NM_003656	Calcium/calmodulin-dependent protein kinase I	CAMK1	Cell differentiation	-1.8	-2.2
NM_003916	Adaptor-related protein complex 1, sigma 2 subunit	AP1S2	Vesicle-mediated transport; establishment of protein localization	-1.8	-2.4
NM_006828	Activating signal cointegrator 1 complex subunit 3	ASCC3	Transcription regulation	-2.0	-1.8
NM_014426	Sorting nexin 5	SNX5	Cell communication; establishment of protein localization	-2.1	-1.8
NM_004548	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 10, 22kDa	NDUFB10	Mitochondrial electron transport, NADH to ubiquinone	-2.1	-1.8

NM_006924	Splicing factor, arginine/serine-rich 1 (splicing factor 2, alternate splicing factor)	SFRS1	Nuclear mRNA splicing, via spliceosome	-2.1	-1.8
NM_017740	Zinc finger, DHHC-type containing 7	ZDHHC7	B cell transcription factor	-2.1	-1.8
NM_015088	Trinucleotide repeat containing 6B	TNRC6B	Unknown	-2.4	-1.8
NM_078483	Solute carrier family 36 (proton/amino acid symporter), member 1	SLC36A1	Amino acid transport	-1.9	-2.7
NM_001688	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit B1	ATP5F1	ATP synthesis coupled proton transport	-1.9	-2.0
NM_178841	Ring finger protein 166	RNF166	Unknown	-1.9	-2.1
NM_004454	Ets variant gene 5 (ets-related molecule)	ETV5	IL12 and Stat4 dependent signaling pathway in Th1 development	-1.9	-2.3
NM_002882	RAN binding protein 1	RANBP1	Positive regulation of mitotic centrosome separation	-2.1	-1.9
NM_002576	p21/Cdc42/Rac1-activated kinase 1 (STE20 homolog, yeast)	PAK1	MAPKKK cascade; apoptosis; ER-nuclear signaling pathway; cytoskeleton organization	-2.2	-1.9
NM_002423	Matrix metallopeptidase 7 (matrilysin, uterine)	MMP7	Involved in the breakdown of extracellular matrix; Wnt signaling; regulation of cell proliferation	-2.2	-1.9
NM_014033	Methyltransferase like 7A	METTL7A	Unknown	-2.4	-1.9
NM_001442	Fatty acid binding protein 4, adipocyte	FABP4	Cytokine production; PPAR signaling pathway; inflammatory response	-2.6	-1.9
NM_015441	Olfactomedin-like 2B	OLFML2B	Unknown	-3.1	-1.9
NM_014014	Activating signal cointegrator 1 complex subunit 3-like 1	ASCC3L1	RNA splicing	-2.0	-2.1
NM_002729	Hematopoietically expressed homeobox	HHEX	Antimicrobial humoral response; regulation of cell proliferation; may play a role in hematopoietic differentiation	-2.0	-2.9
NM_017952	Pentatricopeptide repeat domain 3	PTCD3	Unknown	-2.2	-2.0
NM_005869	Serologically defined colon cancer antigen 10	SDCCAG10	Protein folding	-2.0	-
NM_002890	RAS p21 protein activator (GTPase activating protein) 1	RASA1	Cytokinesis; vasculogenesis; negative regulation of cell-matrix adhesion	-2.0	-
NM_005627	Serum/glucocorticoid regulated kinase 1	SGK1	Sodium ion transport and homeostasis; apoptosis	-	-2.0
NM_025134	Chromodomain helicase DNA binding protein 9	CHD9	Chromatin assembly or disassembly	-2.0	-

NM_030769	N-acetylneuraminate pyruvate lyase (dihydrodipicolinate synthase)	NPL	Carbohydrate metabolism	-2.0	-2.4
NM_015103	Plexin D1	PLXND1	Patterning of blood vessels	-2.0	-
NM_017901	Two pore segment channel 1	TPCN1	Calcium ion transport	-	-2.0
NM_005762	Tripartite motif-containing 28	TRIM28	Epithelial to mesenchymal transition; transcription regulation	-2.0	-
NM_014763	Mitochondrial ribosomal protein L19	MRPL19	Translation	-2.0	-
NM_173694	ATPase, class VI, type 11C	ATP11C	ATP biosynthesis	-2.0	-2.1
NM_033515	Rho GTPase activating protein 18	ARHGAP18	Signal transduction	-2.3	-2.0
NM_014755	SERTA domain containing 2	SERTAD2	Negative regulation of cell growth	-	-2.1
NM_018121	Chromosome 10 open reading frame 6	C10orf6	Unknown	-	-2.1
NM_006100	ST3 beta-galactoside alpha-2,3-sialyltransferase 6	ST3GAL6	Protein amino acid glycosylation	-	-2.1
NM_014650	Zinc finger protein 432	ZNF432	Transcription regulation	-2.1	-
NM_006709	Euchromatic histone-lysine N-methyltransferase 2	EHMT2	Chromatin modification; negative regulation fo transcription	-2.1	-
NM_005000	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 5, 13kDa	NDUFA5	Mitochondrial electron transport, NADH to ubiquinone	-2.1	-
NM_018301	RNA binding motif protein 41	RBM41	Unknown	-2.1	-
NM_013252	C-type lectin domain family 5, member A	CLEC5A	Defense response; cell adhesion; nflammation	-2.1	-5.1
NM_030925	Calcium binding protein 39-like	CAB39L	Unknown	-	-2.1
NM_015918	Processing of precursor 5, ribonuclease P/MRP subunit (S. cerevisiae)	POP5	tRNA processing	-2.1	-
NM_032335	PHD finger protein 6	PHF6	Transcription regulation	-2.1	-
NM_012096	Adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 1	APPL1	Cell proliferation	-	-2.1
NM_014967	Myotubularin related protein 15	MTMR15	DNA repair; cellular response to stress	-2.1	-
NM_003253	T-cell lymphoma invasion and metastasis 1	TIAM1	Regulation of Rho protein signal transduction; ephrin receptor signaling pathway	-	-2.1
NM_020536	CSRP2 binding protein	CSRP2BP	Metabolism	-2.1	-

NM_004331	BCL2/adenovirus E1B 19kDa interacting protein 3-like	BNIP3L	Induction of apoptosis; negative regulation of survival gene product expression	-	-2.1
NM_033050	Succinate receptor 1	SUCNR1	G-protein coupled receptor protein signaling pathway	-	-2.2
NM_005842	Sprouty homolog 2 (Drosophila)	SPRY2	KIndirectly involved in the non-cell autonomous inhibitory effect on fibroblast growth factor two signaling; interacts with Cas-Br-M (murine) ectropic retroviral transforming sequence, and can function as a bimodal regulator of epidermal growth factor receptor/mitogen-activated protein kinase signaling	-	-2.2
NR_003367	Pvt1 oncogene homolog (mouse)	PVT1	Possibly invovled in tumorigenesis	-2.6	-2.2
NM_006335	Translocase of inner mitochondrial membrane 17 homolog A (yeast)	TIMM17A	Protein targeting to mitochondrion	-2.2	-
NM_014112	Trichorhinophalangeal syndrome I	TRPS1	NLS-bearing substrate import into nucleus	-2.2	-2.5
NM_032772	Xinc finger protein 503	ZNF503	Transcription regulation	-	-2.3
NM_004531	Molybdenum cofactor synthesis 2	MOCS2	Mo-molybdopterin cofactor biosynthesis	-2.3	-
NM_004536	NLR family, apoptosis inhibitory protein	NAIP	Regulation of apoptosis	-	-2.3
NM_019043	Amyloid beta (A4) precursor protein-binding, family B, member 1 interacting protein	APBB1IP	Appears to function in the signal transduction from Ras activation to actin cytoskeletal remodeling. suppresses insulin- induced promoter activities through AP1 and SRE; mediates Rap1- induced adhesion	-2.4	-
XM_001720626	Similar to hCG2020760	LOC100131993	Unknown	-	-2.4
NM_001080480	Membrane bound O-acyltransferase domain containing 1	MBOAT1	Acyltransferase activity	-	-2.4
NM_002166	Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	ID2	TGF-beta signaling pathway; negatively regulating cell differentiation	-	-2.5
NM_006095	ATPase, aminophospholipid transporter (APLT), class I, type 8A, member 1	ATP8A1	ATP biosynthesis	-	-2.5
NM_024778	LON peptidase N-terminal domain and ring finger 3	LONRF3	ATP-dependent proteolysis	-	-2.8

NM_000602	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	SERPINE1	Blood coagulation; fibrinolysis; regulation of angiogenesis	-3.0	-

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associated with variations in humoral immunity to smallpox vaccination in Caucasians and African Americans. J Infect Dis 2011;204(3):433-441.

VITA

Terence Edward Hill was born in Richmond, VA on January 18th, 1984. He is the second born and youngest child of Edward and Denise Hill. Terence graduated from Monacan High School in Chesterfield, VA in 2002 and matriculated into Virginia Tech University in August of that year. In 2005 he received his B.S. in biological sciences and entered continued his education and began research on diagnostic platform for *Brucella spp*. and novel vaccine platform for *Yesinia pestis* as a VT-PREP scholar. In 2006 Terence started graduate school at the University of Texas Medical Branch in Galveston, TX, and began under the mentorship of Dr. Peters and Dr. Tseng in 2007. For his dissertation topic Terence characterized the innate host responses against Rift Valley fever virus using high-throughput global transcriptional gene expression profiling and analysis methods. Specific interest surrounded to role of the NSs protein in modulating immune related biological processes and signaling pathways.

Education

B.S., Biological Sciences, Virginia Tech University, Blacksburg, VA. May, 2005.

Peer-Reviewed Publications

Yoshikawa T, Hill TE, Yoshikawa N, Popov VL, Galindo CL, Garner HR, Peters CJ, Tseng CT. Dynamic innate immune responses of human bronchial epithelial cells to severe acute respiratory syndrome-associated coronavirus infection. PLoS One 2010;5(1):e8729.

Yoshikawa N, Yoshikawa T, Hill T, Huang C, Watts DM, Makino S, Milligan G, Chan T, Peters CJ, Tseng CT. Differential virological and immunological outcome of severe acute respiratory syndrome coronavirus infection in susceptible and resistant transgenic mice expressing human angiotensin-converting enzyme 2. J Virol 2009;83(11):5451-65.

Yoshikawa T, Hill T, Li K, Peters CJ, Tseng CT. Severe acute respiratory syndrome (SARS) coronavirus-induced lung epithelial cytokines exacerbate SARS pathogenesis by modulating intrinsic functions of monocyte-derived macrophages and dendritic cells. J Virol 2009;83(7):3039-48.

Abstracts

Hill T. Characterization of Human Innate Immunity to Rift Valley Fever Virus. American Society for Virology annual meeting. Bozeman, MT, 2010.

Hill, T. Impact of Rift Valley Fever Virus NSs Expression on Innate Signaling Events Elicited by Primary Human Macrophages. American Society of Tropical Medicine and Hygeine annual meeting Washington, DC, 2009.

Hill, T. Impact of Cytosoloic RNA Recognition Molecules on Dhori Virus Infection. American

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RESEARCH ACTIVITIES:

A. I am interested in high-throughput analysis of the host-pathogen interactions using affymetrix chip cDNA microarray analysis and subsequent bioinformatics data-mining tools. Particularly, working with live attenuated Rift Valley fever virus strains I investigated the similarities and differences of global host transcriptional responses elicited by the presence or absence of the major viral virulence factor, NSs. From this analysis identification of pathologically important host genes and characterization of patterns of biological process and signaling pathway utilization can be extracted for hypothesis generation. Various pattern recognition receptors in detecting Rift Valley fever and the impact these molecules have on regulation of the subsequent host defense responses.

HONORS: 2002-2004 Minorities Agriculture Opportunities Program (MAOP) Scholarship Recipient

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PUBLISHED:

1. Yoshikawa T, Hill TE, Yoshikawa N, Popov VL, Galindo CL, Garner HR, Peters CJ, Tseng CT. Dynamic innate immune responses of human bronchial epithelial cells to severe acute respiratory syndrome-associated coronavirus infection. PLoS One 2010;5(1):e8729.
2. Yoshikawa N, Yoshikawa T, Hill T, Huang C, Watts DM, Makino S, Milligan G, Chan T, Peters CJ, Tseng CT. Differential virological and immunological outcome of severe acute respiratory syndrome coronavirus infection in susceptible and resistant transgenic mice expressing human angiotensin-converting enzyme 2. J Virol 2009;83(11):5451-65.
3. Yoshikawa T, Hill T, Li K, Peters CJ, Tseng CT. Severe acute respiratory syndrome (SARS) coronavirus-induced lung epithelial cytokines exacerbate SARS pathogenesis by modulating intrinsic functions of monocyte-derived macrophages and dendritic cells. J Virol 2009;83(7):3039-48.

SUBMITTED:

Hill T, Tseng M, Garron T, Yoshikawa T, Galindo C, Ikegami T, Peters CJ, Tseng CT. Impact of Rift Valley Fever Virus NSs Expression on Innate Signaling Events Elicited by Primary Human Macrophages. J. Virol. 2011

ABSTRACTS:

1. Hill T. Characterization of Human Innate Immunity to Rift Valley Fever Virus. ASV annual meeting 2010.
2. Hill, T. Impact of Rift Valley Fever Virus NSs Expression on Innate Signaling Events Elicited by Primary Human Macrophages. ASTMH annual meeting 2009.
3. Hill, T. Impact of cytosoloic RNA Recognition Molecules on Dhori Virus Infection. ASV annual meeting 2009.