Final Report for DOE Agreement# DE-SC0001599 Infectious Disease Proteome Biomarkers

Research for the DOE Infectious Disease Proteome Biomarkers focused on Rift Valley fever virus (RVFV) and Venezuelan Equine Encephalitis Virus (VEEV). RVFV and VEEV are Category A and B pathogens respectively. Among the priority threats, RVFV and VEEV rank high in their potential for being weaponized and introduced to the United States, spreading quickly, and having a large health and economic impact. In addition, they both have live attenuated vaccine, which allows work to be performed at BSL-2. While the molecular biology of RVFV and VEEV are increasingly well-characterized, little is known about its host-pathogen interactions. Our research is aimed at determining critical alterations in host signaling pathways to identify therapeutics targeted against the host.

RVFV Studies

We have utilized a novel proteomics technology, reverse-phase protein arrays (RPMA) to identify phosphoprotein signaling pathways modulated during infection of cultured airway epithelium. ZH-501 infection induced activation of MAP kinases (p38, JNK and ERK) and downstream transcriptional factors [STAT1 (Y701), ATF2 (T69/71), MSK1 (S360) and CREB (S133)]. NFkB phosphorylation was also increased. Activation of p53 (S15, S46) correlated with the increased levels of cleaved effector caspase-3, -6 and -7, indicating activation of the extrinsic apoptotic pathway. RVFV infection downregulated phosphorylation of a major anti-apoptotic regulator of survival pathways, AKT (S473), along with phosphorylation of FOX 01/03 (T24/31) which controls cell cycle arrest downstream from AKT. Consistent with this, the level of apoptosis inhibitor XIAP was decreased. However, the intrinsic apoptotic pathway marker, caspase-9, demonstrated only a marginal activation accompanied by an increased level of the inhibitor of apoptosome formation, HSP27. Concentration of the autophagy marker, LC3B, which often accompanies the pro-survival signaling, was decreased. Cumulatively, our analysis of RVFV infection in lung epithelium indicated a viral strategy directed toward the control of cell apoptosis through a number of transcriptional factors. Analyses of MP-12 titers in challenged cells in the presence of MAPK inhibitors indicated that activation of p38 represents a protective cell response while ERK activation controls viral replication.

As a follow-up to our RPMA studies we have focused on selected signaling pathways indentified, specifically p38 MAPK, DNA damage signaling and p53. We demonstrated that the cellular antioxidant enzyme superoxide dismutase 1 (SOD1) displays altered abundances at early time points following exposure to RVFV. We show that the enzyme is down regulated in cases of both a virulent (ZH501) and a vaccine strain (MP12) exposure. Our data demonstrates that the down regulation of SOD1 is likely to be due to post transcriptional processes and may be related to up regulation of TNFa following infection. We also provide evidence for extensive oxidative stress in the MP12 infected cells. Concomitantly, there is an increase in the activation of the p38 MAPK stress response, which our earlier published study demonstrated to be an essential cell survival strategy. Our data suggests that the viral anti-apoptotic protein NSm may play a role in the regulation of the cellular p38 MAPK response. Alterations in the host protein SOD1 following RVFV infection appears to be an early event that occurs in multiple cell types. Activation of the cellular stress response p38 MAPK pathway can be observed in all cell types

tested. Our data implies that maintaining oxidative homeostasis in the infected cells may play an important role in improving survival of infected cells.

We have extended our studies regarding the role of oxidative stress on liver pathology following RVFV infection by performing specific staining for reactive oxygen species. We have quantified oxidative stress in MP-12 infected liver cells in comparison with uninfected cells and have evidence that it may be an early event during infection. Using RT-PCR, we have determined that specific pro-apoptotic genes are expressed in MP12 infected liver cells. We have determined by ELISA that NFkB dependent cytokine production may play a role in the activation of apoptosis in liver cells. We have performed a more elaborate NFkB based oligonucleotide array and have determine specific cytokines as being upregulated during MP-12 infection that may contribute to liver pathology. We are currently exploring the role of antioxidants in ameliorating apoptotic responses in the liver.

We investigated the regulation of the DNA damage signaling cascades by RVFV infection and found virally inducted phosphorylation of the classical DNA damage signaling proteins, ataxia-telangiectasia mutated (ATM) (Ser-1981), Chk.2 (Thr-68), H2A.X (Ser-139), and p53 (Ser-15). In contrast, ataxia-telangiectasia mutated and Rad3-related kinase (ATR) (Ser-428) phosphorylation was decreased following RVFV infection. Importantly, both the attenuated vaccine strain MP12 and the fully virulent strain ZH548 showed strong parallels in their up-regulation of the ATM arm of the DNA damage response and in the down-regulation of the ATR pathway. The increase in DNA damage signaling proteins did not result from gross DNA damage as no increase in DNA damage was observed following infection. Rather the DNA damage signaling was found to be dependent on the viral protein NSs, as a NSs mutant virus was not found to induce the equivalent signaling pathways. RVFV MP12-infected cells also displayed an S phase arrest that was found to be dependent on NSs expression. Use of ATM and Chk.2 inhibitors resulted in a marked decrease in S phase arrest as well as viral production. These results indicate that RVFV NSs induces DNA damage signaling pathways that are beneficial for viral replication.

We further focused on the role of p53 signaling in RVFV infection and viral replication. Activation of p53 is important for the DNA damage signaling cascade, initiation of apoptosis, cell cycle arrest and transcriptional regulation of multiple genes. Our results show an up-regulation of p53 phosphorylation at several serine sites after RVFV MP-12 infection that is highly dependent on the viral protein NSs. qRT-PCR data showed a transcriptional up-regulation of several p53 targeted genes involved in cell cycle and apoptosis regulation following RVFV infection. Cell viability assays demonstrate that loss of p53 results in less RVFV induced cell death. Furthermore, decreased viral titers in p53 null cells indicate that RVFV utilizes p53 to enhance viral production. Collectively, these experiments indicate that the p53 signaling pathway is utilized during RVFV infection to induce cell death and increase viral production.

We have identified that MP-12 infection induces phosphorylation of the p65 component of the NFκB cascade. We demonstrated that phosphorylation of p65 (serine 536) involves phosphorylation of IκBα and hence occurs through the classical NFkB activation cascade. A unique low molecular weight complex of the IKK-β subunit can be observed in MP-12 infected cells that we have labeled as IKK-β2. The IKK-β2 complex retains kinase activity and is able to phosphorylate an IκBα substrate. Inhibition of the IKK complex using multiple inhibitors impairs viral replication thus alluding to the requirement of an active IKK complex to the viral life cycle. Curcumin, a well-documented inhibitor of the NFkB cascade strongly down-regulates levels of

extracellular infectious virus. Our data demonstrated that curcumin binds to and inhibits the kinase activity of the IKK-β2 complex in infected cells. As curcumin is also considered to be a proteasome inhibitor, we tested additional proteasome inhibitors and demonstrate that other proteasome inhibitors induce modest down regulation of extracellular virus. Finally, our data indicates that curcumin treatment down regulates viral replication in the liver of infected animals. In a broader perspective, our data points to the possibility that RVFV infection may result in the generation of novel versions of host components (such as IKK-β2) that by virtue of altered protein-protein interaction and function, qualify as unique therapeutic targets that can be utilized to down regulate virus replication.

VEEV Studies

There are no current FDA licensed vaccines or specific therapies against VEEV, making identification of potential therapeutic targets a priority. Our studies with VEEV have focused on identification of novel therapeutics and alterations of RNAi machinery.

Alphaviruses, including VEEV cause disease in both equine and humans that exhibit overt encephalitis in a significant percentage of cases. Features of the host immune response and tissue-specific responses may contribute to fatal outcomes as well as the development of encephalitis. It has previously been shown that VEEV infection of mice induces transcription of pro-inflammatory cytokines genes (e.g. IFN-γ, IL-6, IL-12, iNOS and TNF-α) within 6 h. GSK-3β is a host protein that is known to modulate pro-inflammatory gene expression and has been a therapeutic target in neurodegenerative disorders such as Alzheimer's. Hence inhibition of GSK-3β in the context of encephalitic viral infections has been useful in a neuroprotective capacity. Small molecule GSK-3β inhibitors and GSK-3β siRNA experiments indicated that GSK-3β was important for VEEV replication. Thirty-eight second generation BIO derivatives were tested and BIOder was found to be the most potent inhibitor, with an IC_{50} of ~0.5 μM and a CC_{50} of >100 μM. BIOder was a more potent inhibitor of GSK-3β than BIO, as demonstrated through *in vitro* kinase assays from uninfected and infected cells. Size exclusion chromatography experiments demonstrated that GSK-3β is found in three distinct complexes in VEEV infected cells, whereas GSK-3β is only present in one complex in uninfected cells. Cells treated with BIOder demonstrated an increase in the anti-apoptotic gene, survivin, and a decrease in the pro-apoptotic gene, BID, suggesting that modulation of pro- and anti-apoptotic genes contributes to the protective effect of BIOder treatment. Finally, BIOder partially protected mice from VEEV induced mortality. Our studies demonstrate the utility of GSK-3β inhibitors for modulating VEEV infection.

We have also focused on the interactions of VEEV with the host cell microRNA (miRNA) machinery. MiRNA are small non-coding RNA which act as master regulators of the cell by downregulating or degrading messenger RNA, thus suppressing production of the resultant proteins. Recent publications implicate miRNA interaction in the pathogenesis of HIV, rabies virus, HCV, and other viral diseases, leading to the hypothesis that interactions with host miRNA machinery are important in a human cell model of VEEV infection. These interactions were tested by infecting cells in which specific miRNA machinery was inactivated through null mutation, siRNA knockdown, or small-molecule inhibition. Absence of cytoplasmic components Dicer and TRBP caused little to no decrease in viral replication when tested in null-mutant cells or by siRNA knockdown, whereas absence of nuclear components Drosha, DGCR8 (Pasha), or

Exportin-5 resulted in at least 2 log decreases in both viral genomic copies and viral replication. Absence of Ago-2, an important component of the RNA-induced silencing complex (RISC), resulted in approximately 2 log decrease when tested by siRNA knockdown and 50% decrease when tested in null-mutant cells. Acriflavine, a small-molecule inhibitor of Ago-2 binding to the RISC, produced a marked decrease in both viral replication and viral genomic copies, an increase in cell survival when administered pre- or post-infection, and an increase in survival of VEEV-infected mice when administered post-infection. These findings demonstrate the following: 1) that the nuclear processing and mRNA binding steps are the most critical for VEEV infection, 2) the host cell may use redundant or alternate means of cytoplasmic miRNA processing during VEEV infection, and 3) that small-molecules of miRNA machinery may prove therapeutic in cases of VEEV infection.

We have identified that certain inhibitors of the host NFkB cascade increase mean time of survival of animals infected with TC83 virus. We have performed preliminary studies on the phosphorylation of p65 on serine 536 and IκBα. Additionally, we have evidence regarding the formation of IKK-β2 complex in infected cells. More extensive experiments to further characterize the involvement of the host NFkB cascade in alphavirus infection are in progress.

Collectively the research supported by the DOE grant has resulted in the following manuscripts (see attached manuscripts):

Popova, T.G., Turell, M., Espina, V., Kehn-Hall K., Kidd, J., Narayanan, A., Liotta, L., Petricoin, E.F. 3rd , Kashanchi, F., Bailey, C. and Popov, S.G. 2010. Reverse-Phase Phosphoproteome Analysis of Signaling Pathways Induced by Rift Valley Fever Virus in Human Small Airway Epithelial Cells. *PLoS ONE.* 5(11):e13805

Narayanan A, Bailey C, Kashanchi F, Kehn-Hall K. 2011. Developments in antivirals against influenza, smallpox and hemorrhagic fever viruses. Expert Opin Investig Drugs. 20(2):239-54.

Narayanan A, Popova T, Turell T, Kidd J, Chertow J, Popov S, Bailey C, Kashanchi F, Kehn-Hall K. 2011. Alteration in superoxide dismutase 1 causes oxidative stress and p38 MAPK activation following RVFV infection. PLoS ONE. 6(5):e20354.

Baer A, Austin D, Narayanan A, Popova T, Kainulainen M, Bailey C, Kashanchi F, Weber F, Kehn-Hall K. 2012. Induction of DNA Damage Signaling upon Rift Valley Fever Virus Infection Results in Cell Cycle Arrest and Increased Viral Replication. J Biol Chem. Mar 2;287(10):7399-410.

Kehn-Hall K, Narayanan A, Lundberg L, Sampey G, Pinkham C, Guendel I, Van Duyne R, Senina S, Schultz K, Stavale E, Aman MJ, Bailey C, Kashanchi F. 2012. Modulation of GSK3-b Activity in Venezuelan Equine Encephalitis Virus Infection. *PLoS ONE*. [Accepted].

Austin A, Baer A, Lundberg L, Shafagati N, Schoonmaker A, Narayanan A, Popova T, Panthier J, Kashanchi F, Bailey C and Kehn-Hall K. 2012. p53 Activation Following Rift Valley Fever Virus Infection Contributes to Cell Death and Viral Production. PLoS ONE. [Accepted].

In addition, we have additional manuscripts that have been submitted or are in preparation:

Madsen C, Hooper I, Lundberg L, Shafagati N, Guendel I, Sampey G, Senina S, Bailey C, Kashanchi F, Jean KT, Kehn-Hall K. Venezuelan Equine Encephalitis Virus interacts with host cellular micro-RNA processing machinery to facilitate viral replication. *In preparation.*

Aarthi Narayanan, Kylene Kehn-Hall, Svetlana Senina, Lindsay Hill, Rachel Van Duyne, Irene Guendel, Ravi Das, Alan Baer, Michael Turell, Taissia Popova, Bhaskar Das, Charles Bailey, Fatah Kashanchi. Curcumin Inhibits Rift Valley Fever Virus Replication in Human Cells. Submitted to J Biol Chem. *Under revision.*

Aarthi Narayanan, Myung Chung, Kylene Kehn-Hall, Ramin Hakami, Charles Bailey, Sina Bavari, Fatah Kashanchi. Oxidative stress contributes to apoptosis in RVFV infected liver cells. *In preparation.*

The research supported by the DOE grant has resulted in the following scientific presentations:

"Proteomic Characterization of the Rift Valley Fever Virus NSs Protein", ASTMH 59th Annual Meeting, 2010 (Oral Presentation, Kylene Kehn-Hall)

“Membrane Proteomics of Virally Infected Cells for the Identification of Novel Therapeutic Targets”, Chemical and Biological Defense Science and Technology Conference, 2010 (Oral Presentation, Kylene Kehn-Hall)

“Interaction of RVFV Anti-Apoptotic Protein NSm with the Host p38-MAPK Signaling Cascade”, Chemical and Biological Defense Science and Technology Conference, 2010 (Oral Presentation, Aarthi Narayanan)

“Alteration in superoxide dismutase 1 causes oxidative stress and p38 MAPK activation following RVFV infection”, Chemical and Biological Defense Science and Technology Conference, 2010 (Poster Presentation, Aarthi Narayanan)

“Proteomic Characterization of the Rift Valley Fever Virus NSs-Host Protein Interactions”, ASM Biodefense, 2011 (Poster Presentation, Kylene Kehn-Hall)

“Induction of DNA Damage Signaling Cascade upon RVFV Infection”, ASM Biodefense, 2011 (Poster Presentation, Alan Baer)

““Alteration in superoxide dismutase 1 causes oxidative stress and p38 MAPK activation following RVFV infection”, ASM Biodefense, 2011 (Poster Presentation, Aarthi Narayanan)

“Role of NFkB cascade in RVFV infection”, ASM Biodefense, 2011 (Poster Presentation, Aarthi Narayanan)

“Modulation of GSK-3beta activity in encephalitic viral infections”, International Conference and Exhibition on Virology, 2011 (Oral Presentation, Kylene Kehn-Hall)

“Changes in Cellular MicroRNA Following Rift Valley Fever Virus Infection”, Chemical and Biological Defense Science and Technology Conference, 2011 (Oral Presentation, Cathaleen King)

“Oxidative stress and liver pathology in RVFV infection”, Chemical and Biological Defense Science and Technology Conference, 2011 (Poster Presentation, Aarthi Narayanan)

“Activation of host NFkB signaling cascade is a crucial component of RVFV infection”, Chemical and Biological Defense Science and Technology Conference, 2011 (Poster Presentation, Aarthi Narayanan)

“Interactions of Venezuelan Equine Encephalitis Virus with Host MicroRNA Processing Machinery”, ASM Biodefense, 2012 (Oral Presentation, Kylene Kehn-Hall)

“Interplay between the host NFkB cascade and Rift Valley fever virus”, ASM Biodefense, 2012 (Oral Presentation, Aarthi Narayanan)