Curcumin Inhibits Rift Valley Fever Virus Replication in Human Cells*

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Background: Rift Valley fever virus is a single-stranded RNA virus that causes disease in humans and livestock.

Results: Rift Valley fever virus infection activates the host NFκB signaling cascade.

Conclusions: NFκB inhibitors, particularly curcumin, down regulates virus in both in vitro and in vivo models.

Significance: Novel versions of host components resulting from an infection make them ideal therapeutic targets.

SUMMARY

Rift Valley fever virus (RVFV) is an arbovirus that is classified as a select agent, an emerging infectious virus and an agricultural pathogen. Understanding RVFV-host interactions is imperative to the design of novel therapeutics. Here, we report that an infection by the MP-12 strain of RVFV induces phosphorylation of the p65 component of the NFκB cascade. We demonstrate that phosphorylation of p65 (serine 536) involves phosphorylation of IκBα and occurs through the classical NFκB 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 phosphorylates an IκBα substrate. Inhibition of the IKK complex using inhibitors impairs viral replication thus alluding to the requirement of an active IKK complex to the viral life cycle. Curcumin, strongly down regulates levels of extracellular infectious virus. Our data demonstrate that curcumin binds to and inhibits kinase activity of the IKK-β2 complex in infected cells. Curcumin partially exerts its inhibitory influence on RVFV replication by interfering with IKK-β2 mediated phosphorylation of the viral protein NSs and by altering cell cycle of treated cells. Curcumin also demonstrates efficacy against ZH501, the fully virulent version of RVFV. Curcumin treatment down regulates viral replication in the liver of infected animals. Our data point 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 interaction and function, qualify as unique therapeutic targets.

Rift Valley fever virus (RVFV) is a RNA virus that belongs to the genus Phlebovirus, family Bunyaviridae (1). It infects humans and livestock and causes Rift Valley Fever (RVF). RVFV is classified as an emerging infectious agent and as a category A select agent. RVFV is included as an agricultural pathogen by the USDA as it causes 100% abortion in cattle and extensive death of newborns. In humans, RVFV infection causes fever, ocular damage, liver damage, hemorrhagic fever and death in some cases (2). There are currently limited options for vaccine candidates that include MP-12 and Clone 13 versions of RVFV (3). Ribavirin may be used as a treatment option, albeit with suboptimal efficacy (2,4).

Overall, we are lacking in our understanding of host factors that contribute to RVFV-induced pathogenesis which is vital to not only understand disease progression, but also to design better therapeutics.

RVFV is a single-stranded RNA virus with a tripartite genome. The L (large) segment encodes the RNA dependent RNA polymerase. The M (medium) segment codes for the glycoproteins Gn and Gc. Additionally, the M segment encodes a 78kDa protein of unknown function and a small nonstructural protein NSm. NSm has been demonstrated to have an anti-apoptotic function in infected cells (5,6). The S (small) segment encodes the N protein and a second nonstructural protein NSs. NSs, a viral virulence factor, is a transcriptional repressor that controls expression of host genes determines cell fate by regulating numerous host cell events such as apoptosis, survival and cell cycle progression.

Our earlier Reverse Phase protein MicroArray (RPMA) studies revealed that infection of human small airway lung epithelial cells (HSAECs) by the virulent ZH501 strain of RVFV elicited multiple host phospho-signaling events relevant to diverse pathological manifestations such as oxidative stress, activation of stress response MAPKs and DNA damage (17,18). Multiple on-going follow up studies indicated that the signaling responses detected in HSAECs could also be observed in many other cell types such as HepG2 cells, HeLa cells and 293T cells following RVFV infection (17, 48; data not shown). Therefore, our results support the use of HSAECs as a model cell line to study host signaling events after RVFV infection.

One host signaling event that is connected to multiple manifestations including cellular stress and DNA damage following RVFV infection is the p65/Nuclear Factor kappa B (NFκB) signaling cascade (19-24). In fact, Billecocq et al, as part of the study that demonstrated the involvement of NSs in interferon suppression, have shown nuclear presence and DNA binding function of NFκB after RVFV infection (7). Activation of the NFκB response is a multistep process that originates at the plasma membrane in the form of receptor activation and terminates in the nuclear activation of NFκB responsive genes (25). In the classical NFκB activation cascade, a heterotrimeric I kappa B kinase (IKK) complex consisting of IKK-α, IKK-β and IKK-γ (NFκB essential modulator or NEMO) induces phosphorylation of IkBα which is then degraded by the host proteasome. Degradation of IkBα exposes the nuclear localization signal on p65 which is then translocated to the nucleus. Once within the nucleus, p65 forms dimers on κB elements of NFκB responsive genes. Transcription of these genes determines cell fate by regulating numerous host cell events such as apoptosis, survival and cell cycle progression.

We have previously demonstrated that inhibition of the host signaling kinase components such as JNK and MEK inhibited viral replication (18). Along these lines, recent publications by our colleagues have provided evidence that regulation of the host factors in the context of RVFV infection is a viable and attractive therapeutic strategy to down regulate virus replication (26,27). In this manuscript, we sought to expand on the activation of the NFκB signaling cascade following infection by MP-12 virus. Our experiments have resulted in the identification of a novel low molecular form of IKK-β that is enzymatically active and unique only to infected cells. We have labeled this novel complex as IKK-β2. Additionally, our results suggest that the IKK complex may play a role in the viral life cycle because inhibitors that target the IKK complex also result in down regulation of extracellular virus. We have identified curcumin as a candidate inhibitor that displays effective inhibition of virus, both in the case of pre-exposure or post-exposure treatment. We provide evidence that suggest that
Curcumin may exert its inhibitory effect on RVFV replication by influencing cell cycle progression of the host cell. Additionally, we demonstrate that IKK-β2 may phosphorylate NSs which could enhance the ability of NSs to interact with host proteins such as mSin3A which is critical for NSs-induced down regulation of host transcription function. We provide evidence that curcumin prevents phosphorylation of NSs by IKK-β2 thus providing additional mechanistic explanation for curcumin-mediated viral inhibition. Experiments carried out using the virulent ZH501 strain demonstrate that curcumin can inhibit replication of the fully virulent virus as well. Finally, our experiments using the INFAR-/- murine model (28, 29) provide preliminary proof-of-concept validation that curcumin can down regulate virus in the livers of infected animals as well, thus paving the way for further development of novel curcumin-based therapeutic options.

**EXPERIMENTAL PROCEDURES**

**Viruses** - MP-12 strain of RVFV is a live attenuated vaccine derivative of the ZH548 strain. ZH548 was isolated from a patient with uncomplicated RVFV infection in 1977. MP-12 was generated by 12 serial passages in MRC5 cells in the presence of 5-fluorouracil, which induced a total of 25 nucleotide changes across the three viral genome segments. arMP-12-del21/384 has a large deletion in the pre-Gn region of the M segment and as a result does not express NSm, 78 kDa, 75 kDa, or 73 kDa proteins encoded by this region. rMP-12-NSdel completely lacks the NSs ORF. ZH501 strain of RVFV is a fully virulent strain of RVFV.

**Cell culture, viral infection and extract preparation** - HSAECs were cultured, infected with RVFV and whole cell extracts prepared as previously described (18). Briefly, HSAECs were grown in Ham’s F12 medium and infected with MP-12 (MOI: 3). In the case of infections with the NSs and NSm mutant viruses (6,28), cells were infected with the appropriate mutant constructs (MOI: 3). When infections were carried out in 6-well plates, existing medium was removed (and stored as “conditioned medium”), cells washed with PBS and overlaid with 400 µl of medium with virus. After incubation for one hour at 37°C, the overlay was removed, wells washed twice with PBS and replaced with the conditioned medium.

When infections were carried out in 96-well plates, a similar approach was used with the only exception of utilizing an overlay medium (with virus) of 50 µl. HepG2, HeLa cells and A549 cells were also infected following similar methods. To prepare whole cell extracts, supernatant was removed from the wells and cells were lysed in lysis buffer {1:1 mixture of T-PER reagent (Pierce, IL), 2X Tris-glycine SDS sample buffer (Novex, Invitrogen), 2.5% β-mercaptoethanol, and protease and phosphatase inhibitor cocktail (1X Halt cocktail, Pierce} and boiled for 10 minutes prior to electrophoresis.

**Western blot analysis** - Whole cell lysates were separated in 4-20% Tris-glycine gels and transferred to nitrocellulose membranes (iBlot Gel Transfer system, Invitrogen). The membranes were blocked with 1% dry milk solution in PBS-T at room temperature. Primary antibodies to RVFV (ProSci, Cat# 4519), Total p65 (Abcam, Cat# ab7970), phospho-p65 (ser536) (Santacruz biotechnology, Inc., Cat# 33020), phospho-IκBα (Santacruz biotechnology, Inc., Cat# sc21869), HRP conjugated Actin (Abcam, Cat# ab49900), IKK-α (Santacruz biotechnology, Inc., Cat# sc-7182), IKK-β (Santacruz biotechnology, Inc., sc-7329), and IKK-γ (Cell Signaling, Cat#2685P), were used according to manufacturer’s instructions and the blots were incubated overnight at 4°C. The blots were then washed 3X with PBS-T and incubated with secondary HRP coupled goat anti-rabbit antibody (Cell Signaling, Cat# 7074). The blots were visualized by chemiluminescence using Super signal west femto maximum sensitivity substrate kit (ThermoScientific) and a BIO-RAD Molecular Imager ChemiDoc XRS system (BIO-RAD). Band intensities were calculated using Quantity One 4.6.5 software (BIO-RAD).

**Inhibitor studies** - For inhibitor studies, HSAECs were seeded at 50,000 cells per well in a 96-well plate and viral infections were carried out (MOI: 0.1). The inhibitors used in this study are as follows: Curcumin (Santacruz biotechnology, Inc., Cat# sc-200509), Synthetic curcumin (Santacruz biotechnology, Inc., Cat# sc-294110), Dimethoxycurcumin (Santacruz biotechnology, Inc., Cat# 205217), Lactacystin (Calbiochem, Cat# 426100), Genistein (Sigma, Cat# G6649), Resveratrol (Sigma, Cat# R5010), 17-DMAG (Santacruz biotechnology, Inc., Cat# sc-202005), and
SC-514 (Santacruz biotechnology, Inc., Cat# sc-205504), Arctigenin (Santacruz biotechnology, Inc., Cat# sc-202957), IKK2-compound IV (Santacruz biotechnology, Inc., Cat# sc-203083), BAY-11-7082 (Sigma, Cat# B5681), BAY-11-7085 (Sigma, Cat# B5681), RO-106-9920 (Santacruz biotechnology, Inc., Cat# sc-203240), CAPE (Santacruz biotechnology, Inc., Cat# sc-200800), 5,7-dihydroxy-4-methylcoumarin (Santacruz biotechnology, Inc., Cat# sc-254863), o-phenanthroline (Santacruz biotechnology, Inc., Cat# 202256). The inhibitors were dissolved in 100% DMSO and added to the cells at final inhibitor concentration of 10 µm (0.1% DMSO final concentration).

Quantitative RT-PCR (qRT-PCR) analysis- HSAECs were grown at a density of 50,000 cells per well in 96 well plates. Viral RNA from cell culture supernatants was extracted using Ambion's MagMAX™-96 Viral RNA Isolation Kit, and RNA analyzed by qRT-PCR. The primers and probe used for amplification of viral RNA were originally described by Drosten et al (29). qRT-PCR assays were performed using the ABI Prism 7000 and Invitrogen's RNA UltraSense™ One-Step Quantitative RT-PCR System. Cycling conditions were as follows: 1 cycle- 50° C for 15 minutes, 1 cycle 95° C for 2 minutes and 40 cycles- 95° C for 15 seconds and 60° C for 30 seconds. The absolute quantification was calculated based on the threshold cycle (Ct) relative to the standard curve.

Plaque Assays- Neutral red plaque assays were performed by standard procedures. Briefly, vero cells were plated in 6 well plates (10⁶ cells per well). Supernatants were diluted in DMEM media (10² to 10⁶) and used to infect cells in duplicate. After 1 hour infection the media was removed, and overlaid with 2X E-MEM and 0.5% agarose solution. After the overlay solidified, plates were incubated for 48 hours. Plaques were visualized with Neutral Red, E-MEM and 0.5% agarose solution by overlaying Neutral Red on top of the first layer. After the overlay had solidified, plates were placed in the incubator for an additional 24 hours after which plaques were counted and viral titers determined.

Flow cytometry analysis- HSAECs were prepared for flow cytometry analysis by standard procedures. Briefly, the cells were washed twice in 1X PBS (without calcium and magnesium) and trypsinized. Trypsin was neutralized by adding back cold media with 10% serum and the cells were spun down at 2000 rpm for 10 minutes in a refrigerated microcentrifuge. The cell pellet was washed twice with 1X PBS and resuspended in 70% ice cold ethanol. The cells were rehydrated using 1X PBS (without calcium and magnesium) for 15 minutes and pelleted. Cells were then stained with 1 ml of Propidium iodide solution and cell cycle analysis was carried out on an Accuri C6 flow cytometer. Data analysis was performed with Multicycle AV and FCS Express.

Cell Viability Assays- HSAECs were seeded in 96 well plates at 50,000 cells per well and cell viability was measured using CellTiter-Glo Cell Luminescent Viability kit (Promega) as per manufacturer’s instructions. Briefly, an equal volume of room temperature media and CellTiter-Glo reagent was added to the cells. The plate was shaken for 2 minutes on an orbital shaker and after incubation for 10 minutes at room temperature luminescence was detected using the DTX 880 multimode detector (Beckman Coulter).

Size-exclusion chromatography- HSAECs were infected with MP-12 virus (MOI: 10) and cells were pelleted for analysis at approximately 20 hours post infection. In case of TNFα treatment, HSAECs were treated with TNFα (10 and 50 ng/ml). BCBL-1 cells are Kaposi Sarcoma Herpes Virus (KSHV) infected cells and BJAB cells are their uninfected counterparts. In all cases, cell pellets were washed twice with phosphate-buffered saline (PBS) without Ca²⁺ and Mg²⁺ and resuspended in lysis buffer [50 mM Tris– HCl (pH 7.5), 120 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 0.5% NP-40, 50 mM NaF, 0.2 mM Na₃VO₄, 1 mM DTT, and one complete protease cocktail tablet/50 mL] and incubated on ice for 20 minutes, with gentle vortexing every 5 minutes. Lysates were then centrifuged at 4°C at 10,000 rpm for 10 minutes. Supernatants were transferred to a fresh tube and protein concentrations were determined using the Bradford protein assay (BioRad, Hercules, CA). Two milligrams of protein from each treatment was equilibrated and degassed in chromatography running buffer [0.2 M Tris–HCl (pH 7.5), 0.5 M NaCl, and 5% glycerol]. The lysates were run on a Superose 6 HR 10/30 size-exclusion chromatography column using the AKTA purifier system (GE Healthcare, Piscataway, NJ, USA).
The samples were injected over a 1 ml loop into the column and the columns used in this study were used for multiple runs prior to when the actual virus-infected or uninfected control extracts were fractionated. This is an essential step to ensure that the column is conditioned and the flow rates and elution patterns are reproducible. Flow-through was collected at 4°C at a flow rate of 0.3 mL/minute at 0.5 mL for approximately 70 fractions. Every 5th fraction was acetone precipitated using 4 volumes of ice-cold 100% acetone, incubating for 15 minutes on ice. Lysates were centrifuged at 4°C for 10 minutes at 12,000 rpm, supernatants were removed, and the pellets were allowed to dry for a few minutes at room temperature. The pellets were resuspended in Laemmli buffer and analyzed by immunoblotting for IKK-α, IKK-β, IKK-γ and β-actin.

Immunoprecipitation and in vitro kinase assay- Immunoprecipitation (IP) and in vitro kinase assays were carried out as previously described (30). Briefly, for immunoprecipitation, low molecular weight complex fractions from MP-12 or UV-MP-12 infected cells were immunoprecipitated at 4°C overnight with IKK-β antibody. The next day, complexes were precipitated with A/G beads (Calbiochem) for 2 hours at 4°C. Immunoprecipitated samples were washed twice with appropriate TNE buffer and kinase buffer. Reaction mixtures (20 μl) contained final concentrations: 40 mM β-glycerophosphate pH 7.4, 7.5 mM MgCl₂, 7.5 mM EGTA, 5% glycerol, [γ-32P]-ATP (0.2 mM, 1 μCi), 50 mM NaF, 1 mM orthovanadate, and 0.1% (v/v) β-mercaptoethanol. Phosphorylation reactions were performed with immunoprecipitated material and [γ-32P]-labeled GST-IkBα as a substrate in TTK kinase buffer containing 50 mM HEPES (pH 7.9), 10 mM MgCl₂, 6 mM EGTA, and 2.5 mM dithiothreitol. When using a GST-NSs substrate, a similar reaction set up was employed. For reactions involving curcumin, synthetic curcumin (Santacruz biotechnology, Inc., Cat# sc-294110) was included in the reaction at 0.1 and 1 μm concentrations. Reactions were incubated at 37°C for 1 hour and stopped by the addition of 1 volume of Laemmli sample buffer containing 5% β-mercaptoethanol and run on a 4–20% SDS-PAGE. Gels were subjected to autoradiography and quantification using Molecular Dynamics PhosphorImager software (Amersham 6BIOsciences, Piscataway, NJ, USA).

Mass spectrometry- Fractions corresponding to the medium molecular weight IKK-β complex and the low molecular weight IKK-β2 complex were immunoprecipitated with an anti-IKKβ antibody. The immunoprecipitated material was separated on a gel and multiple bands were cut out that spanned the entire length of the gel. The separated material was then subjected to in-gel tryptic digestion (Trypsin, Promega) overnight at 37°C. The digested peptides were eluted using ZipTip purification (Millipore) and identification of the peptides was performed by LTQ-tandem MS/MS equipped with a reverse-phase liquid chromatography nanospray (ThermoFisher). The reverse phase column was slurry-packed in house with 5 μM, 200-A pore size C₁₈ resin (Michrom BioResources) in a 100 um X 10 cm fused silica capillary (Polymicro Technologies) with a laser-pulled tip. After sample injection, the column was washed for 5 min at 200 nL/min with 0.1% formic acid, peptides were eluted using a 50-min linear gradient from 0 to 40% acetonitrile and an additional step of 80% acetonitrile (all in 0.1% formic acid) for 5 minutes. The LTQ-MS was operated in a data-dependent mode in which each full MS scan was followed by five MS-MS scans where the five most abundant molecular ions were dynamically selected and fragmented by collision-induced dissociation using normalized collision energy of 35%. Tandem mass spectra were matched against the National Center for Biotechnology Information mouse database by Sequest Bioworks software (ThermoFisher) using full tryptic cleavage constraints and static cysteine alkylation by iodoacetamide. For a peptide to be considered legitimately identified, it had to be the top number one matched and had to achieve cross-correlation scores of 1.9 for [M+H]⁺, 2.2 for [M+2H]²⁺, 3.5 for [M+3H]³⁺, ΔCn=0.1, and a maximum probability of randomized identification of 0.01.

Curcumin-bead binding assay- Curcumin immobilized in trimethoxysilane based nanoparticles (patent pending) or nanoparticles without curcumin were synthesized as follows: 30 ml of 50 mM PBS buffer at pH 7.5, 1ml of PEG (200) and 4 ml of curcumin (1mg/ml in DMSO) were added sequentially and vortexed thoroughly. Next, 4 ml of previously hydrolyzed TMOS was
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added to the tube, and the contents were vortexed for about 2 minutes. The tube was allowed to sit undisturbed for gelation (2-5 hours). The gel was then lyophilized for 24-48 hours. The resulting particles were ball milled at 150 rpm for 6.5 hours. The particles were incubated with fractions 18-21 (medium molecular weight complex) and 33-36 (low molecular weight complex) from MP-12 infected and UV-MP-12 infected cells at 4° C overnight. The next day, the particles were washed 2X with TNE-50 (0.1% NP-40) buffer, resuspended in 20 µl of Laemmli sample buffer containing 5% β-mercaptoethanol and run on a 4–20% Tris-glycine gels.

Animal studies- Six to eight week old INFAR-/- mice were obtained from the National Center for Biodefense and Infectious Disease’s breeding colony (GMU). All experiments were carried out in bio-safety level 2 (BSL-2) facilities and in accordance with the Guide for the Care and Use of Laboratory Animals (Committee on Care And Use of Laboratory Animals of The Institute of Laboratory Animal Resources, National Research Council, NIH Publication No. 86–23, revised 1996). For infection experiments, all animals were treated subcutaneously with DMSO alone or curcumin every day for 4 days. Animals were pretreated with curcumin 24 hours prior to infection and infected with 10^4 pfu of MP-12 virus by intra-peritoneal injection. Mice were weighed daily and monitored for morbidity and mortality, including lethargy and ruffled fur. Liver tissue was dissected from DMSO treated and curcumin treated animals and analyzed for viral load by standard plaque assay.

Statistical analysis- All quantifications indicated are based on data obtained from triplicate experiments. P-values were calculated by the student’s t-test.

**RESULTS**

The NFκB subunit p65 is phosphorylated at serine 536 in MP-12 infected cells- Using a RPMA approach, we previously demonstrated that infection of HSAECs with the virulent strain of RVFV (ZH501) caused phosphorylation and activation of multiple signal transduction events including p38 MAPK, JNK and ERK and their downstream transcription targets including p65 (NFκB) in the host cell (18). Specific to the NFκB signaling pathway, RPMA studies indicated that following infection by ZH501 virus, p65 was phosphorylated at serine 536 (18). In the current study, we first asked the question whether the attenuated strain (MP-12) also caused phosphorylation of p65 at serine 536 in HSAECs. We carried out western blot analysis of MP-12 infected and mock infected cell extracts obtained at 1 and 6 hours post infection. Activation of the NFκB cascade and p65 phosphorylation (serine 536) has been demonstrated to occur at similar time points after viral infection (31). During the course of these experiments care was taken to maintain cells in “conditioned medium” (the same medium that cells were maintained in prior to infection was returned to the cells post infection) to avoid any p65 phosphorylation event due to medium/serum components. Results demonstrated that p65 is phosphorylated at serine 536 at both 1 and 6 hours post infection when compared to the mock infected control samples (Figure 1A, compare lanes 1 with 2 and 4 with 5). Phosphorylation of p65 was however, not due to an increase in total p65 levels (Figure 1A).

Phosphorylation of IkBα was also detectable at 1 and 6 hours post infection (Figure 1A) suggesting that the activation of p65 occurred by the classical pathway. We performed similar experiments with an UV-inactivated virus in order to address the issue of specificity of the phosphorylation event. Quantification of signal intensities of phosphorylated p65 observed in multiple experiments revealed that MP-12 infection resulted in an approximate 8-9 fold increase in phospho-p65 when compared to the mock-infected control (Figure 1B). This fold increase in phosphorylation was significantly higher than that observed in the case of infection by the UV-inactivated virus, which did not change over the time frame tested. This observation provided specificity to the observed phosphorylation of p65 following MP-12 infection.

We performed plaque assays with the UV-inactivated virus in comparison with wild type MP-12 and were unable to observe any plaques with the inactivated virus (Supplemental Figure 1A). We also tested whether p65 was phosphorylated on additional residues (serine 276) and were unable to detect significant changes in the phosphorylation of serine 276 at the same time points (data not shown).
We then asked whether any viral protein component played a role in the phosphorylation of p65. We infected HSAECs with the wild type MP-12 virus or mutant viruses that lacked the NSs (rMP-12-NSdel) or the NSm (arMP-12-del21/384) coding sequences and checked for p65 phosphorylation. Results demonstrated a modest influence of NSs on the phosphorylation of p65 (an average drop of about 34% as determined from two experiments) suggesting that the viral protein may play a role in inducing p65 phosphorylation (Figure 1C). We then asked if the NSs and the NSm mutant viruses displayed any inherent replication differences in our cell system in order to verify that the observed effects on p65 phosphorylation were not indirect. To that end, we performed quantitative RT-PCR (qRT-PCR) analysis of intracellular viral RNA at 6 hours post infection. We reasoned that a quantitative analysis of intracellular RNA of the wild type and mutant viruses performed at the time point when phosphorylation differences were observed will demonstrate if it is an indirect consequence of decreased replication kinetics of the NSs mutant. The data is shown in Figure 1D where we did not observe any significant difference between replication of the wild type or the mutants at the time point tested suggesting that the observed differences in p65 phosphorylation level following infection by MP-12 and mutant viruses were not a mere reflection of altered replication kinetics.

We next asked whether phosphorylation of p65 on serine 536 was a cell type specific event. HeLa and HepG2 cells were infected with MP-12 virus (MOI: 3) and cell extracts were analyzed by western blot. These experiments revealed that p65 was indeed phosphorylated in both cell types following viral infection without any change in total p65 levels (Supplemental Figure 1B). Cumulatively, these experiments revealed that p65 is phosphorylated on serine 536 following infection by MP-12 virus.

**Presence of a novel IKK complex in RVFV infected cells.** The IKK complex is the upstream component in the NFκB cascade that leads to the phosphorylation of IκBα and p65 (31, 32). The IKK complex that functions as the IκBα and p65 kinase is typically a hetero-trimer that consists of IKK-α, IKK-β and IKK-γ (NEMO) proteins. IKK-α, and IKK-β possess kinase activity while IKK-γ (NEMO), is required to stabilize the heterotrimeric complex.

First, we asked whether the IKK complex is altered due to MP-12 infection. When no major changes in total IKK levels could be detected upon infection (data not shown), we asked whether changes could be manifested as alterations of IKK-higher-order protein complexes. To answer that question, we performed conventional chromatography separation of MP-12 infected cell extracts using a Superose 6 sizing column and analyzed fractions for distribution of individual IKK components by western blots. The Superose 6 sizing column is designed to separate higher-order molecular complexes based on cumulative molecular weight as indicated in Figure 2A. Therefore, if individual components of the IKK complex display alterations in higher-order protein association, the cumulative molecular weight of that component will be expected to change. This will result in a different elution profile which can then be detected by methods such as western blotting. As a control, we utilized extracts from cells infected with the UV-inactivated virus. Whole cell lysates from infected cells were fractionated and every 5th fraction (F# in Figure 2A) was analyzed by western blot for IKK-α, IKK-β and IKK-γ (NEMO) proteins. β-actin was analyzed as a control. Results showed that there was an alteration in the distribution profile of IKK-β in MP-12 infected cells that was not observable in the UV-inactivated virus infected cells (Figure 2A). Among the changes, IKK-β reproducibly displayed unique low molecular weight complexes in MP-12 infected cells that we will refer to as IKK-β2 henceforth (Figure 2A, red squares). Immunoblotting the same extracts with actin demonstrated comparable distribution of actin in fractions 30-40 in both MP-12 and UV-MP-12 infected cell extracts.

IKK-β subunit is one of the kinases involved in phosphorylation of p65 at serine 536 (34). The native IKK complex exhibits a molecular mass of approximately 700-900 kDa. The IKK complex that is common to both MP-12 virus and UV-MP-12 virus infected cells migrated at an approximate molecular range of 600-900 kDa in our sizing columns (Figure 2A). The novel IKK-β2 complex eluted around 300 kDa in the case of MP-12 infected cells (Figure 2; fractions 35-40). The existence of the IKK-β2 complex...
after infection by MP-12 has been confirmed in multiple independent experiments. We did not observe any significant or reproducible alterations in the case of the IKK-α or IKK-γ subunits in the MP-12 and UV-MP-12 infected cells.

We then asked the question if a similar alteration in IKK-β can be observed in the case of an infection by a DNA virus. To that end, we utilized Kaposi Sarcoma Herpes Virus (KSHV / HHV-8) infected cells (BCBL-1) and their uninfected counterparts BJAB (33) and performed a similar fractionation experiment as we did with the RVFV infected cells. KSHV infection is known to activate the host NFκB signaling cascade with involvement of the IKK complex (34-36). As seen in Figure 2B, interestingly, when infected with a DNA virus, we observed that IKK-β distribution had shifted towards the high molecular weight range (> 670 kDa) (compare lanes 4 and 5 between BCBL1 and BJAB samples in Figure 2B). This suggested an altered regulation of IKK-β in the case of at least one DNA virus that was different from that of RVFV infection. Additionally, we also tested whether activation of the NFκB pathway by inducers such as TNFα would result in the formation of IKK-β2. To that end, HSAECs were treated with TNFα. Cells were analyzed 24 hours later by a similar fractionation scheme. As seen in Figure 2C, TNFα treatment does not result in any significant change to the IKK-β profile with almost all detectable IKK-β eluting between fractions 20 and 30. We tested the fractionation profile of TNFα at 5X higher concentration (50 ng/ml) and were still unable to detect any difference in IKK-β distribution profile between untreated and treated cells (data not shown). Therefore, the data from the fractionation studies revealed that a low molecular weight version of IKK-β (IKK-β2) may be a distinctive occurrence during RVFV infection which was not observed during infection by a DNA virus or during NFκB activation by inducers such as TNFα.

We carried out proteomic analysis of the IKK-β and IKK-β2 complex by immunoprecipitating the kinase using an anti-IKK-β antibody from pooled fractions. Specifically, we pooled fractions 19-23 (medium molecular weight complex) and fractions 34-38 (low molecular weight complex) and subjected the immunoprecipitated complexes to LC-MS/MS analysis. The data, as shown in Figure 2D demonstrated multiple common and unique aspects to the IKK-β and IKK-β2 complexes. As for commonalities, in both cases, we observed interaction of chaperone proteins including Hsp70 and 90. Hsp90 is also known to be a strong interacting protein with IKK-γ. Figure 2A shows that IKK-γ co-fractionated with both IKK-β and IKK-β2 in the Superose column. Therefore, both complexes are likely to contain IKK-γ. In fact, based on data shown in Figure 2A, we suspect that IKK-β2 complex may contain slightly more IKK-γ when compared to IKK-β. Additionally, we observed that IKK-β and IKK-β2 contained actin-related proteins and proteins involved in nuclear-cytoplasmic transport. The fact that both complexes contained nuclear export/import related proteins suggested that both complexes were likely to be present in the nuclear and cytoplasmic compartments of the cell; however, it would be of great interest to determine targets of the two kinase complexes in the nuclear and cytoplasmic compartments. In terms of unique aspects to either complex, our results suggested that specific proteasome components may be associated with IKK-β, which were not found to be associated with IKK-β2. This indicates interesting regulatory possibilities with altered stability kinetics between the two enzymatic complexes. The association of proteasome components may extend to inclusion of Diablo in this complex as well. It was also interesting to observe protein phosphatase 2C associated with IKK-β. This may also contribute to differences in kinase-active states of IKK-β versus IKK-β2 with IKK-β2 possibly being more kinase-active than IKK-β. The association of a methyltransferase enzyme component may be suggestive of a nuclear inhibitory function for IKK-β which is absent in the case of IKK-β2, again alluding to IKK-β2 having different kinetic rates and targets from the IKK-β enzyme. Therefore, analysis of the proteomic composition of IKKβ and IKKβ2, while not indicating any note-worthy changes in intracellular distribution, pointed to differences in regulation that will affect function and targets.

Overall, our results indicated that MP-12 infection resulted in the formation of a novel IKK-β2 complex without any major alterations in the other components of the IKK complex which may
be a distinctive consequence of a RNA virus infection.

**Inhibition of the IKK complex resulted in decreased viral replication** - We then investigated whether activation of the NFκB cascade was essential for viral replication. We utilized well-established inhibitors of the NFκB cascade including those that inhibit the IKK complex, IκBα degradation and p65 nuclear translocation (Figure 3A). Briefly, the inhibitors utilized include IKK inhibitors (Geldanamycin, SC514, curcumin, arctigenin and IKK2-compound IV), IκBα inhibitors (BAY-11-7082, BAY-11-7085, RO-106-9920), nuclear translocation inhibitor (CAPE) and those that prevent NFκB dependent transcriptional activation (5, 7-dihydroxy 4-methyl coumarin and O-phenanthroline). HSAECs were pretreated with inhibitors for two hours followed by infection with MP-12 virus. Infected cells were treated with DMSO alone as a negative control. After infection, cells were post-treated with the inhibitor or DMSO for 24 hours and supernatants analyzed for infectious virus by plaque assays. The results of our inhibitor studies demonstrated that activation of the IKK complex and nuclear translocation was necessary for viral replication (Figure 3B; Supplemental Figure 2). Untreated cells (UT), DMSO treated or inhibitor treated cells were also analyzed by CellTiter-Glo (luminescence units) 24 hours post-treatment in order to determine cytotoxic effects. The toxicity studies confirmed that the inhibitors were not toxic to the cells at these concentrations. Among the inhibitors tested, curcumin was the strongest and down regulated RVFV by 3-4 logs (Figure 3B, compare columns 1 and 2 with 3). When we treated cells with a dimethoxy-derivative of curcumin, we found that the level of inhibition of viral replication by the dimethoxy-derivative was comparatively lower than that of synthetic curcumin (Figure 3A, compare columns 4 and 5).

We then determined whether time of addition of curcumin would have an influence on extent of inhibition. We compared extracellular viral genomic copy numbers following pre-treatment (2 hours prior to infection) with curcumin and post-treatment (3 hours post infection) using qRT-PCR. HSAECs were infected with MP-12 and supernatants were collected 24 hours post exposure. DMSO treatments were included as controls. As shown in Figure 4B, treatment of infected cells 3 hours post exposure with curcumin continued to down regulate extracellular virus in a manner comparable to pretreatment with curcumin (compare columns 3 and 5).

As indicated earlier, we observed a unique low molecular weight IKK-β2 complex in MP-12 infected extracts (Figure 2A). We next asked whether the IKK-β2 complex exhibited kinase activity similar to IKK-β and could be inhibited by curcumin. To answer that question, we immunoprecipitated the IKK-β2 complex from fractions 33-36 and performed an in vitro kinase assay using a GST-IκBα as substrate (Figure 5A). An IKK-β immunoprecipitation from HTLV-1 infected cells was used as a positive control (C81) in the kinase assay. The immunoprecipitated IKK-β2 complex demonstrated kinase activity while comparable fractions from UV-MP-12 infected extracts showed no activity (Figure 5A, compare lanes 4 and 6). We performed similar kinase assays from the IKK-β complex that was observed in both MP-12 and UV-MP-12 extracts (fractions 18-21) and detected comparable kinase activity (data not shown). When the kinase assay was carried out using immunoprecipitated IKK-β2 in the presence of increasing concentrations of curcumin, we observed down regulation of kinase activities.
activity at low concentration of curcumin and a complete loss of activity at higher concentrations (Figure 5A, compare lane 4 with lanes 7 and 8), suggesting that curcumin inhibited the IKK-β2 complex in infected cells. As curcumin is known to inhibit the conventional IKK-β complex, we utilized IKK-β immunoprecipitated from fractions 18-21 in the kinase assay in the presence of increasing concentrations of curcumin (lanes 9 and 10). Interestingly, we found that while both IKK-β and IKK-β2 were susceptible to curcumin at high concentrations (lanes 8 and 10) IKK-β2 was more susceptible to curcumin than IKK-β at lower concentrations (compare lanes 7 and 9).

Finally, we asked whether immobilized curcumin could bind to IKK-β2 in vitro. To answer that question, we immobilized curcumin in trimethoxysilane (TMOS)-based nanoparticles and incubated the particles with medium (Fractions 18-21) and low molecular weight fractions (Fractions 33-36) overnight at 4 °C. Next day, bound samples were separated in a 4-20% Tris-glycine gel and western blot analysis was carried out using IKK-β antibody (Figure 5B). Interestingly, immobilized curcumin did not bind to IKK-β in the medium molecular weight complex from the MP-12 infected cell fractions (Fractions 18-21); however, we observed binding of curcumin to the low molecular weight IKK-β2 complex (Fractions 33-36) (Figure 5B, compare lanes 2 and 4). We did not observe similar interactions with immobilized curcumin and IKK-β2 in UV-MP-12 infected cells. This suggested an unique interaction of curcumin with the IKK-β2 complex that is present in infected cells. Taken together, our results demonstrated for the first time that inhibitors of the IKK complex such as curcumin cause down regulation of extracellular virus. Additionally, our functional assays indicated that the kinase activity of the IKK-β2 complex is inhibited by curcumin.

Curcumin, in addition to being an inhibitor of the NFκB cascade, is also a well-documented inhibitor of the host proteasome (37). Relevant to the critical role played by the host proteasome pathway in RVFV biology, Ikegami et al had demonstrated that two proteasome inhibitors lactacystin and MG-132 were capable of reversing the NSs induced post transcriptional down-regulation of PKR (10-12). We extended the studies to see whether proteasome inhibitors other than curcumin could down regulate extracellular virus levels. To answer that question, we pretreated HSAECs with three different proteasome inhibitors, lactacystin (38), resveratrol (39) and genistein (40). Lactacystin is a specific and irreversible inhibitor of the 26S proteasome and has been demonstrated to down regulate the trypsin-like, chymotrypsin-like and peptidyl glutamyl hydrolase-like proteasomal activity of the proteasome (41). Resveratrol has been demonstrated to suppress cytokine-induced proteasome function and degradation of IkBa (42). Genistein is thought to interact with the proteasomal β5 subunit and result in inhibition of the chymotrypsin-like activity of the proteasome (43). After two hours of pretreatment, cells were infected with MP-12 virus. DMSO treated cells were maintained as controls. Supernatants were obtained from inhibitor treated and DMSO treated cells 24 hours post infection and analyzed for extracellular virus by plaque assays. Our results demonstrated that some of the inhibitors could modestly down regulate extracellular virus levels by approximately one log (Supplemental Figure 3A). While treatment with lactacystin down regulated extracellular virus, we observed some level of cytotoxicity associated with inhibitor treatment. We then determined if the observed down regulation of infectious virus was a reflection of low extracellular genomic copy numbers. We carried out qRT-PCR studies with virus specific primers (29). Our studies revealed that treatment with various proteasome inhibitors resulted in comparable decrease in genomic copies (Supplemental Figure 3B). Taken together, these data confirm prior studies that the host proteasomal pathway is a critical component of RVFV-host interaction.

Reduced viral replication correlates with rescue of RVFV induced cell cycle arrest- We have consistently observed that RVFV infection induces a strong S-phase cell arrest of the infected cells in diverse cell types and this arrest is dependent on the viral protein NSs (44) (Figure 6A, B). Here we asked whether inhibition of viral replication could be sufficient to rescue the cells from the infection-induced S-phase arrest. Therefore, we pretreated synchronized cells with curcumin for two hours and then infected the cells with MP-12 virus. Cells were maintained in curcumin post infection. Untreated, infected cells...
were maintained alongside as controls. We performed FACS analysis 24 hours post infection to evaluate cell cycle progression and observed that treatment of cells with curcumin rescued the infected cells from S-phase arrest. The pronounced S-phase peak that is observed in the third histogram (Figure 6A) is reduced to a level that can be compared to the peak observed in the uninfected panel thus suggesting that the infection induced S-phase arrest may be relieved upon curcumin treatment. This is quantitatively demonstrated in Figure 6B. We also observed that treatment of uninfected cells with curcumin decreased the population of cells that were at S-phase (Figure 6A, compare S phase peaks between histograms 1 and 2, Figure 6B, compare bars 1and 3 in the %S-phase panel). Therefore, it may be possible that S-phase cells are better hosts that support RVFV replication and curcumin may inhibit RVFV by lowering the population of susceptible host cells. Additionally, western blot analyses revealed that marker proteins of cell cycle arrest such as Cyclin A and p21/Waf1 accumulated in the MP-12 infected cells while curcumin treatment restored Cyclin A and p21/Waf1 levels to those seen in control mock infected cells (Figure 6C). Rescue of Cyclin A and p21/Waf1 phenotypes suggested that the population of cells that responded to curcumin were likely to be in late G1/ early S phase of the cell cycle. Cyclin D1, which is an early G1 phase marker, did not appear to be influenced significantly by curcumin treatment (in comparison with the DMSO control), thus arguing against curcumin being effective in early G1 phase of the cell cycle. Collectively these experiments suggested that inhibition of viral replication by curcumin may be able to reverse infection-induced host cell phenotypes. Importantly, efficacy of curcumin treatment may be a cell cycle regulated phenomenon with curcumin exerting its antiviral activity and cell cycle rescue in the late G1/early S phase cells.

**Curcumin-based down regulation of RVFV replication is likely due to phosphorylation of NSs and cell cycle changes** We sought to arrive at mechanistic explanations for the inhibition of RVFV replication by curcumin. We approached this question by two independent assays. We first addressed the important issue whether IKK-β2 could function as a kinase for any of the viral proteins. Accordingly, we asked whether IKK-β2 phosphorylated NSs protein. We chose NSs because there is evidence in the literature that nuclear and cytoplasmic forms of NSs can be phosphorylated at 2 different serine residues, 252 and 256 (16). We performed in vitro kinase assays using immunoprecipitated IKK-β2 (fractions from MP-12 infected and UV-MP-12 infected cells as the source of the kinase) and a GST-NSs substrate similar to the studies carried out in Figure 5A. The results shown in Figure 7A demonstrated that NSs can be phosphorylated by IKK-β2 in vitro. Additionally, we observed that this phosphorylation event was sensitive to curcumin as even low concentrations of curcumin could inhibit phosphorylation (compare lanes 4 and 5 to lane 3). Therefore, it is possible that curcumin exerts its inhibitory influence on RVFV replication by interfering with the IKK-β2 mediated phosphorylation of NSs.

We performed a comparative phosphorylation assay of NSs using IKK-β (fractions 20-24) and IKK-β2 (fractions 34-38) to determine relative efficacies of either kinase on the NSs substrate. To that end, GST-NSs was phosphorylated using increasing concentrations (5ng, 50ng and 500ng) of either immunoprecipitated IKK-β or IKK-β2. It was striking to note that there was a strong increase in phosphorylated NSs in the presence of increasing amounts of IKK-β2 (Figure 7B, lanes 5, 6). In comparison, even though we can detect a band corresponding to phosphorylated NSs in the presence of highest amount of IKK-β (Figure 7B, lane 3), the extent of phosphorylation with IKK-β2 was 6X higher (compare lanes 3 and 6) thus suggesting that IKK-β2 is a better kinase when it comes to phosphorylating NSs. The band corresponding to phosphorylated NSs was not observed when GST alone was used as a substrate for IKK-β2 or IKK-β at highest concentration of enzyme (Figure 7B, lanes 1, 2). This experiment provided critical evidence that the phosphorylation of NSs may be more strongly influenced by the IKK-β2 enzyme.

Next, we performed an experiment to address the consequence of NSs phosphorylation. Specifically, we asked the question if phosphorylated NSs would be able to interact with and bind better to other inhibitory proteins such as mSin3A and HDAC3 which are known to co-
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 localize with NSs at the interferon promoter (16). To perform this in vitro binding assay, we utilized IKK-β2 immunoprecipitated from fractions 33-37 in a kinase assay to phosphorylate GST-NSs in the presence of cold ATP. We performed the same reaction with [32P]-ATP alongside to ensure that GST-NSs was indeed phosphorylated by IKK-β2 (data not shown). As a negative control, we carried out the same reaction in the absence of ATP. We then incubated the phosphorylated NSs protein (or unphosphorylated control NSs protein) with HSAEC whole cell extracts (as the source of mSin3A and HDAC3) overnight and then washed the beads 3X with TNE-50 (0.1% NP-40) buffer. The material was then electrophoresed on a 4-20% gel, proteins transferred to nitrocellulose membranes and western blotted using anti-mSin3A and HDAC3 antibodies to compare relative interaction of mSin3A and HDAC3 with either phosphorylated NSs or unphosphorylated NSs. Data shown in Figure 7C indicated that a higher amount of mSin3A was pulled down along with NSs when NSs was phosphorylated (Figure 7C, compare lanes 4 and 5). Interestingly, phosphorylated NSs did not appear to have any significant enhancement of binding with HDAC3 (compare lanes 4 and 5, HDAC3 panel) suggesting that the influence of phosphorylated NSs may be specific to some proteins such as mSin3A. As the second step, to correlate with the observation that the late G1/early S phase cells were effective responders to curcumin, we asked if these cells were effective hosts for viral infection as well. To answer that question, we arrested cells in late G1/early S-phase using hydroxyurea (HU). As controls, we maintained unsynchronized cells and cells that were arrested in G0/G1 using serum starvation. We infected the unsynchronized and synchronized cells with MP-12 virus, maintained them in serum-starved or HU-treated condition for 24 hours and then collected supernatants for plaque assays. As seen in Figure 7D, infection of cells synchronized in late G1/early S-phase resulted in modest increase of virus. Therefore, as suggested earlier, one way by which curcumin may inhibit the virus could be by lowering the population of cells in the late G1/early S transition stage (Figure 6A, B).

As a control, we performed the same experiment as outlined above, but released the cells in complete media after infection for 24 hours after which we collected the supernatants for plaque assays. As seen in Figure 7C, there was a four log drop in the levels of extracellular virus in the presence of curcumin. We carried out validation studies alongside using MP-12 virus in the same cell type and were able to observe a robust inhibition of MP-12 as well (Figure 8B). Based on toxicity studies (Figure 8C), we determined that the inhibition of extracellular virus by curcumin was not a consequence of cell death. Cumulatively, experiments performed with ZH501 virus demonstrated that curcumin treatment can down regulate replication of virulent virus.

Curcumin inhibits ZH501 replication- We then asked if curcumin can exert its inhibitory effect against ZH501, the fully virulent form of RVFV. To test that possibility, we adopted a similar approach as we did with the attenuated strain (MP-12). Briefly, A549 cells were pretreated with curcumin (10 µM) for 2 hours and then infected with ZH501 virus (MOI: 0.1). Infected cells that were not treated with curcumin (UT) were maintained as controls alongside. Supernatants were obtained at 24 hours post infection and analyzed by plaque assays for extracellular virus levels. As shown in Figure 8A, there was a four log drop in the levels of extracellular virus in the presence of curcumin. We carried out validation studies alongside using MP-12 virus in the same cell type and were able to observe a robust inhibition of MP-12 as well (Figure 8B). Based on toxicity studies (Figure 8C), we determined that the inhibition of extracellular virus by curcumin was not a consequence of cell death. Cumulatively, experiments performed with ZH501 virus demonstrated that curcumin treatment can down regulate replication of virulent virus.
Treatment of INFAR-/- mice with curcumin decreases hepatic viral load. Our cell culture studies revealed that curcumin is a strong inhibitor of MP-12 and ZH501 replication. We next asked whether curcumin can exert a similar influence on viral replication in vivo. We used the INFAR-/- mouse model as this is the only model that has been documented to display disease manifestations and mortality following infection by MP-12 virus (8,45) and performed a preliminary proof-of-concept experiment. Our specific question was whether curcumin treatment can down regulate virus in the liver of MP-12 infected mice as the liver is a prominently affected organ in RVFV infection. INFAR-/- mice were pretreated with curcumin 24 hours prior to infection by sub-cutaneous route. Mice were infected by the intra-peritoneal route with MP-12 virus (10^4 pfu/animal) and were continued to be treated with curcumin, once every 24 hours for a period of up to four days. All of the control untreated, infected animals were dead by 3 days post infection while 60% of the treated animals survived for up to 4 days after infection (Figure 9A). We dissected out the liver from the infected animals and measured hepatic viral load by plaque assays. In agreement with our in vitro data, plaque assays revealed strong reduction of viral titers in the livers (up to 90% decrease) of treated animals in comparison with the untreated controls (Figure 9B). Cumulatively, our in vivo experiments using curcumin provides preliminary evidence that curcumin can down regulate viral replication in vivo.

DISCUSSION
RVFV is a category A select agent, an emerging infectious virus and an agricultural pathogen that infects humans and livestock. While in the case of human RVFV infections, mortality up to 45% have been reported in certain instances, infection of cattle and livestock results in an extreme 100% abortion rate. Treatment with generic antivirals such as Ribavirin and supportive therapy are the only options currently available to treat RVF. Understanding the interactions between the virus and the host is an important step towards the design of effective therapeutics. Host-based therapeutic intervention is an advantageous approach to pursue for multiple reasons that include positive influences on the host mean survival time, decreased potential for evolution of resistant viruses and broad-spectrum application potential.

Earlier RPMA studies using both ZH501 and MP-12 viruses revealed that the host NFκB signaling subunit p65 is phosphorylated upon infection (18). This phosphorylation of p65 agrees with the reported nuclear migration and DNA-binding of activated NFκB following RVFV infection as reported by Billecocq et al (7). The term NFκB refers to a family of ubiquitously expressed, structurally related transcription factors namely p65 (RelA), RelB, c-Rel, p50 and p52 (46,47). These five proteins can form homo- or hetero-dimers that are usually held in the cytosol by IκB family of inhibitory proteins. The p65/p50 dimer is the most abundant and well-studied hetero-dimer that regulates a wide array of NFκB responsive genes by binding to the κB site on the promoter. p65 is retained in the cytoplasm by IκBα which, in the classical pathway, is phosphorylated by IKK-β and proteasomally degraded which in turn, leads to nuclear translocation of p65. Our in vitro studies with the MP-12 strain of RVFV demonstrated phosphorylation of p65 on serine 536 in multiple cell types (Figure 1, Supplemental Figure 1B). This phosphorylation event on p65 is known to increase its transactivation potential (46). We have also demonstrated that p65 is phosphorylated on serine 536 via the classical NFκB activation pathway. Similar results have been demonstrated for RSV in a recent publication by Yoboua et al (31). We were unable to detect alternate phosphorylation events on p65, such as phosphorylation of serine 276 within the time frames analyzed. Relevant to that observation, our RPMA studies with MP-12 infected HSAECs revealed that MSK1, one of the upstream kinases that phosphorylates p65 on serine 276 does not display significant changes in phosphorylation at such early time points (data not shown).

The IKK complex is one of the kinase complexes that phosphorylates p65 on serine 536 (47). IKK is a macromolecular signaling complex or the signalosome that consists of two related kinases IKK-α and IKK-β and a third regulatory subunit IKK-γ (NEMO) whose exact mechanistic role in the trimeric complex is still unclear. While both IKK-α and IKK-β kinases can phosphorylate IκBα, IKK-β mediated phosphorylation is more
rapid, dramatically more efficient and corresponds to the classical activation cascade which is what we observed relevant to RVFV infection. Considering that the entire viral life cycle is completed within a matter of a few hours (approximately 8-10 hours between entry and exit), a rapid phosphorylation and activation would be more plausible in contrast to a delayed IKK-α mediated alternative activation cascade. Under stringent isolation parameters, the IKK complex demonstrates an apparent molecular mass of approximately 700-900 kDa. The IKK complex that is common to both MP-12 virus and UV-MP-12 virus infected cells migrated at an approximate molecular range of 600-900 kDa in our sizing columns (Figure 2A). A novel IKK-β containing low molecular weight complex that we refer to as IKK-β2 eluted around 300 kDa in the case of MP-12 infected cells (Figure 2A). Interestingly, such a phenomenon does not happen in the case of infection by a DNA virus or by other NFκB inducers such as TNFα (Figures 2B, C). In contrast to our observation following RVFV infection, IKK-β profile was shifted towards the higher molecular weight range in the case of infection by at least one DNA virus (Figure 2B) suggesting that IKK-β2 may be a phenomenon that is associated with RVFV infection. It would be interesting to determine if IKK-β2 is associated with infection by other RNA viruses as well. Our proteomic analysis of the composition of IKK-β2 indicated that this complex contained multiple chaperones including Hsp70 and Hsp90 that were also associated with the IKK-β complex (Figure 2D). However, interestingly, the IKK-β2 complex appeared to be missing certain regulatory components such as inhibitory enzymes and proteasome units that were seen in association with IKK-β, thus alluding to altered regulation (Figure 2D). IKK-α, in some instances has been suggested to down regulate the kinase activity of IKK-β (48). Additionally, the absence of proteasome subunits and inhibitory enzymatic components hinted to the possibility that the IKK-β2 complex may respond to a different set of activator signaling components and have increased and/or constitutive kinase activity than the regular IKK-α, IKK-β, and IKK-γ containing signalosome. In vitro kinase assay using immunoprecipitated IKK-β2 demonstrated kinase activity alluding to this complex being functional in RVFV infected cells (Figure 5A). In support of such a possibility, screening of multiple inhibitors of the NFκB cascade singled out inhibitors of the IKK-β component as being most effective in the down regulation of RVFV replication (Figures 3, 4). Thus, it is possible that the novel IKK-β2 complex observed in RVFV-infected cells is a hyperactive enzyme that lacks the control that could be normally exerted on the IKK-β complex (43).

The IKK component of the NFκB signaling cascade is a critical node that is often exploited by multiple viruses to either suppress the host innate immune response or enhance their replication potential. Murine Gamma Herpesvirus (gamma HV68), a model system for KSHV and EBV, is known to activate IKK-β. Interestingly, activated, phosphorylated IKK-β promoted viral replication because the viral protein RTA (replication and transcription activator) was a phosphorylation target for IKK-β kinase activity (49). In contrast to this situation, in the case of vaccinia virus infection, the viral protein B14 modulates IKK-β kinase activity in a way that inhibits phosphorylation of its downstream target IκBα without exerting any influence on IKK-α activity (50,51). In a similar manner, Enterovirus 71 2C protein is known to target IKK-β activity by interacting with IKK-β and this is a critical event in the infectious process (52). In the case of EBV infection, the viral protein EBNA1 negatively regulates IKK activation by inhibiting phosphorylation of IKK-α/β (53). There are some interesting lines of evidence in the literature that viruses can influence functionality of IKK-β by altering the composition of the canonical IKK-β complex. A Herpes simplex virus virulence factor γ(49) 34.5 is known to block dendritic cell maturation and hence influence the host innate immune response by associating with IKK-α/β. Importantly, association of γ(49) 34.5 with the canonical IKK-α/β complex now alters the protein composition by recruiting protein phosphatase 1 which, by dephosphorylating IKK-β Influences the host innate immune response (54). Bovine Foamy Virus (BFV), infection by which results in a persistent activation of the NFκB cascade, uses its transactivator protein BTas to keep the NFκB cascade persistently active. To achieve this, the viral transactivator interacts with the IKK-α/β

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complex as demonstrated by co-immunoprecipitation experiments (55).

At this juncture, we hypothesized that the kinase function of IKK-β2 may play a role in the viral replication cycle beyond influencing host transcription. This hypothesis was also based on the observation by Ikegami et al that inhibition of host basal transcription by generic inhibitors such as α-amanitin did not result in down regulation of virus (12). It would be interesting to determine if any of the viral proteins are phosphorylated by IKK-β2. To that effect, our in vitro kinase studies with a GST-NSs substrate revealed that NSs may be phosphorylated by IKK-β2 (Figure 7A, B).

Additionally, phosphorylated NSs appeared to interact better in an in vitro binding assay with specific inhibitory host components such as mSin3A (Figure 7C) while certain other components of transcriptional repressive complexes such as HDAC3 remained unaffected by a phosphorylated NSs. Interaction of NSs with transcription repressor proteins such as mSin3A is a key step in the viral suppression of the host innate immune response. Therefore, we suspect that a critical aspect of an activated NFκB cascade in the context of RVFV replication is the activation of the IKK-β complex, which is likely to exert its effect on NSs in the nucleus. This speculation is supported by our observation that inhibitors that interfered with these two functions (Figure 3B; Supplemental Figure 2) negatively influenced RVFV replication while those that did not influence these two functions had no effect on viral replication. Additionally, while we focused on the interaction between NSs and IKK-β2, it may be possible that such a phenomenon may extend to other viral proteins as well and have influences on virus-induced pathology. With regards to the formation of IKK-β2, while it is interesting to speculate whether the viral protein NSs may have any role to play in the formation of IKK-β2, qRT-PCR studies do not demonstrate any significant difference in replication competencies between the wild type MP-12 virus and the NSs mutant virus at early time points (Figure 1C). We suspect that formation of IKK-β2 may be associated with infection by a replication-competent virus and data shown with UV-inactivatved MP-12 (Figure 2A) provides support to this idea. Therefore, based on current data, we do not believe that formation of IKK-β2 is directly linked to NSs function. However, as shown in Figure 7A, NSs can be phosphorylated by IKK-β2 and is therefore a potential substrate for this kinase.

Among the multiple IKK inhibitors that showed efficacy against RVFV, curcumin emerged as being the most potent with about 3-4 logs of inhibition of virus in treated cells which included reduced total genomic copies and lowered infectivity (Figures 3, 4). Our experiments also demonstrated that curcumin directly bound to IKK-β2 and suppressed its kinase function (Figure 5). Curcumin is a well-documented natural polyphenolic compound which in recent years has been demonstrated to have extensive anti-proliferative, anti-viral, anti-arthritic, anti-amyloid and anti-inflammatory properties (56-58).

We also observed that curcumin down regulated viral replication in the liver of infected animals. Our current preliminary study was performed using well-tolerated doses of curcumin in the context of other murine models (59-63). Our studies revealed that treatment of infected animals with curcumin resulted in down regulation of liver viremia (Figure 9B). Further studies are on-going to explore derivatives of curcumin that may confer better bioavailability at low doses and hence offer increased survival advantage to infected animals.

Collectively our observations indicated that viral infections may cause alterations in macromolecular complexes such as IKK that resulted in the presence of novel versions of enzymes, such as IKK-β2 observed in RVFV infection. These novel host components, by virtue of altered protein-protein interactions and function, may serve as therapeutic targets. There are multiple inhibitors of host signaling components that are currently FDA approved and in the market for treatment of many cancers. Identification of altered host signaling components will be a critical step in drug repositioning to utilize them in the treatment of infectious diseases as well.
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FOOTNOTES

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FIGURE LEGENDS

Figure 1. MP-12 infection caused phosphorylation of p65 by the classical pathway. A) HSAECs were infected with MP-12 or UV-inactivated MP-12 virus and analyzed for phosphorylation of p65 and IκBα by western blot analysis. Mock infected cells were maintained alongside as negative controls. Total p65 and β-actin western blots were carried out as controls. B) Extent of phosphorylation of p65 was quantified by averaging signal intensities observed in three different experiments after infection by MP-12 or UV-MP-12 virus. Phosphorylation is represented as fold increase over that of uninfected cells. C) Phosphorylation of p65 and IκBα after infection by wild type MP-12 virus, NSs mutant (rMP-12-NSdel) and NSm mutant (arMP-12-del21/384) were analyzed by western blot. Total p65 and β-actin levels were analyzed as controls. D) Intracellular RNA levels of MP-12 virus or the NSs and NSm mutant viruses were determined by qRT-PCR using total RNA extracted from infected cells at 6 hours post infection.

Figure 2. IKK complex components were altered after MP-12 infection. A) HSAECs were infected with MP-12 and UV-inactivated MP-12 and cell lysates were analyzed for IKK complex components at approximately 20 hours post infection. Whole cell lysates were fractionated in a Superose 6 HR 10/30 size-exclusion chromatography column using the AKTA purifier system. A total of 70 fractions were obtained and every 5th fraction was analyzed for IKK-α, β, γ complexes and β-actin by western blots. The fraction numbers analyzed are indicated above each panel as F#. The red square in the IKK-β blot shows lower molecular weight complexes (IKK-β2) that eluted in the case of MP-12 infection. B) Total protein extracts from BCBL1 cells (KSHV-infected) and BJAB (uninfected control) cells were fractionated using a new, conditioned Superose 6 size-exclusion column. Fraction numbers (F#) for subsequent analysis were determined based on the elution profiles of known standards. Accordingly, fractions indicated in B match with fractions indicated in A and C in terms of elution profiles. C) Total protein extracts from HSAECs treated with TNFα were fractionated and every 5th fraction was analyzed for IKK-β and β-actin.

Figure 3. Inhibitors of the IKK complex down regulated MP-12 replication. A) Diagrammatic representation of the NFκB activation cascade and the interface of tested inhibitors with the cascade. B) HSAECs were pretreated with inhibitors of the NFκB cascade for 2 hours. Pretreated cells were infected with MP-12 and continued to be post treated with the inhibitors for up to 24 hours. Supernatants were collected from infected- untreated cells, infected- DMSO treated cells and infected- inhibitor treated cells. All supernatants were quantified for infectious progeny virus by plaque assays. Inhibitor toxicity was evaluated by measuring survival of inhibitor treated cells in comparison with DMSO treated and untreated (UT) cells.
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Figure 4. Down regulation of extracellular genomic RNA by curcumin. A) HSAECs were pre- and post-treated with curcumin and supernatants were analyzed for viral genomic RNA copies by qRT-PCR in comparison with untreated and DMSO-treated, MP-12 infected cells. Viral genomic copy numbers in the context of inhibition by synthetic curcumin or dimethoxy-curcumin were determined. Supernatants were obtained from infected, inhibitor treated cells 24 hours post infection and analyzed by qRT-PCR. B) HSAECs were either pretreated (2 hours) or post treated (3 hours) with curcumin and viral genomic copy number in the supernatant was determined by qRT-PCR.

Figure 5. IKK-β2 derived from MP-12 infected cells retained kinetic function and was bound by curcumin. A) Fractions 33-36 from MP-12 infected and UV-MP-12 infected cell extracts were pooled and immunoprecipitated with anti-IKK-β antibody. Phosphorylation reactions were carried out with the immunoprecipitated material and a GST-IκBα substrate. After incubation, samples were separated on a 4-20% Tris-glycine gel, dried, and analyzed by a phosphor-imager (Molecular Dynamics). In lane 2 (C81), total C81 cell extract was used as a positive control for IKK-β activity. In lane 1, GST alone without the IκBα substrate was used as a negative control. In lanes 7 and 8, increasing concentrations of curcumin were added to the phosphorylation reaction. In lanes 9 and 10, fractions 18-21 were used as the source of IKK-β and the effect of curcumin on its kinase activity was determined. B) Fractions 18-21 and 33-36 were independently pooled and subjected to a pull down assay by incubation with immobilized curcumin beads. The washed beads were resuspended in laemelli buffer, separated by SDS-PAGE and analyzed by western blot using an anti-IKK-β antibody.

Figure 6. Curcumin reversed S-phase arrest induced by MP12 infection. A) HSAECs were synchronized by serum starvation for 72 hours prior to infection. After infection, cells were analyzed for cell cycle phenotypes by FACS. Each histogram demonstrates relative distributions of cells in G1, S and G2 phases of the cell cycle. In each histogram, the y-axis refers to collected events (cell counts) and x-axis shows fluorescence from the PI stain. The three histograms show relative distributions of cells in G1, S and G2 phases of the cell cycle in mock infected HSAECs, MP-12 infected HSAECs and curcumin treated HSAECs infected with MP-12. B) Relative percentages of cells in G1, S and G2/M phases were quantified. C) Changes in total protein levels of p21/Waf1, cyclin A and cyclin D were determined by western blot analysis of serum starved, MP-12 infected and curcumin treated cells in comparison with DMSO treated or untreated cells. Actin was determined as a control.

Figure 7. Inhibition by curcumin may be due to its influence on NSs phosphorylation and cell cycle progression. A) Phosphorylation of a GST-NSs substrate by IKK-β2 was determined by in vitro kinase assay. Specifically, fractions 34-38 from MP-12 infected cell extracts were pooled and immunoprecipitated with anti-IKK-β antibody. Phosphorylation reactions were carried out with the immunoprecipitated material and a GST-NSs substrate. To determine effects of curcumin on the phosphorylation reaction, the reaction was carried out in the presence of increasing concentrations of curcumin (0.1 and 1µM). After incubation, samples were separated on a 4-20% Tris-glycine gel, dried, and analyzed by a phosphor-imager (Molecular Dynamics). B) Efficacy of NSs phosphorylation by IKK-β (obtained from pooled fractions 20-25) and IKK-β2 (obtained from pooled fractions 35-40) were compared by titrating increasing concentrations of both enzymes (5ng, 50ng and 500ng) in the presence of a constant amount of GST-NSs substrate. After incubation of the substrate with increasing concentration of enzymes, the substrate was electrophoresed and analyzed by a phosphor-imager. C) Relative binding of phosphorylated NSs with mSin3A and HDAC3 was evaluated by incubating uninfected whole cell extract with phosphorylated GST-NSs (using IKK-β2 and ATP). The same reaction without ATP (lanes 2 and 4) was set up alongside as a negative control. The next day, the reaction was washed 3X with TNE-50 (0.1% NP-40) buffer and run on a 4-20% Tris-glycine gel. The proteins were then transferred to a nitrocellulose membrane and western blotted for mSin3A and HDAC3. D) HSAECs were synchronized at either G0/G1 phase (Sync-G1) by serum starvation or at late G1/early S phase.
Curcumin inhibits Rift Valley fever virus

Curcumin inhibits Rift Valley fever virus (Sync-S) by treatment with hydroxyurea (HU) for up to 48 hours and then infected with MP-12 (MOI: 0.1). Cells were maintained in serum starvation conditions or in HU for 24 hours post infection and infectious progeny determined by plaque assays. E) Similar experiment as described in D) except that following infection, cells were maintained in complete media for 24 hours after which supernatants were analyzed for infectious virus by plaque assays.

**Figure 8. Curcumin inhibits ZH501 replication.** A) A549 cells were infected with ZH501 at a MOI of 0.1 and were either treated with curcumin or left untreated (UT) for 24 hours. Culture supernatants were then analyzed for infectious virus by plaque assays. B) A549 cells were infected with MP-12 virus at a MOI of 0.1 and extent of inhibition was determined by plaque assays. C) Inhibitor toxicity was evaluated by measuring survival of inhibitor treated A549 cells after 24 hour treatment with curcumin.

**Figure 9. Curcumin treatment reduces hepatic viral load in INFAR-/- mice.** A) INFAR-/- mice (n=5) were treated subcutaneously with DMSO or curcumin (60 mg/kg) every day. Concentrations of curcumin up to 300 mg/kg have been used by others in murine models (in some cases, for up to periods of 21 days with daily administration) and no apparent toxicity effects were observed (59-63). All animals were infected with MP-12 virus at a concentration of $10^4$ pfu per animal. All mice were pretreated with inhibitor at 24 hours prior to infection and were post treated for a total of four days post infection. Percentage of survivors is shown in the Y-axis while the number of days post infection is shown in the X-axis. B) Liver tissue from infected, DMSO-treated and infected, curcumin-treated mice were isolated and infectious viral titers were determined by plaque assays.
Curcumin inhibits Rift Valley fever virus

Figure 1

A) h.p.i 1 6

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1 h.p.i to 6 h.p.i

B) Fold change

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C) h.p.i 1 4

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D) Genomic copies
**Figure 2**

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**B)**

![Image of gel electrophoresis results](image)

- IKK-α
- IKK-β
- IKK-γ

**C)**

![Image of gel electrophoresis results](image)

- TNFα

**D)**

**IKKβ complex**

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<tr>
<td>COP9 signalosome subunit 8 isoform 1</td>
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<td>heat shock 70kDa protein 5</td>
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**Proteasome associated proteins**

- ubiquitin specific protease 14 isoform a
- proteasome 26S ATPase subunit 1

**Enzymes**

- p21-activated kinase 2
- p21-activated kinase 3 isoform a
- HMT1 hnRNP methyltransferase-like 2 isoform 2
- integrin-linked kinase-associated protein phosphatase 2C
- diablo isoform 1 precursor
- GTPase Rab14

**Cytoskeleton associated proteins**

- ARP3 actin-related protein 3 homolog
- F-actin capping protein beta subunit
- actin related protein 2/3 complex subunit 4 isoform a

**Nuclear transport associated proteins**

- importin 8
- importin 4
- nuclear protein localization 4
- Ran GTPase activating protein 1
- karyopherin alpha 2

**IKKβ2 complex**

**IKK associated proteins**

- heat shock 90kDa protein 1, beta
- heat shock 70kDa protein 4-like

**Cytoskeleton associated proteins**

- coronin, actin binding protein, 1C isoform 1
- moesin

**Apoptosis associated proteins**

- apoptosis inhibitor 5 isoform b

**Nuclear transport associated proteins**

- exportin 1
- importin 5
- nucleosome assembly protein 1-like 1
- nucleolin
- paraspeckle protein 1
Curcumin inhibits Rift Valley fever virus

Figure 3A

A)

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Curcumin inhibits Rift Valley fever virus

Figure 3B
Figure 4

Curcumin inhibits Rift Valley fever virus

A)

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MP12 MP12+DMSO MP12+Curcumin MP12+Curcumin (synth) MP12+Dimethoxy Curcumin

B)

Genomic Copies

1.0E+00 1.0E+01 1.0E+02 1.0E+03 1.0E+04 1.0E+05 1.0E+06 1.0E+07

MP12 MP12 + DMSO_Pre MP12 + Curcumin_Pre MP12 + DMSO_Post MP12 + Curcumin_Post
Figure 5

A)

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B)

Nanoparticles + curcumin

Input | MP12 (F18-21) | UV-MP12 (F18-21) | MP12 (F33-36) | UV-MP12 (F33-36) |

IKK-β

(P)-GST-IκBα
Curcumin inhibits Rift Valley fever virus

Figure 6

- **B)**
  - Graph showing cell cycle distribution with bars indicating % G1-phase, % S-phase, and % G2-phase for Mock, MP12, Mock + curcumin, and MP12 + curcumin.

- **C)**
  - Western blot images showing proteins Cyclin A, p21/Waf1, Cyclin D1, and β-Actin with lane markers 1, 2, 3, and 4.
Curcumin inhibits Rift Valley fever virus

Figure 7

A) GST
GST-NS$_S$
GST - NS$_S$
IKK$_{\beta}$2 (WT)
IKK$_{\beta}$ (UV)

B) GST
GST-NS$_S$
IKK$_{\beta}$ (50ng)
IKK$_{\beta}$ (500ng)
IKK$_{\beta}$ (50ng)
IKK$_{\beta}$ (500ng)

C) Input (WCE)
WCE
IKK$_{\beta}$
GST
GST-NS$_S$
ATP

D) UT Syn-G1 Syn-C
p=0.05

E) UT Syn-G1 Syn-C
Curcumin inhibits Rift Valley fever virus

Figure 8
Curcumin inhibits Rift Valley fever virus

Figure 9

A)

B)
Curcumin inhibits Rift Valley fever virus replication in human cells.

Aarthi Narayanan, Kylene Kehn-Hall, Svetlana Senina, Lindsay Lundberg, Rachel Van Duyne, Irene Guendel, Ravi Das, Alan Baer, Laura Bethel, Michael Turell, Amy Lynn Hartman, Bhaskar Das, Charles Bailey, and Fatah Kashanchi

PAGE 33201:

The curcumin nanoparticle binding assay described failed to indicate that 1 ml of chitosan (5 mg/ml stock at pH 5) was combined with PEG, curcumin, and phosphate-buffered saline in the first step and that hydrolyzed tetramethylorthosilicate (TMOS) was used, not trimethoxysilane. This protocol was based on the platform developed by Friedman et al. (Friedman, A. J., Han, G., Navati, M. S., Chacko, M., Gunther, L., Alfieri, A., and Friedman, J. M. (2008) Sustained release nitric oxide releasing nanoparticles: characterization of a novel delivery platform based on nitrite containing hydrogel/glass composites. Nitric Oxide 19, 12–20).

PAGE 33213:

The curcumin nanoparticles described in Fig. 5B were developed and synthesized by Mahantesh H. Navati and Joel M. Friedman (Department of Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, NY) and Adam J. Friedman (Division of Dermatology, Department of Medicine, Albert Einstein College of Medicine, Bronx, NY). Madiha Sewani also assisted in synthesizing the nanoparticles.
Curcumin Inhibits Rift Valley Fever Virus Replication in Human Cells
Aarathi Narayanan, Kylene Kehn-Hall, Svetlana Senina, Lindsay Hill, Rachel Van Duyne, Irene Guendel, Ravi Das, Alan Baer, Laura Bethel, Michael Turrell, Amy Lynn Hartman, Bhaskar Das, Charles Bailey and Fatah Kashanchi

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