Identification of Differentially Activated Cell-Signaling Networks Associated with Pichinde Virus Pathogenesis Using Systems Kinomics

Running Title: Kinomics investigation of virus-induced signaling

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Abstract

Phosphorylation plays a key role in regulating many signaling pathways. Although studies investigating the phosphorylated forms of signaling pathways are now commonplace, global analysis of protein phosphorylation and kinase activity has lagged behind genomics and proteomics. We have used a kinomics approach to study the effect of virus infection on host-cell signaling in infected guinea pigs. Delineating the host-responses which lead to clearance of a pathogen requires the use of a matched, comparative model system. We have used two passage variants of the arenavirus Pichinde - used as a BSL-2 model of Lassa fever virus as it produces a similar pathology in guinea pigs as Lassa fever does in humans- to compare the host-cell response between infections which lead to either a mild, self-limiting infection, or to lethal disease. Using this model, we can begin to understand the differences in signaling events which give rise to these markedly different outcomes. By contextualizing these data using pathway analysis, we have identified key differences in cellular-signaling matrices. By comparing these differentially involved networks, we have identified a number of key signaling ‘nodes’ which show differential phosphorylation between mild and lethal infection. We believe that these nodes provide potential targets for the development of antiviral therapies which act at the level of the host-response, rather than by directly targeting viral proteins.
Introduction

The family Arenaviridae includes a number of hemorrhagic fever viruses including Lassa fever virus and Junin virus. Previous findings suggest that arenavirus pathogenesis could well involve the dysregulation of cytokines and immune signaling (5,14,15,41), however we do not understand how arenaviruses induce cell-signaling changes, which lead to this dysregulation and potentially to clinical disease. A characteristic of hemorrhagic arenavirus disease is a perturbation of host cytokine responses. Modulation of the host innate immune response as a countermeasure against infectious agents is a significant area of research (2), with current approaches predominantly focusing on ‘boosting’ the immune response. However, treating a number of infections in this way may lead to an exacerbation of clinical disease, rather than to protection, due to the limited understanding of the molecular basis of how these viruses cause disease. Many viruses have evolved mechanisms to interfere with host-cell signaling, either to evade the immune response (22,33,42), or to activate signaling pathways required for viral replication (3,9). A detailed knowledge of how viruses and the host interact, and how these interactions change over time, would allow researchers to more rationally target the design of immunomodulatory compounds.

Lassa fever virus is endemic in West Africa where it causes significant morbidity and mortality and is responsible for a large proportion of hospitalizations (40). In addition to its threat as an emerging infectious disease, Lassa fever virus and other hemorrhagic fever viruses are CDC and NIAID category A biothreat agents (6,11,21). While there is an effective vaccine available for Junin virus, and ring-vaccination could play an important role in outbreak control, vaccination may not be effective within a suitable timeframe for treatment of primary cases during an
epidemic or in response to intentional release. For these reasons, expansion of the therapeutic armamentarium is urgently required.

Phosphorylation events are critical in the understanding of cell-signaling pathways (26). A number of signaling pathways are controlled in the initial response to activation, not at the transcriptional or translational level, but by a series of phosphorylations and other modifications, such as acetylation, ubiquitination, methylation, citrullination and sumoylation (47). While a number of methodologies are available for the study of protein phosphorylation (28), there have been relatively few investigations into the broad changes in the cellular kinome in response to a stimulus. While genomic and proteomic studies have allowed us to further understand the transcriptional and translational changes induced by activating cell-signaling pathways, the activities of any differentially expressed proteins has often remained undefined due to incomplete characterization of relevant post-translational modifications which may act to modulate their activities.

Unraveling the complex cell-signaling events which lead to clearance of a pathogen or to clinical disease requires a comparative model system in which attenuated and virulent pathogens can be directly compared. While attenuated or vaccine strains of viruses such as Japanese encephalitis virus (38), Rabies virus (45), and Junin virus (1) provide good comparative models, they are limited by the fact that they require higher levels of containment for study. We have used Pichinde virus infection of guinea pigs –which produces a similar pathology to Lassa fever in humans (27)– to study these differences at BSL-2. We have used an attenuated virus variant, P2, and a variant which causes lethal disease, P18, to dissect the differences in cell signaling
networks which lead to differing outcomes of infection. We hypothesized that infection of guinea pigs with P2 virus would lead to activation of signaling pathways which produce a protective immune response, viral clearance and recovery, while infection with P18 virus would induce a differential network of signaling pathways which would lead to an inappropriate response and to lethal disease. We have previously shown that infection of cells with P2 or P18 virus induces differential responses of host transcription factors and signaling intermediates (12,20). We sought to expand these findings by investigating global cell-signaling changes in response to Pichinde virus infection.

Genomic arrays have previously been used to investigate global changes in transcription in response to pathogens. While gene arrays have provided a valuable insight into virally-induced changes in the host cell, they are limited by the fact that they do not address translational or post-translational effects. We have shown (12) that infection with Pichinde virus causes changes in the total levels of a number of proteins when compared to mock-infection. A key regulator of signal transduction is phosphorylation of specific signaling proteins at one or more residues, which can cause activation or inhibition of the protein. The role of phosphorylation in regulating signaling is reviewed extensively in reference (26). A number of viruses have been shown to interfere with the phosphorylation of cellular proteins. For example, Herpes simplex virus and Measles virus have been shown to interfere with interferon signaling by inhibiting STAT phosphorylation (43,49). The NP protein of Lymphocytic choriomeningitis virus –the prototype arenavirus- has recently been shown to inhibit type I interferon signaling by preventing nuclear translocation of IRF-3 (36).
Analysis of signaling pathways in the guinea pig model system has been hampered by the lack of available reagents which cross-react with guinea pig proteins. This, combined with the fact that the genome has only recently been sequenced, has hindered the use of traditional genomic and proteomic approaches in this model. For this reason, we utilized a substrate-based kinomics approach to investigate the effect of virus infection on signaling pathways in guinea pig macrophages: the primary target cell of Pichinde infection, and key mediators of the immune response. We have utilized the PepChip kinase assay system to assay the ability of cytoplasmic extracts from infected macrophages from infected guinea pigs to phosphorylate synthetic peptide kinase-substrates \textit{ex vivo}. This technology has been previously used to assay the effect of lipopolysaccharide on signal transduction in human peripheral blood mononuclear cells (17), and to investigate protein phosphorylation in big mitogen-activated protein kinase 1 knockout cells (24). A kinomics-based approach using an array of phosphorylation state-specific antibodies has also been used to investigate the host response to infection with mycobacteria (25). To our knowledge, this study represents the first use of a broad kinomics approach to investigate global changes in protein phosphorylation and kinase activity in response to viral infection.

**Materials and Methods**

**Cell lines and culture conditions**

P388D1 (murine monocyte-like) cells were maintained in RPMI medium supplemented with 5% fetal bovine serum and 2 mM glutamine. Cells were infected with purified P2 or P18 Pichinde virus at a multiplicity of infection of 1, or mock infected with an equivalent fraction of virus purification medium. Cells were harvested at various times post-infection (2-16 hours) and cytoplasmic and nuclear extracts prepared.
Virus stocks

Viruses were from serial spleen passaged stocks from inbred strain 13 guinea pigs infected with Pichinde Munchique strain CoAn 4763 (53). Virus was quantified in a standard plaque assay on Vero cells as described previously (4). Viruses used for in vitro experiments were purified using PEG (Sigma, St. Louis, MO) gradients to remove potential contamination from cytokines and other soluble factors.

Cytoplasmic and nuclear extract preparation

Cytoplasmic and nuclear extracts were prepared from primary guinea pig macrophages and P388D1 cells as previously described (18) with the addition of a nuclear purification step using Optiprep (Axis-Shield, Oslo, Norway) gradients. Briefly, lysates were underlayered with 10 ml 30% Optiprep, and 5 ml 35% Optiprep and centrifuged at 4 °C for 30 minutes at 4300 x g. The interface was removed and placed in a fresh tube which was filled with sucrose buffer I (described in reference 18) plus 1.5 mM CaCl$_2$. Following centrifugation at 4 °C for 15 minutes at 1900 x g, the pellet was resuspended in sucrose buffer I and the centrifugation repeated. Nuclear lysis was completed following the referenced protocol.

Gel electrophoresis and immunoblotting

5 µg of cytoplasmic (approximately 140 000 cell equivalents) or nuclear (approximately 370,000 cell equivalents) extracts were electrophoresed on standard 10% SDS-polyacrylamide gels and transferred to nitrocellulose (Hybond ECL, Amersham Biosciences, Piscataway, NJ). Phospho-tyrosine residues were detected by immunodetection following standard methods. Anti-
phosphotyrosine PY20 was purchased from BD Biosciences (Rockville, MD). Protein A-HRP conjugate was purchased from Abcam (Cambridge, MA).

Animal protocols

All animal experiments were conducted following approved institutional animal guidelines and protocols. Male outbred Hartley strain guinea pigs, 400 to 500 g, were inoculated intraperitoneally with 1 ml of phosphate-buffered saline (PBS) containing 1,000 PFU of Pichinde virus (P2 or P18 strain), or with mock spleen homogenate diluted in PBS. Weights and rectal temperatures were recorded daily. Animals were sacrificed 1 day or 6 days post-infection. Peritoneal cells were harvested immediately following death by aseptic lavage with 100 ml PBS (calcium and magnesium-free); cells were collected by centrifugation and washed in 10 ml PBS. Cells were counted and used to prepare cytoplasmic extracts following the procedure used for cultured cells.

PepChip kinase assay

Arrays of kinase substrates on glass slides (PepChip kinase v1, PepScan Systems, Netherlands) were incubated with gamma-32P-ATP, kinase buffer, and cell extracts according to the manufacturer’s instructions. Cytoplasmic extracts from four guinea pigs per treatment/timepoint were used to phosphorylate 2 PepChip slides (4 arrays), giving a dataset of 16 arrays per treatment/timepoint. Arrays were used to expose X-ray film which was then analysed by densitometry using the Gel-Pro v.4.5 software (Media Cybernetics, Silver Spring, MD). Background was subtracted on a per-spot basis by subtracting the optical density at each spot edge.
Array data analysis

Data from arrays which showed high background were excluded from analysis. Data were normalized and scaled using the spotting control peptides throughout each array. Following production of a scaled dataset, data were analyzed using S-Plus and Spotfire software packages. Student’s $t$-test was run on all possible data combinations; ANOVA was also performed for each time point and each condition, as well as combining all treatments in one analysis. Hierarchical clustering was performed on the significant peptides ($p \leq 0.05$) resulting from the ANOVA analysis to produce heat maps for P2 vs. mock and P18 vs. mock at day 1 and day 6 post-infection. Spots which showed a significant difference in intensity compared following pairwise comparison ($p<0.05$ by student’s $t$-test) were used for the pathway analysis.

Construction of the Ingenuity knowledge base

The Ingenuity Systems Pathway Analysis knowledge base (Ingenuity Systems, Redwood City, CA) has been described in detail previously (13). Briefly, functions of, and interactions between, cellular proteins are mined from peer-reviewed literature and encoded into an ontology by post-doctoral level scientists. A network analysis of the knowledge base is used to construct interaction-based relationships, both direct and indirect, between proteins in the knowledge base.

A dataset containing SWISS-PROT protein identifiers and their corresponding phosphorylation status values was uploaded as an Excel spreadsheet using the template provided in the application. Each protein identifier was mapped to its corresponding object in the Ingenuity Pathways Knowledge Base. A fold-change cut-off of 1.3 for 1 day post-infection data, and 2 for...
6 days post-infection data was set to identify proteins whose phosphorylation status was significantly differentially regulated. These genes were then used as the starting point for generating biological networks. Networks were constructed using direct interactions only.

Biological functions were assigned to each protein network by using the findings that have been extracted from the scientific literature and stored in the Ingenuity Pathways Knowledge Base. The biological functions assigned to each network are ranked according to the significance of that biological function to the network. A Fischer’s exact test is used to calculate a p-value determining the probability that the biological function assigned to that network is explained by chance alone.

**Transcription factor binding ELISA**

Transcription factor binding assays were performed using the TransAM assay system (Active Motif, Carlsbad, CA) following the manufacturer’s instructions. Briefly, P388D1 cells were infected at a multiplicity of infection of 5 with P2 or P18 Pichinde virus, or mock-infected with PEG purification medium and nuclear extracts prepared following harvesting at various times post-infection. Timecourse infections were performed in triplicate, pooled, and protein concentrations determined using the BCA assay (Pierce, Aalst, Belgium). Equivalent amounts of protein were added in quadruplicate wells in a 96-well plate per treatment/timepoint and allowed to bind to the consensus transcription factor binding site oligonucleotide bound to the well. Following washing, bound transcription factor was detected by antibody binding and colorimetric absorbance.
Phospho-protein cell-based ELISA

Cell-based ELISA was performed using the FACE assay system (Active Motif) following the manufacturer’s instructions. Briefly, a 96 well plate was seeded with P388D1 cells and a quadruplicate timecourse of infection with P2 or P18 Pichinde virus was performed. Control cells were mock-infected with PEG purification medium. Cells were formaldehyde fixed and assayed for the presence of the specific phospho-protein using antibody-binding and detection by colorimetric absorbance.

Results

Phosphorylation changes following arenavirus infection

Although we had previously found that total protein levels of specific transcription factors are similar following P2 and P18 infection, we found that their phosphorylation statuses are different between P2 and P18 infection (SMF & NKH, unpublished results). These findings suggested that, while pathways leading to a generalized increase in specific protein level may be upregulated as a consequence of viral infection, activation of these proteins may be differentially affected between attenuated and lethal infection. We expanded on these specific findings by examining nuclear extracts from Pichinde-infected murine macrophage cells for phosphorylated proteins by immunoblotting against phosphotyrosine residues. We observed a difference in the quantitative and temporal expression of tyrosine-phosphorylated proteins in the nucleus between P2 and P18 infected cells (data not shown). Following infection with both virus variants, we observed reduced nuclear levels of phospho-proteins and a more rapid reduction in these levels following P18 infection compared to infection with P2.
Analysis of high-throughput kinomic data

We used a high-throughput kinase-substrate array to investigate the host-cell response to mild and lethal arenavirus infection. Hierarchical cluster analysis was used to visualize the changes in host-cell phosphorylation changes in response to attenuated and lethal virus infection. Figure 1a shows the results of hierarchical cluster analysis following ANOVA of substrate phosphorylation by macrophage extracts from P2 and P18 infected guinea pigs compared to mock-infection at 1 day and 6 days post-infection. Further analysis of these data by pairwise comparison using the Student’s t-test revealed a number of peptides which were differentially phosphorylated between P2-infection and mock, P18-infection and mock, and between P2 and P18 infection, at one day and six days post-infection (data not shown, available at http://www.utmb.edu/pathology/profiles/?user=nherzog). Figure 1b and 1c show the commonality in protein phosphorylation between P2 and P18 infection. We observed a considerably smaller number of changes at one day post-infection compared to six days-post infection. We believe that, as fewer peritoneal cells are infected at this time point, the majority of the lower-fold changes will be undetectable, due to the presence of a majority of uninfected cells in the sample. We have previously shown that infection does not significantly alter the resident cell population and that monocytic cells were the dominant cell types in the samples (20). We also observed that P2 infection induced a greater number of differences in peptide phosphorylation than P18 infection. This correlates with our observations using other experimental systems and is consistent with our hypothesis that P18 actively suppresses the host-responses that lead to viral clearance. A number of our findings correlated with our previous data, and results from other investigators. For example, we observed that the virulent P18 variant caused a greater reduction in fibrinogen-α phosphorylation than P2 at six days post-infection.
This is consistent with a previous finding which showed increased fibrinogen-α dephosphorylation in patients infected with more highly virulent variants of Junin virus compared to those infected with isolates which cause a milder clinical disease (32).

Network analysis of phosphorylation changes

We sought to integrate these data into functional signaling networks in an attempt to better understand the host-cell pathways involved in response to viral infection. A recent study used a network-based analysis to model the leukocyte response to systemic inflammation using a genomic array approach (13). Using a web-based knowledge base, the authors modeled the cellular pathways involved in the leukocyte response to inflammation. We used a similar approach to contextualize our data into functional upstream to downstream signaling pathways.

We have used the Ingenuity Pathways Analysis Knowledge Base (IPA) (Ingenuity Systems, Redwood City, CA) to model the signaling networks which, based on our identification of differential phosphorylation states, are likely to be involved in the cellular response to attenuated and virulent arenavirus infection. IPA has traditionally been used to analyze genomics datasets, whereby proteins in networks are represented as being up or down-regulated based on their mRNA level following microarray analysis. We have previously used IPA to construct networks from the results of a high-throughput immunoblot (12), in which total protein levels are directly represented in the network. In the present study, we assayed the fold-changes in peptide phosphorylation following attenuated or virulent Pichinde infection relative to phospho-peptide levels induced by mock-infection. Representing these data as simple up and down-regulation in a network may be misleading due to the different effects of phosphorylation on the activity of the
protein. Also, since some proteins have multiple phosphorylation sites represented in the dataset, not all the data could be analysed and represented by the software.

For these reasons, our initial analysis disregarded fold-change and differential phosphorylations at multiple sites. All proteins, represented by individual phosphorylation site containing peptides, which showed a significant difference in phosphorylation (p<0.05) were compiled into a dataset and used to construct signaling networks. Figure 2 shows signaling networks produced following IPA analysis after pairwise comparison between P2 versus mock-infection and P18 versus mock infection at one day and six days post-infection. Identified networks with scores >5 were merged and displayed according to sub-cellular location. Figure 2a shows the network created from differential phosphorylations between P2-infection and mock at one day post-infection; figure 2b shows the differences between P18-infection and mock. Of interest is the identification of pathways leading to p53 following infection with both viruses. Figure 2c+d show the networks identified at six days post-infection following P2 (c) or P18 (d) infection. Of note is the greatly increased complexity of the network induced by P2 infection, compared to that induced by P18 at six days post-infection.

As the focus of our study was to identify the differences in host-cell signaling events between attenuated and lethal infection, we used the IPA application to perform a comparison analysis between our datasets. Figure 3a shows the significance of the involvement of a number of canonical signaling pathways between infections at both time points. As can be seen, P2 induced a higher significance of pathway involvement in the case of most canonical signaling pathways. Interestingly, we observed a higher significance of involvement of the NF-κB and p38 MAP
kinase pathways following P18 infection at six days post-infection. Comparing P2 and P18-induced phosphorylation changes in the context of functional roles of the modified proteins, we observe that, at six days post-infection, P2 infection induces a higher significance of involvement of proteins in all studied categories. However, at one day post-infection, P18 infection alters the phosphorylation status of more proteins involved in intra-cellular signaling and post-translational modification than infection with P2. We next performed pathway analysis on the proteins which showed significant differential phosphorylation between P2 and P18 infection at one day (figure 3c) and six (figure 3d) days post-infection. The networks show that insulin receptor, epidermal growth factor receptor (EGFR), protein kinase Cδ and retinoblastoma protein may all be key factors in regulating the differential response to these virus variants.

Functional analysis of phosphorylation changes

In order to further investigate the role of these phosphorylations, and to improve our understanding of the inter-cellular/intra-cellular signaling interface, we mined the literature to determine the functional effects of phosphorylation changes in cellular receptors. Identified differential phosphorylation changes in receptors were categorized as being indicative of receptor activation or inhibition. Where receptors were represented by multiple phosphorylations in our array, all phosphorylation sites were investigated and we did not observe any conflicting changes, i.e. increased phosphorylation events in both inhibitory and activation domains at a given treatment and timepoint. A summary of these changes is shown in figure 4. We observed differential receptor activity in a number of proteins, including EGFR, platelet-derived growth factor receptor (PDGFR), interleukin-1 receptor and macrophage colony-stimulating factor receptor.
**Validation of involvement of identified signaling pathways**

In order to confirm the involvement of the pathways and the identification of specific proteins following pathway analysis, we investigated the effects on specific signaling intermediates and transcription factors implicated in the signaling networks.

The transcription factor SP1 was implicated in a signaling network following infection with P18 at 1 day post-infection (figure 2b). We performed a transcription factor binding ELISA to confirm the involvement of this protein in Pichinde infection. Figure 5a shows a 1.8-fold increase in SP1 binding following P18 infection compared to P2 infection at 2 hours post infection (p=0.0029). No significant differences were observed at other time points. This is consistent with the lack of SP1 involvement in signaling networks constructed at 6 days post-infection.

The transcription factor c-Myc was a node in a signaling network induced by P18 infection at 6 days post-infection (figure 2d). The ability of c-Myc to bind to its DNA consensus sequence was assayed by transcription factor binding ELISA. Figure 5b shows a number of small, but significant, changes in c-Myc binding between times and infections. c-Myc binding was equivalent in mock, P2-infected and P18-infected cells at 2 hours post-infection. At 4 hours post-infection, activity in P18-infected cells had increased 1.6-fold over mock-infected (p=0.0078), 1.4-fold over P2-infected cells (p=0.0212) and 2.6-fold over c-Myc binding in P18-infected cells at 2 hours post-infection (p<0.0001). At 8 hours post-infection c-Myc binding was increased 1.8-fold compared to mock-infected cells (p=0.0002) following P18 infection. Levels of c-Myc
binding were 1.3-fold higher in P18-infected cells than P2-infected cells (p=0.0069) and 1.4-fold higher in P2-infected cells than mock-infected cells (p=0.0287). At 12 hours post-infection, binding was 1.3-fold lower in P18-infected cells than in mock-infected (p=0.0147) and 1.5-fold lower than P2-infected (p=0.0042) cells. At 16 hours post-infection, binding was very 1.2-fold higher following P18-infection than mock-infection (p=0.013).

The transcription factor p53 was implicated in all analyses in which infected cells were compared to mock-infected cells (figure 2 a-d); it was not incorporated in networks in which P2-induced kinome changes were compared with changes induced by P18 infection (figure 3 c+d). We investigated the binding activity of p53 throughout a timecourse of infection (figure 5c). There were no significant differences in p53 binding between P2 and P18 infection. There were significant increases in p53 binding induced by P18 infection when compared to mock-infection at 4 (p=0.0195), 12 (p=0.0136) and 16 hours (p=0.0027) hours post-infection.

Comparison analysis of P2 versus P18-induced signaling networks revealed a higher significance of involvement of the p38 MAPK pathway following P2 infection at 1 day post-infection, and a higher significance following P18 infection at 6 days post-infection (figure 3a). We used a cell-based ELISA assay to investigate the degree of p38 phosphorylation through a time course of infection (figure 6). While the early times post-infection show a trend for increased phosphorylation of p38 following infection with P2, these differences were not statistically significant. At 16 hours post-infection however, we observed a significant increase in p38 phosphorylation: a 7-fold increase compared to p-p38 induced by P2 infection (p<0.0001).
Discussion

These studies show the complexity of virus-induced cellular signaling networks when investigated at the level of protein phosphorylation. We have used a comparative model system to reveal differentially involved signaling networks between attenuated and virulent arenavirus infection. Of particular interest is the small amount of overlap between the differential phosphorylation events between mock and P2 infection, and those which differed between mock and P18 infection. This contrasts with our previous investigations, in which we observed a significant amount of commonality in total protein expression changes following P2 or P18 infection (12). This finding reveals the importance of understanding the virus-induced host-response at a number of levels, from genomic to post-proteomic.

Our observation that P18 infection induces a much more limited host-response than infection with P2 is consistent with our hypothesis that P18 actively suppresses host-cell signaling. These networks reveal a number of differentially phosphorylated targets, at all levels of signal-transduction pathways, from the plasma membrane to the nucleus, which are targets for further investigation and possible therapeutic intervention. For example, STAT-5A, Jun and ATF-2 are all differentially phosphorylated between P2 and P18 infection and modulation of their activities could be used to ‘push’ a P18-induced response towards that induced by P2 infection in an attempt to elicit a protective immune response. This result is consistent with our hypothesis that P18 infection activates signaling pathways early in infection, but is able to actively inhibit host-cell signaling following replication leading to the suppression of the immune response.
Of particular interest is the observed difference in the receptor activity of EGFR. EGFR is a receptor tyrosine kinase which can activate a number of downstream signaling pathways (reviewed in (10)). It has been shown that inhibition of EGFR increases the expression of pro-inflammatory and apoptotic genes (46). Our finding that EGFR shows decreased activity during P2 infection and increased at six days following P18 infection is consistent with our hypothesis that P2 facilitates a normal, pro-inflammatory response early in infection allowing clearance of the virus. However, it has also been shown that infection of lung epithelial cells with respiratory syncytial virus induced EGFR activation which leads to increased inflammation (37). This apparent contradiction reveals the importance of studying signaling networks in a global, integrated context as cellular responses to pathogens are likely to be a delicate balance between pro- and anti-inflammatory pathways.

Analysis of post-translational modification is likely to be a key next step in our understanding of biological systems. Genomic and proteomic methods have played a crucial role in furthering this understanding, but do not reveal the complete story with respect to biological functionality. Assimilating data from large datasets, from the genomic to the metabonomic, will be fundamental in fully understanding the complexity of the regulation of intra- and inter-cellular signaling. As data analysis tools continue to evolve in parallel with new methodologies, we are in a position where researchers can begin to integrate data from a number of different viral pathogens and from diverse biological disciplines.

Our finding that infection with the lethal P18 passage variant of Pichinde viruses induces a more limited host-response than infection with the attenuated P2 variant is consistent with
observations in other arenavirus systems. It has been shown that the apathogenic arenavirus Mopeia virus activates human macrophages, whereas the pathogenic Lassa virus does not (7,39); Lassa virus infection of macrophages reduces TNF-α and IL-8 production following stimulation of cells with LPS, whereas Mopeia virus infection does not (34). It has also been shown that monocyte-derived dendritic cells infected with Ebola and Lassa viruses do not upregulate costimulatory molecules or secrete pro-inflammatory cytokines and are inhibited in their ability to stimulate T-cells (35). These results suggest that virulent arenaviruses are able to actively suppress host-signaling events which lead to the development of a protective immune response.

We have previously shown a potential mechanism for these observations: infection of macrophages with the P2 variant of Pichinde virus causes an increase in DNA-binding of the activating RelA/p50 dimer of the NF-κB transcription factor in contrast to P18 virus infection, which causes an increase in binding of the repressive p50/p50 homodimeric form (20).

The guinea pig/Pichinde model is a good system for studying the pathology of arenavirus infection. However, there are few reagents available for use in this system, and there is not an available guinea pig monocyte/macrophage cell line in which to confirm in vivo results using an in vitro system. This made validating our array findings problematic. By following canonical signaling pathways from upstream to downstream, we found that the phosphorylation changes we observed made sense in a biological context. We also investigated the effect on downstream transcription factors and a signaling intermediate using conventional in vitro assays using murine monocyte-like cells. Our findings with the transcription factor binding and p38 phosphorylation assays were consistent with the networks and comparisons produced following network analysis of the kinomic data, both in terms of infection-based and temporal observations. These data,
while validating the conclusions drawn from the kinome array results, also provide interesting starting points to focused investigations aimed at understanding the role these pathways play in virus replication and pathogenesis.

The pathways we targeted for verification have previously been shown to be implicated in virus infection. Infection with cytomegalovirus causes an increase in SP1 mRNA and protein levels which results in increased NF-κB expression (50). Virus infection has been shown to activate c-Myc, with a 50-100-fold increase in c-Myc transcription reported following Rous sarcoma virus infection (31). A number of viruses have been shown to activate p38 MAP kinase, including Herpes simplex virus type 1 (51,52) and infectious bursal disease virus (29). Viruses from several families have been shown to modulate p53 activity at a number of different levels. African swine fever virus induces upregulation, stabilization and nuclear accumulation of p53 (23); Adenovirus inhibits p53-mediated transactivation to facilitate replication (48); cells infected with influenza virus show upregulated p53 activity which may play a key role in influenza virus-induced cell death (44).

The c-Myc-binding activities observed do not follow the pathway observations as closely as the SP-1 data, with significant differences observed at 4 hours post-infection. However, no significant changes were observed at the earliest timepoint and the P18 v mock changes then persist through intermediate to late times post-infection. However, the fold changes observed are small and may not be relevant in the host-response to infection, although this could be an artefact of using the murine system. Use of the guinea pig system could reveal larger differences which may be important in infection. It has been shown that EBNA3 of Epstein-Barr virus association
with RBP-Jκ causes down-regulation of c-Myc, suggesting a role for RBP-Jκ in c-Myc regulation (16). Differences in c-Myc activity could perhaps be explained by the difference in RBP-Jκ complex size caused by infection with P2 virus, but not by P18, observed in our previous study (20).

This study, combined with our previous proteomic data, focused transcription factor results and the observations of other investigators, into the functional effects of arenavirus infection, reveals a clear trend which suggests that interference with cellular signaling, potentially at a number of levels of regulation, is involved in arenavirus pathogenesis. Attenuated and apathogenic arenaviruses induce appropriate signaling events which lead to the development of a protective immune response and viral clearance; pathogenic viruses result in a limited response -likely an active process mediated by viral proteins- to inhibit activation of these pathways or activate inhibitory factors. As yet, we do not know the viral proteins responsible for these effects, but, with an increased understanding of the cellular pathways involved, we can rationally target our future investigations to those pathways and proteins which are likely to be key players in mediating pathogenesis.

We have used a novel kinomics assay to investigate the host-cell response to attenuated and virulent arenavirus infection. By integrating our results using pathway analysis we have shown differentially involved signaling networks between these two infections. By employing this approach, we can begin to understand the complex host-response to pathogens and start to unravel the cross-talk between signaling pathways and how these act to cause the cell to respond in one way or another. By studying the phosphorylation state of proteins, we can dissect their
roles in cellular signaling at a more physiological level than by investigating mRNA or total protein levels. A recent editorial has highlighted the importance of understanding the host-response to infection as an alternative approach to anti-microbial drug design (19). By employing a high-throughput assay and integrated signaling network analysis approach, investigators can quickly identify candidate proteins for further investigation and rational targeting of novel anti-viral and anti-bacterial therapeutics.

Many viruses modulate the signaling pathways of the host cell to escape activation of key innate immune mechanisms and establish a productive infection. It is clear that infection with the P18 variant of Pichinde virus suppresses host-cell signaling when compared with an attenuated variant. By understanding molecular events responsible for the clinical disease observed at the tissue, organ and organismal level, we can attempt to elucidate the determinants of pathogenesis at the level of signaling pathways. A number of small-molecule agonists and antagonists to receptors and signaling intermediates are already available due to their use as anti-cancer therapies. By combining these classical treatments, with novel drug-design concepts such as decoy thioaptamers to transcription factors (8,30) we may be able to inhibit microbial pathogenesis at the level of the host-response rather than directly targeting viral or bacterial proteins. This approach could have important consequences with regard to the current problem of microbial resistance to existing therapies.

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Figure Legends

Figure 1. Analysis of PepChip kinase data. Peptide phosphorylation was assayed by densitometry, scaled and normalized using the Spotfire software, analyzed by ANOVA and displayed as a heatmap following hierarchical clustering. Panel a shows the heatmap produced following ANOVA analysis of spots which showed differential phosphorylation between treatments. The scaled and normalized datasets were then analyzed in a pairwise fashion using the student’s t-test. Spots which showed a significant (p<0.05) differential phosphorylation between P2 and mock and P18 and mock were compiled into a dataset. Commonality between spot phosphorylation is represented as Venn diagrams showing number of changes compared to mock-infected samples. Panel b shows the degree of overlap in peptide phosphorylation between P2 and P18 infection at each timepoint. Panel c shows the degree of overlap within an infection between timepoints.

Figure 2. Construction of integrated signaling networks. Datasets compiled from pairwise comparisons of spot data were uploaded to the Ingenuity Pathway Analysis application. The software was used to create functional signaling networks of known interactions based around the differentially phosphorylated proteins we identified using direct interactions only. Pathways with scores >5 were merged to create one global network for each treatment/timepoint. Panel a shows the network built from the P2 day one data; panel b: P18 day one; panel c: P2 day six; panel d: P18 day 6. Proteins shown in blue are those in which we observed significant differential phosphorylation at one or more sites between the infected and control samples.
Figure 3. Comparison analysis of P2 and P18-induced phosphorylation changes. We used the IPA software comparison analysis function to compare the significance of pathway involvement between attenuated and virulent infections at one day and six days post-infection. We compared the significance of the involvement of canonical signaling pathways (a) and functional roles of proteins (b). The y axis represents increasing significance of the pathway or process as determined by the number of differentially phosphorylated proteins; it does not represent a level of activity. We comparing P2 and P18-induced changes with each other by pairwise comparison, we created a dataset of proteins which are differentially phosphorylated between P2 and P18 infection. We uploaded these data to IPA and used this dataset to create signaling networks around the proteins which showed differential phosphorylations between P2 and P18 infection at one day (c) and six days (d) post-infection. Proteins shown in blue are those in which we observed significant differential phosphorylation at one or more sites between the infected and control samples.

Figure 4. Functional characterization of receptor activation. In order to correlate our phosphorylation data with functional consequences, we mined the literature for the roles of various phosphorylation sites of receptors to understand how our identified differential phosphorylations affected the activation of a number of cellular receptors. Phosphorylation sites were categorized as inhibitory or as reflecting the activated receptor. The effects of phosphorylation of multiple sites were consistent, with increased phosphorylation in activation domains correlating with decreased phosphorylation in inhibitory domains and vice versa. A number of differential receptor activities were identified at one day and six days post-infection, and between infection with P2 or P18 viruses. EGFR: epidermal growth
factor receptor; PDGFR: platelet-derived growth factor receptor; IL-1R: interleukin-1 receptor; HGFR: hepatocyte growth factor receptor; M-CSFR: macrophage colony stimulating factor receptor; M/SGFR: mast/stem cell growth factor receptor.

**Figure 5. Transcription factor binding assays.** Murine P388D1 monocyte-like cells were mock-infected or infected with P2 or P18 Pichinde virus for the times indicated and nuclear extracts prepared and protein levels quantified. 5 µg of protein was assayed for transcription factor binding to its consensus sequence. Bound transcription factor was detected by indirect immunoassay. Error bars show the standard error of the mean. Binding of Sp1 (**a**), c-Myc (**b**) and p53 (**c**) was assayed. Bracketed bars are those in which the fold-change was statistically significant (p<0.05); p values are given in the text.

**Figure 6. Phosphorylation of p38 MAP kinase.** Murine P388D1 monocyte-like cells were mock-infected or infected with P2 or P18 Pichinde virus in quadruplicate for the times indicated in a 96-well plate. Cells were fixed and phospho-p38 was detected by indirect immunoassay. Error bars show the standard error of the mean. Bracketed bars are those in which the fold-change was statistically significant (p<0.05); p values are given in the text.
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