EBioMedicine xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

Research Paper

Comprehending a Killer: The Akt/mTOR Signaling Pathways Are Temporally High-Jacked by the Highly Pathogenic 1918 Influenza Virus

Charlene Ranadheera [a,b], Kevin M. Coombs [a,c,d,*], Darwyn Kobasa [a,b,**]

[a] Department of Medical Microbiology, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba R3E 0J6, Canada
[b] Special Pathogens Program, National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, Manitoba R3E 3R2, Canada
[c] Manitoba Centre for Proteomics & Systems Biology, Room 799, 715 McDermot Avenue, Winnipeg, Manitoba R3E 3P4, Canada
[d] Manitoba Institute of Child Health, John Buhler Research Centre, Room 513, 715 McDermot Avenue, Winnipeg, Manitoba R3E 3P4, Canada

ARTICLE INFO

Article history:
Received 15 February 2018
Received in revised form 8 May 2018
Accepted 21 May 2018
Available online xxxx

Keywords:
RNA virus
Virus infection
Host cell alterations
Mass spectrometry
Liquid chromatography
Bioinformatics

ABSTRACT

Previous transcriptomic analyses suggested that the 1918 influenza A virus (IAV1918), one of the most devastating pandemic viruses of the 20th century, induces a dysfunctional cytokine storm and affects other innate immune response patterns. Because all viruses are obligate parasites that require host cells for replication, we globally assessed how IAV1918 induces host protein dysregulation. We performed quantitative mass spectrometry of IAV1918-infected cells to measure host protein dysregulation. Selected proteins were validated by immunoblotting and phosphorylation levels of members of the PI3K/AKT/mTOR pathway were assessed. Compared to mock-infected controls, >170 proteins in the IAV1918-infected cells were dysregulated. Proteins mapped to amino sugar metabolism, purine metabolism, steroid biosynthesis, transmembrane receptors, phosphatases and transcription regulation. Immunoblotting demonstrated that IAV1918 induced a slight up-regulation of the lamin B receptor whereas all other tested virus strains induced a significant down-regulation. IAV1918 also strongly induced Rab5b expression whereas all other tested viruses induced minor up-regulation or down-regulation. IAV1918 showed early reduced phosphorylation of PI3K/AKT/mTOR pathway members and was especially sensitive to rapamycin. These results suggest the 1918 strain requires mTORC1 activity in early replication events, and may explain the unique pathogenicity of this virus.

1. Introduction

The 1918 influenza pandemic was one of the most devastating infectious disease events of the 20th century, resulting in 20–100 million deaths [56,86]. Although the young and the elderly are usually the most susceptible to influenza A virus (IAV) epidemics and many pandemics, the 1918 pandemic was unusual in that a much larger proportion of healthy young adults succumbed to the infection [86], which has been attributed to a dysfunctional host immune response (cytokine storm) [47,53]. A link between the cytokine storm and IAV-induced pathogenesis and poor clinical outcome has long been appreciated [1,47,49,61,87]. Recent attempts to modulate the cytokine storm, including using lipid-modifying compounds such as sphingosine-1-phosphate [60,61,91] have been only partially successful. Because all viruses are obligate parasites that require a host cell in which to replicate, a more complete and detailed understanding of cell signaling and how IAV induces host protein dysregulation is required (recently reviewed in [87]).

The need to better delineate host responses to IAV infection is further underscored by the nature of the virus. IAV is a small, enveloped virus in the family *Orthomyxoviridae*, with a genome of 8 negative-sense single stranded RNA segments that encode for at least 15 proteins [39,62]. IAV have enormous genetic plasticity, mediated by nucleotide (genetic drift) and genome segment exchange (genetic shift), changes that control differences in host range and virulence. IAV are serologically categorized by the hemagglutinin (HA) and neuraminidase (NA) proteins, both of which are located in the viral envelope. There are currently 18 recognized HA (H1–H18) and 11 NA (N1–N11) types [62,65,89]. Various anti-viral strategies, including small molecule inhibitors and vaccines, have been developed to combat IAV. However, the virus' genetic plasticity often leads to resistance rapidly developing to these virus-targeted anti-viral modalities. In addition, because of the virus' enormous host range, spanning avian, marine mammals and numerous land animals including humans, eradication of the virus is extremely unlikely.

Environmental stressors, including virus infection, induce a number of alterations in a host cell's transcriptome and proteome. Previous

* Correspondence to: K. M. Coombs, Manitoba Centre for Proteomics & Systems Biology, University of Manitoba, Winnipeg, MB, Canada.
** Correspondence to: D. Kobasa, National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, MB, Canada.
E-mail addresses: kevin.coombs@umanitoba.ca, (K.M. Coombs), darwyn.kobasa@phac-aspc.gc.ca (D. Kobasa).

https://doi.org/10.1016/j.ebiom.2018.05.027

transcriptomic analyses of cellular responses to IAV have provided some information (for example: [2,28]), including description of innate immune response patterns in macaques infected with the 1918 influenza strain [47]. However, there often is poor concordance between microarray and protein data [2,57,88], partly because mRNA levels cannot provide complete information about extents of post-translational modifications or about levels of effector protein synthesis.Thus, we complemented some of our previous transcriptomic analyses of 1918 virus infection [47] by using a non-biased stable isotope-based quantitative mass spectrometric method to globally assess host proteomic alterations induced by 1918 virus infection in cultured A549 cells.

2. Materials and Methods

2.1. Cells and Viruses

2.1.1. Viruses

All viruses used in this study (Supplementary Table S1), including IAV strain A/South Carolina/1/1918 (H1N1; "1918"), were generated by reverse genetics as previously described [59]. All infectious work was carried out under containment level 4 (CL-4) conditions at the National Microbiology Laboratory in Winnipeg, Canada as outlined in the Health Canada Laboratory Bio-safety Guidelines CL-4 handling procedures (www.hc-sc.gc.ca/pphb-dgspsp/publicat/lbg-ldmbl-96/index.html).

2.1.2. Cells

Human lung A549 cells (American Type Culture Collection # CCL-185) and Madin Darby canine kidney (MDCK) (ATCC # CCL-34) cells were routinely cultured in Dulbecco's modified MEM (DMEM) supplemented with non-essential amino acids, sodium pyruvate, 0.2% (*w*/*v*) glucose, 10% fetal bovine serum (FBS; Invitrogen), and 2 mM L-glutamine as previously described[12]. To label A549 cells with SILAC for global non-biased quantitative proteomic analyses, they were grown in DMEM media provided with a SILAC™ Phosphoprotein Identification and Quantification Kit (Invitrogen Canada Inc.; Burlington, Ontario), supplemented as above (except without non-essential amino acids), and with 10% dialyzed FBS (Invitrogen Canada Inc.; Burlington, Ontario), plus 100 mg each of "light" (**L**; $^{12}C_6/^{14}N_4$) or "heavy" (**H**; $^{13}C_6/^{15}N_4$) L-lysine and l-arginine per liter of D-MEM, such that **H** isotopic forms have 6.0 and 10.0 Da heavier masses than the corresponding **L** forms [12].Virus stocks were generated, and virus titrations were performed, in MDCK cells as previously described [12].

2.1.3. Infection

Once A549 cells destined for SILAC labeling had grown through six doublings, **L** cells in 2 experiments were infected with 1918 at a multiplicity of infection (MOI) of 7 plaque forming units (PFU) per cell and an equivalent number of **H** cells were mock infected as control. Labels were swapped in the 3rd biologic replicate. Cells were overlaid with appropriate media and cultured for 5 (early) and 24 (late) hours.

All other A549 infections were performed in DMEM supplemented with 0.1% BSA and 0.5 μg/ml TPCK-Trypsin.

2.2. Cell Viability Assays

A549 cells were infected with 1918 at a MOI of 7 PFU/cell. At 5, 24 and 48 h post-infection, the media was removed and replaced with fresh OptiMEM (Life Technologies, Burlington, ON, Canada) supplemented with XTT (XTT-based InVitro Toxicology Assay Kit, Sigma-Aldrich, Oakville, Ontario, Canada) as per the manufacturer's directions. The cells were incubated for an additional 3 h. The colorimetric change was read at an absorbance of 450 nm and percent viability was normalized to the control samples. Experiments were performed in triplicate. In parallel, cells were visualized by light microscopy for the presence of cytopathic effect.

2.3. Cell Fractionation

At 5 and 24 h post-infection (hpi), **L** and **H** cells were collected and counted. Equivalent numbers of **L** and **H** cells were mixed together, mixed cells were washed 3× in >50 volumes of ice-cold Phosphate Buffered Saline (PBS), washed cells were lysed with 0.5% NP-40 supplemented with 1.1 μM pepstatin A, incubated on ice for 30 min, and nuclei removed by pelleting at 5000 ×g for 10 min. Nuclei were processed by a previously-described high salt/urea double extraction procedure [48] and both fractions frozen at −80C until further processing. Fractionated samples were probed with antibodies targeting nuclear and cytoplasmic proteins to ensure the method for fractionation was complete (Fig. 5A).Histone H3 is a nuclear protein, Lamin is predominantly found in the nuclear envelope, Actin is typically found in the cytoplasm and tubulin is found in both the nucleus and cytoplasm.

2.4. Mass Spectrometric Sample Preparation and Analysis

Protein content in the various fractions was determined using a BCA™ Protein Assay Kit (Pierce; Rockford, IL) and BSA standards. Samples were then reduced, alkylated and digested with trypsin as previously described[12]. Digested peptides were separated by 2D RP (reversed-phase) high pH – RP low pH peptide fractionation [29,83], and analyzed on a QStar Elite mass spectrometer (Applied Biosystems, Foster City, CA) run in a data-dependent MS/MS acquisition mode as previously described using the manufacturer's "smart exit" (spectral quality 5) settings [12]. Previously targeted parent ions were excluded from repetitive MS/MS acquisition for 60 s (50 mDa mass tolerance). Protein Pilot 2.0 (Applied Biosystems) software was used for protein identification and quantitation.Raw data files (30 in total for each run) were submitted for simultaneous search using standard SILAC settings for QStar instruments. Proteins for which at least 2 fully trypsin digested **L** and **H** peptides were detected at >99% confidence were used for subsequent comparative quantitative analysis. Raw MS data files were analyzed by Protein Pilot®, version 2.0, using the non-redundant human gene database. Proteins, and their confidences and **L:H** ratios, were returned with Geninfo Identifier (gi) numbers. Differential regulation within each experimental dataset was determined by *Z*-score normalization of each dataset, using a confidence of >1.960σ as previously described [12].

2.5. Cellular Protein Expression

A549 cells were seeded 24 h prior to use so that they were 80% confluent at the time of infection. Cells were washed with DMEM supplemented with 0.1%BSA and infected at an MOI of 7 for 1 h. The virus inoculum was removed, cells were washed with PBS, and fresh DMEM supplemented with 0.1% BSA and 0.5 μg/ml TPCK-trypsin was added to the cells. Cells were harvested at 5 h and 24 h post infection. Total cell lysates were collected by washing the cells once with PBS and lysing cells with 2% SDS for immunoblot analysis. Fractionated lysates were harvested by washing the cells with PBS, adding 0.5% NP40 supplemented with Complete Protease Inhibitor (Roche) to the cells and incubating on ice for 30 min. The lysates were then collected and centrifuged at 2500 ×*g* for 10 min to generate soluble cytoplasmic and pelleted nuclear fractions. Each fraction was brought up to equal volumes with a final concentration of 2% SDS for gel electrophoresis and immunoblot analysis. Immunoblots were performed using commercially available primary antibodies coupled with secondary antibodies containing a conjugated IRdye® (Supplementary Table S2). Blots were visualized using a Licor® Odyssey scanner. Band intensities were quantified by densitometry using ImageJ software and normalized to the expression levels of actin. Each experiment was replicated at least 3 times, the means and standard errors are graphically presented. A one-way Anova with a Dunnett post-test was used to determine any significant changes between the various virus strains tested, and a one-way

Please cite this article as: Ranadheera C, et al, Comprehending a Killer: The Akt/mTOR Signaling Pathways Are Temporally High-Jacked by the Highly Pathogenic 1918 Influenza Virus, EBioMedicine (2018), https://doi.org/10.1016/j.ebiom.2018.05.027