Arch Virol (2017) 162:45–55
DOI 10.1007/s00705-016-3072-8

ORIGINAL ARTICLE

G45R on nonstructural protein 1 of influenza A virus contributes to virulence by increasing the expression of proinflammatory cytokines in mice

Challika Kaewborisuth[1,2] · Bryan Kaplan[2] · Mark Zanin[2] · David Finkelstein[3] · Richard J. Webby[2] · Porntippa Lekcharoensuk[4,5]

Received: 6 September 2016 / Accepted: 15 September 2016 / Published online: 23 September 2016

Abstract Nonstructural protein 1 (NS1) is a multifunctional protein that is a viral replication enhancer and virulence factor. In this study, we investigated the effect of the amino acid substitution G45R on the NS1 of A/Puerto Rico/8/1934 (H1N1) (G45R/NS1) on viral virulence and host gene expression in a mouse model and the human lung cell line A549. The G45R/NS1 virus had increased virulence by inducing an earlier and robust proinflammatory cytokine response in mice. Mice infected with the G45R/NS1 virus lost more body weight and had lower survival rates than mice infected with the wild type (WT/NS1) virus. Replication of the G45R/NS1 virus was higher than that of the WT/NS1 virus *in vitro*, but the replication of both viruses was similar in mouse lungs. In A549 cells, the majority of G45R/NS1 protein was localized in the cytoplasm whereas the majority of WT/NS1 protein was localized in the nucleus. Microarray analysis revealed that A549 cells infected with the G45R/NS1 virus had higher expression of genes encoding proteins associated with the innate immune response and cytokine activity than cells infected with the WT/NS1 virus. These data agree with cytokine production observed in mouse lungs. Our findings suggest that G45R on NS1 protein contributes to viral virulence by increasing the expression of inflammatory cytokines early in infection.

Electronic supplementary material The online version of this article (doi:10.1007/s00705-016-3072-8) contains supplementary material, which is available to authorized users.

✉ Richard J. Webby
richard.webby@stjude.org

✉ Porntippa Lekcharoensuk
fvetptn@ku.ac.th

Challika Kaewborisuth
k.challika@hotmail.com

Bryan Kaplan
bryan.kaplan@stjude.org

Mark Zanin
mark.zanin@stjude.org

David Finkelstein
David.finkelstein@stjude.org

[1] Present Address: Interdisciplinary Graduate Program in Genetic Engineering, The Graduate School, Kasetsart University, Bangkok 10900, Thailand

Introduction

Influenza A virus is an important respiratory virus that can cause severe illness in humans and other mammalian species. The emergence and spread of new strains of influenza A virus, such as the highly pathogenic avian influenza

[2] Department of Infectious Diseases, MS 330, Division of Virology, St. Jude Children's Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105-3678, USA

[3] Department of Computational Biology, St. Jude Children's Research Hospital, Memphis, TN 38105-2794, USA

[4] Department of Microbiology and Immunology, Faculty of Veterinary Medicine, Kasetsart University, 50th Ngamwongwan Rd., Chatuchak, Bangkok 10900, Thailand

[5] Center for Advances Studies in Agriculture and Food, KU Institute for Advanced Studies, Kasetsart University, Bangkok 10900, Thailand

(HPAI) H5N1 virus and the swine-origin pandemic influenza (H1N1) 2009 virus, have led to increased mortality rates in poultry and humans [10, 17]. Novel virus strains and genotypes have emerged through gene reassortment and mutation [11, 12, 16, 18, 25].

Viral proteins such as hemagglutinin (HA), polymerase basic protein 1-F2, and non-structural protein 1 (NS1) have been extensively studied for their roles in increasing virus transmissibility and pathogenicity [12, 18, 36, 43, 64]. Increased virus pathogenicity is associated with a greater induction of host proinflammatory cytokine responses, which can lead to severe illness [9]. However, the induction of proinflammatory cytokine responses can vary by viral subtype and host species [19, 29].

NS1 interacts with several cellular proteins in infected cells to increase viral replication and pathogenesis [4, 27, 58]. The deletion of 15 nucleotides from positions 263 to 277 in NS1 of the H5N1 virus, together with the D92E NS1 mutation, contributes to increased virulence of H5N1 viruses in chickens and mice [38]. Moreover, the D92E shift in NS1 of lethal H5N1 influenza viruses can induce severe illness in infected pigs as well as increased production of inflammatory cytokines and chemokines in the lungs and specific B cells and T cells of mice, thereby highlighting the importance of NS1 in increasing virus pathogenesis [35, 57]. NS1 contains 2 conserved nuclear localization signals, NLS1 and NLS2, on the N terminus (aa 34 to 38) and C terminus (aa 203 to 237), respectively, which allow its localization to both the nucleus and the cytoplasm [22, 39]. In the cytoplasm, the double-stranded (ds) RNA-binding domain of NS1 sequesters the dsRNA from retinoic acid–inducible gene I (RIG I) and interferon (IFN)-inducible dsRNA-activated enzymes such as protein kinase R (PKR) and 2′,5′-oligoadenylate synthetase 1 (OAS1), leading to impaired host IFNβ production and antiviral activity [41, 42]. NS1 increases viral mRNA translation by binding to the host translation initiation factor eIF4GI, poly(A)-binding protein 1, and the 5′ untranslated region of viral mRNA, leading to viral protein synthesis [3, 7, 56]. In the nucleus, NS1 can inhibit the export of nuclear mRNA [2, 52, 53]. NS1 likely interacts with the 30-kDa cleavage and polyadenylation specificity factor (CPSF30) and poly(A)-binding protein II, which leads to inhibition of 3′ cleavage and polyadenylation and nuclear export of host pre-mRNA, including pre-mRNA of antiviral proteins such as IFNβ [8, 44, 45, 60].

Our initial studies demonstrated that the G45R mutation on PR8 NS1 facilitates viral replication independent of its dsRNA-binding affinity and type I IFN induction [28]. In this report, we sought to further understand the role of G45R/NS1 in viral replication and virulence in a mouse model and in the human lung adenocarcinoma cell line A549.

Materials and methods

Cell lines

Human lung adenocarcinoma epithelial cells A549 were maintained in Kaighn's Modification of Ham's F-12 Medium (ATCC) supplemented with 5 % fetal bovine serum (Invitrogen), L-glutamine (Invitrogen), and penicillin/streptomycin (Invitrogen). Cells were incubated in a humidified atmosphere of 5 % CO_2 at 37 °C.

Reverse genetics–derived viruses

Viruses possessing HA and neuraminidase (NA) genes from X31, A/Hong Kong/1/1968 (H3N2), in an A/Puerto Rico/8/1934 (H1N1) (PR8) background with PR8 wild-type NS1 (WT/NS1) or G45R mutation NS1 (G45R/NS1) were generated by the 8-plasmid reverse genetics system in 293T and MDCK cells [23]. Briefly, 8 plasmids, 0.5 μg each, were incubated with 200 μL of the X-tremeGENE 9 transfection reagent (Roche) for 15 min before being gently overlaid onto the 293T-MDCK cell mixture. Transfected cells were cultured in Opti-MEM I reduced serum (Invitrogen) containing 1 μg/mL TPCK-treated trypsin (Sigma) and incubated at 37 °C with 5 % CO_2. Viruses were rescued from the supernatant of transfected cells and inoculated in the allantoic cavity of 9-day-old specific pathogen-free embryonated chicken eggs for virus amplification. Virus presence was determined by the hemagglutination assay, as previously described [32] and titrated by plaque assay.

Infection of mice

To determine virus pathogenicity in the mouse model, three groups of five 6-week-old female BALB/c mice (The Jackson Laboratory) were used. Mice were anesthetized with isoflurane, and each mouse was intranasally inoculated with 30 μL of 10^4 plaque forming units (PFUs)/mL of each virus in PBS or mock-infected. Mice were monitored and weighed for up to 8 days post infection (dpi). Mice that lost more than 30 % of their initial body weight were euthanized by using CO_2 according to institution protocol. The mouse study was conducted in a standard biosecurity level 2 (BSL2). All mouse experiments were conducted in accordance with the guidelines of the institutional animal care and use committee.

To determine virus titer as well as cytokines and chemokines levels in the lungs, 3 mice from each group were sacrificed for lung collection on 1, 2, 4, 6, and 8 dpi. Lungs were collected and homogenized in MEM (Invitrogen) containing 0.3 % BSA (Sigma-Aldrich), L-glutamine,

and penicillin/streptomycin (Invitrogen) and frozen at –80 °C until used. After thawing, homogenized lungs were centrifuged at 4000 rpm at 4 °C for 15 min and the supernatant was collected. Viruses were titrated in MDCK cells and the $TCID_{50}$/mL was calculated by the Reed and Muench method [55].

Quantification of cytokines and chemokines in the lung

Levels of cytokines and chemokines in the supernatant from homogenized lung of infected mice were determined by using the MILLIPLEX MAP mouse cytokine/chemokine magnetic bead panel (Millipore) as per the manufacturer's protocol. Cytokine and chemokine levels were determined from 3 individual mice per group on 1, 2, and 4 dpi. Lungs were collected and homogenized in MEM containing 0.3 % BSA (Sigma-Aldrich), L-glutamine, and penicillin/streptomycin (Invitrogen) and frozen at −80 °C until used. After thawing, homogenized lungs were centrifuged at 4000 rpm at 4 °C for 15 min, the supernatant was collected, and the assay was performed by using Luminex 100®/200® assays (Millipore).

Confocal microscopy studies

A459 cells were seeded on glass coverslips in 6-well plates and incubated overnight at 37 °C in 5 % CO_2. A549 cells were infected with WT/NS1 and G45R/NS1 viruses at a multiplicity of infection (MOI) of 2. At the indicated time point, cells were washed with 1× PBS and fixed with 4 % paraformaldehyde (Electron Microscopy Science) in PBS for 20 min at 4 °C. Cells were then washed thrice with 1× PBS for 5 min each. For immunostaining, fixed cells were permeabilized in a blocking buffer containing 1× PBS, 5 % BSA, and 0.3 % Triton X-100 for 60 min. After incubation, cells were incubated with anti-influenza A virus NS1 (Thermo Scientific) primary antibody at a dilution of 1:500 in the antibody dilution buffer containing 1× PBS, 1 % BSA, and 0.3 % Triton X-100 for 1 h at room temperature. After 3 washes with 1× PBS for 5 min each, cells were incubated with goat anti-rabbit IgG conjugated to Alexa Fluor 488 (Molecular Probes) in the dark for 1 h. Coverslips were mounted with ProLong® Gold Antifade Mountant with DAPI (Invitrogen). Cells were imaged with a 60× objective on a Nikon C2 confocal microscope (Nikon). Representative images were processed in NIS-elements viewer 4.2 (http://www.nikon-instruments.jp/eng/service/download/software/imgsfw/index.aspx) and Image J software (http://imagej.nih.gov/ij/).

Microarray analysis

A549 cells were grown in 6-well plates and infected with WT/NS1 or G45R/NS1 viruses (MOI of 2) in 3 biological replicates. At 8 hours post infection (hpi), the infected cells were lysed with TRIzol® reagent (Invitrogen), total cellular RNA was isolated by using Direct-zol™ RNA MiniPrep (Zymo Research) as per manufacturer's instructions, and RNA concentration and purity were quantified by a Spectromax® Plus spectrophotometer (Molecular Devices). RNA quality and integrity were assessed by lab-on-a-chip analysis by using the Agilent 2100 BioAnalyzer (Agilent Technologies). The human gene 2.0 ST array (Affymetrix) was performed by the Hartwell Center for Bioinformatics and Biotechnology, St. Jude Children's Research Hospital. Data from the Affymetrix Gene Chip array was analyzed by using the Gene Chip Operating System Software and converted into expression data. The data was summarized and quality controlled by Robust Multi-Array average (RMA) and principle component analysis (PCA), respectively. Each gene probe set was tested for the effect of infection of WT and mutant viruses by 1 way ANOVA model using Partek Genomics Suite 6.6. Differentially expressed genes were those that had a ≥0.5 log ratio in expression between mock and infected cells ($P < 0.01$). Upregulated and downregulated genes were considered if there was a 2-fold or higher change in expression ($P < 0.01$). [Note that the selected downregulated genes could not be cut off at a 2-fold or higher difference according to their lower expression compared with upregulated genes; thus, those genes were filtered at a 1-fold or higher compared with mock-infected cells.] Gene ontology analysis (available at http://david.abcc.ncifcrf.gov/) was used to characterize biological functions of corresponding genes that had the most significant differential expressions, as previously described [24]. The average expression levels (represented by the *z*-score) of global and selected genes from each pathway were included for hierarchical clustering analysis by the unweighted pair group method with arithmetic mean to visualize differential gene expression between cells infected with WT/NS1 and G45R/NS1 viruses by using the Spotfire® Decision Site software 9.1.2 (Tibco).

Quantitative real-time PCR

Total RNA was isolated from A549 cells infected with WT/NS1 and G45R/NS1 viruses by using the TRIzol reagent (Invitrogen) as per manufacturer's instructions. One microgram of total RNA was reverse transcribed by using a high-capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative real-time PCR (qRT-PCR) was performed by using the 7500 Fast Real-Time PCR System (Applied Biosystems). In a 15-μL reaction

volume, 0.4 mM each of forward and reverse primers specific for each gene and an equal amount of cDNA were mixed with the SYBR® Select Master Mix (Applied Biosystems) as per the manufacturer's instructions. The qRT-PCR cycles were conducted under the following conditions: enzyme activation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 3 sec and annealing and extension at 60 °C for 30 sec. For each sample, 3 biological replicates and 2 technical repeats were performed. The housekeeping gene β-actin was used as an internal control. Normalized data from each sample in relation to that of mock-infected cells were compared by the threshold cycle ($^{\Delta\Delta}CT$) method [37].

Results

G45R on NS1 increases viral virulence in BALB/c mice

Our initial studies indicated that G45R on PR8 NS1 enhances viral replication *in vitro* [28]. Here, we investigated whether G45R affects viral virulence and replication *in vivo*, using X31 viruses containing HA and NA genes from A/Hong Kong/1/1968 (H3N2) in an A/Puerto Rico/8/1934 (H1N1) (PR8) background. We used this backbone because of the intrinsic virulence of the PR8 virus in mice. Mice infected with the G45R/NS1 virus lost an average of 10.52 ± 2.9 % (mean ± standard error of mean) of their body weight from 2 dpi, whereas mice infected with the WT/NS1 virus lost an average of less than 2.24 ± 0.62 % of their body weight (Fig. 1a). Only 40 % of mice infected with the G45R/NS1 virus survived, whereas 100 % of mice infected with the WT/NS1 virus survived (Fig. 1b). There were no significant differences in peak viral titers in the lungs of mice infected with WT/NS1 or G45R/NS1 virus; however, the G45R/NS1 virus was detected until 8 dpi whereas the WT/NS1virus was undetectable at 8 dpi (Fig. 1d), indicating that the G45R/NS1 virus had a more delayed clearance than the WT/NS1 virus.

Cytokines and chemokines levels in the lung revealed that the G45R/NS1 virus induced an early inflammatory response, because the levels of proinflammatory cytokines and chemokines (TNF-α, IP-10, IL-17, RANTES and KC), anti-inflammatory cytokine IL10, and G-CSF that appeared on 1 dpi were higher than those in mice infected with the WT/NS1 virus (Fig. 2).

G45R alters the localization of the PR8 NS1 protein in infected cells

NS1 plays roles in both the nucleus and cytoplasm by interacting with cellular proteins and interfering with their activities to inhibit the host immune response and enhance viral replication. However, the subcellular localization of NS1 is strain and host specific [16, 30]. We determined whether G45R changes the subcellular localization of NS1. In A549 cells inoculated at an MOI of 2, the G45R/NS1 protein accumulated mostly in the cytoplasm whereas the WT/NS1 protein localized mostly in the nucleus (Fig. 3). These differences in localization occurred early in infection (4 to 8 hpi) but not at later time points (20 hpi; Fig 3). The expression of G45R/NS1 was higher than that of WT/NS1. NS1 staining was clearly visible at 4 hpi in cells infected with the G45R/NS1 virus, but only weak reactivity was observed in cells infected with the WT/NS1 virus. Staining became clearly visible in cells infected with the WT/NS1 virus from 8 hpi. This result is consistent with our previous finding that replication of the G45R/NS1 virus is higher than that of the WT/NS1 virus at early time points after infection [28].

G45R/NS1 alters host gene expression in A549 cells

As G45R/NS1 increased viral virulence in mice and promoted cytoplasmic localization in A459 cells, we hypothesized that G45R/NS1 changes the host response to virus infection. Global gene expression levels from infected cells were analyzed in parallel with those relative to mock-infected cells. One-way ANOVA ($P < 0.05$) was used to analyze statistical differences in gene expression levels in the host response among the 3 groups. The transcriptional profiles of genes induced by the WT and G45R/NS1 viruses were different (Fig. 4). The G45R/NS1 virus induced gene upregulation or downregulation to a greater extent than did the WT/NS1 virus, relative to mock-infected cells (Figs. 5a and 6a). The higher gene expression of the host response to the G45R/NS1 virus may be due to higher virus replication (Fig. S1), which is consistent with our previous report that titers for the G45R/NS1 virus and NS1 mRNA expression in infected cells were higher than those for the WT/NS1 virus early in infection [28].

The differentially expressed genes were filtered to select for genes whose expression changed by 2 fold or more, relative to the mock-infected group ($P < 0.01$). These genes were further analyzed by using DAVID (available at http://david.abcc.ncifcrf.gov/) [24] to characterize the biological functions associated with those genes. The extent of upregulation of genes related to cytokine activity and IFN induction was higher in cells infected with the G45R/NS1 virus than those infected with the WT/NS1 virus (Fig. 5b). These findings indicate that the G45R/NS1 virus can induce a stronger host immune response than the WT/NS1 virus early in infection. Furthermore, the extent of downregulation of genes associated with positive regulation of apoptosis and serine-threonine protein kinases activity was

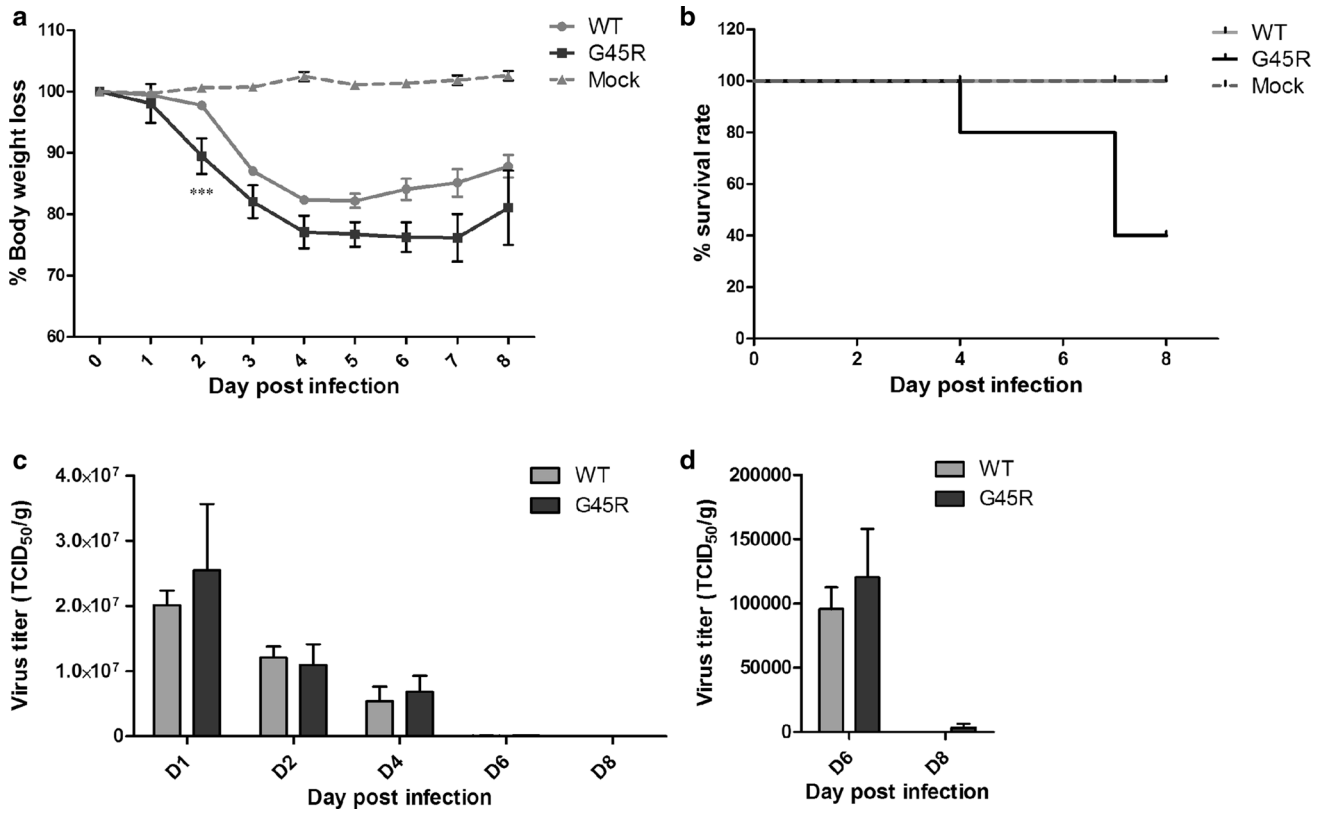

Fig. 1 Survival rate and viral titers in lungs of infected mice. Mice were infected with 10^4 plaque-forming units of viruses possessing HA and NA from A/Hong Kong/1/1968 (H3N2) in the PR8 background encoding PR8 wild-type (WT/NS1) or G45R mutation NS1 genes (G45R/NS1). Body weight loss (a) and survival rate (b) were monitored every day. Lung samples were collected on the indicated days. (c) Viral titers in the lungs were determined in MDCK cells and reported as $TCID_{50}$/g. Error bar indicates the standard error of the mean ($P < 0.05$). (d) The virus titers similar to Fig 1c with a magnified scale on the Y axis to demonstrate the viral titers at 6 and 8 dpi

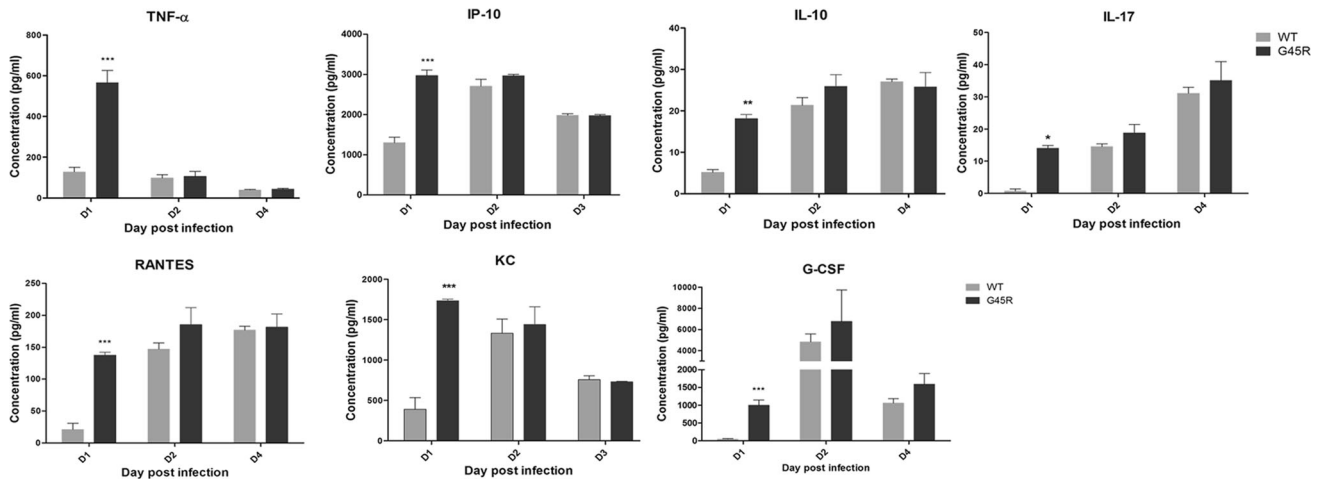

Fig. 2 Cytokine and chemokine levels in infected mice. Mice were infected with 10^4 plaque-forming units of viruses possessing HA and NA from A/Hong Kong/1/1968 (H3N2) in the PR8 background of encoding PR8 wild-type (WT/NS1) or G45R mutation NS1 genes (G45R/NS1). Levels of cytokines and chemokines in the clear supernatant from homogenized lungs were measured by using the MILLIPLEX MAP mouse cytokine/chemokine magnetic bead panel. Error bars indicate the standard error of the mean (***$P < 0.001$)

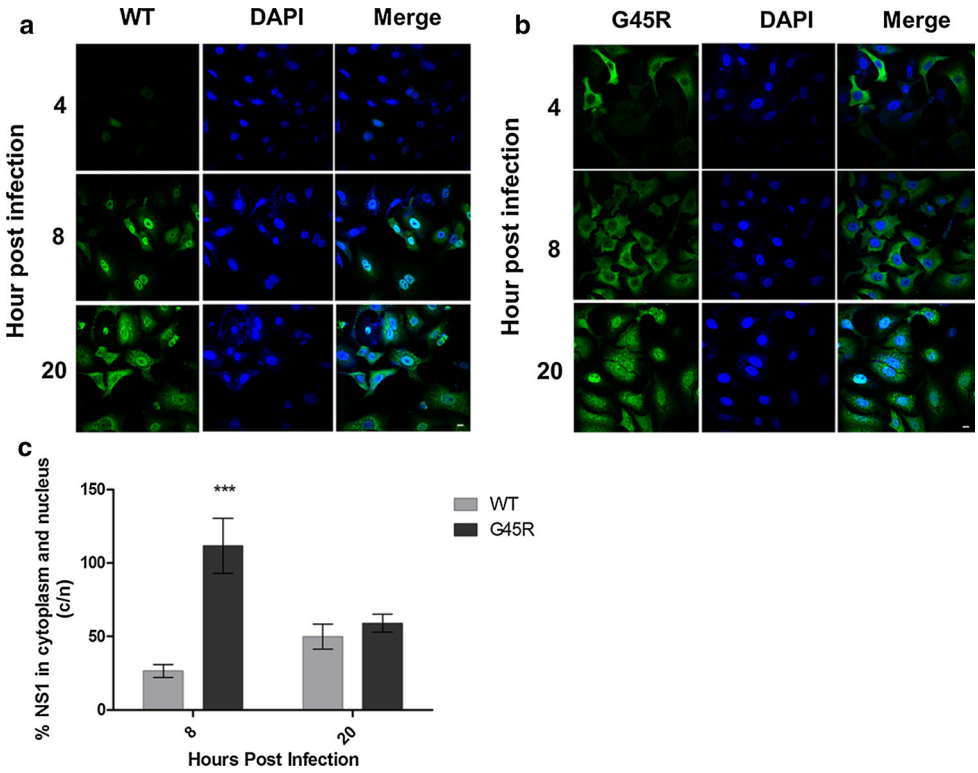

Fig. 3 Intracellular localization of NS1 protein in infected A549 cells. The viruses encoding HA and NA from A/Hong Kong/1/1968 (H3N2) in the PR8 background encoding (a) PR8 wild-type (WT/NS1) or (b) G45R mutation NS1 (G45R/NS1) genes were used to infect A549 cells at an MOI of 2. At the indicated time points, infected cells were fixed and stained with rabbit anti-NS1 polyclonal antibody and goat anti-rabbit IgG conjugated to Alexa Fluor 488. The signals were observed by using a confocal microscope. Scale bar 10 μm. (c) Quantified signals in the nucleus and cytoplasm are shown. Error bars indicate the standard error of the mean (***$P < 0.001$)

higher in cells infected with the G45R/NS1virus than those infected with the WT/NS1 virus (Fig. 6).

Quantitative RT-PCR was performed to determine expression levels of selected genes in the aforementioned pathways. Expression levels of genes related to induction of innate immune response and proinflammatory cytokines and chemokines, such as IFNα/β, IFN-induced protein with tetratricopeptide repeats 1 and 5, IFN-induced protein 44, chemokine (C-X-C motif) ligand (CXCL) 10 and 11, chemokine (C-C motif) ligand (CCL) 5, IL28A, and IL29 were significantly higher in cells infected with the G45R/NS1 virus than cells infected with the WT/NS1 virus (Fig. 5c). In contrast, the expression levels of genes involved in the apoptosis pathway and protein kinase function, such as homeodomain interacting protein kinase 2 (HIPK2), triple functional domain (TRIO), protein kinase C, alpha (PRKCA), and interleukin-1 receptor-associated kinase 1(IRAK1), were significantly lower in cells infected with the G45R/NS1 virus than those infected with the WT/NS1 virus (Fig. 6c).

Discussion

The NS1 of influenza A virus can increase viral replication and pathogenicity through various mechanisms, including inhibition of apoptosis and host innate immune response as well as induction of proinflammatory cytokines [1, 15, 35, 38, 57]. However, these functions vary by viral subtypes and host cell species [4, 39]. We have demonstrated that the non-conservative amino acid mutation, G45R on PR8 NS1 increases virus replication independent of the type I IFN system and induces the activation of type I IFN signaling at an early stage of infection [28]. We therefore hypothesized that G45R may be a determinant of viral virulence and replication *in vivo*.

In the current study, BALB/c mice infected with the G45R/NS1 virus had rapid and robust expression of CXCL5, CXCL10, CXCL11, IFNα/β, TNF-α, IL-17, RANTES, IP-10, MKC, IL28, IL29 and G-CSF, as early as 1 dpi. These cytokines and chemokines are associated with fever, sepsis, and autoimmunity [49]. Hypercytokinemia is associated with HPAI H5N1 virus [35] and pandemic H1N1 2009 virus [54, 62] infection. Glu-92 on NS1 of HPAI H5N1 viruses is essential for increasing pathogenicity by increasing the expression of proinflammatory cytokines IL1-α, IL1-β, and IL6 [35]. Compared with the PR8 virus, the pandemic H1N1 2009 virus also induces higher expression of IL-6, TNF-α, IL-10, and CCL5 in supernatants from macrophage cultures [54]. Consistent with these results, in our study the upregulation of proinflammatory cytokine expression and IFN response preceded the severe illness and loss in body weight in mice infected with the G45R/NS1 virus, suggesting that the G45R/NS1 mutation contributes to increased viral virulence *in vivo*.

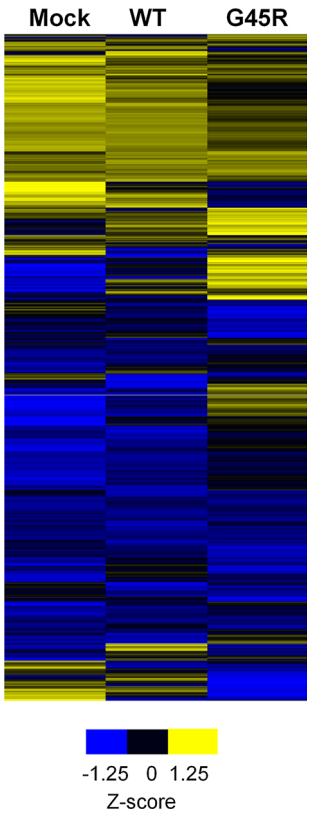

Fig. 4 Global gene expression levels in response to virus infection. The hierarchical clustering of differentially expressed genes from WT/NS1 and G45R/NS1 and mock-infected cells is presented. The relative expression level for each group is shown as the Z-score (−SD to SD) color scale

The NLS1 of NS1 is located on the dsRNA-binding domain (amino acids 34 to 41) [39], and the interaction between NLS1 and cellular importin α induces nuclear import of the protein. The nuclear localization of NS1 is an essential mechanism to block host IFN induction. For example, binding of NS1 to CPSF30 in the nucleus suppresses posttranscriptional processing of the entire cellular pre-mRNA, including antiviral IFN, which in turn facilitates viral replication [8, 44, 60]. However, the localization of NS1 in different intracellular compartments leads to different functions that affect viral pathogenicity [16, 39]. We demonstrated that the majority of the WT/NS1 protein was localized in the nucleus of A549 cells, which is in agreement with the results of a previous study [58], whereas G45R/NS1 increased cytoplasmic localization early in infection (Fig. 3). Increased cytoplasmic localization of NS1 of the mouse-adapted influenza A virus can play a role in influenza A virus pathogenicity and replication and is associated with host-specific adaptive evolution [16]. In the cytoplasm, NS1 counteracts host cell mechanisms by interfering with the activation of RIG-I, OAS1, and PKR [33, 40, 41] and promoting viral mRNA translation [56]. The abundance of the G45R/NS1 protein in this compartment might increase the cytoplasmic functions of NS1, which could increase viral replication or virulence.

Microarray analysis revealed that the level of differential gene expression was higher in cells infected with the G45R/NS1 virus than those infected with the WT/NS1 virus (Fig. 4). Upregulated genes were mostly involved in pathways related to the innate immune response and induction of pro-inflammatory cytokines and cytokines (e.g., IFNα/β, CXCL5, CXCL10, CXCL11) and IFN-inducible genes and ISGs early in infection. Influenza A virus infection activated Type I IFN induction and inflammatory response through various pathogen sensors including RIG-I in the cytoplasm and toll-like receptors (TLR) in the endosome and on the cell surface [14, 31, 46]. RIG-I is one of the cytoplasmic pathogen sensors that recognizes viral RNA, particularly 5'-ppp single stranded RNA, and viral double stranded RNA (replicative intermediates) and triggers type I IFN induction [50, 61]. Although G45R NS1and WT viruses induced RIG-I mediated IFNβ-promoter activity at similar levels in transfected 293T cells [28], the increased G45R/NS1 virus replication and the accumulation of viral RNA in G45R/NS1 infected cells may upregulate the expression of immune stimulated genes through the RIG-I pathway (Fig. 5b and S1). This could lead to the robust production of proinflammatory cytokines and chemokines and high virulence in G45R/NS1 virus infected mice.

Moreover, G45R/NS1 downregulated the expression of genes related to serine-threonine protein kinases such as HIPK2, TRIO, IRAK1, and PRKCA, which play important roles in inducing apoptosis or the innate immune response. For example, HIPK2 phosphorylates p53 to induce apoptotic pathways in cancer cells [13, 51]. However, in virus-infected cells, HIPK2 function is counteracted by the multifunctional viral RNA-binding protein US11, which plays a role in replication of the herpes simplex virus and regulation of cellular functions [20, 48]. Apoptosis is also a crucial cellular response to influenza A virus infection [5]. NS1 can regulate apoptosis during viral infection, which can affect virus pathogenicity and replication cycles [63]. These functions depend on levels of NS1 expression, virus strain, and infected cell type [26, 65]. Therefore, the downregulation of genes associated with apoptosis induction may be an anti-apoptotic activity of G45R/NS1 that is used to delay cell death in order to extend virus survival and increase virus replication early in infection [65].

IRAK1, a key mediator of the innate immune system, plays an important role in the signaling pathways activated by the Toll-like receptors/IL-1 receptors after recognizing pathogen-associated molecular patterns such as dsRNA, ssRNA and dsDNA and is important for rapid initiation of the innate immune response to pathogens early in infection

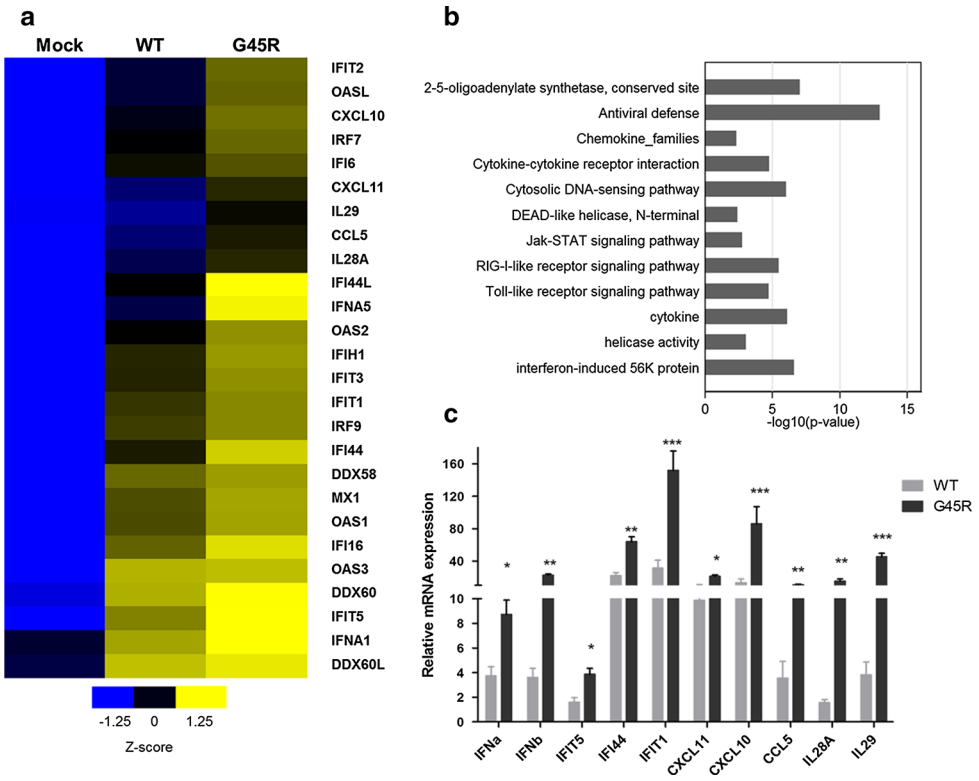

Fig. 5 Differentially upregulated genes and associated biological pathways. (a) The hierarchical clustering of differentially expressed genes within the categories of cytokine activity and innate immune response is presented. The relative expression level for each group is shown as the Z-score (−SD to SD) color scale. (b) Biological pathways of upregulated genes expressed in cells infected with the WT/NS1 or G45R/NS1 viruses, as generated by using DAVID. (c) Relative mRNA expression levels for the selected genes measured by qRT-PCR. Three biological replicates and two technical repeats were performed for each sample. The housekeeping gene β-actin was used as an internal control. Normalized data of each sample was analyzed in relation to that of mock-infected cells by the threshold cycle ($^{\Delta\Delta}$CT) method. Error bars indicate the standard error of the mean. $*P < 0.01$, $**P < 0.01$, $***P < 0.001$, compared to WT, as determined by the t-test

[21, 34, 66]. Influenza A virus infection can increase the production of miRNAs in A549 cells. These miRNAs target cellular mRNAs associated with antiviral response pathways such as IRAK1 and MAPK3, suggesting that influenza A virus can interfere with host immune responses [6, 59]. Therefore, downregulation of gene expression by the G45R/NS1 virus may have had a negative effect, overall, on the immune response early in infection. However, the underlying mechanisms of this process need further investigation.

Analysis of NS1 sequences of influenza A viruses has revealed that the G45R mutation occurs in pandemic 2009 H1N1 viruses such as A/New York/3307/2009 (accession number CY041641), A/Russia/74/2009 (accession number CY053732), and A/Texas/15/2009 (accession number GQ122093). The G45R mutation on NS1 of A/Texas/15/2009 (H1N1) isolated from a patient has been proposed to be related to the increased chemokines and cytokines produced during virus infections in A549 cells [47]. Previously, we demonstrated that G45R on NS1 functioned independently of the dsRNA-binding affinity to accelerate influenza virus replication and elevated the expression of type I IFN at early of infection in A549 cells [28]. In our current study we demonstrate that the G45R mutation on NS1 enhances virus virulence by increasing the proinflammatory chemokine and cytokine response in mice.

Conclusions

Our study shows that G45R on the NS1 protein of influenza A virus contributes to viral virulence by inducing rapid and robust proinflammatory cytokine responses. Furthermore, G45R possibly regulates host cell apoptosis and interferes with immune signaling pathways to facilitate virus replication early during infection, as previously described [28]. Taken together, the findings reveal important roles of a

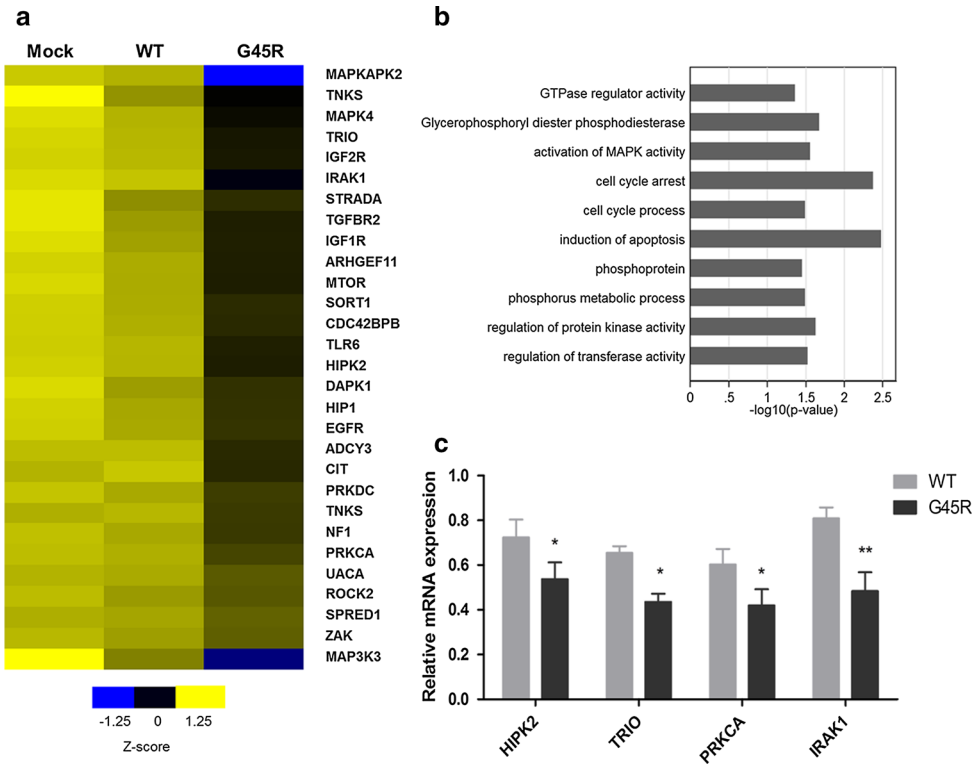

Fig. 6 Differentially downregulated genes and associated biological pathways. (a) The hierarchical clustering of differentially expressed genes within the categories of apoptosis and protein kinase is presented. The relative expression level for each group is shown as the Z-score (−SD to SD) color scale. (b) Biological pathways of downregulated genes expressed in both WT and G45R/NS1 infected cells, as generated by using DAVID. (c) Relative mRNA expression levels for the selected genes measured by qRT-PCR. Three biological replicates and two technical repeats were performed for each sample. The housekeeping gene β-actin was used as an internal control. Normalized data of each sample was analyzed in relation to that of mock-infected cells by the threshold cycle ($^{\Delta\Delta}$CT) method. Error bars indicate the standard error of the mean. $*P < 0.01$, $**P < 0.01$, $***P < 0.001$, compared to WT, as determined by the t-test

non-conserved amino acid, R45, on the NS1 protein in impeding the host antiviral response at multiple levels, particularly the immune response, and provide more insights into its functions to promote pathogenicity.

Acknowledgments This work was supported by the Thailand Research Fund (TRF) through the Royal Golden Jubilee PhD Program and Kasetsart University (Grant No. PHD/0153/2552). This work was also supported by the American Lebanese Syrian Associated Charities (ALSAC).

Compliance with ethical standards

Conflict of interest There is no conflict of interest.

Ethics standard All mouse experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of St. Jude's Children Research Hospital.

References

1. Akarsu H, Burmeister WP, Petosa C, Petit I, Muller CW, Ruigrok RW, Baudin F (2003) Crystal structure of the M1 protein-binding domain of the influenza A virus nuclear export protein (NEP/NS2). EMBO J 22:4646–4655
2. Alonso-Caplen FV, Nemeroff ME, Qiu Y, Krug RM (1992) Nucleocytoplasmic transport: the influenza virus NS1 protein regulates the transport of spliced NS2 mRNA and its precursor NS1 mRNA. Genes Dev 6:255–267
3. Aragon T, de la Luna S, Novoa I, Carrasco L, Ortin J, Nieto A (2000) Eukaryotic translation initiation factor 4GI is a cellular target for NS1 protein, a translational activator of influenza virus. Mol Cell Biol 20:6259–6268
4. Billharz R, Zeng H, Proll SC, Korth MJ, Lederer S, Albrecht R, Goodman AG, Rosenzweig E, Tumpey TM, García-Sastre A, Katze MG (2009) The NS1 protein of the 1918 pandemic influenza virus blocks host interferon and lipid metabolism pathways. J Virol 83:10557–10570
5. Brydon EW, Morris SJ, Sweet C (2005) Role of apoptosis and cytokines in influenza virus morbidity. FEMS Microbiol Rev 29:837–850
6. Buggele WA, Johnson KE, Horvath CM (2012) Influenza A virus infection of human respiratory cells induces primary microRNA expression. J Biol Chem 287:31027–31040
7. Burgui I, Aragon T, Ortin J, Nieto A (2003) PABP1 and eIF4GI associate with influenza virus NS1 protein in viral mRNA translation initiation complexes. J Gen Virol 84:3263–3274
8. Chen Z, Li Y, Krug RM (1999) Influenza A virus NS1 protein targets poly(A)-binding protein II of the cellular 3′-end processing machinery. EMBO J 18:2273–2283

9. Cheung CY, Poon LL, Lau AS, Luk W, Lau YL, Shortridge KF, Gordon S, Guan Y, Peiris JS (2002) Induction of proinflammatory cytokines in human macrophages by influenza A (H5N1) viruses: a mechanism for the unusual severity of human disease? Lancet 360:1831–1837
10. Claas EC, Osterhaus AD, van Beek R, De Jong JC, Rimmelzwaan GF, Senne DA, Krauss S, Shortridge KF, Webster RG (1998) Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet 351:472–477
11. Conenello GM, Zamarin D, Perrone LA, Tumpey T, Palese P (2007) A single mutation in the PB1-F2 of H5N1 (HK/97) and 1918 influenza A viruses contributes to increased virulence. PLoS Pathog 3:1414–1421
12. Conenello GM, Tisoncik JR, Rosenzweig E, Varga ZT, Palese P, Katze MG (2011) A single N66S mutation in the PB1-F2 protein of influenza A virus increases virulence by inhibiting the early interferon response in vivo. J Virol 85:652–662
13. D'Orazi G, Cecchinelli B, Bruno T, Manni I, Higashimoto Y, Saito S, Gostissa M, Coen S, Marchetti A, Del Sal G, Piaggio G, Fanciulli M, Appella E, Soddu S (2002) Homeodomain-interacting protein kinase-2 phosphorylates p53 at Ser 46 and mediates apoptosis. Nat Cell Biol 4:11–19
14. Davis WG, Bowzard JB, Sharma SD, Wiens ME, Ranjan P, Gangappa S, Stuchlik O, Pohl J, Donis RO, Katz JM, Cameron CE, Fujita T, Sambhara S (2012) The 3′ untranslated regions of influenza genomic sequences are 5′PPP-independent ligands for RIG-I. PLoS One 7:e32661
15. Ehrhardt C, Wolff T, Pleschka S, Planz O, Beermann W, Bode JG, Schmolke M, Ludwig S (2007) Influenza A virus NS1 protein activates the PI3K/Akt pathway to mediate antiapoptotic signaling responses. J Virol 81:3058–3067
16. Forbes NE, Ping J, Dankar SK, Jia JJ, Selman M, Keleta L, Zhou Y, Brown EG (2012) Multifunctional adaptive NS1 mutations are selected upon human influenza virus evolution in the mouse. PLoS One 7:e31839
17. Fraser C, Donnelly CA, Cauchemez S, Hanage WP, Van Kerkhove MD, Hollingsworth TD, Griffin J, Baggaley RF, Jenkins HE, Lyons EJ, Jombart T, Hinsley WR, Grassly NC, Balloux F, Ghani AC, Ferguson NM, Rambaut A, Pybus OG, Lopez-Gatell H, Alpuche-Aranda CM, Chapela IB, Zavala EP, Guevara DME, Checchi F, Garcia E, Hugonnet S, Roth C, Collaboration TWRPA (2009) Pandemic potential of a strain of influenza A (H1N1): early findings. Science 324:1557–1561
18. Gao Y, Zhang Y, Shinya K, Deng G, Jiang Y, Li Z, Guan Y, Tian G, Li Y, Shi J, Liu L, Zeng X, Bu Z, Xia X, Kawaoka Y, Chen H (2009) Identification of amino acids in HA and PB2 critical for the transmission of H5N1 avian influenza viruses in a mammalian host. PLoS Pathog 5:e1000709
19. Geiler J, Michaelis M, Sithisarn P, Cinatl J Jr (2011) Comparison of pro-inflammatory cytokine expression and cellular signal transduction in human macrophages infected with different influenza A viruses. Med Microbiol Immunol 200:53–60
20. Giraud S, Diaz-Latoud C, Hacot S, Textoris J, Bourette RP, Diaz JJ (2004) US11 of herpes simplex virus type 1 interacts with HIPK2 and antagonizes HIPK2-induced cell growth arrest. J Virol 78:2984–2993
21. Gottipati S, Rao NL, Fung-Leung W-P (2008) IRAK1: A critical signaling mediator of innate immunity. Cell Signal 20:269–276
22. Greenspan D, Palese P, Krystal M (1988) Two nuclear location signals in the influenza virus NS1 nonstructural protein. J Virol 62:3020–3026
23. Hoffmann E, Krauss S, Perez D, Webby R, Webster RG (2002) Eight-plasmid system for rapid generation of influenza virus vaccines. Vaccine 20:3165–3170
24. da Huang W, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 4:44–57
25. Imai M, Watanabe T, Hatta M, Das SC, Ozawa M, Shinya K, Zhong G, Hanson A, Katsura H, Watanabe S, Li C, Kawakami E, Yamada S, Kiso M, Suzuki Y, Maher EA, Neumann G, Kawaoka Y (2012) Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. Nature 486:420–428
26. Jackson D, Killip MJ, Galloway CS, Russell RJ, Randall RE (2010) Loss of function of the influenza A virus NS1 protein promotes apoptosis but this is not due to a failure to activate phosphatidylinositol 3-kinase (PI3K). Virology 396:94–105
27. Jiao P, Tian G, Li Y, Deng G, Jiang Y, Liu C, Liu W, Bu Z, Kawaoka Y, Chen H (2008) A single-amino-acid substitution in the NS1 protein changes the pathogenicity of H5N1 avian influenza viruses in mice. J Virol 82:1146–1154
28. Kaewborisuth C, Zanin M, Häcker H, Webby RJ, Lekcharoensuk P (2016) G45R mutation in the nonstructural protein 1 of A/Puerto Rico/8/1934 (H1N1) enhances viral replication independent of dsRNA-binding activity and type I interferon biology. Virol J 13:1–10
29. Kuchipudi SV, Tellabati M, Sebastian S, Londt BZ, Jansen C, Vervelde L, Brookes SM, Brown IH, Dunham SP, Chang KC (2014) Highly pathogenic avian influenza virus infection in chickens but not ducks is associated with elevated host immune and pro-inflammatory responses. Vet Res 45:118
30. Lalime EN, Pekosz A (2014) The R35 residue of the influenza A virus NS1 protein has minimal effects on nuclear localization but alters virus replication through disrupting protein dimerization. Virology 458–459:33–42
31. Lee N, Wong CK, Hui DS, Lee SK, Wong RY, Ngai KL, Chan MC, Chu YJ, Ho AW, Lui GC, Wong BC, Wong SH, Yip SP, Chan PK (2013) Role of human Toll-like receptors in naturally occurring influenza A infections. Influenza Other Respir Viruses 7:666–675
32. Lekcharoensuk P, Wiriyarat W, Petcharat N, Lekcharoensuk C, Auewarakul P, Richt JA (2012) Cloned cDNA of A/swine/Iowa/15/1930 internal genes as a candidate backbone for reverse genetics vaccine against influenza A viruses. Vaccine 30:1453–1459
33. Li S, Min JY, Krug RM, Sen GC (2006) Binding of the influenza A virus NS1 protein to PKR mediates the inhibition of its activation by either PACT or double-stranded RNA. Virology 349:13–21
34. Lin KM, Hu W, Troutman TD, Jennings M, Brewer T, Li X, Nanda S, Cohen P, Thomas JA, Pasare C (2014) IRAK-1 bypasses priming and directly links TLRs to rapid NLRP3 inflammasome activation. PNAS 111:775–780
35. Lipatov AS, Andreansky S, Webby RJ, Hulse DJ, Rehg JE, Krauss S, Perez DR, Doherty PC, Webster RG, Sangster MY (2005) Pathogenesis of Hong Kong H5N1 influenza virus NS gene reassortants in mice: the role of cytokines and B- and T-cell responses. J Gen Virol 86:1121–1130
36. Liu Q, Zhou B, Ma W, Bawa B, Ma J, Wang W, Lang Y, Lyoo Y, Halpin RA, Lin X, Stockwell TB, Webby R, Wentworth DE, Richt JA (2014) Analysis of recombinant H7N9 wild-type and mutant viruses in pigs shows that the Q226L mutation in HA is important for transmission. J Virol 88:8153–8165
37. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(−delta delta C(T)) method. Methods San Diego Calif 25:402–408
38. Long J-X, Peng D-X, Liu Y-L, Wu Y-T, Liu X-F (2008) Virulence of H5N1 avian influenza virus enhanced by a 15-nucleotide deletion in the viral nonstructural gene. Virus Genes 36:471–478

39. Melen K, Kinnunen L, Fagerlund R, Ikonen N, Twu KY, Krug RM, Julkunen I (2007) Nuclear and nucleolar targeting of influenza A virus NS1 protein: striking differences between different virus subtypes. J Virol 81:5995–6006
40. Mibayashi M, Martinez-Sobrido L, Loo YM, Cardenas WB, Gale M Jr, Garcia-Sastre A (2007) Inhibition of retinoic acid-inducible gene I-mediated induction of beta interferon by the NS1 protein of influenza A virus. J Virol 81:514–524
41. Min JY, Krug RM (2006) The primary function of RNA binding by the influenza A virus NS1 protein in infected cells: Inhibiting the 2′-5′ oligo (A) synthetase/RNase L pathway. PNAS 103:7100–7105
42. Min JY, Li S, Sen GC, Krug RM (2007) A site on the influenza A virus NS1 protein mediates both inhibition of PKR activation and temporal regulation of viral RNA synthesis. Virology 363:236–243
43. Mok CK, Lee HH, Lestra M, Nicholls JM, Chan MC, Sia SF, Zhu H, Poon LL, Guan Y, Peiris JS (2014) Amino acid substitutions in polymerase basic protein 2 gene contribute to the pathogenicity of the novel A/H7N9 influenza virus in mammalian hosts. J Virol 88:3568–3576
44. Nemeroff ME, Barabino SM, Li Y, Keller W, Krug RM (1998) Influenza virus NS1 protein interacts with the cellular 30 kDa subunit of CPSF and inhibits 3′end formation of cellular pre-mRNAs. Mol Cell 1:991–1000
45. Noah DL, Twu KY, Krug RM (2003) Cellular antiviral responses against influenza A virus are countered at the posttranscriptional level by the viral NS1A protein via its binding to a cellular protein required for the 3′ end processing of cellular pre-mRNAS. Virology 307:386–395
46. Opitz B, Rejaibi A, Dauber B, Eckhard J, Vinzing M, Schmeck B, Hippenstiel S, Suttorp N, Wolff T (2007) IFNbeta induction by influenza A virus is mediated by RIG-I which is regulated by the viral NS1 protein. Cell Microbiol 9:930–938
47. Patel JR, Vora KP, Tripathi S, Zeng H, Tumpey TM, Katz JM, Sambhara S, Gangappa S (2011) Infection of lung epithelial cells with pandemic 2009 A(H1N1) influenza viruses reveals isolate-specific differences in infectivity and host cellular responses. Viral Immunol 24:89–99
48. Peters GA, Khoo D, Mohr I, Sen GC (2002) Inhibition of PACT-mediated activation of PKR by the herpes simplex virus type 1 Us11 protein. J Virol 76:11054–11064
49. Pfeffer K (2003) Biological functions of tumor necrosis factor cytokines and their receptors. Cytokine Growth Factor Rev 14:185–191
50. Pichlmair A, Schulz O, Tan CP, Naslund TI, Liljestrom P, Weber F, Reis e Sousa C (2006) RIG-I-mediated antiviral responses to single-stranded RNA bearing 5′-phosphates. Science 314:997–1001
51. Puca R, Nardinocchi L, Sacchi A, Rechavi G, Givol D, D'Orazi G (2009) HIPK2 modulates p53 activity towards pro-apoptotic transcription. Mol Cancer 8:85
52. Qian XY, Alonso-Caplen F, Krug RM (1994) Two functional domains of the influenza virus NS1 protein are required for regulation of nuclear export of mRNA. J Virol 68:2433–2441
53. Qiu Y, Krug RM (1994) The influenza virus NS1 protein is a poly(A)-binding protein that inhibits nuclear export of mRNAs containing poly(A). J Virol 68:2425–2432
54. Ramírez-Martínez G, Cruz-Lagunas A, Jiménez-Alvarez L, Espinosa E, Ortíz-Quintero B, Santos-Mendoza T, Herrera MT, Canché-Pool E, Mendoza C, Bañales JL, García-Moreno SA, Morán J, Cabello C, Orozco L, Aguilar-Delfín I, Hidalgo-Miranda A, Romero S, Suratt BT, Selman M, Zúñiga J (2013) Seasonal and pandemic influenza H1N1 viruses induce differential expression of SOCS-1 and RIG-I genes and cytokine/chemokine production in macrophages. Cytokine 62:151–159
55. Reed LJ, Muench H (1938) A simple method of estimating fifty percent endpoints. Am J Epidemiol 27:493–497
56. Salvatore M, Basler CF, Parisien JP, Horvath CM, Bourmakina S, Zheng H, Muster T, Palese P, Garcia-Sastre A (2002) Effects of influenza A virus NS1 protein on protein expression: the NS1 protein enhances translation and is not required for shutoff of host protein synthesis. J Virol 76:1206–1212
57. Seo SH, Hoffmann E, Webster RG (2002) Lethal H5N1 influenza viruses escape host anti-viral cytokine responses. Nat Med 8:950–954
58. Steidle S, Martinez-Sobrido L, Mordstein M, Lienenklaus S, Garcia-Sastre A, Staheli P, Kochs G (2010) Glycine 184 in nonstructural protein NS1 determines the virulence of influenza A virus strain PR8 without affecting the host interferon response. J Virol 84:12761–12770
59. Terrier O, Textoris J, Carron C, Marcel V, Bourdon JC, Rosa-Calatrava M (2013) Host microRNA molecular signatures associated with human H1N1 and H3N2 influenza A viruses reveal an unanticipated antiviral activity for miR-146a. J Gen Virol 94:985–995
60. Twu KY, Noah DL, Rao P, Kuo RL, Krug RM (2006) The CPSF30 binding site on the NS1A protein of influenza A virus is a potential antiviral target. J Virol 80:3957–3965
61. Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M, Taira K, Akira S, Fujita T (2004) The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat Immunol 5:730–737
62. Yu X, Zhang X, Zhao B, Wang J, Zhu Z, Teng Z, Shao J, Shen J, Gao Y, Yuan Z, Wu F (2011) Intensive cytokine induction in pandemic H1N1 influenza virus infection accompanied by robust production of IL-10 and IL-6. PLoS One 6:e28680
63. Zhang C, Yang Y, Zhou X, Liu X, Song H, He Y, Huang P (2010) Highly pathogenic avian influenza A virus H5N1 NS1 protein induces caspase-dependent apoptosis in human alveolar basal epithelial cells. Virol J 7:51
64. Zhang Y, Zhang Q, Gao Y, He X, Kong H, Jiang Y, Guan Y, Xia X, Shu Y, Kawaoka Y, Bu Z, Chen H (2012) Key molecular factors in hemagglutinin and PB2 contribute to efficient transmission of the 2009 H1N1 pandemic influenza virus. J Virol 86:9666–9674
65. Zhirnov OP, Konakova TE, Wolff T, Klenk H-D (2002) NS1 protein of influenza A virus down-regulates apoptosis. J Virol 76:1617–1625
66. Zhu J, Mohan C (2010) Toll-like receptor signaling pathways—therapeutic opportunities. Mediators Inflamm 2010:781235