CrossMark

Pandemic H1N1 influenza A directly induces a robust and acute inflammatory gene signature in primary human bronchial epithelial cells downstream of membrane fusion

Stéphane G. Paquette [a,b], David Banner [a], Le Thi Bao Chi [c,d], Alberto J. León [a,e],
Luoling Xu [a], Longsi Ran [a], Stephen S.H. Huang [a,f], Amber Farooqui [a,e],
David J. Kelvin [a,b,e,f,g,h], Alyson A. Kelvin [g,*]

[a] Division of Experimental Therapeutics, Toronto General Hospital Research Institute, University Health Network, Toronto, Ontario, Canada
[b] Institute of Medical Science, Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada
[c] Department of Microbiology, Hue University of Medicine and Pharmacy, Thua Thien Hue, Vietnam
[d] Carlo Urbani Centre, Hue University of Medicine and Pharmacy, Thua Thien Hue, Vietnam
[e] International Institute of Infection and Immunity, Shantou University Medical College, Shantou, Guangdong, China
[f] Department of Immunology, Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada
[g] Immune Diagnostics & Research, Toronto, Ontario, Canada
[h] Sezione di Microbiologia Sperimentale e Clinica, Dipartimento di Scienze Biomediche, Universita' degli Studi di Sassari, Sassari, Italy

ARTICLE INFO

ABSTRACT

Pandemic H1N1 influenza A (H1N1pdm) elicits stronger pulmonary inflammation than previously circulating seasonal H1N1 influenza A (sH1N1), yet mechanisms of inflammatory activation in respiratory epithelial cells during H1N1pdm infection are unclear. We investigated host responses to H1N1pdm/sH1N1 infection and virus entry mechanisms in primary human bronchial epithelial cells *in vitro*. H1N1pdm infection rapidly initiated a robust inflammatory gene signature (3 h post-infection) not elicited by sH1N1 infection. Protein secretion inhibition had no effect on gene induction. Infection with membrane fusion deficient H1N1pdm failed to induce robust inflammatory gene expression which was rescued with restoration of fusion ability, suggesting H1N1pdm directly triggered the inflammatory signature downstream of membrane fusion. Investigation of intra-virion components revealed H1N1pdm viral RNA (vRNA) triggered a stronger inflammatory phenotype than sH1N1 vRNA. Thus, our study is first to report H1N1pdm induces greater inflammatory gene expression than sH1N1 *in vitro* due to direct virus–epithelial cell interaction.

Introduction

In 2009, novel variant pandemic H1N1 influenza A (H1N1pdm) emerged from Mexico and the southwestern United States (Perez-Padilla et al., 2009). The rapid spread of H1N1pdm during the first (Summer 2009) and second (Fall/Winter 2009/2010) pandemic waves (Helferty et al., 2010) led to replacement of the previous antigenically-related seasonal H1N1 influenza A (sH1N1) strains with the seasonal circulation of the H1N1pdm virus worldwide (Pica et al., 2012; Huang et al., 2013). While H1N1pdm infection often does not require hospitalization (Nicoll and Coulombier, 2009; Bautista et al., 2010; Gilsdorf and Poggensee, 2009), the clinical pathology as well as virus localization within the respiratory tract differs significantly from sH1N1 infection (Childs et al., 2009; Shieh et al., 2010; Mauad et al., 2010). Specifically, the H1N1pdm virus has been shown to bind both α2,6-linked sialic acid receptors and α2,3-linked sialic acid receptors which are located in the upper and lower respiratory tract, respectively (Childs et al., 2009) whereas sH1N1 binds preferentially to α2,6-linked sialyl sequences. It has been suggested that increased lower respiratory tract tropism by H1N1pdm may exacerbate disease (Childs et al., 2009; Shieh et al., 2010). Furthermore, severe cases of H1N1pdm infection are characterized clinically by rapidly progressing lower respiratory tract disease leading to multilobar pneumonia, the development of acute respiratory distress

* Correspondence to: Immune Diagnostics and Research, IDR, Toronto Medical Discovery Tower, 101 College Street, 3rd Floor, room 913, Toronto, Canada M5G 1L7. Fax: +1 416 581 7606.
  E-mail address: akelvin@jidc.org (A.A. Kelvin).

syndrome, and impaired lung function (Kumar et al., 2009; Uyeki et al., 2009; Napolitano et al., 2009; Hui et al., 2010). Strikingly, the immune response profile of human H1N1pdm infection differs from that of previously circulating sH1N1, where H1N1pdm infection is associated with stronger, sustained inflammatory responses and pathogenic immune dysregulation (Shieh et al., 2010; Lee et al., 2011a, 2011b). Animal models of H1N1pdm infection have similarly shown increased lung inflammation and pathogenicity compared to sH1N1 infection (Rowe et al., 2010; Itoh et al., 2009; Huang et al., 2011; Fang et al., 2012).

The respiratory epithelium is the primary site of influenza infection (Taubenberger and Morens 2008) with a pivotal role in establishing the inflammatory microenvironment to orchestrate broader pulmonary inflammation (Tam et al., 2011; Bals and Hiemstra 2004; Kato and Schleimer 2007; León et al., 2013; Sanders et al., 2011). Following influenza A infection, epithelial cells release cytokines such as interleukin-6 (IL-6), chemokine (C-X-C motif) ligand 8 (CXCL8/IL-8), and chemokine (C-X-C motif) ligand 10 (CXCL10/IP-10) (León et al., 2013; Sanders et al., 2011), resulting in localized inflammatory activation of surrounding tissues (Teijaro et al., 2011), the recruitment of innate immune cells (Kato and Schleimer 2007; León et al., 2013; Sanders et al., 2011; Teijaro et al., 2011), and further amplification of the inflammatory response (León et al., 2013). Importantly, the respiratory epithelial cell response to influenza A infection is strain-dependent, and robust inflammatory microenvironments induced by certain virus strains, most notably avian H5N1 influenza A, may play critical roles in exacerbating pulmonary inflammation (Chan et al., 2005; Hui et al., 2011). In the case of H1N1pdm infection, the contribution of respiratory epithelial cells to pulmonary inflammation remains unclear (Chan et al., 2010; Patel et al., 2011; Mukherjee et al., 2011; Zeng et al., 2011).

Influenza A virus entry into the cell is mediated by host cell endocytosis (Lakadamyali et al., 2004). Virions are internalized and trafficked along the endocytic pathway until they reach acidified late endosomes. pH-dependent changes following virion internalization allows for conformational changes in the viral hemagglutinin (HA) to mediate fusion of viral and endosomal membranes and allow the release of internal virion contents to the cell cytosol (Lakadamyali et al., 2004; Skehel and Wiley, 2000). Importantly, the viral entry process can initiate widespread changes in host cell gene expression. First, influenza A viruses activate host cell signaling cascades to promote endocytosis and direct late endosomal sorting (Eierhoff et al., 2010; Ehrhardt et al., 2006; Sieczkarski et al., 2003; Marchant et al., 2010). In addition, the virus inadvertently activates various innate pathogen sensing pathways throughout the entry process (Le Goffic et al., 2007; Guillot et al., 2005; Sharma et al., 2011; Rehwinkel et al., 2010; Zhang et al., 2011; Shapira et al., 2009; Xu et al., 2012). Critically, many host-pathogen interactions which occur during entry produce strain-specific changes in host gene expression (Zheng and Bevilacqua, 2004; Nallagatla et al., 2011; Liu et al., 2010; Davis et al., 2012; Saito et al., 2008) and inflammatory responses elicited by viral entry may differ between influenza A viruses.

Early epithelial cell responses in the lower airways may play critical roles in exacerbating pulmonary inflammation during H1N1pdm infection (Childs et al., 2009; Shieh et al., 2010; Tam et al., 2011; Bals and Hiemstra, 2004; Kato and Schleimer 2007; León et al., 2013; Sanders et al., 2011), yet these responses are difficult to detect using *in vivo* infection models. To overcome this limitation, we analyzed host responses to H1N1pdm and sH1N1 infection in normal human bronchial epithelial cells (NHBECs) *in vitro*, primary human cells derived from the tracheal/bronchial epithelia. Here, we report H1N1pdm induced a robust, acute inflammatory gene signature distinct from sH1N1, featuring neutrophil-recruiting chemokines (chemokine (C-X-C motif) ligand 1 (CXCL1), CXCL8) and Th1/Th17-response associated cytokines (CXCL10, interleukin-36γ

(IL-36γ)). Subsequent mechanistic studies revealed H1N1pdm directly elicited inflammatory gene expression during viral entry after membrane fusion and implicated host vRNA sensors in the distinct response to H1N1pdm. Thus, our study revealed a robust and acute inflammatory gene signature to H1N1pdm infection in NHBECs *in vitro* with important implications for H1N1pdm pathogenesis.

Results

### H1N1pdm and sH1N1 infect NHBECs in vitro with similar replication kinetics

Given the critical role lower airway epithelial cells may play in H1N1pdm-induced inflammation (Shieh et al., 2010; Taubenberger and Morens 2008; Tam et al., 2011; Bals and Hiemstra 2004; Kato and Schleimer 2007; León et al., 2013; Sanders et al., 2011), we first investigated sH1N1 and H1N1pdm infection kinetics in normal human bronchial epithelial cells (NHBECs) which are primary human cells derived from the tracheal/bronchial epithelia. Specifically we investigated differences in viral burden and host cell damage, factors which could contribute to increased H1N1pdm pathogenesis.

NHBECs were infected with 0.9 MOI of either H1N1pdm virus A/Mexico/4108/2009 (H1N1) or sH1N1 virus A/Brisbane/59/2007 (H1N1). Changes in vRNA levels were used to monitor viral replication. Total cellular RNA was collected at the specified time-points and the level of vRNA gene segment 7 was quantified by qRT-PCR, normalized to 1.0×10^5 copies of housekeeping gene GAPDH mRNA. Low initial levels of vRNA were detected in NHBECs at the earliest time-point of 2 h post-infection (pi). vRNA levels for both viruses increased approximately 10-fold at 6 h pi and again at 12 h pi before plateauing between 12–24 h pi (Fig. 1A). Peak vRNA levels for both viruses were detected at 12 h pi, with approximately 8.0×10^5 vRNA copies detected during H1N1pdm infection and 9.0×10^5 vRNA copies during sH1N1 infection. Notably, no significant differences in vRNA levels were detected between H1N1pdm and sH1N1 viruses at any of the time-points tested.

NHBEC cell viability remained at 100% following infection with either sH1N1 or H1N1pdm viruses until 24 h pi at which point the cell viability was reduced to 60-80% (Fig. 1B). A trend of greater viability reduction during sH1N1 infection was consistently observed, although differences were not statistically significant. Typical host cell viability after sH1N1 infection was 60–70% relative to uninfected controls, compared to 80% viability in host cells infected with H1N1pdm in the 24 h to 72 h time period. As an assay control for all viability experiments, cells were treated with 5 μM Cytochalasin B. Cytochalasin B treatment consistently caused a reduction in cell viability to approximately 70% relative to untreated cells. In summary, we found NHBECs were similarly permissive to both sH1N1 and H1N1pdm replication *in vitro*.

H1N1pdm elicits robust acute inflammatory signature in NHBECs distinct from sH1N1

Severe H1N1pdm infection is associated with excessive pulmonary inflammation (Shieh et al., 2010; Lee et al., 2011b; Bermejo-Martin et al., 2009) but the role of the respiratory epithelium in modulating these inflammatory responses remains unclear (Chan et al., 2010; Patel et al., 2011; Mukherjee et al., 2011; Zeng et al., 2011). To define the genetic signature of human respiratory epithelial cells during H1N1pdm infection and the differences between H1N1pdm and sH1N1 infections, we infected NHBECs with 0.9 MOI of either H1N1pdm virus A/Mexico/4108/2009 (H1N1) or sH1N1 virus A/Brisbane/59/2007 (H1N1). Total
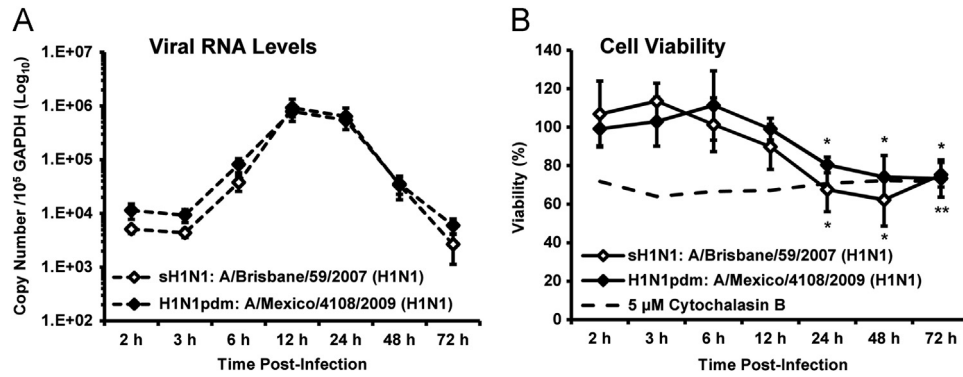
Fig. 1. H1N1pdm and sH1N1 viruses infected NHBECs *in vitro* have similar replication kinetics. NHBECs were infected with 0.9 MOI H1N1pdm virus A/Mexico/4108/2009 (H1N1) or sH1N1 virus A/Brisbane/59/2007 (H1N1). For vRNA measurement, total cellular RNA was collected and viral mRNA segment 7 copy numbers were quantified by qRT-PCR, normalized to host cell GAPDH mRNA copy numbers. $n=3$ for each time-point. Error bars denote ± 1 standard deviation (A). For cell viability, cells were incubated with 500 µg/mL MTT, lysed, and absorbance was measured at 570 nm–670 nm. Viability of infected cells was reported as percentage of mock infected cell viability. Cytochalasin B was included as a control for detection of reduced cell viability. Each condition was assayed in triplicate. Error bars denote ± 1 standard deviation. p-value $< 0.05 =$*, p-value $< 0.01 =$**. Results are representative of three independent experiments (B).
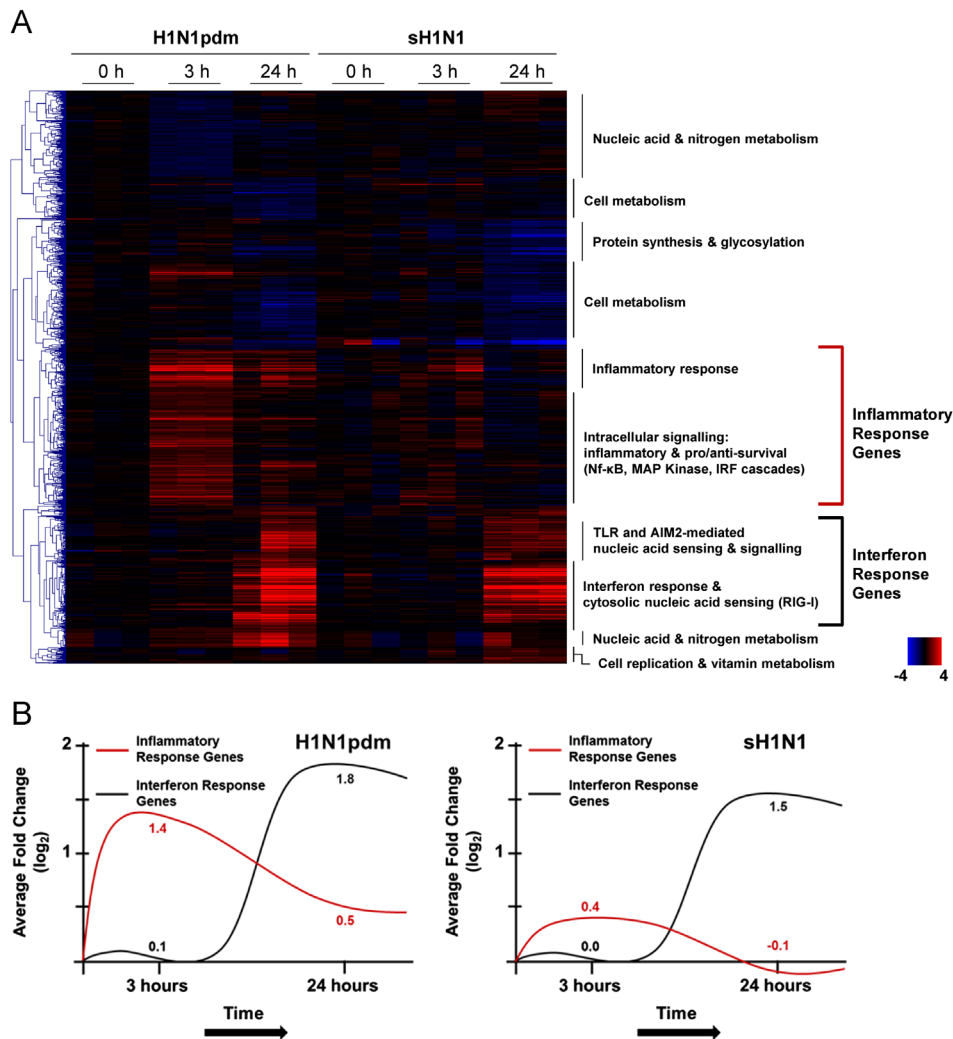
Fig. 2. H1N1pdm triggers stronger acute inflammatory gene expression in NHBECs than sH1N1. Global gene expression profiling of host responses in NHBECs at 3 h and 24 h pi with either 0.9 MOI A/Mexico/4108/2009 (H1N1) or A/Brisbane/59/2007 (H1N1). $n=3$ for each time-point. Probes for significantly differentially regulated genes (p-value < 0.05, fold change ≥ 1.5 fold; total number of genes: 1031) were subjected to one-way hierarchical clustering analysis (Pearson's correlation) and clusters were functionally annotated using DAVID Bioinformatics Resource v6.7. The most prominent functional groups are indicated for each cluster (A). Stylized model of immune activation during H1N1pdm or sH1N1 infection of NHBECs. Approximated gene expression profiles for the inflammatory response (red) and interferon response (black) clusters. Numeric values denote average fold changes for each cluster at 3 h pi and 24 h pi (B).

cellular RNA was then collected at 3 h and 24 h pi for global gene expression analysis by microarray.

Global clustering analysis of mRNA expression data by Pearson's correlation was performed for all significantly differentially regulated genes (p-value < 0.05, fold change $\geq$ |1.5 fold|; total number of genes: 1031) using MultiExperiment Viewer v4.6.2. Two clearly delineated clusters of upregulated genes were identified (Fig. 2A). Detailed summaries of the most prominent functional groups in each of the two clusters are included in Fig. S1. The first cluster was upregulated at 3 h pi only during H1N1pdm infection and was strongly enriched in genes encoding inflammatory cytokines such as CXCL8, IL-6, and IL-36γ, as well as inflammatory signaling intermediates of the Nf-κB (NFKB1, NFKB2, NFKBIA, RIPK2), Jak-STAT (SOCS1, SOCS2, STAM), and TLR (TICAM1) signaling pathways. As a whole, the inflammatory response gene cluster was upregulated approximately 2.6 fold (1.4 log$_2$) by H1N1pdm infection compared to only 1.3 fold (0.4 log$_2$) by sH1N1 infection at 3 h pi (Fig. 2B). Interestingly, while upregulation of inflammatory signaling intermediates had largely dissipated by 24 h pi, expression of many inflammatory cytokine genes, including CXCL8, IL-6, and IL-36γ, remained elevated. The second cluster of upregulated genes was induced by both H1N1pdm and sH1N1 infection at 24 h pi and was heavily enriched in antiviral and interferon response genes which included MX1, ISG15, and IFI44. The magnitude of upregulation for genes in this cluster was similar for both viruses, with average increases in interferon response gene expression of approximately 3.5-fold at 24 h post-H1N1pdm infection and 2.8-fold at 24 h post-sH1N1 infection. Thus, global analysis revealed a much stronger acute inflammatory response to H1N1pdm infection than sH1N1 infection, which was gradually replaced by a dominant interferon response by 24 h pi.

To complement our hierarchical clustering analysis, we also performed gene ontology classification of all significantly upregulated genes (p-value < 0.05, fold change $\geq$ 1.5 fold) at 3 h and 24 h pi with H1N1pdm or sH1N1. Gene Ontology (GO) Biological Process, PANTHER Biological Process, and Kyoto encyclopedia of genes and genomes (KEGG) Pathway databases were used for classification. Similar to above, H1N1pdm infection led to the significant upregulation of more immune response genes (34) than sH1N1 (1) at 3 h pi (GO0006955) and, in particular, inflammatory response genes (H1N1pdm: 25, sH1N1: 3) (GO0006954) (Table S1). Moreover, genes associated with inflammatory signaling cascades such as NF-κB (BP00115) and Jak-STAT (hsa04630) were significantly upregulated in greater numbers by H1N1pdm compared to sH1N1. We also found that H1N1pdm led to the rapid upregulation of genes involved in apoptosis (H1N1pdm: 32, sH1N1: 4) (GO0006915) which were predominantly intermediates of the TNF-α/NF-κB signaling cascade. The individual genes significantly upregulated by H1N1pdm at 3 h pi and included in Table S2 and classifications are listed in Table S2.

Detailed analysis of H1N1pdm-induced inflammatory gene expression at 3 h pi revealed prominent upregulation of neutrophil recruiting chemokines including CXCL1 and CXCL8, as well as Th1/Th17 activating cytokines including IL-36γ and CXCL10. We also found H1N1pdm triggered broad upregulation of pleiotropic inflammatory cytokines and inflammatory signaling cascade intermediates (Fig. 3A). CXCL1, CXCL8, CXCL10, IL-6, and IL-36γ mRNA expression levels were next quantified by qRT-PCR to validate our global analyses. H1N1pdm infection consistently induced greater upregulation of all cytokines measured at both 3 and 24 h pi (Fig. 3B). IL-36γ and CXCL8 were again the most strongly upregulated genes at 3 h pi with H1N1pdm, with increases in expression of 206-fold and 147-fold over baseline, respectively.

To determine whether increased inflammatory gene expression led to secretion of inflammatory cytokines in infected NHBECs, cell culture supernatants were collected at 3 h pi and protein levels of CXCL1 and CXCL8 were measured by ELISA. Consistent with our mRNA expression data, H1N1pdm infected cells secreted significantly higher CXCL1 (116 pg/mL) and CXCL8 (33 pg/mL) than sH1N1 infected cells (CXCL1: 52 pg/mL, CXCL8: 14 pg/mL) (Fig. 3C).

To determine the effect of infection dose on the specific host response to H1N1pdm, we also investigated inflammatory cytokine mRNA expression at 3 h pi with increasing doses of either sH1N1 or H1N1pdm. We found H1N1pdm infection consistently generated stronger inflammatory signatures than sH1N1 infection at all doses tested. For both viruses, the magnitude of the host inflammatory response was dose-dependent (Fig. S2). Taken together, our findings provided evidence at the mRNA and protein level that H1N1pdm infection produced a robust, acute inflammatory gene signature in NHBECs, distinct from sH1N1 infection.

*H1N1pdm directly induces acute inflammatory gene signature*

Our initial *in vitro* studies indicated H1N1pdm induced stronger acute inflammatory gene expression than sH1N1 in NHBECs. We next investigated whether inflammatory gene expression was directly triggered by host interaction with the virus or if the secretion of secondary inflammatory mediators mediated this response. To determine if protein secretion was required for the induction of an acute inflammatory signature, we pretreated NHBECs with 10 µg/mL brefeldin A to block protein secretion and secondary mediator signaling, then subsequently infected with 0.9 MOI of either A/Mexico/4108/2009 (H1N1) or A/Brisbane/59/2007 (H1N1).

To evaluate the effect of protein secretion blockade on the acute inflammatory response, mRNA levels for CXCL1, CXCL8, CXCL10, and IL-36γ were measured, at 3 h pi. H1N1pdm infection resulted in strong, equivalent upregulation of all four genes with and without brefeldin A treatment. In untreated and brefeldin A pretreated cells respectively, H1N1pdm generated 41-fold and 59-fold increases in IL-36γ expression, 26-fold and 35-fold increases in CXCL8 expression, 11-fold and 13-fold increases in CXCL10 expression, and equivalent 12-fold increases in CXCL1 expression. Critically, differences in inflammatory gene expression between H1N1pdm and sH1N1 infections were statistically significant in both untreated and brefeldin A treated NHBECs. To verify that brefeldin A treatment blocked protein secretion during infection, we quantified CXCL1 and CXCL8 levels in cell culture supernatants collected at 3 h pi. As expected, pretreatment with brefeldin A blocked secretion such that CXCL1 and CXCL8 levels were equivalent to levels detected from mock infected untreated NHBECs (Fig. 4B). We also confirmed equivalent amounts of sH1N1 and H1N1pdm virus infected NHBEC by qRT-PCR quantification of intracellular vRNA at 3 h pi (Fig. 4C). Overall, these results suggested the acute inflammatory gene signature H1N1pdm infection was independent of secondary inflammatory mediators and in direct response to host interaction with the virus.

The acute inflammatory gene signature of H1N1pdm infection is initiated downstream of the virus-host membrane fusion event

Our above data suggested H1N1pdm virus infection directly caused stronger acute inflammatory gene expression in NHBECs than sH1N1. We next sought to investigate the molecular events required during H1N1pdm infection to elicit the more robust inflammatory response. We first investigated potential host virus interactions at the cell surface and during H1N1pdm internalization. For this we treated H1N1pdm and sH1N1 viruses with formalin, a cross-linking agent which prevents membrane fusion but allows for virus internalization (Budimir et al., 2012). Thus,
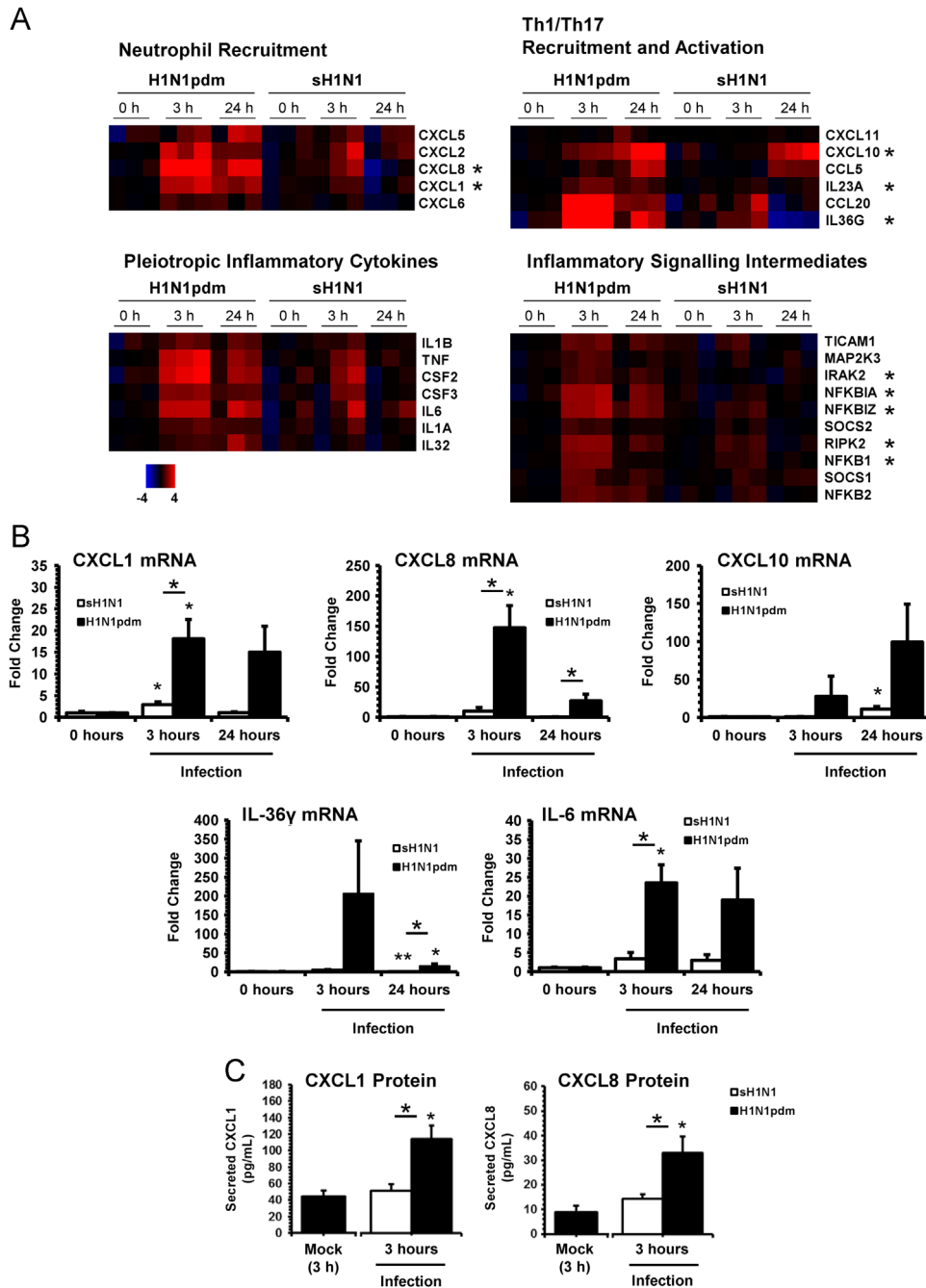
Fig. 3. The acute inflammatory gene signature of H1N1pdm is enriched in neutrophil-recruiting and Th1/Th17-response associated genes. Expression data of inflammatory response genes significantly upregulated at 3 h pi with H1N1pdm, profiled over the course of infection. Inflammatory response genes were classified by immune function: Neutrophil Recruitment, Th1/Th17 Recruitment, Pleiotropic Inflammatory Cytokines, and Inflammatory Signaling Intermediates. Significant differences between H1N1pdm and sH1N1 expression at 3 h pi are denoted by an asterisk (p-value < 0.05, fold-change $\geq 1.5 = ^{*}$) (A). Gene expression data validation by qRT-PCR for CXCL1, CXCL8, CXCL10, and IL-36γ mRNA, expression data reported as fold change over expression at baseline (0 h). Average values ($n = 3$) are reported with $+1$ standard deviation indicated by vertical error bars (B). Protein secretion levels for CXCL1 and CXCL8 in infected cell supernatants as measured by ELISA. Average values are reported, with $n = 3$ for each group. Vertical error bars indicate $+1$ standard deviation (C). Asterisks above data bars indicate significant difference relative to mock controls (3 h). Asterisks over horizontal lines indicate significant difference between H1N1pdm and sH1N1 infections. p-value $< 0.05 = ^{*}$, p-value $< 0.01 = ^{**}$.

formalin inactivated viruses do not have access to the intracellular compartment. NHBECs were stimulated with 0.9 MOI equivalent of formalin-inactivated A/Mexico/4108/2009 (H1N1) or A/Brisbane/59/2009 (H1N1) and inflammatory gene expression was measured at 3 h post-stimulation. Both sH1N1 and H1N1pdm formalin-inactivated viruses produced modest and equivalent increases in CXCL1, CXCL8, CXCL10, and IL-36γ mRNA levels, with 2-5 fold increases in expression detected for all four genes (Fig. 5).

Importantly, no significant differences in expression were detected between sH1N1 and H1N1pdm stimulation for any of the genes tested. This result suggested host-pathogen interactions upstream of membrane fusion were not responsible for the acute inflammatory gene signature specific to H1N1pdm infection.

We next investigated the roles of virus-host membrane fusion and the internal virion release to the cytosol in the host response to H1N1pdm and establishment of an inflammatory gene
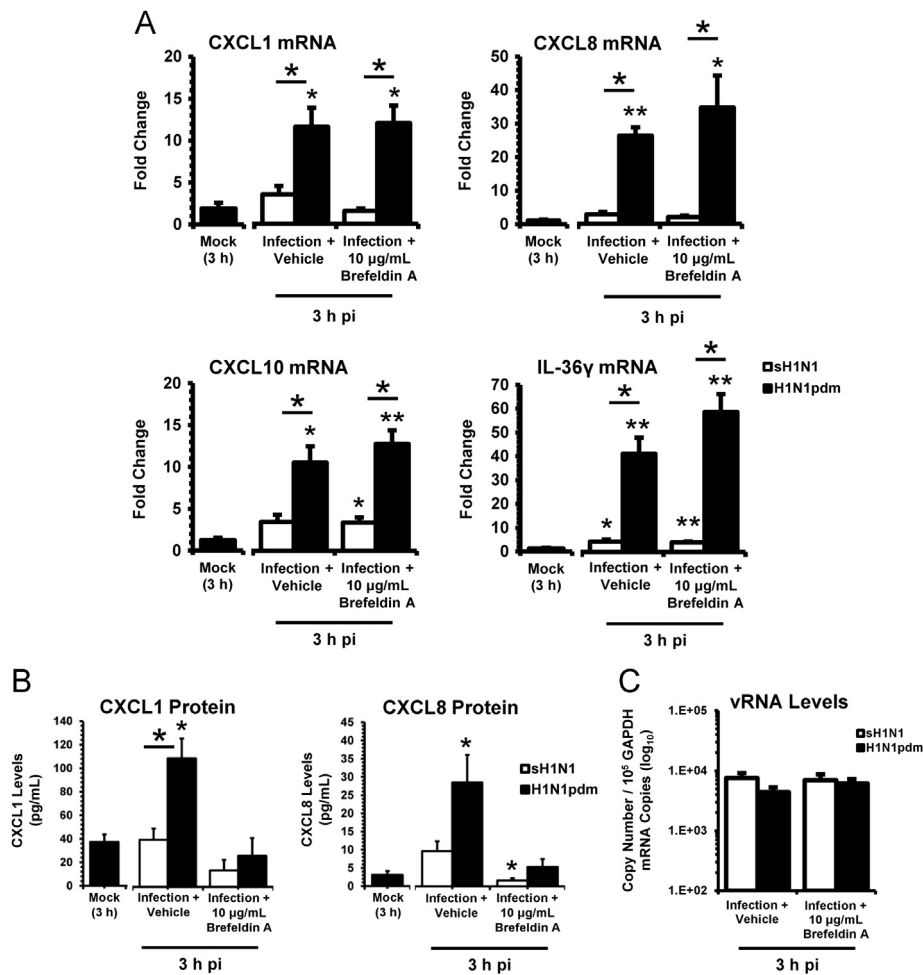
Fig. 4. H1N1pdm virus directly induces stronger acute inflammatory gene expression in NHBECs than sH1N1. NHBECs were pretreated with 10 μg/mL brefeldin A for 1 h prior to infection with either 0.9 MOI A/Mexico/4108/2009 (H1N1) or A/Brisbane/59/2007 (H1N1). Total cellular RNA was collected at 0 h (baseline) and 3 h pi and mRNA levels of inflammatory cytokines CXCL1, CXCL8, CXCL10, and IL-36γ were quantified by qRT-PCR, reported as fold changes over expression in baseline (0 h) controls. (A). To verify inhibition of inflammatory cytokine secretion, cell supernatants were collected at 3 h pi for secreted cytokine protein quantification by ELISA. (B). To verify infection with equivalent quantities of sH1N1 or H1N1pdm virus, viral mRNA segment 7 copy numbers were quantified by qRT-PCR, normalized to host cell GAPDH mRNA (C). $n=3$ for each group. Error bars denote $+1$ standard deviation. p-value $< 0.05 =$*, p-value $< 0.01 =$**. Asterisks above data bars denote significant difference relative to mock controls (3 h). Asterisks over horizontal lines indicate significant difference between H1N1pdm and sH1N1 infections.

signature. For these experiments, viruses were heat-inactivated such that the viruses were capable of viral entry by membrane fusion but incapable of replication (Budimir et al., 2012; Wang et al., 2008). NHBECs were stimulated with 0.9 MOI equivalent of heat-inactivated A/Mexico/4108/2009 (H1N1) or A/Brisbane/59/2009 (H1N1) and gene induction was determined as above. Stimulation with heat-inactivated H1N1pdm resulted in significantly increased expression of all measured genes. Fold changes in expression over baseline were 43-fold for IL-36γ, 23-fold for CXCL8, 10-fold for CXCL1, and 4-fold for CXCL10 at 3 h post stimulation. In contrast, heat-inactivated sH1N1 only produced modest upregulation of those same genes (IL-36γ: 8-fold, CXCL8: 4-fold, CXCL1: 3-fold, CXCL10: 1.5-fold) (Fig. 6A). Critically, H1N1pdm elicited significantly higher expression of CXCL1, CXCL8, and IL-36γ than sH1N1. Thus, H1N1pdm was able to induce stronger inflammatory gene expression independent of viral replication when membrane fusion and cytosolic access were allowed. As control, we verified that equivalent amounts of sH1N1 and H1N1pdm virus were internalized upon NHBEC stimulation by qRT-PCR quantification of intracellular vRNA at 3 h pi (Fig. 6B). Similar results were obtained with β-propiolactone inactivation of H1N1pdm and sH1N1 influenza A viruses, which also prevents viral replication but allows

membrane fusion (Budimir et al., 2012) (Fig. S3). Taken together, our results suggested that the robust, acute inflammatory gene signature of H1N1pdm infection occurred during viral entry downstream of membrane fusion and was independent of viral replication.

H1N1pdm vRNA is a more potent inducer of inflammatory gene expression than sH1N1 vRNA

Inactivated virus stimulation of NHBECs revealed membrane fusion was required to initiate the acute inflammatory gene signature of H1N1pdm. Influenza vRNA is released to the cell cytosol by membrane fusion and is an important trigger of host innate responses (Le Goffic et al., 2007; Guillot et al., 2005; Sharma et al., 2011; Rehwinkel et al., 2010; Zhang et al., 2011). Since innate sensing of viral RNA is partially sequence dependent (Zheng and Bevilacqua 2004; Nallagatla et al., 2011; Davis et al., 2012; Saito et al., 2008), we hypothesized that inherent differences in vRNA motifs between H1N1pdm and sH1N1 viruses were responsible for the stronger inflammatory gene expression observed early during H1N1pdm infection.

NHBECs were then transfected with a final concentration of 100, 300, or 500 ng/mL vRNA extracted from A/Mexico/4108/2009 (H1N1) or A/Brisbane/59/2007 (H1N1) egg allantoic stocks. For

Formalin-Inactivated Virus Stimulations
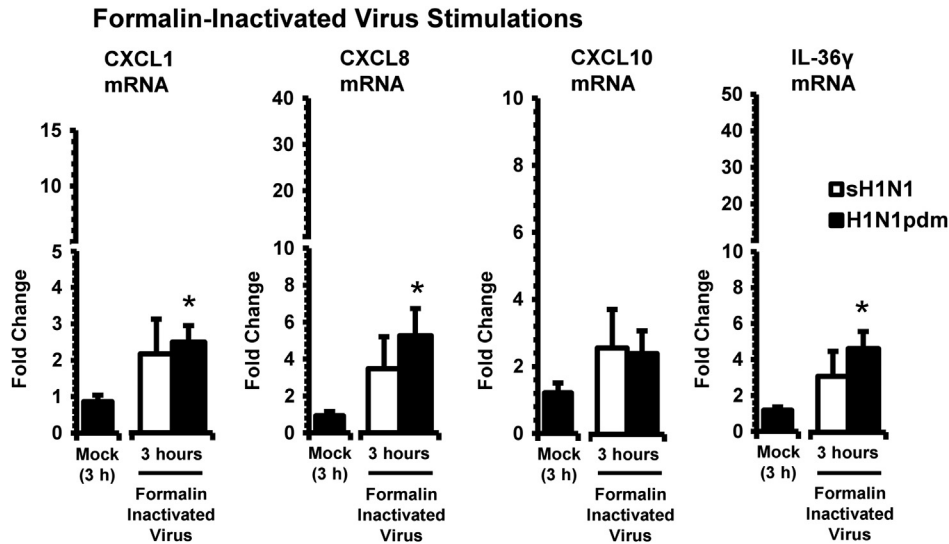
Fig. 5. H1N1pdm and sH1N1 viruses unable to fuse with host cell membrane have equivalent modest upregulation of inflammatory gene expression. NHBECs were stimulated with 0.9 MOI equivalent formalin-inactivated H1N1pdm virus A/Mexico/4108/2009 (H1N1) or formalin-inactivated sH1N1 virus A/Brisbane/59/2007 (H1N1). Total cellular RNA was collected at baseline (0 h) and 3 h post-stimulation, and CXCL1, CXCL8, CXCL10, and IL-36γ mRNA expression was measured by qRT-PCR. Results are expressed as fold changes over expression in baseline (0 h) controls. Reported values for each time point are the average of three samples ($n=3$) with $+1$ standard deviation indicated by vertical error bars. p-value $< 0.05 =$*. Asterisks above data bars indicate significant difference relative to mock controls (3 h). Asterisks over horizontal lines indicate significant difference between inactivated H1N1pdm and sH1N1 stimulations.
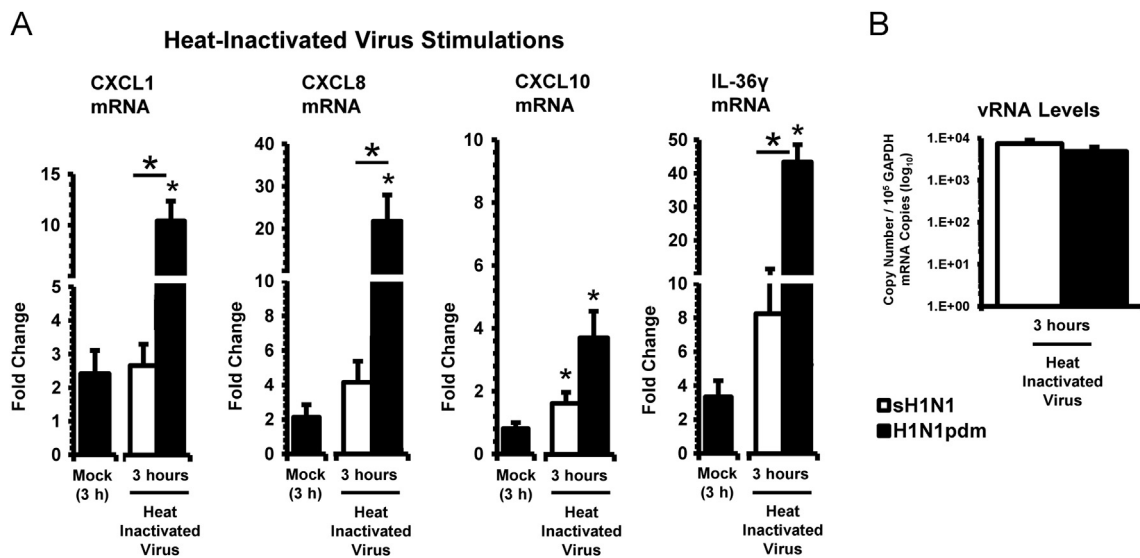
Fig. 6. Infection with membrane fusion competent H1N1pdm inactivated virus leads to significant inflammatory gene induction. NHBECs were stimulated with 0.9 MOI equivalent heat-inactivated H1N1pdm virus A/Mexico/4108/2009 (H1N1) or heat-inactivated sH1N1 virus A/Brisbane/59/2007 (H1N1). Total cellular RNA was extracted and expression of CXCL1, CXCL8, CXCL10, and IL-36γ mRNA was measured by qRT-PCR. Results are shown as fold changes over expression in baseline (0 h) controls (A). To verify stimulation with equivalent quantities of sH1N1 or H1N1pdm virus, viral mRNA segment 7 copy numbers were quantified by qRT-PCR, normalized to host cell GAPDH mRNA (B). Average values ($n=3$) reported with $+1$ standard deviation indicated by vertical error bars. p-value $< 0.05 =$*. Asterisks above data bars indicate significant difference relative to mock controls (3 h). Asterisks over horizontal lines indicate significant difference between inactivated H1N1pdm and sH1N1 stimulations.
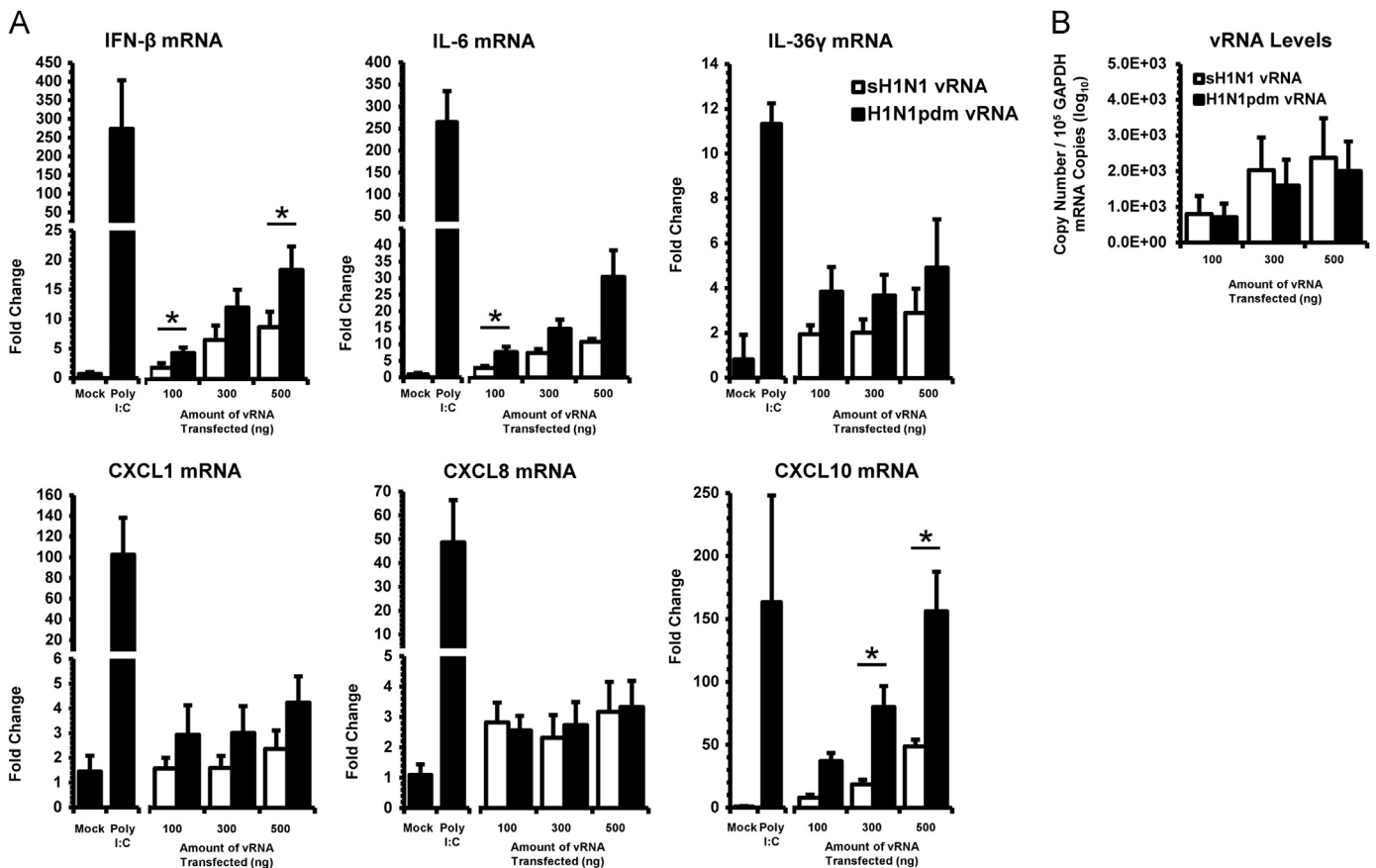
mock stimulations, cells were transfected with RNA extracted from uninfected egg allantoic fluid (500 ng). Cells were also transfected with 1 μg/mL Poly I:C as an assay control. Cytosolic stimulation by influenza vRNA transfection led to the specific upregulation of CXCL10, IL-6, and IFN-β mRNA in a dose-dependent manner. Importantly, H1N1pdm vRNA consistently induced greater upregulation of these genes than sH1N1 vRNA; at the highest vRNA dose of 500 ng/mL, changes in CXCL10, IL-6, and IFN-β expression over baseline were 156-fold, 31-fold, and 18-fold after H1N1pdm transfection, but only 49-fold, 11-fold, and 9-fold after sH1N1 transfection (Fig. 7A). As control, qRT-PCR showed equal amounts of sH1N1 and H1N1pdm RNA had gained entry into the cells (Fig. 7B). These results suggested a partial role for cytosolic vRNA

sensors in generating the stronger inflammatory response to H1N1pdm infection.

Discussion

Severe cases of H1N1pdm infection are characterized by epithelial cell activation/damage, pulmonary inflammation and impaired lung function (Shieh et al., 2010; Mauad et al., 2010; Kumar et al., 2009; Uyeki et al., 2009; Napolitano et al., 2009) and the mechanisms of epithelial cell activation leading to severe disease remain unknown. Here we have shown that H1N1pdm virus infection of human primary bronchial epithelial cells *in vitro* directly induced a

Fig. 7. Intracellular stimulation with H1N1pdm vRNA induces a partial inflammatory gene program. NHBECs were transfected with 100, 300, or 500 ng purified vRNA of A/Mexico/4108/2009 (H1N1) or A/Brisbane/59/2007 (H1N1) origin, or RNA from uninfected allantoic fluid (500 ng) as mock. Total cellular RNA was collected for gene expression analysis and confirmation of transfection at baseline (0 h) and 3 h post-stimulation. CXCL1, CXCL8, CXCL10, IL-6, IL-36γ, and IFN-β mRNA expression was measured by qRT-PCR, reported as fold changes in expression over baseline (0 h) controls (A). Viral mRNA segment 7 copy numbers were quantified by qRT-PCR for confirmation of transfection (B). Reported values for each time point are the average of three samples ($n=3$) with $+1$ standard deviation indicated by vertical error bars. p-value $< 0.05 = *$. Asterisks over horizontal lines indicate significant difference between H1N1pdm- or sH1N1-derived vRNA transfections.

robust, acute inflammatory gene signature enriched in neutrophil recruiting chemokines (CXCL1, CXCL8) and Th1/Th17-response associated cytokines (CXCL10, IL-36γ) which was not seen with sH1N1 infection. Furthermore, we also showed that the H1N1pdm inflammatory phenotype was downstream of virus-host membrane fusion and that H1N1pdm vRNA transfection yielded stronger inflammatory gene up-regulation of a subset of genes *in vitro*. Our findings reveal an acute inflammatory gene signature specific to H1N1pdm virus entry, implicating the respiratory epithelium in exacerbation of pulmonary inflammatory during severe H1N1pdm infection. These findings are significant to the investigation of the immediate early events of influenza virus infection as studying the events following virus infection remains a significant challenge *in vivo*. Our results utilizing primary human epithelial cells and the identification of the direct H1N1pdm induction of an inflammatory gene signature may be extrapolated to the initial epithelial cellular reactions upon H1N1pdm infection in the human trachea and bronchi. Furthermore, the dependence on virus-host cell fusion suggests viral entry and membrane fusion to be possible therapeutic targets for the treatment of severe H1N1pdm infection.

Respiratory epithelial cells are both the first and most frequently exposed cells to the influenza virus during infection, suggesting a central role in establishing broader pulmonary immune responses (Tam et al., 2011; Bals and Hiemstra 2004; Kato and Schleimer 2007; León et al., 2013; Sanders et al., 2011). The gene signature we detected following H1N1pdm infection *in vitro* was highlighted by strong upregulation of genes involved in neutrophil recruitment (CXCL1, CXCL8) and Th1/Th17-type

responses (CXCL10, IL-36γ) consistent with results of human and animal H1N1pdm investigations (Mauad et al., 2010; Itoh et al., 2009; León et al., 2013; Bermejo-Martin et al., 2009; Paquette et al., 2012; Cho et al., 2012; Frisullo et al., 2011; Marcelin et al., 2011; Huang et al., 2012) and was not seen in sH1N1 infection. León et al. recently profiled the infectome of H1N1pdm infection in ferrets and identified a cluster of inflammatory genes upregulated in the lungs of ferrets at Day 5 pi (León et al., 2013). Some of the most strongly upregulated genes *in vivo*, CXCL8, CXCL10, and IL-6, were genes we also found to be preferentially induced during H1N1pdm entry *in vitro*. Similarly, inflammatory cytokines CXCL1 and chemokine (C-X-C motif) ligand 2 (CXCL2), along with CXCL10 and IL-6, were strongly upregulated both during our *in vitro* study as well in the lungs of mice infected with H1N1pdm (Day 3 pi) (Itoh et al., 2009; Paquette et al., 2012). Excessive Th1/Tc1-type responses and neutrophil infiltration have been heavily implicated in H1N1pdm human pathogenesis (Mauad et al., 2010; Bermejo-Martin et al., 2009; Cho et al., 2012; Frisullo et al., 2011; Marcelin et al., 2011; Huang et al., 2012). Furthermore, cytokine profiling studies in patients hospitalized with severe H1N1pdm infection have found CXCL8, IL-6, and Th1/Th17 associated cytokines to be most frequently associated with severe disease (Bermejo-Martin et al., 2009; Paquette et al., 2012). Pulmonary responses to influenza infection *in vivo* are mediated by multiple immune and non-immune cell populations (Taubenberger and Morens 2008; León et al., 2013; Teijaro et al., 2011) and the precise contributions of individual cell populations to H1N1pdm-induced pulmonary inflammation remain to be defined. Accordingly, cytokines

detected *in vivo* may not be entirely generated from the respiratory epithelial cell population. However, close agreement between our *in vitro* gene signature and previous *in vivo* response profiles suggests the epithelial response to H1N1pdm entry to be detectable *in vivo*. Furthermore, this finding supports the possibility that respiratory epithelial cells may play a role orchestrating inflammatory events following initial infection through the release of inflammatory mediators but may not be the only source of cytokine production as other cell types begin to respond to the epithelial cell signals. As well, identification of the direct acute inflammatory phenotype *in vivo* may reflect continued and expanding infection of new epithelial cells as the virus moves throughout the respiratory tract thereby leading to a more sustained inflammatory signature. This, together with the putative role of epithelial cells in initiating pulmonary inflammation (Tam et al., 2011; Bals and Hiemstra 2004; Kato and Schleimer 2007; León et al., 2013; Sanders et al., 2011), may recommend the inflammatory cytokines identified here as suitable therapeutic targets for immunomodulation and/or biomarkers of severe H1N1pdm infection.

Previous *in vitro* studies have reported H1N1pdm infection caused either similar or weaker immune responses than seasonal influenza A, which is in disagreement with our findings that showed the direct induction of an acute inflammatory signature (Chan et al., 2010; Patel et al., 2011; Mukherjee et al., 2011; Zeng et al., 2011). Importantly, our study focused on the immediate responses to viral entry in contrast to previous studies that investigated host responses in cells with established infection. Cytosolic sensing of replication intermediates and accumulation of viral protein both trigger innate responses in respiratory epithelial cells (Ehrhardt et al., 2006; Marjuki et al., 2006; Ichinohe et al., 2010) and may have masked the direct acute host responses during previous studies. In support of this hypothesis, global gene expression analysis in our study suggested sH1N1 and H1N1pdm generated similar interferon-dominated responses at the later stages of infection at the time point of 24 h pi. Prior studies which reported attenuated inflammatory responses to H1N1pdm infection were performed in immortalized cell lines (Patel et al., 2011; Mukherjee et al., 2011; Zeng et al., 2011) which may not be representative of normal human respiratory epithelial cells. Previous *in vitro* studies in primary respiratory epithelial cells have not reported attenuation (Chan et al., 2010). These differences in experimental sampling and design may explain why we are first to identify H1N1pdm as a stronger inducer of inflammation than sH1N1 *in vitro*.

Here we found only membrane fusion competent viruses were able to elicit the acute inflammatory gene signature of H1N1pdm, a finding which prompted us to investigate internal virion contents as potential triggers of the inflammatory signature. We found H1N1pdm vRNA to be a more potent inducer of inflammatory gene expression than sH1N1. Host RNA sensors have been implicated in initiating inflammatory gene expression during influenza infection (Takeuchi and Akira 2010). In the context of our study, the potential contributions of TLR3 and PKR are of particular interest. Both receptors are constitutively expressed (García et al., 2006; Akira and Takeda 2004) and modulate expression of many inflammatory genes we found to be strongly upregulated during H1N1pdm entry *in vitro*, including CXCL1 (Matsukura et al., 2006; Yamashita et al., 2005), CXCL8 (Le Goffic et al., 2007; Matsukura et al., 2006; Gern et al., 2003), IL-6 (Le Goffic et al., 2007; Matsukura et al., 2006), and IL-36γ (Chustz et al., 2011). Although we found H1N1pdm vRNA initiated stronger innate host responses than sH1N1 vRNA, vRNA stimulation did not completely mimic the broader response profile observed during infection. Rather, we found that vRNA stimulation predominantly led to strong expression of interferon response factor (IRF)-associated cytokines IL-6, IFN-β, and CXCL10 (Taniguchi et al.,

2001). Our stimulation experiments investigated the effect of vRNA on the host response in isolation. Since influenza viruses activate multiple signaling cascades during viral attachment and entry which modulate the host immune response (Eierhoff et al., 2010; Ehrhardt et al., 2006; Sieczkarski et al., 2003; Marchant et al., 2010) it is possible that vRNA sensing is only a part of the stimulatory event which leads to the identified inflammatory phenotype in respiratory epithelial cells identified in this study. This hypothesis is supported by previous reports that simultaneous activation of innate RNA sensors in the presence of other proinflammatory stimuli results in synergistic amplification of inflammatory cytokine expression (Chustz et al., 2011). Thus, H1N1pdm vRNA may have a broader effect on inflammatory gene expression in the context of live virus infection. Importantly, further studies are required to conclusively determine whether vRNA sensing pathways alone mediate the acute inflammatory gene signature of H1N1pdm or if additional proinflammatory signals, such as host sensing of membrane fusion, are required. Our data did not allow us to determine if the fusion event itself directly influenced the epithelial inflammatory response. Given previous reports of fusion inducing innate cellular responses during viral infection (Holm et al., 2012), the direct impact of membrane fusion on the acute H1N1pdm signature should be the focus of future studies.

Our results imply that the H1N1pdm virus inherently possesses a molecular motif capable of instigating an inflammatory phenotype which is not contained within the seasonal H1N1 virus and may serve as a therapeutic target. Current antivirals have been designed to target various viral proteins and stages of the viral life cycle of which includes two main classes: M2 inhibitors and neuraminidase (NA) inhibitors (Hayden 2013). Due to the rise of antiviral resistance (Hayden 2013; Samson et al., 2013; Whitley et al., 2013; Govorkova et al., 2013), targeting events other than HA-receptor cleavage (NA activity) and virion acidification (M2 activity) may serve as an alternate therapeutic strategy. Amantadine antiviral drugs target the M2 protein of the influenza virus by inhibiting ion channel activity which ultimately leads to disruption of viral entry and escape. Importantly, the majority of current circulating seasonal influenza A strains and all of influenza B strains are amantadine resistant and having alternative antiviral drugs and drug targets is increasingly important. Our results advocate further investigation of targeting viral proteins involved in viral entry and membrane fusion, including the HA protein. Currently, clinical studies are investigating the efficacy of inhibiting HA conformational changes which are required for fusion (Hayden 2013). Thus, our finding that membrane-fusion competent inactivated-H1N1pdm directly induced an inflammatory gene signature may influence the development of influenza therapeutics and prophylactics. Furthermore, our results suggested the existence of specific host-pathogen interaction which led to the direct induction of the inflammatory phenotype and should be the focus of future studies. Identification of host cell intracellular receptors involved in this interaction may uncover additional possible targets for the development of future immunotherapeutics. While our data suggests induction occurred during viral entry downstream of fusion, our findings do not exclude the possibility that downstream viral replication events may also influence epithelial responses in a strain-specific manner and serve as potential therapeutic targets. The strain-specific effects of replication should also be investigated in the future.

Here we found H1N1pdm infection of NHBECs produced a robust, acute inflammatory gene signature consistent with the clinical manifestations of severe H1N1pdm infection. Furthermore, we established that acute inflammatory gene expression detected during H1N1pdm infection was in direct response to viral entry at a stage downstream of membrane fusion, and mediated in part by

host sensing of vRNA. Thus, our study implicates respiratory epithelial cells in H1N1pdm pathogenesis and suggests a critical role for host sensing of viral entry in amplifying pulmonary inflammation. Early signaling events during viral entry, as well as the membrane fusion event itself, may serve as valuable therapeutic targets for the treatment of severe H1N1pdm infection.

Materials and methods

Cells

Primary human bronchial epithelial cells (NHBECs) (undifferentiated) have previously been used to study host responses to viral infection *in vitro* (Escaffre et al., 2013; Suksatu et al., 2009; Dinwiddie and Harrod, 2008). Cells were purchased from Lonza (Basel, Switzerland) and subcultured as per manufacturer recommended conditions in bronchial epithelial cell growth media (BEGM; Lonza, Basel, Switzerland), which consisted of bronchial epithelial cells basal medium supplemented with bovine pituitary extract, hydrocortisone, human epidermal growth factor, epinephrine, transferrin, insulin, retinoic acid, triiodothyronine, genatmicin sulfate, and amphotericin-B. Subculturing conditions were selected to prevent NHBEC differentiation. Undifferentiated NHBECs were grown submerged in BEGM and passaged once confluence reached 80%. Low passage cells (Huang et al., 2013; Nicoll and Coulombier, 2009; Bautista et al., 2010; Gilsdorf and Poggensee, 2009; Childs et al., 2009; Shieh et al., 2010; Mauad et al., 2010) were used for all experiments.

Viral Infections

All infection experiments were conducted with A/Mexico/4108/2009 (H1N1) as representative H1N1pdm strain and A/Brisbane/59/2007 (H1N1) as representative sH1N1 strain. All viruses were provided by the centers for disease control and prevention ([CDC], Atlanta, GA, USA). Both viruses were propagated in embryonated eggs for no more than one passage. All viral stocks were tested for the presence of endotoxin and confirmed to be endotoxin free (< 0.1 EU/mL). Viral stocks were stored in liquid nitrogen and thawed prior to use. Tissue culture infectious dose 50 ($TCID_{50}$) titers for viral stocks were determined based on infection of Madin–Darby Canine Kidney (MDCK) Cells. Briefly, viral stocks were serially diluted ($0.5 \log_{10}$) in quadruplicate over MDCK cells, cultured at 2.0×10^4 cells/well in 96-well plates. Cells were incubated at 37 °C and 5% CO_2 for 2 h to allow for virus adsorption. Virus was then removed and replaced with fresh assay media. Cells were incubated for 6 days at 37 °C and 5% CO_2. Supernatants were then tested for the presence of virus by hemagglutination assay using 0.5% (v/v) turkey red blood cells (LAMPIRE Biological Laboratories, Pipersville, PA, USA). $TCID_{50}$ values were calculated by the Reed and Muench method. Viral RNA levels for each stock were measured by quantitative real-time reverse-transcription PCR (qRT-PCR) and normalized to stock $TCID_{50}$/mL; all viral stocks contained equivalent vRNA to $TCID_{50}$ levels when compared using one-way ANOVA ($\alpha = 0.05$).

All infections in NHBECs were performed in 6-well or 96-well plates at 0.9 multiplicity of infection (MOI), unless otherwise specified. 6-well plates were seeded with 5.0×10^4 cells/well and 96-well plates were seeded with 1.0×10^4 cells/well. Cells were seeded and incubated overnight prior to infection. Immediately prior to infection, cell culture media was supplemented with 1 µg/mL final concentration TPCK-Trpysin. Virus was then added and cells were incubated at 37 °C and 5% CO_2 for 2 h. After adsorption, media was replaced with fresh growth media, and cells were incubated at 37 °C and 5% CO_2 until sample collection. For protein

secretion inhibition experiments, cells were pretreated with 10 µg/mL brefeldin A (Sigma-Aldrich, St. Louis, MO, USA) for 1 h prior to addition of virus; sample collection was performed at 3 h pi.

Virus inactivation

Influenza viruses were formalin inactivated by incubating the viruses in the presence of 0.02% w/v formalin for 18 h at 37 °C. Viruses were then dialyzed against PBS (1:500) three times to remove residual formalin, twice for 2 h at room temperature and once overnight at 4 °C. Influenza viruses were inactivated by β-propiolactone by incubating the viruses in the presence of 0.094% w/v β-propiolactone for 18 h at 4 °C. Viruses were then incubated for 2 h at 37 °C to hydrolyze residual β-propiolactone. Influenza viruses were heat inactivated by incubating viruses for 30 min at 56 °C. Heat inactivation under these conditions yielded a replication-incompetent virus that retained the ability to enter host cells by membrane fusion. Virus inactivation was verified by inocuity testing in embryonated eggs. To ensure that inactivation did not alter the ability of the virus to bind host cell receptors or cause a significant reduction in viral titer, virus particle quantification was also performed following inactivation by hemagglutination assay.

vRNA stimulation

Genomic vRNA was purified from egg allantoic fluid stocks of A/Mexico/4108/2009 (H1N1) or A/Brisbane/59/2007 (H1N1) by RNEasy Mini Kit (Qiagen, Hilden, Germany). Transfections of NHBECs were performed in 6-well plates. Six-well plates were seeded with 2.0×10^5 cells/well and incubated overnight prior to transfection. NHBECs were then transfected with specified doses of vRNA using Lipofectamine 2000 as per manufacturer's protocol (RNA (µg):Lipofectamine 2000 (µL) ratio of 1:2). For all vRNA experiments, Poly I:C was included as assay control. Cells were incubated for 3 h at 37 °C and 5% CO_2, followed by sample collection.

Recombinant HA stimulation

Purified recombinant HA protein was obtained from Sino Biological Inc. (Beijing, China). Protein was expressed from DNA sequences encoding the extracellular regions of A/Brisbane/59/2007 (H1N1) or A/California/07/2009 (H1N1) HA. Stimulations of NHBECs were performed in 6-well plates. 6-well plates were seeded with 5.0×10^4 cells/well and incubated overnight prior to stimulation. Recombinant HA was diluted in sterile PBS and added to cell supernatant (1 mL BEGM) at 1, 2, or 5 µg/mL. Cells were incubated at 37 °C and 5% CO_2 for 3 h until sample collection.

Cell viability

Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT; Sigma-Aldrich, St. Louis, MO, USA) assay. Cells treated with 5 µM Cytochalasin B (Sigma-Aldrich, St. Louis, MO, USA) were included as assay controls for all experiments. At designated time points following infection, 20 µL of freshly prepared 5 mg/mL MTT were added to infected and mock infected cells in 96-well plates (final MTT concentration: 500 µg/mL). Cells were incubated for 2 h at 37 °C and 5% CO_2, after which 25 µL 0.1 N hydrochloric acid/10% sodium dodecyl sulfate was added to all wells. Cells were incubated and shook at room temperature for 1 h. Absorbance at 570 nm and 670 nm was then measured on plate reader, where OD570–OD670 corresponded to cell viability. Cell viability of infected cells was expressed as percentage of mock infected control cells. Wells containing media only were similarly processed and measured as reference blanks.

Host gene expression and viral load measurement by qRT-PCR

Total cellular RNA was collected and purified by RNEasy Mini Kit (Qiagen, Hilden, Germany), as per manufacturer's instructions. Purified RNA was then reverse transcribed using ImProm-II Reverse Transcription System (Promega, Madison, WI, USA). qRT-PCR was performed using the ABI-Prism 7900HT Sequence Detection Systems (Applied Biosystems, Foster City, CA, USA). Raw data was collected with Applied Biosystems Sequence Detection Systems Version 2.3 software. Each reaction well contained 4 μL of 0.625 ng/uL cDNA, 0.5 μL each of forward and reverse primers (final concentration of 200 nM), and 5 μL SYBR Green Master Mix, for a total reaction volume of 10 uL. Each sample was run in quadruplicate. Host gene expression was normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene, and quantified by the relative standard curve method. Viral load was quantified by the absolute standard curve method, normalized to GAPDH housekeeping. Primer sequences are listed in Table S3.

Microarray gene expression analysis

Total cellular RNA from infected cells was collected and purified by RNEasy Mini Kit (Qiagen, Hilden, Germany), as per manufacturer's instructions. Purified RNA was amplified with Illumina TotalPrep RNA Amplification Kit (Ambion, Austin, TX, USA) as per manufacturer's instructions. cRNA (1.5 μg) was labeled and hybridized to HumanHT-12 v4 Expression BeadChip (Illumina, San Diego, CA, USA) and scanned on Illumina BeadStation 500GX. Raw data was collected with Illumina GenomeStudio V2010.3 software. The data sets were pre-processed with quantile normalization, variance stabilization, and $\log_2$ transformation. Hierarchical clustering by Pearson's correlation and heatmap representations were generated using MultiExperiment Viewer v4.6.2 (Dana–Farber Cancer Institute, Boston, MA, USA). DAVID Bioinformatics Resource v6.7 (Huang da et al., 2008, 2009) was also used to perform functional classification of differentially expressed genes.

Microarray data accession number

Microarray expression data are MIAME compliant and are available publicly in the MIAME-compliant Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo/) under accession number GSE48575.

Cytokine protein measurement

Supernatants were collected from infected cells, supplemented with complete EDTA-free protease inhibitor cocktail (Roche Applied Science, Laval, Canada) to prevent protein degradation, and stored at $-20\,^{\circ}\text{C}$ prior to analysis. Inflammatory cytokine protein levels were quantified by enzyme-linked immunosorbent assay (ELISA) as per manufacturer's instructions. ELISA kits for CXCL1 and CXCL8 were obtained from Aviscera Bioscience (Santa Clara, CA, USA).

Statistical methods

For all two group comparisons, the Student's t-test ($\alpha = 0.05$) was used to ascertain significance. All Student's t-tests assumed two-tailed distributions and two-sample unequal variances.

Acknowledgments

Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2013.09.022.

References

Akira, S., Takeda, K., 2004. Toll-like receptor signaling. Nature Reviews Immunology 4, 499–511.

Bals, R., Hiemstra, P., 2004. Innate immunity in the lung: how epithelial cells fight against respiratory pathogens. European Respiratory Journal 23, 327–333.

Bautista, E., Chotpitayasunondh, T, Gao, Z., Harper, S.A., Shaw, M., Uyeki, T.M., Zaki, S.R., Hayden, F.G., Hui, D.S., Kettner, J.D., Kumar, A., Lim, M., Shindo, N., Penn, C., Nicholson, K.G., 2010. Clinical aspects of pandemic 2009 influenza A (H1N1) virus infection. New England Journal of Medicine 362, 1708–1719.

Bermejo-Martin, J.F., Ortiz de Lejarazu, R., Pumarola, T., Rello, J., Almansa, R., Ramírez, P., Martin-Loeches, I., Varillas, D., Gallegos, M.C., Serón, C., Micheloud, D., Gomez, J.M., Tenorio-Abreu, A., Ramos, M.J., Molina, M.L., Huidobro, S., Sanchez, E., Górdon, M., Fernández, V., del Castillo, A., Marcos, M.Á., Villanueva, B., López, C.J., Rodríguez-Domínguez, M., Galan, J.-C., Cantón, R., Lietor, A., Rojo, S., Eiros, J.M., Hinojosa, C., Gonzalez, I., Torner, N., Banner, D., Leon, A., Cuesta, P., Rowe, T., Kelvin, D.J., 2009. Th1 and Th17 hypercytokinemia as early host response signature in severe pandemic influenza. Critical Care 13, R201.

Budimir, N., Huckriede, A., Meijerhof, T., Boon, L., Gostick, E., Price, D.A., Wilschut, J., de Haan, A., 2012. Induction of heterosubtypic cross-protection against influenza by a whole inactivated virus vaccine: the role of viral membrane fusion activity. PLoS One 7, e30898.

Chan, M., Cheung, C., Chui, W., Tsao, S., Nicholls, J., Chan, Y., Chan, R., Long, H., Poon, L., Guan, Y., Peiris, J., 2005. Proinflammatory cytokine responses induced by influenza A (H5N1) viruses in primary human alveolar and bronchial epithelial cells. Respiratory Research 6, 135.

Chan, M.C., Chan, R.W., Yu, W.C., Ho, CC, Yuen, K.M., Fong, J.H., Tang, LL, Lai, W.W., Lo, A.C., Chui, W., Sihoe, A.D., Kwong, D.L., Wong, D.S., Tsao, G.S., Poon, L.L., Guan, Y, Nicholls, J.M., Peiris, J.S., 2010. Tropism and innate host responses of the 2009 pandemic H1N1 influenza virus in *ex vivo* and *in vitro* cultures of human conjunctiva and respiratory tract. American Journal of Pathology 176, 1828–1840.

Childs, R.A., Palma, A.S., Wharton, S., Matrosovich, T., Liu, Y., Chai, W., Campanero-Rhodes, M.A., Zhang, Y., Eickmann, M., Kiso, M., Hay, A., Matrosovich, M., Feizi, T., 2009. Receptor-binding specificity of pandemic influenza A (H1N1) 2009 virus determined by carbohydrate microarray. Nature Biotechnol 27, 797–799.

Cho, J.L., Roche, M.I., Sandall, B., Brass, A.L., Seed, B., Xavier, R.J., Medoff, BD., 2012. Enhanced Tim3 activity improves survival after influenza infection. Journal of Immunology 189, 2879–2889.

Chustz, R.T., Nagarkar, D.R., Poposki, J.A., Favoreto Jr, S., Avila, P.C., Schleimer, R.P., Kato, A., 2011. Regulation and function of the IL-1 family cytokine IL-1F9 in human bronchial epithelial cells. American Journal of Respiratory Cell and Molecular Biology 45, 145.

Davis, W.G., Bowzard, J.B., Sharma, S.D., Wiens, M.E., Ranjan, P., Gangappa, S., Stuchlik, O., Pohl, J., Donis, R.O., Katz, J.M., Cameron, C.E., 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.

Dinwiddie, D.L., Harrod, K.S., 2008. Human metapneumovirus inhibits IFN-α signaling through inhibition of STAT1 phosphorylation. American Journal of Respiratory Cell and Molecular Biology 38, 661–670.

Ehrhardt, C., Marjuki, H., Wolff, T., Nürnberg, B., Planz, O., Pleschka, S., Ludwig, S., 2006. Bivalent role of the phosphatidylinositol-3-kinase (PI3K) during influenza virus infection and host cell defence. Cellular Microbiology 8, 1336–1348.

Eierhoff, T., Hrincius, E.R., Rescher, U., Ludwig, S., Ehrhardt, C., 2010. The epidermal growth factor receptor (EGFR) promotes uptake of influenza A viruses (IAV) into host cells. PLoS Pathogen 6, e1001099.

Escaffre, O., Borisevich, V., Carmical, J.R., Prusak, D., Prescott, J., Feldmann, H., Rockx, B., 2013. Henipavirus pathogenesis in human respiratory epithelial cells. Journal of Virology 87, 3284–3294.

Fang, Y., Banner, D., Kelvin, A.A., Huang, S.S., Paige, C.J., Corfe, S.A., Kane, K.P., Bleackley, RC, Rowe, T., Leon, AJ, Kelvin, D.J., 2012. Seasonal H1N1 influenza virus infection induces cross-protective pandemic H1N1 virus immunity through a CD8-independent, B cell-dependent mechanism. Journal of Virology 86, 2229–2238.

Frisullo, G., Iorio, R., Plantone, D., Nociti, V., Patanella, A.K., Marti, A., Palermo, C., Valentini, P., Mariotti, P., Batocchi, A.P., 2011. Involvement of type I immune responses in swine-origin H1N1 influenza virus infection. Human Immunology 72, 632–635.

García, M., Gil, J., Ventoso, I., Guerra, S., Domingo, E., Rivas, C., Esteban, M., 2006. Impact of protein kinase PKR in cell biology: from antiviral to antiproliferative action. Microbiology and Molecular Biology Reviews 70, 1032–1060.

Gern, J.E., French, D.A., Grindle, K.A., Brockman-Schneider, R.A., Konno, S.-I., Busse, W.W., 2003. Double-stranded RNA induces the synthesis of specific chemokines by bronchial epithelial cells. American Journal of Respiratory Cell and Molecular Biology 28, 731–737.

Gilsdorf, A., Poggensee, G., 2009. Influenza A (H1N1) v in Germany: the first 10,000 cases. Euro Surveillance, 14.

Govorkova, E.A., Baranovich, T., Seiler, P., Armstrong, J., Burnham, A., Guan, Y., Peiris, M., Webby, R.J., Webster, R.G., 2013. Antiviral resistance among highly pathogenic influenza A (H5N1) viruses isolated worldwide in 2002-2012 shows need for continued monitoring. Antiviral Research 98, 297–304.

Guillot, L., Le Goffic, R., Bloch, S., Escriou, N., Akira, S., Chignard, M., Si-Tahar, M., 2005. Involvement of toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus. Journal of Biological Chemistry 280, 5571–5580.

Hayden, F.G., 2013. Newer influenza antivirals, biotherapeutics and combinations. Influenza and Other Respiratory Viruses 7, 63–75.

Helferty, M., Vachon, J., Tarasuk, J., Rodin, R., Spika, J., Pelletier, L., 2010. Incidence of hospital admissions and severe outcomes during the first and second waves of pandemic (H1N1) 2009. Canadian Medical Association Journal 182, 1981–1987.

Holm, C.K., Jensen, S.B., Jakobsen, M.R., Cheshenko, N., Horan, A., Moeller, H.B., Gonzalez-Dosal, R., Rasmussen, S.B., Christensen, M.H., Yarovinsky, T.O., Rixon, F.J., Herold, B.C., Fitzgerald, K.A., Paludan, S.R., 2012. Virus-cell fusion as a trigger of innate immunity dependent on the adaptor STING. Nature Immunology 13, 737–743.

Huang, S.S., Banner, D., Fang, Y., Ng, D.C., Kanagasabai, T., Kelvin, D.J., Kelvin, A.A., 2011. Comparative analyses of pandemic H1N1 and seasonal H1N1, H3N2, and influenza B infections depict distinct clinical pictures in ferrets. PLoS One 6, e27512.

Huang, S.S., Banner, D., Degousee, N., Leon, A.J., Xu, L., Paquette, S.G., Kanagasabai, T., Fang, Y., Rubino, S., Rubin, B., Kelvin, D.J., Kelvin, A.A., 2012. Differential pathological and immune responses in newly weaned ferrets are associated with a mild clinical outcome of pandemic 2009 H1N1 infection. Journal of Virology 86, 13187–13201.

Huang, S.S., Lin, Z., Banner, D., León, A.J., Paquette, S.G., Rubin, B., Rubino, S., Guan, Y., Kelvin, D.J., Kelvin, A.A., 2013. Immunity toward H1N1 influenza hemagglutinin of historical and contemporary strains suggests protection and vaccine failure. Scientific Reports 3, 1698.

Huang da, W., Sherman, B.T., Lempicki, R.A., 2008. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nature Protocol 4, 44–57.

Huang da, W., Sherman, B.T., Lempicki, R.A., 2009. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Research 37, 1–13.

Hui, D.S., Lee, N, Chan, P.K., 2010. Clinical management of pandemic 2009 influenza A (H1N1) infection. Chest 137, 916–925.

Hui, KP, Lee, SM, Cheung, C-y, Mao, H., Lai, A.K., Chan, R.W., Chan, M.C., Tu, W., Guan, Y., Lau, Y.-L., Peiris, J., 2011. H5N1 influenza virus–induced mediators upregulate RIG-I in uninfected cells by paracrine effects contributing to amplified cytokine cascades. Journal of Infectious Diseases 204, 1866–1878.

Ichinohe, T., Pang, I.K., Iwasaki, A., 2010. Influenza virus activates inflammasomes via its intracellular M2 ion channel. Nature Immunology 11, 404–410.

Itoh, Y., Shinya, K., Kiso, M., Watanabe, T., Sakoda, Y, Hatta, M., Muramoto, Y., Tamura, D., Sakai-Tagawa, Y., Noda, T., Sakabe, S., Imai, M., Hatta, Y., Watanabe, S., Li, C., Kakugawa, S., Ito, M., Takano, R., Iwatsuki-Horimoto, K., Shimojima, M., Horimoto, T., Goto, H., Takahashi, K., Makino, A., Ishigaki, H., Nakayama, M., Okamatsu, M., Takahashi, K., Warshauer, D., Shult, P.A., Saito, R., Suzuki, H., Furuta, Y., Yamashita, M., Mitamura, K., Nakano, K., Nakamura, M., Brockman-Schneider, R., Mitamura, H., Yamazaki, M., Sugaya, N., Suresh, M., Ozawa, M., Neumann, G., Gern, J., Kida, H., Ogasawara, K, Kawaoka, Y., 2009. In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses. Nature 460, 1021–1025.

Kato, A., Schleimer, R.P., 2007. Beyond inflammation: airway epithelial cells are at the interface of innate and adaptive immunity. Current Opinion Immunology 19, 711–720.

Kumar, A., Zarychanski, R., Pinto, R., Cook, DJ, Marshall, J., Lacroix, J., Stelfox, T., Bagshaw, S., Choong, K., Lamontagne, F., Turgeon, A.F., Lapinsky, S., Ahern, S.P., Smith, O., Siddiqui, F., Jouvet, P., Khwaja, K., McIntyre, L, Menon, K., Hutchison, J., Hornstein, D., Joffe, A., Lauzier, F., Singh, J., Karachi, T., Wiebe, K., Olfason, K., Ramsey, C., Sharma, S., Dodek, P., Meade, M., Hall, R., Fowler, R.A., 2009. Critically ill patients with 2009 influenza A (H1N1) infection in Canada. Journal of the American Medical Association 302, 1872–1879.

Lakadamyali, M., Rust, M.J., Zhuang, X., 2004. Endocytosis of influenza viruses. Microbes and Infection 6, 929–936.

Le Goffic, R., Pothlichet, J., Vitour, D., Fujita, T., Meurs, E., Chignard, M., Si-Tahar, M., 2007. Cutting Edge: Influenza A virus activates TLR3-dependent inflammatory and RIG-I-dependent antiviral responses in human lung epithelial cells. Journal of Immunology 178, 3368–3372.

Lee, N., Chan, P., Wong, CK, Wong, K-T, Choi, K.-W., Joynt, G.M., Lam, P., Chan, M., Wong, B., Lui, G., Sin, W.W., Wong, R.Y., Lam, W.-Y., Yeung, A.C., Leung, T.-F., So, H-Y, Yu, A.W., Sung, J.J., Hui, D.S., 2011a. Viral clearance and inflammatory response patterns in adults hospitalized for pandemic 2009 influenza A (H1N1) virus pneumonia. Antiviral Therapy 16, 237.

Lee, N, Wong, C.K., Chan, PK, Chan, MC, Wong, RY, Lun, SW, Ngai, K.L., Lui, G.C., Wong, B.C., Lee, S.K., 2011b. Cytokine response patterns in severe pandemic 2009 H1N1 and seasonal influenza among hospitalized adults. PLoS One 6, e26050.

León, A.J., Banner, D., Xu, L., Ran, L., Peng, Z., Yi, K., Chen, C., Xu, F., Huang, J., Zhao, Z., Lin, Z., Huang, S.S., Fang, Y., Kelvin, A.A., Ross, T.M., Farooqui, A., Kelvin, D.J., 2013. Sequencing, annotation, and characterization of the influenza ferret infectome. Journal of Virology 87, 1957–1966.

Liu, W.-C., Lin, S.-C., Yu, Y.-L., Chu, C.-L., Wu, S.-C., 2010. Dendritic cell activation by recombinant hemagglutinin proteins of H1N1 and H5N1 influenza A viruses. Journal of Virology 84, 12011–12017.

Marcelin, G., Aldridge, J.R., Duan, S., Ghoneim, H.E., Rehg, J., Marjuki, H., Boon, A.C., McCullers, J.A., Webby, R.J., 2011. Fatal outcome of pandemic H1N1 2009 influenza virus infection is associated with immunopathology and impaired lung repair, not enhanced viral burden, in pregnant mice. Journal of Virology 85, 11208–11219.

Marchant, D., Singhera, G.K., Utokaparch, S., Hackett, T.L., Boyd, J.H., Luo, Z., Si, X., Dorscheid, D.R., McManus, B.M., Hegele, R.G., 2010. Toll-like receptor 4-mediated activation of p38 mitogen-activated protein kinase is a determinant of respiratory virus entry and tropism. Journal of Virology 84, 11359–11373.

Marjuki, H., Alam, M.I., Ehrhardt, C., Wagner, R., Planz, O., Klenk, H.-D., Ludwig, S., Pleschka, S., 2006. Membrane accumulation of influenza A virus hemagglutinin triggers nuclear export of the viral genome via protein kinase Cα-mediated activation of ERK signaling. Journal of Biological Chemistry 281, 16707–16715.

Matsukura, S., Kokubu, F., Kurokawa, M., Kawaguchi, M., Ieki, K., Kuga, H., Odaka, M., Suzuki, S., Watanabe, S., Takeuchi, H., Kasama, T., Adachi, M., 2006. Synthetic double-stranded RNA induces multiple genes related to inflammation through Toll-like receptor 3 depending on NF-κB and/or IRF-3 in airway epithelial cells. Clinical and Experimental Allergy 36, 1049–1062.

Mauad, T., Hajjar, L., Callegari, G., da Silva, L., Schout, D., Galas, F., Alves, V., Malheiros, D., Auler, J., Ferreira, A., Borsato, M., Bezerra, S., Gutierrez, P., Caldini, E., Pasqualucci, C., Dolhnikoff, M., Saldiva, P., 2010. Lung pathology in fatal novel human influenza A (H1N1) infection. American Journal of Respiratory Cell and Molecular Biology 181, 72–79.

Mukherjee, S., Vipat, V.C., Mishra, A.C., Pawar, S.D., Chakrabarti, A.K., 2011. Pandemic (H1N1) 2009 influenza virus induces weaker host immune responses in vitro: a possible mechanism of high transmissibility. Virology Journal 8, 140.

Nallagatla, S.R., Toroney, R., Bevilacqua, P.C., 2011. RNA structure and regulation of innate immunity through protein kinase PKR. Current Opinion in Structural Biology 21, 119.

Napolitano, L., Park, P, Sihler, K., Papadimos, T., Chenoweth, C., Cinti, S., Zalewski, C., Sharangpani, R., Somsel, P., Wells, E., 2009. Intensive-care patients with severe novel influenza A (H1N1) virus infection-Michigan, June 2009. MMWR Morbidity and Mortality Weekly Reports 58, 749–752.

Nicoll, A., Coulombier, D., 2009. Europe's initial experience with pandemic (H1N1) 2009-mitigation and delaying policies and practices. Euro Surveill. 29, pii 19279, 14.

Paquette, S.G., Banner, D., Zhao, Z., Fang, Y., Huang, S.S., León, A.J., Ng, D.C., Almansa, R., Martin-Loeches, I, Ramirez, P., Socias, L., Loza, A, Blanco, J., Sansonetti, P., Rello, J., Andaluz, D, Shum, B., Rubino, S., de Lejarazu, RO, Tran, D, Delogu, G., Fadda, G., Krajden, S., Rubin, B.B, Bermejo-Martin, JF, Kelvin, AA, Kelvin, DJ, 2012. Interleukin-6 is a potential biomarker for severe pandemic H1N1 influenza A infection. PLoS One 7, e38214.

Patel, J.R., Vora, K.P., Tripathi, S., Zeng, H., Tumpey, T.M., Katz, J.M., 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 Immunology 24, 89–99.

Perez-Padilla, R., de la Rosa-Zamboni, D., Ponce de Leon, S., Hernandez, M., Quiñones-Falconi, F., Bautista, E., Ramirez-Venegas, A., Rojas-Serrano, J., Ormsby, C.E., Corrales, A., Higuera, A., Mondragon, E., Cordova-Villalobos, J.A., 2009. Pneumonia and respiratory failure from swine-origin influenza A (H1N1) in Mexico. New England Journal of Medicine 361, 680–689.

Pica, N., Hai, R., Krammer, F., Wang, T.T., Maamary, J., Eggink, D., Tan, G.S., Krause, J. C., Moran, T., Stein, CR, Banach, D., Wrammert, J., Belshe, R.B., Garcia-Sastre, A., Palese, P., 2012. Hemagglutinin stalk antibodies elicited by the 2009 pandemic influenza virus as a mechanism for the extinction of seasonal H1N1 viruses. Proceedings of the National Academy of Sciences of the United States of America 109, 2573–2578.

Rehwinkel, J., Tan, C.P., Goubau, D., Schulz, O., Pichlmair, A., Bier, K., Robb, N., Vreede, F., Barclay, W., Fodor, E., Reis e Sousa, C., 2010. RIG-I detects viral genomic RNA during negative-strand RNA virus infection. Cell 140, 397–408.

Rowe, T., León, J., Crevar, CJ, Carter, D.M., Xu, L., Ran, L., Fang, Y., Cameron, C.M., Cameron, M.J., Banner, D., Ng, DC Ran, R., Weirback, H.K., Wiley, C.A., Kelvin, D. J., Ross, T.M., 2010. Modeling host responses in ferrets during A/California/07/2009 influenza infection. Virology 401, 257.

Saito, T., Owen, D.M., Jiang, F., Marcotrigiano, J., Gale Jr, M., 2008. Innate immunity induced by composition-dependent RIG-I recognition of hepatitis C virus RNA. Nature 454, 523–527.

Samson, M., Pizzorno, A., Abed, Y., Boivin, G., 2013. Influenza virus resistance to neuraminidase inhibitors. Antiviral Research 98, 174–185.

Sanders, C.J., Doherty, P.C., Thomas, P.G., 2011. Respiratory epithelial cells in innate immunity to influenza virus infection. Cell and Tissue Research 343, 13–21.

Shapira, SD, Gat-Viks, I., Shum, B.O., Dricot, A., de Grace, M.M., Wu, L., Gupta, PB, Hao, T, Silver, S.J., Root, D.E, Regev, A., Hacohen, N., 2009. A physical and regulatory map of host-influenza interactions reveals pathways in H1N1 infection. Cell 139, 1255–1267.

Sharma, K., Tripathi, S., Ranjan, P., Kumar, P., Garten, R., Deyde, V., Katz, JM, Cox, N.J., Lal, R.B., Sambhara, S., Lal, S., 2011. Influenza A virus nucleoprotein exploits Hsp40 to inhibit PKR activation. PLoS One 6, e20215.

Shieh, W.-J., Blau, D.M., Denison, A.M., DeLeon-Carnes, M., Adem, P., Bhatnagar, J., Sumner, J., Liu, L., Patel, M, Batten, B., Greer, P., Jones, T., Smith, C., Bartlett, J., Montague, J., White, E., Rollin, D., Gao, R., Seales, C., Jost, H., Metcalfe, M., Goldsmith, C.S., Humphrey, C., Schmitz, A., Drew, C., Paddock, C., Uyeki, T.M., Zaki, S.R., 2010. 2009 pandemic influenza A (H1N1): pathology and pathogenesis of 100 fatal cases in the United States. American Journal of Pathology 177, 166–175.

Sieczkarski, S.B., Brown, H.A., Whittaker, G.R., 2003. Role of protein kinase C βII in influenza virus entry via late endosomes. Journal of Virology 77, 460–469.

Skehel, J.J., Wiley, D.C., 2000. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annual Review of Biochemistry 69, 531–569.

Suksatu, A., Sangsawad, W., Thitithanyanont, A., Smittipat, N., Fukuda, M.M., Ubol, S., 2009. Characteristics of stork feces-derived H5N1 viruses that are preferentially transmitted to primary human airway epithelial cells. Microbiology and Immunology 53, 675–684.

Takeuchi, O., Akira, S., 2010. Pattern recognition receptors and inflammation. Cell 140, 805–820.

Tam, A., Wadsworth, S., Dorscheid, D., Man, S.P., Sin, D.D., 2011. The airway epithelium: more than just a structural barrier. Therapeutic Advances in Respiratory Disease 5, 255–273.

Taniguchi, T., Ogasawara, K., Takaoka, A., Tanaka, N., 2001. IRF family of transcription factors as regulators of host defense. Annual Review of Immunology 19, 623–655.

Taubenberger, JK, Morens, DM., 2008. The pathology of influenza virus infections. Annual Review of Pathology 3, 499.

Teijaro, J.R., Walsh, K.B., Cahalan, S., Fremgen, D.M., Roberts, E., Scott, F., Martinborough, E., Peach, R., Oldstone, M., Rosen, H., 2011. Endothelial cells are central orchestrators of cytokine amplification during influenza virus infection. Cell 146, 980–991.

Uyeki, T.M., Sharma, A., Branda, J.A., 2009. Case 40-2009: a 29-year-old man with fever and respiratory failure. New England Journal of Medicine 361, 2558–2569.

Wang, J.P., Bowen, G.N., Padden, C., Cerny, A., Finberg, R.W., Newburger, P.E., Kurt-Jones, E.A., 2008. Toll-like receptor-mediated activation of neutrophils by influenza A virus. Blood 112, 2028–2034.

Whitley, R.J., Boucher, C.A., Lina, B., Nguyen-Van-Tam, J.S., Osterhaus, A., Schutten, M., Monto, A.S., 2013. Global Assessment of Resistance to Neuraminidase Inhibitors: 2008–2011. The Influenza Resistance Information Study (IRIS). Clinical Infectious Diseases 56, 1197–1205.

Xu, W., Chen, M., Ge, N., Xu, J., 2012. Hemagglutinin from the H5N1 virus activates janus kinase 3 to dysregulate innate immunity. PLoS One 7, e31721.

Yamashita, K., Imaizumi, T., Taima, K., Fujita, T., Ishikawa, A., Yoshida, H., Oyama, C., Satoh, K., 2005. Polyinosinic-polycytidylic acid induces the expression of GRO-α in BEAS-2B cells. Inflammation 29, 17–21.

Zeng, H., Pappas, C., Katz, J.M., Tumpey, T.M., 2011. The 2009 pandemic H1N1 and triple-reassortant swine H1N1 influenza viruses replicate efficiently but elicit an attenuated inflammatory response in polarized human bronchial epithelial cells. Journal of Virology 85, 686–696.

Zhang, Z., Kim, T., Bao, M., Facchinetti, V, Jung, S.Y., Ghaffari, AA, Qin, J., Cheng, G, Liu, Y.-J., 2011. DDX1, DDX21, and DHX36 helicases form a complex with the adaptor molecule TRIF to sense dsRNA in dendritic cells. Immunity 34, 866–878.

Zheng, X., Bevilacqua, P.C., 2004. Activation of the protein kinase PKR by short double-stranded RNAs with single-stranded tails. RNA 10, 1934–1945.