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A comparison of interferon gene expression induced by influenza A virus infection of human airway epithelial cells from two different donors

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ABSTRACT

Influenza is an acute respiratory disease that can cause local annual epidemics and worldwide pandemics of different morbidity and mortality. Our understanding of host factors that modulate the frequency and severity of influenza virus infections is less than complete. In this study, we examined the inter-individual variations in the innate immune responses to H1N1 and H3N2 influenza A viruses (IAV) using primary cultures of normal human bronchial epithelial (NHBE) cells derived from two different donors (D1 and D2). Although IAV replication kinetics were similar in cultures derived from these two donors, the levels of type III interferons (IFNs) were significantly higher in D1 cells compared to D2 cells (~31-fold↑ in D1 cells versus D2 cells; $P < 0.05$). The levels of IFN-λ1 protein at individual time points as well as the total amounts of IFN-λ1 secreted over 72 h were also significantly higher in D1 than in D2 NHBE cells (0.7–7.7 − fold↑, $P < 0.05$). The relative levels of IFN-stimulated gene (ISG) expression also differed significantly between D1 and D2 cells. Our data indicate that donor-specific differences can result in significant differences in IFN and ISG induction by human airway epithelium.

1. Introduction

Influenza is a common public health problem that can result in local annual epidemics and worldwide pandemics. About 10–20% of people in the United States experience influenza infection every year, and more than 200,000 individuals per year are hospitalized for complications related to influenza (Wright et al., 2006). Influenza A viruses (IAV) of the H1N1 and H3N2 subtypes cause similar symptoms, including fever, headache, cough, sore throat, nasal congestion, sneezing, and body aches (Frank et al., 1985; Nicholson, 1992; Wright et al., 2006). However, the frequency of severe infections is higher with H3N2 viruses (Thompson et al., 2003). The rate and severity of infection depend on a number of host and viral factors, including the level of pre-existing immunity, the age and medical conditions of an individual, and the virulence of the virus, all of which vary greatly among outbreaks (Fox et al., 1982; Karlas et al., 2010; Wright et al., 2006). Our current understanding of the relative contributions of all possible mechanisms and pathways that modulate the degree of influenza severity is far from complete.

The interferon (IFN) system represents a major element of the innate immune response against influenza infection. Infection by most viruses, including influenza, induces rapid expression of the IFN genes, particularly type I and type III IFNs (Durbin et al., 2000; Hsu et al., 2012; Kotenko et al., 2003; Sheppard et al., 2003). Type I IFNs consist of 14 different IFN-α subtypes as well as single versions of IFN-β, -ε, -κ, and -ω. All of the type I IFN proteins signal through the same cell surface receptor complex, known as the IFNAR complex. Type I IFN receptors are broadly expressed on most somatic cell types (Donnelly et al., 2011; Durbin et al., 2000; Kotenko et al., 2003; O'Brien et al., 2014). Type III IFNs signal via a distinct receptor complex that is composed of the ligand-binding chain, IFN-λR1, and the IL-10R2 chain. Expression of type III IFN receptors is largely restricted to cells of epithelial origin, including epidermal, respiratory, and gastrointestinal epithelial cells (Durbin et al., 2013; Mordstein et al., 2010; Sommereyns et al., 2008). Consequently, type III IFNs (*i.e.*, IFN-λ1, -λ2, -λ3, and -λ4) have a more limited functional range than type I IFNs and are likely to play distinct functional roles in vivo from the type I IFNs (e.g., IFN-α/β).

Although type I and type III IFNs signal via distinct receptors, they trigger strikingly similar intracellular signaling pathways that result in expression of hundreds of IFN-stimulated genes (ISGs) that act to restrict influenza virus infection (Donnelly et al., 2011; Durbin et al., 2000; Kotenko et al., 2003; O'Brien et al., 2014). Because airway epithelial cells are the primary cellular targets of influenza virus infection, it is important to determine if there are differences in the innate immune response of these cells from different individuals. In this study,

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we examined inter-individual variation in the cellular response to IAV infections using primary cultures of normal human bronchial epithelial (NHBE) cells isolated from two different donors (D1 and D2). NHBE cells provide a useful model for evaluating virus-induced IFN and ISG production. Using cultures of primary human airway epithelial cells from two different donors, we found that the magnitude of IAV-induced innate responses was significantly different in NHBE cells derived from these two individuals.

2. Materials and methods

2.1. Cells and viruses

Madin-Darby canine kidney (MDCK) cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and were maintained as described elsewhere (Ilyushina et al., 2008). Primary NHBE cells from human tracheal/bronchial tissues were obtained from Lonza (Walkersville, MD, USA). Cell cultures of passage 2 were grown on membrane supports (6.5-mm Transwell, Corning Inc., Corning, NY, USA) at the air-liquid interface in serum-free and hormone- and growth factor-supplemented medium as described previously (Matrosovich et al., 2004). Fully differentiated 4- to 8-week-old cultures were used for all experiments.

Human A/Nanchang/1/99 (H1N1), A/New Jersey/15/07 (H1N1), A/California/07/09 (H1N1), A/Panama/2007/99 (H3N2), and A/Perth/16/09 (H3N2) strains were kindly provided by Dr. Robert G. Webster at St. Jude Children's Research Hospital, Memphis, TN. Human A/Minnesota/11/10 (H3N2) virus was kindly provided by Dr. Kanta Subbarao at the National Institute of Allergy and Infectious Diseases, Bethesda, MD. Stock viruses were prepared by one passage in MDCK cells for 72 h at 37 °C, and aliquots were stored at −80 °C until used. All experimental work was performed in a biosafety level 2 laboratory approved for use with these strains by the U.S. Department of Agriculture and the U.S. Centers for Disease Control and Prevention.

2.2. Infectivity of influenza viruses

The infectivities of human IAV were determined in MDCK cells by plaque assay and expressed as $\log_{10}$ plaque-forming units (PFU)/ml (Hayden et al., 1980). Briefly, confluent MDCK cells were incubated at 37 °C for 1 h with 10-fold serial dilutions of virus. The cells were then washed and overlaid with minimal essential medium containing 1 μg/ml L-(tosylamido-2-phenyl)ethylchloromethylketone (TPCK)-treated trypsin, 0.3% bovine serum albumin, and 0.9% Bacto agar. After 3 days of incubation at 37 °C, cells were stained with 0.1% crystal violet in 10% formaldehyde solution, and the PFU per milliliter were determined.

2.3. Viral replication kinetics

To determine growth curves in NHBE cells, triplicate cell cultures growing in 6.5-mm-diameter inserts were washed extensively with sterile phosphate-buffered saline to remove mucus secretions on the apical surface prior to infection and then were inoculated via the apical side with each influenza virus at a multiplicity of infection (MOI) of 3 at 37 °C. Because the MOI was determined from a titer measured in MDCK cells, it may not accurately reflect the number of infectious units per susceptible NHBE cells. Approximately 80% of the cells in our NHBE cultures were infected (Mindaye et al., 2017), with a similar proportion of infected cells in cultures from both donors. After 1 h of incubation, the inoculum was removed. Viruses released into the apical compartment of NHBE cells were harvested at the indicated time points by the apical addition and collection of 300 μl of medium allowed to equilibrate for 30 min at 37 °C. The virus titers were determined as $\log_{10}$PFU/ml in MDCK cells. Area under the curve (AUC) of the viral load was defined as the area under the multistep growth curve and calculated by the trapezoidal rule, using exact viral titers at 24, 48, and 72 h post-infection (hpi) as determined by plaque assay in MDCK cells.

2.4. Quantitative real-time polymerase-chain reaction (qPCR) of IFN, IFN-stimulated, and viral matrix (M1) genes

Quantification of changes in gene expression was carried out by qPCR analyses of individual IFNs and ISGs using RT^2 qPCR primer assays (Qiagen, Germantown, MD, USA). The following gene-specific qPCR primers were used: *IFNA1* (PPH01321B), *IFNB1* (PPH00384 F), *IFNL1* (PPH05849 A), *IFNL2/3* (PPH05847B), *IFIT1* (PPH01332 F), *IFIT3* (PPH02856 A), *IRF7* (PPH02014 F), *MX1* (PPH01325 A), and *OAS1* (PPH01324 A). Viral *M1* gene was amplified using the specific primers as described previously by Spackman et al. (2002). Total cellular RNA was isolated from virus-infected NHBE cell cultures using RNeasy Minikit (Qiagen), treated with DNase, and 1 μg of the purified RNA was reverse-transcribed to cDNA with Quantiscript reverse transcriptase (Qiagen). The cDNA was mixed with RT^2 SYBR® green qPCR Mastermix (Qiagen) and qPCR was performed using the ViiA™7 system (Applied Biosystems, Beverly, MA, USA). Changes in gene expression levels were analyzed using ViiA™ 7 software v.1.2.2 (Applied Biosystems), and the results were expressed as the mean-fold increase relative to the untreated control gene expression levels after normalization to the housekeeping gene, *GAPDH* (PPH00150 F). Graphing and statistical analysis of qPCR results were performed using Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA). Values represent the mean ± standard deviation (SD) of at least triplicate determinations. AUC of IFNs and ISG expression was calculated by the trapezoidal rule, using exact levels of respective mRNAs expression at 24, 48, and 72 hpi as determined by qPCR.

Additionally, *IFNB1*, *IFNL1*, and *IFNL2/3* genes copy numbers were assayed by using Taqman gene expression assay primer/probe sets and master mix (Life Technologies, Carlsbad, CA, USA) and values were determined by running respective standard curves.

2.5. Enzyme-linked immunosorbent assay (ELISA)

The secreted levels of IFN-λ1 and IFN-λ2/3 from NHBE cell culture supernatants were analyzed using ELISA kits supplied by BioLegend (San Diego, CA, USA). ELISA to detect human IFN-β was supplied by PBL Biomedical Laboratories (Piscataway Township, NJ). The IFNs levels from cell culture supernatants isolated from two different donors were analyzed in one experiment.

2.6. Statistical analysis

The peaks of IFNs and ISGs expression, cumulative total amounts of IFN and ISG levels, virus yield, AUCs of the viral load, secreted levels of IFN-λ1, were compared by analysis of variance (ANOVA) with Dunnett's multiple comparisons test. A probability value of 0.05 was prospectively chosen to indicate that the findings were not the result of chance alone.

3. Results

3.1. Replication kinetics of IAV in NHBE cells derived from two different donors

We first evaluated the quantities of viral RNA M1 gene transcripts (Supplementary Fig. 1) together with the replication kinetics of IAV infections in D1 and D2 NHBE cells (MOI = 3, Fig. 1). Although H1N1 IAV exhibited significantly higher peak viral titers at some time points in D1 than in D2 NHBE cells (˜1.5 logs, $P < 0.05$), all three H1N1 isolates replicated to a similar extent in both D1 and D2 cell cultures, as measured by the total amount of virus shedding over 72 h (i.e., AUC). The replication kinetics of H3N2 IAV did not differ from each other, and

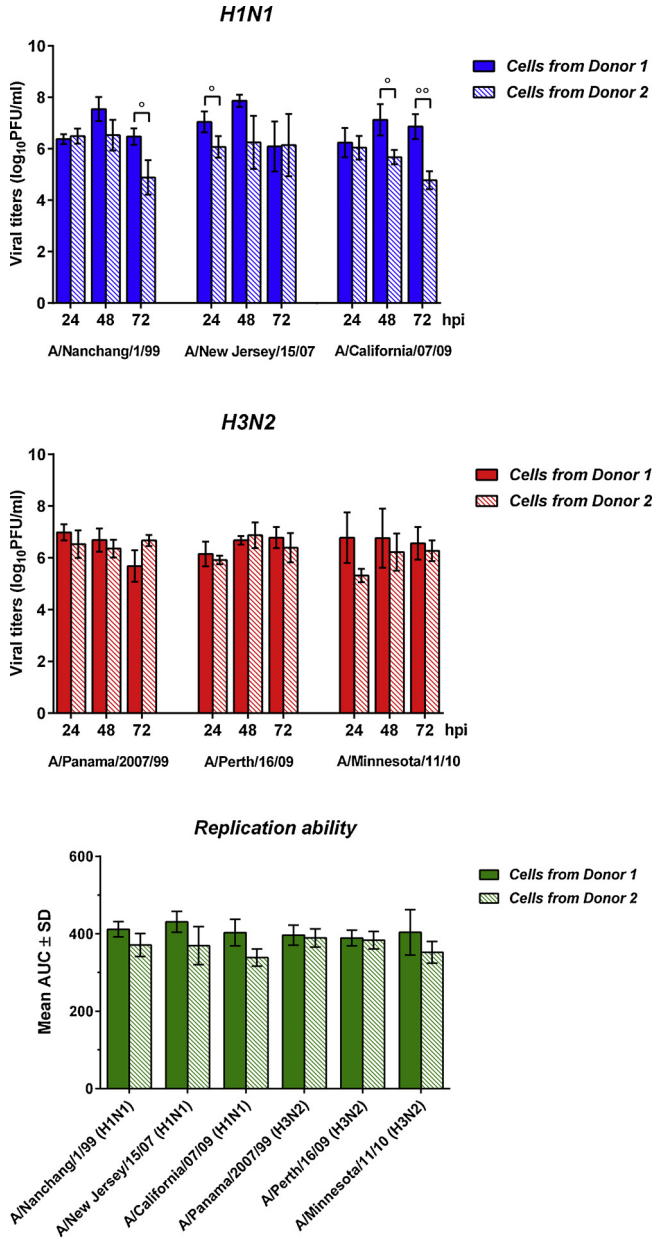

Fig. 1. Replication kinetics and total viral loads of IAV in D1 and D2 NHBE cells. Cultures were infected with viruses at a MOI of 3. Supernatants were collected at 24, 48, and 72 hpi, and the virus titers were determined as log_{10}PFU/ml in MDCK cells. °, $P < 0.05$, °°, $P < 0.01$ compared with the respective value in D2 NHBE cells (one-way ANOVA).

these viruses shed similar amount of virus from both D1 and D2 cells (Fig. 1). These results showed that IAV exhibited almost identical replicative kinetics in NHBE cells derived from two different donors.

3.2. IAV-induced IFN expression in NHBE cells isolated from two donors

We next examined the ability of several IAV strains to induce expression of the IFN genes in D1 and D2 NHBE cells. The cells were infected with H1N1 or H3N2 viruses at an MOI of 3, and IFN gene expression levels were assayed by qPCR at several time points throughout 72 h (Fig. 2). As shown in Fig. 2A, infection of D1 cells by the H1N1 viruses induced rapid expression of the *IFNB1* gene, which was maximal at 24 hpi. In contrast, mRNA levels for *IFNA1*, *IFNL1*, and *IFNL2/3* peaked somewhat later at 48 hpi. It is noteworthy that the pandemic 2009 strain, A/California/07/09 (H1N1), induced high levels

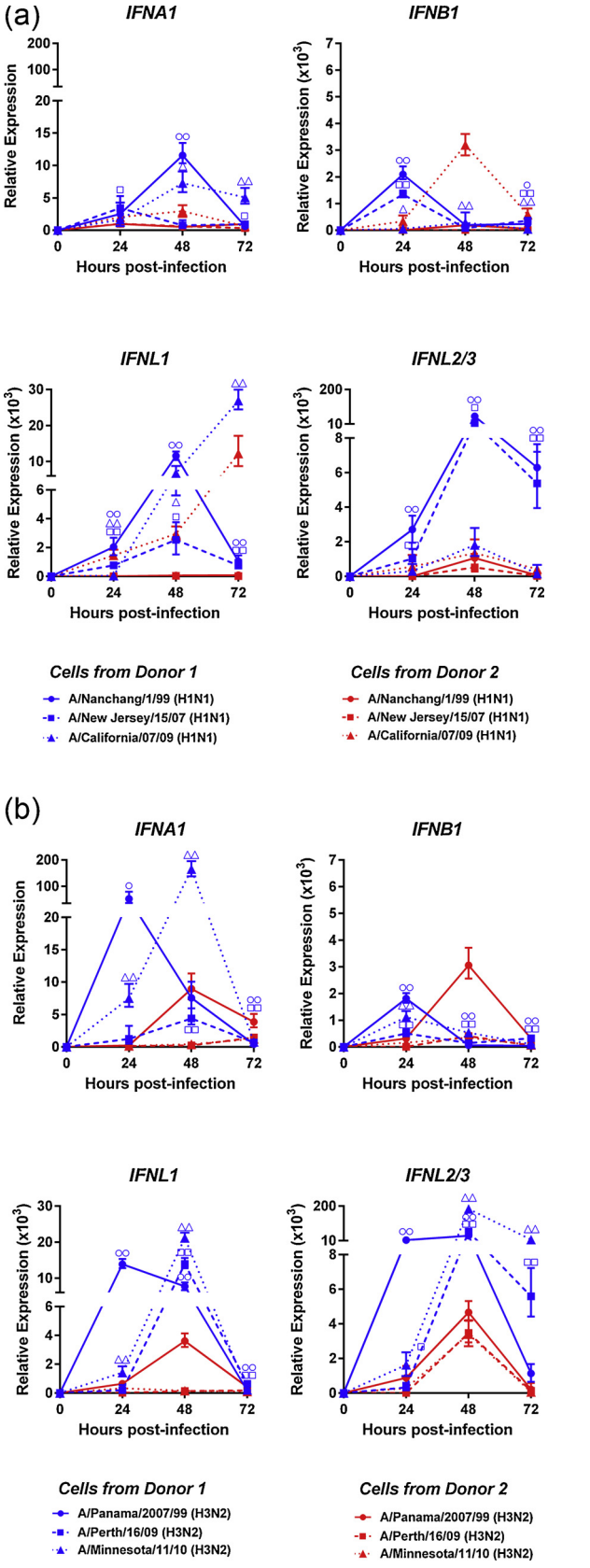

Fig. 2. H1N1- (A) and H3N2-induced (B) IFN expression in D1 and D2 NHBE cells as measured by qPCR. NHBE cells were infected at a MOI of 3 with the indicated IAV and the levels of IFNs were quantified by qPCR or by using Taqman gene expression assay at indicated time points. Values represent the mean ± SD of at least triplicate determinations. °, $P < 0.05$, °°, $P < 0.01$ compared with the respective value in D2 NHBE cells (one-way ANOVA).

of *IFNL1* expression at 72 hpi and lower levels of *IFNB1* and *IFNL2/3* expression in D1 cells. In contrast to the response of D1 cells, infection of D2 NHBE cells with the same H1N1 IAV strains induced a more latent induction of *IFNB1* expression that was maximal at 48 hpi and lower expression levels of the IFN-λ genes, *IFNL1* and *IFNL2/3* (Fig. 2A). Although IFN gene expression levels induced by the A/Nanchang/1/99 (H1N1) and A/New Jersey/15/07 (H1N1) viruses were significantly lower in D2 cells than in D1 cells, induction of *IFNL2/3* expression in D2 cells by the pandemic 2009 strain, A/California/07/09, was similar to the response of D1 cells.

The H3N2 strains induced a similar pattern of IFN gene expression to that triggered by the H1N1 strains in D1 NHBE cells, with the notable exception of A/Panama/2007/99 (H3N2), which induced maximal mRNA expression of all IFN genes at 24 hpi (Fig. 2B). Infection of D2 cells with the same H3N2 IAV strains induced lower levels of the *IFNA1*, *IFNL1* and *IFNL2/3* genes than we observed with D1 cells. Comparison of the three H3N2 viruses that we tested (A/Panama/2007/99, A/Perth/16/09, and A/Minnesota/11/10) showed that the A/Panama/2007/99 strain induced the highest levels of IFN gene expression by D2 cells. Overall, the results in Fig. 2A and B demonstrates that the induction of type III IFN gene expression by both H1N1 and H3N2 influenza viruses was stronger in cells derived from donor D1 than in cells derived from donor D2.

We performed pairwise comparisons of the cumulative total amounts of IFN transcripts expressed after IAV infections over 72 h (i.e., AUC) in NHBE cells from the two donors (Supplementary Fig. 2). Although the majority of IAV strains induced significantly higher levels of total *IFNB1* expression in D1 cultures, two viruses (A/California/07/09 (H1N1) and A/Panama/2007/99 (H3N2)) induced ~6-fold higher *IFNB1* levels in D2 NHBE cells ($P < 0.05$). The levels of the type III IFN genes were usually significantly higher in D1 cells than in D2 cells (~31-fold; $P < 0.05$). However, the pandemic H1N1 strain induced equivalent levels of *IFNL2/3* gene expression in NHBE cells derived from both donors (Supplementary Fig. 2).

3.3. IAV-induced IFN-β, IFN-λ1, and IFN-λ2/3 protein expression in NHBE cells isolated from two donors

We next measured IFN-β, IFN-λ1, and IFN-λ2/3 protein levels in the culture supernatants of D1 and D2 NHBE cells after IAV infections. The levels of IFN-β and IFN-λ2/3 proteins induced by the IAV strains were negligible at 24, 48, and 72 hpi (data not shown). In contrast, all the viruses induced measurable amounts of IFN-λ1 protein by NHBEs derived from either D1 or D2 donor at all time points tested (Fig. 3). The levels of IFN-λ1 protein (Fig. 3) correlated well with the levels of *IFNL1* gene expression (Fig. 2A and B). The IFN-λ1 protein levels at individual time points as well as the total amounts of IFN-λ1 secreted over 72 h were significantly higher in D1 than in D2 NHBE cells (0.7–7.7 – fold, $P < 0.05$).

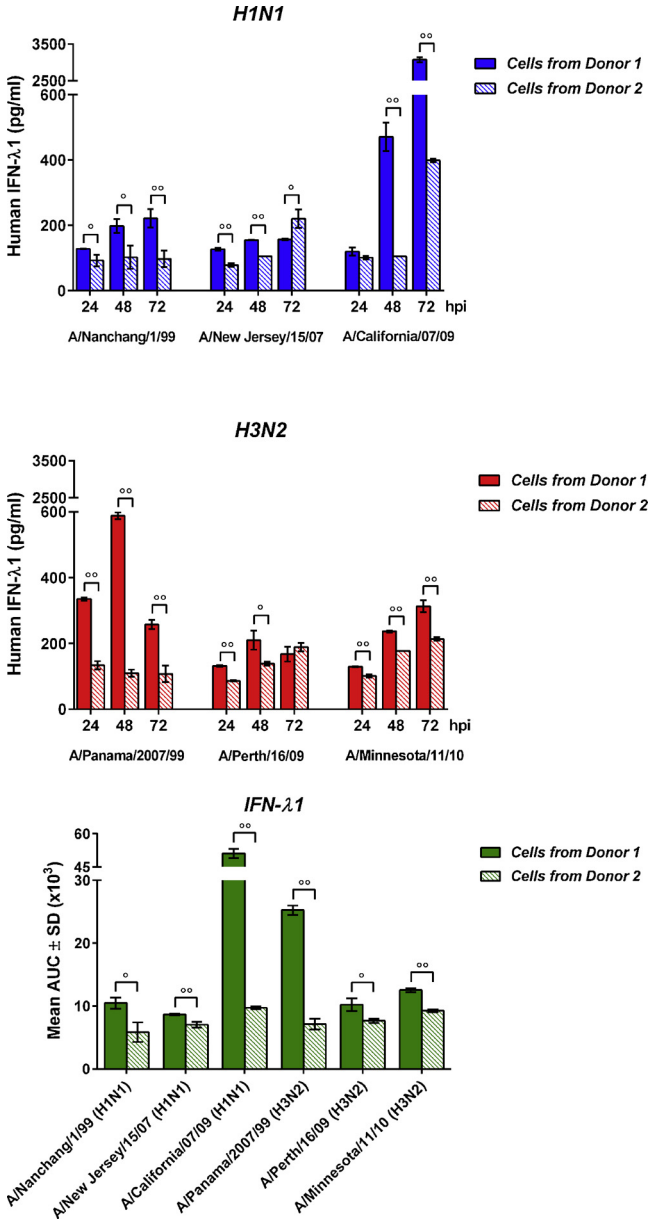

Fig. 3. IFN-λ1 protein production in D1 and D2 NHBE cells. Cultures were infected with IAV at a MOI of 3. Supernatants were collected at 24, 48, and 72 hpi, and the levels of secreted IFN-λ1 protein were determined by ELISA. °, $P < 0.05$, °°, $P < 0.01$ compared with the respective value in D2 NHBE cells (one-way ANOVA).

3.4. IAV-induced ISG expression in NHBE cells derived from two different donors

Since IFN production induces consequent expression of various ISGs, we examined expression levels of several ISGs, including *IFIT1*, *IFIT3*, *IRF7*, *MX1*, and *OAS1*, induced by IAV in D1 and D2 NHBE cells at 24, 48, and 72 hpi (Fig. 4). The kinetics of ISG expression differed markedly between IAV infections. Two H1N1 isolates, A/Nanchang/1/99 and A/New Jersey/15/07, induced significant, rapid, and biphasic *IFIT3* expression, while the *IFIT3* expression response of D1 NHBEs infected with either H3N2 viruses or A/California/07/09 (H1N1) was much weaker (Fig. 4A and B). A/California/07/09 (H1N1) exhibited a very different kinetic pattern of ISG expression as well as the lowest levels of the *IFIT3* and *MX1* genes relative to the other strains in D1 cells. In contrast, D2 NHBE cells infected with A/California/07/09 (H1N1) virus expressed the highest levels of *IFIT1*, *IFIT3*, *IRF7*, and *OAS1* compared to other H1N1 infections. All H3N2 viruses induced maximal *IFIT3*, *IRF7*, and *OAS1* responses at 48 hpi in D2 NHBE cells (Fig. 4B).

Side-by-side comparisons of cumulative levels of ISG expression revealed that the majority of the viruses tested induced significantly higher levels of *IFIT1* in D1 compared to D2 NHBE cultures (~8-fold↑, $P < 0.05$). The total levels of *OAS1* gene expression were significantly higher in D2 cells ($P < 0.05$, Supplementary Fig. 3). The levels of expression of the *IFIT3* and *MX1* genes varied for the different IAV strains in D1 and D2 NHBE cells. Overall, our data demonstrated statistically significant differences in the levels of ISG expression by IAV-infected NHBE cells derived from two different individuals.

4. Discussion

In this study, we compared the effects of different IAV strains on

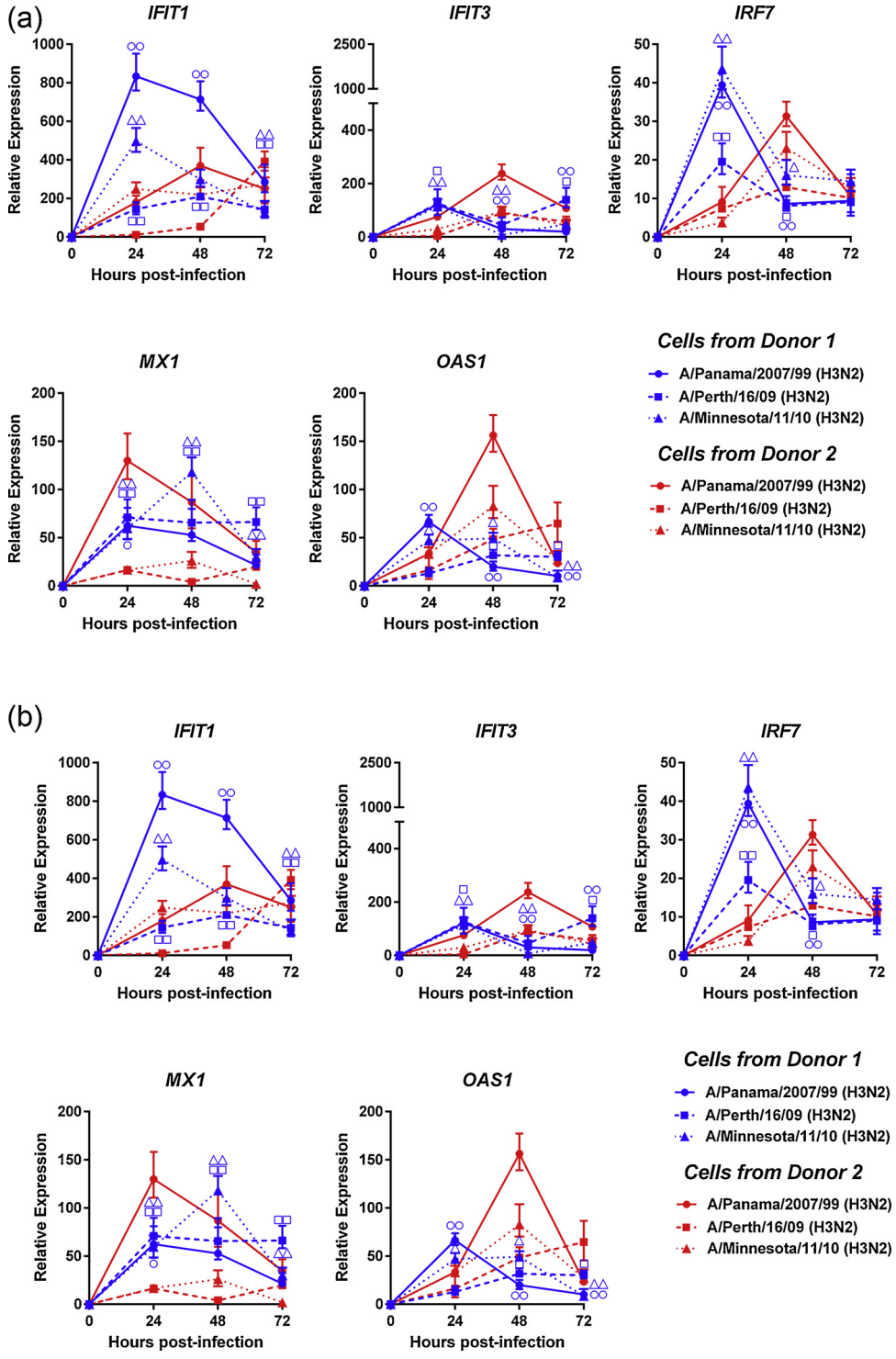

Fig. 4. H1N1- (A) and H3N2-induced (B) ISG expression in D1 and D2 NHBE cells. NHBE cells were infected at a MOI of 3 with the indicated IAV and the levels of ISG were quantified by qPCR at 24, 48, and 72 hpi. Results are expressed as fold over mock infected cells and normalized to a housekeeping gene. Values represent the mean ± SD of at least triplicate determinations. °, $P < 0.05$, °°, $P < 0.01$ compared with the respective value in D2 NHBE cells (one-way ANOVA).

expression of IFNs and ISGs by primary NHBE cells derived from two different donors. Evaluation of the kinetics of IFN induction following IAV infections demonstrated that the first IFN gene expressed was *IFNB1* followed by the type III IFN genes, *IFNL1* and *IFNL2/3*. *IFNL1* and *IFNL2/3* were the most highly expressed IFN genes in cultures derived from both donors. These findings correlate well with previous reports demonstrating that IFN-λs primarily act on epithelial cells (Mordstein et al., 2010; Sommereyns et al., 2008). We observed little to no *IFNA1* gene expression by human airway cells derived from the two donors that we examined. This finding was expected because IFN-α is expressed predominantly by hematologic cell types instead of epithelial cells (Mangan and Fung, 2012; Siegal et al., 1999).

The pandemic 2009 A/California/07/09 (H1N1) virus induced a very distinct kinetic profile of IFN gene expression compared to the other H1N1 strains. It triggered only a weak antiviral response relative to other IAV strains, as evidenced by a weak induction of *IFNA1*, *IFNL2/*

3, *IFIT3*, and *MX1* genes in the NHBEs derived from both cell donors. This finding is consistent with previously published data demonstrating that pandemic 2009 IAV strains do not induce high levels of inflammatory cytokines/chemokines neither in primary human monocyte-derived dendritic cells and macrophages (Osterlund et al., 2010) nor in NHBE cells and type I-like pneumocytes as compared with other seasonal strains (Chan et al., 2010). Our data provided additional support for the fact that pandemic 2009 isolates are unlikely to demonstrate an intrinsic capacity for cytokine hyperinduction and enhanced immunopathogenesis in humans.

Our findings show that not only strain differences between the selected viruses but also between individuals can affect the ability of human epithelial cells to elicit anti-influenza cytokine responses. Despite the similar replication competence of H1N1 and H3N2 IAV seen in D1 and D2 cultures, we observed statistically significant differences in the IFN and ISG levels induced by the same influenza strain in NHBE cells derived from two different donors. The levels of ISG expression induced by all viruses tested varied significantly between D1 and D2 cells. The levels of type III IFNs, *IFNL1* and *IFNL2/3*, induced by most IAV strains were significantly higher in D1 cells compared to D2 cells ($P < 0.05$). Furthermore, IFN-λ1 protein levels at each time point and the total amounts of secreted IFN-λ1 after infection with either isolate were also significantly higher in D1 than in D2 cells ($P < 0.05$).

In a separate but related study (Mindaye et al., 2017), we compared the proteomic profiles of NHBE cells derived from three different donors before and after infection with the pandemic 2009 virus for 24 or 48 h. Our data showed that there was limited overlap of the identified proteins, with only 24% of all proteins shared between cells from the three donors before infection. We also observed significant differences in the repertoire and levels of antiviral proteins that are expressed by NHBE cells from these three individuals after infection with the A/California/07/09 (H1N1) strain. The differences in the proteomic profiles of cells derived from these three donors included significant differences in the levels of type I and type III IFNs and IFN-stimulated proteins such as MX1, IFIT1, IFIT3, and IFITM1 (Mindaye et al., 2017). Differentially expressed proteins affecting interferon and chemokine signaling could partially explain the marked differences in innate immune responses by cells from the two donors evaluated in the present study.

Previous studies by others showed that IRF7 expression is critical for development of effective innate immune responses to influenza virus infection, including IFN production (Ciancanelli et al., 2015; Crotta et al., 2013). We observed that infection with either H1N1 IAV or H3N2 IAV induced higher levels of *IRF7* expression in D1 cells than in D2 cells. The higher levels of *IRF7* expression by D1 cells correlated with higher levels of type I and type III IFN expression by D1 cells. These findings indicate that differences in the magnitude of *IRF7* expression by D1 versus D2 cells might explain why interferon expression levels were higher in D1 cells compared to D2 cells.

The severity of influenza virus infection is determined by the balance between the virulence of the particular virus strain and the magnitude of antiviral gene expression by the host target cells. This balance is influenced by several viral characteristics, including the viral polymerase and the viral NS1 protein that antagonizes the host antiviral response (Plant et al., 2017; Schmolke and Garcia-Sastre, 2010). Although viral factors linked to the severity of influenza have been well studied (Karlas et al., 2010; Schmolke and Garcia-Sastre, 2010; Wright et al., 2006), host factors that may be linked to differences in antiviral responses in different individuals are not well understood. Our data provide additional evidence that genetic differences of IAV strains as well as donor-specific differences of the host cells can lead to significant variations in IFN and ISG induction in human airway epithelium.

Competing interests

The authors declare that they have no competing interests. This article reflects the views of the authors and should not be construed to represent FDA's views or policies.

Acknowledgments

We are grateful to Dr. Robert G. Webster for providing influenza A/Nanchang/1/99 (H1N1), A/New Jersey/15/07 (H1N1), A/California/07/09 (H1N1), A/Panama/2007/99 (H3N2), and A/Perth/16/09 (H3N2) viruses. We thank Dr. Kanta Subbarao for providing influenza A/Minnesota/11/10 (H3N2) virus.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2019.02.002.

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