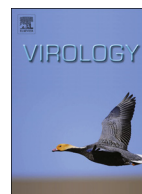




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RIG-I and TLR3 are both required for maximum interferon induction by influenza virus in human lung alveolar epithelial cells



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ABSTRACT

Pattern recognition receptors, such as retinoic acid-inducible protein I (RIG-I), Toll-like receptors 3 and 7 (TLR3 and 7), and nucleotide-binding oligomerization domain containing protein 2 (NOD2), play important roles in the recognition of influenza A virus (IAV), but their role in interferon (IFN) induction is still unclear, particularly in human lung. We investigated IFN induction by IAV in the A549 cell line as well as in primary human alveolar epithelial cells (AEC). TLR3/7, NOD2, RIG-I, and IFN expression levels were measured by qRT-PCR and ELISA in cells infected with IAV PR8. We found that TLR7 and NOD2 were not involved in IFN induction by IAV in these cells. Neither RIG-I nor TLR3 siRNA alone completely blocked IFN induction. However, double knockdown of RIG-I and TLR3 completely inhibited IFN induction by influenza. Thus, signaling through both RIG-I and TLR3 is important for IFN induction by IAV in human lung AEC.

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Introduction

Influenza A virus (IAV) continues to pose an important public health threat globally each year. The virus triggers intra- and extracellular receptors during infection to elicit an innate immune response that serves as a first line of protection against intrusion of any infectious pathogens. Induction of interferon (IFN) is a critical component of the host response to influenza infection. IFNs are further divided into type I (mainly IFN- α and β), II (IFN- γ) and III (IFN- λ) subtypes, based in part on the differential use of unique receptors through which they mediate signal transduction to induce anti-viral activity. Rapid production of type I IFN and recently discovered type III IFN is a central and essential component of the anti-viral response (Kotenko et al., 2003; Stetson and Medzhitov, 2006).

The innate immune receptors, which detect viral infection, are called pattern recognition receptors (PRRs). Previous studies have demonstrated that PRRs, such as retinoic acid-inducible protein I (RIG-I), toll-like receptors 3 and 7 (TLR3 and 7), nucleotide-binding oligomerization domain containing protein 2 (NOD2), all play important roles in the recognition of IAV (Guo et al., 2007; Malathi et al., 2007; Mibayashi et al., 2007; Sabbah et al., 2009; Thomas et al., 2009). RIG-I is believed to play an important role in the recognition of, and response to, IAV in most types of cells. RIG-I is a highly inducible cytoplasmic RNA helicase that activates antiviral responses to IAV leading to IFN production. RIG-I associates with and is activated by IAV ssRNA, which is uncapped and has a 5'-triphosphate moiety (Pichlmair et al., 2006). Stimulation of RIG-I activates specific signaling pathways that lead to activation of NF- κ B which is crucial for inflammatory cytokine induction, and/or IRF3/7 which are then translocated into the nucleus where they act as transcription factors for type I and III IFNs (Fitzgerald et al., 2003; Kotenko et al., 2003). RIG-I interacts with mitochondrial antiviral signaling protein (MAVS, also known as IPS-1), followed by the activation of IRF3/7 via TBK1- and IKKi-dependent phosphorylation. Signaling events regulated by RIG-I during IAV infection are important in the antiviral IFN response

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and for modulation, either directly or indirectly, of proinflammatory cytokine responses. TLR3/7 trigger antiviral responses in a cell type-specific manner. In humans, TLR7 expression is confined to plasmacytoid dendritic cells (pDC) including those in human lung (Di Domizio et al., 2009; Lund et al., 2004). Double-stranded RNA (dsRNA) is produced during viral replication and is recognized by endosomal TLR3 (Guillot et al., 2005). Le Goffic reported that the sensing of IAV by TLR3 primarily regulates a proinflammatory response, whereas RIG-I mediates both a type I IFN-dependent antiviral signaling and a proinflammatory response in human epithelial cells (Le Goffic et al., 2007).

Epithelial cells are the primary site of viral replication for IAV, although monocytes/macrophages and other leukocytes can also be infected (Ronni et al., 1997; Wang et al., 2009). IAV infection of the lower respiratory tract of lung is associated with very high morbidity and mortality (Hers, 1966; Hers et al., 1958; Hers and Mulder, 1961). This can progress to fatal pneumonia (Cox and Subbarao, 1999). The interactions between host immune responses and IAV determine the outcome of infection. Alveolar epithelial cells (AEC), located in the lower respiratory tract, are comprised of two main cell types: type I and type II AEC. IAV specific antigen has been found in both types of AEC, as well as in alveolar macrophages. AEC respond in several ways to limit viral spreading. The IFN responses are the central component of innate immune control of viral infection. Once released, type I and III IFNs bind to their respective receptors on the same and neighboring cells. Receptor engagement subsequently leads to the activation of the IFN-stimulated regulatory factor 3 (ISGF3) transcription complex (Kotenko et al., 2003). The activation of this transcriptional activator complex leads to the increased expression of interferon-stimulated genes (ISGs), including 2',5'-oligoadenylate synthetase (OAS), Mx proteins, and protein kinase R (PKR), inducing an antiviral state (Zhou et al., 1999). PKR, OAS, and MxA have similar roles in their

ability to sense and degrade viral RNAs, and induce apoptosis of the infected cells (Jewell et al., 2010; Stark et al., 1998).

We have shown that RIG-I is critical for the initiation of the early antiviral cytokine response in the human lung using a human lung organ culture model (Wu et al., 2010, 2011, 2012). We have recently mastered the technique of isolation of highly purified human AEC (Langer et al., 2012). In this study, we will use our highly purified human AEC, as well as A549 cells, a widely used in vitro model for human type II alveolar epithelial cells, to examine the role of PRRs in the important type I and type III IFN anti-viral responses to IAV.

Results

RIG-I and TLR3, but not TLR7 and NOD2, are induced by IAV infection in A549 and type II AEC

We isolated and purified human AEC using standard methods as previously described (Langer et al., 2012). Isolation of human AEC is known to result in an initial culture consisting mainly of surfactant-producing AEC II cells (Fig. 1A), which differentiate into AEC I phenotype over 8–10 days in culture (Fig. 1B). Our isolated AEC I-like cells expressed the tight junction component zonula occludens-1 (ZO-1) (Fig. 1B). Differentiation of AEC II resulted in detection of the purinergic receptor P2X7, which was previously shown to be expressed primarily in rat AEC I (Chen et al., 2004). Flow cytometry data of AEC staining with α -human P2X7 determined that 73.2% of cells were positive for P2X7 (Fig. 1D). In subsequent experiments described below, AEC were cultured for

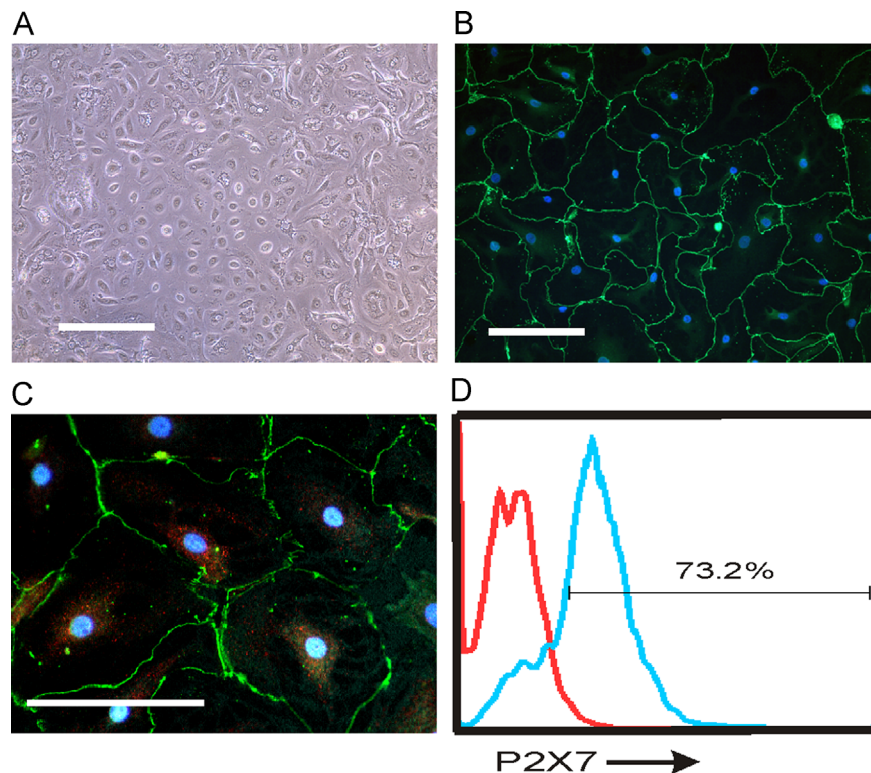


Fig. 1. Isolation and culture of primary human AEC. (A) Culture of isolated AEC cells at 4 days. The cells have AEC II morphology and produce surfactant, as expected. Bar = 250 μ m. (B) AEC II differentiate into AEC I-like cells. At 10 days in culture, cells show an increase in diameter, are confluent in areas, exhibit an AEC I-like morphology, and stain positive for the tight junction protein ZO-1. A representative field is shown with ZO-1 stained using α -human ZO-1 antibody labeled with Alexa Fluor-488 (green) and DNA stained with DAPI (blue). Bar = 100 μ m. (C) AEC II identity is confirmed by differentiation into primary human AEC I-like cells expressing the AEC I purinergic receptor P2X7. A representative field is shown with ZO-1 stained with α -human ZO-1 (green); DNA stained with DAPI (blue); and P2X7 stained with α -human purinergic receptor P2X7 antibody labeled with Alexa Fluor-555 (red). (D) Representative flow cytometry histogram of AEC staining with α -human P2X7 determined that 73.2% of cells were positive for P2X7. Blue line, P2X7-FITC labeled cells; Red line, isotype control labeled cells. Bar = 100 μ m.

approximately four days before viral infection in order to study the effects of IFN response to IAV in a primarily type II AEC phenotype.

To determine which PRRs are essential for the IFN response in human lung alveolar epithelia, A549 cells were infected by IAV PR8 at the MOI of 1. Viral diluent (PBS) was used for mock infection. After 24 h, RIG-I, TLR3, TLR7 and NOD2 mRNA induction in collected cells were assessed by qRT-PCR. PR8 infection of A549 cells (Fig. 2A) caused a 49 and 13 fold increase above mock-infected cells for RIG-I and TLR3 mRNA, respectively. There was minimum mRNA induction for TLR7 and NOD2. As A549 cells were originally derived from an alveolar adenocarcinoma, it cannot be excluded that they respond differently to external stimuli than primary cells. Therefore, to put our results with A549 cells into perspective, we repeated the experiments with our isolated primary human type II AEC. Similar to A549 cells, PR8 infection of AEC (Fig. 2B) caused a 27 and 12 fold increase above mock-infected cells for RIG-I and TLR3 mRNA, respectively. TLR7 and NOD2 were not induced by IAV in these cells. A dose response curve using different MOI of PR8 on A549 cells demonstrated that RIG-I and TLR3 mRNA induction was increased when higher IAV amounts were added to the cells, but TLR7 and NOD2 induction

did not occur at any IAV dose (Fig. 2C, and TLR7, NOD2 not shown). These experiments suggest that RIG-I and TLR3 gene expression is likely necessary for the cytokine response to IAV PR8 in human AEC, while TLR7 and NOD2 are not involved in the response in human lung alveolar epithelia.

Type III IFNs are the major antiviral cytokines induced by IAV infection in A549 and type II AEC

In order to determine the major IFN induced by IAV, IFN subtype and antiviral cytokine IP-10 gene expression was assessed in PR8 infected A549 cells. Although IFN- β mRNA was significantly induced by the virus, 27 fold over mock, IFN- λ 1 and IFN- λ 2/3 were induced to a much greater degree at 375 and 587 fold over mock, respectively (Fig. 3A). Again, we repeated the experiments with primary human type II AEC. PR8 infection induced IFN- β mRNA 7 fold over mock. However, IFN- λ 1 and IFN- λ 2/3 induction was more significant, and had a 63 and 27 fold increase over mock, respectively (Fig. 3B). In the same experiment, IP-10 mRNA induction in A549 and primary AEC was 45 and 18 fold increase over mock, respectively.

To confirm the result, we determined the IFN protein level in the supernatant of cultured cells. We were not able to detect any IFN- β protein in the supernatants using ELISA. Since the detection range of the ELISA is between 60 and 4000 pg/ml, the concentration of IFN- β is below 60 pg/ml in the supernatants. IFN- λ 2/3 protein was significantly increased in the supernatants at 190 and 290 pg/ml from A549 and AEC, respectively (Fig. 4A). The other important antiviral cytokine IP-10 was also detected in the supernatant at 163 and 142 pg/ml in A549 and AEC, respectively

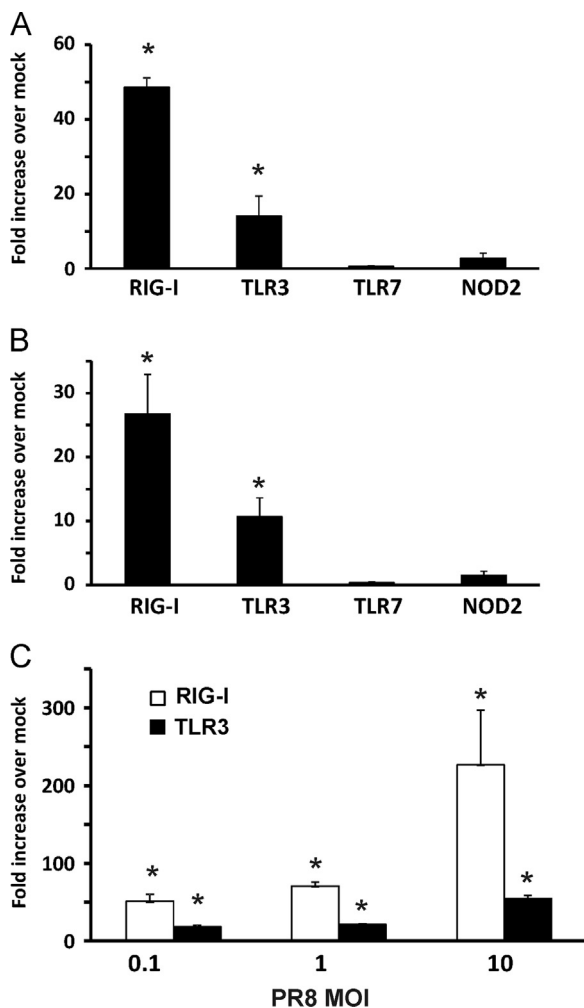


Fig. 2. RIG-I and TLR3, but not TLR7 and NOD2, are induced by influenza virus infection in A549 (A), type II AEC (B), and (C) PR8 Dose Response. After infection of A549 or AEC cells with IAV PR8 at an MOI of 1 for 24 h (A and B), total RNA was extracted and cell PRR mRNA expression was assessed by qRT-PCR. (C) A549 cells were infected by PR8 at increasing MOIs. Transcript levels of PRR were normalized relative to the constitutively expressed β -actin gene. Data were expressed as the means \pm SEM from three separate experiments. Statistical significance was determined by ANOVA. * denotes significant difference compared to data from the mock treated group, $P < 0.05$.

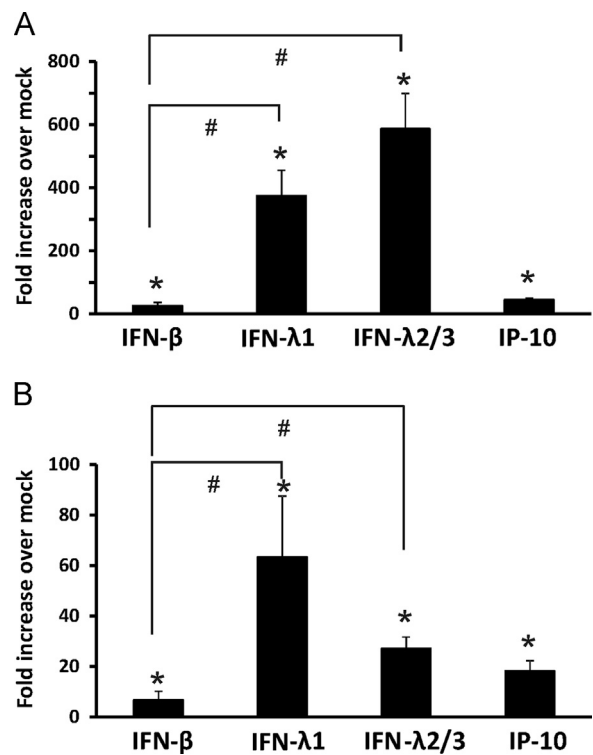


Fig. 3. Type III IFNs are the major antiviral cytokines induced by influenza virus infection in A549 (A) and type II AEC (B). After infection of A549 or AEC cells with influenza virus PR8 at the MOI of 1 for 24 h, total RNA was extracted and cell cytokine mRNA expression was assessed by qRT-PCR. Transcript levels of the cytokines were normalized relative to the constitutively expressed β -actin gene. Data were expressed as the means \pm SEM from three separate experiments. Statistical significance was determined by ANOVA. * denotes significant difference compared to data from the mock treated group, $P < 0.05$. # denotes significant difference between the indicated groups, $P < 0.05$.

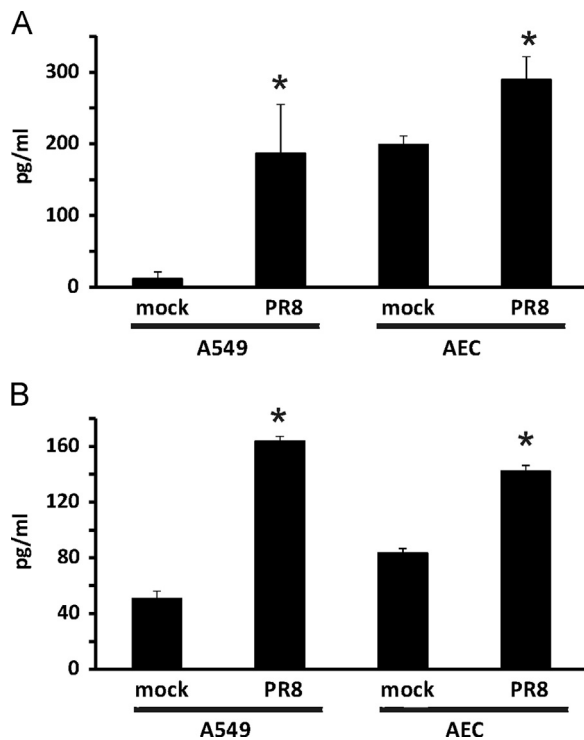


Fig. 4. IFN- λ 2/3 (A) and IP-10 (B) are the major antiviral cytokines detected in the supernatant of influenza virus infected A549 and type II AEC. After infection of A549 or AEC cells with influenza virus PR8 at the MOI of 1 for 24 h, supernatants were collected and cytokine expression was assessed by ELISA. Data were expressed as the means \pm SEM from three separate experiments. Statistical significance was determined by ANOVA. * denotes significant difference compared to data from the mock treated group, $P < 0.05$.

(Fig. 4B). In terms of the interferon response to IAV infection, these results demonstrate that type III IFN- λ responses are more prominent than type I IFN responses in human AEC.

Both RIG-I and TLR3 are required for IFN mRNA induction by IAV in human lung epithelial cells

In order to specifically silence RIG-I and TLR3 mRNA production in previously transfected A549 cells, we made use of siRIG-I and siTLR3 to knock down gene expression of these PRRs. First, we tested the inhibition efficiency of siRNA against RIG-I and TLR3. RIG-I and TLR3 mRNA induction by PR8 was blocked 89% and 85%, respectively, in the corresponding siRNA treated cells (Fig. 5A and B). We also confirmed RIG-I protein knockdown using western blot in the siRIG-I treated cells (Fig. 5C).

With regards to the role of specific PRR activation in cytokine induction, IFN- β mRNA induction was decreased only 55% in RIG-I siRNA treated cells compared to control siRNA treated cells (Fig. 6A). Surprisingly, IFN- β mRNA induction in TLR3 siRNA treated cells only showed 14% decrease compared to control siRNA treated cells.

Similarly, IFN- λ 1 mRNA induction in RIG-I and TLR3 single siRNA treated cells was decreased 75% and 41%, respectively, compared to control siRNA treated cells (Fig. 6B). Thus, single siRNA treatment of the cells did not completely block IFN production. However, double knockdown of RIG-I and TLR3 almost completely blocked the IFN induction by IAV, with 93% and 88% reduction of IFN- λ 1 and IFN- β mRNA induction, respectively. Regarding IFN- λ 2/3, IFN- λ 2/3 mRNA induction by PR8 appeared to be inhibited to a greater degree by double knockdown of RIG-I and TLR3 than by single knockdown of either PRR although the difference did not reach statistical significance (Fig. 6C). The results suggested that either RIG-I or TLR3 initiated IFN mRNA induction

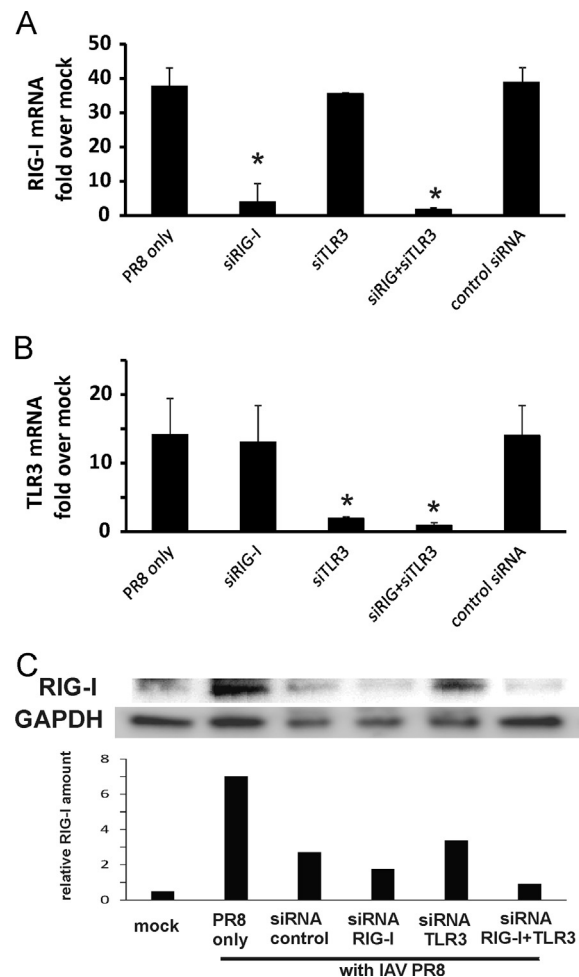


Fig. 5. Specific siRNA knocked down influenza-mediated RIG-I (A) and TLR3 mRNA (B) induction. A549 cells were first transfected with RIG-I or TLR3 siRNA for three days and then infected with influenza virus PR8 at a MOI of 1 for 24 h. Total RNA was extracted and mRNA expression was assessed by qRT-PCR. Transcript levels of mRNA were normalized relative to the constitutively expressed β -actin gene. (C) RIG-I protein in cells was detected by immunoblotting, normalized to GAPDH. Data were expressed as the means \pm SEM from three separate experiments. Statistical significance was determined by ANOVA. * denotes significant difference compared to data from the mock treated group, $P < 0.05$.

during IAV infection. For maximum induction of IFN, both RIG-I and TLR3 were required to be activated.

We confirmed the IFN- λ 1 and 2/3 induction at level of translation using ELISA. The protein levels for the most part reflected mRNA expression with a few differences. For instance, IFN- λ 1 protein in the supernatant was not inhibited by TLR3 siRNA, suggesting there may be a role for posttranscriptional regulation of IFN- λ 1 by TLR3. However, IFN- λ protein induction by IAV also required both RIG-I and TLR3 activation, as knockdown of both PRRs further inhibited IFN- λ (Fig. 7).

Both RIG-I and TLR3 contribute to activation of interferon regulatory factor 3 (IRF3) during IAV infection of human lung epithelial cells

Influenza virus-activated RIG-I binds to its adapter, MAVS, and activates specific signaling pathways that lead to phosphorylation of interferon regulatory factor 3 (IRF3) and subsequent production of type I and III IFN. The phosphorylated IRF3 (phospho-IRF3) then homodimerizes and enters the nucleus to activate IFN in a highly cooperative manner with NF- κ B (Seth et al., 2005; Wu et al., 2011). Thus, IRF3 is a major regulator of IFN production at the level of transcription. We examined phospho-IRF3 and total IRF3 protein

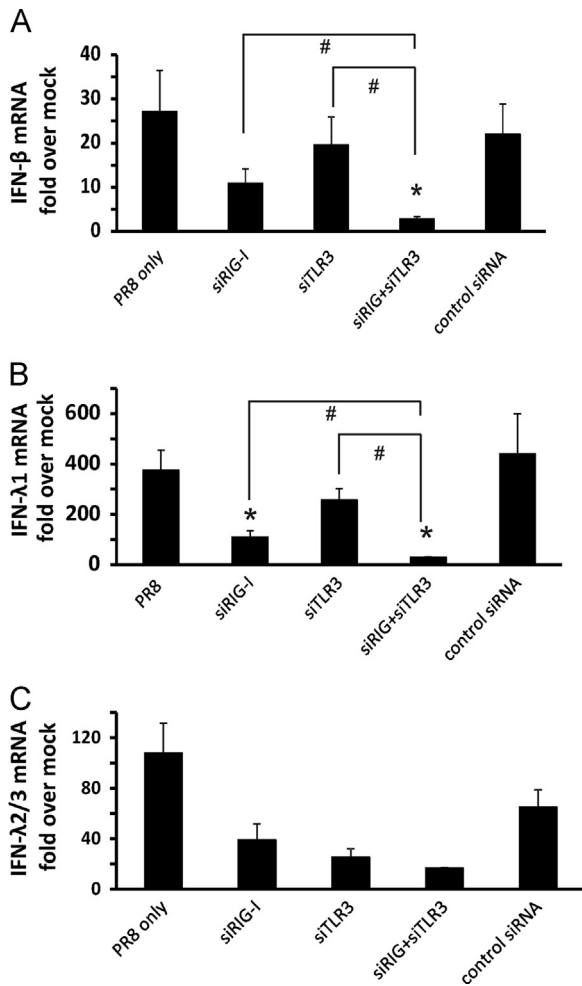


Fig. 6. Both RIG-I and TLR3 are required for IFN mRNA induction by influenza virus in human lung epithelial cells. A549 cells were first transfected with RIG-I or TLR3 siRNA for three days and then infected with influenza virus PR8 at the MOI of 1 for 24 h. Total RNA was extracted and mRNA expression was assessed by qRT-PCR. Transcript levels of mRNA were normalized relative to the constitutively expressed β -actin gene. Data were expressed as the means \pm SEM from three separate experiments. Statistical significance was determined by ANOVA. * denotes significant difference compared to data from the mock treated group, $P < 0.05$. # denotes significant difference between the indicated groups, $P < 0.05$.

levels by immunoblotting in siRNA treated IAV infected A549 cells. Phosphorylation of IRF3 occurred after IAV PR8 infection in control siRNA treated cells. The amount of phospho-IRF3 was reduced by RIG-I and TLR3 single siRNA treatment. However, maximal inhibition of IRF3 phosphorylation occurred only when RIG-I and TLR3 double knockdown was performed (Fig. 8). Thus, induction of the downstream pathway leading to IFN production involves both RIG-I and TLR3.

Discussion

Interaction of IAV with the innate immune system involves the induction of multiple PRR pathways triggered simultaneously by various pathogen-associated molecular patterns (PAMP) and different life cycles of the virus. RIG-I is identified as the central regulator of IAV-induced expression of antiviral cytokines in human lung epithelial cells (Matikainen et al., 2006). TLR3, in contrast, triggers proinflammatory cytokines in response to IAV in epithelial cells (Le Goffic et al., 2007). The detrimental contribution of TLR3 is supported by an animal experiment showing that

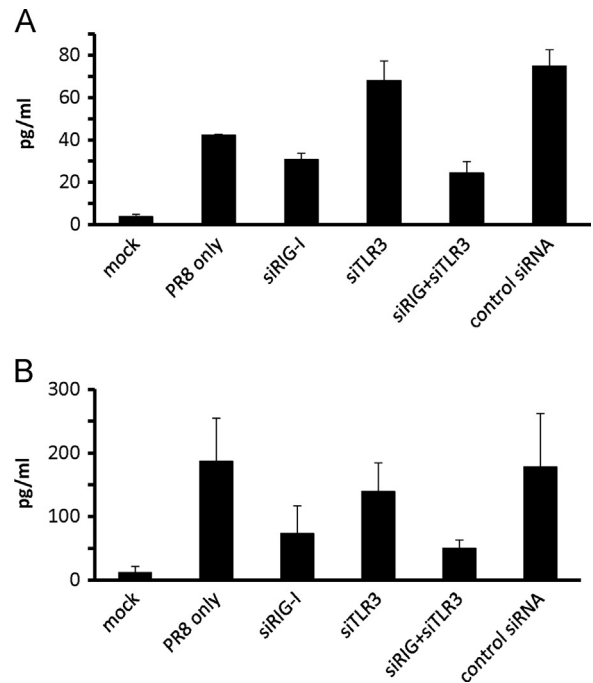


Fig. 7. Both RIG-I and TLR3 are required for IFN- λ 1 (A) and 2/3 (B) protein induction by influenza virus in human lung epithelial cells. A549 cells were first transfected with RIG-I or TLR3 siRNA for three days and then infected with influenza virus PR8 at an MOI of 1 for 24 h. Supernatants were collected and cytokine expression was assessed by ELISA. Data were expressed as the means \pm SEM from three separate experiments. Statistical significance was determined by ANOVA.

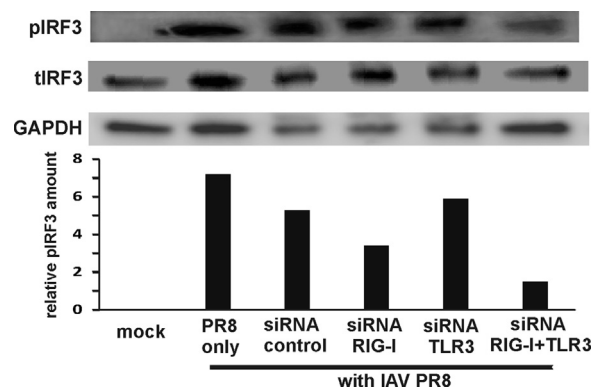


Fig. 8. Both RIG-I and TLR3 contribute to activation of interferon regulatory factor 3 (IRF3) during IAV infection in human lung epithelial cells. A549 cells were first transfected with RIG-I or TLR3 siRNA for three days and then infected with influenza virus PR8 at a MOI of 1 for 24 h. IRF3 phosphorylation in cells was detected by immunoblotting, normalized to GAPDH.

TLR3-deficient mice survive longer than wild-type mice following lethal IAV infection likely due to TLR3 induction of uncontrolled inflammation (Le Goffic et al., 2006). It is known that TLR3 is highly expressed in mouse innate immune cells, but has a low level of expression in human monocytes, macrophages and dendritic cells (DC) (Heinz et al., 2003). This may be responsible for conflicting results demonstrated in studies examining the role of TLR3 in the innate immune response using human and mouse models. Here, we have demonstrated that RIG-I and TLR3 are the two major PRRs induced by IAV infection in human A549 and type II AEC. Single knockdown of either RIG-I or TLR3 does not completely abolish IFN induction. Similarly, Slater and colleagues also found that inhibition of TLR3 and RIG-I by siRNA reduced IFN induction using rhinovirus infected human primary bronchial

epithelial cells (Slater et al., 2010). Our data differs in that we did not find that TLR3 induces RIG-I gene and protein expression. The data suggest that the possible interaction of RIG-I or TLR3 signaling pathways against IAV might be potentiating (synergistic), instead of weakening (hampering), in human AEC. Either of them may function as a backup for antiviral immunity when the other signaling mechanism is shut down. Single knockdown of TLR3 did not have as strong an effect on IFN induction as knockdown of RIG-I. There are two possibilities. First, TLR3 may play a lesser role in sensing flu as RIG-I. Alternately, RIG-I silencing overall may be more efficient than TLR3 silencing although this was not reflected at the level of transcription in the RIG-I and TLR3 mRNA assays.

All type I IFNs bind to a specific cell surface receptor complex known as the IFN- α receptor (IFNAR). Type III IFNs signal through a heterodimeric cell surface receptor composed of two chains: IFNLR1 and IL10R2 (Kotenko et al., 2003; Sheppard et al., 2003). The IFNAR complex is present on most if not all nucleated cells, whereas expression of the IFNLR1 subunit seems to be restricted to cells of epithelial origin (Brand et al., 2005). Consequently, antiviral protection by type I IFN is observed in most cell types, whereas antiviral protection mediated by type III IFN is restricted to cells that express functional IFNLR1/IL10R2 complexes. Recent experiments suggest that epithelial cells are the main targets of type III IFN in the mouse (Sommerreyns et al., 2008). An important issue to be addressed currently is whether type III IFN might serve a similar role in human epithelial cells as in mice. When type I and type III IFN receptors are expressed by a single cell line, triggering either receptor complex leads to the upregulation of the same set of ISGs (Zhou et al., 2007). Therefore, a major question is which types of IFNs are more redundantly produced in humans. In this report, we demonstrate that type III IFNs (IFN λ 1 and λ 2/3) are the major antiviral cytokines induced by IAV infection in human AEC. Combined with earlier reports from other investigators, type III IFNs are likely the principal IFNs produced during innate responses to RNA viruses in human respiratory epithelia (Khaïtov et al., 2009; Wang et al., 2009). The results suggest that human AEC might play a vitally important role in detection (through RIG-I and TLR3) and elimination (through type III IFN) of IAV.

Taken together, we have found that IAV-induced antiviral IFN responses require both RIG-I and TLR3 induction in human lung epithelia. TLR3 and RIG-I cooperate in mediating innate antiviral IFN responses in human AEC cells, which may contribute to the defense of the lung against viral infections. More work is needed in the future to address whether RIG-I and TLR3 compensation plays a role in influenza infection.

Materials and methods

Cell culture

The human pulmonary epithelial cell line, A549, was obtained from the American Type Culture Collection (ATCC, Manassas, Va., CCL-185). The A549 cell line has the characteristics of alveolar type II cells. A549 cells were propagated in Dulbecco's modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 80 μ g of gentamicin/ml.

Isolation of primary pneumocytes

One lobe of human lung obtained from the National Disease Research Interchange (NDRI, Philadelphia, PA) or the International Institute for the Advancement of Medicine (IIAM, Jessup, PA) under a protocol approved by the University of Oklahoma Health Sciences institutional review board, was perfused with 2 L Plasma-Lyte A Injection (Baxter Healthcare Corp., Deerfield, IL) followed by lavage

with 2 L physiological saline containing 100 μ g/ml of crude DNase from bovine pancreas (Sigma-Aldrich Co., St. Louis, MO). Approximately 55 g of tissue were injected with a digestion solution comprised of 8 U/ml porcine elastase (Worthington Biochemical Corp, Lakewood, NJ) and 150 KU/ml DNase in 150 ml Hanks Balanced Salt Solution (HBSS) without Ca^{2+} , Mg^{2+} or phenol red (Mediatech, Inc., Manassas, VA). After incubation at 37 °C for 60 min in a shaking water bath, the enzyme was inactivated by addition of 48 ml HyClone FBS (Thermo Fisher Scientific, Inc., Waltham, MA) plus 200 mg DNase. The softened tissue was cut into fine bits, and the slurry was passed sequentially through 1-ply gauze, 2-ply gauze, 150 μ m mesh (Small Parts, Inc., Logansport, IN), and 30 μ m mesh. Following concentration into 40 ml plain DMEM, 150 μ g/ml DNase, cells were panned for 60 min at 37 °C in 2 \times T-175 flasks previously coated with 20 μ g/cm² human IgG. Unattached cells were pelleted and resuspended in DMEM/DNase solution, and 2 ml were overlaid into each of 4 \times 30 ml Corex tubes, all containing 11 ml of Percoll solution $\rho = 1.040$ layered over 11 ml $\rho = 1.089$ (GE Healthcare Biosciences, Piscataway, NJ). The gradient was formed by centrifugation at 400 \times g for 20 min, 16 °C, without braking. The predominant band was negatively selected against CD45+ Dynabeads (Invitrogen Corp., Carlsbad, CA) for 40 min at 4 °C. Average yield was 5.7×10^7 live, single cells with 91% viability.

All cells were grown in AEC media consisting of DMEM, 10% Hyclone FBS, 1% pen/strep/Amphotericin B (Mediatech, Inc., Manassas, VA), 0.5 μ g/100 ml vancomycin, and 1.0 μ g/100 ml ceftazidime (both from Sigma-Aldrich Co., St. Louis, MO). Media was changed every 2 days. Freshly cultured cells were used for all experiments because freezing, storage and thawing led to low recovery of viable cells.

Preparation of influenza virus stock

H1N1 influenza virus, A/PR/34/8 (PR8), was passaged in Madin–Darby canine kidney (MDCK) cells. Virus was grown in MDCK cells in DMEM/F12 with ITS+ (BD Biosciences, Franklin Lakes, NJ) and trypsin, harvested at 72 h postinfection and titered by plaque assay in MDCK cells. There was no detectable endotoxin in the final viral preparations used in the experiments as determined by limulus amoebocyte lysate assay (Cambrex, Walkersville, MD). The lower limit of detection of this assay is 0.1 EU/ml or approximately 20 pg/ml LPS.

siRNA transfection into A549 cells

For siRNAs treatment, A549 cells were plated 24 h before treating with siRNAs (100 nM; Ambion). siRNA was diluted in 250 μ l of Opti-MEM medium and mixed gently. 5 μ l of Lipofectamine 2000 (Invitrogen) were added in 250 μ l Opti-MEM medium and incubated for 5 min. Diluted siRNA and Lipofectamine 2000 were combined and mixed gently and incubated for 20 min at RT. The siRNA-Lipofectamine 2000 complexes were added to each well and mixed gently. siRNA final concentration was 100 nM. The cells were incubated at 37 °C for 24 h. On-target plus siRNA to human RIG-I was from Thermo Scientific Dharmacon (Cat#J-012511-08). TLR3 silencer select siRNA was from Ambion (Cat#4390824).

Measurement of mRNA expression by quantitative real-time PCR (qRT-PCR)

Total RNA from cells was extracted using a modified TRIzol (Invitrogen, Carlsbad, CA) protocol, spectrophotometrically quantitated, and the integrity verified by formaldehyde agarose gel electrophoresis. Equal amounts (1 μ g) of RNA from each sample were used with oligo (dT) as primers for production of cDNA (SuperScript II First-Strand Synthesis System for RT-PCR, Invitrogen, Carlsbad, CA). Gene

specific primers for the PRRs, cytokines and the β -actin house-keeping genes were used. qRT-PCR was performed using 100 ng sample RNA and SYBR Green (Quanta Biosciences, Gaithersburg, MD) in a Bio-Rad CFX96™ Touch Real-Time PCR Detection System. Results were calculated and graphed from the Δ CT of the target gene and normalizer. The primers' sequences were as follows: RIG-I forward 5'-TCCTTTATGAGTATGTGGCA-3'; RIG-I reverse 5'-TCGGGCACAGAA-TATCTTTG-3'; IFN- β forward 5'-GCTCTCCTGTGTGCTTCTCCAC-3'; IFN- β reverse 5'-CAATAGTCTCATTCCAGCCAGTGC-3'; β -actin forward 5'-GCCAACCCGCGAGAAGATGACC-3'; β -actin reverse 5'-CTCCTAATG-TCACGCACGATTTC-3'; TLR3 forward 5'-GTCTGCGGAACATTTCTTTC-3'; TLR3 reverse 5'-GATTTAAACATTCCTTCTCGC-3'; NOD2 forward 5'-GAAGTACATCCGCACCGAG-3'; NOD2 reverse 5'-GACACCATCCAT-GAGAAGACAG-3'; TLR7 forward 5'-TGGAAATTGCCCTCGTTGTT -3'; TLR7 reverse 5'-GTCAGCGCATCAAAAGCATT-3'; IFN- λ 1 forward 5'-CGCCTTGAAGAGTCACTCA-3'; IFN- λ 1 reverse 5'-GAAGCCTCAGGTC-CCAATTC-3'; IFN- λ 2/3 forward 5'-AGTTCGGGGCTGTATCCAG-3'; IFN- λ 2/3 reverse 5'-GAGCCGGTACAGCCAATGGT-3'; IP-10 forward 5'-TCTAGAACCGTACGCTGTACTGC-3'; IP-10 reverse 5'-CTGGTTTA-AGGAGATCT-3'.

ELISA and multiplex immunoassay

ELISAs of IP-10 and IFN- β , λ 1, λ 2/3 cytokine protein levels in the supernatants were all performed using commercially available kits (R&D system, Minneapolis, MN).

RIG-I and IRF3 protein determination by immunoblotting

The cells were harvested and homogenized, and then lysed in 500 μ l of cold lysis buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 10 mM EDTA, NaF, sodium pyrophosphate, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 μ g of leupeptin/ml). Cell homogenates were clarified by centrifugation at 10,000 \times g, at 4 °C for 10 min, and the clarified lysates were mixed with SDS-PAGE sample buffer (60 mM Tris, pH 6.8, 10% glycerol, 2.3% SDS) and heated to 95 °C for 5 min. The samples were separated by 4–15% gradient gel and electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes. For the detection of proteins, the membranes were immunoblotted with rabbit polyclonal antibody specific for RIG-I, phospho-IRF3, total IRF3 (Abcam, Cambridge, MA) and GAPDH (R&D Systems). The membranes were viewed with horseradish peroxidase-labeled goat anti-rabbit IgG (Cell Signaling Technology, Beverly, MA) and chemiluminescent reagents (Pierce Biotechnology, Rockford, IL). Blots were developed using the Syngene G:box Bioimaging System and GeneTools software (Syngene, Frederick, MD) and quantified using ImageQuant software (BD/Molecular Dynamics, Bedford, MA).

Statistical analysis

Where applicable, the data have been expressed as the mean \pm standard error of the mean (SEM). Statistical significance was determined by one-way ANOVA with Student-Newman-Keuls *post-hoc* correction for multiple comparisons. Significance was considered as $P < 0.05$.

Disclosures

The authors have no financial conflicts of interest.

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