Innate Immune Response to Influenza A Virus in Differentiated Human Alveolar Type II Cells

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Alveolar Type II (ATII) cells are important targets for seasonal and pandemic influenza. To investigate the influenza-induced innate immune response in those cells, we measured the global gene expression profile of highly differentiated ATII cells infected with the influenza A virus at a multiplicity of infection of 0.5 at 4 hours and 24 hours after inoculation. Infection with influenza stimulated a significant increase in the mRNA concentrations of many host defense-related genes, including pattern/pathogen recognition receptors, IFN, and IFN-induced genes, chemokines, and suppressors of cytokine signaling. We verified these changes by quantitative real-time RT-PCR. At the protein level, we detected a robust virus-induced secretion of the three glutamic acid-leucine-arginine (ELR)-negative chemokines CXCL9, CXCL10, and CXCL11, according to ELISA. The ultraviolet inactivation of virus abolished the chemokine and cytokine response. Viral infection did not appear to alter the differentiation of ATII cells, as measured by cellular mRNA and concentrations of surfactant proteins. However, viral infection significantly reduced the secretion of surfactant protein (SP)-A and SP-D. In addition, influenza A virus triggered a time-dependent activation of phosphatidylinositol 3-kinase signaling in ATII cells. The inhibition of this pathway significantly decreased the release of infectious virus and the chemokine response, but did not alter virus-induced cell death. This study provides insights into influenza-induced innate immunity in differentiated human ATII cells, and demonstrates that the alveolar epithelium is a critical part of the initial innate immune response to influenza.

Keywords: human Type II cell; influenza; chemokine; PI3k; differentiation

Influenza A virus (IAV) is a major respiratory pathogen, and can cause widespread viral pneumonia as well as secondary bacterial pneumonia. Probably the major public health concern is avian H5N1 influenza, which produces a mortality rate of 50% in young adults. Avian influenza is thought to target alveolar epithelial cells because of the type of sialic acid linkages on the surface of epithelial cells (1, 2). However, seasonal as well as the recent swine-origin H1N1 of 2009 can also target alveolar epithelial cells (3, 4).

Most studies of respiratory viruses focus on bronchial epithelial cells, cell lines, or inflammatory cells. However, we recently developed a system to study respiratory viruses with primary cultures of adult human alveolar type II (ATII) cells (5). These cells produce surfactant, transport sodium from the

CLINICAL RELEVANCE

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apical surface into the interstitium to keep the alveoli relatively free of fluid, restore the epithelium after injury, and participate in the innate immune response. Our initial study with influenza infection in human ATII cells (6) showed that these cells secrete proinflammatory chemokines such as IL-8, IL-6, CCL5, and IFN- λ 1 (IL-29), a Type III IFN, in response to infection with influenza (6). However, this approach was narrowly focused, and did not examine many other components of the innate immune response.

Influenza also activates a variety of signaling pathways that may be subject to pharmacologic intervention, and these include mitogen-activated protein kinase, protein kinase C, Janus kinase and signal transducers and activators of transcription (JAK-STAT), and phosphatidylinositol 3-kinase (PI3k)/Akt pathways. Of these, the PI3k/Akt pathway may be the most specific for influenza, and is the most controversial (7). During infection with influenza, the PI3k/Akt pathway was initially described as an antiviral pathway, activated by IFN and participating in the regulation of mRNA translation in response to IFNs (7, 8). However, PI3k/Akt also favors the virus and promotes viral replication. For example, in A549 cells, a lung cancer cell line, the activation of PI3k/Akt was reported to promote viral entry and propagation (9). In the early stage of viral infection, PI3k interacts with viral nonstructural protein 1 (NS1) to prevent premature apoptosis, and in the late stage of infection, PI3k promotes apoptosis to facilitate the release of virus from infected cells (7, 10). Little is known about the role of the PI3k/Akt pathway in the response by human ATII cells to influenza.

To further our understanding of the role of human ATII cells in innate immunity and to define more fully their response to influenza, we performed a genome-wide microarray study in highly differentiated ATII cells with or without IAV infection. We verified changes in mRNA concentrations of pathogen recognition receptors (PRRs), IFN, IFN responsive genes, chemokines, cytokines, suppressors of cytokine signaling (SOCS), and surfactant genes. We found that IAV induced an extensive innate immune response in human ATII cells as early as 4 hours post inoculation (PI), which indicates that these cells play an important role in the early response to infection. Interestingly, infection with IAV did not alter mRNA and protein concentrations of surfactant proteins. However, the infection significantly reduced the secretion of surfactant protein (SP)-A and SP-D from virus-infected cells. To our knowledge, this is the first report of genome-wide changes induced by IAV in differentiated human ATII cells, which are important targets for both seasonal and pandemic flu. Because of the specific interaction of NS1 with

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the PI3k pathway, we further determined the effects of inhibiting the PI3k pathway on viral infection. PI3k signaling was found to be important for the production of chemokines and for the release of infectious virus, but not for IAV-induced cell death.

MATERIALS AND METHODS

Patients

De-identified patient lungs that were not suitable for transplantation and donated for medical research were obtained through the International Institute for the Advancement of Medicine (Edison, NJ) and the National Disease Research Interchange (Philadelphia, PA). The Committee for the Protection of Human Subjects at National Jewish Health approved this research. The patients used in this article included 14 men and 19 women with an average age of 46.5 years, comprising 13 current smokers, 3 ex-smokers, and 17 nonsmokers. The three patients for microarray experiments were nonsmokers (two men and one woman), whose ages were 46, 53, and 87 years.

Isolation and Culture of Human ATII Cells

Human ATII cells were isolated as previously described (5, 6, 11). The isolated cells were cultured as described previously (5, 6, 11), with modifications as outlined in the online supplement.

Virus Preparation

IAV A/PR/8/34 (PR/8) was grown in 10-day-old embryonated eggs, and prepared as described previously (6). For the ultraviolet (UV) inactivation of virus, 500 μ l of diluted PR/8 were placed in a 35-mm² Petri dish on ice and irradiated in a UV Stratalinker (Stratagene, La Jolla, CA) at a cumulative dose of 120 mJ/cm² twice. Inactivation of virus was confirmed by a plaque assay on the Mardin-Darby canine kidney epithelial cells (MDCK) monolayer, as described in the online supplement.

IAV Infection with Differentiated ATII Cells

Human ATII cells were cultured to achieve a differentiated phenotype. On Day 7 of culture, these cells were washed twice with Dulbecco's minimum essential medium, and inoculated with PR/8 at a multiplicity of infection (MOI) of 0.5 or the same amount of UV-inactivated PR/8. After 1 hour of inoculation, cells were washed twice, fresh medium were added to the culture, and cells were harvested at 4 hours and 24 hours PI. For time-course experiments, cells were infected with PR/8 at an MOI of 5 or 0.5, and cellular protein was harvested at designated times for the detection of signaling proteins.

For the inhibition experiments, cells were treated with the PI3k inhibitor LY294002 (20 μ M) or the extracellular signal-regulated kinase (ERK) inhibitor PD98059 (20 μ M) or DMSO (0.1%) as a vehicle control (Cell Signaling Technology, Danvers, MA), 1 hour before viral inoculation (MOI = 0.5). After the 1-hour viral inoculation, cells were washed and then cultured with the medium including inhibitor for an additional 24 hours.

Affymetrix Microarray Experiments

Total RNA from virus-infected and noninfected ATII cells was extracted using Trizol reagent, and purified through the digestion of genomic DNA. Samples were run on HG-U133 Plus 2.0 chips (Affymetrix, Santa Clara, CA), and processed as indicated by the manufacturer at the Microarray Core of the University of Colorado at Denver. A detailed description of the analyses of microarray data is provided in the online supplement.

Statistical Analyses

Statistical analyses were conducted in GraphPad Prism, version 5.0 (GraphPad Software, San Diego, CA). Pairwise comparisons were tested between infected and noninfected groups for significance, using a paired *t* test or Wilcoxon matched-pairs test. Comparisons among three or more groups were performed using one-way ANOVA with the Tukey *post hoc* test. Differences were considered significant at *P* < 0.05. Additional materials and methods are described in the online supplement.

RESULTS

Human ATII Cells Maintain a Differentiated Phenotype *In Vitro*

Previously, we developed a primary culture system to maintain human ATII cells (5). In the present study, we slightly modified the culture system, as described in the online supplement. We decreased the proportion of Matrigel from 60% to 20%, and changed the addition of differentiation factors to form a complete monolayer with minimal contamination of fibroblasts, alveolar macrophages (AMs), or endothelial cells (Figure 1A). According to immunocytochemistry, most cells expressed SP-A, proSP-B, and E-cadherin (Figures 1B–1D). These cells were negative for vimentin, CD68, CD45 and CD31 (data not shown).

Figure 1. Primary cultured human alveolar Type II (ATII) cells develop a differentiated phenotype *in vitro*. ATII cells were suspended in Dulbecco's minimum essential medium with 10% FBS and antibiotics, plated on inserts coated with 20% Matrigel and 80% rat-tail collagen for 2 days, and cultured with keratinocyte growth factor (K) for 2 days and KIAD for an additional 2 days. Cells were processed for phase microscopy (A, $\times 20$) and immunofluorescent staining with surfactant protein (SP)–A (B, red, $\times 40$), proSP-B (C, green, $\times 40$), and E-cadherin (D, red, $\times 40$). (E) The expression of ATII cell differentiation proteins were evaluated by Western blotting, and compared with freshly isolated ATII cells and cells cultured with 1% charcoal-stripped (CS)–FBS alone. Lane 1, freshly isolated ATII cells; lane 2, cells cultured with 1% CS–FBS; lane 3, cells cultured with K for 2 days, and KIAD for an additional 2 days. SP-B was run under nonreduced conditions. Protein loading was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Figure 2. Heat maps of influenza-induced cytokine and IFN-related genes in human ATII cells from microarray experiments. ATII cells isolated from lungs of three patients were cultured, and infected with influenza A/PR/8/34 (PR/8) virus. RNA from virus-infected and noninfected cells at 4 hours and 24 hours post inoculation (PI) were extracted and subjected to Affymetrix HG-U133 Plus 2.0 gene chip analyses. Responses of innate immune genes were clustered using the “heat map” function from R statistical software (R Foundation, Vienna, Austria). (A) Cytokine-related genes at 4 hours PI. (B) Cytokine-related genes at 24 hours PI. (C) IFN-related genes at 4 hours PI. (D) IFN-related genes at 24 hours PI.

The amount of contaminating cells varied with the different isolations, but the purity of epithelial cells was above 95%. Consistent with previous work, alveolar epithelial cells exhibited apical microvilli, and the lamellar bodies appeared preferentially distributed toward the apical surface according to electron microscopy (6). At the mRNA level, according to the microarrays, these cultured cells also expressed Clara cell secretory protein, also termed SCGB1A1 or CC10 (12), but they did not express podoplanin, the well-known alveolar Type I cell marker (13) (data not shown). To evaluate more fully the status of differentiation of cultured ATII cells, we measured the expression of proteins associated with pulmonary surfactant and lamellar bodies in freshly isolated ATII cells and cultured ATII cells. As shown in Figure 1E, the addition of keratinocyte growth factor for 2 days, and of keratinocyte growth factor, isomethy-

butylxanthine, 8Br-cAMP, and dexamethasone (KIAD) for an additional 2 days significantly increased the expression of all ATII markers, compared with basal media alone. The cultured cells expressed similar concentrations of SP-A, pro-SP-B, SP-B, pro-SP-C, SP-C, and pepsinogen II, an ATII cell-specific protease, important for the processing of SP-B (14), compared with freshly isolated cells. They also expressed a higher amount of ATP-binding cassette sub-family A member 3 (ABCA3) protein, which is required for lamellar body formation (15).

Infection with IAV Does Not Alter Surfactant Protein Expression, but Reduces Secretion of SP-A and SP-D from Human ATII Cells

To investigate whether infection with IAV alters the differentiation status of human ATII cells, we used Western blotting to

Figure 3. Verification of influenza A virus (IAV)-induced innate immune response in ATII cells at the mRNA and protein levels. ATII cells were cultured and infected with or without PR/8 virus. Cells were harvested at 4 hours and 24 hours PI for evaluation of the mRNA expression of innate immune response genes by real-time RT-PCR and the secretion of ELR-negative chemokine at 24 hours PI by ELISA. (A–E) Real-time RT-PCR. (A) ELR-negative CXC chemokines. (B) Suppressors of cytokine signaling (SOCSs). (C) Pathogen recognition receptors (PRRs). (D) IFN genes. (E) IFN-stimulated genes. The data show the expression level of each gene relative to the constitutive probe 36B4 (5). (F) Protein secretion of CXCL9–11 by ELISA. Open bars, noninfected cells; solid bars, virus-infected cells. * $P < 0.05$ and ** $P < 0.01$ versus noninfected cells; $n = 8–17$.

measure the protein expression of SP-A, SP-B, proSP-B, SP-C, pro-SP-C, SP-D, ABCA3, and pepsinogen II in virus-infected cells. We failed to detect consistent changes in expression of these

proteins at an MOI of 0.5 at 24, 48, and 72 hours PI in four different patients (data not shown). At the mRNA level, we did not find significant changes in these markers according to either

Figure 4. Ultraviolet (UV) inactivation of virus eliminates the IAV-induced response in ATII cells. ATII cells were infected with live PR/8 or UV-inactivated PR/8, and were harvested for the evaluation of mRNA changes of innate immune response-related genes at 4 hours and 24 hours by real-time RT-PCR, and for the evaluation of the secretion of IL-29 and CXCL10 at 24 hours PI by ELISA. (A) Real-time RT-PCR. Open bars, noninfected cells; solid bars, live virus-infected cells; striped bars, UV-inactivated virus-infected cells. (B) ELISA. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, versus noninfected cells; $n = 4$.

real-time RT-PCR or microarray experiments (data not shown). However, we observed a significant decrease in the secretion of SP-A and SP-D by ELISA at 24 hours PI (Figure E1).

Infection with IAV Significantly Increases mRNA Expression of Innate Immune Response Genes

To investigate the regulation of the host response induced by IAV infection in ATII cells, we performed global gene profiling in differentiated human ATII cells from three individuals at 4 hours and 24 hours PI with Affymetrix HG-U133 Plus 2.0 chips. Infection with IAV induced an extensive increase in mRNA concentrations of many genes involved in innate immunity, metabolism, RNA transcription, and cell signaling. Although more genes were changed by infection with IAV at 24 hours PI than at 4 hours PI (see gene lists in online supplement), we observed a consistent increase in most host defense-related genes at both time points, and therefore we clustered those genes into groups of cytokine-related and IFN-related genes (Figure 2). For selected genes related to chemokine regulation and IFN signaling, we verified the changes in mRNA concentrations by real-time quantitative RT-PCR in samples from 6–14 different patients (Figure 3).

As shown in Figures 2A and 2B, detailed analyses of increased host defense genes revealed that infection with IAV increased the mRNA concentrations of CC chemokines CCLs 2–5, C–X–C chemokines CXCL1, CXCL3, CXCL5, IL-8, CXCL10, and CXCL11, and CX3C chemokine CX3CL1 (fractalkine) in ATII cells at both 4 hours PI and 24 hours PI. Among these, CXCL11 and CXCL10 were the two genes with a more than 1,000-fold induction at both time points, as depicted in

Figure 3A. An increase was also evident in cytokines IL-6, IL-32, and IL-15 (Figures 2A and B). Infection with IAV also significantly increased the mRNA concentrations of three SOCS genes (SOCS1, SOCS2, and SOCS3) in ATII cells. However, the degree of increase was much lower than in the chemokine and IFN genes (Figures 2 and 3B). Moreover, infection with IAV increased IFN gene expression, as shown by IFNB1 (IFN- β) and three IFN- λ family genes (IL-29, IL-28A, and IL-28B) (Figures 2A, 2B, and 3D). Consistent with our previous results (6), infection with IAV did not induce any IFN- γ or IFN- α genes in ATII cells. As reported previously (6), we detected a significant amount of IL-29, but were unable to detect IFN- β protein in IAV-infected ATII cells with commercially available ELISAs (Figure 4 and data not shown). In contrast, we detected a specific increase in the IFN- α group genes in AMs from the same patient (data not shown). These results further demonstrate that IFN- λ is the main IFN protein produced by ATII cells in response to infection with IAV (6). As shown in Figures 2C and 2D, infection with IAV stimulated the high transcription of genes related to IFN signaling (which include PRRs) and IFN-induced genes such as IFN-induced protein with tetratricopeptide repeats (IFITs), guanylate binding protein, and the well-known antiviral genes myxovirus (influenza virus) resistance 1 (MX1) and 2'5' Oligoadenylates (OASs). Among three well known PRRs, the mRNA of retinoic-acid-inducible gene 1 (RIG-I), also termed DDX58, was increased the most, with a more than 100-fold increase after viral infection. Melanoma differentiation associated gene-5 (MDA-5), also termed IFIH1, and Toll-like receptor 3 (TLR3) were significantly increased (Figure 3C). Among antiviral genes,

Figure 5. Infection with IAV stimulates the phosphorylation (p) of Akt and ERK in human ATII cells. Cultured human Type II cells were infected with PR/8 virus at MOI of 5. At designated time points, the cellular protein was harvested and evaluated for the expression of phosphorylated and total Akt and ERK signaling by Western blotting. (A) Representative results from one of four patients. Lane 1, mock (no virus) or time 0; lane 2, 15 minutes after addition of virus; lane 3, 30 minutes after addition of virus; lane 4, 1 hour after addition of virus; lane 5, 4 hours after addition of virus; lane 6, 24 hours after addition of virus. (B) Quantification of relative expression of signal proteins in virus-infected ATII cells. ** $P < 0.01$, versus noninfected cells; *** $P < 0.001$, versus noninfected cells; $n = 4$.

IFIT1, also named ISG56, was increased more than 1,000-fold by infection with IAV at 4 hours PI, and the increase continued to 24 hours PI (Figure 3E). We also detected a significant increase of ISG56 protein according to Western blotting and immunofluorescent staining as early as 4 hours PI (data not shown).

IAV Infection Induces the Secretion of ELR-Negative Chemokines

Because the ELR-negative CXC chemokine family genes CXCL10 and CXCL11 are among the most induced genes by IAV infection in ATII cells, we chose this family for further study at the protein level. Consistent with observations from our microarray experiments, a significant induction of secretion of CXCL10 and CXCL11 proteins occurred in ATII cells after infection with IAV, although substantial variation is evident in the amount from patient to patient (Figure 3F). We also detected a significant induction of CXCL9 mRNA and protein in virus-infected ATII cells. However, the concentration of CXCL9 was much lower than of CXCL10 and CXCL11 (Figures 3A and 3F).

Viral Replication Is Required for IAV-Induced Responses

To determine whether viral replication is required to induce an innate immune response, we inactivated the infectivity of PR/8

by UV irradiation. As shown in Figure 4A, no significant increase was evident in the expression of the mRNA of IL-29, RIG-I, CXCL11, SOCS1, and ISG56 from ATII cells infected by UV-inactivated virus compared with mock control. Consistent with observations at the mRNA level, the secretion of IL-29 and CXCL10 was abolished by the UV inactivation of virus (Figure 4B).

Infection with IAV Activates PI3k Signaling, and PI3k Inhibitor Significantly Decreases Infectious Virus Release and Virus-Induced Chemokine Secretion

To investigate whether the IAV-induced innate immune response is dependent on the activation of PI3k signaling, we measured the phosphorylation of Akt, a commonly used marker for the activation of PI3k, in virus-infected ATII cells, using a higher MOI of infection. As shown in Figure 5, IAV (MOI = 5) increased the phosphorylation of Akt as early as 15 minutes after inoculation at both serine 473 and threonine 308, and the activation reached a peak at 24 hours PI. At MOI of 0.5, we observed similar effects (data not shown). IAV also induced the phosphorylation of ERK in ATII cells. However, we did not detect a significant activation of stress-activated protein kinase/c-Jun NH2-terminal kinase signaling (data not shown). Treatment with the PI3k-specific inhibitor LY294002 significantly

Figure 6. Inhibition of phosphatidylinositol 3-kinase (PI3k) decreases the release of influenza viral particles and the virus-induced chemokine response, but does not impair viral infection in ATII cells. Cultured human Type II cells were treated with PI3k inhibitor LY294002 and ERK inhibitor PD98059, 1 hour before inoculation with PR/8 virus. After infection, cells were continually cultured with inhibitors. At 24 hours PI, cells were fixed for fluorescent staining with murine anti-nucleoprotein of IAV. Cell supernatants were evaluated for the release of infectious virus by standard plaque assay and secretion of CXCL9, CXCL10, CXCL11, and IL-29 by ELISA. (A) Secretion of chemokines. Data are expressed as percentage of secretion with virus alone. (B) Release of infectious virus. (C) Immunofluorescent stain with influenza A nucleoprotein. * $P < 0.05$, versus virus treatment alone. *** $P < 0.001$, versus virus treatment alone.

decreased the secretion of ELR-negative chemokines CXCLs 9–11, but not IL-29 (Figure 6A). The inhibition of PI3k also significantly decreased the release of infectious virus from infected ATII cells at 24 hours PI (Figure 6B). The ERK inhibitor PD98059 decreased the secretion of three ELR-negative

chemokines to a lesser degree than the PI3k inhibitor, but did not reduce viral release (Figures 6A and 6B). We did not find any effects of either inhibitor on the percentage of cells infected with influenza (data not shown). However, as indicated in Figure 6C, more nuclear accumulation of viral nucleoprotein (NP) may occur in PI3k inhibitor-treated cells than in cells inoculated with PR/8 alone or in ERK inhibitor-treated ATII cells.

Infection with IAV Induces Activation of Caspase-3 and Poly (ADP-Ribose) Polymerase Cleavage in ATII Cells, Independent of PI3k Activation

Beyond the IFN and inflammatory signaling pathways, detailed analyses of gene chip data revealed that infection with IAV also activated the cell-death pathway in human ATII cells, including an increase of mRNA concentrations of several poly (ADP-ribose) polymerase (PARP) family members: PARP12 (8.2-fold), PARP14 (5-fold), PARP8 (2.3-fold), and PARP9 (3.9-fold). Therefore, we evaluated the activation of caspase-3 and its downstream molecule PARP in IAV-infected human ATII cells at 24 hours PI by Western blotting. As shown in Figure 7A, we detected a consistently active form of caspase-3 (cCasp3) in live virus-infected cells only, although we did not see a change of caspase-3 mRNA attributable to viral infection (data not shown). Significant variation was evident in the activation of PARP between patients. However, the ratio of cleaved/uncleaved forms of PARP (cPARP/PARP) was much higher in live virus-infected wells than in control wells (Figure 7B). No difference was evident between UV-inactivated virus-infected cells and control cells, and we did not see any difference with or without treatment using the PI3k inhibitor. These results suggest that infection with IAV activated caspase-3-involved cell death, independent of the activation of PI3k signaling. In addition, UV-inactivation abolished viral NP expression, but the PI3k inhibitor did not (Figure 7), further supporting the conclusion that PI3k inhibition did not alter the rate of infection, as shown in Figure 6C.

DISCUSSION

ATII cells play an important role in protecting the alveolar microenvironment through synthesizing and secreting pulmonary surfactant, regulating alveolar fluid volume and acting as progenitors for Type I cells. In addition, alveolar and bronchial epithelial cells provide an important part of the innate immune system in the lung. These cells are likely the first cells to encounter inhaled or aspirated organisms or particles. They express a wide variety of PRRs, and can initiate a significant chemokine or IFN response. Finally, they are ultimately responsible for restoring the epithelium after injury.

In the present study, we slightly modified the system described previously (5, 6), so that cells formed an intact monolayer but retained their Type II phenotype. We used a genome-wide approach to define more completely the innate immune response of ATII cells to IAV. To our knowledge, this is the first report to investigate global gene expression in differentiated human ATII cells in response to a viral infection. At this level of infection (MOI = 0.5), IAV does not appear to alter the differentiation of ATII cells, as evidenced by the absence of change in mRNA and protein levels of surfactant genes, ABCA3, and pepsinogen II at 24 hours PI. However, infection decreased the secretion of SP-A and SP-D (Figure E1), which are important pulmonary collectins (16). Because of the well-known functions of SP-A and SP-D against diverse microbes (16, 17), this result suggests that low-dose infection with IAV may weaken the host defense of the lung to other pathogens, and perhaps promote secondary infections.

Figure 7. UV inactivation of virus, but not PI3k inhibitor, abolishes IAV-induced activation of caspase-3 and poly (ADP-ribose) polymerase (PARP). Cultured human Type II cells were infected with live and UV-inactivated PR/8 virus. The cellular protein from attached and detached cells from the same well was harvested and evaluated for the expression of viral nucleoprotein (NP), caspase-3 (Casp3), and PARP at 24 hours PI. Some cells were treated with PI3k inhibitor LY294002, 1 hour before inoculation, and were incubated with the inhibitor throughout the infection. (A) Representative results from one of three patients. Lane 1, control cells; lane 2, live PR/8 virus-infected cells; lane 3, UV-inactivated PR/8 virus-infected cells; lane 4, PI3k inhibitor and live PR/8 virus-treated cells. (B) Quantification of relative expression of proteins after they were normalized to expression of GAPDH in each condition ($n = 3$).

The secretion of chemokines and cytokines to recruit circulating neutrophils, mononuclear cells, and lymphocytes into the lung comprises an important strategy by the host to clear lung infection. Chemokines constitute a superfamily of small proteins, and can be divided into four functionally distinct groups: CC, CXC, C, and CX3C. CXC chemokines can be further subdivided into ELR-positive and ELR-negative, according to the existence of a specific amino-acid sequence of glutamic acid-leucine-arginine. ELR-positive chemokines, such as IL-8, specifically induce the migration of neutrophils. ELR-negative CXC chemokines, such as CXCL10, tend to be chemoattractants for lymphocytes. We and other researchers reported that alveolar epithelial cells secrete CCL5, IL-8, IL-6, CCL2, macrophage inflammatory protein (MIP)-1, and CXCL10 in response to infection with IAV (18, 19). In this study, infection with IAV significantly increased or induced the expression of many genes from all four chemokine groups in human ATII cells, and the effect was dependent on viral replication. Specifically, infection with IAV induced all three ELR-negative CXC chemokines, CXCL9–11, at both the mRNA and protein levels. These three CXC chemokines bind to a common receptor chemokine (C–X–C motif) receptor 3 (CXCR3), and activate multiple functions of CD8⁺ T cells. Recently, the importance of CXCR3 signaling was shown in the pathogenesis of several viral infections, including influenza (20–24). Among those chemokines, CXCL10 was highly expressed in avian flu virus H5N1-infected ferrets, macaques, monkeys, and human cells (19–21), and is viewed as a prognostic marker for several viral infections (23–26). The actual role of CXCL11 in viral infections remains unclear. In mice during infection with IAV, the peak level of CXCL11 mRNA coincides with the peak of viremia, and the CXCL11 protein was reported to inhibit viral growth (27). CXCL11 also directly inhibits respiratory adenovirus serotypes Ad3 and Ad5 (28). The induction of CXCL9 was also reported in similar viral infections (21–23, 25). Interestingly, recent investigations indicate a differential regulation of CXCL9–11 chemokines in hepatitis C virus in-

fection, and the differential expression of CXCL9–11 correlates with different disease phenotypes (21, 25). Therefore, the role of each CXCR3 chemokine in IAV infection in human ATII cells deserves further study. In addition, human fetal ATII cells express the ELR-negative chemokine receptor CXCR3. All three CXCR3 ligands, and especially CXCL11, can induce epithelial cell chemotaxis and proliferation and perhaps epithelial wound repair during the resolution of viral infections (29). CXCL11 also stimulates re-epithelization of the skin during wound repair (30). In addition, recent reports showed that ELR-negative chemokines have antimicrobial activity against Gram-positive and Gram-negative bacteria (31, 32). Taken together, the high induction of ELR-negative chemokines in ATII cells suggests that this family of proteins may play an important role in viral infection, secondary bacterial infection after influenza, and epithelial repair in the human lung.

The inhibition of PI3k by infection with IAV significantly decreased the secretion of all three ELR-negative chemokines CXCL9–11 in ATII cells. However, we did not observe a consistent effect on the secretion of IL-29. ELR-negative chemokines are known to be induced by IFN, and infection with IAV induces an abundant IFN response, especially by IFN- λ 1 (IL-29) (6) (Figures 3 and 4). Treatment with IL-29 also induces the secretion of CXCL10 in human Type II cells (data not shown). To investigate the possible mechanism of the PI3k inhibitor in virus-induced chemokine regulation, we measured the mRNA expression of IFN and SOCS genes in inhibitor-treated cells, and did not find significant differences between inhibitor-treated cells and control cells. These results suggest that the inhibition of CXCL9–11 production in IAV-infected ATII cells by the PI3k inhibitor may be more attributable to the direct decrease of viral propagation, and independent of IFN signaling in human ATII cells.

PRRs are critical elements in the pathways for stimulating the production of IFN and an antiviral immune response. TLR3 and TLR7 and RNA helicases RIG-I and MDA-5 are the best-characterized sensors of RNA viruses, including influenza (33).

We compared the expression of these four PRRs in ATII cells and AMs from the same patient. In ATII cells, infection with IAV increased the mRNA of three PRRs: RIG-I, MDA-5, and TLR3 (Figures 2 and 3). The increase of RIG-I was most prominent compared with MDA-5 and TLR3, suggesting that RIG-I may be the main PRR responsible for stimulating the production of IFN in ATII cells. TLR7 was increased by viral infection in AMs, but not ATII cells (data not shown). Consistent with our previous study (6), IAV stimulated the production of predominant Type III IFN, as also observed in human ATI-like cells (34). Infection also upregulated many IFN-stimulated genes, including the well-known antiviral MX1, OAS, and ISG genes (Figures 2 and 3). Among those IFN-stimulated genes, ISG56 showed the largest increase at the mRNA level after infection with IAV in ATII cells (Figure 3E). We also detected the induction of ISG56 protein as early as 4 hours after infection with IAV or treatment with IFN- λ 1 (IL-29) (data not shown). Although ISG56 is one of the first identified proteins induced by viruses and Type I IFN (35), the function of ISG56 remains unclear. ISG56 was implicated in antiviral actions against hepatitis C virus, West Nile virus, lymphocytic choriomeningitis virus, and human papillomavirus (36–38). However, a recent study by Li and colleagues (39) suggests that ISG56 is a negative regulator of IFN production and antiviral response.

In fighting against the host defense, a number of viruses evolved mechanisms that interfere with the induction of cytokines or the function of IFN-induced antiviral proteins (40–42). As a negative regulatory mechanism used by viruses, SOCSs play a pivotal role in the control of cytokine response (42–44). The SOCS gene family includes eight members (SOCS1–SOCS7 and CIS) that bind to tyrosine kinases of the JAK elements, resulting in the reduction of JAK enzymatic activity and inhibition of the tyrosine phosphorylation of JAK/STAT factors (40). As expected, infection with IAV induced the mRNA expression of SOCS family members SOCS 1–3 in ATII cells after infection. However, the specific role of each SOCS remains unclear.

NS1 is a critical protein that IAV uses to hijack host defense factors (45). NS1 suppresses the IFN response by interfering with signaling inducers and mediators (42, 46, 47). Although PI3k/Akt is an important pathway involved in the regulation of cell growth, proliferation, and survival (7), during infection with influenza, this pathway is used by NS1 to promote infection (7, 45, 48). To investigate whether PI3k plays an important role in influenza infection in differentiated ATII cells, we measured the phosphorylation of Akt, a commonly used marker for the activation of PI3k, in virus-infected cells. Consistent with other studies (8, 48), we detected a time-dependent activation of PI3k signaling in IAV-infected ATII cells (Figure 5). The inhibition of PI3k by chemical inhibitor LY294002 significantly decreased the release of infectious viral particles (Figure 6B), suggesting that the activation of PI3k is required for the release of progeny virus. However, the inhibition of PI3k did not inhibit viral infection or viral NP protein expression (Figures 6C and 7), implying that PI3k may not be important for viral entry into human ATII cells. This result differs from that in a previous report on IAV-infected A549 cells, where the inhibition of PI3k decreased the expression of NP (8). In addition, infection with IAV induced the activation of caspase-3 and the cleavage of its downstream protein PARP in ATII cells. In contrast with previous reports that NS1 activates PI3k to prevent cell apoptosis (49), our results (Figure 7) suggest that PI3k may not be involved in IAV-induced cell death in ATII cells, a finding supported by Shin and colleagues (8) and Jackson and colleagues (50). The controversial finding of PI3k signaling in IAV infection in our study and elsewhere suggests the

complexity of the influenza-induced innate immune response. Although the precise role of PI3k/Akt signaling needs to be determined further, our results from highly differentiated human ATII cells shed light on the mechanism of human lung alveolar epithelium in response to infection with IAV.

In conclusion, we studied the gene profile of highly differentiated human ATII cells in response to a low-dose influenza infection. Infection with IAV induced a robust innate immune response, with a predominant induction of ELR-negative CXC chemokines in human ATII cells. Infection with IAV at MOI of 0.5 did not appear to alter the differentiation of ATII cells, but it reduced the secretion of SP-A and SP-D. The PI3k/Akt pathway appears to play a critical role in virus-induced chemokines response and virus release, but may not involve virus-induced cell death in ATII cells.

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References

1. Ibricevic A, Pekosz A, Walter MJ, Newby C, Battaile JT, Brown EG, Holtzman MJ, Brody SL. Influenza virus receptor specificity and cell tropism in mouse and human airway epithelial cells. *J Virol* 2006;80:7469–7480.
2. Nicholls JM, Chan MC, Chan WY, Wong HK, Cheung CY, Kwong DL, Wong MP, Chui WH, Poon LL, Tsao SW, *et al.* Tropism of avian influenza A (H5N1) in the upper and lower respiratory tract. *Nat Med* 2007;13:147–149.
3. Chan MC, Chan RW, Yu WC, Ho CC, Yuen KM, Fong JH, Tang LL, Lai WW, Lo AC, Chui WH, *et al.* 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. *Am J Pathol* 2010;176:1828–1840.
4. Itoh Y, Shinya K, Kiso M, Watanabe T, Sakoda Y, Hatta M, Muramoto Y, Tamura D, Sakai-Tagawa Y, Noda T, *et al.* *In vitro* and *in vivo* characterization of new swine-origin H1N1 influenza viruses. *Nature* 2009;460:1021–1025.
5. Wang J, Edeen K, Manzer R, Chang Y, Wang S, Chen X, Funk CJ, Cosgrove GP, Fang X, Mason RJ. Differentiated human alveolar epithelial cells and reversibility of their phenotype *in vitro*. *Am J Respir Cell Mol Biol* 2007;36:661–668.
6. Wang J, Oberley-Deegan R, Wang S, Nikrad M, Funk CJ, Hartshorn KL, Mason RJ. Differentiated human alveolar Type II cells secrete antiviral IL-29 (IFN- λ 1) in response to influenza A infection. *J Immunol* 2009;182:1296–1304.
7. Ehrhardt C, Ludwig S. A new player in a deadly game: influenza viruses and the PI3k/Akt signalling pathway. *Cell Microbiol* 2009;11:863–871.
8. Shin YK, Liu Q, Tikoo SK, Babiuk LA, Zhou Y. Effect of the phosphatidylinositol 3-kinase/Akt pathway on influenza A virus propagation. *J Gen Virol* 2007;88:942–950.
9. Kaur S, Sassano A, Joseph AM, Majchrzak-Kita B, Eklund EA, Verma A, Brachmann SM, Fish EN, Platanius LC. Dual regulatory roles of phosphatidylinositol 3-kinase in IFN signaling. *J Immunol* 2008;181:7316–7323.
10. Hale BG, Kerry PS, Jackson D, Precious BL, Gray A, Killip MJ, Randall RE, Russell RJ. Structural insights into phosphoinositide 3-kinase activation by the influenza A virus NS1 protein. *Proc Natl Acad Sci USA* 2010;107:1954–1959.
11. Mossel EC, Wang J, Jeffers S, Edeen KE, Wang S, Cosgrove GP, Funk CJ, Manzer R, Miura TA, Pearson LD, *et al.* SARS-CoV replicates in primary human alveolar Type II cell cultures but not in Type I-like cells. *Virology* 2008;372:127–135.
12. Boers JE, Amberg AW, Thunnissen FB. Number and proliferation of Clara cells in normal human airway epithelium. *Am J Respir Crit Care Med* 1999;159:1585–1591.
13. Ramirez MI, Millien G, Hinds A, Cao Y, Seldin DC, Williams MC. T1alpha, a lung Type I cell differentiation gene, is required for normal

- lung cell proliferation and alveolus formation at birth. *Dev Biol* 2003; 256:61–72.
14. Foster C, Aktar A, Kopf D, Zhang P, Guttentag S. Pepsinogen C: a Type 2 cell-specific protease. *Am J Physiol Lung Cell Mol Physiol* 2004; 286:L382–L387.
 15. Cheong N, Zhang H, Madesh M, Zhao M, Yu K, Dodia C, Fisher AB, Savani RC, Shuman H. ABCA3 is critical for lamellar body biogenesis *in vivo*. *J Biol Chem* 2007;282:23811–23817.
 16. Kuroki Y, Takahashi M, Nishitani C. Pulmonary collectins in innate immunity of the lung. *Cell Microbiol* 2007;9:1871–1879.
 17. Orgeig S, Hiemstra PS, Veldhuizen EJ, Casals C, Clark HW, Haczku A, Knudsen L, Possmayer F. Recent advances in alveolar biology: evolution and function of alveolar proteins. *Respir Physiol Neurobiol* 2010;173:S43–S54.
 18. Herold S, von Wulffen W, Steinmueller M, Pleschka S, Kuziel WA, Mack M, Srivastava M, Seeger W, Maus UA, Lohmeyer J. Alveolar epithelial cells direct monocyte transepithelial migration upon influenza virus infection: impact of chemokines and adhesion molecules. *J Immunol* 2006;177:1817–1824.
 19. Chan MC, Cheung CY, Chui WH, Tsao SW, Nicholls JM, Chan YO, Chan RW, Long HT, Poon LL, Guan Y, *et al.* Proinflammatory cytokine responses induced by influenza A (H5N1) viruses in primary human alveolar and bronchial epithelial cells. *Respir Res* 2005;6:135.
 20. Cameron CM, Cameron MJ, Bermejo-Martin JF, Ran L, Xu L, Turner PV, Ran R, Danesh A, Fang Y, Chan PK, *et al.* Gene expression analysis of host innate immune responses during lethal H5N1 infection in ferrets. *J Virol* 2008;82:11308–11317.
 21. Baskin CR, Bielefeldt-Ohmann H, Tumpey TM, Sabourin PJ, Long JP, Garcia-Sastre A, Tolnay AE, Albrecht R, Pyles JA, Olson PH, *et al.* Early and sustained innate immune response defines pathology and death in nonhuman primates infected by highly pathogenic influenza virus. *Proc Natl Acad Sci USA* 2009;106:3455–3460.
 22. Helbig KJ, Ruskiewicz A, Lanford RE, Berzsenyi MD, Harley HA, McColl SR, Beard MR. Differential expression of the CXCR3 ligands in chronic hepatitis C virus (HCV) infection and their modulation by HCV *in vitro*. *J Virol* 2009;83:836–846.
 23. Larrubia JR, Benito-Martinez S, Calvino M, Sanz-de-Villalobos E, Parra-Cid T. Role of chemokines and their receptors in viral persistence and liver damage during chronic hepatitis C virus infection. *World J Gastroenterol* 2008;14:7149–7159.
 24. Thapa M, Carr DJ. CXCR3 deficiency increases susceptibility to genital herpes simplex virus Type 2 infection: uncoupling of CD8⁺ T-cell effector function but not migration. *J Virol* 2009;83:9486–9501.
 25. Zeremski M, Dimova R, Brown G, Jacobson IM, Markatou M, Talal AH. Peripheral CXCR3-associated chemokines as biomarkers of fibrosis in chronic hepatitis C virus infection. *J Infect Dis* 2009;200: 1774–1780.
 26. Fife BT, Kennedy KJ, Paniagua MC, Lukacs NW, Kunkel SL, Luster AD, Karpus WJ. CXCL10 (IFN- γ -inducible protein-10) control of encephalitogenic CD4⁺ T cell accumulation in the central nervous system during experimental autoimmune encephalomyelitis. *J Immunol* 2001;166:7617–7624.
 27. Hamilton NH, Mahalingam S, Banyer JL, Ramshaw IA, Thomson SA. A recombinant vaccinia virus encoding the IFN-inducible T-cell alpha chemoattractant is attenuated *in vivo*. *Scand J Immunol* 2004;59:246–254.
 28. Harvey SA, Romanowski EG, Yates KA, Gordon YJ. Adenovirus-directed ocular innate immunity: the role of conjunctival defensin-like chemokines (IP-10, I-TAC) and phagocytic human defensin- α . *Invest Ophthalmol Vis Sci* 2005;46:3657–3665.
 29. Ji R, Lee CM, Gonzales LW, Yang Y, Aksoy MO, Wang P, Brailoiu E, Dun N, Hurford MT, Kelsen SG. Human Type II pneumocyte chemotactic responses to CXCR3 activation are mediated by splice variant A. *Am J Physiol Lung Cell Mol Physiol* 2008;294:L1187–L1196.
 30. Yates CC, Whaley D, A YC, Kulesekar P, Hebda PA, Wells A. ELR-negative CXC chemokine CXCL11 (IP-9/I-TAC) facilitates dermal and epidermal maturation during wound repair. *Am J Pathol* 2008; 173:643–652.
 31. Cole AM, Ganz T, Liese AM, Burdick MD, Liu L, Strieter RM. Cutting edge: IFN-inducible ELR⁺ CXC chemokines display defensin-like antimicrobial activity. *J Immunol* 2001;167:623–627.
 32. Tohyama M, Sayama K, Komatsuzawa H, Hanakawa Y, Shirakata Y, Dai X, Yang L, Tokumaru S, Nagai H, Hirakawa S, *et al.* CXCL16 is a novel mediator of the innate immunity of epidermal keratinocytes. *Int Immunol* 2007;19:1095–1102.
 33. Thompson AJ, Locarnini SA. Toll-like receptors, RIG-I-like RNA helicases and the antiviral innate immune response. *Immunol Cell Biol* 2007;85:435–445.
 34. Lee SM, Chan RW, Gardy JL, Lo CK, Sihoe AD, Kang SS, Cheung TK, Guan YI, Chan MC, Hancock RE, *et al.* Systems-level comparison of host responses induced by pandemic and seasonal influenza A H1N1 viruses in primary human Type I-like alveolar epithelial cells *in vitro*. *Respir Res* 2010;11:147–156.
 35. Sen GC, Sarkar SN. The IFN-stimulated genes: targets of direct signaling by IFNs, double-stranded RNA, and viruses. *Curr Top Microbiol Immunol* 2007;316:233–250.
 36. Wang C, Pflugheber J, Sumpter R Jr, Sodora DL, Hui D, Sen GC, Gale M Jr. Alpha IFN induces distinct translational control programs to suppress hepatitis C virus RNA replication. *J Virol* 2003;77:3898–3912.
 37. Wachter C, Muller M, Hofer MJ, Getts DR, Zabarar S, Ousman SS, Terenzi F, Sen GC, King NJ, Campbell IL. Coordinated regulation and widespread cellular expression of IFN-stimulated genes (ISG) ISG-49, ISG-54, and ISG-56 in the central nervous system after infection with distinct viruses. *J Virol* 2007;81:860–871.
 38. Terenzi F, Saikia P, Sen GC. IFN-inducible protein, p56, inhibits HPV DNA replication by binding to the viral protein E1. *EMBO J* 2008;27: 3311–3321.
 39. Li Y, Li C, Xue P, Zhong B, Mao AP, Ran Y, Chen H, Wang YY, Yang F, Shu HB. ISG56 is a negative-feedback regulator of virus-triggered signaling and cellular antiviral response. *Proc Natl Acad Sci USA* 2009;106:7945–7950.
 40. Hashimoto K, Ishibashi K, Ishioka K, Zhao D, Sato M, Ohara S, Abe Y, Kawasaki Y, Sato Y, Yokota S, *et al.* RSV replication is attenuated by counteracting expression of the suppressor of cytokine signaling (SOCS) molecules. *Virology* 2009;391:162–170.
 41. Katze MG, He Y, Gale M Jr. Viruses and IFN: a fight for supremacy. *Nat Rev Immunol* 2002;2:675–687.
 42. Garcia-Sastre A. Inhibition of IFN-mediated antiviral responses by influenza A viruses and other negative-strand RNA viruses. *Virology* 2001;279:375–384.
 43. Alexander WS, Hilton DJ. The role of suppressors of cytokine signaling (SOCS) proteins in regulation of the immune response. *Annu Rev Immunol* 2004;22:503–529.
 44. Yoshimura A, Naka T, Kubo M. SOCS proteins, cytokine signalling and immune regulation. *Nat Rev Immunol* 2007;7:454–465.
 45. Hale BG, Randall RE, Ortin J, Jackson D. The multifunctional NS1 protein of influenza A viruses. *J Gen Virol* 2008;89:2359–2376.
 46. Wolff T, Ziebeck F, Abt M, Voss D, Semmler I, Matthaei M. Sabotage of antiviral signaling and effectors by influenza viruses. *Biol Chem* 2008;389:1299–1305.
 47. Krug RM, Yuan W, Noah DL, Latham AG. Intracellular warfare between human influenza viruses and human cells: the roles of the viral NS1 protein. *Virology* 2003;309:181–189.
 48. Ehrhardt C, Marjuki H, Wolff T, Nurnberg B, Planz O, Pleschka S, Ludwig S. Bivalent role of the phosphatidylinositol-3-kinase (PI3k) during influenza virus infection and host cell defence. *Cell Microbiol* 2006;8:1336–1348.
 49. Zhironov OP, Klenk HD. Control of apoptosis in influenza virus-infected cells by up-regulation of Akt and p53 signaling. *Apoptosis* 2007;12: 1419–1432.
 50. Jackson D, Killip MJ, Galloway CS, Russell RJ, Randall RE. Loss of function of the influenza A virus NS1 protein promotes apoptosis but this is not due to a failure to activate phosphatidylinositol 3-kinase (PI3k). *Virology* 2010;396:94–105.