

Viral Replication and Innate Host Responses in Primary Human Alveolar Epithelial Cells and Alveolar Macrophages Infected with Influenza H5N1 and H1N1 Viruses^{†‡}

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Highly pathogenic influenza H5N1 virus continues to pose a threat to public health. Although the mechanisms underlying the pathogenesis of the H5N1 virus have not been fully defined, it has been suggested that cytokine dysregulation plays an important role. As the human respiratory epithelium is the primary target cell for influenza viruses, elucidating the viral tropism and innate immune responses of influenza H5N1 virus in the alveolar epithelium may help us to understand the pathogenesis of the severe pneumonia associated with H5N1 disease. Here we used primary cultures of differentiated human alveolar type II cells, alveolar type I-like cells, and alveolar macrophages isolated from the same individual to investigate viral replication competence and host innate immune responses to influenza H5N1 (A/HK/483/97) and H1N1 (A/HK/54/98) virus infection. The viral replication kinetics and cytokine and chemokine responses were compared by quantitative PCR (qPCR) and enzyme-linked immunosorbent assay (ELISA). We demonstrated that influenza H1N1 and H5N1 viruses replicated productively in type II cells and type I-like cells although with different kinetics. The H5N1 virus replicated productively in alveolar macrophages, whereas the H1N1 virus led to an abortive infection. The H5N1 virus was a more potent inducer of proinflammatory cytokines and chemokines than the H1N1 virus in all cell types. However, higher levels of cytokine expression were observed for peripheral blood monocyte-derived macrophages than for alveolar macrophages in response to H5N1 virus infection. Our findings provide important insights into the viral tropisms and host responses of different cell types found in the lung and are relevant to an understanding of the pathogenesis of severe human influenza disease.

The recent influenza H1N1 virus (H1N1pdm) of swine origin, which recently emerged to become pandemic, highlighted the rapidity with which an influenza virus can spread worldwide. While its virulence for humans so far remains modest in comparison with that seen for zoonotic H5N1 disease (8), the magnitude of its extensive spread challenged health care systems in many parts of the world. Highly pathogenic avian influenza (HPAI) H5N1 virus has zoonotically transmitted repeatedly to humans, with over 500 diagnosed human cases, associated with an overall case-fatality rate of about 60% (http://www.who.int/csr/disease/avian_influenza/country/cases_table_2010_06_08/en/index.html). Patients with H5N1 disease manifest with rapidly progressing primary viral pneumonia leading to acute respiratory distress syndrome (ARDS), multiple-organ dysfunction, lymphopenia, and hemophagocytosis (6, 39, 48). These syndromes have previously been associated with cytokine dysregulation (11, 14). The H5N1 virus therefore continues to pose a major public

health concern, and it is important to understand its pathogenesis in humans. If a virus with virulence comparable to that of H5N1 acquires efficient transmissibility in humans to become pandemic, either by adaptation or through reassortment with other influenza viruses, its impact could be devastating. It is important to understand the determinants of virus tropism and pathogenesis of H5N1 infection in humans.

The pathogenesis of human influenza virus can be investigated with humans, with relevant animal models, and with *in vitro* or *ex vivo* primary human cells (2–4, 9, 23, 26, 27, 40, 43). Autopsy studies of lungs from H5N1-infected patients and infection of *ex vivo* lung tissues have shown that alveolar epithelial cells and macrophages are targets for the virus (42). Experimental infection of primates showed that alveolar type I epithelial (ATI) and alveolar type II epithelial (ATII) cells and alveolar macrophages (AMs) contain the H5N1 viral antigen (19, 33), and more recently, tracheal and bronchial epithelial cells, type I and type II alveolar epithelial cells, and macrophages were found to be the key target cells for H1N1pdm infection (35).

The human alveolar epithelial surface is a large interface for gas exchange. The alveolar epithelium is comprised predominantly of two specialized epithelial cell types, ATI cells (31) and ATII cells. ATI cells are specialized for the key lung function of gas exchange and play a role in fluid and ion transport (16), whereas ATII cells play an important role in

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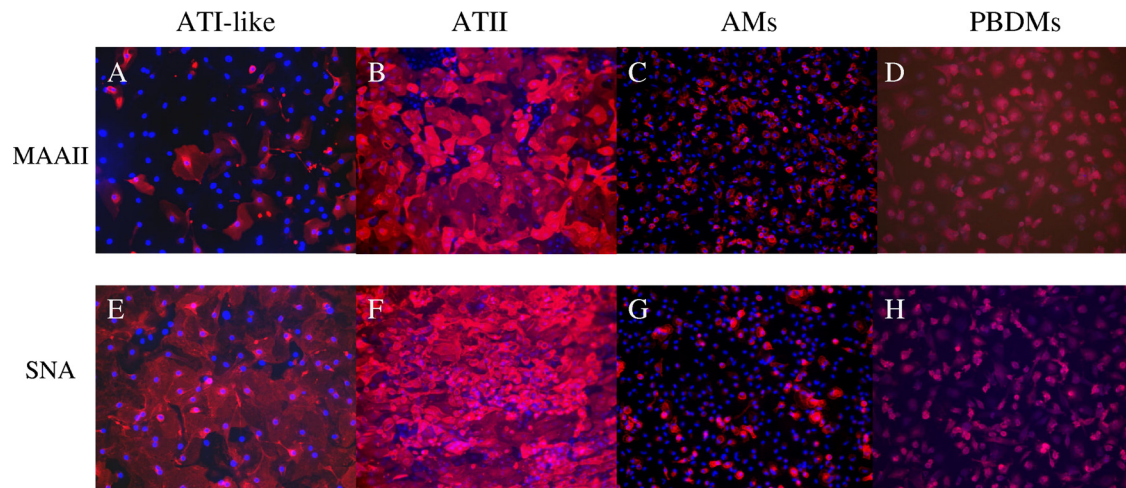


FIG. 1. Sia expression on ATI-like cells, ATII cells, AMs, and PBDMs. (A to D) Sia in α 2-3 linkages was stained by using MAAII lectin binding. (E to H) Sia in α 2-6 linkages was stained by using SNA lectin binding. Magnification, $\times 200$.

alveolar defense by producing and secreting surfactant proteins (surfactant protein A [SP-A], SP-B, SP-C, and SP-D) (7), are also involved with ion and fluid transport, and are key for the repair of alveolar damage by producing new alveolar epithelial cells to replace damaged ones. AMs are involved in the host defense system of the respiratory tract, mediating the release of both proinflammatory cytokines (e.g., tumor necrosis factor alpha [TNF- α]) and anti-inflammatory cytokines (e.g., interleukin-10 [IL-10]) in response to invading pathogens. In the resting normal lung, alveolar macrophages are usually suppressive and minimize inflammatory responses caused by exterior stimuli (38). As ATI cells, ATII cells, and AMs are all target cells for influenza A viruses, it is important to study the viral replication competence and host innate immune responses following influenza virus infection in these cell types. We have previously demonstrated that infection with HPAI H5N1 virus leads to the hyperinduction of proinflammatory cytokines in human primary alveolar epithelial type I-like cells and peripheral blood monocyte-derived macrophages (PBDMs), compared to human seasonal influenza H1N1 or H3N2 viruses (2, 5). The tropisms, replication competences, and innate immune responses of influenza virus in AMs have not been directly compared with those in PBDMs, the more easily accessible primary human macrophage for experimental study. Recently, we also demonstrated the cytokine responses induced by influenza H1N1pdm virus in well-differentiated bronchial epithelial cells and alveolar type I-like cells *in vitro* compared to other seasonal influenza viruses, suggesting that the cytokine dysregulation seen for influenza H5N1 virus infection is not a feature of the H1N1pdm virus (3).

As the most serious complication of human influenza, whether caused by H5N1 or by the H1N1pdm virus, is primary viral pneumonia, we employed ATII cells, ATI-like cells, and AMs as models to examine the viral replication competence and host innate immune responses induced by influenza H5N1 virus compared with those induced by seasonal influenza H1N1 virus. ATI-like cells refer to ATII cells that are differentiated toward the ATI cell phenotype and express ATI cell markers

but no longer express the surfactant proteins SP-A, SP-B, and SP-C (46). Human ATI cells have not been reported to be isolated to our knowledge. We aimed to identify the differential viral tropisms and host responses induced by these two influenza viruses in different cell types from same individual to provide insights for an understanding of virus pathogenesis, which may be relevant to therapeutic strategies for the treatment of influenza.

In order to minimize genetic and other host variabilities, we cultured and maintained differentiated human ATII cells, ATI-like cells, and AMs from the same individual *in vitro*. Viral replications and host responses elicited by low-pathogenic (LP) human seasonal influenza H1N1 virus (A/HK/54/98) and HPAI H5N1 virus (A/HK/483/97) were compared. We found that both influenza H1N1 and H5N1 viruses can infect and replicate in alveolar epithelial cells (ATII and ATI-like cells). We also demonstrated that while H5N1 productively replicates in AMs, the H1N1 virus replicates poorly in these cells. As we previously reported (2, 4, 5), in comparison to influenza H1N1 virus, the H5N1 virus was a more potent inducer of cytokines and chemokines (e.g., monocyte chemoattractant protein 1 [MCP-1] and RANTES) in alveolar epithelial cells and macrophages. Interestingly, AMs are less responsive than PBDMs in proinflammatory cytokine induction. Our data may provide important insights into the unusual severity of H5N1 disease in humans.

MATERIALS AND METHODS

Isolation, culture, and differentiation of human ATII cells, ATI-like cells, and AMs. ATII cells were isolated from human lungs that were not suitable for transplantation and donated for medical research. The lungs were obtained through the National Disease Research Interchange (Philadelphia, PA) and the International Institute for the Advancement of Medicine (Edison, NJ). The Committee for the Protection of Human Subjects at National Jewish Health approved this research. The isolation method was reported previously (44, 45). Briefly, the middle lobe was perfused, lavaged, and then instilled with elastase (12.9 units/ml; Roche Diagnostics, Indianapolis, IN) and incubated for 50 min at 37°C. The lung was minced, and the cells were isolated by filtration and partially purified by centrifugation on a discontinuous density gradient made of Optiprep (Accurate Chemical Scientific Corp., Westbury, NY) with densities of 1.080 and 1.040 and by negative selection with CD-14-coated magnetic beads (Dyna Bio-

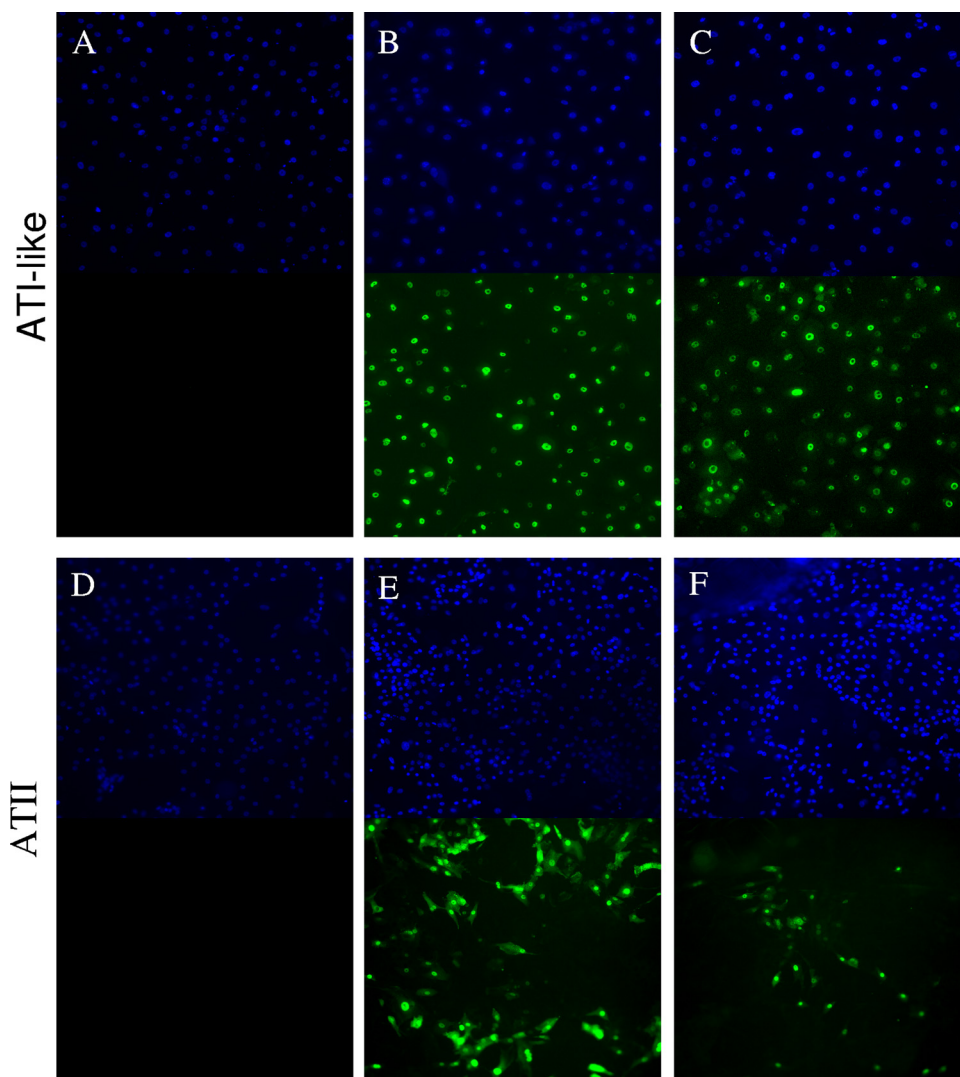


FIG. 2. Immunofluorescence staining of ATI-like cells (A to C) and ATII cells (D to F) at 24 h postinoculation with mock infection (A and D), HK98/H1N1 infection (B and E), and HK97/H5N1 infection (C and F). The influenza virus nucleoprotein and matrix protein were stained in green (bottom) with FITC-conjugated mouse antibody, while cell nucleus DNA was stained in blue with DAPI (top). Magnification, $\times 200$.

tech ASA, Oslo, Norway) and binding to IgG-coated petri dishes (Sigma Chemicals Inc., St. Louis, MO). The cells were isolated at National Jewish Health, Denver, CO, frozen down in 10% dimethyl sulfoxide (DMSO) and 90% fetal bovine serum (FBS), and shipped to Hong Kong, where the experiments were performed.

Isolated cells were plated onto 6-well or 24-well Millicell inserts (Millipore) coated with a mixture of rat tail collagen and Matrigel (BD Biosciences) in 10% FBS in Dulbecco's modified Eagle's medium (DMEM) with 1×10^6 and 5×10^5 cells per well, respectively, and were maintained in a humidified atmosphere (5% CO_2 at 37°C). The cells were then cultured with 1% charcoal-stripped FBS along with keratinocyte growth factor, isobutylmethylxanthine, 8-bromo-cyclic AMP (cAMP), and dexamethasone (KIAD) and antibiotics to achieve their differentiated phenotypes.

To transdifferentiate ATII cells into ATI-like cells, ATII cells were plated onto rat tail collagen-coated glass coverslips with 1×10^5 cells per well on the 24-well tissue culture plates in DMEM with 10% FBS. After 24 to 48 h for adherence, the medium was changed to DMEM with 5% FBS without the KIAD additives described above and cultured for an additional 6 days (45).

The procedure for the isolation of AMs was reported previously (44, 45). AMs were isolated from the same donor from which the corresponding ATII cells were obtained. Briefly, the lung was lavaged with HEPES-buffered saline and 2 mM EDTA, and the lavage fluid was centrifuged at 4°C for 10 min. If there was

a significant number of red blood cells (RBCs), the RBCs were lysed with Pharm Lyse (BD Biosciences). The macrophages were resuspended in DMEM supplemented with 10% FBS, 2 mM glutamine, 2.5 $\mu\text{g}/\text{ml}$ amphotericin B, 100 $\mu\text{g}/\text{ml}$ streptomycin, 100 U/ml penicillin G, and 10 $\mu\text{g}/\text{ml}$ gentamicin (Life Technologies), and 1×10^5 cells were plated per well in the 24-well tissue culture plates. After adherence for 4 h or overnight, the cells were washed with DMEM to remove the nonadherent cells and were then cultured for two more days before infection. The purity was measured by CD-68 staining and was nearly 100%, as previously described (44, 45).

Isolation of human peripheral monocytes and generation of primary macrophages (PBDMs). The isolation method was reported previously (5). Briefly, peripheral blood leukocytes were separated from buffy coats of healthy blood donors (provided by the Hong Kong Red Cross Blood Transfusion Service) by centrifugation on a Ficoll-Paque density gradient (Pharmacia Biotech) and were purified by adherence. PBDMs were seeded onto tissue culture plates in RPMI 1640 medium (Sigma-Aldrich) supplemented with 5% heat-inactivated autologous plasma. The cells were allowed to differentiate for 14 days before virus challenge.

Lectin cytochemistry. Sialic acid (Sia) receptor distribution was detected by lectin cytochemistry. Human ATII cells cultured in transwell inserts and ATI-like cells, AMs, and PBDMs cultured on coverslips in culture plates were fixed with 2% paraformaldehyde, followed by washing with 0.1 M Tris-buffered saline

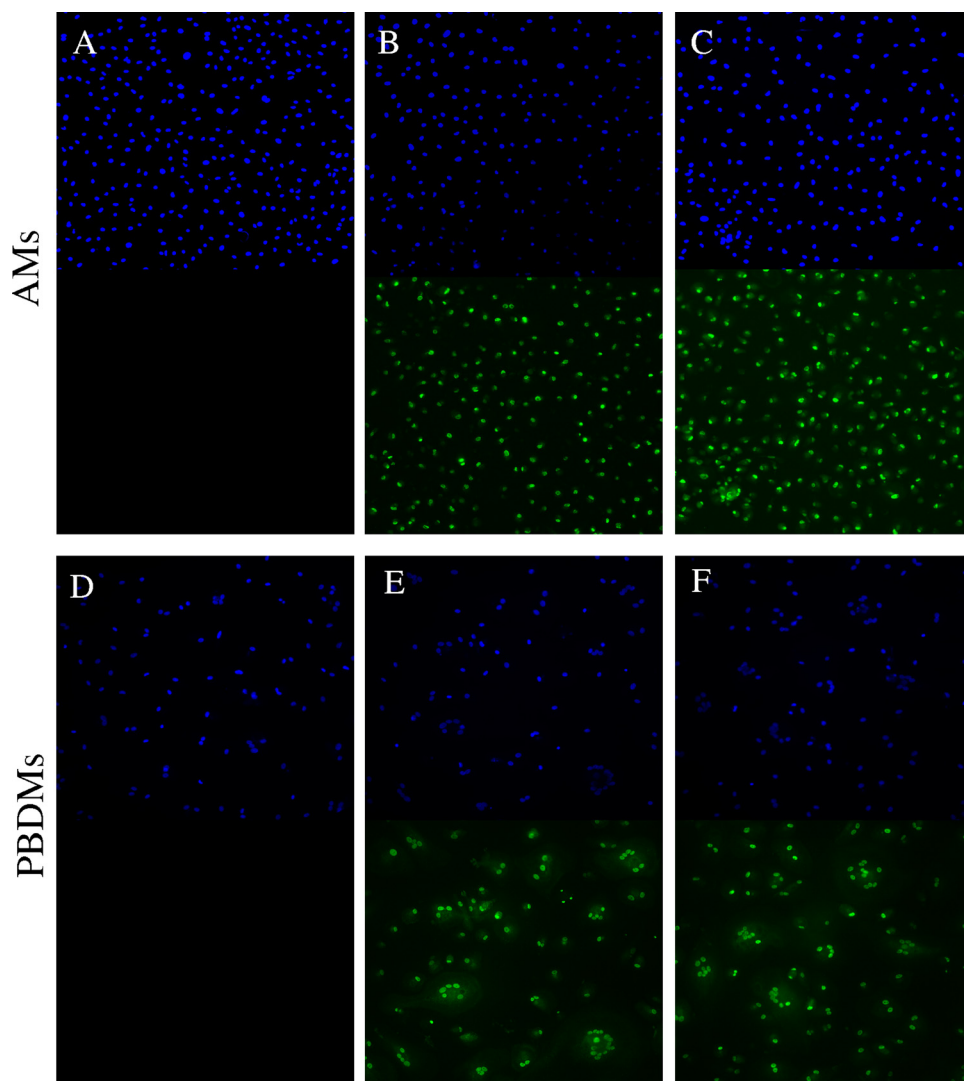


FIG. 3. Immunofluorescence staining of AMs (A to C) and PBDMs (D to F) at 16 h postinoculation with mock infection (A and D), HK98/H1N1 infection (B and E), and HK97/H5N1 infection (C and F). The influenza virus nucleoprotein and matrix protein were stained in green (top) with FITC-conjugated mouse antibody, while cell nucleus DNA was stained in blue with DAPI (bottom). Magnification, $\times 200$.

(TBS) three times. For sialidase (sialidase A, catalog no. GK80040; Glyko) treatment, ATII cells cultured in transwell inserts, and ATI-like cells, AMs, and PBDMs on coverslips were treated with 10 $\mu\text{g}/\text{ml}$ of sialidase for 3 h in a 37°C incubator, followed by the blocking of the cells with an avidin-biotin blocking kit for 30 min at room temperature. The cells were incubated with a 1/2,000 dilution of 594 lectin biotinylated with streptavidin, *Maackia amurensis* agglutinin II (MAAII), or *Sambucus nigra* agglutinin (SNA) (both from Vector) for 1 h at room temperature in the dark. After the cells were washed, they were then incubated with streptavidin and Alexa 594 for 30 min at room temperature. The transwell membrane and coverslips were then washed again with TBS three times and mounted by using Dako fluorescent mount (catalog no. S3023; Dako Cytomation).

Viruses. An HPAI influenza H5N1 virus, A/Hong Kong/483/97 (HK97/H5N1), and a seasonal human influenza H1N1 virus, A/Hong Kong/54/98 (HK98/H1N1), were used for our comparative studies. Viruses were initially isolated and subsequently maintained in Madin-Darby canine kidney (MDCK) cells. Virus seed stocks were prepared in MDCK cells. Virus infectivity was assessed by titration of the 50% tissue culture infection dose (TCID_{50}) in MDCK cells. The influenza H5N1 viruses used in this study were handled in biosafety level 3 facilities at the Department of Microbiology, the University of Hong Kong.

Influenza virus infection of ATII cells, ATI-like cells, AMs, and PBDMs. Primary ATII cells, ATI-like cells, AMs, and PBDMs were cultured as described above and inoculated with mock, A/HK/54/98 (HK98/H1N1), and A/HK/483/97

(HK97/H5N1) at 2 multiplicities of infection (MOIs); we used an MOI of 0.01 to evaluate the release of newly formed virus and an MOI of 2 to determine the percentage of cell infection and cytokine release. After 1 h of virus adsorption, the inoculum was aspirated, and the cells were washed three times with warm phosphate-buffered saline (PBS). The infected cell culture was then replenished by the appropriate growth medium that corresponded to the cell type and was incubated at 37°C in an atmosphere of 5% CO_2 . At the designated times after inoculation, samples of the culture supernatant were collected for virus titration and cytokine analysis.

Virus replication analysis. Viral infection was analyzed by several ways: by assaying viral matrix mRNA at designated hours postinoculation by quantitative reverse transcription (RT)-PCR, by detecting viral antigen expression by immunofluorescence staining with mouse anti-influenza virus nucleoprotein and matrix antibody conjugated with fluorescein isothiocyanate (FITC) (Dako Imagen; Dako Diagnostics Ltd., Ely, United Kingdom), and also by assaying infectious virus in cell culture supernatant by a TCID_{50} assay to demonstrate complete virus replication.

Immunofluorescence staining. The cell layer was fixed at 16 h or 24 h postinoculation by using 4% paraformaldehyde for immunofluorescence staining with mouse anti-influenza virus nucleoprotein and matrix antibody conjugated with FITC (Imagen Influenza, Dako Cytomation; Dako Diagnostics, Denmark). The fixed cell layers were mounted onto glass slides with a drop of mounting medium

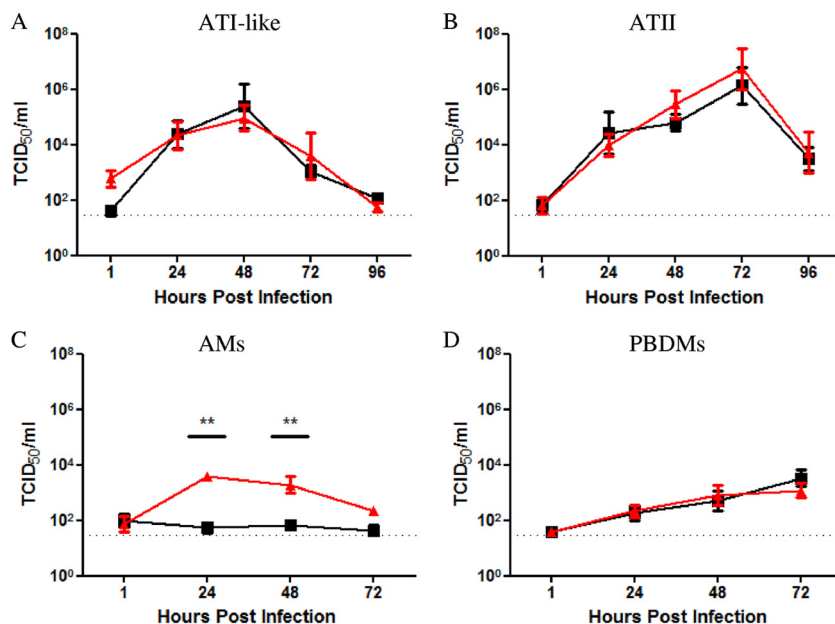


FIG. 4. Virus titer detected in the supernatants of influenza virus-infected ATI-like cells (A), ATII cells (B), AMs (C), and PBDMs (D) at an MOI of 0.01. Black lines represent virus titers of HK98/H1N1, and red lines represent virus titers of HK97/H5N1. Double asterisks indicate statistically significant differences of means with P values of <0.01 . Dotted lines represent the detection limit of the TCID₅₀ assay.

with 4',6-diamidino-2-phenylindole (DAPI) (Vectashield mounting medium with DAPI; Vector Laboratories Inc.).

Virus titration by TCID₅₀ assay. A confluent 96-well tissue culture plate of MDCK cells was prepared 1 day before the virus titration assay. Cells were washed once with PBS and replenished with serum-free minimal essential medium (MEM) supplemented with 100 units/ml penicillin, 100 μ g/ml streptomycin, and 2 μ g/ml of tosylsulfonyl phenylalanylchloromethyl ketone (TPCK)-treated trypsin. Serial dilutions of virus-infected culture supernatants from 0.5 logs to 7 logs were performed before the addition of the culture supernatant onto the plates in quadruplicate. The plates were observed for a cytopathic effect (CPE) daily. The endpoint of viral dilution leading to CPE in 50% of inoculated wells was estimated by using the Karber method.

Quantification of cytokine and chemokine mRNAs by quantitative RT-PCR. DNase-treated mRNA from infected cells was extracted at the designated hours postinoculation by using an RNeasy minikit (Qiagen, Hilden, Germany). The cDNA was synthesized from mRNA with oligo(dT) primers and Superscript III reverse transcriptase (Invitrogen). The mRNA levels of selected genes were quantified by real-time quantitative PCR (qPCR) analysis with a LightCycler instrument (Roche, Mannheim, Germany). The gene expression profiles for cytokines (beta interferon [IFN- β], IL-6, and tumor necrosis factor alpha [TNF- α]), chemokines (gamma interferon-inducible protein 10 [IP-10], RANTES, and MCP-1), and the viral matrix gene were normalized by using the housekeeping gene product β -actin mRNA. The absolute copy numbers of the cytokine, chemokine, and β -actin gene were determined from the standard curve generated from a standard plasmid with a known copy number, which was included in the qPCR simultaneously, as previously described (5).

Quantification of cytokine and chemokine proteins by ELISA. The secretion of MCP-1, RANTES, IL-6, IP-10, and TNF- α from influenza virus-infected ATII cells, ATI-like cells, AMs, and PBDMs was measured by specific enzyme-linked immunosorbent assays (ELISAs) (R&D Systems, Minneapolis, MN). Samples of culture supernatants were irradiated with UV light (CL-100 ultraviolet cross-linker) for 15 min to inactivate any infectious virus before the ELISAs were done. Previous experiments confirmed that the dose of UV light used did not affect the cytokine concentration, as measured by ELISAs (5).

Statistical analysis. The differences of log₁₀-transformed viral titers among different viruses at different time points postinfection and the quantitative cytokine and chemokine mRNAs and proteins of influenza virus-infected cells were compared by using one-way analysis of variance followed by a Bonferroni multiple-comparison test. Differences were considered significant at a P value of <0.05 . The statistical analysis was performed by using Graph-Pad Prism 5 software.

RESULTS

Sia receptor distribution on human differentiated ATII cells, ATI-like cells, and AMs. Lectin cytochemistry staining (Fig. 1) was performed on primary cultures of human ATI-like cells (Fig. 1A and E), ATII cells (Fig. 1B and F), AMs (Fig. 1C and G), and PBDMs (Fig. 1D and H). We showed that MAAII, which recognizes the accepted avian influenza receptor Sia α 2-3Gal β 1-3GalNAc (25, 26), while it will pick up some nonspecific glycan (25), bound strongly to ATII cells and AMs (Fig. 1B and C), and there was less MAAII binding of the ATI-like cells (Fig. 1A), which is similar to data in studies reported previously (36, 43). In contrast, SNA (which recognizes the human influenza receptor Sia α 2-6) binds strongly to both ATI-like and ATII cells compared to MAAII binding (Fig. 1E and F), and this is consistent with data from previous reports by Shinya et al. (36, 37) and ourselves (2). When ATI-like and ATII cells were compared, ATI-like cells showed a higher level of binding of SNA than did ATII cells. On the other hand, the Sia receptor-binding profile for the AMs and PBDMs showed that there was binding of both MAAII and SNA to AMs (Fig. 1C and G) and PBDMs (Fig. 1D and H), respectively. Thus, AMs and PBDMs have similar Sia receptor profiles and would be expected to bind both human and avian influenza viruses.

On the other hand, with the treatment with sialidase A, no fluorescent signal was detected in ATI-like cells, ATII cells, AMs, and PBDMs compared to the control (buffer and no-treatment) cells, inferring the specificities of lectin, MAAII (see Fig. S1A in the supplemental material), and SNA (Fig. S1B) binding to the sialic acid (Fig. S1) used.

Differential replication kinetics of influenza H1N1 and H5N1 viruses in human ATII cells, ATI-like cells, AMs, and PBDMs. Influenza virus protein expression was detected by

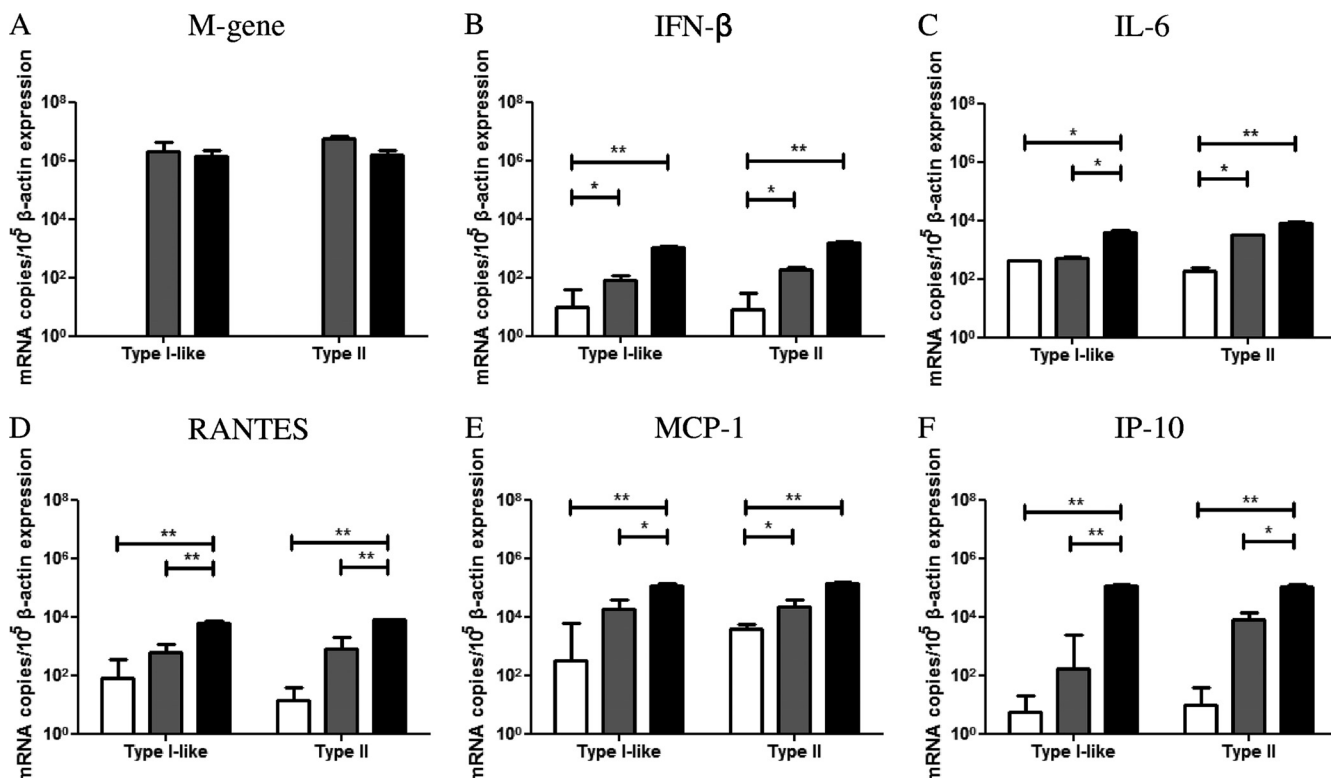


FIG. 5. (A) Influenza virus matrix gene expression and cytokine and chemokine gene expression levels in ATI-like and ATII cells after mock infection (white bars), influenza HK98/H1N1 virus infection (gray bars), and influenza HK97/H5N1 virus infection (black bars). (B to F) The cytokines IFN- β (B) and IL-6 (C) and the chemokines RANTES (D), MCP-1 (E), and IP-10 (F) from ATI-like and ATII cells infected with influenza A viruses at 24 h postinfection. The graph shows the means and standard deviations (SD) of mean mRNA copies expressed per 10⁵ β -actin copies from five representative experiments. Single asterisks indicate statistically significant differences of means with P values of <0.05, and double asterisks indicate statistically significant differences of means with P values of <0.01.

immunofluorescence staining for viral proteins in ATI-like cells (Fig. 2A to C) and ATII cells (Fig. 2D to F) 24 h after infection at comparable MOIs (MOI = 2) with HK98/H1N1 or HK97/H5N1 virus. The number of HK98/H1N1-infected ($42\% \pm 8\%$) or HK97/H5N1-infected ($26\% \pm 4\%$) ATII cells at 24 h was significantly lower ($P < 0.01$) than the number of ATI-like infected cells, which had over 95% infected cells following infection with either virus. On the other hand, both AMs (Fig. 3A to C) and PBDMs (Fig. 3D to F) were equally susceptible to HK98/H1N1 and HK97/H5N1 virus infection, with over 95% of cells expressing virus protein at 16 h postinoculation.

The culture supernatants of HK98/H1N1- and HK97/H5N1-infected ATI-like cells (Fig. 4A), ATII cells (Fig. 4B), AMs (Fig. 4C), and PBDMs (Fig. 4D) were titrated by using TCID₅₀ assays to compare infectious virus yields and viral replication kinetics. Both influenza A viruses can infect and productively replicate in ATI-like cells and ATII cells (Fig. 4A and B) with similar virus replication kinetics in the first 48 h postinoculation. However, the levels of viral replication of influenza H5N1 and H1N1 viruses continued to increase for a longer period and peaked at 72 h postinfection in ATII cells (Fig. 4B). Interestingly, although both influenza viruses infected AMs, as judged by immunofluorescence staining for virus protein expression (Fig. 3B and C, respectively), productive viral replication was observed only with HPAI H5N1 virus-infected

AMs, while the level of replication of seasonal HK98/H1N1 virus was marginal (Fig. 4C). In contrast, both influenza viruses replicated comparably in PBDMs (Fig. 4D) and demonstrated peak viral titers similar to those seen for H5N1-infected AMs.

These preliminary experiments were performed by using freshly prepared PBDMs and frozen-resuscitated AMs. In order to confirm that the observed differences in the viral replication kinetics of H5N1 and H1N1 were not a result of the storage history of the cells, we performed further experiments by infecting both fresh and frozen-resuscitated PBDMs with HK98/H1N1 and HK97/H5N1 viruses. We found that both influenza viruses replicated efficiently in both frozen-stored and freshly isolated PBDMs, suggesting that the difference in the viral replication kinetics of H5N1 (which showed productive replication) and H1N1 (which showed abortive replication) was due to the AM phenotype and not to the use of frozen cells.

Interestingly, when the yields of infectious virus and the replication kinetics of influenza HK97/H5N1 virus in ATII cells, ATI-like cells, and AMs isolated from the same individual were compared (Fig. 4), we found that influenza H5N1 virus replicated to an approximately 2-log-higher titer in alveolar epithelial cells than in alveolar macrophages. The replication kinetics of influenza H5N1 virus in AMs and PBDMs from different donors were comparable and reached similar peak titers.

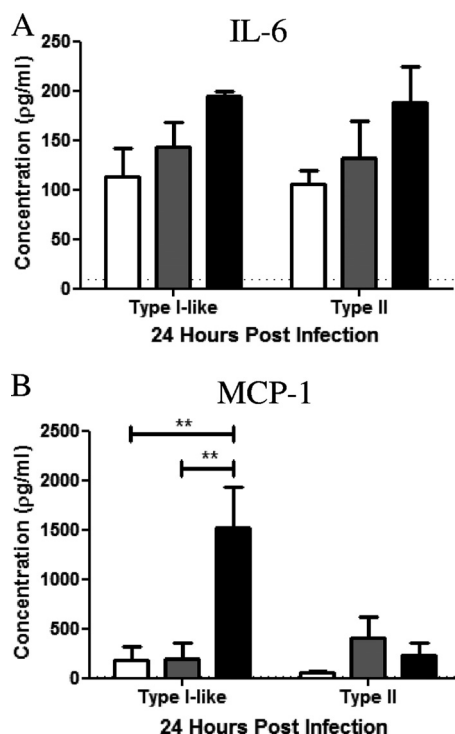


FIG. 6. Cytokine and chemokine protein secretion of IL-6 (A) and MCP-1 (B) in ATI-like and ATII cells infected with mock (white bars), influenza HK98/H1N1 virus (gray bars), and influenza HK97/H5N1 virus (black bars) at 24 h postinoculation. The graph shows the means and SD of means from five representative experiments. Double asterisks indicate statistically significant differences of means with P values of <0.01 . Horizontal dotted lines indicate the detection limit of the ELISA.

Cytokine and chemokine gene and protein expressions by influenza virus-infected ATI-like cells and ATII cells. We first compared influenza virus matrix (M) gene copy numbers using quantitative PCR as a measure of viral replication for each virus and cell type. In ATI-like cells and ATII cells, M gene transcription levels for both influenza H1N1 and H5N1 viruses were comparable at 24 h postinoculation (Fig. 5A). We next compared the expression levels of cytokine and chemokine genes by the low-pathogenic HK98/H1N1 virus and the HPAI HK97/H5N1 virus. In general, the IFN- β (Fig. 5B), IL-6 (Fig. 5C), RANTES (Fig. 5D), MCP-1 (Fig. 5E), and IP-10 (Fig. 5F) genes were induced following infection with influenza H1N1 and H5N1 viruses in both ATI-like cells and ATII cells, but the HK97/H5N1 virus led to significantly higher IFN- β ($P = 0.008$), IL-6 ($P = 0.041$), RANTES ($P = 0.007$), MCP-1 ($P = 0.002$), and IP-10 ($P = 0.008$) gene expression levels in ATI-like cells than in mock-infected cells at 24 h postinoculation. In addition, the HK97/H5N1 virus led to significantly higher RANTES ($P = 0.002$) and IP-10 ($P = 0.012$) gene expression levels in ATII cells. However, the HK97/H5N1 virus tended to induce higher IFN- β ($P = 0.056$), IL-6 ($P = 0.075$), and MCP-1 ($P = 0.057$) gene expression levels in ATII cells than did HK98/H1N1, but this did not reach statistical significance.

We next used ELISAs to measure the secretion of cytokine and chemokine proteins from ATI-like and ATII cell culture supernatants infected by influenza H1N1 and H5N1 viruses at

24 h postinfection. HK97/H5N1 infection led to significantly higher levels of MCP-1 ($P < 0.009$) (Fig. 6B) protein secretion in ATI-like cells than in HK98/H1N1-infected cells, but the MCP-1 protein secretion levels were comparable between HK97/H5N1- and HK98/H1N1-infected ATII cells, which contrasts with the mRNA data (Fig. 6B). In addition, HK97/H5N1 tended to induce higher levels of IL-6 secretion in both ATI-like cells ($P = 0.078$) and ATII cells ($P = 0.113$) than those in HK98/H1N1-infected cells, although these results did not achieve statistical significance. ($P = 0.189$) (Fig. 6A). We failed to detect any IFN- β protein in the supernatants of AMs, ATII cells, and ATI-like cells after influenza virus infection (data not shown), but this is likely because of the poor sensitivity of the IFN- β ELISA (the limit of detection was 250 pg/ml).

The inactivation of the virus by UV irradiation or high temperatures (70°C for 15 min) prior to infection abolished cytokine induction (data not shown). This finding suggests that virus replication is required for cytokine induction and also rules out the possibility that endotoxin contamination in the virus stocks contributed to the observed cytokine responses. In addition, an increase in the MOI of up to 5 did not result in changes in the profiles of the cytokine and chemokine gene expressions induced by the influenza H1N1 and H5N1 viruses in both cell types (data not shown).

Cytokine and chemokine gene and protein induction by influenza virus-infected AMs and comparison with PBDMs. The influenza virus matrix (M) gene copy number was measured by quantitative PCR as a measure of viral replication after infection by both influenza viruses. In AMs, the M gene transcription levels for both the influenza H1N1 and H5N1 viruses were comparable from 8 h to 24 h postinoculation (Fig. 7A). At 1 h postinoculation, the M gene transcription level for influenza H5N1 virus was about 2 logs higher ($P = 0.226$) than that for influenza H1N1 virus-infected AMs, which may be due to the intrinsic replication kinetic capacities of the two viruses. Similar differential cytokine and chemokine gene expression profiles were obtained from HK98/H1N1- and HK97/H5N1-infected AMs from 8 h to 24 h postinoculation. In general, TNF- α (Fig. 7B), IFN- β (Fig. 7C), IL-6 (Fig. 7D), RANTES (Fig. 7E), MCP-1 (Fig. 7F), and IP-10 (Fig. 7G) mRNAs were induced following infection with influenza H1N1 and H5N1 viruses in AMs. The HK97/H5N1 virus led to significantly higher IFN- β , IL-6, RANTES, MCP-1, and IP-10 (all with a P value of <0.05) mRNA expression levels in AMs than those with HK98/H1N1 or mock infection from 8 to 24 h postinfection. At 1 h postinoculation, both cytokine and chemokine gene expression levels were comparable in HK97/H5N1-, HK98/H1N1-, and mock-infected AMs. A high level of TNF- α gene expression was demonstrated for influenza A virus-infected AMs, whereas this was not found for ATII cells and ATI-like cells.

We next used ELISAs to measure the secretion of cytokine and chemokine proteins from AM culture supernatants infected by influenza H1N1/98 and H5N1/97 viruses (Fig. 8). Markedly higher levels of TNF- α were secreted at 8, 16, and 24 h postinoculation (Fig. 8A) by HK97/H5N1-infected than by HK98/H1N1-infected or mock-infected cells. A significantly higher RANTES protein secretion level was observed for HK97/H5N1-infected AMs than for mock-infected ($P = 0.024$) or HK98/H1N1-infected ($P = 0.016$) AMs at 8 h postinfection

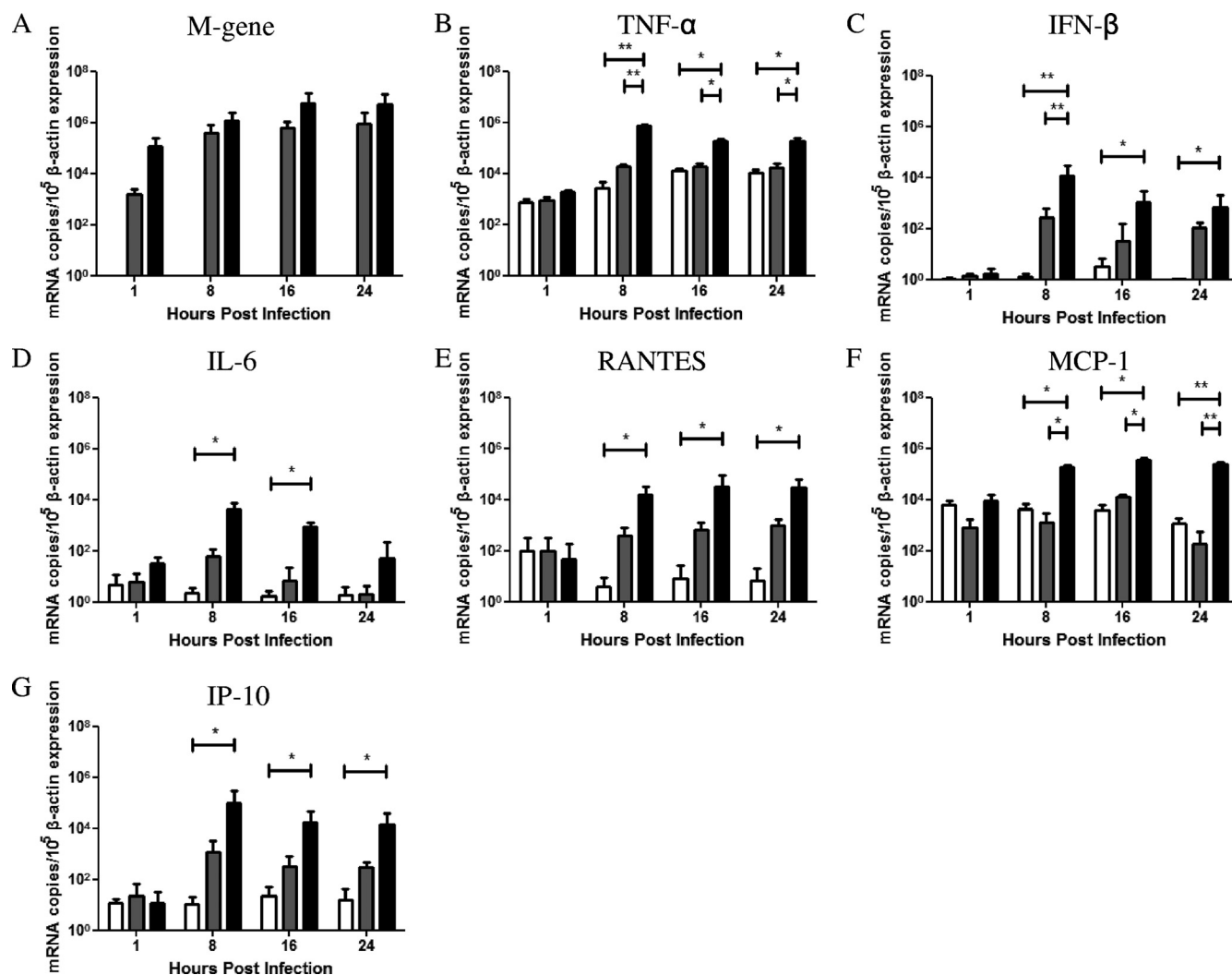


FIG. 7. (A) Influenza virus matrix gene expression and cytokine and chemokine gene expression in AMs after infection of mock (white bars), influenza HK98/H1N1 virus (gray bars), and influenza HK97/H5N1 virus (black bars). (B to G) Levels of the cytokines TNF- α (B), IFN- β (C), and IL-6 (D) and the chemokines RANTES (E), MCP-1 (F), and IP-10 (G) from AMs infected with influenza A viruses from 1 to 24 h postinoculation. The graph shows the means and SD of mean mRNA copies expressed per 10⁵ β -actin copies from five representative experiments. Single asterisks indicate statistically significant differences of means with P values of <0.05 , and double asterisks indicate statistically significant differences of means with P values of <0.01 .

(Fig. 8C). A significantly higher IP-10 protein secretion level was also demonstrated for HK97/H5N1-infected AMs than for HK98/H1N1-infected AMs ($P = 0.042$) (Fig. 8D). However, the levels of IL-6 protein secretion induced by both influenza viruses were low and just above the detection level of the ELISA kit (9.25 pg/ml) (Fig. 8B). We failed to detect any IFN- β proteins in the supernatants of infected AMs, possibly because of the poor sensitivity of the IFN- β ELISA (the limit of detection was 250 pg/ml) (data not shown).

In order to compare the host innate immune responses in human AMs and PMDMs, cytokine and chemokine protein secretion levels were measured in HK97/H5N1- and HK98/H1N1-infected cells (Fig. 9). The HK97/H5N1 virus led to significantly higher TNF- α (Fig. 9A), RANTES (Fig. 9B), and IP-10 (all with P values of <0.05) (Fig. 9C) protein secretion levels than those in mock- and HK98/H1N1-infected macrophages at 24 h postinfection. The levels of TNF- α (Fig. 9A),

RANTES (Fig. 9B), and IP-10 (Fig. 9C) secretion were significantly higher in PMDMs than in AMs ($P = 0.047$, 0.009, and 0.020, respectively) following infection with comparable virus MOIs.

DISCUSSION

Immunohistopathology of autopsy lung samples from patients with viral pneumonia due to H5N1; data for the pandemic viruses of 1918, 1957, and 2009; and the limited data for seasonal influenza viruses reveal that alveolar epithelial cells as well as AMs are the key target cells infected by influenza virus (35, 41, 42). Similar conclusions have also been drawn from experiments where *ex vivo* lung tissue has been infected with H5N1 virus (26). A massive infiltration of macrophages has been observed for the lungs of H5N1-infected humans and mice (28, 29). It is therefore important to understand in more

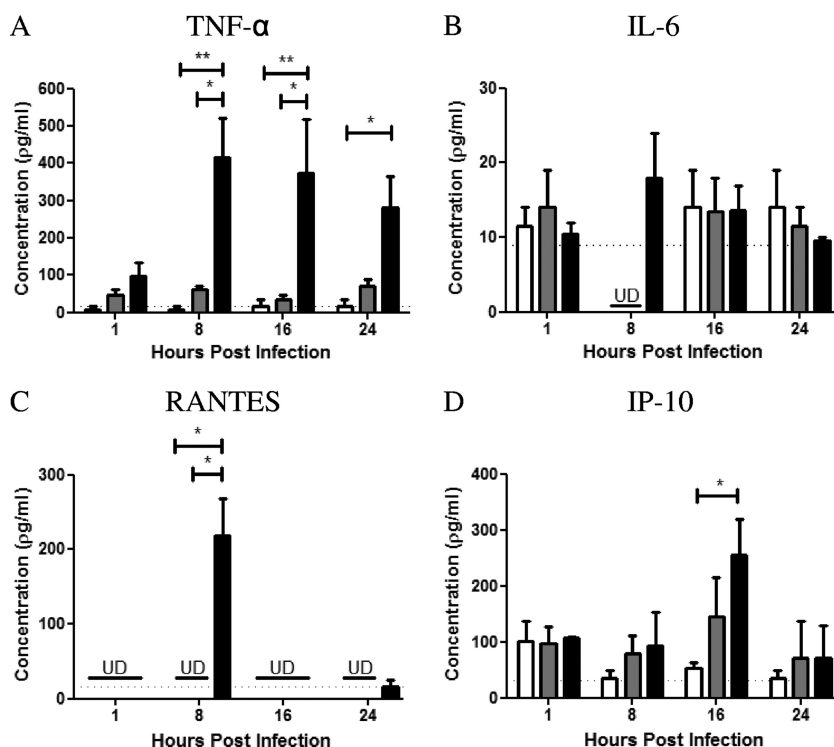


FIG. 8. Cytokine and chemokine protein secretion of TNF- α (A), IL-6 (B), RANTES (C), and IP-10 (D) in AMs after infection of mock (white bars), influenza HK98/H1N1 virus (gray bars), and influenza HK97/H5N1 virus (black bars) from 1 to 24 h postinoculation. The graph shows the means and SD of means from five representative experiments. Single asterisks indicate statistically significant differences of means with P values of <0.05 , and double asterisks indicate statistically significant differences of means with P values of <0.01 . Horizontal dotted lines indicate the detection limit of the ELISA. UD represents undetectable (below the detection limit).

detail the aspects of influenza virus infection and replication in alveolar epithelial cells and AMs and the innate immune responses elicited as a consequence of such an infection. Rather than studying transformed cell lines (e.g., A549 cells), in this study, we established primary cultures of human ATII cells, ATI-like cells, and AMs from the lung of the same individual so that we could compare the effects of virus-cell interactions in a physiologically relevant manner while minimizing the variability that could be imposed by genetic and other variations that may be present between different donors.

We compared the replication competences and innate immune responses induced by HPAI H5N1 virus with that of LP human seasonal influenza H1N1 virus in primary human alveolar epithelial cells (ATII and ATI-like cells) and macrophages (AMs and PBDMs). While both influenza viruses replicated in ATII and ATI-like cells, the ATII cells maintained virus replication for longer periods, with virus titers peaking at 72 h (Fig. 4). The H5N1 virus was a more potent cytokine and chemokine inducer than was seasonal influenza H1N1 virus (Fig. 5 and 6) in both ATII and ATI-like cells. These inflammatory mediators are likely to be important for initiating neutrophil and macrophage recruitment into the lung following infection. The overly exuberant chemokine responses elicited by H5N1 may explain the massive macrophage infiltration seen in the lungs of H5N1-infected patients and contribute to the severe viral pneumonia and acute lung injury associated with this virus (10).

ATII and ATI cells have distinct physiological and func-

tional properties within the lung. The alveolar surface is critical to gaseous exchange and is under continuous exposure to environmental and microbial insults, and 95% of the alveolar epithelium is covered by flattened ATI cells (21, 47). Thus, ATI cells are likely to be a cell type first infected by influenza viruses within the lung parenchyma and critical for effective gas exchange. Studies with freshly isolated type I cells and cultured type I cells will have to await the development of isolation and culture techniques, and the results with primary type I cells may differ from the results seen with type II cells that differentiate into type I-like cells *in vitro*. ATII cells have the capacity to proliferate, differentiate into ATI cells, and restore the epithelium after damage. ATII cells secrete surfactant proteins A and D, which play an important role in innate immunity. Both cell types play an important role in maintaining the alveolar fluid equilibrium by transporting sodium and fluid from the apical to the basolateral surface of the alveolar epithelium (44). Thus, the dynamic interplay between ATII and ATI cells is crucial for the maintenance of the fragile physiological balance crucial to efficient gas exchange in the lung.

Macrophages play an important role in innate and acquired immunity within the respiratory tract and have a role in lung defense against viruses, bacteria, mycobacteria, and fungi (13). Previous studies have shown that alveolar macrophages may be critical for inhibiting influenza viruses and reducing mortality and clinical signs in pigs, even though they may contribute to some damage in the lungs by producing proinflammatory cytokines such as IFN- γ and TNF- α (18). While influenza H5N1

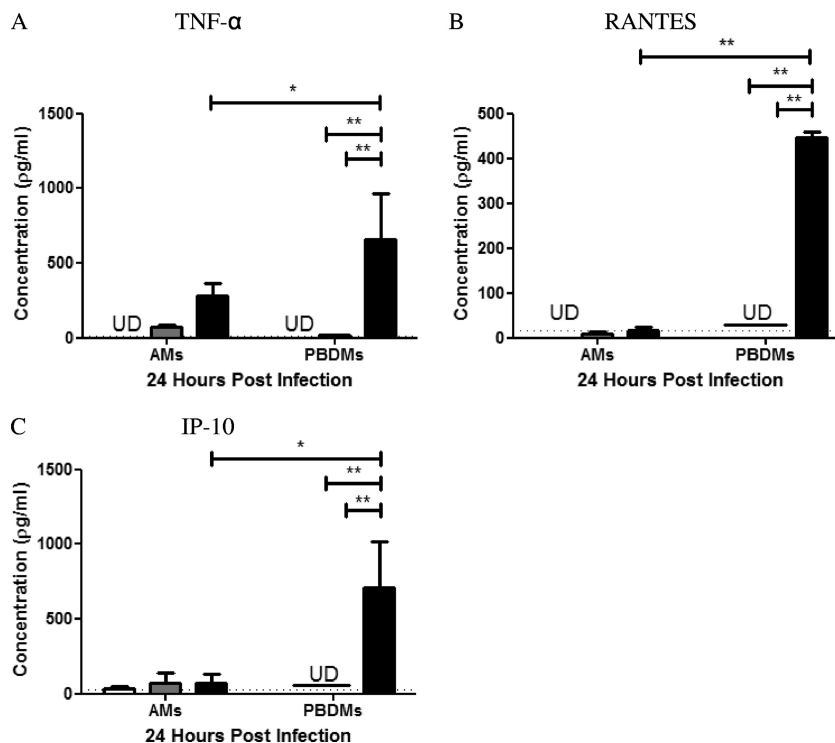


FIG. 9. Cytokine and chemokine protein secretion in AMs and PBDMs after infection of mock (white bars), influenza HK98/H1N1 virus (gray bars), and influenza HK97/H5N1 virus (black bars). Shown are levels of the cytokine TNF- α (A) and the chemokines RANTES (B) and IP-10 (C) from AMs and PBDMs infected with influenza A viruses at 24 h postinoculation. The graphs show the means and SD of means from five representative experiments. Single asterisks indicate statistically significant differences of means with P values of <0.05 , and double asterisks indicate statistically significant differences of means with P values of <0.01 . Horizontal dotted lines indicate the detection limit of the ELISA. UD represents undetectable (below the detection limit).

virus replicated equally well in AMs and PBDMs, seasonal influenza H1N1 virus replicated poorly within AMs and more efficiently in PBDMs (Fig. 3 and 4). This remained true even after the addition of exogenous trypsinlike proteases and is in agreement with our previous findings on seasonal influenza H1N1 and H3N2 virus infection in AMs (45). These findings highlight the importance of AMs in the virus-host interaction of influenza viruses. Although we find that both H5N1 and H1N1 influenza viruses replicate to higher titers in alveolar epithelial cells than in alveolar macrophages, reinforcing the importance of the epithelial cells as a key amplifier of virus within the lung parenchyma (Fig. 4), recruited macrophages, represented by PBDMs, may contribute significantly to both viral amplification and the innate immune cascades that occur later in infection with seasonal influenza virus. Furthermore, avian viruses such as H5N1 appear to differ from seasonal influenza viruses in their abilities to replicate in resting AMs, a finding that may be significant for the pathogenesis of influenza virus pneumonia. Furthermore, while the initial macrophages encountered by a virus invading the lung parenchymal spaces would be AMs, once initial infection in epithelial cells and macrophages leads to the release of chemokines, circulating monocytes will be recruited to the lung parenchyma and are likely to play an important role in the subsequent pathogenesis. It is thus important to study the innate immune responses in AMs and PBDMs following viral infection.

AMs are a key component of the innate defenses of the lung

and are a rich source of chemokines and cytokines such as TNF- α , IFN- β , IL-6, MCP-1, and IP-10 after viral infection (15, 30, 34). AMs secrete TNF- α in response to viral infection (Fig. 7), whereas ATII and ATI-like cells did not. TNF- α is a key cytokine that regulates innate immune responses and has been extensively documented in viral infection-induced inflammation, including infection with influenza A virus (22, 40), and it is known as a key mediator of the pathogenesis of ARDS (20). H5N1 stimulated an especially robust secretion of TNF- α by AMs. Chemokines such as MCP-1 and IP-10 are chemoattracted to monocytes and lymphocytes and will lead to the influx of PBDMs from the peripheral circulation (Fig. 7).

Macrophages from different anatomical sites vary considerably in their physiologies and in their innate immune responses (1), and AMs resident in the alveolar spaces play a role in immune homeostasis (17). While AMs are themselves potent producers of inflammatory mediators, PBDMs are even more potent producers of chemokines such as RANTES and IP-10 (Fig. 9). These blood-derived macrophages are likely to be recruited to the lung via chemokines secreted by infected alveolar epithelial cells and AMs. Once within the alveolar spaces, the PBDMs, which produce even more potent innate immune responses following influenza virus infection, amplify the inflammatory cascade.

Our results showed that HPAI H5N1 virus was a more potent inducer of cytokines and chemokines than was LP H1N1 virus in human alveolar epithelial cells (Fig. 5 and 6),

AMs (Fig. 7 and 8), and PBDMs (Fig. 9), which is comparable to data from previous reports (2, 4–6, 45). Similar observations were found for the lungs of H5N1-infected mice, where elevated levels of macrophage-tropic chemokines, including MCP-1, were found, compared to those in lungs of mice infected with influenza H1N1 virus (29). Impaired alveolar epithelial fluid clearance (AFC) (i.e., the resolution of alveolar edema) is a common characteristic feature of patients with ALI and acute respiratory distress syndrome (ARDS) (24, 46). In the early phase of acute lung injury (ALI), pulmonary edema fluid contains high levels of proinflammatory cytokines, including IL-1 β , IL-8, TNF- α , and transforming growth factor β 1 (TGF- β 1) (12, 32). In a recent study by Lee et al. (20), pulmonary edema fluid from an ALI patient stimulated the gene expressions of all major inflammatory cytokines and chemokines in cultured human ATII cells compared to plasma controls (20). These changes suggest that human ATII cells were capable of propagating an inflammatory response once stimulated in an autocrine or paracrine manner. The exact nature of the altered alveolar fluid clearance in the pathogenesis of human H5N1 disease in the human alveolar epithelium requires further investigation.

In summary, we demonstrate that both influenza H1N1 and H5N1 viruses efficiently infect human ATII and ATI-like cells and induce robust innate immune responses. However, in contrast to PBDMs, which are permissive to the productive replication of both H5N1 and H1N1 viruses, only the highly pathogenic influenza H5N1 virus can infect and productively replicate in AMs. Compared with the H1N1 virus, HPAI H5N1 virus induced a higher level of cytokines from both PBDMs and AMs. However, PBDMs were more active in responding to H5N1 virus infection with cytokine and chemokine responses than were AMs. Our findings may provide insights into the viral tropisms and host responses of the main cell types found within the lung and may be relevant to an understanding of the pathogenesis of severe human influenza disease.

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