Human pulmonary microvascular endothelial cells support productive replication of highly pathogenic avian influenza viruses: possible involvement in the pathogenesis of human H5N1 virus infection.

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Running title: Replication of H5N1 virus in lung endothelial cells

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Abstract

Highly pathogenic avian influenza (HPAI) H5N1 viruses continue to cause sporadic human infections with a high fatality rate. Respiratory failure due to acute respiratory distress syndrome (ARDS) is a complication among hospitalized patients. Since progressive pulmonary endothelial damage is the hallmark of ARDS, we investigated host responses following HPAI virus infection of human pulmonary microvascular endothelial cells. Evaluation of these cells for the presence of receptors preferred by influenza virus demonstrated that avian-like (α2-3 linked) receptors were more abundant than human-like (α2-6 linked) receptors. To test the permissiveness of pulmonary endothelial cells to virus infection, we compared the replication of selected seasonal, pandemic (2009 H1N1 and 1918), and potentially pandemic (H5N1) influenza strains. We observed that these cells only support productive replication of HPAI H5N1 viruses which preferentially enter through and are released from the apical surface of polarized human endothelial monolayers. Furthermore, A/Thailand/16/2004 and A/Vietnam/1203/2004 (VN/1203) H5N1 viruses, which exhibit heightened virulence in mammalian models, replicated to higher titers than less virulent H5N1 strains. VN/1203 infection caused a significant decrease in endothelial cell proliferation compared to other subtype viruses. VN/1203 virus was also found to be a potent inducer of cytokines and adhesion molecules known to regulate inflammation during acute lung injury. Deletion of the H5 hemagglutinin (HA) multi-basic cleavage site did not impact virus infectivity but resulted in decreased virus replication in endothelial cells. Our results highlight remarkable tropism and infectivity of the H5N1 viruses for human pulmonary endothelial cells resulting in the potent induction of host inflammatory responses.
INTRODUCTION

Since 2003, highly pathogenic avian influenza (HPAI) A H5N1 viruses have spread to Asia, the Middle East, Africa, and Europe, and present a continuing threat to global public health. As of October 2011, more than 565 laboratory–confirmed human cases of H5N1 virus infection have been reported with a high fatality rate of approximately 60% (http://www.who.int/en/). In humans, common symptoms of H5N1 infection are fever, cough, and pneumonia with impairment of gas exchange (1, 18, 53). The disease can progress to acute respiratory distress syndrome (ARDS), multiorgan failure including combined hepatic/renal dysfunction, and death (24, 33, 53, 56). High viral load and exacerbated cytokine production in the lower respiratory tract of patients has been shown to be associated with fatal cases (15, 24, 37, 43). Although pulmonary endothelial injury is expected to contribute to the complication of H5N1-induced ARDS, H5N1 infection of human pulmonary endothelial cells has not been well studied.

Receptor specificity is an important determinant of host range restriction among influenza viruses (41). The influenza virus hemagglutinin (HA) protein is responsible for binding to sialic acid (SA) containing cell surface receptors for virus entry. In general, human influenza viruses express HA that preferentially bind to α-2,6 linked SA receptors which are the predominant linkage expressed in the upper respiratory tract (URT) of humans, whereas avian influenza viruses, such as contemporary avian H5N1 viruses, preferentially bind to α-2,3 linked SA receptors (21, 39, 52). Higher proportions of α-2,3 linked SA receptors in the human lower respiratory tract compared with the URT may partially explain the severity of H5N1 viral pneumonia in humans resulting from H5N1 viral attachment deep in the lungs (21, 39, 52). Detailed studies of virus attachment to
human respiratory tissue have shown that H5N1 viruses bind to type II pneumocytes, alveolar macrophages, and non-ciliated epithelial cells in the terminal bronchioles of the lower respiratory tract (47, 48). Therefore, the composition of a particular SA species present on cells can influence influenza virus tropism and pathogenesis.

The cleavage properties of HA$_0$ and the distribution of functional proteases in the host are main factors for tissue tropism and systemic virus spread. Cleavage of the HA precursor molecule HA$_0$ is required for the release of the fusion peptide and the conformational changes necessary for viral infectivity. Human influenza viruses contain a single basic amino acid at the cleavage site and can be cleaved by extracellular trypsin-like proteases present in certain respiratory cell types, restricting virus spread beyond the respiratory tract (8, 10, 23, 28). More recently, Type II transmembrane serine proteases (TTSPs), such as TMPRSS2 (transmembrane protease, serine 2), have been identified in the human lung and may also support the spread of human influenza viruses by intracellular cleavage activation of HA (10). HA cleavability is a critical determinant of HPAI H5N1 influenza virus pathogenicity in poultry and mammals (16, 20, 32, 40). The HA of HPAI H5N1 viruses invariably contain multiple basic amino acid residues (-RRRKK-) at the cleavage loop and can be cleaved intracellularly by ubiquitously expressed furin-like proteases, which are expressed in most organs of mammals enabling the virus to infect many cell types and causing systemic infections (16, 20, 32, 40).

Systemic spread of virus to multiple extrapulmonary organs is a characteristic of HPAI H5N1 virus infection in mammals (29, 30). In limited autopsy cases, viral genomic sequences or antigens have been detected not only in lung pneumocytes but also in tissue...
samples from spleen, brain, liver, and placenta (19, 24, 42, 56). Viral RNA has also been
detected in blood samples of several fatal H5N1 cases (12, 14, 15, 42).
The nature of the interactions between H5N1 influenza virus and the host
pulmonary vasculature are largely unknown. Pulmonary endothelium, the intimal lining
of blood vessels, is the barrier between the blood and interstitium and has important
regulatory functions (reviewed in (2)). The alveolar capillaries are terminal networks in
the pulmonary circulation, with microvascular endothelial cells present in the alveolar gas
exchange units. The gross pulmonary pathology of H5N1-virus infected lung shows that
the diffuse alveolar damage and extensive consolidation with varying degree of
hemorrhage is usually caused by alveolar capillary damage (24, 33). Moreover, the
interaction of leukocytes and mediators, such as cytokines, oxygen radicals, and
complement, are believed to be responsible for endothelial damage and increased
permeability to fluid and proteins (13). Recently published papers reported replication of
HPAI H5N1 virus in lung microvascular endothelial cells (11, 35). However, the
interaction of HPAI H5N1 viruses and those of other subtypes with pulmonary
endothelial cells which comprise one-third of the lung cell population warrants further
investigation.
In this study, we characterized the influenza virus receptors on human pulmonary
microvascular endothelial cells and used an in vitro model to assess the infection and
replication of selected seasonal, pandemic (2009 H1N1 and 1918), and potentially
pandemic H5N1 influenza strains. The data demonstrated that pulmonary endothelial
cells only support productive replication of HPAI H5N1 viruses and that the polybasic
amino acids at the cleavage site play an important role in H5N1 virus replication.
Furthermore, in comparison to infection with H1N1 subtype virus, HPAI H5N1 virus infection results in substantial induction of cytokines and adhesion molecules. These differences shed light on understanding the pathogenic mechanisms of pulmonary endothelial injury associated with H5N1 virus infections.

MATERIALS AND METHODS

**Viruses.** The viruses used in this study are listed in Table 1. HPAI H5N1 and H7 subtype viruses were grown in the allantoic cavities of 10-day-old embryonated hen’s eggs for 24-40 hours at 37°C. Seasonal human H1N1 and H3N2 viruses were grown in eggs for 48 h at 33.5°C. Allantoic fluid was clarified by centrifugation, aliquoted, and stored at –70°C. The 2009 H1N1 virus, recombinant 1918 virus and Tx/91 virus were propagated in MDCK cells for 48 h at 37°C. The supernatants were clarified by centrifugation, aliquoted, and stored at -70°C. Virus titers were determined by standard plaque assay (54). The identity of virus genes was confirmed by sequence analysis to verify that no inadvertent mutations were present during the generation of virus stocks.

All research with HPAI H5 and H7 subtype viruses were conducted under biosafety level 3 containment, including enhancements required by the U.S. Department of Agriculture and the Select Agent Program (Wilson and Chosewood in 5th ed BMBL and http://www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm)

**Generation of recombinant virus.** The HAdeIVN/1203 mutant (virus lacking the polybasic cleavage site), wild type HAVN/1203 virus, and the HA and neuraminidase (NA) of VN/1203 in the 6-gene backbone of A/Puerto Rico/8/34 (PR/8) virus were generated using reverse genetics plasmids (kindly provided by Adolfo Garcia-Sastre,
Mount Sinai School of Medicine, New York) (36). The construction of plasmids and the removal of nucleotides that encode the polybasic amino acids at the cleavage site of VN/1203 HA gene are described by Park et al. (36). For virus rescue, plasmids were transfected into 293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, Calif., USA). Forty eight hours later, the supernatants were transferred to MDCK cells and virus was harvested upon observation of cytopathic effect (CPE). The rescued viruses were propagated in MDCK cells and the identity of virus genes was confirmed by sequence analysis.

**Cells and viral infection.** Primary human lung blood microvascular endothelial cells (HMVEC-LBI) (Lonza, Walkersville, MD) and an immortalized human lung microvascular endothelial cell line (HULEC) were grown in Endothelial Cell Basal Medium-2 (EBM-2) (500ml) containing the following growth supplements: hEGF, 0.5ml; Hydrocortisone, 0.2 ml; GA-1000, 0.5ml; FBS, 25 ml; VEGF, 0.5ml; hFGF-B, 2ml; R-IGF-1, 0.5ml; Ascorbic Acid, 0.5 ml (Lonza). The HULEC cell line possesses many features of primary pulmonary microvascular endothelial cells (9, 31). Cells were seeded onto six-well (5x10^5/well) or 12-well (3x10^5/well) plates and cultured for 24 h. Monolayers were then washed with EBM-2 supplemented with 0.3% bovine serum albumin (EBM-2/BSA) and infected with virus at a multiplicity of infection (MOI) ranging from 0.01-5 for one hour. After washing, EBM-2/BSA was added to each well for the duration of the experiment. Human bronchial epithelium cells, Calu-3 were grown and inoculated as previously described (54). Rat pulmonary microvascular endothelial cells (PMVEC) and rat pulmonary arterial endothelial cells (PAEC) (22) were grown in Dulbecco's Modified Eagle Medium.
(DMEM), supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. The cells were seeded onto six-well plates at 5x10^5/well and infected similarly as described above using DMEM supplemented with 0.3% BSA.

In experiments that required supplementation of exogenous protease for cleavage of the HA protein, either 1 µg/ml of N-p-tosyl-l-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Sigma-Aldrich, St. Louis, MO) or 10% chicken egg allantoic fluid was used.

**Immunofluorescence staining and microscopy.** For surface receptor analysis, endothelial cells were seeded onto collagen-coated 8-well chamber slides. After 30 min fixation with 3.7% formaldehyde in PBS, cell monolayers were blocked with 3% BSA in PBS for 30 min and then sequentially incubated with either biotinylated *Maackia amurensis* lectin I and II (MAA I and II, 20 µg/ml) or biotinylated *Sambucus nigra* lectin (SNA, 20µg/ml) (Vector Laboratories, Burlingame, CA) for one hour, followed by the addition of fluorescein-conjugated avidin D (Vector Laboratories). To detect influenza A virus nucleoprotein (NP) antigen, cells seeded on chamber slides were infected with influenza virus at an MOI 1. At 10 and 24 h post-inoculation (p.i.), cells were fixed, permeabilized with 0.5% Triton X-100 in PBS for 20 min, and incubated with mouse anti-NP monoclonal antibody A-3 (50) followed by Rhodamine-conjugated secondary antibody (Becton Dickinson (BD) Biosciences, San Diego, CA). Immunostained cells were mounted with 4',6-Diamidino-2-phenyindole (DAPI; Sigma-Aldrich) and examined under a Zeiss Axioskop 2 fluorescent microscope.

**Detection of α-2,6 and α-2,3 linked sialic acid residues by flow cytometry.** Endothelial cells grown on tissue culture flasks were trypsinized and the cell suspension
was passed through a cell strainer (BD Biosciences) to obtain single cells. After counting, cells were separated to 1.5 ml tubes containing 1x10^6 cells each, centrifuged at 1000 x g for 10 min, and washed twice with EBM-2 medium and once with FACS washing buffer (PBS with 2% FBS). A 200 μl aliquot of SNA-FITC or MAA-FITC (1mg/ml, EY Laboratory Inc, San Mateo, CA) in different dilutions was added to the cells and incubated for 1 h at 4°C. Cells were washed and resuspended in FACS washing buffer. Data acquisition was performed on a BD LRS II flow cytometer and analyzed using BD FACSDiva software (BD Biosciences).

**Virus binding assay.** Labeling of virus was modified and adapted from previously published methods (6, 47). Concentrated virus was labeled using the FluoroTag fluorescein isothiocyanate (FITC) conjugation kit (Sigma-Aldrich) according to the manufacturer's protocols. FITC-labeled virus was diluted and passed through a Millex-HV 0.45 μm filter, followed by hemagglutination units (HAU) determination. The confluent HMVEC-LBI cells grown on collagen-coated chamber slides were fixed with 2% paraformaldehyde for 30 mins and blocked with 3% BSA. FITC-labeled virus with comparable concentration was added to the cell monolayer and incubated overnight at 4°C in humidified chamber. After PBS wash, cell monolayers were incubated with 3% H_2O_2 for 10 mins and anti-FITC-HRP antibody for 1 h, followed by AEC solution for 10 mins in dark. The slides were mounted and evaluated under the Zeiss Axioskop 2 microscope.

**Real-time quantitative PCR and human endothelial cell biology PCR Array.** Total RNA from virus-infected or uninfected cells were extracted using the RNeasy Mini Kit (Qiagen; Carlsbad, CA) with DNase digestion, and 1.0 μg of total RNA was reverse
transcribed with QuantiTect Reverse Transcription Kit (Qiagen). The cDNA products were subjected to Real-Time PCR assay using QuantiTect SYBR Green PCR Kit (Qiagen) and analyzed using previously published methods (54). Additionally, cDNAs were analyzed using Human Endothelial Cell Biology RT² Profiler™ PCR Array (Qiagen), containing 84 genes involved in endothelial cell permissibility and vessel tone, angiogenesis, endothelial cell activation and injury, according to the manufacturer’s instructions. The expression data were analyzed through the vendor’s web module, and fold change in differential expression with significant p-values were calculated and presented.

**Cytokine quantification.** Endothelial cells were infected with virus at an MOI of 1. Supernatants were collected at 24 h p.i. and cytokine levels were examined using Bio-Plex Pro Assay, according to the manufacturer’s instructions (Bio-Rad, Hercules, CA). The cytokines included in the assay were IL-6, IL-7, IL-8, GM-CSF, IFN-γ, IP-10, MCP-1, RANTES, TNF-α, and VEGF.

**Cell proliferation assay.** Endothelial cells were seeded onto a 96-well plate at 5x10^4 per well 24 h before cells were inoculated with virus at different MOIs. Uninfected and infected cells (44 h p.i.) were incubated with 10 µl of the Cell Proliferation Reagent (WST-1) (Roche, Indianapolis, IN) for 4h. The stable tetrazolium salt WST-1 is cleaved to a soluble formazan through a complex cellular mechanism, as a marker for cellular metabolic activity. Absorbance (450 nm) of each sample was then measured against a background control using a microplate reader, according to the manufacturer’s instructions. The measured absorbance directly correlates to the number of viable cells.
RESULTS

α-2,3 sialic acid (SA) configuration is more abundant than α-2,6 SA configuration on the surface of pulmonary endothelial cells. Influenza virus infection is initiated due to interactions between the HA protein and sialic acid-containing glycans on the surface of host cells. To determine the susceptibility of lung endothelial cells to influenza virus infection, we first examined the distribution of SA receptors on the cell surface using lectin staining. *Sambucus nigra* (SNA) lectin binds predominantly to α-2,6 SA configuration, the receptor preferred by human influenza viruses. *Maackia amurensis* lectin I (MAA I) and II (MAA II) are two isoforms that identify α-2,3 SA configuration, the preferred receptors of avian influenza viruses. MAA I is specific for SAα2-3Galβ1-4GalNAc whereas MAA II is specific for SAα2-3Galβ1-3GalNAc (34). Both α-2,6 and α-2,3 SA receptors were detected on the surface of HMVEC-LBI (primary cells) and HULEC (cell line) by lectin staining, however the intensity of MAA (I and II) staining was visually stronger than SNA staining (Fig. 1A). To perform a relative quantification of lectin binding on pulmonary endothelial cell surfaces, human endothelial cells were removed from the culture flasks by a brief trypsinization, stained with FITC-conjugated lectin at different concentrations, and analyzed using flow cytometry. At a concentration of 20 μg/ml, almost 100% of human endothelial cells were stained positive for both α-2,6 (FITC-SNA) and α-2,3 (FITC-MAA) SA receptors, however the Mean Fluorescence Intensity (MFI) of MAA was three times higher than that of SNA. Using a lower concentration of 5 μg/ml labelled lectin, 53.8% and 99.5% of HMVEC-LBI cells stained positive for FITC-SNA and FITC-MAA, with MFI as 422 and 1,275, respectively. At the same concentration, 57.8% and 93% of HULEC cells stained...
positive for FITC-SNA and FITC-MAA, with MFI as 389 and 1,944, respectively (Fig. 1B). To further investigate the binding preference of different influenza viruses, we assessed the attachment of FITC-labelled virus to HMVEC-LBI cells. As shown in Fig. 1C, the HPAI H5N1 virus (Thai/16) demonstrated extensive attachment to the cell surface, whereas attachment was much less abundant with human influenza viruses, 1918 HA/NA:Tx/91 (SC/18 HA/NA and internal genes of A/Texas/36/91 virus) (7, 45, 54, 55) or 2009 H1N1 (Mexico/4482) virus.

To confirm our observations made with human endothelial cells, these initial studies were repeated using well-defined endothelial cells derived from nonhuman mammalian species (22). Primary rat pulmonary microvascular endothelial cells (PMVEC) and primary rat pulmonary arterial endothelial cells (PAEC) seeded on chamber slides were stained by the lectins as described above. Similar to binding pattern of human pulmonary endothelial cells, much greater amounts of \(\alpha\)-2,3 linked SA were observed on the cell surface of both PAEC and PMVEC in comparison to \(\alpha\)-2,6 linked SA levels (Fig. 1D). Taken together, these data demonstrate that the distribution of \(\alpha\)-2,3 linked SA configuration is much more abundant than \(\alpha\)-2,6 linked SA configuration on the surface of pulmonary endothelial cells.

**HPAI H5N1 viruses replicate productively in human pulmonary microvascular endothelial cells.** Targeted influenza virus infection of pulmonary vascular endothelial cells may acutely impair normal lung function and contribute to the pathogenesis of severe disease. Here, we compared the replication kinetics of multiple subtypes of influenza A viruses (listed in Table 1) in HMVEC-LBI and HULEC cells grown on six-well plates. As shown in Fig. 2A and B, both types of human endothelial...
cells tested supported productive replication of the HPAI H5N1 viruses, VN/1203 and Thai/16. The H5N1 viruses reached high titers of $10^6$ to $10^7$ PFU/ml at 24 and 48 h p.i. In contrast, despite the addition of a protease supplement to the culture medium to promote HA cleavage, the peak infectious titers of human H3N2 viruses (Pan/99 and Wis/05) only reached $10^{3.6}$ PFU/ml (Fig 2A-B). Comparatively poor replication was also observed among human H1N1 influenza viruses, including the pandemic 1918 (SC/18) virus. Similar replication kinetics were obtained in rat pulmonary endothelial cells, PMVEC and PAEC following virus inoculation at a MOI of 0.01. Rat PMVEC and PAEC cultures were found to support productive replication of VN/1203 H5N1 virus (peak titers of $10^6-7$ PFU/ml), whereas comparatively low virus titers ($<10^2$ PFU/ml) were observed following inoculation with seasonal influenza H1N1 and H3N2 subtypes (data not shown).

We next determined the replication kinetics of a 2009 H1N1 pandemic virus that began circulating in the spring of 2009. For this experiment, primary HMVEC-LBI cell medium was supplemented with 10% normal allantoic fluid to promote extracellular HA cleavage. Only subtle differences were observed among the influenza viruses tested and in comparison to the first data point (2 h) of the growth curve, cultures did not demonstrate substantial virus titer increases up to 48 h p.i. (Fig. 2C). Human seasonal H3N2 viruses (Pan/99 and Wis/05) and 2009 pandemic H1N1 virus (Mexico/4482) virus titers reached approximately $10^3$ PFU/ml at 24 h p.i. with no further increases measured at 48 h p.i. Seasonal H1N1 virus (Tx/91 and Brisbane/59) cultures showed no increase in viral titers at any time point. Because the growth of influenza viruses in many cell lines requires the addition of trypsin to the culture medium to ensure HA cleavage and multicycle infection, replication...
kinetics of selected viruses were measured in the presence or absence of exogenous protease (TPCK-treated trypsin). In the absence of TPCK-trypsin, only H5N1 VN/1203 virus replicated to substantial titers >10^5 PFU/ml (Fig. 2D). Conversely, even in the presence of TPCK-trypsin, the 1918 (SC/18) virus failed to replicate above input titer and Pan/99 virus failed to reach viral titers above 10^{3.5} PFU/ml.

To further test the permissiveness of pulmonary endothelial cells for influenza virus infection, cells grown on chamber slides were inoculated with VN/1203, SC/18 (1918), or Pan/99 virus at an MOI of 1 and intracellular expression of influenza virus NP was evaluated at 10 h and 24 h p.i. Overall, influenza virus infection of pulmonary endothelial cells was less efficient compared with infection of airway epithelial cells, which possess a reported infection rate of 30% to 40% within 24 h p.i. (7, 45, 54, 55). In this study, greater numbers of NP-positive cells (8-24% infection rate) were observed at 24 h p.i in endothelial cells infected with H5N1 VN/1203 virus compared with SC/18- or Pan/99-virus infected cells (3-7% infection rate); SC/18 virus showed the least amount (0.5-1.1%) of NP positive cells (Fig. 2E). HULEC cells, which can reach a higher cell density, allowed the visualization of more NP-positive cells, some of which were observed in clusters in VN/1203 virus infected cultures at 24 h p.i. (Fig. 2E; bottom-left panel).

In a detailed side-by-side comparison experiment of avian influenza subtype viruses possessing α-2,3 SA-binding preference, we next compared the replication kinetics of avian H1N1, H5N1, and H7 subtype viruses in HULEC and HMVEC-LBI cultures. H7 viruses were included for comparison purposes because they represent avian influenza viruses of another subtype and they largely maintain the classic avian-binding
preference for α-2,3 linked SA. However, the North American lineage H7N2 (NY/107) virus was found to possess increased affinity toward α-2,6 linked SA and reduced binding to α-2,3 linked SA (5). Although possessing an HA with a polybasic cleavage site (Table 1), both SP/83 and Ck/Korea HPAI H5N1 viruses exhibited a low-pathogenicity phenotype in ferrets and mice (30). As shown in Fig. 3A, SP/83 and Ck/Korea HPAI H5N1 viruses replicated significantly less efficiently than the highly virulent H5N1 viruses, VN/1203 and Thai/16 (p <0.05). The LPAI avian H1N1 (Dk/NY) virus exhibited minimal growth in HULEC cells. In HMVEC-LBI, Thai/16 virus showed a clear distinction in replication efficiency compared to H7 subtype viruses, for which productive replication was generally not observed (Fig. 3B). Only the highly pathogenic H7N7 (NL/219) virus, isolated from a fatal case, replicated to a moderate level of $10^2$ to $10^3$ PFU/ml at 24 and 48 h p.i. Taken together, the data suggest that endothelial cells support entry and efficient replication of HPAI H5N1 viruses, whereas human and other avian viruses, including HPAI H7 subtype viruses tested showed poor infectivity in these cells.

**HPAI H5N1 virus preferentially enter through and are released from the apical surface of human pulmonary endothelial cells.** To assess the polarity of HPAI H5N1 virus infection and release of progeny virus particles, we compared the replication kinetics of VN/1203 virus following infection of the apical or basolateral side of the cell monolayer. HULEC cells were seeded onto 12-well transwell membrane inserts with pore size of 0.3 µm and cultured for 1 week for polarization of the pulmonary endothelial cells. Unlike human bronchial epithelial cells that exhibit high transepithelial resistance (54), HULEC endothelial cells displayed very low transepithelial resistance. Cells were
inoculated with virus either from the apical surface of the monolayers or the basolateral surface at an MOI of 0.01. Supernatants from both compartments were collected for virus titer determination. When VN/1203 virus was inoculated via the apical surface of endothelial cells, the H5N1 strain replicated to a much higher titer compared to virus titers detected following basolateral inoculation (Fig. 4). Moreover following apical surface inoculation, virus was released from the apical side at significant higher titers (p <0.05) than those released from basolateral side at 24 h p.i. These results demonstrate that avian H5N1 viruses enter and release primarily from the apical surface of polarized human pulmonary microvascular endothelial cells.

**Removal of the HA polybasic cleavage site diminished HPAI H5N1 virus replication in human pulmonary microvascular endothelial cells.** We next evaluated the contribution of the multi-basic HA cleavage site on H5N1 virus replication in pulmonary endothelial cells. Using NP staining of HMVEC-LBI cells, a similar rate of infection was observed between the mutant H5N1 virus (HAdelVN/1203) with deletion of the HA polybasic cleavage site and the wild-type rescued VN/1203 virus (Fig. 5A). The replication kinetics of this pair of viruses was further evaluated in HMVEC-LBI cells and using an established human bronchial epithelial (Calu-3) cell line which possesses HA-activating host proteases capable of cleaving monobasic HA cleavage sites (54). Calu-3 cell cultures were found to support productive replication of either H5N1 virus containing the wild-type or mutant HA (peak titers of $10^8$ PFU/ml) (Fig. 5B), however in HMVEC-LBI cultures, the HAdelVN/1203 virus displayed significantly (p<0.001) lower viral titers compared to wild-type VN/1203 virus at 24, 48, and 72 h p.i. (Fig. 5C). Although HAdelVN/1203 virus displayed reduced replication capacity in pulmonary
endothelial cells, a relatively similar infection rate was observed for the HA mutant virus based on M1 gene expression levels (Fig. 5D). At 1 and 24 h p.i., M1 gene levels were comparable for both viruses in HMVEC-LBI cultures, suggesting that H5N1 viruses could successfully infect pulmonary endothelial cells independent of the cleavage site motif, but efficient H5N1 replication and production of infectious progeny virus in these cells requires a multibasic cleavage site.

Because the expression of interferon β (IFNβ) and Mx1 (an interferon-stimulated antiviral gene) are well characterized components of the innate immune response directed against viral infections, we also compared the expression levels of these IFN signature genes in pulmonary endothelial cell cultures collected at 24 h p.i. The 24-hour time point was selected because previous studies have demonstrated low expression of IFNβ at earlier time points (such as 8 h) following H5N1 virus infection of cultured cells (54). Consistent with replication data, endothelial cells elicited a significantly higher induction of IFNβ and Mx-1 genes to wild-type VN/1203 virus compared to the mutant HAdelVN/1203 virus (Fig. 5E). Taken together, these data support an important role for the HA polybasic cleavage site in both HPAI H5N1 virus replication and host innate antiviral defense in HMVEC-LBI cells. However other viral genes, or combination of genes, are most likely contributing to the replication efficiency of HPAI H5N1 virus in human pulmonary endothelial cells.

HPAI H5N1 virus elicits a higher level of gene expression and production of proinflammatory cytokine/chemokines and adhesion molecules in human pulmonary microvascular endothelial cells. We further examined host responses to infection with HPAI H5N1 viruses in human pulmonary endothelial cells and compared
the results with those responses induced by the 1918 H1N1 virus. As indicated above, dramatic differences in replication were observed between H5N1 (VN/1203) and 1918 (SC/18) viruses in pulmonary endothelial cells (Fig. 2). In this experiment, HULEC cultures grown on six-well plates were infected with virus (MOI = 1) and at 24 h p.i., total RNA from uninfected (mock) or virus-infected cells was extracted and analyzed for host gene expression using a Human Endothelial Cell Biology RT² Profiler™ PCR Array. Detailed gene expression data for genes with greater than 3-fold induction/reduction over mock are presented in Table 2. With few exceptions, VN/1203 virus infection induced the greatest numbers of host genes with the highest level of expression; VN/1203 virus infection induced 24 host genes whereas SC/18 virus infection resulted in the induction of 5 genes with greater than 3-fold induction over mock-infected HULEC cultures (Table 2). In particular, VN/1203 virus induced expression of key cytokines/chemokines, including IFNβ, IL7, TNF, CCL2 and CCL that were at least 10-fold higher than the host responses observed following SC/18 virus infection. Significant increases in gene expression of several adhesion molecules, including members of the selectin family (E-Selectin, L-Selectin, and P -Selectin ligand), ICAM1, and VCAM1, were observed following VN/1203, but not SC/18 virus infection. Genes related to endothelial cell function regulation were also up-regulated, including genes involved in angiogenesis (TYMP, FGF1), coagulation (PF4), homeostasis (NPPB), cell survival (SPHK1), and thrombosis (SERPINE1). During VN/1203 virus infection, the transcriptional level of endothelial NO synthase (eNOS) did not change significantly, however, inducible nitric oxide synthase 2A was highly induced. Among genes related to
apoptosis, TNFAIP3, FASLG, and CASP1 were up-regulated; CASP6 and TNFRSF10C were down-regulated 3-fold.

We further investigated differences in host responses to VN/1203 virus compared with 1918 H1N1 infection by measuring cytokine protein levels released into pulmonary endothelial cell cultures. HULEC cells were infected with virus at an MOI of 1 and supernatants were collected at 24 h p.i. for Bio-Plex assay (Fig 6). The results showed that VN/1203 virus infected cultures released substantial amounts of cytokines/chemokines (RANTES, IP-10, IL-6, IL-8, MCP-1, VEGF, TNF and IFNγ) were significantly (> 4-fold) higher than that induced by the pandemic SC/18 virus (p<0.05). These data further demonstrate that in comparison to H1N1 virus, HPAI H5N1 virus infection resulted in a substantial induction of innate immune response in human pulmonary endothelial cells.

**Proliferation and viability of human pulmonary microvascular endothelial cells is affected by HPAI H5N1 virus infection.** Overall, influenza virus infection of pulmonary endothelial cells (HULEC and HMVEC-LBI) resulted in less CPE and cell death microscopically than that previously observed in infected airway epithelial cells (7, 45, 54, 55). In fact, following VN/1203 virus infection, HMVEC-LBI cell monolayers remained generally intact for up to 72 h p.i. (data not shown). To investigate the impact of influenza virus infection on the growth and survival of pulmonary endothelial cells, infected HMVEC-LBI cells (MOI of 5.0, 1.0, and 0.2) were subjected to the WST-1 proliferation/viability assay at 44 h p.i. (Fig 7). In comparison to uninfected controls, which showed an absorbance reading of 0.997, influenza virus infection of HMVEC-LBI cultures resulted in a decrease in cell proliferation/viability with the greatest decrease
observed at the highest MOI. VN/1203 virus infection caused a significant decrease in cell proliferation/viability at all inoculation doses in comparison to pandemic 2009 H1N1 and seasonal influenza viruses. The seasonal H3N2 (Pan/99) virus induced an intermediate decrease, whereas virus infection with both H1N1 (Brisbane/59 and Mexico/4482) viruses resulted in a minimal decrease in cell proliferation/viability following virus infection of pulmonary endothelial cells. These results demonstrate that one outcome of HPAI H5N1 viral infection of pulmonary endothelial cells is an accelerated loss of cell viability compared with other influenza strains of lesser virulence.
In contrast to seasonal influenza virus, human infection with HPAI H5N1 viruses causes severe disease of the lower respiratory tract and many patients progress rapidly to ARDS and multi-organ failure (15, 24, 37, 43). Although endothelial cell injury is a general characteristic feature of ARDS (51), the role of pulmonary endothelial cells in H5N1 pathogenesis is still largely unknown. Here, we provide data using a pulmonary endothelial cell line and primary cells isolated from human lung tissue and compared host cell permissiveness to infection with influenza viruses of multiple subtypes. The human pulmonary endothelial cell model was also utilized to evaluate the cell receptors for influenza virus and innate immune responses induced by HPAI H5N1 virus compared with other influenza virus subtypes. We found that unlike human bronchial epithelial cells, which are permissive to productive replication of both avian and human influenza viruses and express avian-like α-2,3- and human-like α-2,6-linked SA receptors equally well (7, 45, 54, 55), pulmonary endothelial cells possess α-2,3-linked SA configuration as the dominant receptor type and only support efficient replication of HPAI H5N1 virus. The polybasic HA cleavage site was found to be necessary but not sufficient for the high replication efficiency of HPAI H5N1 virus in pulmonary endothelial cells. The H5N1 virus replication correlated with high level expression of proinflammatory cytokines/chemokines and adhesion molecules and also resulted in a marked decrease in cell viability and proliferation which could be attributable to virus-mediated cell death and/or a cytokine-mediated process. The clinical course of H5N1 virus infection in humans often results in the progression of severe lower respiratory tract disease, which includes dyspnea, chest pain
and pulmonary infiltrates (1, 18, 53). Infiltration of neutrophils, alveolar edema,
endothelial hypertrophy and necrosis among H5N1 infected patients is thought to
contribute to the severe lung pathology of fatal human infection. Although in-situ
hybridization failed to show positive viral RNA or influenza NP antigen staining in lung
endothelial cells from postmortem examination of patients who succumbed to H5N1
infection (19), the detection of IFN-β-positive endothelial cells in lungs of H5N1-infected
patients suggests that these cells are responding to viral infection (17). However, in situ
evidence for H5N1 virus infection of pulmonary endothelial cells in humans is limited
due to the paucity of postmortem tissues for study. In the macaque model, endothelial
cells appeared to be productively infected and displayed necrosis in H5N1-challenged
animals (4, 25). In standard cultures, we observed that both human and rat pulmonary
microvascular endothelial cells could be productively infected by VN/1203 (H5N1) virus,
which was highly lethal in ferrets and mice (29, 30). Utilizing a polarized endothelial cell
model, in which cells were seeded on transwells creating two distinct surfaces: apical and
basolateral domains, we found preferential viral entry and release from the apical surface.
HPAI H5N1 virus particles bud from the apical (luminal) side of endothelial cells, in
contact with blood, so that released virus could enter the general circulation and cause
viremia. Unlike seasonal influenza viruses, which are confined mainly to the upper
respiratory tract of mammals, viral RNA has been detected in the blood of H5N1-infected
patients with extrapulmonary complications, including multiorgan failure, which are
common in fatal cases (reviewed in (46). It is reasonable to speculate that efficient
replication of H5N1 virus in pulmonary endothelial cells may be promoting systemic
spread of virus, and contributing to the pathogenicity of HPAI H5N1 viruses in the
mammalian host. Since transport of H5N1 virus from the basolateral to the apical chamber was minimal in the polarized endothelial cell model, it is not entirely clear how initial apical infection occurs in these cells. 

In this study, we have addressed the possible contribution of the HA glycoprotein in the replication efficiency of H5N1 virus in pulmonary endothelial cells. The two main functions of the HA are to facilitate i) virus binding to target host cells, via sialic acid-containing receptors, ii) virus entry into the cell which is dependent on HA cleavage. Because the availability of suitable receptors on the host cell surface can determine efficiency of influenza virus infection and replication (41), the abundance of surface-associated SA on pulmonary endothelial cells was analyzed through careful flow cytometric analysis coupled with lectin staining in a dose-dependent manner. It has been reported that α-2,3 SA and α-2,6 SA receptors were homogenously expressed on microvascular endothelial cells (52). However, we found that α-2,3 SA configuration was much more abundant than α-2,6 SA receptors which corresponded to a lower rate of infectivity for human influenza viruses possessing the α-2,6 SA receptor binding preference. Moreover avian-origin viruses with preferential α-2,3 SA binding, attached and infected pulmonary endothelial cells at a higher frequency compared to human influenza viruses. Taken together, the data suggest that the receptor specificity is most likely contributing to the efficient H5N1 virus replication in these cells; however, additional data are needed to definitively assess the role of this molecular determinant. The generation of mutant influenza viruses that carry deletions in H5 HA receptor-binding site conferring a change in binding preference from avian- to human-type receptor are required.
The cleavage properties of HA₀ and the distribution of functional proteases in the host contribute to tissue tropism (16, 20, 32, 40). Unlike airway epithelial cells (54), pulmonary endothelial cells do not appear to produce sufficient endogenous proteases capable of cleaving HA at a monobasic cleavage site. We found that these cells only support multiple-cycle growth of H5N1 viruses containing a polybasic cleavage site. Removal of the polybasic cleavage site in VN/1203 virus did not impact initial viral infectivity in pulmonary endothelial cells; however, it greatly attenuated viral growth kinetics relative to wild-type virus. The results lend support to the concept that in vivo, the polybasic HA cleavage site is a molecular determinant for not only extrapulmonary virus replication, but also enhancing H5N1 virus replication in pulmonary endothelial cells. H5N1 infection of endothelial cells may contribute to the high viral load in lung tissue and potential virus spread beyond the respiratory tract.

Although HA appears to play an important role in efficient H5N1 virus replication through its receptor binding specificity and polybasic cleavage site, additional virulence determinants of the virus most certainly exist. We observed that a reassortant virus containing six internal genes from A/PR/8/34 (H1N1) and the HA and NA genes from VN/1203 virus exhibited attenuated replication in pulmonary endothelial cells compared with wild-type VN/1203 virus despite comparable efficiencies of initial infectivity (based on NP staining) (data not shown). Moreover, H7 subtype viruses and the less virulent SP/83 and Ck/Korea H5N1 viruses, which possess “avian-like” α-2,3 SA binding preference and a polybasic cleavage site, could not productively replicate as efficiently as HPAI H5N1 viruses in human pulmonary endothelial cells, further suggesting that additional molecular factors are associated with HPAI H5N1 virus virulence.
ARDS is characterized by diffuse alveolar damage usually following an intense inflammatory response to infection (51). The suggestion that the fatal outcome in patients with H5N1 virus infection may be attributed to elevated levels of proinflammatory cytokines (4, 15, 30, 45) prompted us to characterize the mediators of inflammation produced by human pulmonary endothelial cells. We found that H5N1 virus infection resulted in elevated production of multiple cytokines, including TNF, IP-10, IL-6, IL-8, MCP-1, IFNγ, VEGF, and RANTES. TNF and IL-8 are of particular interest because of their association with ARDS and can instigate a cascade of physiological changes including recruitment of neutrophils leading to alveolar capillary damage (51). Moreover, it has been demonstrated that H5N1, but not H1N1-infected TNFR-1-deficient knockout mice exhibit a substantial reduction in lung inflammation and delay in mortality compared to wild-type mice, suggesting that TNF contributes to H5N1-induced inflammation of lung tissue (38). Blocking TNF has been shown to decrease vascular permeability through the destabilization of endothelial cell cytoskeleton (49) and affects subsequent immune cell transmigration across the endothelium (26). When exposed to TNF, a normally “quiescent” endothelium becomes activated and expresses additional proinflammatory factors, including chemokines and adhesion molecules (51). In our analysis we also observed that human pulmonary endothelial cell cultures generated high levels of adhesion molecules, including selectin P ligand, selectin L & E, ICAM1, and VCAM1, in response to H5N1, but not to H1N1 virus infection. Production of chemokines and adhesion molecules by endothelial cells could contribute to the tissue damage by causing vascular injury and destruction of the parenchymal cells through the accumulation of inflammatory cells (3, 27). The resulting loss of functional alveolar...
surface area could result in inadequate gas exchange, lower respiration, and ultimately death. The pulmonary endothelium is strategically located within the lung and it’s functional and structural integrity are essential for adequate pulmonary function. The majority of patients with confirmed H5N1 virus infection has an aggressive clinical course and often present with respiratory failure frequently complicated with ARDS (44). ARDS induced by H5N1 viral infection is most likely to be linked to patient death and pulmonary endothelial injury is expected to contribute to the abnormalities seen in H5N1-induced ARDS. Our results demonstrate a unique virulence trait of HPAI H5N1 following virus infection of pulmonary endothelial cells which leads to high virus load, an overwhelming immune reaction and marked decrease in cell viability. Future investigation of the interaction between H5N1-induced inflammation and the pulmonary endothelium, especially in in vivo models, is warranted.

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REFERENCES


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<table>
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<tr>
<th>Virus</th>
<th>Abbreviation</th>
<th>Subtype</th>
<th>Cleavage site</th>
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<tbody>
<tr>
<td>A/Vietnam/1203/04</td>
<td>VN/1203</td>
<td>H5N1</td>
<td>*PQRERRRKKR/GLF</td>
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<tr>
<td>A/Thailand/16/2004</td>
<td>Thai/16</td>
<td>H5N1</td>
<td>*PQRERRRKKR/GLF</td>
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<tr>
<td>A/Thailand/SP/83/2004</td>
<td>SP/83</td>
<td>H5N1</td>
<td>*PQRERRRKKR/GLF</td>
</tr>
<tr>
<td>A/Chicken/Korea/ES/2003</td>
<td>CK/Korea</td>
<td>H5N1</td>
<td>*PQRE-KKRRR/GLF</td>
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<tr>
<td>A/Netherlands/219/2003</td>
<td>NL/219</td>
<td>H7N7</td>
<td>*PEIP-KRRRRR/GLF</td>
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<td>H7N7</td>
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<td>A/Canada/504/2004</td>
<td>Can/504</td>
<td>H7N3</td>
<td>PENPKQAYQKRMTR/GLF</td>
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* indicated the polybasic cleavage site on HA protein.
Table 2. Genes induced in HULEC cells infected with influenza viruses

<table>
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<tr>
<th>Gene</th>
<th>Symbol</th>
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<th>SC/18</th>
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<tr>
<td><strong>Cytokines/chemokines</strong></td>
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<td>Interferon, beta 1, fibroblast</td>
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<td>Interleukin 7</td>
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<td>Tumor necrosis factor</td>
<td>TNF</td>
<td>53.3</td>
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</tr>
<tr>
<td>Interleukin 6 (interferon, beta 2)</td>
<td>IL6</td>
<td>35.3</td>
<td>5.3</td>
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<td>CCL2</td>
<td>28.6</td>
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<td>Chemokine (C-C motif) ligand 5</td>
<td>CCL5</td>
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<td>Colony stimulating factor 2</td>
<td>CSF2</td>
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<td>Selectin P ligand</td>
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<td>1.7</td>
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<td>Selectin E</td>
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<td>Intercellular adhesion molecule 1</td>
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<td>Vascular cell adhesion molecule 1</td>
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<td><strong>Endothelial cell function regulation</strong></td>
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<td>Nitric oxide synthase 2, inducible</td>
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<td>Thymidine phosphorylase</td>
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<td>Natriuretic peptide precursor B</td>
<td>NPPB</td>
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<tr>
<td>Serpin peptidase inhibitor, clade E, member 1</td>
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<td>Fibroblast growth factor 1 (acidic)</td>
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<td>Sphingosine kinase 1</td>
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<td>Platelet factor 4</td>
<td>PF4</td>
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<td>Placental growth factor</td>
<td>PGF</td>
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<td><strong>Apoptosis</strong></td>
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<td>Tumor necrosis factor receptor superfamily, member 10c</td>
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Fold changes in host gene expression between infected and uninfected cultures are represented. The subset of 29 genes were induced (bold) or suppressed (underlined) by VN/1203 virus infection at 24 h p.i. using 3-fold difference over mock with a p-value <0.01 as the cutoff.
**FIGURE LEGENDS**

Fig. 1. Detection and quantification of α-2,3 and α-2,6 linked sialic acid (SA) receptors on the cell surface of pulmonary microvascular endothelial cells. (A) Fluorescence lectin staining. Primary human lung blood microvascular endothelial cells (HMVEC-LBI) and human lung microvascular endothelial cells (HULEC) were grown on collagen-coated chamber slides overnight. The cells incubated with no lectin (control), biotinylated SNA, MAA I and II, followed by FITC-conjugated avidin D (green), and DAPI (blue) for nuclei. (B) Trypsinized endothelial cells were incubated with FITC-conjugated SNA or MAA (5μg/ml) and analyzed using flow cytometry. Fluorescent intensity for α-2,6 SA configuration (SNA, green) and α-2,3 SA configuration (MAA, blue) were compared to unstained cells (red). (C) Virus binding assay. HMVEC-LBI cells grown on collagen-coated chamber slides were incubated with FITC-labeled influenza virus at comparable HAU titer, followed by anti-FITC-Horseradish Peroxidase (HRP) antibody and AEC detection. The images were taken using the same exposure. (D) Fluorescent lectin staining. Primary rat pulmonary microvascular endothelial cells (PMVEC) and primary rat pulmonary arterial endothelial cells (PAEC) were stained green.

Fig. 2. Replication of influenza viruses in human pulmonary microvascular endothelial cells. Endothelial cells grown on six-well plates were infected with influenza virus at an MOI of 0.01. Culture supernatants were collected and viral titers were determined by a standard plaque assay. Values represent mean of three independent experiments plus standard deviation. (A) (C) Virus replication in HMVEC-LBI cells. 10% of normal
chicken egg allantoic fluid was added to the medium during virus infection. (B) Virus replication in HULEC cells. TPCK-trypsin (0.3μg/ml) was added to the medium during infection of H1 and H3 subtype viruses. (D) Virus replication in HULEC cells in presence or absence of TPCK-trypsin. (E) Immunofluorescence detection of nucleoprotein (NP) antigen in infected HMVEC-LBI and HULEC. Cells seeded on chamber slides were infected with virus at an MOI of 1 and fixed for NP detection at 10 and 24 h p.i.

Fig. 3. Replication of avian influenza viruses in human pulmonary microvascular endothelial cells. Endothelial cells grown on six-well plates were infected with influenza virus at an MOI of 0.01. Culture supernatants were collected and viral titers were determined by a standard plaque assay. Values represent the mean of three independent experiments plus standard deviation. (A) Virus replication in HULEC cells. TPCK-trypsin (0.3μg/ml) was only added to the medium for cells infected with Dk/NY virus. (B) Virus replication in HMVEC-LBI cells. 10% of normal chicken egg allantoic fluid was added to the medium during virus infection. * indicated that H5N1 virus replicated to a significant higher titer comparing to H7 subtype viruses (p<0.05) at 48 h and 72 h p.i.

Fig. 4. HPAI H5N1 viruses preferentially enter from apical side of polarized human pulmonary microvascular endothelial cells. HULEC monolayers grown on transwells for one week were infected with VN/1203 virus apically or basolaterally at an MOI of 0.01. The supernatant from both apical and basolateral compartments was collected for virus titration using standard plaque assay. Values represent the mean of three independent
experiments plus standard deviation. aa: apical infection and apical release, ab: apical infection and basolateral release, ba: basolateral infection and apical release, bb: basolateral infection and basolateral release.

Fig. 5. The contribution of HA multiple basic amino acid cleavage site to virus replication in human endothelial cells. Cells were infected with virus at an MOI of 0.01 and supernatants were collected for virus titration using plaque assay. (A) Immuno-fluorescence detection of nucleoprotein (NP) antigen in infected HMVEC-LBI. Cells seeded on chamber slides were infected with virus at an MOI of 1 and fixed for NP detection at 8 h p.i. (B) Virus replication in polarized bronchial epithelial cells (Calu-3). (C) Virus replication in HMVEC-LBI cells. 10% of normal chicken egg allantoic fluid was added into medium. For real-time PCR analysis, cells were infected with each virus at an MOI of 1 and RNA was collected at 24 h p.i. (D) Relative M1 gene levels in infected cells. (E) Relative fold change of gene expression of type-I interferon genes in infected cells.

Fig. 6. Evaluation of cytokine production in virus infected endothelial cells. HULEC cells were infected with virus at an MOI of 1 and supernatants were collected at 24 h p.i. for Bio-Plex assay. Values represent the mean of three independent experiments plus standard deviation.

Fig. 7. Examination of endothelial cell proliferation/viability during influenza virus infection. HMVEC-LBI cells were seeded onto 96-well plates, inoculated with virus at an
MOI of 5, 1, and 0.2 and examined at 44 h p.i. using WST-1 reagent. * Indicates infection with VN/1203 virus resulted in significantly lower levels of cell variability compared to other tested viruses at all virus dilutions (p<0.05). Values represent the mean of three independent experiments plus standard deviation.