Hemagglutinin-Dependent Tropism of H5N1 Avian Influenza Virus for Human Endothelial Cells[∇]

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Although current H5N1 highly pathogenic avian influenza viruses (HPAIV) are inefficiently transmitted to humans, infected individuals can suffer from severe disease, often progressing rapidly to acute respiratory distress syndrome and multiorgan failure. This is in contrast with the situation with human influenza viruses, which in immunocompetent individuals usually cause only a respiratory disease which is less aggressive than that observed with avian H5N1 viruses. While the biological basis of inefficient transmission is well documented, the mechanisms by which the H5N1 viruses cause fatal disease remain unclear. In the present study, we demonstrate that human pulmonary microvascular endothelial cells (hPMEC) had a clearly higher susceptibility to infection by H5N1 HPAIV than to infection by human influenza viruses. This was measurable by de novo intracellular nucleoprotein production and virus replication. It was also related to a relatively higher binding capacity to cellular receptors. After infection of hPMEC, cell activation markers E-selectin and P-selectin were upregulated, and the proinflammatory cytokines interleukin-6 and beta interferon were secreted. H5N1 virus infection was also associated with an elevated rate of cell death. Reverse genetics analyses demonstrated a major role for the viral hemagglutinin in this cell tropism. Overall, avian H5N1 viruses have a particular receptor specificity targeting endothelial cells that is different from human influenza viruses, and this H5N1 receptor specificity could contribute to disease pathogenesis.

Certain highly pathogenic avian influenza viruses (HPAIV) expressing the H5 and H7 hemagglutinins (HA) have acquired the capacity to infect humans. Particularly, HPAIV with the H5 HA and the neuraminidase (NA) type 1 (H5N1) can cause severe disease, often with a fatal outcome in humans and other mammals (27). With such infections in humans, there are two striking differences compared to infection by human influenza A viruses (IAV). First, bird-to-human and human-to-human transmission has been considered inefficient, and second, the mortality rate of H5N1 virus infections has been unexpectedly high. There is a lot of experimental evidence that inefficient transmission rate is related to several viral gene products not optimally adapted to facilitate infection and replication in the primary target cells, the epithelial cells of the respiratory tract. Of particular importance is the HA determining receptor specificity with human viruses preferentially recognizing sialic acid (SA)-α-2,6-Gal-terminated saccharides (α-2,6-SA), abundantly expressed in the upper respiratory tract, and avian viruses preferentially binding to α-2,3-SA, expressed mainly in the lower respiratory tract and on ciliated epithelial cells (23, 33, 39). In addition, the viral polymerases determining the rate of replication as well as the NS1 protein involved in multiple processes enabling efficient viral replication and evasion of cellular antiviral responses are of importance in determining host tropism (17, 26).

However, in contrast to infections with human influenza viruses, avian H5N1 virus infections more often cause severe pneumonia. These are associated with high levels of proinflammatory cytokines and chemokines in the respiratory tract, severe inflammatory reactions, and infiltration of leukocytes. Furthermore, a generalized inflammatory reaction with elevated cytokine and chemokine levels in the circulation, together with leukopenia and multiorgan failure, indicates that an aberrant immunological reaction is an important factor contributing to the fatality of H5N1 virus infections (19). This is supported by in vitro studies of human macrophages, dendritic cells, and epithelial cells, in which it was demonstrated that H5N1 viruses can induce higher levels of inflammatory cytokine and chemokine responses than human IV isolates (2, 3, 37). Based on this, it was proposed that factors of the innate and adaptive immune response are of central importance for the outcome of disease (8, 26).

Endothelial cells (EDC) are abundant in all organs, particularly the lung, and play an important role in inflammatory processes through the regulation of leukocyte extravasation, the production of inflammatory cytokines and chemokines, and the regulation of coagulation (4). During systemic disease in chickens infected with H5N1 isolates, the cardiovascular system can be affected with coagulopathy and viral antigen detectable in EDC (15, 25, 36). This also relates to a report demonstrating a targeted infection of EDC in chicken embryo by A/FPV/Rostock/34 (H7N1) virus (6). In this study, the in-

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TABLE 1. Reverse genetics viruses

Designation	Subtype	Basic strain	Segment substitutions	Segment(s) donor strain	Additional mutations	Cleavage site
R1	H3N2	H1N1 WS33	HA/NA	H3N2 HK68	None	Monobasic
R2	H3N2	H1N1 WS33	HA/NA	H3N2 HK68	HA is mutated to change its receptor specificity from α-2,6-SA to α-2,3-SA	Monobasic
LP H5N1 Vac	H5N1	LP A/duck/ Mongolia/54/01 (H5N2)	NA/NS	A/duck/Mongolia/47/01 (H7N1)	None	Monobasic
Vac-R1_HA	H3N1	LP H5N1 Vac	HA	R1	None	Monobasic
Vac-R2_HA	H3N1	LP H5N1 Vac	HA	R2	None	Monobasic
Vac-Y_HA	H5N1	LP H5N1 Vac	HA	HP H5N1 Yamaguchi	None	Polybasic
NIBRG23	H5N1	H1N1 PR8	HA/NA	HP H5N1 T/T	Polybasic cleavage site in the HA was removed	Monobasic

fection of human umbilical vein EDC is also reported. Finally, in humans, various degrees of hemorrhages as well as signs of disseminated intravascular coagulation have been found (1).

Accordingly, the present study compared influenza virus isolates of avian and human origin with respect to their characteristics of interaction with human EDC. To this end, we infected primary human lung EDC with different naturally occurring virus isolates as well as viruses created by reverse genetics. Viruses expressing the H5 clearly possessed the greatest potency to infect and replicate in EDC, resulting in activation and inflammatory responses.

MATERIALS AND METHODS

Cells. Madin-Darby canine kidney (MDCK) cells were propagated in minimal essential medium (Invitrogen. Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS) (Biowest), nonessential amino acids (Invitrogen), and 1 mM sodium pyruvate (Invitrogen). Human embryonic kidney 293T cells were propagated in Dulbecco MEM GlutaMax without phenol red (Invitrogen) supplemented with 10% FBS. The swine kidney cell line SK-6 (kindly provided by M. Penseart, Faculty of Veterinary Medicine, Ghent, Belgium) was cultured in MEM supplemented with 7% horse serum (SVA, Hatunaholm, Sweden). Human pulmonary microvascular EDC (hPMEC) (ScienCell) were propagated in 1 μg/cm² human fibronectin-coated (Becton Dickinson, Basel, Switzerland) cell culture flasks with complete EDC medium (ScienCell). hPMEC were classified by ScienCell as EDC by CD31 and FVIII expression as well as by low-density lipoprotein uptake.

Viruses. The following virus isolates were used (Tables 1 and 2): A/New-Caledonia/20/99 (H1N1) and A/Wisconsin/67/05/9187 (H3N2) (kindly obtained from Werner Wunderli, National Reference Center for Influenza, University Hospital, Geneva, Switzerland), A/PR/8/34 (H1N1) (kindly obtained from Georg Kochs, University of Freiburg, Freiburg, Germany), and HPAIV isolates A/cygnus olor/Italy/742/06 (H5N1), A/turkey/Turkey/1/2005 (H5N1), and A/ostrich/Italy/2332/2000 (H7N1) (kindly provided by William Dundon, IZSV Istituto Zooprofilattico Sperimentale delle Venezie, Venice, Italy). The following viruses created by reverse genetics were used: NIBRG23, with the HA (polybasic cleavage site of HA removed) and NA from H5N1 A/turkey/Turkey/1/2005 and the remaining genes from H1N1 A/PR/8/34 (kindly obtained from Jim Robertson, National Institute for Biological Standards and Control, United Kingdom); R1, with the HA and NA derived from A/Hong Kong/68 (H3N2) and the remaining

genes from A/WS/33 (H1N1); R2, identical to R1 but modified for α-2,3-SA binding specificity (22); A/duck/Hokkaido/Vac-1/04 (LP H5N1 Vac), composed of H5 from a low-pathogenic avian influenza virus (LPAIV), isolate A/duck/Mongolia/54/01 (H5N2), and N1 from isolate A/duck/Mongolia/47/01 (H7N1); and Yamaguchi (Y), generated from an HPAIV, A/chicken/Yamaguchi/7/04 (H5N1) (20, 34) (Table 1). All viruses were propagated in 10-day-old embryonated, specific-pathogen-free chicken eggs. We followed the protocol of the *WHO Manual on Animal Influenza Diagnosis and Surveillance* with some minor changes. Instead of 0.1 ml we inoculated 0.2 ml of different dilutions of virus. The inoculated eggs were incubated for 24 h (HPAIV) or 48 h (other influenza viruses).

Reverse genetics. 293T and MDCK cells were mixed (1:1) in MDCK medium at a concentration of $1.5 \cdot 10^5$ cells/ml and plated in six-well plates at 2 ml/well. The set of eight plasmids (kindly provided by R. Webster, St. Jude Children's Research Hospital, Memphis, TN) (11) was used to create the LP H5N1 Vac virus (LPAI H5N1 A/duck/Hokkaido/Vac-1/04) (34). In this set, the HA and NA were exchanged with those from an HPAIV, H5N1 A/chicken/Yamaguchi/7/04, and those of the R1 and R2 viruses described above (Table 1). For plasmid DNA (1 μg) transfection, we used Lipofectamine (Invitrogen) according to the manufacturer's instructions. Before transfection, cells were washed and 1.5 ml fresh medium containing 5% FBS was added per well. At 6 h after transfection, the medium was removed and replaced by MEM containing 1× nonessential amino acids, 1 mM sodium pyruvate, 1 mM HEPES, 0.125% bovine serum albumin (BSA), and 2 μg/ml L-1-tosylamide-2-phenylethyl chloromethyl ketone trypsin (Sigma-Aldrich, Buchs, Switzerland). Supernatants were harvested 72 h later and tested for virus by an HA test and titration on MDCK cells. For HA tests, supernatants were serially diluted 1:2 in 96-well plates. Then, 100 μl of supernatants were mixed with 100 μl 2% chicken erythrocytes.

Virus titrations. Calculations of multiplicities of infection (MOI) were based on numbers of infectious units (IU) on MDCK cells, determined using a protocol adapted from Matrosovich et al. (21). At 6 h after titration, cells were washed with phosphate-buffered saline (PBS), and then the plates were dried and frozen at −20°C overnight. The next day, cells were fixed with 4% paraformaldehyde (Sigma) solution in PBS for 15 min at room temperature (RT), washed, and permeabilized with 100 μl/well of PBS containing 0.5% Triton X-100 and 20 mM glycine. Cells were immunostained for viral nucleoprotein (NP) by incubating for 1 h with monoclonal antibody HB65 (ATCC) diluted in PBS supplemented with 10% horse serum (Häst) and 0.05% Tween 80. Cells were washed five times with PBS–0.05% Tween 80, followed by 1 h of incubation with peroxidase-labeled rabbit anti-mouse antibodies (Dako, Zug, Switzerland) diluted 1:1,000 in PBS supplemented with 10% horse serum and 0.05% Tween 80. Cells were washed again five times with PBS–0.05% Tween 80 and then incubated for 15 min with

TABLE 2. Virus isolates used in this study

Name	Subtype	Designation	Cleavage site	Origin
A/NewCaledonia/20/99	H1N1	H1N1 NC	Monobasic	Human
A/PR/8/34	H1N1	H1N1 PR8	Monobasic	Human
A/Wisconsin/67/05/9187	H3N2	H3N2 WS67	Monobasic	Human
A/cygnus olor/Italy/742/06	H5N1	HP H5N1 742	Polybasic	Avian
A/turkey/Turkey/1/2005	H5N1	HP H5N1 T/T	Polybasic	Avian
A/ostrich/Italy/2332/2000	H7N1	HP H7N1	Polybasic	Avian

TrueBlue substrate (KPL). Plates were washed with tap water and then dried. Single infected cells were counted under the microscope to determine numbers of IU/ml. Viral titers in cell culture supernatants were determined as 50% tissue culture infectious doses (TCID$_{50}$)/ml. To this end, supernatants were titrated on MDCK cells in 96-well plates in the presence of 1 μg/ml L-1-tosylamide-2-phenylethyl chloromethyl ketone trypsin (Sigma). At 72 h postinfection (p.i.), cells were washed and stained with crystal violet. The TCID$_{50}$/ml was calculated according to the Reed-Muench formula (*WHO Manual on Animal Influenza Diagnosis and Surveillance*).

Infection of EDC. hPMEC and MDCK cells were seeded out in 12-well plates the day before infection at a concentration of 10^5 cells/well. For infection, growth medium was removed and replaced by RPMI supplemented with 0.125% BSA (Intergen), 1 mM HEPES (Invitrogen), and 1× GlutaMax (Invitrogen). Cells were infected with an MOI of 1 IU/cell. Alternatively, hPMEC/MDCK cell infectivity ratios were determined by parallel infection of the two cell types with three different 10-fold dilutions starting with a 1:10 dilution of the original virus stock, and after 1 h of incubation at 4°C, the medium was removed and replaced by growth medium. Then, cells were incubated for 6 h at 37°C and 5% CO$_2$. To calculate the infectivity ratios, the conditions giving 30 to 90% NP$^+$ MDCK cells were used.

To follow the infection over longer time periods, hPMEC were seeded out in 12-well plates the day before infection at a concentration of 10^5 cells/well. For infection, growth medium was removed and replaced by RPMI supplemented with 0.125% BSA (Intergen), 1 mM HEPES (Invitrogen), and 1× GlutaMax (Invitrogen). After infection at an MOI of 1 IU/cell, the cells were incubated at 4°C to let the virus attach. As a negative control for infection, hPMEC were incubated with control allantoic fluid. After 1 h, cells were washed extensively with PBS (Invitrogen) to remove all unbound viruses. Then, growth medium was added and cells were incubated at 37°C and 5% CO$_2$ until harvesting. As a positive control for activation, we added 10 μg/ml poly(IC) (Sigma) to hPMEC when shifting cells from 4 to 37°C.

Virus inactivation. A 1 M stock solution of 2-bromoethylamine hydrobromide (BEI) was prepared and pH controlled to ensure the BEI circularization into the active form at pH 9. The solution was incubated for 1 h at 37°C, and BEI was added to virus preparations at a final concentration of 3 mM. After 24 h at RT, BEI was neutralized by the addition of 10% (vol/vol) 1 M Na$_2$S$_2$O$_3$ for 1 h at RT. Inactivation was controlled on MDCK cells checked for cytopathogenic effects after 72 h of culture.

Virus binding assay. MDCK cells and hPMEC were seeded out in 24-well plates at 10^5 cells/well. After 24, the medium was replaced with RPMI (4°C) supplemented with 0.125% BSA (Intergen, NY), 1 mM HEPES (Invitrogen), and 1× GlutaMax (Invitrogen). Cells were kept on ice and infected with H1N1 NC, HP H5N1 T/T, LP H5N1 Vac, and Vac-Y_HA at an MOI of 1 IU/cell. After 1 h of incubation at 4°C, cells were washed five times with PBS (4°C) and lysed with 400 μl lysis buffer containing a defined amount of enhanced green fluorescent protein (EGFP) RNA (Machete-Nagel kit nucleospin multi 96-virus). The RNA extraction was done by a Tecan Freedom EVO Roboter, and M1 and EGFP RNA were amplified by real-time reverse transcriptase PCR. Primers and probe with the following sequences were used: 5′-TCAGGCCCCCTCAAAGC CGA-3′ (probe), 5′-AGATGAGYCTTCTAACCGA-3′ (forward primer), and 5′-GCAAAGACATCTTCAAGTYTC-3′ (reverse primer). RNA was amplified using the following protocol: 30 min at 48°C, 10 min at 95°C, 15 s at 95°C, 60 s at 54°C, and 60 s at 70°C. The last three steps were repeated 45 times in total. Threshold cycle (C_T) values were corrected to the amount of EGFP RNA. To compare the virus binding, the dilutions at which virus binding to the cells was not saturated and with C_T values below 30 were chosen. The relative amount of viral RNA was calculated by the ΔC_T method, and the amount of viral RNA relative to EGFP RNA was expressed as 2^{-C_T} (18). RNA levels detected on MDCK cells were set to 1 and compared to the RNA levels bound to hPMEC.

Cytokine analysis. Supernatants of infected hPMEC were analyzed using the interleukin-6 (IL-6) duo kit reagents from R&D Systems (United Kingdom) and a beta interferon (IFN-β) enzyme-linked immunosorbent assay (ELISA) kit from PBL (R&D Systems) following manufacturers' instructions. IFN-α/β bioactivity was quantified using a porcine kidney cell line expressing firefly luciferase under the control of the murine Mx1 promoter (9). To this end, SK-6 cells (14) were transfected with a mixture of plasmids pGL3-Mx1P-Luc (kindly provided by Georg Kochs, Department of Virology, Institute of Medical Microbiology and Hygiene, Freiburg, Germany) (13) and pCI-neo (Promega) at a 20:1 (wt/wt) ratio using FuGENE 6 transfection reagent (Roche). After 48 h, the cell culture medium was replaced with fresh complete medium supplemented with 500 μg/ml G418-sulfate (Calbiochem-Novabiochem). Three rounds of end point dilution were applied to isolate G418-resistant colonies from single cells. The clones with the highest rates of porcine IFN-β-mediated luciferase induction and lowest background activity were selected. The purified SK6-MxLuc clones were maintained under selection with 250 μg/ml G418-sulfate. For IFN-α/β bioactivity, cells from an SK6-MxLuc clone were seeded in 24-well plates at 3 · 10^5 cells/well. The next day, test supernatants of hPMEC infected at an MOI of 1 IU/cell were collected and inactivated by UV treatment to avoid direct stimulation of the luciferase transcription by the virus. Supernatant (500 μl) was added to one well of a six-well plate. Six-well plates were put on ice for UV treatment with 9,999 mJ UV. The standard was treated the same way. SK6-MxLuc cells were washed, and 150 μl of fresh medium was added, together with 150 μl of supernatants. Eighteen hours later, supernatants were removed and cells were lysed with 200 μl cell culture lysis reagent from the Promega luciferase assay kit (Promega). Luciferase activity was measured in triplicate with the luminometer.

Flow cytometry. For E/P-selectin staining, cells were harvested with PBS-A (Invitrogen)–0.94 mM EDTA, washed with Cell Wash (Becton Dickinson), and incubated with CD62E/P antibody (Serotec) for 20 min at 4°C. Cells were washed and then incubated with fluorescein isothiocyanate-conjugated goat-anti mouse immunoglobulin G1 (IgG1) antibody (Southern Biotech) for 15 min at 4°C before a final wash. For NP detection, cells prepared as described above were fixed and permeabilized with Fix & Perm cell permeabilization kit (An der Grub) according to the manufacturer's instructions and labeled using anti-NP clone HB65 (ATCC). Then, the cells were washed and incubated with R-phycoerythrin-conjugated goat-anti mouse IgG2a antibody (Southern Biotech) for 10 min at RT. After a final washing step, the cells were resuspended in 200 μl Cell Wash. The percentage of NP$^+$ hPMEC was divided by the percentage of NP$^+$ MDCK cells to obtain the infectivity ratio. Apoptotic cells were quantified using the AnnexinV/PI kit from BenderMed following the manufacturer's instructions. To fix and permeabilize AnnexinV-stained cells for intracellular NP staining, cells were fixed for 10 min at RT in 4% paraformaldehyde diluted in AnnexinV staining buffer (140 mM NaCl, 2.5 mM CaCl$_2$, 10 mM HEPES in 500 ml double-distilled water). After fixation, cells were permeabilized by washing with 0.3% saponin in AnnexinV buffer. NP antibody was diluted in 0.6% saponin in AnnexinV buffer and incubated with the cells for 20 min on ice. Cells were washed with 0.3% saponin in AnnexinV buffer, and then R-phycoerythrin-conjugated goat anti-mouse IgG2a antibody was added to the cells diluted in 0.6% saponin in AnnexinV buffer. After 15 min on ice, cells were washed in 0.3% saponin in AnnexinV buffer and then once more with AnnexinV binding buffer before acquisition. The expression of α-2,3-SA and α-2,6-SA receptors on hPMEC was detected using the biotinylated lectins *Maackia amurensis* lectin II (MAL II) (Vector Laboratories) and *Sambucus nigra* lectin (SNA) (Vector Laboratories). The cells were harvested with PBS-EDTA and then washed with 10 mM Tris (pH 7.5), 1 mM CaCl$_2$, 1 mM MgCl$_2$, 1 mM MnCl$_2$, and 0.5% BSA in double-distilled water. Cells were incubated with lectins at 20 μg/ml in the above buffer for 20 min on ice, washed, and stained with streptavidin-Spectral-Red conjugated (Southern Biotech) for 15 min on ice. After a final washing step, fluorescence was measured by flow cytometry. All data were acquired with a FACSCalibur (Becton Dickinson) and analyzed with FlowJo software (Treestar Inc., Ashland, OR).

For enzymatic removal of SA, 10^6 cells were incubated with NA from *Vibrio cholerae* (Sigma) at 37°C at the indicated time points and concentrations.

Statistical analyses. P values were calculated by an unpaired t test in a sigma plot for normally distributed data or by the Mann-Whitney rank sum test if normal distribution of data was not given. Error bars representing the standard deviations of at least two experiments were calculated by Excel. Box plots displaying data as the median and 25th and 75th percentiles were done in SigmaPlot.

RESULTS

hPMEC are highly susceptible to H5N1 HPAIV. To investigate the susceptibility of hPMEC to influenza virus, we first investigated NP expression at 6 h p.i. To avoid a possible error source of imprecise viral titers, we infected hPMEC and MDCK cells in parallel with three different virus dilutions and calculated infectivity ratios by dividing the percentage of NP$^+$ hPMEC by the percentage of NP$^+$ MDCK cells (Fig. 1A and B). For these calculations, virus dilutions giving 30 to 90% NP$^+$ MDCK cells were used. This corresponded to MOIs of 0.3 to 0.9 IU/cell, depending on the experiment and the virus used. Figure 1A shows representative flow cytometry data used
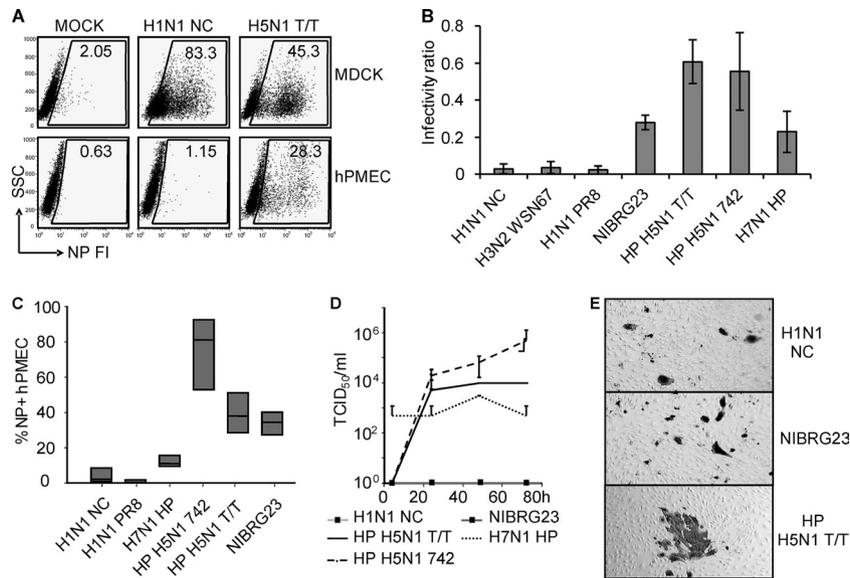
FIG. 1. Infection and replication of influenza A viruses in hPMEC. (A and B) hPMEC/MDCK cell infectivity ratios. hPMEC and MDCK cells were infected in parallel with different dilutions of virus, and at 6 h p.i., cells were harvested and stained for NP. Virus dilutions giving 30 to 90% NP$^+$ MDCK cells were selected. This corresponded to MOIs of 0.3 to 0.9 IU/cell (determined with MDCK cells) (A) Side scatter/NP dot plots from a representative experiment with the percentage of NP$^+$ cells are shown (MOIs selected for the graphs: H1N1 NC, 0.9 IU/cell; H5N1, 0.7 IU/cell). (B) Infectivity ratios, determined by dividing the percentage of NP$^+$ hPMEC by the percentage of NP$^+$ MDCK cells. Means and standard deviations of data from three independent experiments are shown. The differences between all human isolates and the H5N1 viruses were statistically significant ($P < 0.02$). HP H5N1 T/T also differed significantly from NIBRG23 and H7N1 HP ($P < 0.05$). (C) hPMEC were infected at an MOI of 1 IU/cell and analyzed at 30 h p.i. Box plots showing median values (black line) and 25th and 75th percentiles of the percentage of NP$^+$ cells were calculated from five independent experiments. P values calculated for human and all avian viruses and P values for avian H7N1 and H5N1 viruses were below 0.01, indicating statistically significant differences between these groups. The P value for HP H5N1 T/T and NIBRG23 was above 0.05. (D) Supernatants of infected hPMEC were collected at different time points after infection and then titrated on MDCK cells to determine TCID$_{50}$/ml. The error bars represent the standard deviations from two independent experiments. (E) hPMEC were infected at low MOIs (0.01 to 0.5 IU/cell, depending on the virus) to enable the visualization of infectious foci. At 24 h p.i., cells were stained for NP.

for the infectivity ratio shown in Fig. 1B. These results demonstrated that the human A/NewCaledonia/20/99 (H1N1 NC), A/PR/8/34 (H1N1 PR8), and A/Wisconsin/67/05/9187 (H3N2 WS67) viruses infected hPMEC considerably less efficiently than MDCK cells. Avian H7N1 A/ostrich/Italy/2332/2000 (H7N1 HP) virus showed an intermediate infectivity ratio, while A/cygnus olor/Italy/742/06 (HP H5N1 742) and A/turkey/Turkey/1/2005 (HP H5N1 T/T) viruses infected hPMEC almost as efficiently as MDCK cells. NIBRG23 is a genetically engineered virus, representing a vaccine strain composed of the HA and NA of HP H5N1 T/T and the internal genes of H1N1 PR8 (Table 1). Interestingly, NIBRG23 virus was much more efficient than the H1N1 PR8 virus in infecting hPMEC (Fig. 1B), pointing to an important role for HA and/or NA for the EDC tropism of HPAIV. It is important to note that the HA of NIBRG23 virus does not have a polybasic cleavage site and is nonpathogenic, indicating that the efficiency of avian H5N1 viruses in infecting hPMEC does not depend on the cleavage site of the HA.

After infection of hPMEC with avian and human viruses at an MOI of 1 IU/cell, at 30 h p.i. there was clearly more NP detectable in hPMEC infected with HP H5N1 742, HP H5N1 T/T, and NIBRG23 viruses than in those infected with H1N1 NC or PR8 (Fig. 1C and D). H7N1 virus showed an intermediate infection. The differences were found in terms of both the percentage of NP$^+$ cells and the NP fluorescence intensity. The equal percentage of NP$^+$ hPMEC 30 h p.i. with HP H5N1 T/T

and NIBRG23 indicated that the polybasic cleavage site in the HA of HPAIV was not crucial for efficient infection of hPMEC by IAV.

Supernatants of hPMEC infected at an MOI of 1 IU/ml were titrated to determine viral replication. An increase of infectious virus was found in the supernatants of hPMEC infected with the avian virus H5N1 HP isolates, while no infectious virus was detectable after infection with human isolates or NIBRG23 (Fig. 1D). The viral titers after H7N1 HPAIV did not clearly increase. These characteristics of infection in the hPMEC were obtained in the absence of trypsin, indicating that hPMEC are inefficient at cleaving HA with a monobasic cleavage site, although initiation of the replicative cycle was possible in terms of NP production. It was not possible to investigate the impact of trypsin on growth of human influenza virus and LPAIV, because hPMEC cultures were intolerant to trypsin in the culture medium, even at low concentrations. To confirm that hPMEC are unable to cleave HA without a polybasic cleavage site, HP H5N1 T/T, human H1N1 NC, and reverse genetics virus NIBRG23 were titrated on hPMEC, and the plates were then stained for NP 24 h later and analyzed for the formation of infectious foci. Only HP H5N1 T/T with an HA containing a polybasic cleavage site was able to spread in the cell cultures (Fig. 1E). With H1N1 NC, H3N2 WSN 67, H1N1 PR8, and NIBRG23, only single infected cells were found (data shown only for H1N1 NC, NIBRG23, and HP
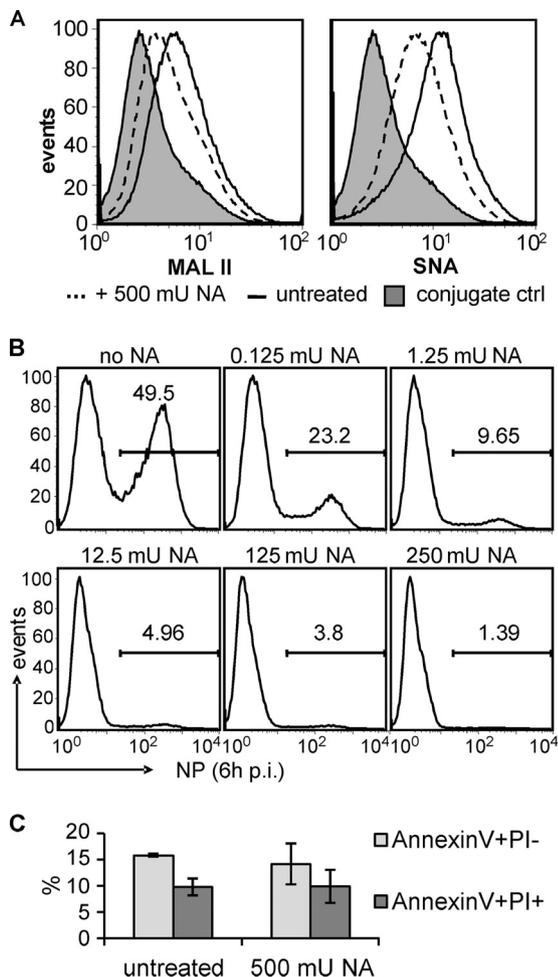
FIG. 2. SA expressed on hPMEC serves as a receptor for infection. (A) hPMEC were harvested with PBS-EDTA and then stained for SA using MAL II (left) and SNA (right) lectins. As a control, hPMEC were treated with 250 mU NA/ml for 3.5 h before being harvested (dotted lines). (B) hPMEC were treated overnight with the indicated amount of NA/ml. Then, cells were infected with HP H5N1 T/T for 1 h at 4°C (MOI of 5 IU/cell). After being washed, the cells were incubated for 6 h at 37°C, harvested, and stained for intracellular NP. (C) hPMEC were treated overnight with 250 mU NA/ml cells and then stained for AnnexinV and PI to determine cell viability. Error bars represent the standard deviations of two independent experiments.

H5N1 T/T). These experiments suggested that hPMEC are unable to cleave HA with a monobasic cleavage site.

Infection of hPMEC is SA dependent. Using lectin staining, we determined that hPMEC express avian and human type SA receptors for influenza virus. Both α-2,3-SA and α-2,6-SA (stained by MAL II and SNA, respectively) were homogenously expressed on hPMEC (Fig. 2A). After treatment with NA, the staining intensity decreased, confirming the lectin-staining specificity for SA.

In order to demonstrate that HPAIV infection of hPMEC was SA dependent, we treated hPMEC with different concentrations of NA. This treatment reduced the infection of hPMEC by HP H5N1 T/T virus in a dose-dependent manner (Fig. 2B). With a high dose of NA, which did not influence the
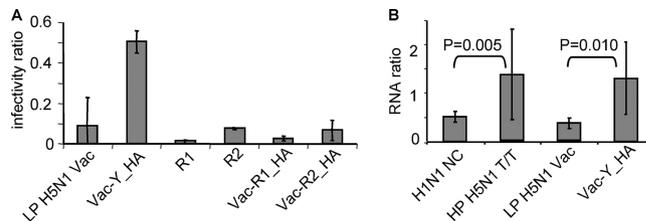
FIG. 3. Role of H5 for infection of hPMEC. (A) Comparison of the R1/R2 virus pair and impact of insertion of the HA of R1, R2, and H5N1 Yamaguchi (Y) within the LP H5N1 Vac backbone on infectivity. Bar graphs with infectivity ratios determined as described for Fig. 1A and B and standard deviations from three independent experiments are shown. R1 differed significantly from R2 ($P < 0.01$) but not from LP H5N1 Vac, Vac-R1_HA, and Vac-R2_HA ($P > 0.05$). Vac-Y_HA differed from all other viruses ($P < 0.01$). (B) To quantify viral particles containing viral RNA attached to the cells, hPMEC and MDCK cells were incubated for 1 h at 4°C with virus (MOI of 1 IU/cell), and M1 RNA was quantified. The ratios of viral RNA bound to hPMEC compared to MDCK cells are shown. P values were calculated with the Mann-Whitney rank sum test and showed significant differences between H1N1 NC and HP H5N1 T/T ($P = 0.005$) and between LP H5N1 Vac and Vac-Y_HA ($P = 0.010$).

viability of hPMEC (Fig. 2C), the infection was almost abrogated.

H5 plays a major role in conferring infectivity of influenza virus for hPMEC. In order to investigate the contribution of the viral surface glycoproteins HA and NA in the infection of hPMEC relative to the internal genes, the role of HA was studied further by infecting hPMEC with a pair of viruses possessing specific mutations in the HA. These mutations alter the receptor binding site specificity from α-2,6-SA (R1 virus) to α-2,3-SA (R2 virus) (22). R1 virus behaved like other human viruses, and infection led to a low ratio of infectivity (Fig. 3A). In contrast, R2 was significantly more efficient in infecting hPMEC, resulting in a ratio of infectivity similar to that of the H7N1 virus shown in Fig. 1B.

This analysis was elaborated using a different set of viruses composed of genes from the avian isolates A/duck/Mongolia/54/01 (H5N2) and A/duck/Mongolia/47/01 (H7N1) (LP H5N1 Vac). This LP H5N1 Vac virus containing an HA from an LPAIV isolate of a duck was relatively inefficient at infecting hPMEC (Fig. 3A). However, exchanging the HA of the LP H5N1 Vac virus with the HA of R1 and R2 did reduce the capacity of the reassortant to infect hPMEC, compared to the parent LP H5N1 Vac virus (Fig. 3A, Vac-R1_HA, Vac-R2_HA). In contrast, when the HA of the LP H5N1 Vac was exchanged with an HA from the HP H5N1 A/chicken/Yamaguchi/7/04 isolate (Fig. 3A, Vac-Y_HA), a high infectivity ratio similar to that found with the HP H5N1 742 and T/T isolates (shown in Fig. 1B) was obtained.

To determine if the receptor binding of the HA was responsible for the observed differences between avian and human viruses, we investigated viral attachment to hPMEC. Binding of human H1N1 NC and avian HP H5N1 T/T virus and reverse genetics viruses LP H5N1 Vac and Vac-Y_HA to hPMEC and MDCK cells was measured by reverse transcriptase PCR (Fig. 3B). The results showed that H1N1 NC virus did bind less efficiently to hPMEC than did HP H5N1 T/T. The same observation was made for the two reverse genetics viruses LP H5N1 Vac and Vac-Y_HA. Therefore, receptor binding spec-
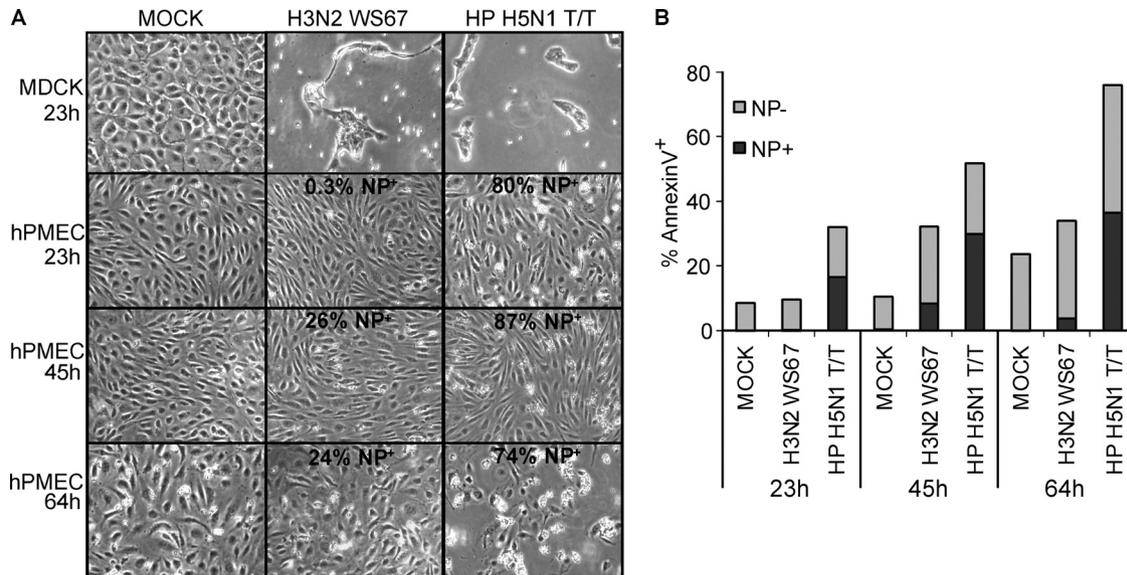
FIG. 4. Cell death and apoptosis in infected hPMEC. (A) Microphotographs of hPMEC and MDCK cells, infected at an MOI of 1 IU/cell at indicated time points. The cells were treated with 10 µg/ml mitomycin C to prevent cell division. (B) Bar graphs of AnnexinV and NP staining, showing the percentage of AnnexinV$^+$ NP$^+$ (dark gray) and AnnexinV$^+$ NP$^-$ (light gray) cells for the hPMEC cultures shown in panel A.

ificity of the HA represents a crucial factor in determining tropism of H5N1 for EDC.

Virus-induced hPMEC apoptosis. Despite the large NP amounts detected in hPMEC infected with HPAIV, we could not observe a strong increase in cell death by light microscopy. The cell monolayer remained intact during the observation period (data not shown). Since it appeared that dead cells were replaced by healthy, dividing cells, hPMEC and MDCK cells were treated with 10 µg/ml mitomycin C overnight in order to inhibit cell proliferation. Cells were then infected at an MOI of 1 IU/cell and observed by microscopy and AnnexinV/NP double staining over a time period of 64 h. Already after 23 h p.i., the MDCK cell monolayer was almost destroyed after infection with H3N2 WS67 or HP H5N1 (Fig. 4A). In contrast, infected hPMEC did not show any sign of virus-induced cytopathogenicity at this time point. At 64 h p.i., a reduction of viable cells was detectable under all conditions, including mock, most probably due to the cytotoxic effects of the mitomycin C. By AnnexinV/NP staining it was possible to detect virus-induced cytopathogenic effects in the HP H5N1 T/T-infected cells already at 23 h p.i. (Fig. 4B). Around 40 to 50% of the AnnexinV positive cells were NP$^-$ during the whole observation period, indicating that apoptosis was also induced in uninfected cells. AnnexinV staining in H3N2 WS67-infected cells did not differ from that in mock controls at 23 h p.i., and only a small percentage of AnnexinV-positive cells were also positive for NP. These results indicate that HP H5N1 virus infection does induce apoptosis in hPMEC although at relatively low levels and late time points considering the high percentage of infected cells and in comparison to MDCK cells.

HPAIV induces E/P-selectin upregulation in hPMEC. EDC play an important role in inflammatory responses through regulation of leukocyte migration, which is mediated by an activation-dependent expression of adhesion molecules such as E/P-selectin. Therefore, we were interested in the expression patterns of E/P-selectin following infection of hPMEC. After stimulation of hPMEC with poly(IC) as a positive control, E/P-selectin expression was upregulated (Fig. 5A). Infection with H1N1 NC virus induced only a minor upregulation (Fig. 5A). In contrast, HPAIV H7N1 virus was more potent at inducing E/P-selectin expression, and the highest rate of induction was found with H5N1 virus infection, where most of the hPMEC population showed an increased amount of E/P-selectin expression (Fig. 5A). Double staining showed that not all E/P-selectin-expressing cells were NP$^+$, indicating a role for indirect effects such as cytokines. Nevertheless, the addition of IL-6 or IFN-β alone to the cell culture was not sufficient to induce E/P-selectin upregulation in hPMEC (data not shown).

HPAIV-induced cytokine responses in hPMEC. EDC also participate in inflammatory responses through secretion of cytokines and chemokines, for example IL-6. Therefore, we analyzed the supernatants of infected hPMEC for cytokines. HPMEC secreted IL-6 in response to poly(IC). Compared with mock controls, the viruses efficiently infecting hPMEC also induced increased levels of IL-6. This included the H5N1 viruses NIBRG23, T/T, and 742, as well as the H7N1, LP H5N1 Vac, and R2 viruses (Fig. 5B). When we inactivated NIBRG23 virus with BEI before infection, induction of IL-6 secretion by hPMEC was abolished, showing that viral replication is essential in order to induce IL-6 secretion.

IFN type I is secreted only after infection with H5N1 viruses. IFN type I is an important innate defense against virus infection, particularly with respect to the induction of an antiviral state in infected and neighboring cells. Considering that hPMEC seem to control influenza virus infection, in particular with human viruses, we looked at IFN type I activity in supernatants by bioassay and by ELISA. Surprisingly, only H5N1 viruses were able to induce production of IFN type I (Fig. 5C). This was confirmed using an ELISA specific for IFN-β. Only the supernatants that were tested positive in the bioassay in-
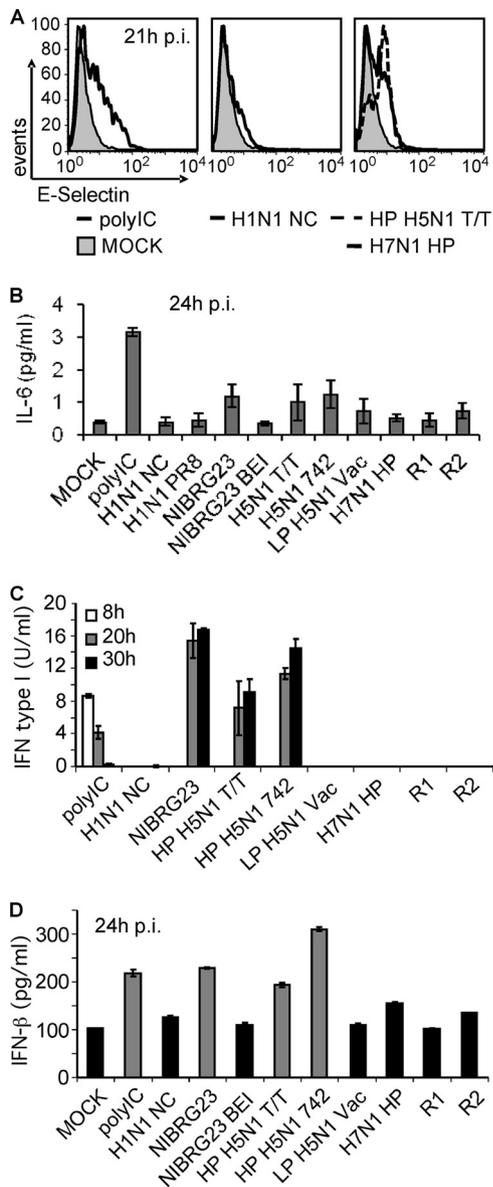
FIG. 5. hPMEC activation by influenza virus. (A) E/P-selectin upregulation in hPMEC infected at an MOI of 1 IU/cell, measured 21 h p.i. by flow cytometry. (B to D) Cytokines in supernatants harvested 24 h p.i. (B) IL-6 response determined by ELISA. Standard deviations of two independent experiments are shown. (C) IFN type I tested by bioassay. (D) IFN-β analyzed by ELISA. For panels C and D, error bars represent standard deviations of triplicates of one representative experiment.

duced IFN-β responses clearly above the mock control (highlighted in gray in Fig. 5D) and at levels similar to or higher than that of the poly(IC) positive control (Fig. 5D).

DISCUSSION

Based on the expression of both α-2,6-SA and α-2,3-SA demonstrated here and recently also in vivo (41) human EDC could be susceptible to both human and avian influenza viruses. However, we found clear differences with respect to the capacity of human and avian viruses to infect hPMEC, particularly in terms of NP detection and viral attachment to the cell. The avian viruses were clearly more efficient at both infecting and replicating in hPMEC. H5 viruses produced large amounts of intracellular NP and infectious progeny virus in cell culture supernatants. The latter was found only with viruses possessing a polybasic amino acid cleavage site in the HA, indicating that hPMEC cannot cleave the HA of LPAIV and human influenza viruses. Relating to its role in virus attachment and fusion with the host cell, our results demonstrate a major importance of HA in the tropism of influenza viruses for EDC. We also show that infection of these cells by influenza virus was SA dependent. Our results relate to the EDC tropism of FPV H7N1 in the chicken embryo described by Feldmann et al. (6). Also with this HPAIV, the infection of EDC was dependent on the expression of the hemagglutinin H7 but independent of proteolytic activation. While one viral feature responsible for enhanced infectivity was α-2,3-SA receptor specificity, as demonstrated by the R1/R2 virus pair specifically differing in this characteristic, this alone was not sufficient to explain the particularly high capacity of H5N1 viruses to infect EDC. A likely explanation could be additional binding characteristics of certain H5 viruses. Interestingly, particular avian viruses isolated from domestic poultry have a high binding affinity for sulfate sialylglycopolymers and also bind to 6-sulfo sialyl Lewis X (7). This could possibly relate to our finding that the LP H5N1 Vac virus, from which the H5 gene was originally isolated from a duck, was not as efficient as H5 from viruses isolated in chicken (Yamaguchi virus) or turkey (HP H5N1 T/T virus), with respect to infection of hPMEC. EDC also express these 6-sulfo sialyl Lewis X groups at high endothelial venules and after activation during inflammation. Leukocytes bind to these structures via L-selectin to extravasate into inflamed tissue (24). It is thus tempting to speculate that the expression of 6-O-sulfotransferases by EDC (38) could explain the susceptibility of EDC to H5N1 isolates. Future studies are required to relate the data obtained from glycan arrays to the capacity of influenza viruses to infect EDC.

Future studies are required to confirm the biological importance of our findings. To our knowledge, there is only one immunohistochemistry study available describing the absence of viral RNA or NP in EDC in two patients who died of H5N1 infections (10) and one study describing avian IAV-like particles in the cytoplasm of EDC by electron microscopy in a patient who died from H5N1 infection (16). Considering that EDC infection could be limited to certain areas and more prominent at earlier phases of the infection, more detailed studies are required to address the question of human EDC infection by avian IAV. In contrast, in poultry, EDC represent a clear in vivo target for HPAIV. It is thus possible that adaptation of HPAIV in domestic poultry to this peculiar receptor specificity could contribute to the high efficiency of infection and replication in hPMEC.

The pathology observed in patients with fatal H5N1 virus infection is associated with an overwhelming cytokine secretion and inflammatory reaction, both in the lung and in other organs (5). E/P-selectin upregulation, as observed with hPMEC in vitro, could contribute to the recruitment of leukocyte to the lung. Together with induced IL-8, which is a chemoattractant for neutrophils, this could enhance the pathology of H5N1 virus infections (35, 43, 44). Activated neutrophils can

cause damage to the tissue by secreting proinflammatory mediators and therefore possibly contribute to the acute respiratory distress syndrome often related to the fatal outcome of H5N1 virus infections (12, 42). IL-6 is a proinflammatory cytokine that can contribute to activation of macrophages and EDC themselves. Macrophages again can play a role in extensive tissue damage during inflammation (28). Enhanced leukocyte extravasation as a consequence of EDC activation could also partially explain the typical leukopenia associated with H5N1 virus infections. Finally, infected and activated EDC may promote blood coagulation, explaining the hemorrhages and coagulopathy found in humans (1), cats (29), and chickens (25) infected with H5N1 virus. This process could also be triggered by virus-induced apoptosis in EDC, resulting in damaged blood vessels (40). Of course, other cells of the innate immune system, such as infected macrophages or dendritic cells, are likely to contribute to these inflammatory processes induced by an influenza virus infection.

Of course, the immune system operates as a "double-edge sword," and the response described above could be necessary to limit the spread of viral infection (30). While this would be particularly expected for the IFN-β released from H5N1 virus-infected hPMEC, there is a study showing that some H5N1 isolates from 1997 are IFN resistant (31, 32). This requires clarification for the H5N1 isolates used in the present study. An explanation for the IFN response after infection with H5N1 viruses but not the other viruses could be the higher level of infection and replication, which will result in a higher threshold of activation. While we cannot exclude a role for a different efficiency of the NS1 protein in counteracting IFN induction, our experiments with reverse genetics viruses did not point in this direction.

Taken together, our results demonstrate that H5 viruses can have a pronounced tropism for human EDC, at least in vitro. Compared to human influenza virus, avian H5N1 viruses are not well adapted for infection and replication in the epithelial cells of the upper respiratory tract, which represent their main target cells and a prerequisite for efficient transmission. However, once the virus has passed this barrier, it appears to have a clearly higher tropism not only for macrophages and dendritic cells (3, 37, 45) but also for EDC. This particular receptor specificity targeting cells of the reticuloendothelial system could explain the inefficient transmission of avian influenza viruses to humans, as well as the high fatality rate once an infection is established.

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