The Hemagglutinin Protein of Highly Pathogenic H5N1 Influenza Viruses Overcomes an Early Block in the Replication Cycle to Promote Productive Replication in Macrophages

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Abstract

Macrophages are known to be one of the first lines of defense against influenza virus infection. However, they may also contribute to severe disease caused by the highly pathogenic avian (HPAI) H5N1 influenza viruses. One reason for this may be the ability of certain influenza virus strains to productively replicate in macrophages. However, studies investigating the productive replication of influenza viruses in macrophages have been contradictory and results may depend on both the type of macrophages used and the specific viral strain. In this work, we investigated the ability of H1-H16 viruses to productively replicate in primary murine alveolar macrophages and RAW264.7 macrophages. We show that only a subset of HPAI H5N1 viruses, those that cause high morbidity and mortality in mammals, can productively replicate in macrophages, as measured by the release of newly synthesized virus particles into the cell supernatant. Mechanistically, we found that these H5 strains can overcome a block early in the viral lifecycle leading to efficient nuclear entry, viral transcription, translation, and ultimately replication. Studies with reassortant viruses demonstrated that expression of the hemagglutinin gene from an H5N1 virus rescued replication of H1N1 influenza virus in macrophages. This study is the first to characterize H5N1 influenza viruses as the only subtype of influenza virus capable of productive replication in macrophages and establishes the viral gene that is required for this characteristic. The ability to productively replicate in macrophages is unique to H5N1 influenza viruses and may contribute to their increased pathogenesis.
Introduction

Highly pathogenic avian (HPAI) H5N1 influenza viruses have caused over 600 human infections with a 60% mortality rate since 2003 (World Health Organization; http://www.who.int). These viruses remain a serious public health threat because they are endemic in domestic poultry populations on three continents (5), increasing the likelihood of continued epidemic outbreaks leading to human infection as well as the opportunity for the virus to continue its adaptation to mammals. Thus, it is imperative that we gain a better understanding of how H5N1 influenza viruses cause severe disease.

The exacerbated disease severity and high mortality rates associated with human H5N1 infection correlates with high viral load, tropism for alveolar epithelium, and dysregulation of the host cytokine response (1, 6, 8, 24). A number of molecular determinants of H5N1-induced pathogenesis have been described. For example, specific amino acid residues in the hemagglutinin (HA) protein of avian influenza viruses have been correlated with increased pathogenicity (3, 12, 16, 20). Much less is known, however, about how H5N1 influenza viruses interact with specific components of the host immune response.

Macrophages are a critical component of the host response to infection, playing an important role in phagocytosis of pathogenic agents and interaction with cells of the adaptive immune response (18, 37). Particularly for respiratory pathogens, alveolar macrophages represent an early point of contact at the host-pathogen interface. Pathogen recognition by alveolar macrophages initiates the host response to infection and the quantitative and qualitative nature of this response is important in determining the outcome of infection. During influenza virus infection, macrophages are an important source of antiviral and proinflammatory cytokines, which serve to control early virus replication and regulate the progression of an effective
antiviral response (33). The importance of macrophages for protection against influenza virus infection is clear from several studies wherein clodronate liposome-mediated depletion of macrophages resulted in greater virus replication in the lungs, systemic dissemination of the virus, and exacerbated disease severity (17, 33).

Despite this demonstrated role for macrophages in preventing severe influenza virus-mediated disease, an excessive cytokine response is thought to be one of the causes of death in patients experiencing an H5N1 infection and macrophages have been implicated in this response (6, 8, 24). H5N1 influenza virus infection results in an early infiltration of excessive numbers of macrophages into the lungs, which correlates with increased expression of proinflammatory cytokines (24). Further, infection of macrophages in vitro with H5N1 influenza viruses results in the induction of greater levels of proinflammatory cytokines when compared with seasonal influenza viruses (13, 19, 22).

Taken together, these studies suggest a fundamental difference in the interaction of macrophages with highly pathogenic and seasonal influenza viruses. Human autopsy studies and ex vivo infections demonstrate that the primary targets of influenza virus infection are the respiratory epithelium and alveolar macrophages (23). However, infection of alveolar macrophages with influenza viruses is believed to be abortive, failing to result in the release of infectious virus progeny (26, 30, 36). Recent investigation into the nature of H5N1 infection of macrophages with regard to virus replication has produced inconsistent results (10, 31, 35, 39). While van Riel et. al. demonstrated a failure of HPAI H5N1 viruses to productively replicate in alveolar macrophages, work by Yu et. al. demonstrated that productive replication of H5N1 influenza viruses in human alveolar macrophages correlated with increased expression of various
proinflammatory cytokines, highlighting the impact that replication of H5N1 influenza viruses may have on the course of infection (35, 39).

To address these seemingly contradictory reports and to determine which viral gene(s) contributes to the ability of H5N1 influenza viruses to productively infect macrophages and the cellular mechanism for replication, we used primary murine alveolar macrophages and RAW264.7 immortalized murine macrophages to study the interaction of seasonal and H5N1 influenza viruses with macrophages in vitro. Our results demonstrate that productive replication in macrophages is unique to a subset of HPAI H5N1 avian influenza viruses. These viruses overcome a block early in the virus lifecycle, allowing entry of the viral ribonucleoprotein (vRNP) into the nucleus followed by transcription, translation, and replication of the viral genes. Further, we demonstrate that the ability to overcome this block to productive replication maps to the hemagglutinin (HA) gene. Expressing the HA of the H5N1 influenza virus, A/Hong Kong/483/97 (HK/483), on the background of A/California/04/09 (CA/09) H1N1 conferred the ability of the virus to replicate in macrophages. Our studies demonstrate that the HA gene from the HPAI H5N1 viruses supports productive replication of influenza viruses in macrophages.
**Materials and Methods**

**Ethics statement.** All procedures were approved by the St. Jude Children’s Research Hospital Institutional Biosafety Committee (IBC) and Institutional Animal Care and Use Committee (IACUC) and were in compliance with the Guide for the Care and Use of Laboratory Animals. These guidelines were established by the Institute of Laboratory Animal Resources and approved by the Governing Board of the U.S. National Research Council.

**Laboratory facilities.** All experiments using parental HPAI H5N1 and CA/09 H1N1 containing the H5 hemagglutinin (HA) gene were conducted in a Biosafety level 3 enhanced containment laboratory (29). Investigators were required to wear appropriate respirator equipment (RACAL, Health and Safety Inc., Frederick, MD). Mice were housed in HEPA-filtered, negative pressure, vented isolation containers. All other viruses were used under enhanced biosafety level 2 conditions by vaccinated personnel.

**Viruses.** The H1N1 influenza A/New Caledonia/20/99 (New Caledonia), and A/Mallard/Wisconsin/8/76 (Mal/WI); the H2N3 influenza A/Chicken/Ohio/494832/07 (Ck/OH); H3N2 influenza A/Aichi/2/68 (Aichi), A/Wyoming/3/03 (Wyoming), A/Fujian/411/02 (Fujian), and A/Brisbane/10/07 (Brisbane); the H5N1 influenza A/Hong Kong/483/97 (HK/483), A/Vietnam/1194/04 (VN/1194), A/Vietnam/1203/04 (VN/1203), and A/Hong Kong/156/97 (HK/156); and the H5 influenza A/Mallard/Alberta/85/76 (H5N2; Mallard/Alb), A/Duck/Hong Kong/820/80 (H5N3; Duck/HK), A/Duck/Potsdam/2216-4/84 (H5N6; Duck/Potsdam), and A/Shorebird/Delaware/35/98 (H5N8; Shorebird/DE) were propagated in Madin-Darby canine kidney (MDCK) cells as described previously (4, 14). The H1N1 influenza viruses A/Puerto Rico/8/34 (PR8) and A/California/04/09 (CA/09); H4N4 A/Grey Teal/Australia/2/79, H6N1 A/Teal/Hong Kong/W312/97, H7N3 A/Duck/Alberta/48/76, H8N4 A/Turkey/Ontario/6118/68,
H9N2 A/Chicken/Bangladesh/659/08, H10N7 Chicken/Germany/N/49, H11N6 A/Duck/Memphis/546/74, H12N5 A/Duck/Alberta/60/76, H13N6 A/Gull/Maryland/704/77, H14N5 A/Mallard/Astrakhan/263/82, H15N8 A/Shearwater/Australia/2576/79, and H16N3 A/shorebird/Delaware/172/06 were propagated in 10-day-old specific pathogen-free embryonated chicken eggs at 37°C. Allantoic fluid was harvested, clarified by centrifugation, and stored at -70°C.

Reverse genetics. The CA/09 viruses expressing genes from A/HK/483/97, A/Turkey/Egypt/06, A/Duck/Hunan/02, or A/VN/1203/04 were generated using the eight-plasmid system as described (11) and the viruses were confirmed by sequence analysis. Viral titers were determined by tissue culture infectious dose 50 (TCID$_{50}$) analysis in MDCK cells as described (27). The limit of detection for the TCID$_{50}$ assay is 100 TCID$_{50}$/ml. All in vitro experiments were performed with at least two different preparations of the reassortant virus (7).

Cells and culture media. MDCK cells were cultured in Eagle’s minimum essential medium (MediaTech, Manassas, VA) supplemented with 2 mM glutamine and 10% fetal bovine serum (FBS, Gemini BioProducts, West Sacramento, CA). A549 cells were cultured in Dulbecco’s minimum essential medium (DMEM, Lonza, Walkersville, MD) supplemented with 4.5 g/L glutamine and 10% FBS. RAW264.7 murine macrophages were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 4.5 g/L glutamine and 10% FBS. All cells were grown at 37°C under 5% CO$_2$.

Alveolar macrophage isolation and culture. Six week-old C57Bl/6 mice were euthanized by CO$_2$ asphyxiation and lungs were gently infused three times with 1ml of PBS supplemented with 1% FBS and 0.5mM EDTA. Cells from multiple mice were pooled and pelleted by centrifugation for 10 minutes at 400 x g, 4°C then resuspended in DMEM and 3x10$^5$ cells/well
were plated into a 24-well cell culture plate (Corning, Corning, NY). Purity was determined by
the Quik-Dip differential staining kit (Mercedes Medical, Sarasota, FL) to be >95%. Twenty-
four hours after plating, the cells were recounted and used for subsequent experiments.

In vitro infections. RAW264.7 or primary murine alveolar macrophages were infected in
triple at the indicated multiplicity of infection for 1 hour at 37°C. Unbound virus was
removed and the cells were washed in PBS and maintained in RPMI 1640 media containing
0.075% BSA in the presence (non-H5 viruses and low pathogenicity H5 viruses) or absence
(HPAI H5 viruses) of 1μg/ml tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin
(Pierce, Rockford, IL). Cell culture media was removed at the indicated times and stored at -
80°C for determination of viral titers by TCID₅₀ analysis on MDCK cells as described (7). All
TCID₅₀ titers are normalized to background levels of residual virus remaining in the culture wells
after the inoculum was washed off.

Quantitation of vRNA, cRNA, and mRNA by real time RT-PCR. Total RNA was isolated
from RAW264.7 macrophages and A549 cells at the indicated time points by TRIzol extraction
(Ambion, Carlsbad, CA) according to manufacturer’s instructions. cDNA complementary to the
three species of viral RNA were synthesized similar to Kawakami et al. (15). Primers specific to
the viral NP gene segment and containing a nucleotide tag that is unrelated to the viral sequence
were used for cDNA synthesis (Table 1). A 13μl mixture containing 200ng of total RNA,
10pmol of tagged primer, 1μl of 10mM dNTP mix (Invitrogen, Carlsbad, CA) and 8μl of
RNase-free water was heated to 65°C for 5 minutes and then returned to ice. After 1 minute,
cDNA synthesis was carried out using the SuperScriptIII First-strand synthesis system for RT-
PCR kit (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions.
Real-time PCR was performed with SsoFast EvaGreen Supermix on a CFX96 Real-time system (BioRad, Hercules, CA), similar to Kawakami et al. (15). Four microliters of a 1:10 dilution of cDNA was added to a master mix containing 10μl of 2x SsoFast EvaGreen Supermix, 1.5μl forward primer (10μM), 1.5μl reverse primer (10μM), and 3μl sterile water. Human or mouse GAPDH levels were also measured using GAPDH control reagents (Invitrogen, Carlsbad, CA or Applied Biosystems, Foster City, CA). Viral gene levels were normalized to GAPDH levels. The primers used are listed in Table 2.

Preparation of Cell Lysates. Mock- or influenza virus-infected RAW264.7 or MDCK cells were disrupted in RIPA buffer (150μM NaCl, 1% TritonX-100, 0.5% SDS, 0.5% deoxycholate in PBS) supplemented with Halt Protease and Phosphastase Inhibitor Cocktail (Thermo Scientific, Rockford, IL). Lysates were incubated on ice for 15 minutes, followed by centrifugation at 12,000rpm for 10 minutes at 4°C and frozen at -80°C until further use.

Western Blot. Cell lysates were quantitated using the Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL). Ten micrograms of total cell lysate was separated on a 4%–20% SDS-PAGE gel under reducing conditions. After transferring to nitrocellulose, blots were blocked in 5% non-fat dry milk in Tris-buffered saline plus 1% Tween 20 (TTBS) overnight at 4°C and probed for the influenza virus non-structural protein 1 (NS1) with mouse-anti-NS1 (1:1000, generous gift of Dr. Robert Webster) in TTBS for 1 h at room temperature. Blots were washed and incubated with goat anti-mouse-HRP (1:10000, Jackson Laboratories, Bar Harbor, ME). Blots were stripped and re-probed for actin with goat anti-actin (1:500) in TTBS for 1 h at room temperature. Blots were washed an incubated with donkey anti-goat-HRP (Jackson Laboratories, Bar Harbor, ME).
Immunofluorescence. RAW264.7 macrophages (3x10⁵ cells) or MDCK cells (1.5x10⁵ cells) seeded onto sterile glass coverslips were inoculated with medium alone, HK/483, or CA/09 (MOI=3.0 and 1.0 for RAW cells and MDCK cells respectively) for one hour at 4°C and then washed with cold PBS to remove unbound virus. Cells were shifted to 37°C for 30 minutes, 90 minutes, 2 hours, or 4 hours, fixed with 4% paraformaldehyde, and permeabilized in 0.1% TritonX-100 in PBS for 10 minutes at room temperature. After permeabilization, the cells were blocked in 1% BSA in PBS for 30 minutes at room temperature and stained for nucleoprotein (ATCC Clone HB-65; Manassas, VA) and DNA (4',6'-diamidino-2-phenylindole [DAPI], 1:1000; Sigma). The secondary antibody was anti-mouse IgG-Alexa 488 (Invitrogen, Eugene, OR) diluted 1:200 in 1% BSA/PBS overnight at 4°C. Coverslips were mounted in ProLong Gold Anti-fade Reagent (Molecular Probes, Eugene, OR) and fluorescence was examined on a Nikon TE2000 E2 microscope equipped with a Nikon C1Si confocal scanhead. Excitation was with 404nm and 488nm DPSS lasers, and the emission was collected through 450/35 and 515/60 band pass filters. Images were acquired with a Nikon 40X 1.3 NA Plan Fluor objective using Nikon EZC1 software. All images were acquired under the same condition.

Statistics. Statistical significance of data was determined by using analysis of variance (ANOVA) or Student’s t-test on GraphPad Prism (San Diego, CA). All assays were run in triplicate and are representative of at least two separate experiments. Error bars represent standard deviation, and statistical significance was defined as a p value of less than 0.05.
Results

Highly pathogenic H5N1 influenza viruses replicate productively in macrophages.

Influenza virus infection of macrophages was thought to be an abortive process in mammalian cells, failing to result in the release of progeny virus (33, 36). However, recent studies on alveolar macrophages infected with HPAI H5N1 influenza viruses have been contradictory; one group showing productive replication (39) and the other group demonstrating abortive infection (35). To address this issue and to get a complete picture of which influenza virus HA subtypes are capable of productive replication in macrophages, RAW264.7 murine macrophages were infected with a panel of influenza viruses representative of the 16 known HA subtypes (Table 1) and viral titers were determined in the cell culture supernatant by TCID_{50} assay at 24 hours post-infection (hpi). In support of previous findings (33, 36), the majority of influenza virus subtypes failed to productively replicate and yield infectious virus into the supernatant (Fig. 1A). However, infectious virus could be detected in the supernatant of macrophages infected with HK/483, a representative HPAI H5 virus. To rule out the possibility that our results in Fig. 1A were a result of the specific virus strains that we chose, we infected macrophages with a broader panel of human and avian H1, H3, and H5 influenza viruses. Similar to our observations in Fig. 1A, none of the H1 or H3 viruses that we tested productively replicated in macrophages (Fig 1B). In contrast, a subset of H5N1 influenza viruses replicated in macrophages and, with one exception (HK/156), replication was either sustained, or continued to increase as late as 72 hpi (Fig. 1B).

All strains of influenza virus except the HPAI influenza H5 and H7 viruses require exogenous TPCK-trypsin to productively replicate in vitro. To rule out an inhibitory effect of TPCK-trypsin on the replication of non-H5 influenza viruses in macrophages, macrophages were
infected with HPAI H5N1 influenza viruses in the presence and absence of TPCK-trypsin and viral titers were determined as described above. We observed no inhibition of H5N1 virus replication in the presence of trypsin (data not shown), demonstrating that the trypsin is not inhibiting non-H5 viruses from replicating in macrophages.

To address the possibility that non-H5 influenza viruses complete a productive replication cycle in the cells that are initially infected, but are limited in their ability to spread and initiate a subsequent round of infection, macrophages were infected at a higher MOI (MOI=3) and titers were measured over time. Similar to the low dose infection, non-H5 viruses failed to productively replicate in macrophages (Fig. 1C). Finally, to confirm our findings and rule out the possibility that our results are unique to RAW cells, primary alveolar macrophages were isolated from C57Bl/6 mice and infected with CA/09 H1N1 virus or the HPAI H5N1 viruses HK/483 or A/Vietnam/1203/2004 (VN/1203). Similar to our observations in RAW cells, only the H5N1 influenza viruses productively replicated in primary alveolar macrophages (Fig. 1D). Based on these findings, we used RAW cells in all subsequent experiments to investigate the mechanism of differential replicative capacity. In summary, our results support those of Yu et al. (39) demonstrating that macrophages support productive replication of H5N1 influenza viruses. Intriguingly, we further show that only a subset of the H5 influenza viruses can productively replicate in macrophages, specifically those associated with high pathogenicity in mammals (21, 38).

H5N1 influenza viruses overcome a block early in the viral lifecycle. Influenza viruses must gain access to the cellular replication machinery in the nucleus in order for productive infection to occur. Previously published reports have demonstrated that influenza viruses differ in their ability to gain entry into macrophages (26). Thus, to determine if macrophages restrict the
uptake of non-H5 influenza viruses, RAW cells were incubated with CA/09 or HK/483, strains that represent viruses that fail to replicate or productively replicate in macrophages respectively, at an MOI=5 for one hour at 4°C, followed by incubation at 37°C for infection to proceed. At the indicated time points, the cells were fixed, stained for the viral nucleoprotein (NP), and visualized by confocal microscopy. At 30 minutes post-infection, both viruses were internalized with equal efficiency (98.5% and 97.4% NP+ for CA/09 and HK/483 respectively) and NP was localized to the cytoplasm (Fig. 2A, left panels), indicating that the initial stage of virus entry is not blocked during H1N1 influenza virus infection. At 90 minutes post-infection, ~89% of the HK/483-infected cells were positive for NP (Fig. 2A, lower middle panel) and by 4 hpi ~60% of HK/483-infected cells were NP-positive with staining localizing to the nucleus (Fig. 2A lower right panel). In contrast, at 90 minutes post-infection, only 43% of the CA/09-infected macrophages remained NP-positive (Fig. 2A, upper middle panel). This continued to decrease and by 4 hpi only 14% of the cells had nuclear NP staining while viral antigen was not detected at all in the remaining cells (Fig. 2A, upper right panel). Differences in NP staining in infected macrophages were not due to a failure of the NP antibody to recognize the CA/09 NP protein as equivalent staining was observed in CA/09- and HK/483-infected MDCK cells at all time-points (Fig. 2B). Thus, abortive infection of macrophages with an H1N1 influenza virus is not due to restricted entry, but may be associated with a rapid loss of viral antigen in infected cells upstream of nuclear entry.

RNA synthesis is disrupted during H1N1 influenza virus infection of macrophages. We hypothesized that the decrease in nuclear NP localization in CA/09-infected macrophages would be associated with less viral transcription and ultimately replication. To test this hypothesis, we infected macrophages with CA/09 or HK/483 viruses at an MOI=5, synchronizing the infections
at 4°C as described above, and isolated total RNA at 30 minutes and 3, 6, or 12 hpi. The viral NP gene levels were then quantitated using a strand-specific, real-time RT-PCR assay to distinguish viral RNA (vRNA), messenger RNA (mRNA), and complementary RNA (cRNA) in infected cells as described by Kawakami, et al (15). The oligonucleotide sequences of the primers are provided in Table 2. Human respiratory epithelial A549 cells were infected in parallel as a positive control.

As shown in Figure 3A, all three RNA species could be detected in A549 cells infected with either the CA/09 or HK/483 viruses, consistent with the fact that A549 cells support productive viral infection. Synthesis of new vRNA and cRNA was detected by 12 hpi with both viruses, while synthesis of mRNA occurred earlier at 3 hpi. Higher levels of vRNA and cRNA were detected in HK/483-infected A549 cells (Fig. 3A). In HK/483-infected macrophages, synthesis of new vRNA and cRNA was detected as early as 6 hpi and mRNA synthesis had begun by 3 hpi. In contrast, there was no synthesis of cRNA or new vRNA in CA/09-infected macrophages as late as 24 hpi (Fig. 3B and data not shown), consistent with a lack of productive replication (Fig. 1). Synthesis of mRNA in CA/09-infected macrophages was detected, but to lower levels and delayed kinetics relative to HK/483-infected macrophages and CA/09-infected A549 cells, consistent with decreased NP in the nucleus in the CA/09-infected macrophages (Fig. 2). In summary, while NP protein is detected in a minority of CA/09-infected macrophages, the synthesis of new vRNA and cRNA is not detected, suggestive of a block downstream of entry of the vRNP into the nucleus in addition to the block which causes decreased NP staining in the nucleus relative to HK/483-infected macrophages.

**Replication of CA/09 H1N1 influenza virus in macrophages is blocked upstream of translation.** Finally, to determine if the decrease in RNA synthesis in CA/09-infected
macrophages was associated with less viral protein synthesis, we monitored the level of the viral non-structural protein (NS1) by western blot. Briefly, macrophages (MOI=5) or MDCK cells (MOI=1) were incubated in triplicate with the CA/09, or HK/483 viruses on ice for one hour followed by incubation at 37°C. A lower MOI was used in MDCK cells to avoid destruction of the monolayer that could affect interpretation of the results. Total cell lysates were prepared at the indicated time points and NS1 levels were determined by western blot. We could not detect NS1 expression in MDCK or macrophage lysates infected with either virus at 30 minutes or 6-hpi, consistent with the fact that NS1 is not a structural component of the virus and must be synthesized de novo in the infected cell (Fig. 4). By 24 hpi, NS1 was detected in MDCK cells infected with both viruses, consistent with the fact that MDCK cells support productive replication of influenza viruses. NS1 was also detected in macrophages infected with HK/483 at 24 hpi. In contrast, NS1 protein was not detected in CA/09-infected macrophages at any time post-infection (Fig. 4). Actin protein levels were monitored in parallel as a loading control (Fig. 4, lower panels). In summary, our studies demonstrate that certain HPAI H5 influenza viruses can productively replicate in macrophages by overcoming a block early in the viral lifecycle leading to efficient nuclear entry, and viral transcription and translation.

The hemagglutinin protein confers the ability of influenza viruses to productively infect macrophages. Our results indicate that the primary restriction to replication of non-H5 influenza viruses in macrophages occurs between 30 and 90 minutes post-infection. At this early stage of infection, the viral HA protein has an important role in mediating the escape of internalized virions from the uptake vesicle in order for the viral RNP to traffic to the nucleus.

To directly determine which viral protein(s) are important for productive replication in macrophages, reverse genetics reassortant CA/09 viruses expressing individual HK/483 viral
genes were generated (7). The reassortant viruses all replicated to similar levels in MDCK cells (7). Macrophages were infected with the reassortant or reverse genetics parental CA/09 and HK/483 viruses (MOI=0.01) and viral titers were measured at 24 hpi. As shown in Figure 5, the reverse genetics-derived parental CA/09 and HK/483 viruses replicated similar to the wild-type viruses (compare Figure 5A and Figure 1). Of the eight reassortant viruses, only the CA/09 virus expressing the HA gene of HK/483 (CA/09-HK/483HA) productively replicated in macrophages (Fig. 5A). A replication kinetics assay showed that, although the CA/09-HK/483HA virus productively replicated in macrophages, at 24 and 48 hpi it was not as efficient as the reverse genetics-derived HK/483 parental virus (Fig. 5B). However, at 72 hpi, the titers of the CA/09-HK/483HA virus were still increasing while those of the HK/483 control were beginning to decrease (Fig. 5B). These data demonstrate that the HA gene of an HPAI H5N1 influenza virus is sufficient to rescue productive replication of the CA/09 virus in macrophages.

The HK/483 virus was isolated from a fatal human infection during the initial outbreak of avian H5N1 influenza virus in humans in 1997 and this clade (clade 0) no longer appears to be circulating in nature. To determine if the HA protein of contemporary H5N1 influenza viruses also promotes replication in macrophages, we generated CA/09 viruses expressing the HA of A/duck/Hunan/795/2002 (clade 2.1), A/Vietnam/1203/2004 (clade 1), and A/turkey/Egypt/2006 (clade 2.2.1). All of these viruses replicated in macrophages (Fig. 5C). Overall, these data confirm the role of the HA gene of H5N1 influenza viruses in mediating productive replication of influenza virus in macrophages.
Discussion

Early investigation into the interaction of influenza viruses with mammalian alveolar and peritoneal macrophages demonstrated that infection is abortive, failing to yield infectious virus into the cellular supernatant (2, 30, 36). More recent studies, however, demonstrate that the fate of viral infection may be dependent on the viral strain and the source of the macrophages, but all studies are consistent in demonstrating that alveolar macrophages do not support productive replication of seasonal influenza viruses or the 2009 pandemic H1N1 virus (10, 24, 35, 39). The potential contribution of macrophages to severe H5N1 disease is now recognized, leading to several recent investigations into the replicative capacity of H5N1 influenza viruses in this cell type. The resulting studies present conflicting evidence, with some groups suggesting that H5N1 influenza viruses do productively replicate in alveolar macrophages (24, 39), while others report abortive infection (10, 31, 35). In the studies reported here, we address these conflicting results and demonstrate that certain H5 influenza viruses are unique among the 16 known HA subtypes of influenza virus in their capacity to replicate productively in macrophages. Further, our work extends that of others by investigating the nature of the restriction of influenza virus replication in macrophages and demonstrating which gene from H5N1 influenza viruses is required for productive replication. We show that the replication of most influenza virus strains is blocked early in the course of the viral lifecycle leading to decreased NP levels in the nucleus, less viral transcription, translation, and ultimately viral replication as determined by monitoring the release of infectious virus. However, the exact step in the process that is blocked and the role of cellular host proteins remains under investigation.

Previously published studies suggest that influenza viruses are differentially capable of infecting macrophages (26, 35). In contrast, and in support of the work of others (10, 39), we
demonstrate here using the A/CA/04/2009, A/Puerto Rico/8/1934 H1N1 viruses and the 
A/Aichi/2/1968 H3N2 virus that non-H5 influenza viruses are not restricted in their ability to be 
internalized by macrophages (Figure 2 and data not shown). While the source of macrophages or 
choice of viral strain may, in some cases, explain the disparity between these results, other work 
which observes differential infection based on visualization of the viral NP at 8-10 hpi (26) is 
unlikely to be representative of how well the initial uptake of the virus occurs as we observe a 
rapid decrease in NP levels by 90 minutes post-infection following efficient uptake.

Many cell surface lipids and proteins are sialylated and the specific molecule that serves 
as the primary entry receptor for influenza viruses is not known. Two C-type lectins expressed 
on macrophages, the macrophage mannose receptor (MMR) and macrophage galactose-type 
lectin (MGL), were shown to be critical for infection of macrophages by influenza viruses (25, 
34). Entry through these receptors results in a non-productive infection as none of the viruses 
used in these studies were able to productively replicate in macrophages. A possible explanation 
for our results is that a subset of H5 influenza viruses binds to a different receptor, one allowing 
entry through a productive pathway. Studies are underway to determine whether influenza 
viruses that productively infect macrophages enter the cell through a different pathway than 
those viruses which do not replicate productively.

In addition to binding to the target cell, HA mediates post-internalization fusion of the 
viral envelope with the endosomal membrane to release the viral ribonucleoprotein (RNP) 
complexes into the cytoplasm. Exposure of the HA molecule to the increasingly acidic 
environment of the endosome triggers a conformational change that permits the fusion event to 
take place (32). Internalized viral particles must escape the endosome prior to its fusion with the 
lysosome in order to avoid degradation by the acid hydrolases present in the lysosome. The pH
at which the HA molecule is triggered is virus strain-specific and variations in the pH of fusion are correlated with disease severity in animal models of influenza virus infection (9, 28). We detected a gradual decrease in the number of CA/09 virus-infected macrophages between 30 minutes and 4 hours post-infection (Fig. 3), suggestive of a possible degradation of the virus after uptake. Experiments ongoing in the laboratory are addressing the role of the pH of fusion on replication of influenza viruses in macrophages.

An alternative explanation for the restricted nuclear entry of the CA/09 virus is that the viral RNPs, rather than not being released from the uptake vesicle, are not properly shuttled to the nucleus. This hypothesis is not as attractive to us because replication of CA/09 is rescued by expression of the HK/483 HA gene and HA is not known to play a role in nuclear import of the viral RNPs. However, our data do not allow us to rule out this possibility and future studies will differentiate between impaired fusion of the viral and endosomal membranes and impaired nuclear transport of the viral RNPs.

Previous studies have reported the importance of macrophages to the host response during influenza virus infection. Indeed, dysregulation of cytokine production by infected macrophages has been implicated in the hypercytokinemia which is linked to high mortality rates in humans infected with avian H5N1 influenza viruses (1, 6, 8). The observation, made by us and by other laboratories, that H5N1 influenza viruses productively infect macrophages is, to our knowledge, the first qualitative difference in the interaction of macrophages with influenza viruses to be reported. It is an intriguing hypothesis to consider that productive replication in macrophages may account for the virulence of HPAI H5N1 viruses by contributing to the excessive production of proinflammatory cytokines. However, unpublished results generated in our lab give no indication that live H5N1 influenza virus stimulates greater cytokine secretion.
from macrophages than infection with an H5N1 virus that has been UV-inactivated (data not shown). However, further investigation is needed to determine if there are differences in the cytokine response between macrophages infected with an influenza virus which can replicate in macrophages and one that cannot.

Does productive replication of macrophages lead to more severe disease? Although this question remains under investigation, our previous work suggests this may be a possibility. We have previously demonstrated that the CA/09 virus expressing the HA of HK/483 exhibits greater pathogenicity in mice relative to the wild-type CA/09 virus (7). While we are unsure of the mechanism of heightened disease severity during \textit{in vivo} infection with CA/09-HK/483HA, the results presented here suggest that replication in macrophages may be a contributing factor to the virulence of this reassortant virus. In order to address this question we are planning future studies in the lab to determine whether the productive replication of influenza viruses that we observe in immortalized cell lines and primary alveolar macrophages can be detected during \textit{in vivo} infection.

In summary, we demonstrate that a subset of HPAI H5N1 influenza viruses is unique among influenza viruses in their capacity to replicate in a mammalian macrophage cell line and alveolar macrophages. These viruses overcome a block early in the replication cycle in an HA-dependent manner to promote transcription, translation, and replication of the viral genes, leading to the assembly and release of newly formed virus particles. Our findings may provide insight into the mechanisms of virulence of H5N1 influenza viruses.
Acknowledgments

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References


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Figure Legends

Figure 1 - H5N1 influenza viruses productively infect RAW cells. RAW264.7 cells were infected in triplicate with a panel of influenza viruses representing all 16 HA subtypes, (A; viruses are listed in Table 1) or with the indicated viruses (B) at MOI=0.01. At the indicated times post-infection, the media was collected and viral titers were determined by TCID$_{50}$ analysis in triplicate. (C) RAW cells were infected with the indicated influenza viruses (MOI=3). The media was collected at 24 and 48 hpi and viral titers were determined by TCID$_{50}$ analysis. (D) Primary murine alveolar macrophages were infected with the indicated viruses (MOI=3), cell supernatants were collected at 24 and 48 hours post-infection, and viral titers were determined as above (limit of detection = 10$^2$ TCID$_{50}$/ml). The data are representative of duplicate experiments. Error bars represent the mean TCID$_{50}$ value +/- standard deviation (SD). Titers determined at 1 hpi were below the limit of detection for the H5 viruses and ranged from below the limit of detection to 10$^3$ TCID$_{50}$/ml for H1 and H3 viruses.

Figure 2 – NP staining decreases in macrophages infected with CA/09. RAW264.7 macrophages (A) or MDCK cells (B) seeded onto coverslips were incubated with CA/09 or HK/483 (MOI=5) on ice for one hour. At time zero, warm infection media was added and the cells were then incubated at 37°C. At the indicated time-points, the cells were fixed and processed for immunofluorescent staining to detect viral NP. The slides were viewed by confocal microscopy as described in Materials and Methods. The mean percent NP+ macrophages was quantified from three independent images and is indicated in the lower right corner of each panel (A). Nuclei were visualized by DAPI staining and representative images from two independent experiments are shown.
Figure 3 – RNA synthesis is inhibited in CA/09-infected macrophages. A549 (A) or RAW 643 cells (B) were incubated with HK/483 or CA/09 at 4°C for one hour. At time zero, warm media was added and the cells were incubated at 37°C. At the indicated time points, total RNA was isolated and the levels of vRNA, mRNA, and cRNA were determined in triplicate using primers that amplify the NP gene as described in Materials and Methods. The RNA level in HK/483-infected cells at 12 hpi was set as a value of 1.0 and all other samples are presented relative to that amount. Viral RNA levels were normalized to GAPDH and to the amount of RNA present at 30 minutes post-infection. The data are representative of two independent experiments. Primer sequences are presented in Table 2.

Figure 4 – Viral protein synthesis is blocked during CA/09 infection of macrophages. RAW264.7 (left panels) or MDCK cells (right panels) were infected with HK/483 or CA/09 and cells were lysed at the indicated times post-infection. Proteins were separated by SDS-PAGE under reducing conditions, transferred to nitrocellulose and probed with anti-NS1 (top panels) or anti-actin (bottom panels) by Western blot.

Figure 5 - The HA gene mediates replication of influenza viruses in macrophages. (A) RAW cells were infected with the parental reverse genetics (rg) viruses or with rgCA/09 expressing individual genes from HK/483 (MOI=0.1). Cell culture supernatants were collected at 24 hpi and viral titers were determined by TCID<sub>50</sub> analysis. (B) RAW cells were infected with the indicated viruses and viral titers determined as described for A. (C) RAW cells were infected with the indicated reverse genetics viruses as described above. The media was collected 24 hpi and viral titers were determined by TCID<sub>50</sub> analysis on MDCK cells. Error bars represent the mean value +/- SD.
Table 1. Influenza virus strains tested for productive replication in macrophages

<table>
<thead>
<tr>
<th>HA Subtype</th>
<th>Virus Name</th>
<th>HA Subtype</th>
<th>Virus Name</th>
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<tbody>
<tr>
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<td>A/California/04/2009</td>
<td>H9</td>
<td>A/Chicken/Bangladesh/659/2008</td>
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<tr>
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<td>H10</td>
<td>A/Chicken/Germany/N/1949</td>
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<td>A/Teal/Hong Kong/W312/1997</td>
<td>H14</td>
<td>A/Mallard/Astrakhan/263/1982</td>
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Table 2. Primer sets for strand-specific RT-PCR

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<thead>
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<th>Reaction</th>
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<td>HK/483 NP</td>
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<td>mRNA</td>
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