Highly pathogenic H5N1 influenza virus alters pH-mediated fusion properties, overcomes blocks to lysosome escape and replication, and impairs macrophage function

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CONTENTS

ABSTRACT

Highly pathogenic H5N1 influenza virus alters pH-mediated fusion properties, overcomes blocks to lysosome escape and replication, and impairs macrophage function

by

Carlos Theodore Huerta

Highly pathogenic H5N1 influenza virus infections are associated with severe respiratory damage and high morbidity and mortality in infected humans. Due to the critical role that macrophages play in response to respiratory pathogens, investigating such viruses' ability to evade or exploit the innate immune response can provide greater insight into macrophage function at the host-pathogen interface. Unlike H5 viruses, we found that all non-H5 influenza virus strains including the 2009 H1N1 pandemic virus experience a block upstream of nuclear entry and escape and are unable to replicate in macrophages. However, only highly pathogenic H5N1 strains could successfully produce new viral protein synthesis and progeny release. Furthermore, it was found that the pH of fusion of the hemagglutinin (HA) protein, not sialic acid receptor binding, influenced replication. On a functional level, it was shown that while reactive oxygen species (ROS) were not produced during viral infection, macrophage phagocytosis was significantly impaired by productive viral replication. Future studies will focus on elucidating the molecular mechanism(s) of these blocks in differential H5 virus-specific replication, and the overall effect of viral replication in *in vivo* model systems.

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INTRODUCTION

Influenza A viruses are known to be an important cause of death among both humans and animals alike. Particularly, highly pathogenic avian H5N1 influenza viruses exhibited zoonotic potential by crossing the species barrier and infecting humans in 1997 (Schultz-Cherry and McCullers 2006). These viruses are now present in domestic poultry on three continents, which can result in further adaption of these viruses to humans and more outbreaks (Schultz-Cherry and McCullers 2006). These viruses are associated with severe respiratory damage and a 60% fatality rate (World Health Organization). Previous studies have determined that a minimum of 4-5 mutations in viral genes, several of which these viruses have already acquired naturally, confers aerosol droplet transmission in a ferret model of infection (Imai et al. 2012; Herfst et al. 2012). Due to the heightened prevalence and clinical relevance of these influenza virus subtypes, more studies regarding the pathology and immune responses associated with H5N1 infections will be necessary to improve antiviral and vaccination strategies.

Influenza initiates infection (Figure 1) by binding to its cellular receptor, sialic acid-containing receptor protein. Influenza viruses of avian origin bind preferentially to terminal sialic acids bound to galactose via an α-2,3 linkage while human viruses prefer α-2,6-linked sialic acids (Ito 2000). Receptor binding triggers endocytosis via clathrin-dependent and clathrin–independent pathways (Sieczkarski, 2002). Endosomal maturation results in an increasingly acidic environment, eventually achieving a pH threshold at which the viral hemagglutinin (HA) protein undergoes a conformational change that triggers fusion of the viral envelope with the endosomal membrane (Reed et al. 2009; Reed et al. 2010; DuBois et al. 2011). This optimal pH of fusion varies between

strains (Reed et al. 2009; DuBois et al. 2011; Reed et al. 2010). Acidification of the interior of the virus particle, mediated by the virally-encoded M2 ion channel, releases the viral ribonucleoprotein complexes (vRNPs) into the cytoplasm (Skehel and Wiley 2000). The vRNPs must enter the nucleus for subsequent transcription, translation and replication of viral genes. Progeny virions are assembled and released from the cellular membrane. During the course of this infection cycle, the host immune response is mounted to limit viral replication and spread with varying degrees of success (Dash and Thomas 2014).

Macrophages, phagocytic cells responsible for destroying foreign microbes and triggering the adaptive immune response, are a required component of the host innate immune response against influenza viruses (Tate et al. 2010). Alveolar macrophages, due to their location in the lungs, act as one of the earliest points of contact in the host to influenza infection and release vital proinflammatory cytokines to trigger the antiviral response (Tate et al. 2010). However, previous work has shown that an excessive proinflammatory cytokine response induced by alveolar macrophages is associated with increased mortality in patients infected with H5N1 viruses (de Jong et al. 2006). As such, understanding the variances in interactions between different influenza A viruses and macrophages may provide insight into the basis for this increased mortality.

Previously, our laboratory demonstrated the unique ability of H5N1 viruses to actively replicate in murine macrophages, which may provide a mechanistic understanding for the severity of these infections (Cline et al. 2012). Through comparison of H5N1 and H1N1 viruses both in macrophages harvested from live mice (primary alveolar macrophages) and macrophage cell lines, this work has shown that there is a

unique difference in the ability of H5N1 viruses to actively replicate in murine macrophages (RAW264.7 macrophages) compared to H1N1 viruses (Cline et al. 2012). Using quantitative real-time PCR (qRT-PCR) analysis of the levels of influenza M gene, a gene actively transcribed in the nucleus during successful replication, and immunofluorescent microscopy for the viral nucleoprotein (antibody is specific to NP alone not to be confused with the vRNA-NP complex referred to as vRNP), we determined that H1N1 viruses are unable to enter the macrophage nucleus to replicate despite their normal patterns of cellular attachment and entry (Cline et al. 2012). To confirm these results, H1N1 strains were developed by reverse genetics to express the H5 HA protein, a protein known to play a role in the cellular entry of influenza viruses (Cline et al. 2012). Reverse genetics-derived viruses are recombinant viruses that can be manipulated to express different combinations of genes from varying influenza subtypes (see Materials & Methods). The engineered viruses were compared against both reverse genetics-derived and wild type H5N1 and H1N1 viruses (Cline et al. 2012). Expressing the H5N1 HA protein in otherwise unmodified H1N1 viruses allowed replication to proceed in macrophages, which supports the model that replication in macrophages occurs in an HA-dependent mechanism (Cline et al. 2012). Intriguingly, expression of the H5 HA in an H1N1 virus also increased the pathogenicity of this virus *in vivo* and resulted in a lower survival rate in mice infected with this virus compared to an H1N1 wild-type CA/09 (Figure 2; Cline et al. 2011). These results seem to indicate that the ability of an influenza virus to efficiently replicate in macrophages correlates with higher mortality and disease severity in the infected host (Cline et al. 2011).

Given the different phenotypes of replication of influenza viral strains in macrophages, the current study was undertaken to ascertain what aspects of the HA protein drive differential influenza replication and what effect replicating virus strains have upon macrophage biological function. Specifically, the aims of this study were threefold: to understand where H1N1 viruses are blocked in the replication cycle, to elucidate whether the mechanism(s) for macrophage replication is regulated by the specific pH of fusion or receptor binding of the H5 HA, and to determine if H5 replication in macrophages affects macrophage function. For the purpose of these studies, we define productive replication as the ability to produce infectious progeny that can spread to neighboring cells, not simply the production of viral protein or nucleic acids as described by other investigators. Our results demonstrate that only highly pathogenic H5N1 viruses are capable of nuclear entry and replication, while non-macrophage replication competent influenza viruses are capable of nuclear entry only. Specifically, H5 nuclear entry is mediated by the ability of H5 viruses to overcome a block in lysosomal escape, which non-H5 viruses are unable to overcome. Furthermore, stabilization of the pH of fusion of highly pathogenic H5 viruses (to make them more H1-like) appeared to inhibit replication and nuclear entry, indicating that the pH of fusion of the viral HA may regulate the ability of strains to replicate in macrophages. Conversely, altering receptor binding of an H1N1 virus from α-2,6 to α-2,3 sialic acid linkages did not rescue nuclear entry or replication, demonstrating that HA receptor binding is not a determinate of replication. Finally, it was found that H5 virus infection of macrophages decreased phagocytic ability of these cells compared to H1 viruses.

MATERIALS AND METHODS

Ethics statement. All procedures were approved by the St. Jude Children's Research Hospital Institutional Biosafety Committee, Institutional Review Board, and Institutional Animal Care and Use Committee 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 highly pathogenic avian H5N1 influenza (HPAI) viruses and CA/09 H1N1 containing the H5 HA gene were conducted in a biosafety level 3 enhanced containment laboratory. 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 virus strains influenza A/New Caledonia/20/99 and A/Swine/NE/2/92, the H3N2 virus strains influenza A/Brisbane/10/07, A/Swine/NC/0668/2011, and A/Swine/TX/4119-2/1998, the H4N4 strain A/Grey Teal/Australia2/79, the H5N1 virus strains influenza A/Hong Kong/486/97 (HK/486), A/Hong Kong/483/97 (HK/483), A/Vietnam/1203/04 (VN/1203), and A/Hong Kong/213/03, and the H5 virus strains influenza A/Mallard/Alberta/85/76 (H5N2; Mallard/Alb), A/Duck/Potsdam/2216-4/84 (H5N6; Duck/Potsdam), and A/Turkey/WI/1/1968 (Turkey/WI), (H5N8; Shorebird/DE) were all propagated in Madin-Darby canine kidney (MDCK) cells as described previously (Carlson et al, 2010; Jones et

al, 2006). The influenza virus H1N1 A/California/04/09 (CA/09), H6N1 A/Teal/Hong Kong/W312/97, and H7N9 A/Anhui/01/2013 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/486/97 or A/VN/1203/04 were generated by using the eight-plasmid system as described previously (Hoffmann et al., 2002), and the viruses were confirmed by sequence analysis. Virus titers were determined by 50% tissue culture infectious dose ($TCID_{50}$) analysis in MDCK cells as described previously (Reed and Muench, 1938). 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 (Cline et al, 2011).

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). RAW264.7 murine macrophages were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 4.5 g of glutamine/liter and 10% FBS. All cells were grown at 37°C under 5% CO_2.

Primary human macrophage isolation and culture. Peripheral blood mononuclear cells (PBMCs) were isolated on a ficoll gradient spin. Lymphocytes were removed by pelleting through a 50% Percoll cushion (Amersham Biosciences) and spun at 2000 x g for 20 minutes. Lymphocytes pellet to the bottom while the remaining cells are at the media-Percoll interface. Cells were plated in Isocove's modified Dulbecco's minimum

essential medium supplemented with glutamax, sodium pyruvate, and 10% human serum. Non-adherent cells were washed away after overnight incubation. Cells were incubated for 7 days prior to infection with washing and media changes every 2-3 days. Cells were stained with an Alexa-555 conjugated CD11 for purity. Cells from at least 2 different donors were used for experimental repeats.

***In vitro* infections.** RAW264.7, MDCK or primary human macrophages were infected in duplicate at the indicated multiplicity of infection for 1 hour (1 h) at 37°C. Unbound virus was removed, and the cells were washed in PBS and maintained in the appropriate medium containing 0.075% bovine serum albumin (BSA) in the presence (non-H5 viruses and low-pathogenicity H5 viruses) or absence (HPAI H5 viruses) of 1 μg of TPCK (tolylsulfonyl phenylalanyl chloromethyl ketone)-treated trypsin (Pierce, Rockford, IL)/ml. Cell culture medium was removed at the indicated times and stored at −80°C for the determination of virus titers by TCID50 analysis on MDCK cells as described previously (21917948). All $TCID_{50}$ titers were normalized to background levels of residual virus remaining in the culture wells after the inoculum was washed off.

Immunofluorescence and colocalization. RAW264.7 macrophages, primary human macrophages or MDCK cells seeded onto 96 well plates or sterile glass coverslips 1 day prior to inoculation (7 days prior for primary human macrophages). Cells were inoculated with medium alone or the indicated viruses (multiplicity of infection [MOI] = 2.0 and 5.0 for nuclear localization and LAMP1 colocalization, respectively) for 1 h at 4°C and then washed with cold PBS to remove unbound virus. The cells were shifted to 37°C for 30 min, 1h, 2 h, or 4 h, and then fixed with 4% paraformaldehyde. For nucleoprotein nuclear localization, cells were permeabilized in 0.5% Triton X-100 in

PBS for 10 min, blocked in 10% FBS in PBS for 1h, and stained for nucleoprotein (NP, clone HB-65; American Type Culture Collection, Manassas, VA) overnight. Anti-mouse IgG-Alexa 488 secondary antibody (Invitrogen) was used at 1:200 in 10% FBS in PBS for 1h. For NP-LAMP1 colocalization, cells were blocked for 1 hr in PBS with 10% FBS and 0.5% saponin (Sigma). Staining for NP and LAMP1 (Sigma, 1:1000) was done in 10% FBS with 0.5% saponin overnight. Anti-mouse IgG-Alexa 488 and anti-rabbit IgG-Alexa 555 (Invitrogen) were both used at 1:200 in PBS with 10% FBS and 0.5% saponin for 1h. All cells were stained for DNA (4′,6′-diamidino-2-phenylindole [DAPI], 1:1,000; Sigma). All steps were performed at room temperature with the exception of overnight incubations, which were performed at 4°C. Wells were imaged on an Evos microscope (Advanced Microscopy Group) at a magnification of ×40, using identical parameters for each treatment. Nuclei and fluorescein isothiocyanate-positive (FITC+) cells were counted using ImageJ software. The percent of nuclear NP is expressed as the percentage of nuclear NP in macrophages standardized to the percent nuclear NP in MDCK cells from the same experiment: (% nuclear NP+ macrophage/% nuclear NP+ MDCK). For NP-LAMP1 colocalization experiments, coverslips were mounted in ProLong Gold antifade reagent (Molecular Probes, Eugene, OR), and fluorescence was examined on a Nikon TE2000 E2 microscope equipped with a Nikon C1Si confocal scanhead. Excitation was performed with 404-nm, 488-nm and 555-nm DPSS lasers, and the emission was collected through 450/35 and 515/60 band-pass filters. Images were acquired with a Nikon ×60 1.3 NA Plan Fluor objective lens using Nikon EZC1 software. All images were acquired under the same conditions. NP three-dimensional surfaces were acquired using IMARIS software. Colocalization was determined by the

fluorescence intensity of the LAMP1 channel at each three-dimensional surface compared to the fluorescence intensity of the secondary-only antibody intensity.

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 a transfer to nitrocellulose, blots were blocked in 5% nonfat dry milk in Tris-buffered saline plus 1% Tween 20 (TTBS) overnight at 4°C and probed for the influenza virus nonstructural protein 1 (NS1) with mouse anti-NS1 (1:1,000; a generous gift from Robert Webster) in TTBS for 1 h at room temperature. Blots were washed and incubated with goat anti-mouse-HRP (1:10,000; Jackson Laboratories, Bar Harbor, ME). The blots were stripped and reprobed for actin with goat anti-actin (1:500) in TTBS for 1 h at room temperature. The blots were washed and incubated with donkey anti-goat HRP-conjugated antibody (Jackson Laboratories, Bar Harbor, ME).

Quantitation of vRNA, cRNA, and mRNA by real-time RT-PCR. Total RNA was isolated from RAW264.7 macrophages at the indicated time points by TRIzol extraction (Ambion, Carlsbad, CA) according to the manufacturer's instructions. cDNA complementary to the three species of viral RNA were synthesized by a procedure similar to that described by Kawakami et al (2011). 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. A 13-μl mixture containing 200 ng of total RNA, 10 pmol of tagged primer, 1 μl of 10 mM deoxynucleoside triphosphate mix (Invitrogen), and 8 μl of RNase-free water was heated to 65°C for 5 min and then returned to ice. After 1 min, cDNA synthesis was carried out using the SuperScript III First-Strand Synthesis System

for Reverse Transcription-PCR (RT-PCR) kit (Invitrogen) according to the manufacturer's instructions. Real-time PCR was performed with SsoFast EvaGreen Supermix on a CFX96 real-time system (Bio-Rad, Hercules, CA) by a method similar to that described by Kawakami et al (2011). Four microliters of a 1:10 dilution of cDNA was added to a master mix containing 10 µl of 2× SsoFast EvaGreen Supermix, 1.5 µl of forward primer (10 µM), 1.5 µl of reverse primer (10 µM), and 3 µl of sterile water. Human or mouse GAPDH (glyceraldehyde-3-phosphate dehydrogenase) levels were also measured using GAPDH control reagents (Invitrogen or Applied Biosystems [Foster City, CA]). Viral gene levels were normalized to GAPDH levels.

Phagocytosis Assay. Macrophages were seeded into 6 well plates 1 day (for RAW264.7) or 7 days (for primary cells) prior to infection. Macrophages were infected with the indicated viruses at an MOI of 1. pHrodo-*Staphylococcus aureus* bioparticles (Invitrogen) were incubated with a 1:100 dilution of rabbit IgG (Invitrogen) for 1h at 37°C. Twenty four hours post-infection, media was removed and bioparticles were added in HBSS (Life Technologies) containing 20mM Hepes (Life Technologies) and incubated on ice or at 37°C for 30 minutes. Cells were placed on ice, washed with PBS, and then removed by scraping. Cells were then fixed in 2% paraformaldehyde for 10 minutes. FITC-positive cells were measured by flow cytometry (Canto-II or FacsCalibur-III).

Reactive Oxygen Species Production. Macrophages were plated in a black 96-well plate (Costar). Prior to infection, cells were incubated with the ROS-sensitive fluorophore H2DCFDA (DCF; Invitrogen) for 30 min followed by two washes with PBS. Cells were inoculated with media, virus (MOI: 5) or 10mM ethanol. Fluorescence

intensity was measured over time at an excitation wavelength of 485nm and an emission wavelength of 520nm on a fluorescent plate reader. Data are expressed as the relative fluorescence intensity (RFI) as standardized to the time zero read for that individual well.

Statistics. The statistical significance of the data was determined by using analysis of variance or the Student t test on GraphPad Prism (GraphPad, San Diego, CA). All assays were run in triplicate and are representative of at least two separate experiments. Error bars represent standard deviations, and statistical significance was defined as a P value of <0.05.

RESULTS AND DISCUSSION

The H5 HA confers the ability of H5N1 viruses to escape lysosomal compartments and produce new viral protein in macrophages

To initially characterize replication differences in influenza strains, we compared a panel of influenza subtypes and demonstrated that only specific H5N1 viruses productively replicate in macrophages (Table 1). Conversely, some H5 viruses such as A/Duck/Potsdam/2216-4/84 and A/Turkey/WI/1/1968 (non-H5N1 viruses) show no irregularities in cellular and nuclear entry (as shown by immunofluorescent staining of viral NP) but are unable to produce new viral progeny. Additionally, viruses of human origin in general displayed much lower rates of nuclear colocalization compared to avian viruses that tend to have a lower pH of fusion. Overall, these results seem to indicate two major findings: that all H5 viruses which we have tested are capable of nuclear entry and that full replication in macrophages is limited to only two of these H5N1 influenza viruses (Table 1). We theorized that both of these differences could be due to unique aspects of the H5 HA protein based on our earlier work concerning H5N1 replication. Given this information, we first investigated the common feature of all H5 viruses' ability to enter macrophage nuclei, and then determine the differences between replicating and non-replicating H5 virus strains.

Since this panel of viruses demonstrated that all the H5 viruses tested are capable of nuclear entry even though some do not replicate, we next investigated whether the H5 HA protein specifically confers the ability of these H5 viruses to escape from the endo-lysosomal pathway and accounts for their nuclear entry in macrophages. Through immunofluorescent staining for lysosomal (LAMP1) and viral NP proteins in murine

macrophages infected with an H1N1 virus (A/California/04/2009) or an H1N1 virus containing a deselected H5 HA (A/California/04/2009+A/VN/1203/03 dHA) protein, we found that H1N1s accumulate within lysosomes while viruses containing an H5 HA are able to escape lysosomes (Figure 2). ("Deselecting" a virus refers to removing the multi-basic cleavage site of the H5 HA, which only affects this strain's transmissibility and allows it to be used in a lower biosafety laboratory.) Furthermore, H1N1 viruses had much lower rates of NP positive infected macrophages compared to the H5N1 strains studied (Table 1). Finally, we demonstrated that both H1N1 and H1N1 virus containing the H5 HA protein are unable to synthesize the influenza nonstructural protein 1 (NS1), which is a protein manufactured *de novo,* in all infected cells (Figure 3A). Since influenza viruses do not contain NS1 protein when they enter the cell, any detected production of NS1 can be used to monitor the ability of the virus to produce new viral proteins (Samji 2009). Production of the NS1 protein in Madin-Darby Canine Kidney (MDCK) cells served as a positive control for protein replication. Production of the NS1 protein by WSN infection, an atypical H1 influenza virus capable of replicating in almost all cell types including macrophages, served as a positive control for viral protein replication. Through qRT-PCR analysis of infected cells we found that H1N1 virus containing the H5 HA protein produces relatively similar amounts of mRNA compared to influenza infection of epithelial cells, unlike the H1N1 virus that produces no mRNA during macrophage infection (Figure 3B). Taken together, these results suggest that there is a secondary block after nuclear entry, but prior to protein translation, that is overcome by the H5N1 HA (Figure 1).

The pH of fusion of the H5 HA confers escape from the endosome-lysosome pathway and subsequent replication of viral progeny in macrophages

With the idea that the H5 HA allows H5 viruses to escape from the endosome-lysosome pathways, we next investigated whether this could be due to differences in the pH of fusion of the H5 HA. During the course of influenza infection, the viral HA protein fuses with the endosomal membrane allowing the release of viral nucleic acid to induce replication (Figure 1). The optimal pH at which HA fusion is triggered is virus strain-dependent, and has been described as a determinant of pathogenicity and transmissibility (Reed et al. 2009; DuBois et al. 2011; Reed et al. 2010). For example, the HA of the H1N1 virus is triggered at pH~5.0 while that of the H5N1 virus is triggered at pH~6.0 (unpublished observations). Thus, the H1 HA is said to be more acid stable than an H5 HA. While all influenza viruses can bind to and be internalized by macrophages, the higher pH of macrophage endosome-lysosome compartments, (relative to epithelial cells) may prevent H1N1 but not H5N1 viruses from undergoing membrane fusion. In turn, this would inhibit subsequent trafficking to the nucleus (Figure 4). For example, the H1N1 HA at a lower pH of fusion (pH~5.0) would fuse to the endosomal membrane in an MDCK epithelial cell prior to encountering lysosomes whereas in a macrophage the pH would not be low enough for HA fusion until the virus reached the lysosome where active proteases can destroy the virus (Figure 4). Based on this work, we hypothesized that the lower acid stability of the H5 HA protein determines influenza NP nuclear entry. The high acid stability of non-H5 influenza viruses keeps the virus isolated to the endosome for sufficient time for lysosomal fusion to occur, leading to inactivation of the virus particle prior to HA activation and endosomal escape. To determine whether

altering the pH of fusion of an H5N1 virus could inhibit replication, we generated several recombinant H5N1 influenza viruses with point mutations in the HA gene that alter the acid stability to varying degrees and result in the loss of function of the virus. Macrophages were infected with the HA mutants or parental viruses and virus titers in the supernatant was monitored at 24 hours post-infection. While the viruses replicated to similar titers in MDCK cells (data not shown), the K58I mutant, a loss of function mutation (more H1-like), displayed a 1-2 log decrease in viral titers, suggesting a potential role for the acid stability of the HA protein in mediating replication in macrophages (Figure 5A). Furthermore, examination of nuclear NP colocalization of the K58I mutant in murine macrophages revealed that nuclear entry was reduced compared to infection with wild-type infection to a statistically significant level (Figure 5B). Ultimately, these data support the concept that the pH of fusion of the HA determines the ability of the virus to escape lysosomal compartments, thereby determining subsequent nuclear import and replication.

α-2,6 versus α-2,3 binding by influenza viruses to sialic acid linkages does not determine nuclear entry or replication in macrophages

However, since our previous comparison of replication of all influenza subtypes demonstrated that human viruses binding via α-2,6 receptors demonstrated lower rates of nuclear entry compared to avian viruses binding via α-2,3 receptors, one could argue that differences in replication could be due to differential sialic acid receptor binding. To investigate if receptor binding plays a role in replication, an H1N1 human virus A/Tennessee/1-560/09 was generated by reverse genetics to express an HA that

preferentially binds to α-2,3 (more avian like) receptors instead of its wild-type α-2,6 (more human like) receptor counterparts. Swapping the viral α-2,6 receptor to an α-2,3 binder did not rescue this strain's ability to enter macrophage nuclei or produce new progeny virus as measured by immunofluorescent microscopy of nuclear NP and $TCID_{50}$ analysis (Figure 6). Given these results, we were able to dismiss the idea that differential sialic acid linkage binding determines replication of influenza viruses in macrophages.

Influenza infection of murine and human primary macrophages does not induce ROS production

An excessive inflammatory response caused by overproduction of proinflammatory cytokines is thought to be a cause of death during H5N1 infection and macrophages are a major contributor of cytokine production. Macrophages infiltrate lung alveoli early during H5N1 infection, which correlates with increased expression of cytokines (Perrone et al. 2008). *In vitro* infection of macrophages showed higher levels of proinflammatory cytokines with H5N1 infection compared to seasonal strains (Hui et al. 2009; Lee et al. 2009; Mok et al. 2009). Higher ROS levels, which also contribute to the inflammatory response, are produced in the lungs during H5N1 infection compared to non-H5 infections (Perrone et al. 2013). However, the cell type(s) responsible for this increased ROS production have yet to be identified (Perrone et al. 2013). Thus, we finally investigated if replication competent viruses could affect macrophage function by examining production of reactive oxygen species (ROS) and phagocytic levels of macrophages.

To determine if H5 replication in macrophages increases ROS production, macrophages were infected with different influenza viruses using the ROS-sensitive dye DCF as described previously (McGuire et al. 2011). Infection of RAW264.7 macrophages with an H1N1 or non-replicating H5 virus (A/Duck/Potsdam/2216/4-84) did not induce higher ROS species compared to uninfected controls (Figure 7A). In fact, there was a significant reduction in baseline ROS levels. Since ROS species have been correlated with increased pathogenesis, the reduced levels of ROS indicated by our data during H1N1 and non-replicating H5 viral infection could account the lessened severity of these infections compared to highly pathogenic H5N1 infection (Perrone et al. 2013). Similar results were obtained with primary human macrophages (Figure 7B). Furthermore, even when ethanol, a chemical scavenger known to reduce ROS production, was added to uninfected cells, none of the viruses tested induced ROS production above this artificially lowered threshold (Billany et al. 1996). These results allowed us to hypothesize that H5N1 infection of macrophages does not induce ROS production. Recent studies suggest that a more likely culprit of ROS production during influenza infection are lung epithelial cells due to influenza's ability to activate ROS intracellular pathways by NOX-4 (Amatore et al. 2015). Future studies could target the ability of macrophages to clear infected epithelial cells from the respiratory tract as a potential mechanism to reduce ROS-induced inflammation.

H5 influenza virus infection of murine and human primary macrophages significantly reduces phagocytic function

To elucidate if influenza replication alters macrophage phagocytosis, murine and human macrophages were infected with a panel of differentially replicating influenza

viruses for 24 hours and monitored for their ability to phagocytose opsonized pHrodo-*Staphylococcus aureus* bioparticles by flow cytometry as described previously (Brubaker et al. 2011). While H1N1 viral infection of murine macrophages did not appear to reduce the ability of these cells to phagocytose new particles, infection with every H5 virus significantly altered macrophage phagocytosis (average of phagocytosis levels of all H5 species; Figure 8A). Furthermore, there did not appear to be any differences in phagocytic levels between non-replication competent H5 viruses and the replication competent WSN strain (data not shown in subdivisions in 8A). Infection of human primary macrophages demonstrated similar results, showing that all H5 viruses (average of phagocytosis levels of all H5 species tested) significantly reduced phagocytosis levels compared to all H1N1 viruses (Figure 8B). As generally known, reducing macrophage phagocytosis significantly impairs the host's ability to mount an effective immune response since it reduces the ability of these cells to clear microbes. These data could provide a partial explanation of the high rate of secondary bacterial infections after influenza infection (Robinson et al. 2015). Future studies could focus specifically on the ability of macrophages to uptake and destroy *Streptococcus pneumoniae* bacteria as a potential mechanism to explain virally-associated pneumonia (Robinson et al. 2015).

CONCLUSION

Overall, these studies represent a significant advancement of knowledge with regards to the interactions of influenza viruses and macrophages. The fact that H5N1 viruses are able to escape lysosomal compartments and fully replicate unlike H1N1 species provides a previously unrecognized mechanism that may account for the increased disease severity and spread associated with H5N1 species (Cline et al. 2011). While all H5 viruses are able to escape lysosomal compartments and enter macrophage nuclei, only highly pathogenic H5 viruses are capable of new viral protein synthesis and progeny release, implying another mechanistic explanation for the severity of H5N1 infections. More studies in the future will be conducted to disseminate the molecular mechanism(s) of this post-transcriptional block. Furthermore, we demonstrated that the pH of fusion of the H5 HA accounts for the nuclear import and replicative ability of a highly pathogenic H5N1 strain. Future studies will be focused on disseminating which differences between replication competent and non-replication competent H5 HA proteins produce these phenotypes. While it did not seem that virally infected macrophages were significant producers of ROS species, we were able to demonstrate that all H5 viruses significantly impair macrophage phagocytosis. Decreased phagocytosis of new bacterial particles after viral infection could be a potent mechanism by which the host becomes susceptible to secondary bacterial infections, and future experiments will focus on delineating what impacts this could have in *in vivo* models. Ultimately, these studies help to explain the differences of influenza infection severity as related to differences in the ability of these viruses to replicate in macrophages. In the future, therapeutics or other clinical interventions could be aimed at disrupting the

replication cycle of these viruses in macrophages, such as at a macrophage specific lysosomal protein marker.

LIST OF FIGURES AND TABLES

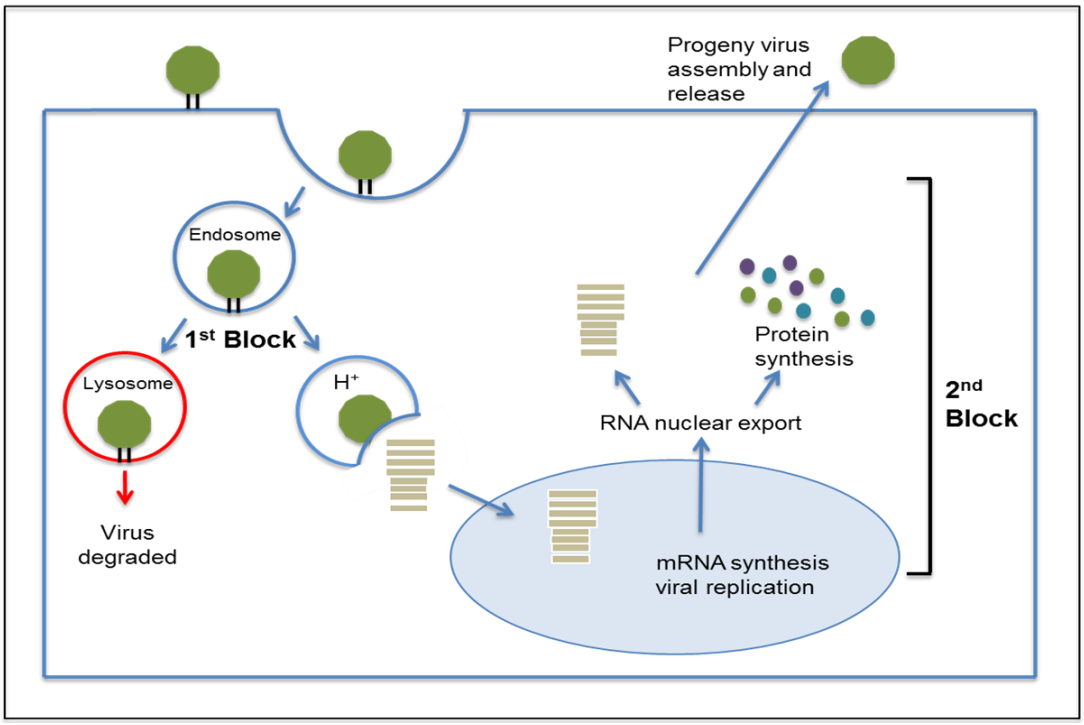

Figure 1: Model of influenza replication in macrophages. After receptor binding, influenza enters the cell via endocytosis. As the endosome acidifies, hemagglutinin (HA)-mediated fusion occurs. Hypothesized Block 1: Viruses with a more acid-stable HA do not reach their pH of fusion until the lysosome compartment, which contains activated proteases that degrade the virus. Viruses that do fuse and uncoat import their viral ribonucleoproteins (vRNPs) into the nucleus. After import, mRNA synthesis, viral replication, protein production, and progeny viral assembly and release of a subset of highly pathogenic H5N1 viruses occurs. Hypothesized Block 2: Other viruses encounter a second block post-nuclear entry.

Virus	Subtype	Receptor	Block in NP Nuclear Entry (1st Block)?	Nuclear NP	Replicate in RAW Cells?
A/CA/04/09	H1N1	2,6	Yes	-	No
A/New Caledonia/20/99	H1N1	2,6	Yes	-	
A/Tennessee/F1052/2010	H1N1	2,6	Yes	-	
A/Brisbane/10/07	H3N2	2,6	No	++	
A/Tennessee/F4008/2012	H3NY	2,6	Yes	-	
A/HK/68	H3N2	2,3/2,6	No	++	
A/Wuhan/359/95	H3N2	2,6	No	++	
A/Puerto Rico/8/1934	H1N1	2,3	Yes	-	No
A/WSN/1933	H1N1	2,6	No	++++	Yes
A/Swine/NE/2/92	H1N1	2,6	Yes	+	No
A/Swine/NC/0668/2011	H3N2	2,3	Yes	+	
A/Swine/TX/4119-2/1998	H3N2	2,6	No	+++	
A/GreyTeal/Australia/2/79*	H4N4	2,3	No	++	No
A/HK/486/97*	H5N1	2,3	No	ND	No
A/HK/213/03*	H5N1	2,3	No	ND	No
A/VN/1203/03*	H5N1	2,3	No	ND	Yes
A/HK/483/97*	H5N1	2,3	No	ND	Yes
A/Mallard/Alberta/85/76	H5N2	2,3	No	++++	No
A/Duck/Potsdam/2216-4/84	H5N6	2,3	No	++++	No
A/Turkey/WI/1/1968	H5N9	2,3	No	+++	No
A/Teal/HK/W312/97	H6N1	2,3	No	++++	No
A/Anhui/01/2013*	H7N9	2,3	ND	ND	No

Table 1: Panel of influenza viruses tested for replication in macrophages. MDCK cells and macrophages were infected with the human (black), swine (blue), avian (red), and other **(**green**),** viruses listed. Twenty-four hours post infection, virus titers in the cell supernatant were determined by $TCID_{50}$ analysis. $TCID_{50}$ calculated using the Reed-Muench method is used as a measure of the limiting dilution of a virus sample capable of infection. Alternatively, cells were fixed at 4 HPI and stained for viral nucleoprotein (NP). The percent nuclear NP was determined and standardized as the percent of MDCK cells: - <10%, + 10-25%, ++ 26-50%, +++ 51-75%, ++++ >75%. High scores in nuclear NP correlate with high rates of viral entry since NP is present within the initial viral envelope. Asterisks (*) indicate highly pathogenic H5N1 influenza viruses. Data are representative of at least two independent experiments for each virus. Several viruses' nuclear entry and percent nuclear NP are not determined (ND) as of yet.

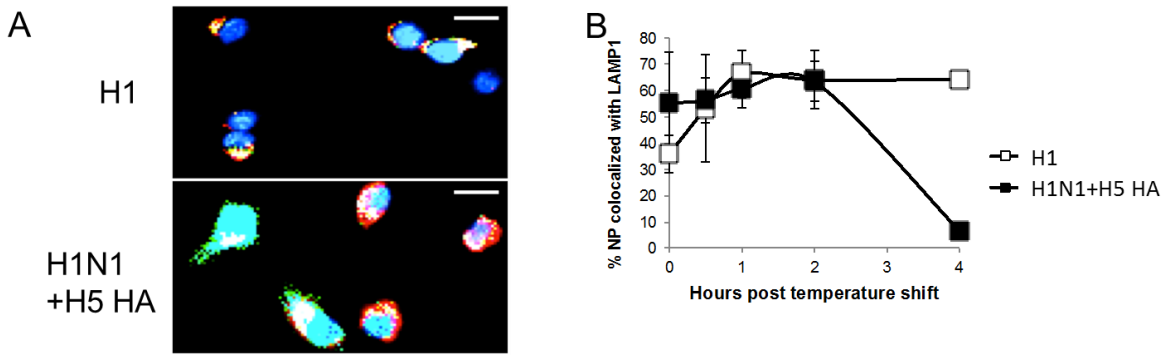

Figure 2: H1N1 viruses accumulate in lysosomes in macrophages. Raw264.7 macrophages were synchronously infected with an H1N1 (A/California/04/2009; H1) or an H1N1 containing the hemagglutinin of the H5N1 A/VN/1203/03 virus (H1N1+H5 HA). The multibasic cleavage site of the H5 HA was removed, allowing us to use the virus in an ABSL2+ facility versus ABSL3+. Cells were fixed at different time points and stained for nucleoprotein (NP) and the late endosome/lysosome marker LAMP1. Colocalization was determined by defining individual 3-dimensional NP surfaces using IMARIS© software, comparing the fluorescence intensity of the LAMP1 channel to the fluorescence intensity of cells that were only stained with the secondary antibody. As shown by immunofluorescent staining in Figure 1A and quantification of staining in 1B, H1N1+H5 HA virus NP particles were mainly located in the nucleus or the cytoplasm at 4 hours post-infection in macrophages. In contrast, H1 virus was significantly colocalized with LAMP1 in Raw264.7 macrophages more than H5 HA containing viruses (~95% LAMP1 colocalization H1 vs. ~2% of H1N1+H5 HA).

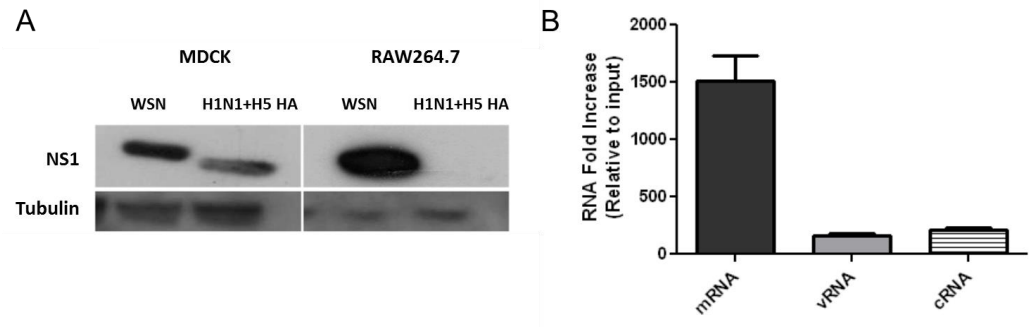

Figure 3: NS1 viral protein synthesis but not mRNA synthesis is inhibited in CA/09+VN/1203 dHA infected macrophages. (A) MDCK (left panels) or RAW264.7 (right panels) cells were infected with WSN (positive control for replication in both cell types) or an H1N1 (CA/09) containing the hemagglutinin of the H5N1 A/VN/1203/03 virus (H1N1+H5 HA). Cells were lysed at 24 HPI. Proteins were separated by SDS-PAGE under reducing conditions, transferred to nitrocellulose, and probed with anti-NS1 (top panels) or anti-tubulin (bottom panels) by Western blotting. NS1 protein, as an indicator of replication, was produced by both WSN and CA/09+VN/1203 dHA infection of MDCK cells. However, infection of Raw macrophages with H1N1+H5 HA failed to produce new NS1 viral protein compared to the replication competent positive control WSN virus. These results indicate non-replicating H5 viruses (H5 viruses that are not H5N1 subtype) seem to experience a block in replication prior to protein synthesis but after mRNA has been produced. Raw264.7 macrophages were infected with H1N1+H5 HA (MOI=5). (B) RNA levels were quantified in triplicate at 6 HPI by qRT-PCR and standardized to GAPDH. Levels are presented as fold increase relative to input (30 minutes post infection). Infection with H1N1+H5 HA rescued mRNA synthesis with vRNA and cRNA levels also comparable to macrophage infections with highly pathogenic H5N1 viruses (B; Cline et al. 2012). Error bars represent standard deviations.

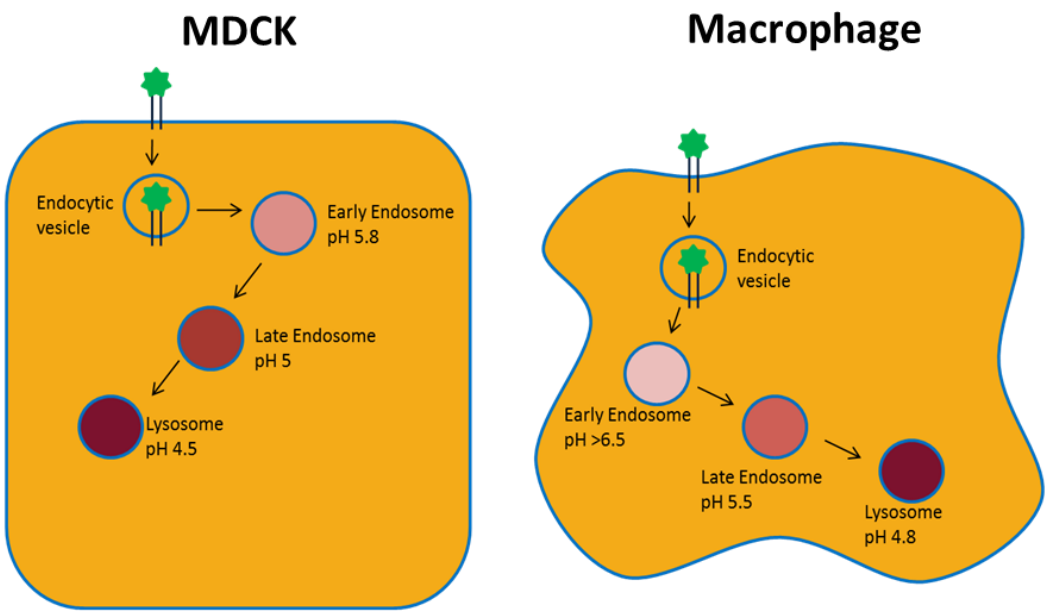

Figure 4: Model of varying cellular endosomal pH pathways utilized in viral entry. After viral attachment and entry, influenza viruses traffic towards the nucleus. As the intracellular pH changes due to the viral M2 ion pump gradually concentrating hydrogen protons within the compartment, the virion's HA coat protein becomes increasingly unstable until an optimal pH induces fusion of the viral HA and the endosomal membrane promoting release of viral nucleic acid. It has been shown by various studies that the overall endosomal pH pathway within macrophages (right) is higher than epithelial cells such as Madin-Darby Canine Kidney (MDCK; left) cells.

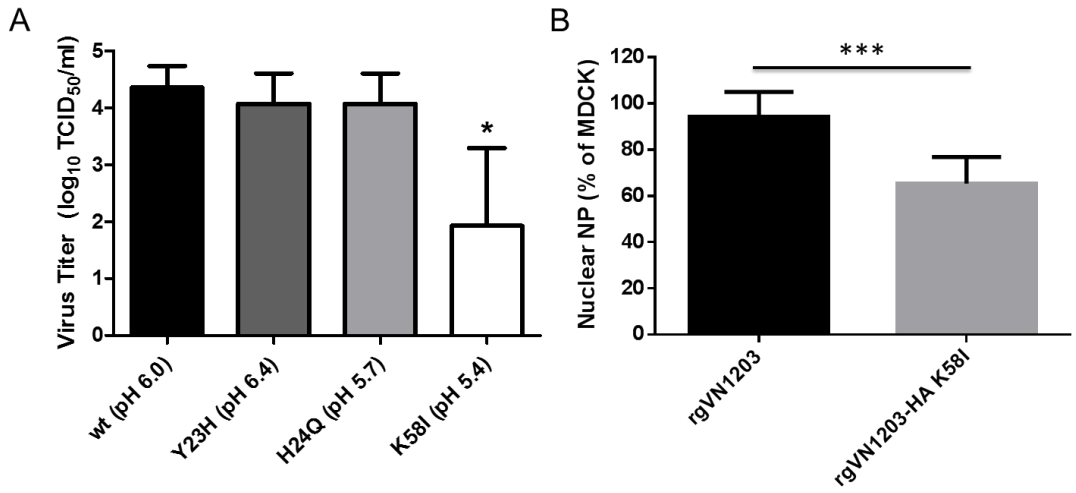

Figure 5: Destabilization of the H5 HA (more H1-like) reduces viral replication and nuclear entry in macrophages. (A) Raw264.7 macrophages were infected with wild-type (wt) VN/1203 (highly pathogenic H5N1 virus) or VN/1203-derived viruses containing the indicated mutants in their HA gene (MOI=0.01). At 24 HPI, virus titers in the cell supernatant were determined by $TCID_{50}$ analysis. The pH of HA fusion for each virus is indicated in parentheses. (B) Raw 264.7 macrophages and MDCK cells were infected with reverse genetics (rg) derived wild-type VN/1203 or a VN/1203 virus expressing the pH of fusion mutant HA K58I (MOI=0.01). At 24 HPI, viral NP colocalization was determined by immunofluorescent microscopy as described previously, and the percent of nuclear NP of each virus in Raw macrophages was quantified as the percent of Nuclear NP against its respective MDCK control infection (Nuclear NP % of MDCK). Since MDCK cells can be productively infected by virtually any influenza virus, the amount of virus in the nucleus of macrophages can be standardized and considered as a fraction of normal nuclear entry in MDCK epithelial cells. Error bars represent standard deviations, and asterisks represent statistical significance (*$p<0.05$, ***$p<0.001$).

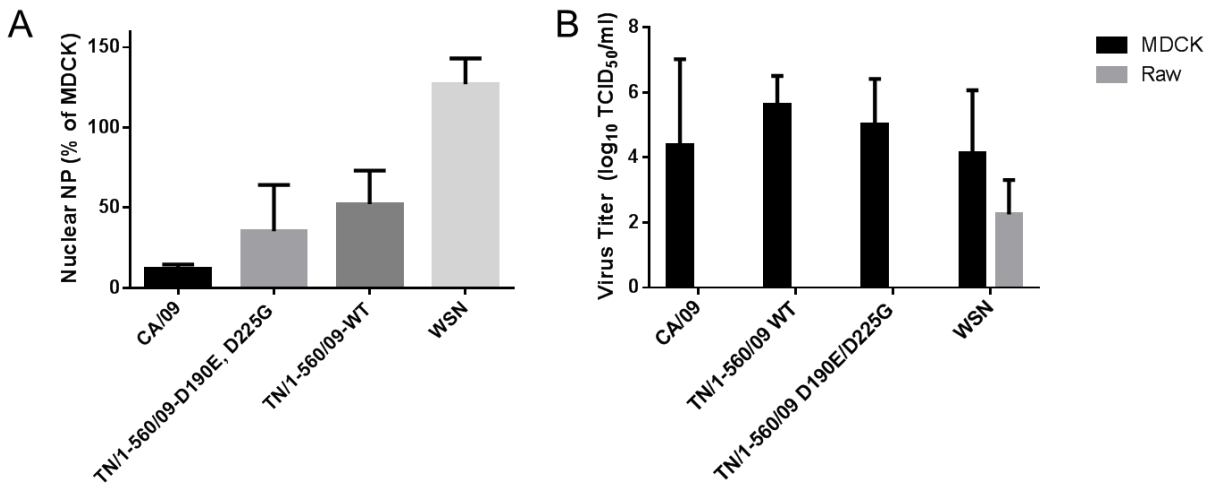

Figure 6: Virus recombination from α-2,6 to α-2,3 receptor binding does not rescue nuclear NP localization or replication in murine macrophages. Raw264.7 macrophages and MDCKs were infected with wild-type (WT) H1N1 virus (TN/1-560/09) or an H1N1 virus (TN/1-560/09) containing receptor mutations (D190E, D225G) in addition to infection with CA/09 (H1N1 that cannot replicate in macrophages) and WSN (atypical H1 virus capable of replication) as negative and positive controls for replication, respectively (A MOI=1; B MOI=0.01). (A) At 24 HPI, viral nucleoprotein (NP) colocalization was determined by immunofluorescent microscopy as described previously, and the percent of nuclear NP of each virus in Raw macrophages was quantified as the percent of Nuclear NP against its respective MDCK control infection (Nuclear NP % of MDCK). (B) At 24 HPI, virus titers in the cell supernatant were determined by $TCID_{50}$ analysis. Error bars represent standard deviations.

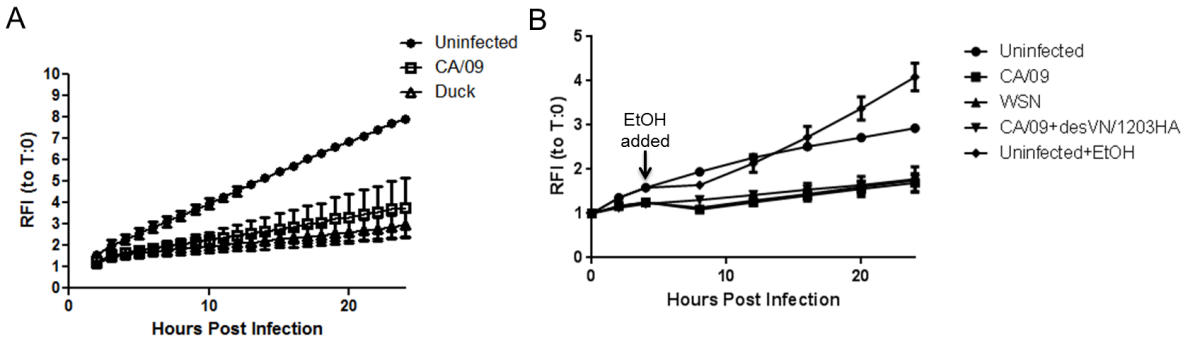

Figure 7: ROS species are not induced during by non-replication competent H5 viruses in macrophages. (A) Raw264.7 macrophages or (B) human primary macrophages were incubated for 30 minutes with DCF dye followed by two washes with PBS. Cells were infected with media or virus (MOI=5) at time zero, and fluorescence intensity was measured every half hour at an excitation wavelength of 485 nm and an emission wavelength of 520 nm on a fluorescence plate reader. After 1 hour, uninfected samples did or did not receive 10 mM ethanol, which acted as a scavenger control for ROS species. CA/09 (H1N1) served as a negative control for replication, while WSN (atypical H1 virus) served as a positive control for replication. Data are expressed as the relative fluorescence intensity (RFI) as standardized to the time zero read for that individual well. Error bars represent standard deviations.

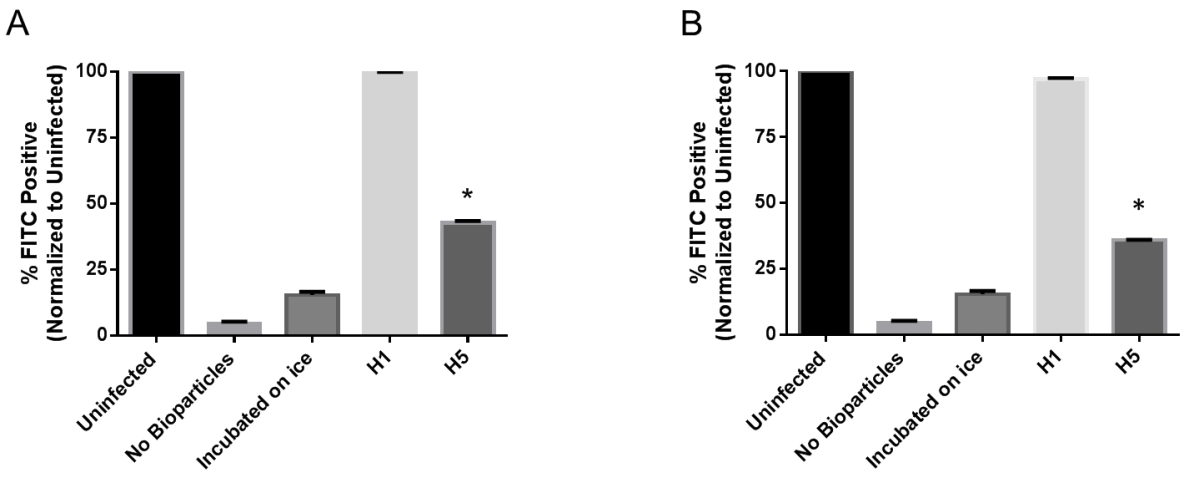

Figure 8: Macrophage phagocytosis is significantly reduced by infection with replication competent viruses. (A) Raw264.7 or (B) human primary macrophages were infected with media or virus (MOI=1) as previously described. At 24 HPI, cells were washed twice with PBS and incubated for 30 minutes with pHrodo-*Staphylococcus aureus* bioparticles opsonized with rabbit IgG at 37°C. Uninfected controls consisted of macrophages incubated with bioparticles, macrophages incubated with bioparticles while on ice to inhibit phagocytosis, and macrophages incubated with media only (no bioparticles). Cells were placed on ice, removed by scraping, and fixed in 2% paraformaldehyde for 10 minutes FITC-positive cells were measured by flow cytometry (Canto-II or FacsCalibur-III). Error bars represent standard deviations, and asterisks indicate statistical significance relative to the uninfected control (*$p<0.05$).

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