Journal of Virology

Virology | Full-Length Text

Neuraminidase-dependent entry of influenza A virus is determined by hemagglutinin receptor-binding specificity

Louisa E. Wallace,[1] Erik de Vries,[1] Frank J. M. van Kuppeveld,[1] Cornelis A. M. de Haan[1]

AUTHOR AFFILIATION See affiliation list on p. 14.

ABSTRACT Influenza A viruses (IAVs) contain sialoglycan-binding hemagglutinin (HA) and sialoglycan-cleaving neuraminidase (NA) proteins, the concerted action of which is needed for escape from decoy receptors and for virion motility ultimately resulting in infection of epithelial cells of the respiratory tract. The importance of NA in egress of newly assembled virions has been well established, whereas its role in entry has yet to be fully elucidated. In this study, we systematically analyzed the role of NA in viral entry in relation to HA receptor-binding preference, the receptor repertoire displayed on cells and the presence of mucus decoy receptors. Utilizing recombinant viruses that differ only in their HA-NA composition, it was observed that the dependence on NA activity for IAV entry largely depends on HA and not NA, with entry of α2–6 sialoglycan-binding viruses being inhibited more by NA inhibitor (oseltamivir carboxylate; OsC) than α2–3 sialoglycan-preferring viruses. In agreement with this, inhibition of virus entry by OsC could be modified by altering the sialoglycan receptor repertoire of cells. Entry inhibition by OsC correlated with the ability of mucus to inhibit infection, with the combination of the two having the largest effect. Our results indicate that the dependency of IAV on NA activity and, thus, virion motility for entry are determined by the receptor-binding properties of HA in combination with the receptor repertoire present on cells. This dependency is larger when fewer preferred receptors are displayed, which coincides with increased inhibition by mucus decoy receptors.

IMPORTANCE Influenza A viruses (IAVs) contain hemagglutinin (HA) proteins involved in sialoglycan receptor binding and neuraminidase (NA) proteins that cleave sialic acids. While the importance of the NA protein in virion egress is well established, its role in virus entry remains to be fully elucidated. NA activity is needed for the release of virions from mucus decoy receptors, but conflicting results have been reported on the importance of NA activity in virus entry in the absence of decoy receptors. We now show that inhibition of NA activity affects virus entry depending on the receptor-binding properties of HA and the receptor repertoire present on cells. Inhibition of entry by the presence of mucus correlated with the importance of NA activity for virus entry, with the strongest inhibition being observed when mucus and OsC were combined. These results shed light on the importance in virus entry of the NA protein, an important antiviral drug target.

KEYWORDS influenza A virus, hemagglutinin, neuraminidase, virus entry, mucus

Respiratory viruses including influenza A viruses (IAVs) target the epithelium of the respiratory tract. To infect the host, the virus must first penetrate a viscous gel-like mucus layer to reach the underlying susceptible epithelial cells. Mucus is a protective barrier comprising a myriad of biological components many of which may play a protective role against invading viruses including heavily glycosylated mucins abundant in terminal sialic acids (Sias), immune components such as antibodies and complement

Editor Anice C. Lowen, Emory University School of Medicine, Atlanta, Georgia, USA

Address correspondence to Cornelis A. M. de Haan, c.a.m.dehaan@uu.nl.

The authors declare no conflict of interest.

See the funding table on p. 14.

Received 21 April 2023
Accepted 9 August 2023
Published 27 September 2023

proteins, extracellular vesicles, and other peptides and proteins, some of which have antiviral properties (1). Sialoglycans also constitute the functional IAV receptors found on permissive airway cells to allow Sia-dependent entry. To avoid being trapped by binding to unproductive decoy Sia receptors present on mucins and cells, IAV particles carry both receptor-binding (hemagglutinin; HA) and receptor-destroying (neuraminidase; NA) glycoproteins in their envelope. A functional balance between receptor-binding and receptor-destroying activities is required to escape decoy receptors and to efficiently infect target cells (2, 3).

IAV HA protein is involved in two stages of viral infection—receptor binding and virus-cell fusion. Avian viruses generally prefer binding to α2–3-linked Sia receptors, while human viruses prefer binding to α2–6-linked Sias (4–6). Upon binding of a virion via HA to functional sialoglycan receptors on the target cell surface, the virus is internalized into an endosome through receptor-mediated endocytosis (7–12). Subsequently, HA mediates the fusion of the viral envelope with the endosomal membrane when endosomal pH becomes acidic allowing delivery of the viral genome into the cytoplasm of the target cell. On the other hand, NA hydrolyzes terminal Sias of sialoglycans, and it is generally accepted that NA promotes the release of progeny virus from an infected host cell by destroying receptors on the host cell and the virus itself (13, 14).

Besides its aforementioned traditional role in virion release, NA has also been proposed to be involved in the viral entry phase (15–19). The concerted action of HA and NA allows virion motility (18, 20) and escape from decoy receptors in respiratory mucus (15, 17, 21, 22) enabling the virus particle to traverse the mucus layer to reach the airway epithelium (15, 23). Also, in the absence of soluble decoy receptors, NA activity has been reported to be important for efficient cell entry (15, 17–19, 24, 25); however, inhibition of NA activity was shown not to appreciably affect virus entry in several other studies (10, 26–28). The use of different cell models and viruses makes it difficult to compare the outcomes of these different investigations.

In view of the disputed role of the NA protein in virus entry, we systematically analyzed the role of NA in viral entry in the absence and presence of mucus. To this end, we utilized a set of (isogenic) IAVs containing HAs with different sialoglycan-binding affinity profiles and cells with different sialoglycan receptor repertoires. The results indicate that a critical role for NA activity in IAV entry depends on the combination of HA receptor-binding specificity, cell surface sialoglycan repertoire, and the presence/absence of mucus. We propose a model in which IAV particles move on the cell surface in an NA-dependent fashion until they can bind to an appropriate set of receptors allowing endocytic uptake. With higher amounts of functional receptors on the cell surface, virions depend less on NA activity and are inhibited less by the presence of mucus.

MATERIALS AND METHODS

Cells

Madin-Darby canine kidney-II (MDCK-II) cells, human adenocarcinoma alveolar basal epithelial (A549) cells, and human cervical carcinoma cells (HeLa R19) were cultured in Dulbecco's modified Eagle's medium (Capricorn Scientific) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific), 100 IU/mL penicillin, and 100 µg/mL streptomycin at 37°C, 5% CO_2. MDCK-SIAT1 cells were obtained from Mikhail Matrosovich (29) and maintained in the aforementioned culture medium with the addition of 400 µg/mL G418 (Gibco).

Influenza A viruses

Recombinant influenza A viruses generated in the background of A/Puerto Rico/8/34 H1N1 by reverse genetics were kindly provided by Ron Fouchier, Erasmus MC,

Rotterdam, the Netherlands, (30) or Ben Peeters, Wageningen BioVeterinary Research, Lelystad, the Netherlands (31, 32). Recombinant viruses contain either only the gene encoding HA (7:1) or both HA and NA genes (6:2) of A/Bilthoven/1791/1976 (H3N2 6:2 and H3N1 7:1) or A/duck/Hunan/795/2002 (H5N1 6:2). The wild-type (WT) multi-basic cleavage site of the H5 is modified to encode a low-pathogenic cleavage site (31, 33). Stocks of the recombinant and WT viruses A/Netherlands/602/09 (H1N1pdm09), A/Puerto Rico/8/34/Mount Sinai (H1N1 PR8), and A/Bilthoven/1791/1976 (H3N2) were propagated in MDCK-II cells in Opti-MEM (Thermo Fisher Scientific) containing 1 µg/mL TPCK-treated trypsin (Sigma-Aldrich) and stored at −80˚C until use. All experiments were performed under BSL-II conditions.

Infection assays

Cells were seeded in 96-well plates and, for luciferase read-outs, were then transfected at a 3:1 ratio of FuGENE 6 (Promega) to one of two pHH-GLuc plasmids expressing Gaussia luciferase (GLuc; 100 ng/well), under the control of either human (using HeLa R19 cells) or canine (using MDCK-II or MDCK-SIAT1 cells) pol I promoter (11, 34). At 24 or 48 h post-transfection, respectively, cells were infected with IAV at MOI 0.1–1 $TCID_{50}$ units (as determined on MDCK-II cells) per cell (unless indicated otherwise) in the absence of TPCK-treated trypsin resulting in a single round of replication, with or without 1 µM oseltamivir carboxylate (OsC). For immunofluorescence analysis, cells were fixed at 8 or 19 h post-infection (HPI). Care was taken so that multiplicities of infection (MOIs) used resulted in approximately similar numbers of infected cells in the absence of OsC. For a luciferase read-out, supernatant samples were collected at 16 HPI to assay for luciferase activity using the *Renilla* Luciferase Assay system (Promega) as per the manufacturer's instructions. Luminescence readings as a measure of viral replication were obtained using a GloMax Discover System GM3000 luminometer microplate reader (Promega).

For mucus-infection inhibition assays, viruses were diluted in Opti-MEM with or without 2 µM OsC to 1.2×10^6 $TCID_{50}$/mL. A mixed healthy donor nasal mucus sample (Epithelix Sàrl) was serially diluted twofold in Opti-MEM to give mucus concentrations of 8%–0.5 %. For comparison with desialylated mucus, the mucus sample was first treated with *Arthrobacter ureafaciens* NA (AUNA; Roche) as per the manufacturer's protocol. Virus and mucus dilutions were mixed 1:1, resulting in the final concentrations of 6.0×10^5 $TCID_{50}$/mL virus, with or without 1 µM OsC, and 4%–0.25 % mucus. The mixes were left to incubate at room temperature for 20 min before addition to confluent 96-well plates of pHH-GLuc-transfected MDCK-II and MDCK-SIAT1 cells.

Immunofluorescence analysis

Cells were fixed with 4% formaldehyde for 30 min at room temperature, before permeabilization and blocking with 3% bovine serum albumin (BSA), 0.2% Triton X-100 in Dulbecco's phosphate-buffered saline (PBS; Capricorn Scientific), or blocking only with 3% BSA in PBS (for lectin staining) for 1 h at 4°C. Primary mouse monoclonal antibody HB65 (11) was utilized along with secondary Alexa Fluor 488-conjugated goat anti-mouse IgG to visualize IAV nucleoprotein (NP). Biotinylated lectins *Maackia amurensis* lectin I (MALI; recognizing α2–3Sia-Galβ1–4GlcNAc) and *Sambucus nigra* lectin (SNA; recognizing α2–6Sia-Galβ1–4GlcNAc) from VectorLabs and Alexa Fluor 568-conjugated streptavidin were used for glycotope immunofluorescence. Primary antibodies or lectins were diluted in blocking buffer, and cells were incubated in the dilutions overnight at 4°C. Cells were washed three times in PBS before incubating with secondary antibody and 4',6-diamidino-2-phenylindole (DAPI) dilutions in PBS for 1 h at room temperature. Cells were washed three times in PBS and imaged with an EVOS FL microscope (Invitrogen).

NA enzymatic activity analysis

NA activity of IAVs was assessed using the synthetic monovalent substrate 4-methylumbelliferyl-*N*-acetyl-α-D-neuraminic acid (4-MU-NANA; Sigma Aldrich) in a fluorometric assay as previously described (35) in the presence and absence of 1 µM OsC.

Biolayer interferometry

Biolayer interferometry (BLI) assays to assess real-time virus binding were performed using an Octet RED348 (ForteBio) as described previously (36). All experiments were performed using PBS containing calcium and magnesium (Capricorn Scientific) at 30°C with plates shaking at 1,000 rpm. Streptavidin biosensors (SA, Pall-FortBio) were loaded to saturation with biotinylated synthetic glycans 3'S(LN)$_2$ (α2–3-sialyl-*N*-acetyllactosamine-*N*-acetyllactosamine) or 6'S(LN)$_2$ (α2–6-sialyl-*N*-acetyllactosamine-*N*-acetyllactosamine) that were kindly provided by Geert-Jan Boons, synthesized at the Department of Chemical Biology and Drug Discovery, Utrecht University, Utrecht, the Netherlands, and used previously to analyze IAV-receptor binding by BLI (37). Virus binding was monitored by moving receptor-loaded sensors to wells containing virus, 1 mM trypsin-like protease inhibitor (Sigma Aldrich; T6552), and 10 µM OsC (Roche) to inhibit all NA activity (20). Virus binding was calculated from virus association curves up to 300 s, at which point the curves are linear, and plotted relative to the highest binding level between 3'S(LN)$_2$ and 6'S(LN)$_2$ per virus.

RESULTS

NA-dependency for virus entry

Conflicting results have previously been reported concerning the role of NA in virus entry (10, 15, 17–19, 24–28), prompting us to study the importance of NA in virus entry using a panel of IAVs consisting of a range of subtypes; recombinant H5N1 (6:2; HA and NA of stated virus subtype in the backbone of PR8), laboratory-adapted human-derived H1N1 PR8, pandemic human H1N1pdm09, and WT and recombinant 6:2 human H3N2 viruses. MDCK-II cells were infected in the absence or presence of the NA inhibitor OsC at a concentration of 1 µM that is expected to completely block NA activity. Infections were performed in the absence of trypsin, which is required to activate the HA proteins, resulting in a single round of replication (38) and in the number of infected cells—visualized by IAV NP-staining—being a measure of virus entry at 19 HPI (Fig. 1A). It was observed that the number of H5N1(6:2)- and PR8-infected cells was not affected by the presence of OsC (Fig. 1B). However, in the presence of OsC, the number of H1N1pdm09-infected cells was severely reduced as well as that for the H3N2 WT and (6:2) viruses, suggesting that these viruses have a greater dependency on NA activity for entry than the H5N1(6:2) and PR8 viruses. Similar results were obtained when PR8 and H3N2(6:2) viruses were used to infect A549 cells in that H3N2 was more inhibited by OsC than PR8 (Fig. 1C). To confirm that the inhibitory effect of OsC resulted from inhibition of virus entry, cells were also infected and fixed at 8 HPI (Fig. 1D). Similar, albeit smaller, virus-dependent effects of OsC on entry were observed. As the H5N1(6:2), PR8, and H3N2(6:2) viruses only differ in their HA and NA proteins, these results indicate that the observed difference in the number of infected cells does not result from the OsC preparation somehow inhibiting NP expression at a post-entry stage and rather reflects a differential NA-dependency for entry, which can be attributed to one or both of the glycoproteins.

IAV HA binding profiles

To confirm that the NA protein of the different viruses was completely inhibited by the concentration of OsC used in these experiments, IAV NA activity was assessed by MUNANA fluorometric assay in the absence and presence of 1 µM OsC using non-saturating amounts of virus (Fig. 2A). The results, indeed, show that the NA activity of all viruses

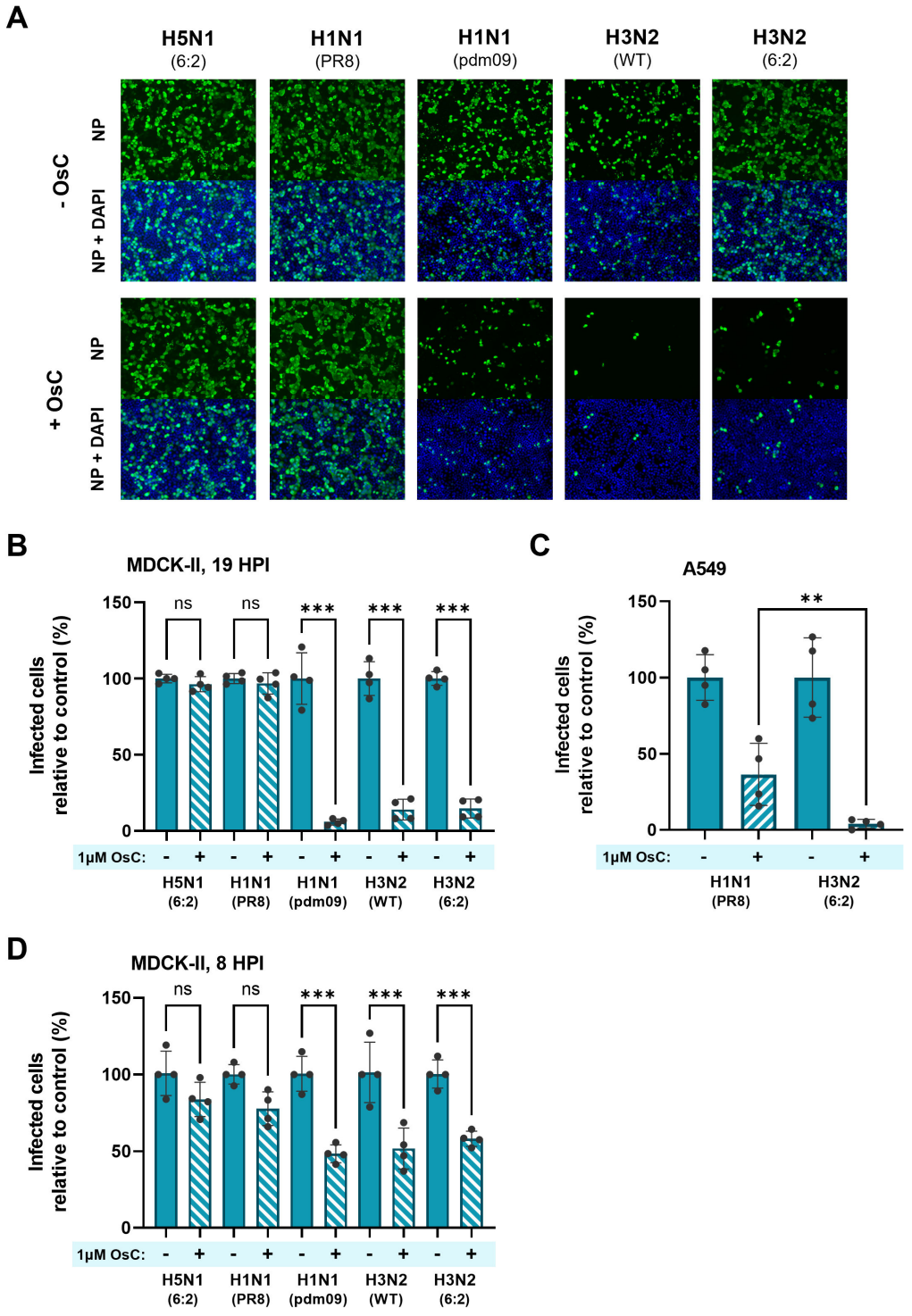

FIG 1 IAVs differ in their NA-dependence for entry. (A) Representative images of single-round IAV infections in MDCK-II cells with and without 1 µM OsC. At 19 HPI, cells were fixed and stained for IAV nucleoprotein (NP) and nuclei using DAPI. NP-positive and total cells were quantified and shown in (B). The experiment was repeated in A549 cells for H1N1(PR8) and H3N2(6:2) and quantified in (C). (D) Similar experiment as in (B) except cells were fixed at 8 HPI. Bars represent means ± standard error of two experiments each with two independent infections. Statistical significance (*P*) was calculated by one-way ANOVA using Tukey's multiple comparison test (*$P = < 0.05$; **$P = < 0.01$; ***$P = < 0.001$).

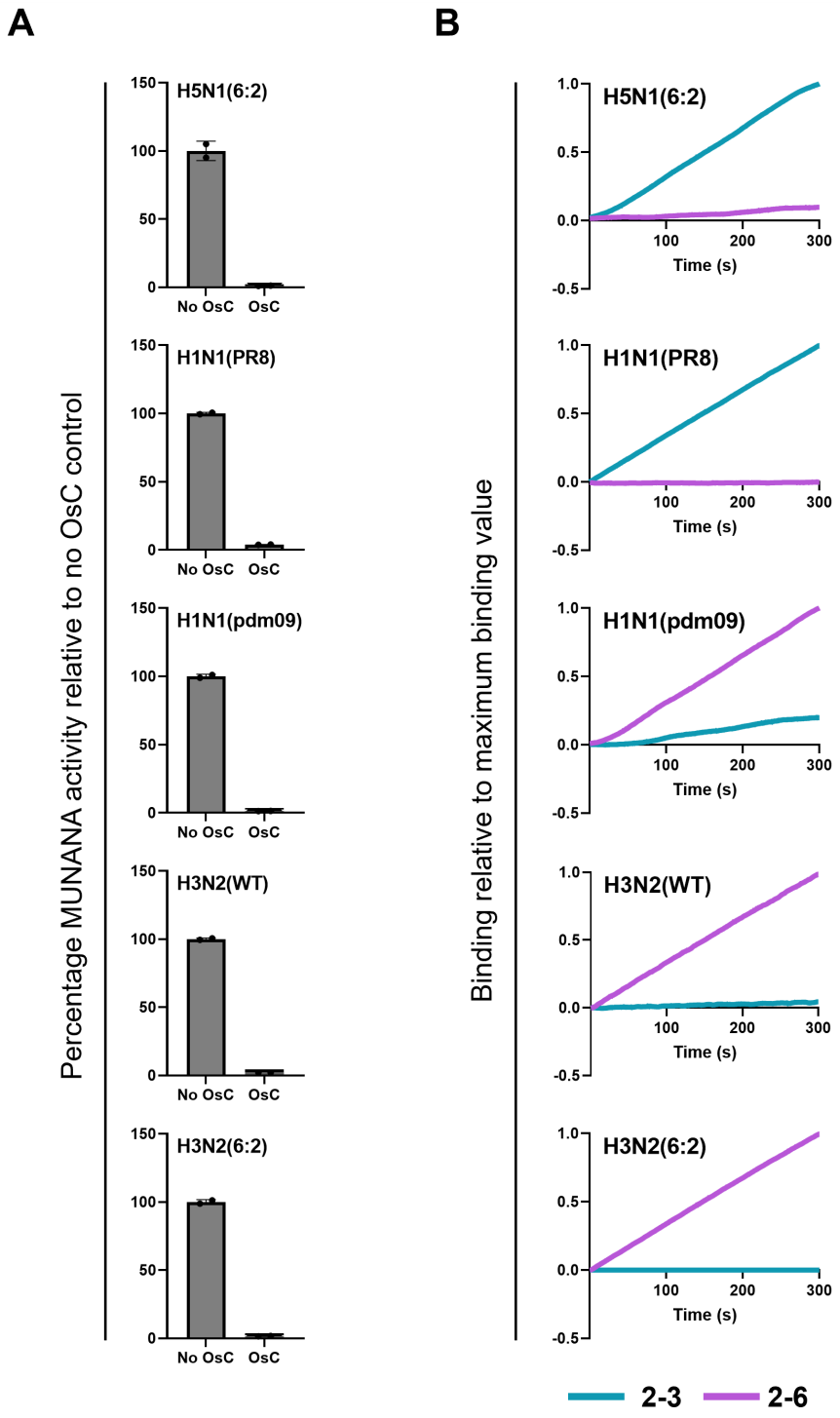

FIG 2 Characterization of IAV NA and HA activity. (A) NA proteins are efficiently inhibited by OsC. MUNANA assay using virus dilutions with highest NA activity, showing NA activity with and without 1 µM OsC relative to the no-OsC control. Bars represent means (n = 2) ± standard deviation of a representative experiment. (B) Relative binding preferences of IAVs to 3′S(LN)$_2$ (indicated by 2–3) and 6′S(LN)$_2$ (indicated by 2–6) synthetic glycans as determined by biolayer interferometry. Binding levels for each virus preparation for both glycans (loaded to saturation) were normalized to the highest binding level observed after 300 s.

was effectively inhibited to the same extent by 1 µM OsC, suggesting that the difference in NA-dependent entry can be attributed to HA rather than NA. Therefore, we next analyzed to what extent the IAVs displayed differing affinities to α2–3- and α2–6-linked Sias. To determine the binding profiles of our viruses, we analyzed virus binding to synthetic glycans 3′S(LN)$_2$ and 6′S(LN)$_2$ by BLI. The binding data indicated that H5N1(6:2) and PR8 preferably bind α2–3-linked sialoglycans, while H1N1pdm09, H3N2(6:2), and H3N2 WT prefer binding to α2–6-linked sialoglycans (Fig. 2B), in agreement with previously published results (20, 32, 39). These differential binding preferences correlated with the viruses' NA-dependency for entry into MDCK-II cells shown in Fig. 1, with the entry of the viruses that display preferential binding to α2–3-linked Sia—H5N1(6:2) and PR8—not being significantly affected with the addition of OsC, while the remaining viruses that preferably bind to α2–6-linked Sia are sensitive to the addition of OsC.

HA is responsible for OsC sensitivity

To further decipher which viral glycoprotein is responsible for the observed sensitivity to OsC in virus entry, MDCK-II cells were also infected with recombinant virus H3N1(7:1) which only contains the H3 protein of the H3N2 WT virus in the backbone of PR8. Again, based on NP-staining quantification, H3N1(7:1) virus entry was significantly more inhibited compared to PR8 in the presence of OsC, compared to their respective no-OsC controls (Fig. 3A). To confirm the results based on NP-staining of infected cells, single-round infections of MDCK-II cells (Fig. 3B) and HeLa R19 cells (Fig. 3C) were also quantified using a GLuc-reporter system. GLuc expression driven by IAV infection was severely affected by inhibition of NA activity for H3N1(7:1) but not for PR8 in the presence of OsC. Together, these results indicate that the differential OsC sensitivity and, thus, NA-(in)dependent entry observed for these viruses can be attributed to their HA protein.

Preferred HA-receptor abundance influences NA-dependency for entry

If the observed difference in NA-dependent entry, indeed, depends on the HA protein and particularly on the receptor-binding preference thereof, one would predict that it should be possible to manipulate the OsC sensitivity of virus entry by changing the receptor repertoire displayed on the cell surface. To this end, we utilized MDCK-II cells and MDCK-SIAT1 cells, the latter which overexpresses α2–6-sialyltransferase ST6Gal1 (29). To confirm the increased presence of α2–6-linked Sia on MDCK-SIAT1 cells, cells were fixed, blocked, and incubated with MAL I which binds to α2–3-linked Sia or SNA which preferentially binds to α2–6-linked Sia. The lectin stains show that α2–6 Sia glycotopes are much more abundant on MDCK-SIAT1 than on MDCK-II cells and α2–3 sialoglycans appear to be slightly more abundant on MDCK-II than MDCK-SIAT1 cells (Fig. 4A) in agreement with previous reports (29, 40).

We then compared the difference in the OsC sensitivity of the viruses when infecting cells with differing α2–3 and α2–6 Sia abundances. Similar to Fig. 1A and B, IAV entry was studied in MDCK-II and MDCK-SIAT1 cells with and without OsC and at 19 HPI, cells were fixed and stained for NP and nuclei in order to determine the percentage of infected cells relative to controls. The amount of virus in the inoculum was adjusted so that approximately similar numbers of cells were infected in the absence of OsC. Comparing the infected cells in the presence of OsC to the no-OsC controls per cell line, it was observed that there was significantly higher H5N1(6:2) and PR8 entry in MDCK-II cells than in MDCK-SIAT1 cells in the presence of OsC (Fig. 4B). All viruses that had preferential binding for α2–6 Sia over α2–3 Sia infected a higher percentage of MDCK-SIAT1 cells compared to MDCK-II cells in the presence of OsC, which was significant for H1N1pdm09 and H3N1(7:1). Similar findings were observed when infection was quantified using the GLuc system and measuring luciferase activity at 16 HPI. Inhibition of NA activity by OsC did not significantly affect the entry of H5N1(6:2) and PR8 in both cell lines. In contrast, infection with H3N1(7:1) in MDCK-SIAT1 cells is significantly less affected by the inhibition of NA activity than in MDCK-II cells (Fig. 4C).

Increasing MOI overcomes NA-dependency for entry

Until now, all infections were performed with an MOI ranging from 0.1 to 1 $TCID_{50}$ units per cell. To see whether a high MOI would allow the virus to overcome the NA-dependency for entry, HeLa R19 cells transfected with pHH-GLuc were infected at 24 h post-transfection with different dilutions of H3N1(7:1) and PR8 (resulting in MOIs ranging from 0.05 to 50), with and without 1 µM OsC. For both viruses, the NA-dependency for entry decreased with higher amounts of virus, with H3N1(7:1) being more NA-dependent for entry than PR8 as observed previously (Fig. 5). At the lowest virus dilution, increased entry of PR8 is observed with the inhibition of NA compared to the no-OsC control, perhaps a result of the inhibition of cleavage of functional Sia receptors, in accordance with previous findings (41–43).

Entry inhibition by mucus reflects that of OsC

IAVs must be able to penetrate the gel-like mucus barrier overlaying the epithelium of the respiratory tract to reach the underlying cellular receptors, for which it is known that NA activity plays a crucial role (15, 17, 21, 23). To assess how mucus affects IAV NA-dependent and -independent entry, MDCK-II and MDCK-SIAT1 cells transfected with pHH-GLuc were infected at 48 h post-transfection with 6.0×10^5 $TCID_{50}$/mL virus incubated with respiratory mucus dilutions with and without 1 µM OsC. Entry of H5N1(6:2) was not affected much by the presence of OsC regardless of the cell line used (Fig. 6A) in accordance with previous results (Fig. 1B and 4C). The presence of mucus resulted in a relatively small but consistent non-significant decrease in entry (Fig. 6A). There was no significant difference in the effect of NA-inhibition on entry of PR8 between the MDCK-II and MDCK-SIAT1 cells (Fig. 6B) in agreement with previous results (Fig. 4C). The addition of increasing concentrations of mucus had a small effect in the absence of OsC, which was significant on MDCK-II cells but not MDCK-SIAT1 cells. Inhibition was more pronounced in the presence of both mucus and OsC on both cell lines. NA-independent H3N1(7:1) entry was lower in MDCK-II cells compared to MDCK-SIAT1 cells in the absence of mucus (Fig. 6C) in agreement with previous results (Fig. 4B and C). In MDCK-II cells, concentration-dependent inhibition by mucus was observed in the absence of OsC, which was much more pronounced in the presence of OsC. In α2–6 Sia-abundant MDCK-SIAT1 cells, increasing levels of entry inhibition were observed with increasing mucus concentrations but in the presence of OsC only. To compare the

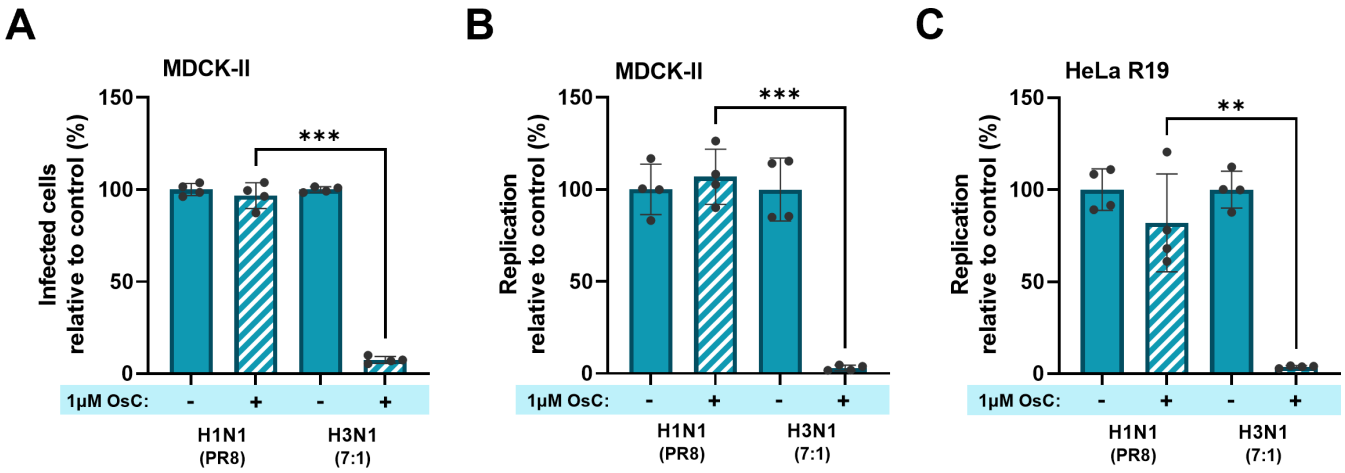

FIG 3 Differences in IAV OsC sensitivity largely depends on HA and not NA. (A) Single-round IAV infections in MDCK-II cells with and without 1 µM OsC. At 19 HPI, cells were stained for IAV nucleoprotien (NP) and nuclei using DAPI and quantified. (B,C) Single-round IAV infections in (B) MDCK-II and (C) HeLa R19 cells transfected with the Gaussia luciferase reporter plasmid, with and without 1 µM OsC. Cells were infected at 24 h (HeLa R19) or 48 h post-transfection (MDCK-II) and luminescence read at 16 HPI. Bars represent means ± standard error of two experiments each with two independent infections. Statistical significance (*P*) was calculated using Student's *t*-test (*$P = < 0.05$; **$P = < 0.01$; ***$P = < 0.001$).

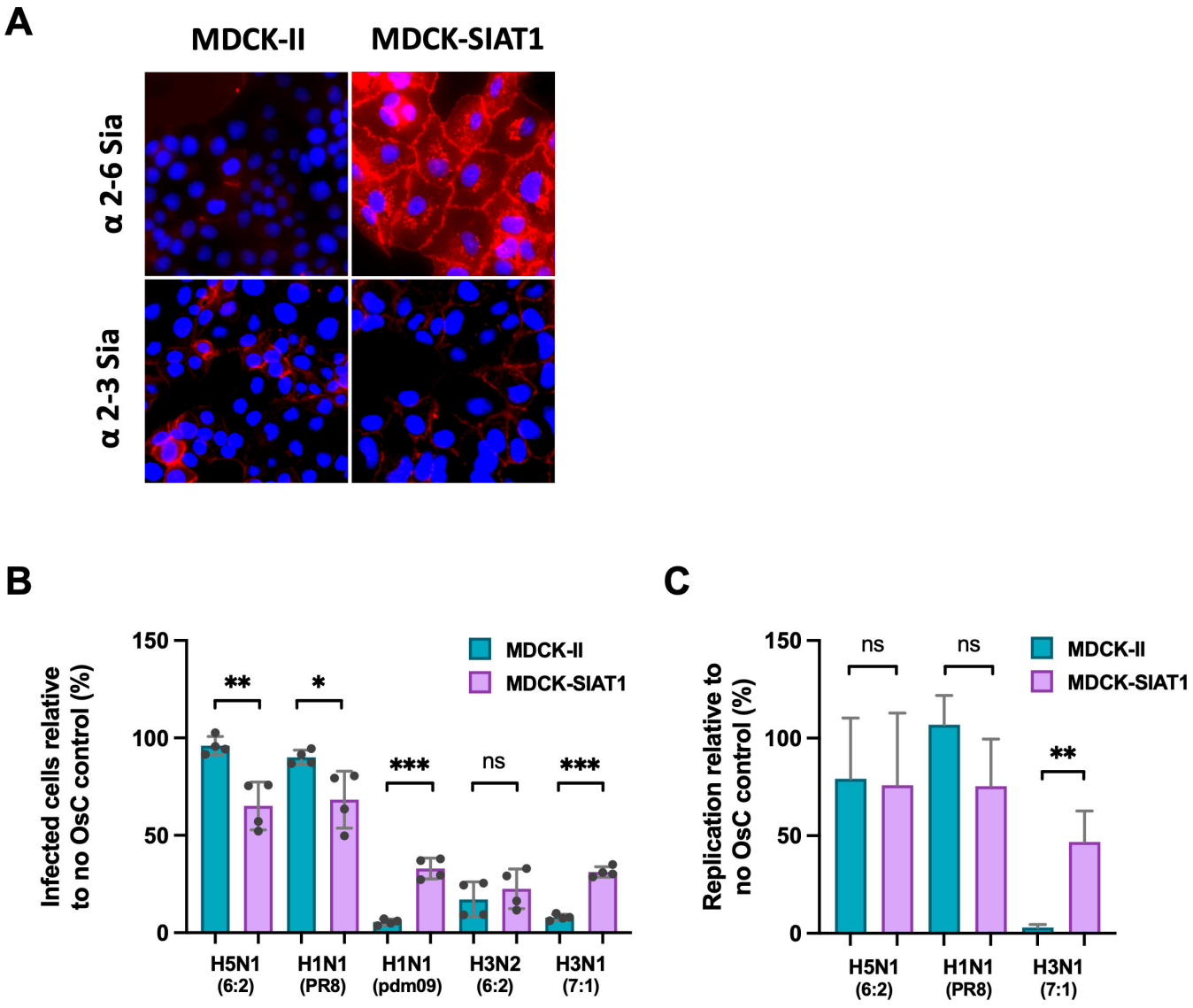

FIG 4 Cell lines with different receptor repertoires affect IAV OsC sensitivity. (A) Lectin stains showing the comparison of α2–6 Sia (SNA) and α2–3 Sia (MALI) abundances between MDCK-II and MDCK-SIAT1 cells. (B) Single-round IAV infections in MDCK-II and MDCK-SIAT1 cells with and without 1 µM OsC. At 19 HPI, cells were stained for IAV nucleoprotein (NP) and nuclei using DAPI. NP-positive and total cells were quantified. (C) Single-round IAV infections in MDCK-II and MDCK-SIAT1 cells transfected with the Gaussia luciferase reporter plasmid, with and without 1 µM OsC. Cells were infected at 48 h post-transfection and luminescence read at 16 HPI. Two experiments each with two independent infections were performed. Bars represent means ± standard error. Statistical significance (*P*) was calculated by one-way ANOVA using Tukey's multiple comparison test. (*$P = < 0.05$; **$P = < 0.01$; ***$P = < 0.001$).

different virus-cell combinations, we determined the IC_{50} values of mucus (vol/vol %) in the absence of OsC (Fig. 6D), which confirmed that inhibition by mucus was limited for H5N1(6:2) and PR8 viruses and not significantly different between the cell lines used. In contrast, infection with H3N1(7:1) virus was much more inhibited by mucus on MDCK-II cells compared to the MDCK-SIAT1 cells and to infection with the other viruses on MDCK-II cells. Treatment of mucus with AUNA to remove Sia moieties from mucus decoy receptors reduced virus infection inhibition by mucus significantly ($P < 0.001$; Fig. 6E) in agreement with the hypothesis that sialylated mucus decoy receptors are inhibitory. The remaining inhibitory effect of AUNA-treated mucus may be attributed to incomplete desialylation of mucus constituents and/or the presence of other inhibitory molecules. In conclusion, the inhibitory capacity of mucus for the different virus-cell line combinations reflects the inhibition observed by OsC.

DISCUSSION

The role of NA in IAV egress has been well established and acknowledged for decades although the full extent of—and the factors that influence—its role in entry has yet to be fully elucidated. In this study, we systematically analyzed the role of NA in viral entry in relation to HA receptor-binding preference. Taken together, our results depict that a virus' dependency on NA for entry is determined by a complicated interplay between the virus' HA receptor-binding preference, the receptor repertoire present on cells, and the presence/absence of mucus decoy receptors. Based on these results and previous studies [reviewed in references (1, 3)], we propose a model for this interplay (Fig. 7). When the activities of HA and NA are balanced to the receptor repertoire present, this will allow efficient virion motility through the mucus layer and escape from decoy receptors while enabling the binding of virions to the cell surface. In the absence of NA activity, virus entry may be inhibited even in the absence of mucus decoy receptors, particularly for α2–6 Sia-specific viruses on cells limited in the corresponding Sia receptors. This suggests that virion motility on the cell surface is required for these viruses to find the appropriate set of functional receptors, binding to which will enable the virus to signal its own endocytic uptake. Mucus by itself may already inhibit virus entry, particularly for α2–6 Sia-specific viruses on cells limited in the corresponding Sia receptors probably by being a more efficient competitor when available receptors are scarce. The greatest inhibition of virus entry is observed when the NA inhibitor is combined with mucus decoy receptors as in the presence of the NA inhibitor, self-elution of the virus from mucus decoy-receptors is inhibited.

NA is important for the release of virus progeny from infected cells (13) and decoy receptors (15) and for virion motility on receptor-coated surfaces and cells (18, 20). In view of the vast abundance of sialoglycans *in vivo* (44), virions are presumably always in a receptor-bound state via multiple HA-receptor interactions (3). The low

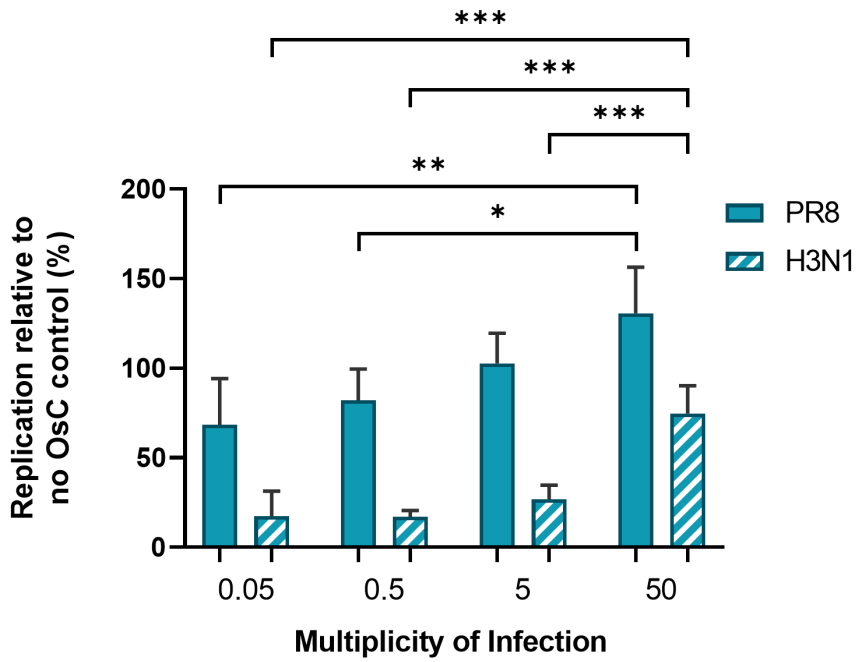

FIG 5 NA-dependency for IAV entry is overcome by increasing MOI. Single-round H1N1(PR8) and H3N1(7:1) infections at different MOIs in HeLa R19 cells transfected with the Gaussia luciferase reporter plasmid, with and without 1 µM OsC. Cells were infected at 24 h post-transfection and luminescence read at 16 HPI. Bars represent means ± standard error of two experiments each with two independent infections. Statistical significance (*P*) was calculated by one-way ANOVA using Tukey's multiple comparison test. (*$P = < 0.05$; **$P = < 0.01$; ***$P = < 0.001$).

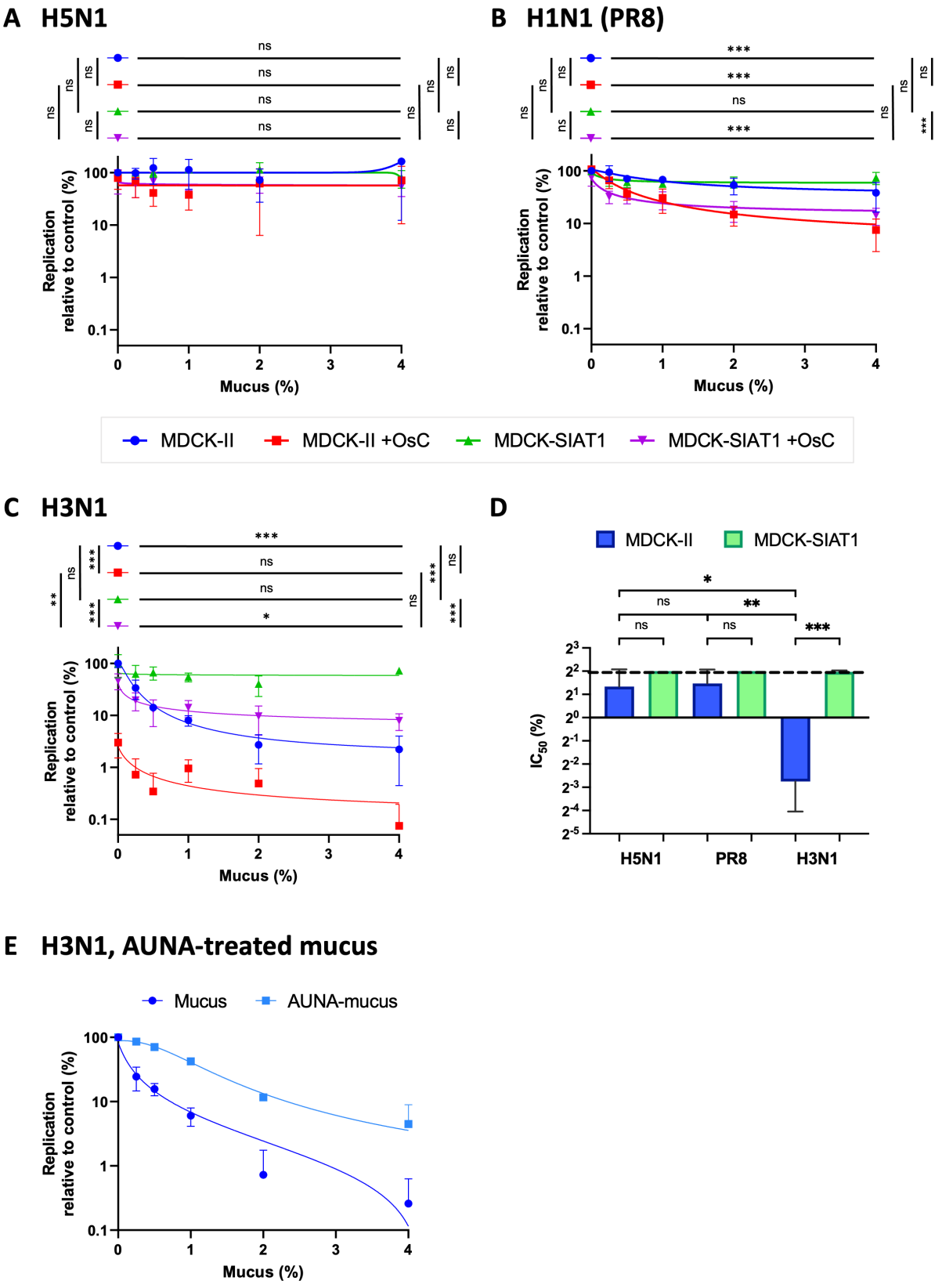

FIG 6 Mucus inhibition of IAV entry is receptor repertoire- and virus-dependent and reflects OsC sensitivity. Single-round (A) H5N1(6:2), (B) H1N1(PR8), and (C) H3N1(7:1) infections at MOI 1 in MDCK-II and MDCK-SIAT1 cells transfected with the Gaussia luciferase reporter plasmid, in the presence of 0%–4% mucus, with and without 1 µM OsC. Cells were infected at 48 h post-transfection and luminescence read at 16 HPI. Results were normalized to control infections in

(Continued on next page)

FIG 6 (Continued)

the absence of both mucus and OsC. Means ± standard error of two experiments each with two independent infections are shown. (D) Non-linear regression analysis using GraphPad Prism software was used to determine the IC_{50} values of mucus (in vol/vol %) for each virus-cell combination in the absence of OsC. (E) Single-round H3N1(7:1) infections at MOI 1 in MDCK-II cells transfected with the Gaussia luciferase reporter plasmid, comparing inhibition between the presence of 0%–4% mucus and *Arthrobacter ureafaciens* NA (AUNA)-treated mucus. Means ± standard error of two experiments each with two independent infections are shown. Statistical significance (*P*) was calculated by one-way ANOVA using Tukey's multiple comparison test (*$P = < 0.05$; **$P = < 0.01$; ***$P = < 0.001$).

affinity of these interactions results in a dynamic-binding mode and access of NA to these previously bound receptors. The combination of multivalent low-affinity receptor interactions together with receptor-destroying activity results in virion motility similar to a burnt bridge molecular motor (18, 45, 46). Thus, the catalytic activity of NA reduces the local density of sialoglycans, resulting in virion motility toward higher receptor densities, while essentially no motility is observed in the absence of NA activity.

Our results indicate that NA activity and, hence, virion motility are required for efficient IAV entry, the extent of which is dependent on the HA receptor-binding

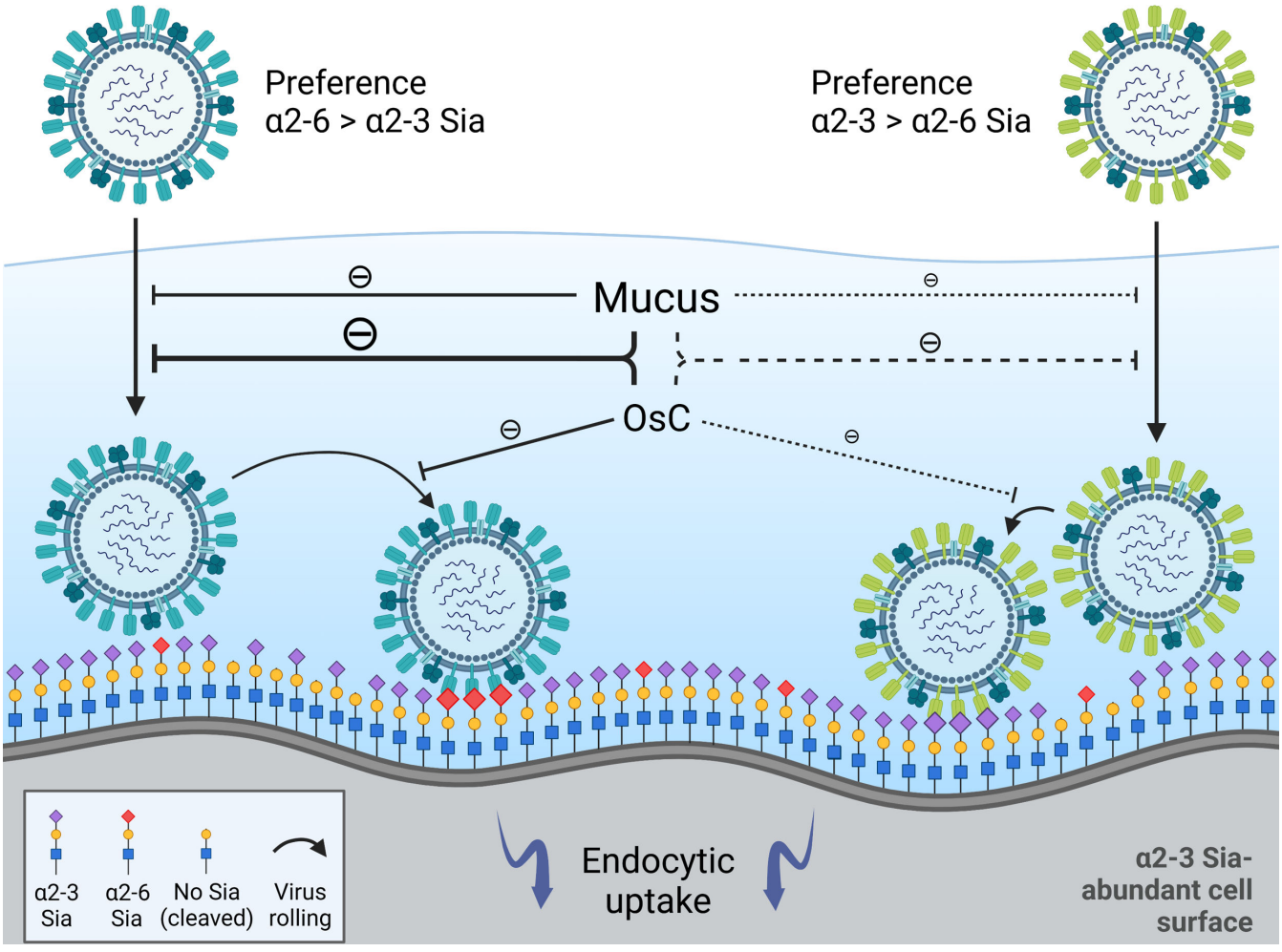

FIG 7 Model for NA-dependent IAV entry. Inhibition of virus entry in MDCK-II cells by NA inhibitor OsC is more pronounced for viruses preferring binding to less-abundantly present α2–6-linked sialoglycans compared to viruses preferentially binding to α2–3-linked sialoglycans. Inhibition by decoy receptor-rich mucus correlates with sensitivity toward OsC and is increased in the presence of both mucus and OsC. Increased preferred-receptor abundance results in reduced inhibition of virus entry by decoy receptors and by OsC. The latter implies that NA-dependent virion mobility (virus rolling) is required more for efficient entry when fewer preferred receptors are present. Presumably mobility of IAV particles allows increased interaction with functional receptors, which is required for endocytic uptake. Inhibition of virus entry is generally more pronounced when NA inhibitor is combined with mucus, hindering self-elution of virus particles from mucus decoy receptors. Figure was generated using BioRender.

preference and abundance of preferred receptors present (Fig. 7). These findings are consistent with previous studies that have also shown that NA is required for viral entry on cell lines (15–19, 24, 25) and may provide an explanation for the apparent lack of effect of NA inhibition on virus entry reported by others (10, 26–28). We conclude that virion motility plays a larger role in virus entry when limited numbers of preferred sialoglycan receptors are present on the cell surface. The need for virion motility can be overcome by increasing preferred receptor abundance, by using an HA with altered receptor-binding specificity, or by increasing the MOI. Furthermore, we hypothesize that the smaller inhibitory effect of OsC after 8 HPI compared to 19 HPI (Fig. 1B and 1D) can be attributed to asynchronous infection. Prolonged infection of some viruses was more efficient in the absence of OsC, when viruses are motile. Presumably virion motility is less important for entry when the probability of finding an appropriate set of functional receptors is increased, binding to which will result in endocytic uptake and fusion. In agreement herewith, NA inhibitors do not suppress the binding of virus particles to receptors (19, 20) but rather inhibit fusion (18).

Here, we show that inhibition of virus entry by mucus derived from primary differentiated epithelial cell cultures corresponds with that of OsC. Somewhat surprisingly, in view of the presumed high abundance of α2–3 sialoglycans on mucins (1, 47), viruses with an HA-receptor-binding preference to α2–3 Sia (PR8 and H5N1[6:2]) were less inhibited by mucus (and by OsC) compared to a virus with a α2–6 Sia-binding preference (H3N1[7:1]) (Fig. 6 and 7). The limited inhibition of PR8 and H5N1(6:2) can be explained by the limited competition of mucus components with receptor binding of these viruses and/or by these viruses containing an NA that efficiently cleaves sialoglycans in mucus to which these viruses bind. When NA activity was inhibited in the presence of mucus, a slightly greater but still limited inhibition was observed, indicating that mucus components acted as decoy receptors to a limited extent under these conditions. The absence of infection inhibition by OsC is not linked to the presence of a second sialic acid binding site in NA as such a site is present in H5N1(6:2) but not in PR8. In contrast, H3N1(7:1) virus preferring binding to α2–6 sialoglycans was severely inhibited by the presence of mucus unless the number of α2–6 sialoglycans was increased on the cell surface. The combination of mucus and OsC resulted in strong inhibition also on MDCK-SIAT1 cells. Due to the complexity of mucus, it is not yet known which component—or more likely multiple components—is responsible for entry inhibition. Mucus is not only abundant in *O*-glycan-rich mucins MUC5AC and MUC5B but also in *N*-glycan-rich exosomes, both of which have been suggested to inhibit viruses due to their high-Sia content (17, 48). To what extent each component may be contributing to IAV entry inhibition individually remains largely unexplored. Interestingly, it was previously shown using a similar H3N2 virus as used here, that the α2–6 sialoglycan-rich exosome fraction of mucus inhibited virus infection in MDCK cells (48).

Presumably, IAVs must evolve their HA-NA balance within conflicting binding interests to penetrate and escape respiratory mucus while still being able to bind to (functional) receptors on cells. The complexity is further increased by recent observations that low-affinity receptors may contribute to the binding of IAVs to surfaces displaying a heterogenous receptor repertoire (32). The HA-NA balance, which may be regarded as a host tropism determinant, will be finely tuned to the host species glycome, i.e. the combined abundances of functional and decoy sialoglycans present. As different species have differing sialoglycomes (1), re-tuning of this balance will be needed for viruses to successfully breach the species barrier.

ACKNOWLEDGMENTS

This work is funded by OrganoVIR (Grant 812673) in the European Union's Horizon 2020 program.

AUTHOR AFFILIATION

[1]Section of Virology, Division of Infectious Diseases and Immunology, Department of Biomolecular Health Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

AUTHOR ORCIDs

Louisa E. Wallace http://orcid.org/0000-0002-6210-8645
Erik de Vries http://orcid.org/0000-0003-0763-8202
Frank J. M. van Kuppeveld http://orcid.org/0000-0001-5800-749X
Cornelis A. M. de Haan http://orcid.org/0000-0002-4459-9874

FUNDING

Funder	Grant(s)	Author(s)
EC | Horizon 2020 Framework Programme (H2020)	OrganoVIR (Grant 812673)	Erik de Vries Frank J. M. van Kuppeveld Cornelis A. M. de Haan

AUTHOR CONTRIBUTIONS

Louisa E. Wallace, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review and editing | Erik de Vries, Conceptualization, Writing – review and editing | Frank J. M. van Kuppeveld, Funding acquisition, Supervision, Writing – review and editing | Cornelis A. M. de Haan, Conceptualization, Formal analysis, Funding acquisition, Supervision, Writing – review and editing

REFERENCES

1. Wallace LE, Liu M, van Kuppeveld FJM, de Vries E, de Haan CAM. 2021. Respiratory mucus as a virus-host range determinant. Trends Microbiol 29:983–992. https://doi.org/10.1016/j.tim.2021.03.014
2. Gaymard A, Le Briand N, Frobert E, Lina B, Escuret V. 2016. Functional balance between neuraminidase and haemagglutinin in influenza viruses. Clin Microbiol Infect 22:975–983. https://doi.org/10.1016/j.cmi.2016.07.007
3. de Vries E, Du W, Guo H, de Haan CAM. 2020. Influenza A virus hemagglutinin–neuraminidase–receptor balance: preserving virus motility. Trends Microbiol 28:57–67. https://doi.org/10.1016/j.tim.2019.08.010
4. Connor RJ, Kawaoka Y, Webster RG, Paulson JC. 1994. Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. Virology 205:17–23. https://doi.org/10.1006/viro.1994.1615
5. Rogers GN, D'Souza BL. 1989. Receptor binding properties of human and animal H1 influenza virus isolates. Virology 173:317–322. https://doi.org/10.1016/0042-6822(89)90249-3
6. Rogers GN, Paulson JC. 1983. Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin. Virology 127:361–373. https://doi.org/10.1016/0042-6822(83)90150-2
7. Yoshimura A, Kuroda K, Kawasaki K, Yamashina S, Maeda T, Ohnishi S-I. 1982. Infectious cell entry mechanism of influenza virus. J Virol 43:284–293. https://doi.org/10.1128/JVI.43.1.284-293.1982
8. Patterson S, Oxford JS, Dourmashkin RR. 1979. Studies on the mechanism of influenza virus entry into cells. J Gen Virol 43:223–229. https://doi.org/10.1099/0022-1317-43-1-223
9. Matlin KS, Reggio H, Helenius A, Simons K. 1981. Infectious entry pathway of influenza virus in a canine kidney cell line. J Cell Biol 91:601–613. https://doi.org/10.1083/jcb.91.3.601
10. Rust MJ, Lakadamyali M, Zhang F, Zhuang X. 2004. Assembly of endocytic machinery around individual influenza viruses during viral entry. 6. Nat Struct Mol Biol 11:567–573. https://doi.org/10.1038/nsmb769
11. de Vries E, Tscherne DM, Wienholts MJ, Cobos-Jiménez V, Scholte F, García-Sastre A, Rottier PJM, de Haan CAM, Pekosz A. 2011. Dissection of the influenza A virus endocytic routes reveals macropinocytosis as an alternative entry pathway. PLoS Pathog 7:e1001329. https://doi.org/10.1371/journal.ppat.1001329
12. Sempere Borau M, Stertz S. 2021. Entry of influenza A virus into host cells - recent progress and remaining challenges. Curr Opin Virol 48:23–29. https://doi.org/10.1016/j.coviro.2021.03.001
13. Palese P, Tobita K, Ueda M, Compans RW. 1974. Characterization of temperature sensitive influenza virus mutants defective in neuraminidase. Virology 61:397–410. https://doi.org/10.1016/0042--6822(74)90276-1
14. McAuley JL, Gilbertson BP, Trifkovic S, Brown LE, McKimm-Breschkin JL. 2019. Influenza virus neuraminidase structure and functions. Front Microbiol 10:39. https://doi.org/10.3389/fmicb.2019.00039
15. Matrosovich MN, Matrosovich TY, Gray T, Roberts NA, Klenk H-D. 2004. Neuraminidase is important for the initiation of influenza virus infection in human airway epithelium. J Virol 78:12665–12667. https://doi.org/10.1128/JVI.78.22.12665-12667.2004
16. Byrd-Leotis L, Cummings RD, Steinhauer DA. 2017. The interplay between the host receptor and influenza virus hemagglutinin and neuraminidase. Int J Mol Sci 18:1541. https://doi.org/10.3390/ijms18071541
17. Cohen M, Zhang X-Q, Senaati HP, Chen H-W, Varki NM, Schooley RT, Gagneux P. 2013. Influenza A penetrates host mucus by cleaving sialic acids with neuraminidase. Virol J 10:321. https://doi.org/10.1186/1743-422X-10-321
18. Sakai T, Nishimura SI, Naito T, Saito M. 2017. Influenza A virus hemagglutinin and neuraminidase act as novel motile machinery. Sci Rep 7:45043. https://doi.org/10.1038/srep45043
19. Ohuchi M, Asaoka N, Sakai T, Ohuchi R. 2006. Roles of neuraminidase in the initial stage of influenza virus infection. Microbes Infect. 8:1287–1293. https://doi.org/10.1016/j.micinf.2005.12.008

20. Guo H, Rabouw H, Slomp A, Dai M, van der Vegt F, van Lent JWM, McBride R, Paulson JC, de Groot RJ, van Kuppeveld FJM, de Vries E, de Haan CAM, Lowen AC. 2018. Kinetic analysis of the influenza a virus HA/NA balance reveals contribution of NA to virus-receptor binding and NA-dependent rolling on receptor-containing surfaces. PLoS Pathog 14:e1007233. https://doi.org/10.1371/journal.ppat.1007233
21. Yang X, Steukers L, Forier K, Xiong R, Braeckmans K, Van Reeth K, Nauwynck H, Bouvier NM. 2014. A beneficiary role for neuraminidase in influenza virus penetration through the respiratory mucus. PLoS ONE 9:e110026. https://doi.org/10.1371/journal.pone.0110026
22. McAuley JL, Corcilius L, Tan H-X, Payne RJ, McGuckin MA, Brown LE. 2017. The cell surface mucin MUC1 limits the severity of influenza A virus infection. Mucosal Immunol 10:1581–1593. https://doi.org/10.1038/mi.2017.16
23. Vahey MD, Fletcher DA. 2019. Influenza a virus surface proteins are organized to help penetrate host mucus. Elife 8:e43764. https://doi.org/10.7554/eLife.43764
24. Su B, Wurtzer S, Rameix-Welti M-A, Dwyer D, van der Werf S, Naffakh N, Clavel F, Labrosse B, Geraghty RJ. 2009. Enhancement of the influenza a hemagglutinin (HA)-mediated cell-cell fusion and virus entry by the viral neuraminidase (NA). PLoS One 4:e8495. https://doi.org/10.1371/journal.pone.0008495
25. Gulati S, Smith DF, Cummings RD, Couch RB, Griesemer SB, St George K, Webster RG, Air GM. 2013. Human H3N2 influenza viruses isolated from 1968 to 2012 show varying preference for receptor substructures with no apparent consequences for disease or spread. PLoS One 8:e66325. https://doi.org/10.1371/journal.pone.0066325
26. Bucher D, Palese P, Kilbourne ED. n.d. The biologically active proteins of influenza virus: neuraminidasethe influenza viruses and influenza. Academic Press, New York, NY.
27. Liu C, Eichelberger MC, Compans RW, Air GM. 1995. Influenza type a virus neuraminidase does not play a role in viral entry, replication, assembly, or budding. J Virol 69:1099–1106. https://doi.org/10.1128/JVI.69.2.1099-1106.1995
28. Hughes MT, Matrosovich M, Rodgers ME, McGregor M, Kawaoka Y. 2000. Influenza a viruses lacking sialidase activity can undergo multiple cycles of replication in cell culture, eggs, or mice. J Virol 74:5206–5212. https://doi.org/10.1128/jvi.74.11.5206-5212.2000
29. Matrosovich M, Matrosovich T, Carr J, Roberts NA, Klenk H-D. 2003. Overexpression of the α-2,6-sialyltransferase in MDCK cells increases influenza virus sensitivity to neuraminidase inhibitors. J Virol 77:8418–8425. https://doi.org/10.1128/jvi.77.15.8418-8425.2003
30. Koel BF, Burke DF, Bestebroer TM, van der Vliet S, Zondag GCM, Vervaet G, Skepner E, Lewis NS, Spronken MIJ, Russell CA, Eropkin MY, Hurt AC, Barr IG, de Jong JC, Rimmelzwaan GF, Osterhaus ADME, Fouchier RAM, Smith DJ. 2013. Substitutions near the receptor binding site determine major antigenic change during influenza virus evolution. Science 342:976–979. https://doi.org/10.1126/science.1244730
31. Du W, Wolfert MA, Peeters B, van Kuppeveld FJM, Boons G-J, de Vries E, de Haan CAM. 2020. Mutation of the second sialic acid-binding site of influenza a virus neuraminidase drives compensatory mutations in hemagglutinin. PLoS Pathog 16:e1008816. https://doi.org/10.1371/journal.ppat.1008816
32. Liu M, Huang LZX, Smits AA, Büll C, Narimatsu Y, van Kuppeveld FJM, Clausen H, de Haan CAM, de Vries E. 2022. Human-type sialic acid receptors contribute to avian influenza a virus binding and entry by hetero-multivalent interactions. Nat Commun 13:4054. https://doi.org/10.1038/s41467-022-31840-0
33. Peeters B, Reemers S, Dortmans J, de Vries E, de Jong M, van de Zande S, Rottier PJM, de Haan CAM. 2017. Genetic versus antigenic differences among highly pathogenic H5N1 avian influenza a viruses: consequences for vaccine strain selection. Virology 503:83–93. https://doi.org/10.1016/j.virol.2017.01.012
34. Lutz A, Dyall J, Olivo PD, Pekosz A. 2005. Virus-inducible reporter genes as a tool for detecting and quantifying influenza a virus replication. J Virol Methods 126:13–20. https://doi.org/10.1016/j.jviromet.2005.01.016
35. Dai M, Guo H, Dortmans JCFM, Dekkers J, Nordholm J, Daniels R, van Kuppeveld FJM, de Vries E, de Haan CAM. 2016. Identification of residues that affect oligomerization and/or enzymatic activity of influenza virus H5N1 neuraminidase proteins. J Virol 90:9457–9470. https://doi.org/10.1128/JVI.01346-16
36. de Vries E, Guo H, Du W, Liu M, van Kuppeveld FJM, de Haan CAM. 2022. Quantification of receptor association, dissociation, and NA-dependent motility of influenza A particles by biolayer interferometry, p 123–140. In Suzuki Y (ed), Glycovirology: methods and protocols. Springer US, New York, NY. https://doi.org/10.1007/978-1-0716-2635-1
37. Du W, Guo H, Nijman VS, Doedt J, van der Vries E, van der Lee J, Li Z, Boons G-J, van Kuppeveld FJM, de Vries E, Matrosovich M, de Haan CAM, Krammer F. 2019. The 2nd sialic acid-binding site of influenza a virus neuraminidase is an important determinant of the hemagglutinin-neuraminidase-receptor balance. PLoS Pathog 15:e1007860. https://doi.org/10.1371/journal.ppat.1007860
38. Klenk HD, Rott R, Orlich M, Blödorn J. 1975. Activation of influenza a viruses by trypsin treatment. Virology 68:426–439. https://doi.org/10.1016/0042-6822(75)90284-6
39. de Vries RP, de Vries E, Martínez-Romero C, McBride R, van Kuppeveld FJ, Rottier PJM, García-Sastre A, Paulson JC, de Haan CAM. 2013. Evolution of the hemagglutinin protein of the new pandemic H1N1 influenza virus: maintaining optimal receptor binding by compensatory substitutions. J Virol 87:13868–13877. https://doi.org/10.1128/JVI.01955-13
40. Lin S-C, Kappes MA, Chen M-C, Lin C-C, Wang TT, Krammer F. 2017. Distinct susceptibility and applicability of MDCK derivatives for influenza virus research. PLoS One 12:e0172299. https://doi.org/10.1371/journal.pone.0172299
41. de Vries E, de Vries RP, Wienholts MJ, Floris CE, Jacobs M-S, van den Heuvel A, Rottier PJM, de Haan CAM. 2012. Influenza a virus entry into cells lacking sialylated N-glycans. Proc Natl Acad Sci U S A 109:7457–7462. https://doi.org/10.1073/pnas.1200987109
42. Baigent SJ, McCauley JW. 2001. Glycosylation of haemagglutinin and stalk-length of neuraminidase combine to regulate the growth of avian influenza viruses in tissue culture. Virus Res. 79:177–185. https://doi.org/10.1016/s0168-1702(01)00272-6
43. Byrd-Leotis L, Jia N, Matsumoto Y, Lu D, Kawaoka Y, Steinhauer DA, Cummings RD. 2022. Sialylated and sulfated N-glycans in MDCK and engineered MDCK cells for influenza virus studies. 1. Sci Rep 12:12757. https://doi.org/10.1038/s41598-022-16605-5
44. Lewis AL, Chen X, Schnaar RL, Varki A. 2022. Chapter 15, Sialic acids and other Nonulosonic acids, In Varki A, Cummings RD, Esko JD, Stanley P, Hart GW, Aebi M, Mohnen D, Kinoshita T, Packer NH, Prestegard JH, Schnaar RL, Seeberger PH (ed), Essentials of Glycobiology, 4th ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
45. Ziebert F, Kulić IM. 2021. How influenza's spike motor works. Phys Rev Lett 126:218101. https://doi.org/10.1103/PhysRevLett.126.218101
46. Blanchard AT. 2021. Burnt bridge ratchet motor force scales linearly with polyvalency: a computational study. Soft Matter 17:6056–6062. https://doi.org/10.1039/d1sm00676b
47. Xia B, Royall JA, Damera G, Sachdev GP, Cummings RD. 2005. Altered O-glycosylation and sulfation of airway mucins associated with cystic fibrosis. Glycobiology 15:747–775. https://doi.org/10.1093/glycob/cwi061
48. Kesimer M, Scull M, Brighton B, DeMaria G, Burns K, O'Neal W, Pickles RJ, Sheehan JK. 2009. Characterization of exosome-like vesicles released from human tracheobronchial ciliated epithelium: a possible role in innate defense. FASEB J 23:1858–1868. https://doi.org/10.1096/fj.08-119131