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Influenza D virus utilizes both 9-*O*-acetylated *N*-acetylneuraminic and 9-*O*-acetylated *N*-glycolylneuraminic acids as functional entry receptors

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ABSTRACT Influenza D virus (IDV) utilizes bovines as a primary reservoir with periodical spillover to other hosts. We have previously demonstrated that IDV binds both 9-*O*-acetylated *N*-acetylneuraminic acid (Neu5,9Ac$_2$) and 9-*O*-acetylated *N*-glycolylneuraminic acid (Neu5Gc9Ac). Bovines produce both Neu5,9Ac$_2$ and Neu5Gc9Ac, while humans are genetically unable to synthesize Neu5Gc9Ac. 9-*O*-Acetylation of sialic acids is catalyzed by CASD1 via a covalent acetyl-enzyme intermediate. To characterize the role of Neu5,9Ac$_2$ and Neu5Gc9Ac in IDV infection and determine which form of 9-*O*-acetylated sialic acids drives IDV entry, we took advantage of a CASD1 knockout (KO) MDCK cell line and carried out feeding experiments using synthetic 9-*O*-acetyl sialic acids in combination with the single-round and multi-round IDV infection assays. The data from our studies show that (i) CASD1 KO cells are resistant to IDV infection and lack of IDV binding to the cell surface is responsible for the failure of IDV replication; (ii) feeding CASD1 KO cells with Neu5,9Ac$_2$ or Neu5Gc9Ac resulted in a dose-dependent rescue of IDV infectivity; and (iii) diverse IDVs replicated robustly in CASD1 KO cells fed with either Neu5,9Ac$_2$ or Neu5Gc9Ac at a level similar to that in wild-type cells with a functional CASD1. These data demonstrate that IDV can utilize Neu5,9Ac$_2$- or non-human Neu5Gc9Ac-containing glycan receptor for infection. Our findings provide evidence that IDV has acquired the ability to infect and transmit among agricultural animals that are enriched in Neu5Gc9Ac, in addition to posing a zoonotic risk to humans expressing only Neu5,9Ac$_2$.

IMPORTANCE Influenza D virus (IDV) has emerged as a multiple-species-infecting pathogen with bovines as a primary reservoir. Little is known about the functional receptor that drives IDV entry and promotes its cross-species spillover potential among different hosts. Here, we demonstrated that IDV binds exclusively to 9-*O*-acetylated *N*-acetylneuraminic acid (Neu5,9Ac$_2$) and non-human 9-*O*-acetylated *N*-glycolylneuraminic acid (Neu5Gc9Ac) and utilizes both for entry and infection. This ability in effective engagement of both 9-*O*-acetylated sialic acids as functional receptors for infection provides an evolutionary advantage to IDV for expanding its host range. This finding also indicates that IDV has the potential to emerge in humans because Neu5,9Ac$_2$ is ubiquitously expressed in human tissues, including lung. Thus, results of our study highlight a need for continued surveillance of IDV in humans, as well as for further investigation of its biology and cross-species transmission mechanism.

KEYWORDS influenza D, receptor, CASD1, Neu5,9Ac$_2$, Neu5Gc9Ac

The Orthomyxoviridae family consists of four influenza virus-related genera: alpha influenza (influenza A), beta influenza (influenza B), gamma influenza (influenza C),

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and delta influenza (influenza D) (1). Influenza A (IAV) and B (IBV) viruses contain an eight single-stranded, negative-sense RNA genome, while influenza C (ICV) and D (IDV) viruses have a seven-segmented RNA genome with negative polarity. Both IAV and IBV contain two major surface glycoproteins: the hemagglutinin (HA) and the neuraminidase (NA). In contrast, ICV and IDV have only one major surface glycoprotein, the hemagglutinin-esterase-fusion (HEF) protein, which communicates with the cell surface receptor and drives viral entry.

IDV as a new type of influenza virus is the latest addition to the Orthomyxoviridae family after its first identification from a diseased pig experiencing influenza-like symptoms. Subsequent studies identified the cattle as a primary reservoir of IDV (2, 3). IDV has a broad host range as antibodies against IDV have been found in sheep, goat, horses, and camels (4–8). More recently, serological studies in wildlife showed the IDV antibody prevalence in wild boar, deer, and hedgehogs (6, 9), indicating that the host range of IDV may be extremely broad. Although no human infection with IDV is reported clinically, antibodies against IDV were detected in people in Italy and U.S., and viral RNA were found in the nasal washes of cattle handlers (2, 10–12) and swine farmers (13). Furthermore, IDV genome was detected in the bio-aerosol sampling of respiratory viruses in the airport (14) and hospital emergency room setting (15). Experimentally challenged small animals like ferret, mice, and guinea pigs also demonstrated robust IDV replication, as well as its transmission to naïve animals (2, 16–18). All these laboratory animals have been used extensively as model systems to study human IAVs and their pathogenesis. Thus, the zoonotic spillover potential and transmission of IDV in humans cannot be excluded (19).

Influenza viruses are known to use cell surface sialic acid-containing glycoconjugates as entry receptors for infection (20). Sialic acids are 9-carbon α-keto aldonic acids that are usually present at the terminal positions of glycoproteins or glycolipids extending outward from the cell surface (21, 22). There is a great diversity in sialic acids due to different substitutions that can occur at carbon 4, 5, 7, 8, and 9 positions (23). *N*-acetyl neuraminic acid (Neu5Ac) is the predominant form of sialic acid species in humans (24). Agricultural animals, such as cattle and swine, have the ability to convert it to *N*-glycolylneuraminic acid (Neu5Gc) (25). Neu5Gc is synthesized via the addition of a hydroxyl group to the *N*-acetyl group of Neu5Ac in its sugar nucleotide form by cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH) encoded by *CMAH* gene (26). Neu5Gc differs from Neu5Ac by an additional oxygen atom in the *N*-acyl moiety. Humans lack a functional CMAH enzyme due to a naturally occurred 92-bp deletion in *CMAH* gene and thus cannot synthesize Neu5Gc (27). Addition of "*O*-acetyl" group at the C-4/7/8/9 position of sialic acids is a common modification widely occurring in nature (28). This modification takes place inside the Golgi system, which is believed to be catalyzed by an enzyme termed CAS1 domain containing 1 (CASD1) encoded by *CASD1* gene (29).

ICV, distantly related to IDV, is known to utilize 9-*O*-acetylated *N*-acetylneuraminic acid (Neu5,9Ac$_2$) for entry (30). Interestingly, ICV has a narrow tropism, while IDV has a broad host range (31). Using the glycan microarray-based approach, we have previously shown that IDV bound to both Neu5,9Ac$_2$- and Neu5Gc9Ac-terminated glycans (32). The binding specificity of IDV to the 9-*O*-acetylated sialic acid-containing glycans observed in the glycan array was further confirmed by the receptor analog-mediated inhibition in the traditional red blood cell (RBC) agglutination and cell-based replication assays (32). However, there is no absolute correlation between receptor binding and its functionality in driving viral entry. Binding assays with the HA proteins of influenza A viruses from avian, human, and equine showed that these influenza viruses were capable of binding both Neu5Ac and Neu5Gc; however, only equine H7N7 is known to exclusively utilize Neu5Gc as a receptor (33, 34). We have previously demonstrated that IDV has evolved with the ability to bind both Neu5,9Ac$_2$ and Neu5Gc9Ac. However, whether binding can mediate functional entry of IDV and whether both forms of 9-O-acetylated sialic acids

(Neu5,9Ac$_2$ and Neu5Gc9Ac) serve as functional entry receptors to promote IDV infection have remained unknown, which is the primary focus of this work.

In this study, we took advantage of a CASD1 (sialate *O*-acetyltransferase gene) knockout (KO) MDCK cell line and carried out feeding experiments with exogenous synthetic 9-*O*-acetyl sialic acids, in combination with the single-round and multi-round IDV infection assays, to delineate the role of Neu5,9Ac$_2$ and Neu5Gc9Ac in IDV entry and infection. The results of our experiments showed that IDV can utilize both Neu5,9Ac$_2$- and Neu5Gc9Ac-containing glycan receptors for infection. Our findings provide evidence that IDV has acquired the ability to infect and transmit among agricultural animals, such as bovines that are relatively enriched in Neu5Gc9Ac, in addition to posing a zoonotic risk to humans expressing only Neu5,9Ac$_2$.

MATERIALS AND METHODS

Cells, virus, and antibodies

HEK-293T (Human embryonic kidney) and MDCK-ATL cells were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco, Invitrogen, USA) supplemented with 10% (vol/vol) fetal bovine serum (FBS; Gibco, USA) and 100 U/mL penicillin-streptomycin (Life Technologies, Carlsbad, CA, USA). The CASD1-KO MDCK and its parental MDCK-NBL-II were cultured in DMEM supplemented with 5% (vol/vol) FBS and 100 U/mL penicillin-streptomycin. Cells were cultured at 37°C under 5% CO_2. Influenza D/swine/Oklahoma/1314/2011 (D/OK), D/bovine/Oklahoma/660/2013 (D/660), D/bovine/California/0363/2019 (D/CA), and D/swine/Kentucky/17TOSU1262/2017 (D/swine/Kentucky) were propagated in MDCK-ATL cells at 33°C. Anti-D/OK, anti-D/CA, and anti-D/660 rabbit polyclonal sera were generated in house following the standard immunization procedure with the inactivated viral cultures. Influenza C/Victoria/2/2012 was propagated in MDCK-ATL cells at 33°C. The Six-His tag mouse monoclonal antibody was obtained from Invitrogen (MA1-21315). Alexa Fluor 488 goat anti-mouse (A-11001) and Alexa fluor 488 goat anti-rabbit (A-11008) antibodies were purchased, respectively, from Invitrogen.

Development of single-round IDV reporter assays

Two single-round reporter assays were developed to study the receptor biology of IDV. The first assay is the green fluorescent protein (GFP)-based reporter assay. The GFP coding sequence was inserted between the 5′- and 3′-end sequences of the D/OK HEF viral cDNA flanked with human polymerase I promoter and polymerase I terminator sequences, respectively. Terminal 240 nucleotide sequences from both directions of the HEF segment were retained in the reporter plasmid for the efficient package of this reporter segment into virus particles, while the native initiation codon (ATG) in the HEF was changed into CTG. To produce IDV GFP reporter virus, seven plasmids (1 µg each) encoding each of seven D/OK genomic segments together with HEF-based GFP reporter plasmid were co-transfected in the cell monolayer comprising co-culture of HEK-293T and MDCK-ATL cells. The supernatant was collected 4–5 days post-transfection for further analysis.

A seven-plasmid Nano Luciferase (Nluc)-based single-round assay (D/OK-NLuc) was also developed for this study. Seventh segment of IDV genome produces two proteins NS1 and NS2 (also called NEP, nuclear export protein). The unspliced mRNA is translated into NS1 protein, while a splicing event produces NS2 protein. To generate a D/OK luciferase reporter virus, a single non-overlapping transcript expressing D/OK NS1 and NEP was separated by a porcine teschovirus 1 (PTV-1) self-cleaving element 2A. NLuc was linked to the C-terminal end of NS1 followed by the PTV-2A element (Fig. 4B). The reporter plasmid (D/OK-NLuc) replaced wild-type D/OK NS segment during the transfection. To produce IDV Nano-Luc reporter virus, seven plasmids (1 µg each) of D/OK (NS NLuc plasmid with the other six wild-type plasmids) were co-transfected into the

co-culture consisting of HEK-293T and MDCK-ATL cells. At 4–5 days post-transfection, viral supernatant was collected for further analysis.

Flow cytometry assay and quantification of D/OK-GFP reporter virus

D/OK-GFP reporter virus was quantified using a conventional flow cytometry assay, and its titer was expressed as fluorescence units per milliliter (FU/mL). Briefly, 10^6 MDCK cells were seeded in a six-well plate. Next day, 1 mL of 10-fold serial dilutions of D/OK-GFP reporter virus was used to inoculate cells. After 1 hour of incubation at 37°C, inoculum was discarded, and cells were washed three times with 1× PBS. Cells were then incubated for 24 hours at 37°C in 1 mL of DMEM media containing 1 µg/mL TPCK-treated trypsin (Thermo Fisher: 20233). After 24 hours post-infection (hpi), cells were washed three times with 1× PBS followed by the digestion with trypsin-EDTA (Thermo Fisher: 25200056). Cells were pelleted down, washed once in 1× PBS, and resuspended in 2% paraformaldehyde. The percentage of GFP-positive cells was calculated using BD FACSymphony A3 cell analyzer (BD Biosciences) and the associated software. For further analysis, 10,000 cells were gated. Reporter virus titer was calculated as: FU/mL = (initial cell count × % GFP positive)/dilution, where initial cell count is the number of cells seeded, dilution is the corresponding virus dilution used for flow cytometry analysis, and percentage of GFP-positive cells is obtained from flow cytometry. For all the assays involving the D/OK-GFP reporter virus, 10^6 FU of reporter virus was used.

9-*O*-Acetyl sialic acid feeding assay

Synthetic Neu5,9Ac_2 and Neu5Gc9Ac (35, 36) were purchased from ChemBind (custom synthesis) and were shown to have more than 95% purity by mass spectrometry and nuclear magnetic resonance spectroscopy. Approximately 10^5 CASD1 KO MDCK cells were seeded in a 96-well plate in DMEM complete media (5% FBS, 1% Pen-strep). Similar numbers of WT MDCK cells were seeded in separate wells. After overnight incubation, cells were washed once with 1× PBS and fed with various concentrations of Neu5,9Ac_2 or Neu5Gc9Ac, or a mixture containing both in equal concentrations in DMEM complete media (5% FBS, 1% Pen-strep). Cells were incubated for 3 days without changing media. On day 3, cells were washed three to four times with 1× PBS and infected with reporter or wild-type virus.

Cell-binding assay with the recombinant receptor-binding domain of D/OK HEF

D/OK HEF receptor-binding domain (RBD) with a histidine (His) tag at the C-terminus was expressed in *E. coli* BL21 using a T7 RNA polymerase-IPTG induction system. The expressed RBD protein was purified by a nickel column (Qiagen), and the protein concentration was measured by the Bio-Rad Protein Assay. To determine viral HEF RBD protein binding to the plasma membrane of various cells, CASD1-KO or WT MDCK cells were seeded in 96-well plates. CASD1-KO cells were exogenously supplemented with 200 µM of Neu5,9Ac_2 and/or Neu5Gc9Ac. On the third day, cells were washed and sequentially incubated with the recombinant His-tagged RBD (20 µg/mL) and the His-tag-specific mouse antibody (5 µg/mL) at room temperature. After three washes with 1× PBS, cells were incubated with Alexa-Fluor 488-labeled anti-mouse antibody (1:300 dilution; Abcam) for 30 min at room temperature. The nuclei were stained with 4′,6-diamidino-2- phenylindole (DAPI) for 5 min. The samples were imaged on a Nikon Ti eclipse fluorescence microscope, and the images were merged using the Image J software.

Single-round reporter virus infection assay

After 3 days of exogenous receptor feeding, cells were washed three times with 1× PBS. D/OK-GFP reporter virus was inoculated at 10^6 FU. After 1 hour of incubation at 37°C, cells were washed three times with 1× PBS and maintained in DMEM containing

1 µg/mL TPCK-treated trypsin. After 24 hours of incubation at 37°C, D/OK-GFP-infected cells were processed for flow cytometry analysis, as described above. For D/OK-NLuc reporter infection assay, cells were infected at the multiplicity of infection (MOI) of 0.1. Receptor-fed CASD1-KO cells infected with D/OK-NLuc were assessed for the luciferase activity using the Nano-Glo Luciferase Assay Kit (Promega). After 24 hpi, virus-infected cells in white culture plates and assay reagents were equilibrated at room temperature prior to the assay. Freshly prepared assay reagents were mixed with equal volume of cell culture media. Nluc was measured in a Synergy HT BioTek plate reader. Each individual experiment assayed samples in triplicate. The D/OK-GFP reporter assay was repeated nine times ($n = 9$), while the D/OK-NLuc reporter assay was conducted six times ($n = 6$).

Multi-round replication assay

CASD1-KO cells fed with 200 µM Neu5,9Ac$_2$ and/or Neu5Gc9Ac were incubated for 3 days. Treated cells were washed and infected with 1 MOI of D/OK, D/660, D/CA, and D/swine/Kentucky. The 50% tissue culture infection dose per milliliter ($TCID_{50}$/mL) of the inoculum was $10^{6.33}$, $10^{6.5}$, 10^{6}, and $10^{6.83}$, respectively, for D/OK, D/660, DD/CA, and D/swine/Kentucky. Final infection volume was adjusted with DMEM to make the equal amount of infectious dose across different virus strains (1.0 MOI). After 1 hour of incubation at 33°C, cells were washed and maintained in 100 µL of DMEM containing 1 µg/mL TPCK trypsin at 33°C. The 100-µL media were collected every day and replaced with fresh DMEM containing 1 µg/mL TPCK trypsin during the 5-day experiment. Three independent experiments were conducted with each sample analyzed in triplicate. The inoculum was titrated using the traditional 50% tissue culture infection dose ($TCID_{50}$) assay. Briefly, 1.5×10^4 MDCK ATL cells were seeded in 96-well plates. Serial 10-fold dilutions of viral supernatants were prepared in DMEM containing 1 µg/mL TPCK trypsin, and 100 µL was transferred into pre-seeded cell plates. The plate was then incubated at 33°C for 5 days. After 5 days, 25 µL of supernatant was used for the measurement of viral hemagglutination using 1% turkey RBC. Virus titer was calculated using the classical Reed-Muench method (37).

Immunofluorescence assay

WT cells, CASD1 KO, and receptor-fed CASD1-KO cells were fixed at 72 hours post virus infection with 80% acetone. These acetone-fixed virus-infected cells were incubated with 1% BSA in 1× PBS to block the non-specific binding. Rabbit polyclonal antibody against IDV D/OK, D/CA, and D/660 was used at 1:200 dilution. For D/swine/Kentucky infection, anti-D/OK rabbit antibody was used. After 1 hour of incubation at 37°C, cells were washed three times with 1× PBS followed by additional incubation with goat-anti rabbit Alexa-Fluor 488 antibody at 1:400 dilution (Thermo Fisher: A11008) for 45 min at 37°C. After three washes with 1× PBS, cells were counterstained with DAPI. Images were taken by the visualization under Nikon Ti eclipse fluorescence microscope, and images were merged using ImageJ software.

Statistical analysis

Statistical analyses were performed in GraphPad Prism version 9. To test the statistical significance, two-tailed *t*-test assuming equal variance was performed for the reporter virus infection assay. For multi-round replication assay, two-way analysis of variance followed by Tukey's multiple comparison test was performed. Statistically significant data are indicated by asterisk (*, $P < 0.05$; **, $P < 0.01$). Data are represented as mean ± standard error of mean (SEM).

RESULTS

CASD1-KO MDCK cells are resistant to IDV infection

To investigate what form of 9-*O*-acetylated sialic acids is utilized by IDV for entry into cells, we first developed an IDV D/OK-reporter virus by taking advantage of the

reverse genetics system (RGS) we have already developed for the virus (38). Briefly, the pUC57mini-D/OK-HEF-reporter plasmid was created by inserting the GFP-coding sequence between the 5′- and 3′-end sequences of the D/OK HEF segment flanked with human polymerase I promoter and polymerase I terminator sequences, respectively (Fig. 1A). The native HEF sequences of 240 nucleotides at both the 5′ and 3′ ends were retained to ensure the efficient package of the reporter segment (Fig. 1A). All the ATG codons before GFP translation initiation codon were changed into CTG codons, so the downstream GFP reporter protein can be expressed properly. Seven WT RGS plasmids and the GFP reporter plasmid (1 µg each) were co-transfected into cell monolayers of HEK-293T and MDCK-ATL co-cultured cells. Supernatants were collected after 4–5 days (passage 0) followed by further passaging in fresh MDCK-ATL cells. Fluorescence microscopy showed successful rescue and propagation of D/OK-GFP reporter virus (Fig. 1B); however, further passaging showed a progressive loss of reporter gene expression (Fig. 1B), which is likely caused by the inability of seven-segmented IDV to stably package the eighth segmented genome. Nevertheless, these data demonstrate that the IDV D/OK-GFP reporter establishes a single round of infection in target cells, thus providing a valid reporter virus to detect IDV infection in host cells.

Based on the above observation, we selected passage 1 (P1) IDV D/OK-GFP reporter virus for this study. The titer of the D/OK-GFP was calculated by flow cytometry, and 10^6 FU/mL of the reporter virus was used in our assays. Following infection, WT MDCK cells showed strong GFP expression (Fig. 1C) under a fluorescence microscope. However, infection of CASD1-KO cells under the identical conditions resulted in no detectable fluorescence signals, indicating that CASD1-KO cell is refractory to IDV infection (Fig. 1D).

IDV requires 9-*O*-acetylated sialic acid for cell attachment

To further determine whether the failure of IDV infection of CASD1-KO cells was caused by the absence of a functional IDV receptor in our assay system, we performed the cell-binding assay with the receptor-binding domain of IDV D/OK HEF protein, which was produced and purified from *E. coli*. As shown in Fig. 2, binding of IDV RBD to WT MDCK cells resulted in strong fluorescence signals (Fig. 2A), which was in marked contrast with RBD binding to CASD1-KO cells where only background signals were observed (Fig. 2B). These data indicate that CASD1-KO cells lose the ability to express IDV's 9-*O*-acetylated sialic acid receptors as reported previously (39), which explains the lack of IDV RBD binding to the cell surface of CASD1 KO cells and no detectable IDV D/OK-GFP reporter virus replication in these cells (Fig. 1D).

Next, we examined whether exogenous feeding of CASD1-KO cells with IDV's 9-*O*-acetylated sialic acid receptors rescues the IDV RBD binding to these cells. For this experiment, cells were fed with exogenously added synthetic receptors followed by surface binding assay with the IDV D/OK RBD. CASD1-KO cells were seeded in 96-well plates and treated with 200 µM of synthetic Neu5,9Ac$_2$ or Neu5Gc9Ac. After 3 days, cells were stained using His-tagged D/OK RBD. As shown in Fig. 2C and D, both exogenous receptor analogs restored the IDV RBD binding to CASD1-KO cells to levels similar to that of RBD binding to WT cells (Fig. 2A). These results indicate that CASD1-KO cells are capable of metabolic uptake of external 9-*O*-acetylated sialic acids and that these sialic acids are efficiently displayed on the cell surface, accessible to binding by the IDV RBD. This feeding assay with exogenous 9-*O*-acetylated sialic acids was further explored in experiments below to study IDV receptor biology. Our results also indicate that IDV D/OK specifically binds to 9-*O*-acetylated sialic acids, and this interaction is essential for viral binding/entry.

IDV utilizes both Neu5,9Ac2 and Neu5Gc9Ac receptors for entry into host cells

Feeding experiments demonstrated that D/OK-RBD binds specifically to CASD1-KO cells only when synthetic 9-*O*-acetylated sialic acids were added in the culture media (Fig. 2C and D). Next, we asked whether binding to these receptors can lead to productive IDV

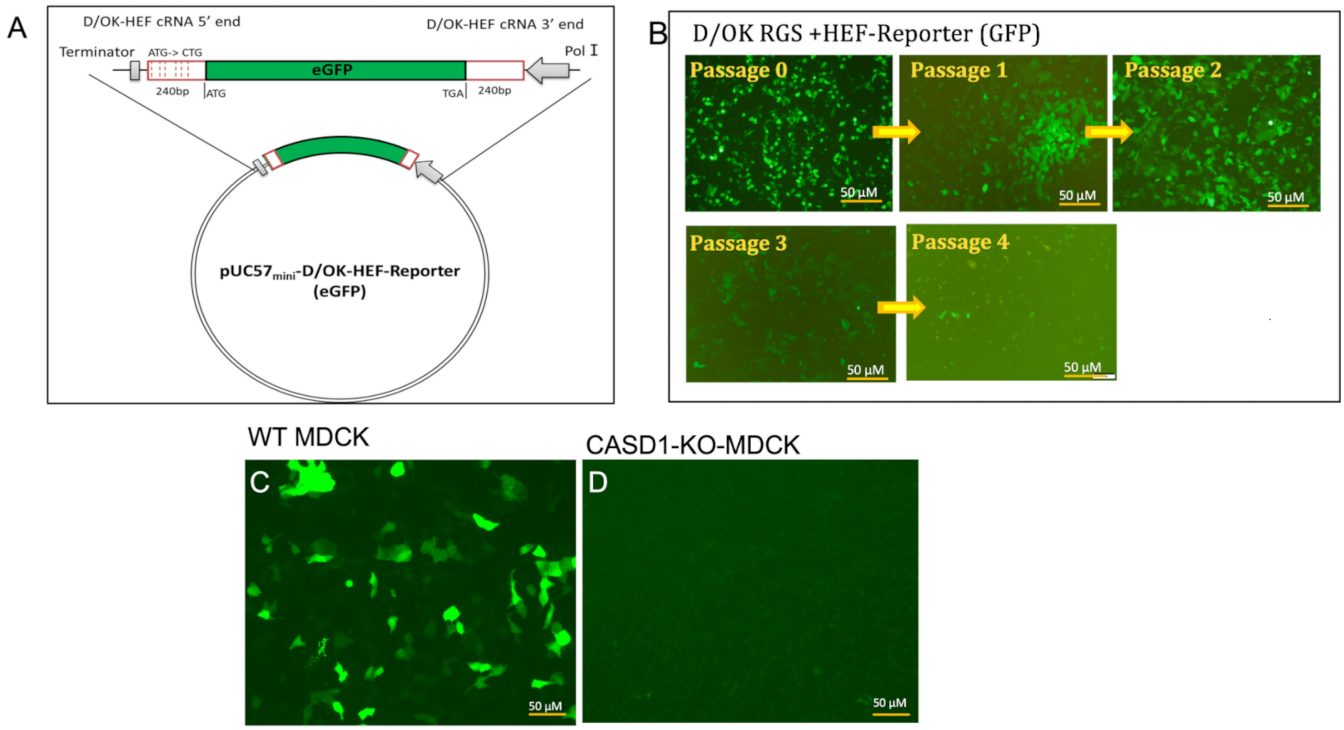

FIG 1 Generation of D/OK-GFP reporter virus and infection of WT and CASD1-KO MDCK cells. (A) Schematic representation of D/OK-GFP plasmid. The GFP open reading frame is inserted between the 5′- and 3′-ends of the D/OK-HEF cDNA, which is flanked by the Pol I terminator and the human RNA Pol I promoter. All ATG codons before the native GFP translation initiation codon in the construct were mutated to CTG so that GFP gene can be expressed in native form. (B) Microscopy images showing a successful rescue and propagation of D/OK-GFP reporter virus in passages 1–4. Infection of WT (C) and CASD1-KO (D) MDCK cells with the D/OK-GFP reporter virus is shown with images representative of three independent experiments. Scale bar represents 50 µm.

entry and replication. In this experiment, we used the single-round D/OK-GFP reporter virus in combination with receptor feeding, as described above. CASD1-KO cells were fed with Neu5,9Ac$_2$ or Neu5Gc9Ac at four different concentrations (100, 200, 400, and 600 µM), followed by extensive washings and infection with 10^6 FU of D/OK-GFP virus. After 18–24 hpi, percentage of GFP-positive cells was quantified using flow cytometry. Flow cytometry analysis showed that D/OK-GFP reporter virus utilized both receptor analogs effectively for entry and replication. Mean percentage of GFP-positive cells for Neu5,9Ac$_2$ was 4.5% (100 µM), 19.1% (200 µM), 38.7% (400 µM), and 46.5% (600 µM) (Fig. 3A). Similarly, in Neu5Gc9Ac feeding experiment, mean percentage of GFP-positive cells was 1.9% (100 µM), 8.3% (200 µM), 22.6% (400 µM), and 31.5% (600 µM) (Fig. 3A). The increasing concentration of fed receptor resulted in a proportionate increase in the percentage of GFP-positive cells for both Neu5,9Ac$_2$ and Neu5Gc9Ac receptors. A dose-dependent increase in GFP-positive cells was also qualitatively captured under fluorescence microscopy. With increasing Neu5,9Ac$_2$ concentration, we observed a proportional increase in GFP-expressing cells (Fig. 3C through F). Similarly, GFP-expressing cells increased proportionally as concentration of Neu5Gc9Ac increased (Fig. 3G through J). As expected, CASD1-KO cells remained uninfected (Fig. 3A and L). As a negative control for the 9-*O*-acetyl sialic acid feeding experiments, CASD1-KO cells fed with synthetic Neu5Ac without 9-*O*-acetyl modification resulted in no detectable infection (Fig. 3M). It should be noted that infectivity levels in CASD1-KO cells fed with 400 µM (Fig. 3A and E) or 600 µM (Fig. 3A and F) of Neu5,9Ac$_2$ or 600 µM of Neu5Gc9Ac (Fig. 3A and J) were very comparable to that observed in infection of WT MDCK cells (Fig. 3A and K). We noticed that D/OK-GFP reporter virus appeared to replicate more efficiently in CASD1-KO cells fed with Neu5,9Ac$_2$ than with Neu5Gc9Ac at all four different concentrations tested (Fig. 3A and B), which was supported by statistical

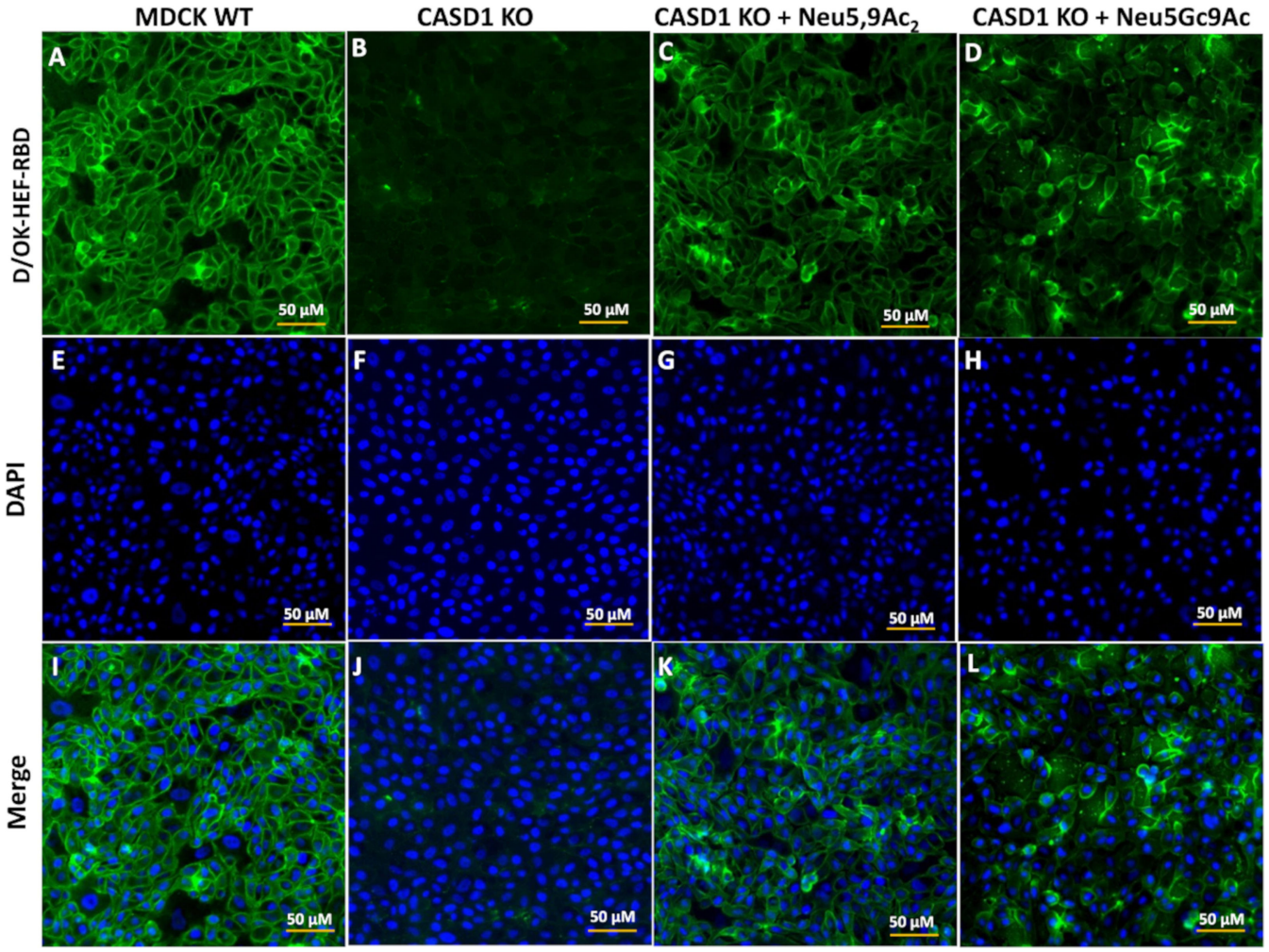

FIG 2 Cell binding of the D/OK-HEF RBD to MDCK cells. Images in the top panel represent immunostaining of WT MDCK cells (A), CASD1-KO MDCK cells (B) without feeding with exogenous *O*-acetylated sialic acids, Neu5,9Ac$_2$-fed CASD1-KO MDCK cells (C), and Neu5Gc9Ac-fed CASD1-KO MDCK cells (D). Note that following the incubation with D/OK-HEF RBD containing His-tag (20 µg/mL), washed cells were further incubated with mouse anti-His antibody and Alexa-Fluor 488-labeled anti-mouse antibody. Nuclear staining of WT MDCK (E), CASD1-KO MDCK cells without external receptor feeding (F), Neu5,9Ac$_2$-fed CASD1-KO MDCK cells (G), and Neu5Gc9Ac-fed CASD1-KO MDCK cells (H), is shown in the middle panel. The bottom panel shows merged DAPI and fluorescence for WT MDCK (I), CASD1-KO MDCK (J), Neu5,9Ac$_2$-fed (K), and Neu5Gc9Ac-fed CASD1-KO cells (L). Images representative of three independent experiments. Scale bar represents 50 µm.

analysis ($P < 0.04$, $P < 0.02$, $P < 0.08$, $P < 0.02$, respectively, for 100, 200, 400, and 600 µM groups). Taken together, these data suggest that IDV D/OK can utilize both Neu5,9Ac2 and Neu5Gc9Ac receptors for entry into host cells.

Combination of Neu5,9Ac$_2$ and Neu5Gc9Ac receptors enhances IDV replication

GFP expression levels within cells are not uniform, and analysis is often confounded by the inability to distinguish low GFP expression levels and autofluorescence that may arise due to cellular components like flavin. Thus, we generated a more sensitive and specific Nluc-based reporter virus (D/OK-NLuc). The seventh segment of IDV genome produces two proteins, NS1 and NEP, through a canonical splicing event. The unspliced mRNA is translated as NS1, while the spliced transcript gives rise to NEP (Fig. 4A). To generate IDV D/OK-Nluc reporter virus, a single non-overlapping transcript expressing D/OK NS1 and NEP was separated by using the PTV-1 self-cleaving element 2A peptide. Nluc was linked

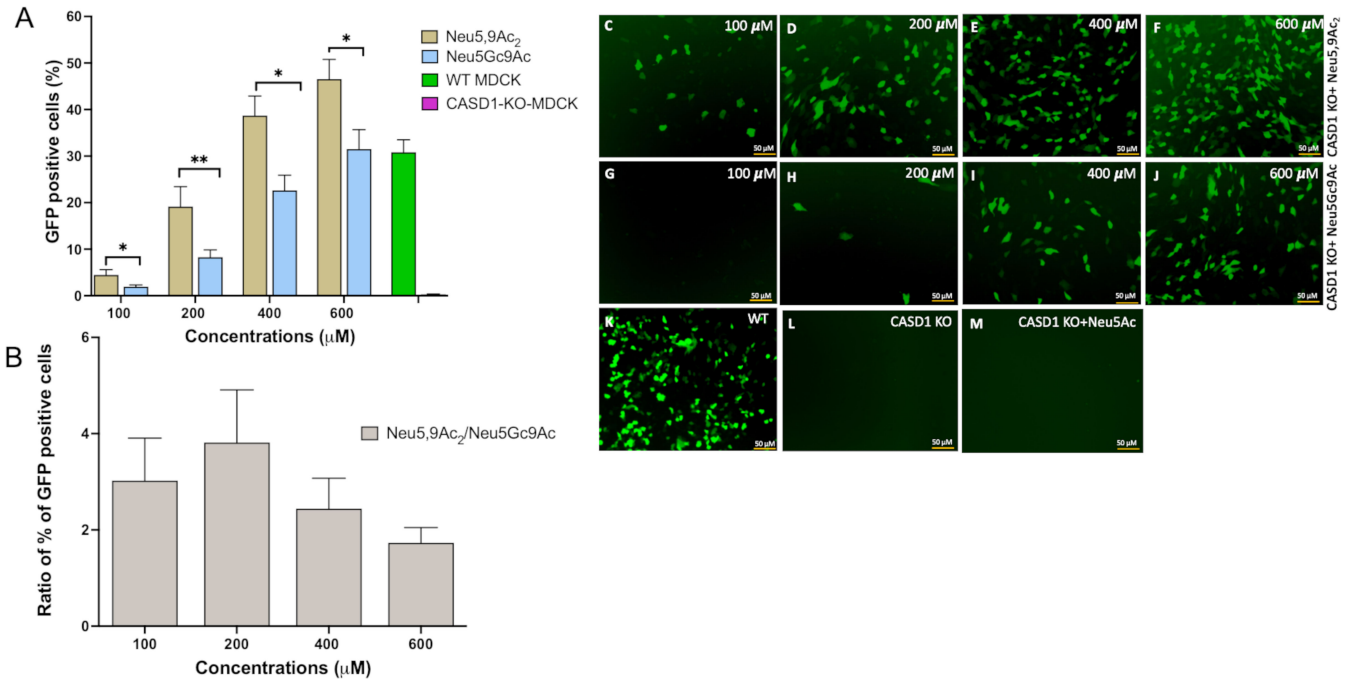

FIG 3 Dose-dependent enhancement of D/OK-GFP infectivity in 9-*O*-acetyl sialic acid-fed CASD1-KO MDCK cells. (A) Percentages of GFP-positive cells infected with IDV D/OK GFP reporter virus at 24 hpi with exogenous Neu5,9Ac$_2$ and Neu5Gc9Ac treatment. WT- and CASD1-KO (without exogenous receptor feeding) MDCK cells were used as internal controls. (B) Ratio of percentage of GFP-positive cells observed between Neu5,9Ac$_2$-fed and Neu5Gc9Ac-fed CASD1-KO cells (Neu5,9Ac$_2$/Neu5Gc9Ac). Fluorescence images of D/OK-GFP reporter virus-infected CASD1-KO cells in the presence of 100 (C), 200 (D), 400 (E), and 600 µM (F) of Neu5,9Ac$_2$ or 100 (G), 200 (H), 400 (I), and 600 µM (J) of Neu5Gc9Ac. Fluorescence images of D/OK-GFP reporter virus-infected WT (K), CASD1-KO (L), and CASD1-KO fed with Neu5Ac (M) are also shown. Bar graphs denote the mean of nine independent experiments with error bar representing SEM. Statistical significance is indicated by * ($P < 0.05$) and ** ($P < 0.001$).

to the C-terminal end of NS1 followed by PTV-2A element (Fig. 4B) and the entire open reading frame of NEP. D/OK-NLuc reporter plasmid in substitution of WT NS plasmid was used together with six other RGS plasmids (PB2, PB1, PA, HEF, NP, and M) for transfection of co-cultures of HEK-293T and WT MDCK cells to generate D/OK-NLuc reporter virus, which was subsequently used for infection of 9-*O*-acetyl sialic acid-fed CASD1 KO cells to determine how the presence of both Neu5,9Ac$_2$- and Neu5Gc9Ac-containing receptors on cell surface influences IDV D/OK replication and infectivity. In this experiment, CASD1-KO cells were fed with a range of concentrations of individual Neu5,9Ac$_2$ or Neu5Gc9Ac or an equimolar mixture of both (with an identical total concentration across conditions) for 3 days, followed by infection with D/OK-NLuc reporter virus (0.1 MOI) and the measurement of NanoLuc luciferase (RLU) as a surrogate of viral titer. For example, 200 µM single 9-*O*-acetylated sialic acid was used for cell feeding, while 200 µM mixed 9-*O*-acetylated sialic acids (combination) for cell feeding consisted of 100 µM of each of the two.

D/OK-NLuc titers (RLU) increased in CASD1-KO cells in a dose-dependent fashion in response to exogenous 9-*O*-acetylated sialic acid feeding. Mean RLUs were 2,844, 16,340, 40,973, and 61,744 for cells fed with Neu5,9Ac$_2$ at 100, 200, 400, and 600 µM concentrations, respectively (Fig. 4C). Similarly, in the Neu5Gc9Ac feeding experiment, the mean RLUs were 2,851, 11,145, 40,382, and 64,628 for cells fed with Neu5Gc9Ac at 100, 200, 400, and 600 µM concentrations, respectively (Fig. 4C). Notably, in the presence of 600 µM Neu5,9Ac$_2$ or Neu5Gc9Ac, viral titers in CASD1-KO cells reached to a level similar to that observed in WT MDCK cells infected with D/OK-Nluc reporter virus. An interesting note is that the observed differences in viral titers between Neu5,9Ac$_2$- and Neu5Gc9Ac-fed cells became insignificant ($P < 0.99$, $P < 0.059$, $P < 0.93$, and $P < 0.69$ for 100, 200, 400, and 600 µM, respectively) for the D/OK-Nluc reporter virus when

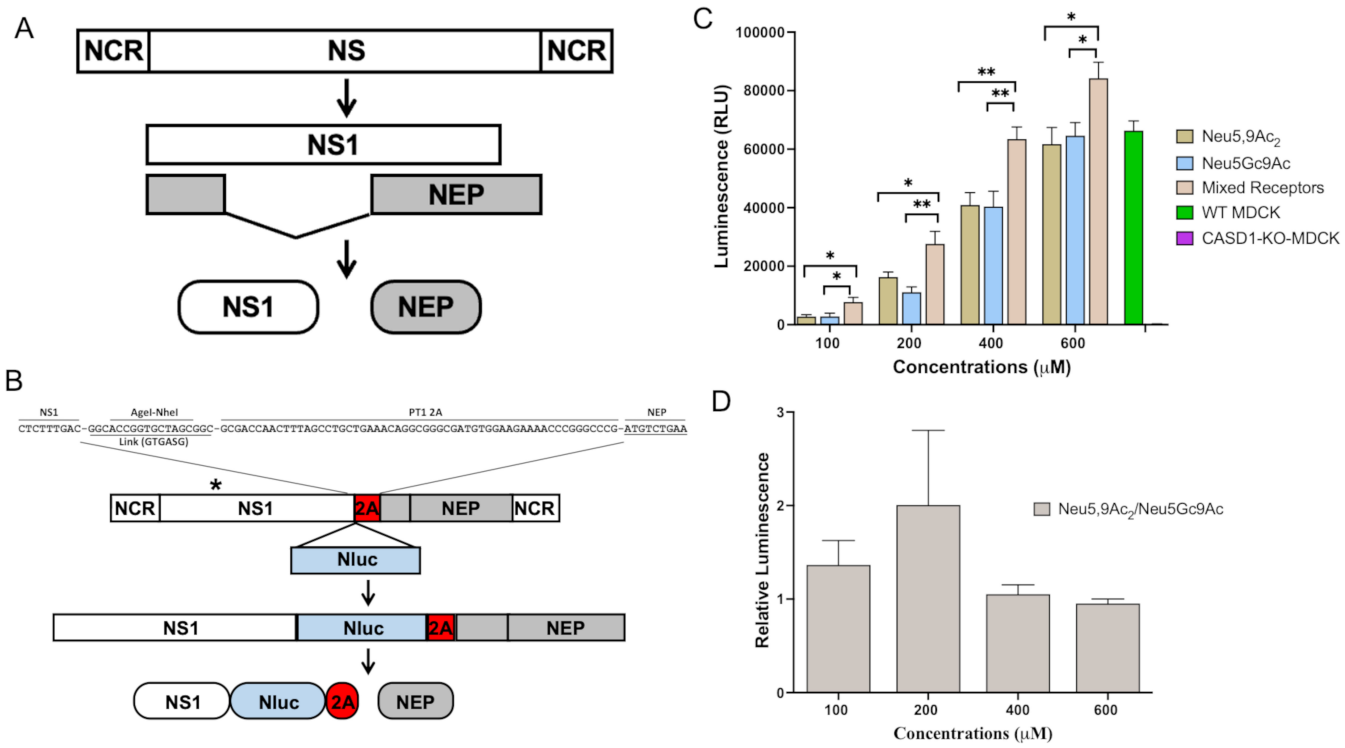

FIG 4 D/OK-NLuc reporter virus infection in 9-*O*-acetyl sialic acid-fed CASD1-KO MDCK cells. Schematic representation of WT (A) and modified (B) IDV NS segment. (C) Levels of Nluc expression detected in cell culture supernatants of CASD1-KO MDCK cells in the absence or in the presence of different 9-*O*-acetyl sialic acids. WT MDCK cells infected with IDV D/OK-NLuc reporter virus were included as control. (D) Ratio of RLUs observed between Neu5,9Ac$_2$-fed and Neu5Gc9Ac-fed CASD1-KO cells (Neu5,9Ac$_2$/Neu5Gc9Ac) following infection with D/OK-NLuc reporter virus. Bar graphs denote the mean of six independent experiments with error bar representing standard error of mean. Statistical significance is indicated by * ($P < 0.05$) and ** ($P < 0.001$). NCR, non-coding region. 2A: porcine teschovirus 2A sequence.

compared to the D/OK-GFP reporter virus system, especially when higher concentrations of 9-*O*-acetyl sialic acids were used (400 and 600 µM) (Fig. 4C and D versus Fig. 3A). Nevertheless, D/OK-Nluc reporter virus appeared to replicate slightly better in cells fed with lower concentrations of Neu5,9Ac$_2$ (100 and 200 µM) than cells fed with Neu5Gc9Ac (Fig. 4C and D), similar to the results observed using the D/OK-GFP reporter virus system (Fig. 3A). Finally, we observed that the combination of two 9-*O*-acetyl sialic acids in exogenous feeding significantly enhanced IDV D/OK-Nluc reporter virus replication in CASD1-KO cells when compared to cells individually treated with each of the compounds at all four different concentrations tested (100, 200, 400, and 600 µM) (Fig. 4C). The viral titer differences between the mixtures of the two and single compound (Neu5,9Ac$_2$ and Neu5Gc9Ac) groups were statistically significant at all concentration groups (Fig. 4C), which indicate that the combination of two receptors can confer the replication advantage to IDV D/OK-Nluc reporter virus in CASD1-KO cells. Overall, these data recapitulated what has been observed previously using the IDV D/OK GFP reporter virus (Fig. 4), thereby emphasizing that IDV can utilize either receptor for productive entry.

IDVs of swine and bovine origin use both Neu5,9Ac$_2$ and Neu5Gc9Ac equally well in a multi-round replication assay

To investigate if diverse IDV strains differ in their ability to utilize Neu5,9Ac$_2$ or Neu5Gc9Ac for entry and infection, we used a traditional multi-round replication assay with four representative IDV strains isolated from swine or bovine. D/OK and D/swine/KY originated from swine, while D/660 and D/CA were isolated from cattle. CASD1-KO cells were fed with 200 µM of a single compound (Neu5,9Ac$_2$ or Neu5Gc9Ac) and

then infected (1.0 MOI) with different IDVs. D/OK virus replicated to higher titers in Neu5,9Ac$_2$-fed cells than Neu5Gc9Ac-fed cells. Although mean viral titers were similar until day 2 for both Neu5,9Ac$_2$- and Neu5Gc9Ac-treated cells, D/OK had nearly one-log higher titers from day 3 onward in Neu5,9Ac$_2$-fed cells. Statistically significant differences were observed (P < 0.02) at day 4 for D/OK viral titer with Neu5,9Ac$_2$ (mean titer of 10^5) than Neu5Gc9Ac (mean titer of $10^{4.2}$) (Fig. 5A). D/OK in WT cells had the highest titer at day 5 (mean titer of 10^6) (Fig. 5A). Like swine-originated D/OK virus, D/swine/KY had slightly better replication kinetics in Neu5,9Ac$_2$ than Neu5Gc9Ac-fed cells during the initial 3 days, although no statistical difference was observed. The highest viral titer was observed at day 3 with mean titer of $10^{6.9}$ for Neu5,9Ac$_2$ and $10^{6.6}$ for Neu5Gc9Ac-fed cells (Fig. 5B). D/swine/KY propagated to high titers in WT MDCK cells, with peak of infection at day 2 (mean titer of $10^{7.4}$) (Fig. 5B). D/660, at day 1, had slightly better replication in Neu5,9Ac$_2$-fed cells (Fig. 5C). However, at days 2 and 3, viral titers were similar between Neu5,9Ac$_2$- and Neu5Gc9Ac-fed cells. Interestingly, at days 4 and 5, Neu5Gc9Ac-fed cells showed higher replication of D/600 virus (Fig. 5C). D/660 propagated on WT MDCK with peak titer of 10^6 on day 2 post-infection (Fig. 5C). Despite notable differences in viral titers between Neu5,9Ac$_2$- and Neu5Gc9Ac-fed cells, no statistically significant differences were revealed at all time points. D/CA showed higher replication in Neu5Gc9Ac compared to Neu5,9Ac$_2$-fed cells after day 3 (Fig. 5D). Notably, at day 5, there was significant difference in replication of D/CA in Neu5Gc9Ac-treated cells than Neu5,9Ac$_2$-fed cells (P < 0.00007) with mean viral titers of $10^{5.5}$ and $10^{5.1}$, respectively, for Neu5Gc9Ac- and Neu5,9Ac$_2$-fed cells (Fig. 5D). The peak viral titer was at day 3 with mean titers of $10^{5.7}$ and $10^{5.6}$ for Neu5Gc9Ac- and Neu5,9Ac$_2$-fed cells, respectively (Fig. 5D), while peak titers of D/CA in WT MDCK were observed at day 2 (mean viral titer of $10^{5.5}$) (Fig. 5D). CASD1-KO cells without any receptor feeding were refractory to infections

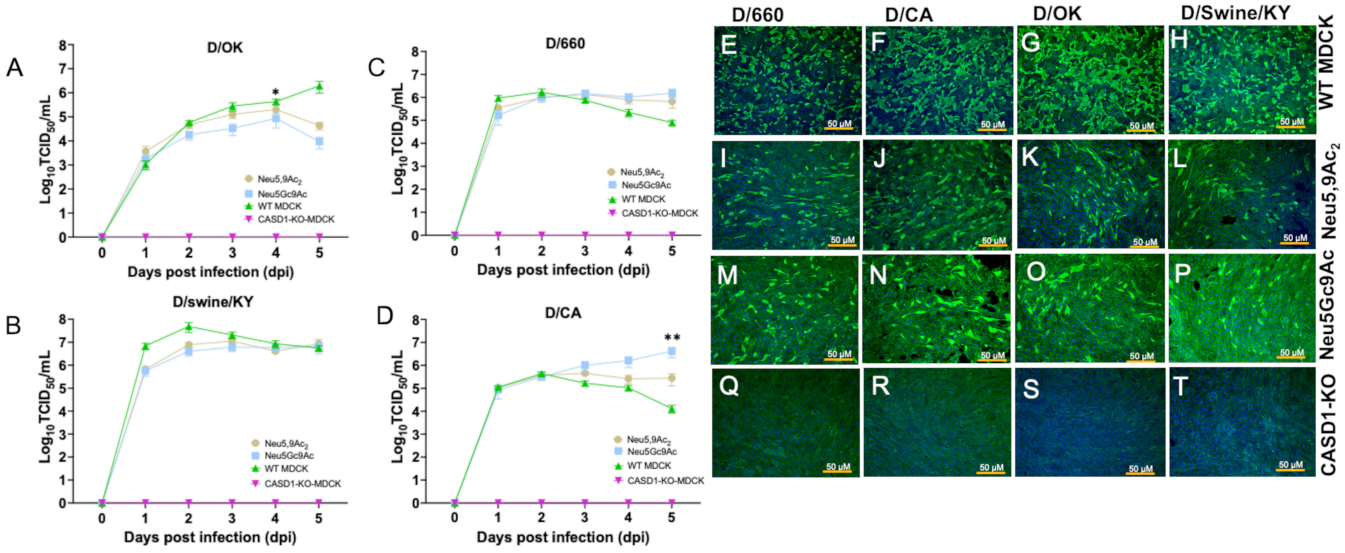

FIG 5 Replication kinetics of swine and bovine origin IDV strains in receptor-treated CASD1-KO MDCK cells. CASD1-KO MDCK cells were fed with synthetic Neu5,9Ac$_2$ or Neu5Gc9Ac (200 µM) for 3 days. Treated cells were then infected (1 MOI) with D/OK (A), D/swine/KY (B), D/660 (C), and D/CA (D), respectively. After 1 hour of incubation at 33°C, cells were washed and maintained in 100 µL of DMEM containing 1 µg/mL of TPCK trypsin at 33°C. The 100-µL media were collected every day and replaced with fresh DMEM containing 1 µg/mL of TPCK trypsin during 5-day experiment. Supernatants were used to determine viral titers (TCID$_{50}$/mL) using a traditional infectivity assay. Immunofluorescence images of WT MDCK cells infected (1 MOI) with D/660 (E), D/CA (F), D/OK (G), and D/swine/KY (H) are shown. Immunofluorescence images of Neu5,9Ac$_2$-fed CASD1-KO MDCK cells infected (1 MOI) with D/660 (I), D/CA (J), D/OK (K), and D/swine/KY (L) are also provided. Similarly, immunofluorescence images of Neu5Gc9Ac-fed CASD1-KO MDCK cells infected (1 MOI) with D/660 (M), D/CA (N), D/OK (O), and D/swine/KY (P) are also shown. CASD1-KO MDCK cells without exogenous receptor feeding were refractory to infections by D/660 (Q), D/CA (R), D/OK (S), and D/swine/KY (T) natural isolates. Viral titers are represented as mean of six individual replicates, and error bar represents the standard error of mean. Statistical significance is indicated by * (P < 0.05). Merged (fluorescence and DAPI) immunofluorescence images are representative of six independent replicates. Scale bar represents 50 µm.

by all four IDV strains (Fig. 5A through D). Taken together, multi-round replication results confirmed the results of D/OK-based single-round replication assay that diverse IDVs utilize both receptors for productive entry and infection in host cells.

To validate the above finding through qualitatively demonstrating the presence of viral proteins in infected CASD1-KO cells, 9-*O*-acetylated sialic acid-fed cells infected with four representative strains of IDV were assessed by immunofluorescence at day 3 following infection using the whole virus-specific rabbit polyclonal sera. Consistent with measured viral titer data, WT MDCK cells infected with D/660 (Fig. 5E), D/CA (Fig. 5F), D/OK (Fig. 5G), and D/swine/KY (Fig. 5H) showed higher abundance of viral proteins. Both Neu5,9Ac$_2$- (Fig. 5I through L, respectively, for D/660, D/CA, D/OK, and D/swine/KY) and Neu5Gc9Ac-fed cells (Fig. 5M through P, respectively, for D/660, D/CA, D/OK, and D/swine/KY) also showed the presence of viral proteins in infected receptor-treated CASD1-KO cells. CASD1-KO cells were refractory to D/660 (Fig. 5Q), D/CA (Fig. 5R), D/OK (Fig. 5S), and D/swine/KY (Fig. 5T) viruses.

Influenza C virus, like IDV, can utilize both Neu5,9Ac$_2$ and Neu5Gc9Ac receptors for infection

Influenza C virus, like IDV, is a seven-segmented influenza virus with the negative polarity genome, and ICV has HEF that can bind to both Neu5Gc9Ac and Neu5,9Ac$_2$ (32). Using influenza C/Victoria/2/2012 isolated from human (40), we performed the multi-round replication assay for ICV Victoria strain in CASD1-KO cells with and without feeding with 200 µM of Neu5,9Ac$_2$ or Neu5Gc9Ac. We found that ICV, like IDV, utilized both receptors (Fig. 6), and there were no statistical differences in viral titers between Neu5,9Ac$_2$- and Neu5Gc9Ac-fed cells. Viral titers of ICV in Neu5,9Ac$_2$- or Neu5Gc9Ac-fed cells were approximately two logs less than those observed in WT MDCK cells with a native CASD1 (Fig. 6).

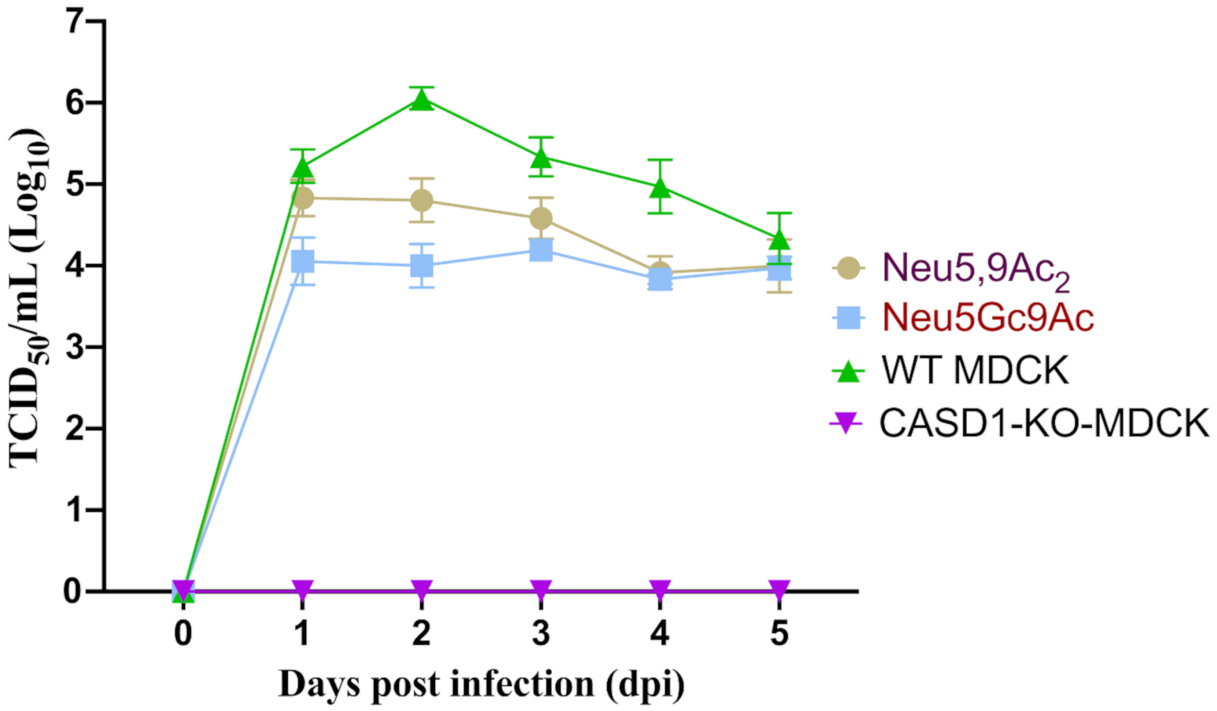

FIG 6 Replication kinetics of human ICV C/Victoria/2/2012 strain in receptor-treated CASD1-KO MDCK cells. CASD1-KO MDCK cells were fed with synthetic Neu5,9Ac$_2$ or Neu5Gc9Ac (200 µM) for 3 days. Treated cells were then infected with 1 MOI of C/Victoria/2/2012. After 1 hour of incubation at 33°C, cells were washed and maintained in 100 µL of DMEM containing 1 µg/mL of TPCK trypsin at 33°C. The 100-µL media were collected every day and replaced with fresh DMEM containing 1 µg/mL of TPCK trypsin during a 5-day experiment. Supernatants were used to determine viral titers (TCID$_{50}$/mL) using a traditional infectivity assay. Viral titers are represented as mean of six individual replicates, and error bar represents the standard error of mean.

DISCUSSION

IDV was first isolated from a diseased pig with influenza-like symptoms in 2011 (2) and was officially designated as new subtype of influenza by the International Committee of Taxonomy of Viruses in 2016 (3, 41). Since its initial isolation, IDV has been shown to infect a variety of farm animals. Although direct human infection has not been established, antibodies against IDV have been reported in both swine and cattle handlers (2, 42, 43). Viral genome has also been detected in farmers who were in close contact to swine and bovine species (11, 13). As such, IDV has the potential to emerge into human population and cause influenza-like respiratory disease. To date, little is known regarding which form of naturally occurring 9-*O*-acetylated sialic acids (Neu5,9Ac$_2$ and Neu5Gc9Ac) serves as a functional receptor for IDV entry into target cells.

Sialic acid *O*-acetylation occurs in the Golgi (29). Two classes of enzymes regulate *O*-acetylation: the sialic acid *O*-acetyltransferases (SOATs) and the sialic acid esterase (28). The only known mammalian SOAT is CASD1 (44). CASD1 enzyme encoded by *CASD1* gene is known to catalyze the addition of *O*-acetyl group to sialic acids (29). *O*-Acetylation can occur at C-4, C7, C-8, and C-9, and reversible migration may occur under neutral-to-mild basic pH conditions (45, 46). MDCK cells are the most common cell line used for influenza research (47). The cell line with the *CASD1* gene that was knocked out using CRISPR/Cas9 was validated for the lack of 9-*O*-acetyl sialic acid expression by using 9-*O*-acetyl sialic acid-specific probes in a previous study (39). This work also revealed that the CASD1-KO MDCK cells were resistant to IDV infection, while WT MDCK showed high infection levels. By leveraging this MDCK CASD1-KO cell lacking sialic acid 9-*O*-acetylation activity in combination with the exogenous 9-*O*-acetyl sialic acids feeding experiments involving single- and multiple-round viral replication assays, we demonstrate that IDV can utilize cell surface receptors containing Neu5,9Ac$_2$ or Neu5Gc9Ac as a functional receptor for infection.

In this study, we demonstrated that IDV RBD binds to both Neu5,9Ac$_2$ and Neu5Gc9Ac on cell surface, which correlates with IDV replication in CASD1-KO cells receiving exogenous 9-*O*-acetyl sialic acid feeding. Treatment of cells with exogenous sialic acid to restore sialic acid levels in hyposialylated cells has been employed in many published studies (48–52). Several studies have shown that humans, who do not have the ability to convert CMP-Neu5Ac to CMP-Neu5Gc, can incorporate diet-derived Neu5Gc into cells (25, 49, 50, 53–55). Thus, metabolic incorporation of sialic acid can occur both *in vitro* and *in vivo*. More recent studies (48, 56) showed that under hyposialylated conditions, cells can uptake exogenous Neu5,9Ac$_2$ and display it in the plasma membrane, as confirmed by 9-*O*-acetylated sialic acid-binding probes. External sialic acids, such as Neu5,9Ac$_2$, can reach the maximum concentration at the cell surface probably at day 3 post-feeding (56). By harnessing this system, we showed that receptor feeding of Neu5,9Ac$_2$ and/or Neu5Gc9Ac can successfully restore infectivity of IDV in CASD1-KO cells. In addition to revealing the receptor usage by IDV, results of these experiments also validated the specificity of CASD1 inactivation by CRISPR-Cas9 since other enzymatic activities, such as the sialic acid conjugation to the glycans of glycoproteins and glycolipids by sialyltransferases, are apparently unaffected in CASD1-KO cells. In the single-round replication assays, we observed that IDV reporter viruses replicated slightly more efficiently in Neu5,9Ac$_2$-fed cells than in Neu5Gc9Ac-fed cells. This could be due to the preference of IDV for Neu5,9Ac$_2$ over Neu5Gc9Ac or the differential uptake and turnover rates between Neu5,9Ac$_2$ and Neu5Gc9Ac, which warrants further investigation.

In the multi-round replication assays, using two representative IDV strains isolated from bovine and swine, we showed that both bovine- and swine-originated IDV can utilize Neu5,9Ac$_2$ and Neu5Gc9Ac for entry and infection. IDV of swine origin showed higher replication titers in Neu5,9Ac$_2$-fed cells, while bovine origin IDV strains showed higher viral titers in Neu5Gc9Ac-fed cells. Similarly, human ICV replicated well in both Neu5,9Ac$_2$- and Neu5Gc9Ac-fed cells. Viral titers were slightly higher in Neu5,9Ac$_2$-fed

cells, which is congruent to our previously reported ICV glycan microarray data and ICV replication inhibition result involving the synthetic receptor analogs (32).

Both swine and bovine species have a functional CMAH enzyme, so they can convert Neu5Ac to Neu5Gc. Since this conversion is unidirectional (27), in swine and bovine, there is a higher expression of Neu5Gc than Neu5Ac. For IAV, the differential expression of sialic acids across various species plays a crucial role in determining the species specificity of influenza viruses and their tissue tropisms. Dogs, ferrets, seals, hedgehogs, certain species of new world monkey, and white-tailed deer have non-functional *CMAH* genes (57, 58) and thus cannot convert Neu5Ac to Neu5Gc. A study quantifying Neu5Gc expression in the lungs of cattle and swine showed that cattle possess higher levels of Neu5Gc expression (59). In wild pigs, there is a higher expression of Neu5Gc than Neu5Ac in the upper respiratory tract, transitioning to higher Neu5Ac and lower Neu5Gc in the lower respiratory tract. In contrast to wild pigs, adult domestic pig lung tissues express a higher amount of Neu5Gc throughout the respiratory tract, including high Neu5Gc in pig saliva (60–62). Elucidating the delicate interplay between Sia diversity (Neu5Ac and Neu5Gc) and selection for influenza viruses with high replication fitness and cross-species transmission efficiency in various animal species clearly warrants further work.

To date, limited studies are available on the quantification of *O*-acetylation expression in animal species. Mouse trachea and lung tissues were found to express high level of 9-*O*-acetylated Sias, while human submucosal glands and lung tissues contain significant amounts of 7,9- and 9-*O*-Ac Sias (63). Interestingly, mouse sera also have higher Neu5Gc, while rat sera contain both Neu5Ac and Neu5,9Ac_2 in significant amounts (64). 7,9- and 9-*O*-Ac Sias are also expressed abundantly in the tracheal epithelium of pigs, horses, and dogs. Ducks were found to express 9-*O*-acetylated Sias in trachea and lung alveolar tissues as well as 7,9- and 9-*O*-Ac Sias in the intestine tissue (63). Despite progress, in-depth quantitative analysis of sialic acid species with various forms of *O*-acetylation in the respiratory tissues of humans and animals is needed in future study toward better understanding of the impact of modified Sias on evolution, replication fitness, and transmission effectiveness of influenza and other respiratory viruses utilizing this group of Sias as functional receptors for infection.

Results of our experiments reported in this study showed a general trend, in that two bovine IDVs replicated slightly more efficiently in Neu5Gc9Ac-treated cells, while an opposite trajectory was observed for the two swine IDVs. Whether the genetic polymorphisms in the HEF protein between two groups confer this phenotype (probably through receptor-binding preference or entry/fusion steps post-receptor binding) will be investigated in future study. Both IDV and ICV utilize Neu5Gc9Ac and Neu5,9Ac_2 as functional receptors for their entry. The only notable difference between Neu5Gc9Ac and Neu5,9Ac_2 is that the former molecule has an extra oxygen in the *N*-acyl group at the C-5 position. Our study indicates that this extra atom has no impact on the IDV binding to the 9-*O*-acetyl group in Neu5Gc9Ac. Thus, exploiting natural diversity of sialic acids with 9-*O*-acetylation may give the IDV or ICV evolutionary advantage to infect multiple host species, which has been already demonstrated in field study. 9-*O*-Acetylated Neu5Ac is ubiquitously expressed throughout the human body (28). In addition to the respiratory tract, including lung, Neu5,9Ac_2 has been also found in brain, salivary gland, kidney, pancreas, circulation, and intestine human tissues (63). Furthermore, a growing number of studies have shown that Neu5,9Ac_2 expression is significantly upregulated in patients with cancer, autoimmunity, and infection (65, 66). Based on the evidence presented in this study establishing that Neu5,9Ac_2 serves as a functional receptor for IDV entry and infection, the potential emergence of IDV in humans, especially with underlying conditions, cannot be underestimated, which highlights a need for continued surveillance of IDV in farm animals and humans, as well as for further investigation of its biology and cross-species transmission mechanism.

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