HCoV-HKU1 Spike protein uses *O*-acetylated sialic acid as an attachment receptor determinant and employs HE protein as a receptor-destroying enzyme

Short title*:* HCoV-HKU1 virus receptor and RDE function of HE

Xingchuan Huang[1], Wenjuan Dong[1,2], Aleksandra Milewska[3], Anna Golda[3], Yonghe Qi[1,4], Quan K. Zhu[5], Wayne A. Marasco[5], Ralph S. Baric[6,7], Amy C. Sims[7*], Krzysztof Pyrc[3,8*], Wenhui Li[1*#] and Jianhua Sui[1*#]

[1] National Institute of Biological Sciences, No. 7 Science Park Road, Zhongguancun Life Science Park, Changping, Beijing, 102206, China.

[2] China Agricultural University Graduate Program, National Institute of Biological Sciences, Beijing, 102206, China.

[3] Microbiology Department, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Gronostajowa 7, 30-387 Krakow, Poland

[4] Beijing Normal University Graduate Program, National Institute of Biological Sciences, Beijing, 102206, China

[5] Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute; Department of Medicine, Harvard Medical School, Boston, Massachusetts, 02115, USA

[6] Department of Microbiology and Immunology, University of North Carolina, Chapel Hill, North Carolina, 27599, USA

[7] Department of Epidemiology, University of North Carolina, Chapel Hill, North Carolina, 27599, USA

[8] Malopolska Centre of Biotechnology, Jagiellonian University, Gronostajowa 7, 30–387 Krakow, Poland

* A.C.S., K.P., W.L. and J.S. are co-senior authors of the paper

[#] Address correspondence to W.L. (liwenhui@nibs.ac.cn) or J.S. (suijianhua@nibs.ac.cn)

Word count for the abstract: 241

Word count for the text: 6370

Number of figures: 4 figures

Key words: Coronavirus, hCoV-HKU1, receptor, hemagglutinin-esterase, HE, Spike protein, neuraminidase, sialic acid

Abstract

Human coronavirus (hCoV) HKU1 is one of six hCoVs identified to date and the only one with an unidentified cellular receptor. HCoV-HKU1 encodes a hemagglutinin-esterase (HE) protein that is unique to the betacoronaviruses group a (group 2a). The function of HKU1-HE remains largely undetermined. In this study, we examined binding of the S1 domain of hCoV-HKU1 spike to a panel of cells and found that the S1 could specifically bind on the cell surface of a human rhabdomyosarcoma cell line, RD. Pretreatment of RD cells with neuramidase (NA) and trypsin greatly reduced the binding, suggesting that the binding was mediated by sialic acids on glycoproteins. However, unlike other group 2a CoVs, e.g. hCoV-OC43 for which 9-*O*-acytylated sialic acid (9-*O*-Ac-Sia) servers as a receptor determinant, HKU1-S1 neither bound with 9-*O*-Ac-Sia containing glycoprotein(s), nor rat and mouse erythrocytes. Nonetheless, the HKU1-HE was similar to OC43-HE, also possessed sialate-*O*- acetylesterase activity and acted as a receptor-destroying enzyme (RDE) capable of eliminating the binding of HKU1-S1 to RD cells, whereas the *O*-acetylesterase inactive HKU1-HE mutant lost this capacity. Using primary human ciliated airway epithelial cell cultures (HAE), the only in vitro replication model for hCoV-HKU1 infection, we confirmed that pretreatment of HAE cells with HE but not the enzymatically inactive mutant blocked hCoV-HKU1 viral infection. These results demonstrate that hCoV-HKU1 exploits O-AC-Sia as a cellular attachment receptor determinant to initiate the infection of host cells, and its HE protein possesses the corresponding sialate-*O*-acetylesterase RDE activity.

66 **Importance statement**

67

68 Human coronaviruses (hCoV) are important human respiratory pathogens. Among the six
69 hCoVs identified to date, only hCoV-HKU1 has no defined cellular receptor. It is also unclear
70 whether hemagglutinin-esterase (HE) protein plays a role in viral entry. In this study, we found
71 that, similar to other members of the group 2a CoVs, sialic acid moieties on glycoproteins are
72 critical receptor determinants for the hCoV-HKU1 infection. Interestingly, the virus seems to
73 employ different type of sialic acid than other group 2a CoVs. In addition, we determined that
74 the HKU1-HE protein is an *O*-acetylesterase and acts as a receptor-destroying enzyme (RDE)
75 for hCoV-HKU1. This is the first study to demonstrate that hCoV-HKU1 uses certain types of
76 *O*-acytylated sialic acid residues on glycoproteins to initiate the infection of host cells, and
77 HKU1-HE protein possesses sialate-*O*-acetylesterase RDE activity.

78

Introduction

Human coronaviruses (hCoV) are enveloped RNA viruses. They are usually associated with mild to moderate respiratory tract illnesses, but can also cause severe and highly lethal disease depending on the virus strain (1). Six hCoV strains have been identified to date and belong to four different groups, including hCoV-229E and hCoV-NL63 in the alphacoronaviruses (group 1); hCoV-OC43 and hCoV-HKU1 in the betacoronaviruses group a (group 2a); severe acute respiratory syndrome CoV (SARS-CoV) in the betacoronaviruses group b (group 2b); and Middle East Respiratory Syndrome CoV (MERS-CoV) in the betacoronaviruses group c (group 2c). Infections by viruses in groups 1 and 2a are common worldwide, and can also cause severe disease in young children or immunocompromised adults. SARS-CoV (2-4) and MERS-CoV (5, 6) are two highly virulent hCoVs causing severe respiratory diseases with high morbidity and mortality (7); the latter strain is still circulating in human populations.

Cellular receptor specificity plays an important role in viral cell and tissue tropism, pathogenesis, interspecies transmission and adaptation. The CoV Spike (S) glycoprotein is generally responsible for binding to cellular receptors and mediating viral entry. S protein is a large type-I transmembrane glycoprotein that exists as a trimer protruding from the surface of virions (8). S proteins have an amino-terminal (NT) S1 domain that mediates binding with cellular receptors and a carboxy-terminal (CT) S2 domain that mediates subsequent virus-cell membrane fusions. A wide-range of diverse cellular receptors specifically recognized by the S1 domains has been identified for all the aforementioned hCoVs except hCoV-HKU1. Human aminopeptidase N (CD13) is the cellular receptor for hCoV-229E (9). 9-*O*-acytylated sialic acid (9-*O*-Ac-Sia) is the cellular receptor determinant for hCoV-OC43 (10). hCoV-NL63 and SARS-CoV both employ human angiotensin-converting enzyme 2 (ACE2) to mediate cellular entry (11, 12), while hCoV-NL63 utilizes heparan sulfate proteoglycans for attachment to target cells (13). MERS-CoV utilizes dipeptidyl peptidase 4 (DPP4 or CD26) receptor to enter host cells (14).

Journal of Virology

HCoV-HKU1 was initially identified in 2005 from a pneumonia patient in Hong Kong (15). It was subsequently found to be as common and widespread as previously known hCoVs, hCoV-229E, hCoV-OC43 and hCoV-NL63 (16-19). Characterization of hCoV-HKU1 has been challenging due to the lack of a convenient cell line-based culture system. It was recently demonstrated that hCoV-HKU1 replicates to high titer in an *in vitro* culture system that uses primary human ciliated airway epithelial cells (HAE) or type II alveolar epithelial cells (20-22), however the functional receptor(s) of hCoV-HKU1 and other important aspects of virus-host interaction remain unknown. Being a member of group 2a CoVs, HKU1-CoV also carry another viral surface protein hemagglutinin-esterase (HE) encoding gene that is present exclusively in this group of CoVs genomes (23). The HE protein is also a type-I transmembrane glycoprotein comprised of two functional domains: an *O*-acetylated sialic acid binding domain and a corresponding sialate *O*-acetylesterase domain (24). HE protein functions primarily as a receptor-destroying enzyme (RDE) for CoVs, e.g. hCoV-OC43 and its proposed zoonotic ancestor bovine coronavirus (BCoV) (25). Both viruses bind to receptor 9-*O*-Ac-sia via their S proteins, and their HE proteins mediate RDE activity late in the infection cycle via the sialate-9-*O*-acetylesterases domain to facilitate the release of viral progeny and escape from attachment on non-permissive host cells (23, 26). In contrast, mouse hepatitis virus (MHV), another member of group 2a CoVs infects cells via the interaction of S protein with its principle receptor carcinoembryonic antigen-related cell adhesion molecule (CEACAM1a), while the MHV HE protein functions at very early viral attachment steps through a concerted action of its *O*-acetylated sialic acid binding and RDE activities (27, 28). To date the function and role of the hCoV-HKU1 HE protein remains undefined.

In this study, we found that hCoV-HKU1 S protein mediated viral attachment by utilizing *O*-acytylated sialic acids on glycoprotein(s) as a receptor determinant or initial attachment factors. The HE protein of hCoV-HKU1 did not exhibit sialic acid binding activity but instead mediated sialate-*O*-acetylesterase RDE activity specific to the *O*-acytylated sialic acids recognized by the S protein. Interestingly, HKU1-HE protein displayed similar sialate-9-*O*-acetylesterase RDE activity as OC43-HE and BCoV-HE. In the hCoV-HKU1 in

vitro replication model, we further demonstrated that the HE protein but not an enzymatically inactive HE mutant acted as a RDE and completely blocked or greatly reduced infection depending on the dose of inoculating hCoV-HKU1. These findings revealed that early viral entry steps for hCoV-HKU1 are similar to but also distinct from other members of group 2a CoVs. Like hCoV-OC43 and BCoV, hCoV-HKU1 employs *O*-acytylated sialic acids as a primary receptor determinant or attachment factor and its HE protein as corresponding RDE; however, hCoV-HKU1 also uniquely requires additional receptor determinants than hCoV-OC43 and BCoV.

Materials and Methods

Construction of expression plasmids. A synthetic codon-optimized sequence for the HKU1-S1 gene (Genebank accession number NC_006577.2) encoding aa15-600 was cloned into a mammalian expression vector containing a CD5 signal peptide and a C-terminal Fc tag from mouse IgG2a (mFc). The expression cassette was under a CAG (CMV early enhancer/chicken β actin) promoter. The resulting construct, pCAGGS-HKU1-S1(600)-mFc, encodes a chimeric S1 protein with an N-terminal CD5 signal peptide and mFc at its C terminus. Similarly, plasmids encoding other proteins were constructed, including the NT domain of HKU1-S1 (aa15-268), the NT domain of hCoV-OC43-S1 (aa15-268) (ATCC VR-759 strain, AAT84354), NT domain of S1 of CoV-HKU3 (aa16-323) (DQ022305). The extracellular domain of HE proteins from different CoVs were also similarly constructed, including HKU1-HE (aa14-358, NC_006577.2), hCoV-OC43-HE protein (aa19-376, AAX85668.1), BCoV-HE (aa19-377, AAA92991.1), and MHV-S-HE (aa 25-393, AAX08110.1). Plasmids encoding mutants of HKU1-HE were generated by site-directed QuikChange mutagenesis method (Stratagene). All mutations were confirmed by DNA sequencing, in which the codon for the esterase catalytic residue Ser40 was substituted by Ala (S40A mutant) or the catalytic triad S40, H329, and D326 were all substituted by Ala (S40A/H329A/D326A).

Expression and purification of recombinant proteins. HEK293T cells were transiently

Journal of Virology

transfected with the expression plasmids using polyethyleneimine (Polysciences). At 12 h after transfection, the medium was replaced by 293 SFM II expression medium (Life Technology). Tissue culture supernatants were harvested 3 days after transfection, and the recombinant proteins were purified by protein A-affinity chromatography.

Flow cytometry (FACS) analysis. HKU1-S1(600)-mFc or other proteins at different concentrations were diluted in FACS buffer (PBS containing 0.5% BSA and 0.1% NaN_3) and then incubated with 0.5-1x10^6 of RD cells or red blood cells (RBCs) from mouse or rat blood samples at 4ºC for 0.5-1 hr. Cells were then washed three times with FACS buffer and followed by incubation with FITC-labeled anti-mouse Fc antibody at a dilution following the manufacturer's instruction (Sigma or Pierce) at 4 ºC for 30 mins. Cells were washed as above, and the binding of proteins to cells was analyzed by a BD FACS LSRII (Beckon,Dickinson) flow cytometer and FCS Express software (De NoVo Software). For FACS analysis to examine the inhibition of HKU1-S1 binding to RD cells, the cells were pretreated with indicated HE proteins or enzymes at different concentrations, then incubated with HKU1-S1 and analyzed binding as described above. The NA was from *clostridium perfringens* (Sigma), trypsin was from bovine pancreas and TPCK-treated (Sigma). For both NA and trypsin, the pretreatment was carried out at 37 ºC for 1 hr ; for HE proteins the pretreatment was carried out at 4 ºC for 1 hr.

Indirect immunofluorescence. RD cells were seeded on glass cover slips one day before staining. Cells were washed three times with PBS, blocked with 0.5%BSA/PBS at 37 ºC for 30 mins, incubated with HKU1-600-mFc or HKU3-323-mFc at 20 μg/mL in PBS at 4 ºC for 1 hr, followed by washing three times with PBS, then incubated with FITC-Goat anti-Mouse Fc antibody (Sigma) at 4 ºC for 1 hr. Cells were washed three times, then incubated with 5 μg/mL Hoechst 33258 at 37 ºC for 10 mins, followed by three additional washes and finally incubated with 5 μg/mL FM-4-64 on ice for 1 min. Cells were analyzed and imaged with a 63x oil objective using an Zeiss LSM510 Meta Confocal Microscope. Representative images are shown.

Hemagglutination assay. About 0.25-0.5% suspension of RBCs prepared from mouse (BALB/c) or rat (Sprague Dawley) blood were added to a round-bottom 96-well plate at 50 μL/well. S1 proteins were two-fold serial diluted with 0.5%BSA/PBS and added at 50 μL/well to the wells containing RBCs. For HE protein inhibition assay, RBCs were preteated with two-fold serial diluted HE first, washed by PBS and followed by adding 50 μL/well of 10μg/mL of hCoV-OC43-S1 to the wells containing the HE pretreated and washed RBCs. The plates were left at room temperature for 60 minutes or longer until hemagglutination developed or RBCs gradually settled. Positive hemagglutination results formed a uniform reddish color across the well, whereas negative results appeared as dots in the center of round-bottomed plates due to RBCs sedimentation.

ELISA assay. The binding of S1 proteins to BSM was determined by an ELISA assay as previously described with modification (28). Maxisorp™ 96-well plates (NUNC) were coated overnight at 4° C with BSM (Sigma) at 10 μg/mL at 100 μL/well. The wells were washed with washing buffer (PBST, 0.05% Tween-20 in PBS) and treated with blocking buffer (PBS, 0.05% Tween-20, 2% nonfat milk) for 1 hr at RT. Serial diluted S1 proteins were prepared in blocking buffer (starting concentration 20 μg/mL) and then added to the BSM-coated wells at 100 μl/well. Incubation was continued for 1 hr followed by washing with PBST for six times. Binding was detected using an HRP-conjugated goat anti-mouse IgG (1:10,000 in blocking buffer; Pierce) followed by washing again. The optical density at 450 nm was measured after incubation of the peroxidase tetramethylbenzidine (TMB) substrate and stop solution.

Acetylesterase activity assay. Chromogenic p-nitrophenyl acetate (pNPA, Sigma) substrate was serially diluted in two-fold, and then incubated with 1 or 2 μg/mL HE-mFc protein or its mutants in 100 μL volume in PBS (pH7.4) at 37 ºC for different time period as indicated. The acetylesterase activity was determined by measuring the release of para-nitrophenol (OD at 405 nm) at the end of each reaction in microtiter plates with a microplate spectrophotometer (Bio-RAD). An unrelated protein was used as a control in the

enzymatic assay, the OD_{405} for HE protein or mutants was subtracted with that of this control. The Km value of HE protein was calculated from the Michaelis-Menten Enzyme Kinetics using Graphpad Prism 5 software.

Neuraminidase activity assay. An Amplex red neuraminidase assay kit (Molecular Probes, Invitrogen) was used to measure NA activity. Briefly, 25 μg/mL HKU1-HE protein was serially diluted in 50 μL of 1x reaction buffer followed by addition of 50 μL of 2x working solution containing of 100 μM Ample Red reagent containing 0.2 U/mL of HRP, 4 U/mL of galactose oxidase and the fetuin substrate serially diluted 100-fold from 2.5 mg/mL to 2.5 pg/mL. The mixture was incubated at 37 °C for 10 mins under dark, the fluorescence signal was then measured by at 595 nm wavelength and the values were used to indicate relative NA activity.

HKU1 infection of HAE. HAE culture system has been described previously (20). Briefly, the apical surface of HAE were washed three times in situ with phosphate-buffered saline (PBS) and then treated with testing reagents or controls by incubation at 32°C for 1 hr followed by washing with PBS to remove testing reagents. The treatment and washing were repeated two more times. Then HAE was inoculated with 100 μl of viral stock. Following incubation for 2 hrs at 32°C, the unbound virus was removed by washing with 500 μL for 10 min at 32°C for three times, and the HAE were maintained at an air-liquid interface for the remainder of the experiment at 32°C. HKU1 replication kinetics were determined at specific time points post-inoculation as indicated, 120 μL of PBS was applied to the apical surface of HAE, and after 10 mins of incubation at 32°C the apical sample was harvested for RNA isolation. The RNA was then analyzed by real-time reverse transcriptase (RT)-PCR to determine viral genomic mRNA copies (20).

Results

S1 domain of hCoV-HKU1 binds to RD cells.

As CoV S1 domains generally mediate the interactions with cellular receptor(s) to trigger subsequent viral-host cell membrane fusion to initiate viral entry, we first expressed codon optimized soluble HKU1 S1 domain (amino acids (aa) 15 to 600) and fused it to the Fc domain from murine IgG2a (HKU1-S1(600)-mFc) (**Fig. 1A**) to identify the cellular receptor/attachment factor for hCoV-HKU1. As a control, we also expressed the NT of the bat coronavirus HKU3 (29) S1 domain (aa16-323) fused to mFc, HKU3-S1(323)-mFc. To determine, which if any, immortalized cell lines expressed the cellular receptor for hCoV-HKU1, we probed cell lines that were isolated from several different species and tissues with our HKU1-S1 protein using flow cytometry. These cell lines included 293T (human embryonic kidney cells), HeLa (human cervical adenocarcinoma), CHO (Chinese hamster ovary cells), A549 (human lung epithelial adenocarcinoma cells), Caco2 (human epithelial colorectal adenocarcinoma cells), HepG2 (human liver hepatocellular carcinoma cell line), Huh-7 (human hepatoma cells), RD (human rhabdomyosarcoma/muscle tumor cells), HRT-18 (human colon adenocarcinoma cells), Lovo (human colon adenocarcinoma cells), MDCK (Madin-Darby canine kidney cells), Vero (African green monkey kidney cells). Interestingly, only RD cells showed specific strong binding with 5 μg/mL of HKU1-S1(600)-mFc as compared with the control protein (**Fig. 1B - left**), no specific binding was found for all other cell lines tested, a representative negative staining result on HeLa cells was shown in **Fig.1B - right**. To independently confirm that HKU1-S1 binds to the surface of RD cells, we incubated cells with either HKU1-S1(600) or HKU3-S1(323) followed by fluorescent-labeled secondary antibody and then used FM-4-64, a lipophilic probe that fluoresces intensely upon binding to the outer leaf of the plasma membrane. As shown in **Fig. 1C - left,** HKU1-S1(600) and FM-4-64 had similar staining patterns on the cell membrane of RD cells, suggesting that both were labeling the surface of the cells. In contrast, no HKU3-S1(323) could be detected on the surface of the RD cells **(Fig. 1C - right)**. FACS analysis also showed that the binding of HKU1-S1 with RD cells was in a dose-dependent manner **(Fig. 1D)**, the binding can be detected at a low concentration of HKU1-S1(600)-mFc protein at 0.61 μg/mL. These results indicate that a cellular attachment factor or receptor(s) for hCoV-HKU1 is present on the surface of the RD cells. We further tested whether the HKU1-S1's NT can bind with RD cells. The NT aa15-268 of HKU1-S1 was

Journal of Virology

expressed as a mFc-fusion protein (HKU1-S1(268)-mFc) (**Fig. 1A**), and analyzed for binding with RD cells by FACS analysis. As shown in **Fig. 1E - left**, the NT domain did not bind to RD cells even at a high concentration of 10 μg/mL, suggesting that the binding of HKU1-S1 to RD cells requires regions beyond the NT of S1, the NT domain alone is not sufficient to support the binding. In contrast, the NT of hCoV-OC43-S1(268) protein could bind to RD cells in a dose dependent manner **(Fig. 1E - right),** but the binding activity was relatively weaker than that of HKU1-S1(600) to RD cells.

Binding of HKU1-S1 to RD cells is sialic acid dependent.

Sialic acids serve as attachment factors or receptors for a number of viruses (28, 30, 31). 9-*O*-Ac-sia was found to be essential for viral entry of BCoV and hCoV-OC43 (10, 30). To investigate whether sialic acids are also involved in the binding of HKU1-S1 to RD cells, we first pretreated cells with neuraminidase (NA), a sialidase that removes terminal free or modified sialic acids which are α2,3-, α2,6- or α2,8-linked to the subterminal residue of a sugar chain. Treated cells were then incubated with 5 μg/mL of HKU1-S1(600)-mFc. As shown in **Fig. 2A - left,** pretreatment with NA ranging from 20- 500 mU/mL markedly reduced the binding of HKU1-S1(600) to RD cells in a dose-dependent manner; 500 mU/mL of NA reduced the binding to about 3-5% of the levels observed in the mock treated cells. Similarly, NA treatment of RD cells resulted in the reduction of OC43-S1(268)-mFc binding (**Fig. 2A –middle**). Whereas a control protein SIRPα binding to RD cells via its protein receptor CD47 (32) expressed on RD cell surface was not affected by NA treatment (**Fig. 2A –right**). This result indicated that cell surface sialic acids participated in the binding of HKU1-S1(600) protein to RD cells. To further test whether sialic acids involved in the binding are attached to glycoprotein(s), RD cells were pretreated with TPCK-trypsin protease and then inoculated with 5 μg/mL of HKU1-S1(600)-mFc. As shown in **Fig. 2B**, pretreatment with trypsin dose-dependently reduced the binding of HKU1-S1 to RD cells, the maximum dose tested at 20 μg/mL trypsin reduced the binding to about 20% of the levels in the mock treated cells. Trypsin treatment also similarly reduced binding of OC43-S1 to RD cells. Taken together, these findings suggest that HKU1-S1 protein, similar to OC43-S1, can bind to sialic acids that

Journal of Virology

311 are attached to glycoprotein(s).

312

313 **Unlike S1 of hCoV-OC43, HKU1-S1 cannot bind to 9-*O*-acetylated sialic acid containing**
314 **glycoprotein or RBCs.**

315 To understand the sialic acid specificity and preference of HKU1-S1, bovine submaxillary
316 mucin (BSM), which mainly contains 9-*O*-Ac-sia and 8,9-di-*O*-Ac-sia (33), was first tested for
317 binding with HKU-S1 by ELISA. As shown in **Fig. 2C**, no binding was observed for BSM
318 coated on ELISA plate, whereas the positive control OC43-S1(268)-mFc bound to BSM under
319 the same condition tested**.**

320 Engagement of sialic acid by viruses usually correlates with the capacity to agglutinate
321 red blood cells (RBCs) from different animal species. hCoV-OC43 and influenza C viruses use
322 9-*O*-Ac-sia for attachment, and consequently both viruses have hemagglutination (HA)
323 activity specific for rat and mouse RBCs, which have a high concentration of 9-*O*-Ac-sia on
324 their surface (33, 34), but not for human, sheep, or horse RBC as they have little to no
325 9-*O*-Ac-sia expressed (10, 35, 36). Using OC43-S1(268)-mFc protein as a positive control,
326 both rat and mouse RBCs could be agglutinated by the OC43-S1 protein dose-dependently
327 as expected, but HKU1-S1(600) protein showed no HA activity on RBCs from both species
328 (**Fig. 2D**). Consistently, HKU1-S1 showed no binding whereas OC43-S1 strongly bound to
329 both RBCs dose-dependently in a FACS analysis (**Fig. 2E**). These results suggest that
330 hCoV-HKU1 is different from influenza C, hCoV-OC43 and BCoV in using 9-*O*-Ac-sia as a
331 binding determinant, 9-*O*-Ac-sia alone at least is not sufficient to mediate entry for
332 hCoV-HKU1.

333

334 **HKU1-HE is an O-acetylesterase and possesses RDE activity**

335 By sequence similarity analysis, the HE protein of HKU1 was predicted to have a
336 hemagglutinin domain and a putative sia-*O*-acetyl-esterase active site (15). However there is
337 only 50-57% amino acid conservation between the HKU1-HE and those of other group 2a
338 CoVs. Currently no function for HKU1-HE during infection has been demonstrated.
339 Sia-*O*-acetylesterases and NAs are two types of viral RDEs identified so far.

Sia-*O*-acetylesterases, e.g. 9-*O*-acetylesterase, which was originally found in influenza C viruses and also represented by the HE proteins of CoVs: hCoV-OC43, BCoV, and porcine Toroviruses (23); NAs are present in influenza A and B viruses. NA removes both free and modified terminal sialic acids from a sugar chain, whereas sia-O-acetylesterases removes the O-acetyl modifications from sailic acids. To investigate whether HKU1-HE has similar functions as other CoVs, acting as lectin and/or RDE, we first expressed the extracellular domain of HKU1 HE protein (aa14-358) and fused it to the Fc domain from murine IgG2a (HKU1-HE-mFc) (**Fig. 3A**). When HKU1-HE-mFc protein was incubated with RD cells, no direct binding was detected (**Fig. 3B**). This result suggests that HKU1-HE is unlikely to mediate viral attachment. To determine if the HKU1-HE protein has RDE activity, RD cells were pretreated with HKU1-HE-mFc protein, and then incubated with HKU1-S1(600)-mFc, followed by FACS to determine how much HKU1-S1(600) could still bind to the RD cells. Remarkably, pretreatment of RD cells with HKU1-HE dramatically reduced the binding of S1 to RD cells in a dose-dependent manner (**Fig. 3C)**, similar to the reduction seen followed NA pretreatment. This indicates that the HKU1-HE serves as a RDE for hCoV-HKU1, possessing enzymatic activities capable of cleaving off the binding determinants for the S protein of HKU1 from the surface of the host cell. We next expressed OC43-HE, BCoV-HE and MHV-S-HE proteins (**Fig. 3A**) and compared their RDE activities on RD cells to HKU1-HE's activity in eliminating HKU1-S1 or OC43-S1 binding to the cells. Similar to HKU1-HE, these HE proteins showed no direct binding to RD cells by FACS analysis (Data not shown). Interestingly, HKU1-HE pretreatment also reduced OC43-S1 binding to RD cells; and vice versa pretreatment of cells with OC43-HE not only blocked OC43-S1 binding but also the binding of HKU1-S1 (**Fig. 3C**); BCoV-HE had the same activities as OC43 in acting as a RDE for both HKU1-S1 and OC43-S1 on RD cells; whereas pretreatment of RD cells with MHV-S-HE, which is a 4-*O*-acetylesterase, had no effects on the binding of both HKU1-S1 and OC43-S1 (**Fig. 3C**). Furthermore the HA activity of OC43-S1 on rat and mouse RBCs were not only inhibited by OC43- and BCoV-HE but also HKU1-HE, whereas MHV-S-HE had no effect (**Fig. 3D**). These results suggest that HKU1-HE has similar 9-O-acetylesterase activity as OC43-HE and BCoV-HE, but different from MHV-S-HE.

To further confirm that HKU1-HE indeed acts as a RDE solely by its sia-O-acetylesterase activity, we first determined if HKU1-HE had any neuraminidase activity using an Amplex red neuraminidase assay kit. As expected HKU1-HE had no detectable NA activity (data not shown). On the other hand, HKU1-HE protein showed strong acetylesterase activity as measured by using chromogenic p-nitrophenyl acetate (pNPA) as substrate (**Fig. 3E**). HKU1-HE hydrolyzed pNPA, released para-nitrophenol (pNP) product with a Km=0.28±0.1 mM, Vmax=0.77±0.03 in the presence of 2 μg/mL of HKU1-HE protein. Similarly, OC43-HE, BCoV-HE and MHV-S-HE were measured for acetylesterase activity, they all showed stronger acetylesterase activity than HKU1-HE (**Fig. 3E**) with parameters Km=1.20 ± 0.06mM, Vmax=3.32±0.00 for OC43-HE, Km=1.50±0.09mM, Vmax=3.42±0.04 for BCoV-HE and Km=1.25 ± 0.01mM, Vmax=3.34 ± 0.04 for MHV-S-HE, respectively. Considering that HKU1-HE treatment has the same effect as NA treatment of RD cells on the HKU1-S1 binding and it has no NA activity, it is conceivable that the HKU1-HE mediates RDE through its sialate-9-*O*-acetylesterase activity similar to BCoV- and OC43-HE.

Sequence alignment of HKU1-HE protein against HEF of influenza C, HEs of hCoV-OC43, BCoV, MHV and Toroviruses revealed that the conserved sialate-*O*-acetylesterase catalytic active sites (S40, H329 and D326 catalytic triad (24)) are also present in HE protein of HKU1 (**Fig. 4A**). BCoV-HE mutant protein containing S40A substitution has been demonstrated to be enzymatically inactive (24). To determine if the three amino acids were critical for the acetylesterase activity of HKU1-HE, HE mutants containing single S40A or triple S40A/H329A/D326A mutations were expressed and tested for their capacity to hydrolyze pNPA substrate. As shown in **Fig. 4B,** both HE mutants completely lost esterase activity. Furthermore, unlike the wild type HE protein, pretreatment of RD cells with these acetylesterase inactive mutant HEs had no effect on HKU1-S1 protein binding to RD cells (**Fig. 4C)**. This result suggests that HKU1-S1 binding required specific type(s) of *O*-acetylated sialic acids to be present on the cell surface, which corresponds to the sialic acid acetylesterase specificity of the HKU1-HE. Considering that HKU1-S1 did not bind to 9-*O*-Ac containing BSM and RBCs, combined with the result that HKU1-HE and OC43-HE mutually served as RDE for their S1 proteins binding to RD cells, it is very likely that the acetyl modification at 9-O position

of sialic acid is a necessary but not sufficient binding determinant for HKU1-S protein. Accordingly, HKU1-HE protein possesses sialate-9-*O*-acetylesterase activity or even broader sialate-*O*-acetylesterase activity.

HKU1-HE inhibited hCoV-HKU1 infection in human tracheobronchial epithelial cultures (HAE) via its RDE activity.

Though HKU1-S protein can bind to RD cells, the lentivirus-based HKU1 spike protein pseudovirus was not able to enter RD cells (data not shown), and previous attempts to culture clinical isolates of HKU1 in RD cells also failed (20). To date no cell line has been found to be permissive for hCoV-HKU1 infection. Only the HAE primary cell culture system utilizing well-differentiated human bronchial epithelial cells has been demonstrated to be a robust *in vitro* model for hCoV-HKU1 infection and propagation (20). HAE cultures have also been successfully used as a model system for studying SARS-CoV, hCoV-NL63, hCoV-229E, MERS-CoV and hCoV-OC43 (22, 37-39). The infection and propagation of hCoV-HKU1 in the HAE cultures models natural infection of the human upper respiratory tract. To determine whether sialic acids are important during hCoV-HKU1 infection and whether HKU1-HE protein possesses RDE activity in a natural infection model, HAE were incubated with NA, HKU1-HE or control proteins for 1 hr and removed by washing prior to hCoV-HKU1 virus inoculation. Apical washes of infected cultures were collected over time until 96 hrs post inoculation for RNA isolation and the number of viral genomic RNA copies was analyzed by real-time RT-PCR to determine the level of viral infection. As shown in **Fig. 4D**, both NA and HKU1-HE pretreatment of HAE markedly inhibited hCoV-HKU1 infection. HKU1-HE at 100 μg/mL reduced viral titers by 2-3 logs at 48, 72 and 96 hrs post viral inoculation. NA also showed dose-dependent inhibition of viral infection though not as efficient as HE protein. Pre-incubation of HAE with HKU1-HE protein inhibited viral replication suggesting that HKU1-HE can destroy sialic acid moieties required for hCoV-HKU1 entry. When a lower titer of HKU1 virus inocula was used, HKU1-HE protein at 100 μg/mL completely blocked hCoV-HKU1 replication in HAE, whereas an enzymatic inactive HE variant HKU1-HE-S40A showed no inhibition activity (**Fig. 4E**). These results demonstrate that hCoV-HKU1 uses

427 *O*-acytylated sialic acids as an attachment factor and that this interaction is required for
428 efficient infection of the target cell; HKU1-HE protein possesses sialate-*O*-acetylesterase
429 RDE activity.
430
431 **Discussion**
432 Sialic acid, a 9-carbon monosaccharide, includes a large number of derivatives arising
433 from differential modifications of the parental molecule as well as various glycosidic linkage
434 (e.g., α2,3 or α2,6) to the subterminal residue of a sugar chain. *O*-acetylation is one of the
435 most common types of sialic acid modification. It can occur at all the four hydroxyl groups of
436 sialic acids at positions of C4, C7, C8 and C9, generates mostly mono-*O*-acetylated but also
437 oligo-*O*-acetylated sialic acids at more than one position. *O*-AC-Sia plays fundamental roles in
438 many biological and pathophysiological events (40). The 9-*O*-Ac-Sia serves as a receptor
439 determinant for several members of Group 2a CoVs, including the closely related BCoV,
440 hCoV-OC43, and porcine hemagglutinating encephalomyelitis virus (PHEV) (10, 30, 41). The
441 binding with 9-*O*-Ac-Sia is essential for these viruses to initiate infection and their S protein is
442 the major viral protein responsible for the binding. In this study, we found that the S protein of
443 hCoV-HKU1 can also recognize *O*-Ac-Sia but only those presented on RD cells among the
444 many cell lines tested. Different from the aforementioned CoVs in the same group, no binding
445 of hCoV-HKU1 S1 with 9-*O*-Ac-Sia containing BSM as well as RBCs from mouse and rat
446 could be detected. In addition, a previous study (42) and our data (Fig. 1E) both demonstrated
447 that the NT of HKU1-S1 was unable to bind with carbohydrate moieties, in contrast the
448 carbohydrate receptor binding domains for hCoV-OC43 and BCoV were located in the NT of
449 S1 (Fig. 2) (42). On the other hand, for both hCoV-HKU1 and other 9-*O*-Ac-Sia-recognition
450 dependent CoVs, the question remains as to whether in addition to *O*-Ac-Sia, they also
451 interact with a protein receptor during the entry process. *O*-Ac-Sia on RD cells can be
452 recognized by hCoV-HKU1 S1 protein, however the cells are not permissive for viral infection.
453 One explanation for this is the lack of a protein receptor for hCoV-HKU1 on RD cells. In line
454 with this, our attempts using HKU1-S1(600) as a viral ligand protein for immunoprecipitation
455 and combining with mass spectrometric identification did not find a protein(s) specific binding

456 to HKU1-S1 (data not shown). It is also possible that there are other molecule(s) present only
457 in HAE cultures but not on RD cells, which are important for viral infection at a later stage, e.g.
458 membrane fusion or viral replication; or a restriction factor(s) that may exist in RD cells to limit
459 viral infection.
460
461 Among CoVs, HE protein is only present in members of group 2a CoVs. Sequence and
462 structural similarity suggest that CoV HE evolved from the HEF protein of influenza C virus
463 (24). Although the dual function of HEF (*O*-Ac-Sia receptor binding and
464 sialate-*O*-acetylesterase activity) was maintained in some CoVs, the HE appears to mainly
465 function as a RDE in these CoVs. Comparing to the essential role of S protein in sia-receptor
466 binding and mediating viral entry, the sia-binding activity of HE seems to be an accessory
467 function and varies in its affinity and sia-preference among CoV strains (23, 43). HEs of two
468 closely related MHV field strains, MHV-DVIM and MHV-S, recognize two different types of
469 *O*-Ac-Sia, 9-*O*-Ac-Sia and 4-*O*-Ac-Sia, respectively, whereas many MHV laboratory strains
470 carry defective HE genes (27, 28). HCoV-OC43 HE lost its sia-binding activity although it has
471 high sequence identity (97%) with the HE of BCoV (Mebus stain) which exhibits high
472 sia-binding affinity (43). Similarly, we did not find that HKU1-HE has *O*-Ac-Sia binding
473 activity. This is consistent with the observation described previously by Langereis et al. that
474 the HE of hCoV-HKU1 failed to hemagglutinate erythrocytes and bind to *O*-Ac-Sia (43).
475 Sequence comparison of HKU1-HE with influenza C's HEF and HEs of CoVs with known
476 protein structures demonstrated that HKU1-HE was the most divergent one at the Sia-binding
477 loops (23, 43), whereas the Sia-*O*-acetylesterase domain is highly conserved among them.
478 The key residues contributing to the catalytic activity of HEF and other CoV HEs are
479 completely conserved in hCoV-HKU1, including the Ser-His-Asp catalytic triad, the oxyanion
480 hole contributing residues Gly_{85} and Asn_{117} in HEF, and an Arg_{322} residue in HEF important for
481 Sia substrate binding (23). In our study, HKU1-HE indeed showed strong *O*-AC-esterase
482 activity with pNPA substrate, and the Ser-His-Asp catalytic triad mutant HEs completely lost
483 this activity. We demonstrated that the sialic acids expressed by RD cells are not only
484 specifically recognized by the HKU1-S1 protein but also are substrates for HE protein.

Wild-type but not mutant HE treatment of RD cells showed similar effect as neuraminidase and abolished the subsequent binding of S1 to RD cells. These results indicate that the S1 protein binds with *O*-Ac-Sia and the HE has the matching Sia-*O*-Ac or even broader esterase activity.

Langereis et al (43) reported that the HKU1-HE like the BCoV-HE displayed Sia-9-*O*-Ac-esterase activity using a synthetic 4,9-di-*O*-Ac-Sia substrate analogue. Consistently, we demonstrated that HKU1-HE had similar RDE activity as OC43-HE and BCoV-HE to remove the binding moiety from RD cells for S1 proteins of both HKU1 and OC43. These results strongly support that HKU1-HE has similar Sia-9-*O*-Ac-esterase activity as OC43-HE and BCoV-HE. However, unlike the S1 of OC43, Sia-9-*O*-AC binding activity was not detected for the S1 of hCoV-HKU1 by BSM binding and erythrocytes hemagglutination assays which are standard methods for examining the usage of Sia-9-*O*-AC as a receptor by other CoVs (**Fig. 2C-E**). Thus it is likely that HKU1 is different than OC43 and BCoV in terms of using Sia-9-*O*-AC as a receptor via its Spike. Sia-9-*O*-AC may be required but not sufficient to support the HKU1-S1 binding. In addition of Sia-9-*O*-AC, RD cells may express other type of *O*-Acetylated sialic acid that is lacking or at lower level in BSM and erythrocyte cell surface but required for HKU1-S1 recognition. One may also speculate that certain di-*O*-Ac-Sia, tri-*O*-Ac-Sia or oligo-*O*-acetylated Sia in which all have an acetyl group at C9 (9-*O*-AC) in common; or certain particular sugar chain core structure(s) to which Sia-9-*O*-Ac attached or the linkage of sialic acid to the penultimate residue of a sugar chain may also be required.

Finally, in HAE cultures, we demonstrated that NA and HKU1-HE but not enzymatic inactive HE mutant dramatically reduced virus infection or completely blocked infection when lower viral challenge dose was applied. Treatment of HAEs with NA and HKU1-HE prior to infection strongly suggests that the sia-9-O-AC-esterase activity of HE acted as a RDE and removed the critical receptor binding moieties so that the early viral entry was impaired. Considering the HAE cells continuously secret large amount of mucus, the effect of inhibition of viral infection by pretreatment of the cells with NA and HE is remarkable and suggests an

Journal of Virology

514 essential role for sialic acids to initiate infection. Our study also suggests that acetyl
515 modification at 9-O of sialic acid may be a necessary but not sufficient receptor or attachment
516 factor determinant, and warrants further investigation to determine the fine specificity and
517 preference of sialic acids recognized by HKU1-S protein. Nevertheless, for the first time this
518 study provided experimental evidence to support *O*-Ac-Sia, by interacting with S1 of
519 hCoV-HKU1, serves as an essential determinant for viral attachment during the early entry
520 step, and HE possesses 9-O-AC-esterase or even broader activity and primarily acts as a
521 RDE for hCoV-HKU1 infection. HCoV-HKU1 is similar to BCoV and hCoV-OC43 employing its
522 two surface proteins S and HE to complete the viral infection cycle in a concerted manner with
523 S protein mediating receptor-binding and entry, HE protein mediating RDE activity late in the
524 infection cycle to facilitate viral progeny release and achieve efficient virus dissemination (23,
525 26).
526
527 **Figure Legends**
528
529 **Fig. 1. Specific binding of HKU1-S1 to RD cells**. **(A)** SDS-PAGE of expressed
530 recombinant S1 or control proteins. All proteins were expressed in 293T cells and purified by
531 protein A sepharose beads. Purified proteins were run on SDS-PAGE and stained by
532 coomassie blue. **(B)** FACS analysis of HKU1-S1(600)-mFc (5 μg/mL) binding to RD and Hela
533 cells. HKU3-323-mFc was used a negative control protein. **(C)** HKU1-S1(600)-mFc
534 binding to a molecule(s) located on RD cell surface. Immunofluorescence microscopy imaging
535 of RD cells stained by HKU1-S1(600)-mFc or control protein, HKU3-323-mFc. Cells
536 membrane was stained with FM-4-64 in red, Hoechst dye 33258 stained the nuclei (blue), and
537 the HKU1-S1 staining was detected by a FITC-labeled anti-mouse Fc antibody. **(D)**
538 HKU1-S1(600)-mFc binding to RD cells in a dose dependent manner in a FACS analysis. (**E**)
539 FACS analysis of the N-terminals of HKU1-S1 and OC43-S1 binding to RD cells.
540 HKU3-323-mFc at 10 μg/mL was used as a control (labeled as Ctrl.) in panels D and E.
541 Graphs shown in panel B-E are reprehensive of at least two independent experiments for
542 each panel.

Fig. 2. Characterization of the binding receptor of HKU1-S1 protein on RD cells by FACS. (A) Pretreatment of RD cells with Neuramidase (NA) greatly reduced HKU1-S1 binding in a dose-dependent manner. RD cells were pretreated with NA at different concentrations as indicated and then stained with 5 μg/mL of HKU1-S1(600)-mFc, OC43(268)-mFc or SIRPα-mFc control protein, respectively, then followed by detecting with a FITC-labeled anti-mouse Fc antibody. **(B)** Pretreatment of RD cells with TPCK-treated Trypsin reduced HKU1-S1(600) and OC43-S1(268) binding to RD cells. The FACS assay method was similar as described in panel A. Ctrl. indicates RD cells were stained with HKU1-S1(268)-mFc only in panel A and B. **(C)** HKU1-S1(600)-mFc did not bind to BSM (containing 9-*O*-Ac-sia) directly coated on ELISA plate. OC43-S1 served as a positive control. **(D)** HKU-S1 did not hemagglutinate mouse or rat RBCs. OC43-S1(268)-mFc served as a positive control and showed HA activity dose-dependently. **(E)** Binding of HKU1-S1 and OC43-S1 to rat and mouse RBCs by FACS analysis. Data shown are reprehensive of at least two independent experiments for each panel.

Fig. 3. HKU1-HE is an *O*-acetylesterase and RDE for HKU1-S1 binding to RD cells. (A) SDS-PAGE of expressed recombinant HE proteins. All proteins were expressed in 293T cells and purified by protein A sepharose beads. Purified proteins were run on SDS-PAGE and stained by coomassie blue. **(B)** HE-mFc protein itself did not bind to RD cells. **(C)** HKU1-HE acted as a RDE on RD cells for HKU1- and OC43-S1 protein. Pretreatment of RD cells with HKU1-HE as well as OC43- and BCoV-HE greatly reduced HKU1-S1 or OC43-S1 (at 5 μg/mL) binding to RD cells in a dose-dependent manner. MHV-S-HE showed no effect. Graphs shown in panel B-C are reprehensive of at least two independent experiments. **(D)** HKU1-HE acted as a RDE on RBCs for OC43-S1 protein. Pretreatment of rat or mouse RBCs with HKU1-, OC43-, BCoV-HE inhibited OC43-S1 mediated hemagglutination activity, whereas MHV-S-HE had no effect. Representative data or images of at least two independent experiments were shown for panels A-D. **(E)** HKU1-HE is an acetylesterase. HEs of HKU1, OC43, BCoV and MHV-S at 2 μg/mL were used to hydrolyze pNPA (2-fold serially diluted) at

room temperature for 15 mins. Enzyme activity was assessed by measuring optical density at 405 nm (OD_{405}). The Km and Vmax values were calculated from the Michaelis-Menten Enzyme Kinetics curve fitting of the two independent repeats.

Fig. 4. Treatment of HAE with HE or NA but not the enzymatic inactive HE mutant inhibited HKU1 infection. (A) Esterase catalytic active site residues in HE. Sequence alignment of amino acids around the catalytic active site (in red) was shown. BCoV-HE amino acid numbering scheme was used (22). GenBank accession numbers of HE proteins of BCoV, OC43, HKU1, MHV-DVIM and influenza C virus are AAA92991.1, AAX85668.1, NC_006577.2, AAC63044.1 and AJ872181, respectively. For HE proteins of MHV-S, bovine Torovirus (BToV), and porcine Torovirus (PToV) stain p10, the Uniprot accession numbers are P31614, P0C0V9, and Q70KP1, respectively. **(B)** HKU1-HE proteins with substitutions at catalytic active sites are enzymatic inactive. HKU1-HE or the mutants at 1 μg/mL were incubated with 2-fold serially diluted pNPA at room temperature for 15 mins prior to measuring OD_{405}. Each data points represent OD_{405} of HE or its mutants subtracted with that of a negative control. **(C)** Esterase inactive mutants of HE did not block HKU1-S1 protein binding to RD cells. RD cells were treated with 10 μg/mL of HE or HE mutant proteins prior to HKU1-S1 staining (5 μg/mL). **(D)** HE and NA pretreatment of HAE inhibited HKU1 infection. Prior to HKU1 virus inoculation, HAE cells were pretreated with HE proteins or NA at different concentrations as indicated for 1 hr, followed by HKU1 virus inoculation at high dose as indicated. Replication kinetics of HKU1 virus was assessed in apical washes from infected HAE cultures by real-time RT-PCR. Virus yield is presented as number of virus RNA copies/mL. **(E)** HE pretreatment blocked HKU1 infection of HAE cells. HAE were pretreated with HE protein or enzymatic inactive HE mutant (S40A) for 1 hour prior to viral challenge at lower dose than used panel D. Replication kinetics of HKU1 virus was assessed as in panel D. Dotted line indicates the detection limit of the assay for panel D-E.

Acknowledgement:

This work was supported by the Ministry of Science and Technology, China to JS

(2012CB837602), to WL (2010CB530101) and the Science and Technology Bureau of Beijing Municipal Government to WL and JS, and by the Thousand Young Talents Program, China to JS, the National Centre for Research and Development, Poland (Lider/27/55/L-2/10/2011) and the National Science Center, Poland (UMO-2012/07/E/NZ6/01712) to KP, and NIH (U19 AI109761) to RSB and NIH (AI085524) to WAM and RSB.

References:

1. **Graham RL, Donaldson EF, Baric RS.** 2013. A decade after SARS: strategies for controlling emerging coronaviruses. Nature reviews. Microbiology **11:**836-848.
2. **Rota PA, Oberste MS, Monroe SS, Nix WA, Campagnoli R, Icenogle JP, Penaranda S, Bankamp B, Maher K, Chen M-h, Tong S, Tamin A, Lowe L, Frace M, DeRisi JL, Chen Q, Wang D, Erdman DD, Peret TCT, Burns C, Ksiazek TG, Rollin PE, Sanchez A, Liffick S, Holloway B, Limor J, McCaustland K, Olsen-Rasmussen M, Fouchier R, Gunther S, Osterhaus ADME, Drosten C, Pallansch MA, Anderson LJ, Bellini WJ.** 2003. Characterization of a Novel Coronavirus Associated with Severe Acute Respiratory Syndrome. Science **300:**1394-1399.
3. **Ksiazek TG, Erdman D, Goldsmith CS, Zaki SR, Peret T, Emery S, Tong S, Urbani C, Comer JA, Lim W, Rollin PE, Dowell SF, Ling AE, Humphrey CD, Shieh WJ, Guarner J, Paddock CD, Rota P, Fields B, DeRisi J, Yang JY, Cox N, Hughes JM, LeDuc JW, Bellini WJ, Anderson LJ, Group SW.** 2003. A novel coronavirus associated with severe acute respiratory syndrome. The New England journal of medicine **348:**1953-1966.
4. **Anderson LJ, Tong S.** 2010. Update on SARS research and other possibly zoonotic coronaviruses. International journal of antimicrobial agents **36 Suppl 1:**S21-25.
5. **Assiri A, McGeer A, Perl TM, Price CS, Al Rabeeah AA, Cummings DA, Alabdullatif ZN, Assad M, Almulhim A, Makhdoom H, Madani H, Alhakeem R, Al-Tawfiq JA, Cotten M, Watson SJ, Kellam P, Zumla AI, Memish ZA.** 2013. Hospital outbreak of Middle East respiratory syndrome coronavirus. N Engl J Med **369:**407-416.
6. **Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus AD, Fouchier RA.** 2012. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. N Engl J Med **367:**1814-1820.
7. **Coleman CM, Frieman MB.** 2014. Coronaviruses: important emerging human pathogens. Journal of virology **88:**5209-5212.
8. **Heald-Sargent T, Gallagher T.** 2012. Ready, set, fuse! The coronavirus spike protein and acquisition of fusion competence. Viruses **4:**557-580.
9. **Yeager CL, Ashmun RA, Williams RK, Cardellichio CB, Shapiro LH, Look AT, Holmes KV.** 1992. Human aminopeptidase N is a receptor for human coronavirus 229E. Nature **357:**420-422.
10. **Vlasak R, Luytjes W, Spaan W, Palese P.** 1988. Human and bovine coronaviruses recognize sialic acid-containing receptors similar to those of influenza C viruses. Proceedings of the National Academy of Sciences of the United States of America **85:**4526-4529.
11. **Hofmann H, Pyrc K, van der Hoek L, Geier M, Berkhout B, Pohlmann S.** 2005. Human coronavirus NL63 employs the severe acute respiratory syndrome coronavirus receptor for cellular entry. Proceedings of the National Academy of Sciences of the United States of America **102:**7988-7993.

12. **Li W, Moore MJ, Vasilieva N, Sui J, Wong SK, Berne MA, Somasundaran M, Sullivan JL, Luzuriaga K, Greenough TC, Choe H, Farzan M.** 2003. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. Nature **426:**450-454.
13. **Milewska A, Zarebski M, Nowak P, Stozek K, Potempa J, Pyrc K.** 2014. Human coronavirus NL63 utilize heparan sulfate proteoglycans for attachment to target cells. Journal of virology.
14. **Lu G, Hu Y, Wang Q, Qi J, Gao F, Li Y, Zhang Y, Zhang W, Yuan Y, Bao J, Zhang B, Shi Y, Yan J, Gao GF.** 2013. Molecular basis of binding between novel human coronavirus MERS-CoV and its receptor CD26. Nature **500:**227-231.
15. **Woo PC, Lau SK, Chu CM, Chan KH, Tsoi HW, Huang Y, Wong BH, Poon RW, Cai JJ, Luk WK, Poon LL, Wong SS, Guan Y, Peiris JS, Yuen KY.** 2005. Characterization and complete genome sequence of a novel coronavirus, coronavirus HKU1, from patients with pneumonia. Journal of virology **79:**884-895.
16. **Jevsnik M, Ursic T, Zigon N, Lusa L, Krivec U, Petrovec M.** 2012. Coronavirus infections in hospitalized pediatric patients with acute respiratory tract disease. BMC infectious diseases **12:**365.
17. **Dominguez SR, Shrivastava S, Berglund A, Qian Z, Goes LG, Halpin RA, Fedorova N, Ransier A, Weston PA, Durigon EL, Jerez JA, Robinson CC, Town CD, Holmes KV.** 2014. Isolation, propagation, genome analysis and epidemiology of HKU1 betacoronaviruses. The Journal of general virology **95:**836-848.
18. **Lau SK, Woo PC, Yip CC, Tse H, Tsoi HW, Cheng VC, Lee P, Tang BS, Cheung CH, Lee RA, So LY, Lau YL, Chan KH, Yuen KY.** 2006. Coronavirus HKU1 and other coronavirus infections in Hong Kong. Journal of clinical microbiology **44:**2063-2071.
19. **Woo PC, Lau SK, Yip CC, Huang Y, Yuen KY.** 2009. More and More Coronaviruses: Human Coronavirus HKU1. Viruses **1:**57-71.
20. **Pyrc K, Sims AC, Dijkman R, Jebbink M, Long C, Deming D, Donaldson E, Vabret A, Baric R, van der Hoek L, Pickles R.** 2010. Culturing the unculturable: human coronavirus HKU1 infects, replicates, and produces progeny virions in human ciliated airway epithelial cell cultures. Journal of virology **84:**11255-11263.
21. **Dominguez SR, Travanty EA, Qian Z, Mason RJ.** 2013. Human coronavirus HKU1 infection of primary human type II alveolar epithelial cells: cytopathic effects and innate immune response. PLoS One **8:**e70129.
22. **Dijkman R, Jebbink MF, Koekkoek SM, Deijs M, Jonsdottir HR, Molenkamp R, Ieven M, Goossens H, Thiel V, van der Hoek L.** 2013. Isolation and characterization of current human coronavirus strains in primary human epithelial cell cultures reveal differences in target cell tropism. Journal of virology **87:**6081-6090.
23. **de Groot RJ.** 2006. Structure, function and evolution of the hemagglutinin-esterase proteins of corona- and toroviruses. Glycoconjugate journal **23:**59-72.
24. **Zeng Q, Langereis MA, van Vliet AL, Huizinga EG, de Groot RJ.** 2008. Structure of coronavirus hemagglutinin-esterase offers insight into corona and influenza virus evolution. Proceedings of the National Academy of Sciences of the United States of America **105:**9065-9069.
25. **Vijgen L, Keyaerts E, Moes E, Thoelen I, Wollants E, Lemey P, Vandamme AM, Van Ranst M.** 2005. Complete genomic sequence of human coronavirus OC43: molecular clock analysis suggests a relatively recent zoonotic coronavirus transmission event. Journal of virology **79:**1595-1604.
26. **Desforges M, Desjardins J, Zhang C, Talbot PJ.** 2013. The acetyl-esterase activity of the hemagglutinin-esterase protein of human coronavirus OC43 strongly enhances the production of infectious virus. Journal of virology **87:**3097-3107.
27. **Langereis MA, van Vliet AL, Boot W, de Groot RJ.** 2010. Attachment of mouse hepatitis virus to

O-acetylated sialic acid is mediated by hemagglutinin-esterase and not by the spike protein. Journal of virology **84:**8970-8974.

28. **Langereis MA, Zeng Q, Heesters BA, Huizinga EG, de Groot RJ.** 2012. The murine coronavirus hemagglutinin-esterase receptor-binding site: a major shift in ligand specificity through modest changes in architecture. PLoS pathogens **8:**e1002492.

29. **Lau SK, Woo PC, Li KS, Huang Y, Tsoi HW, Wong BH, Wong SS, Leung SY, Chan KH, Yuen KY.** 2005. Severe acute respiratory syndrome coronavirus-like virus in Chinese horseshoe bats. Proceedings of the National Academy of Sciences of the United States of America **102:**14040-14045.

30. **Schwegmann-Wessels C, Herrler G.** 2006. Sialic acids as receptor determinants for coronaviruses. Glycoconjugate journal **23:**51-58.

31. **Stroh LJ, Stehle T.** 2014. Glycan Engagement by Viruses:Receptor Switches and Specificity. Annu. Rev. Virol. **1:**285-306.

32. **Barclay AN, Van den Berg TK.** 2014. The interaction between signal regulatory protein alpha (SIRPalpha) and CD47: structure, function, and therapeutic target. Annual review of immunology **32:**25-50.

33. **Ravindranath MH, Higa HH, Cooper EL, Paulson JC.** 1985. Purification and characterization of an O-acetylsialic acid-specific lectin from a marine crab Cancer antennarius. The Journal of biological chemistry **260:**8850-8856.

34. **Traving C, Schauer R.** 1998. Structure, function and metabolism of sialic acids. Cellular and molecular life sciences : CMLS **54:**1330-1349.

35. **Rogers GN, Herrler G, Paulson JC, Klenk HD.** 1986. Influenza C virus uses 9-O-acetyl-N-acetylneuraminic acid as a high affinity receptor determinant for attachment to cells. The Journal of biological chemistry **261:**5947-5951.

36. **Bulai T, Bratosin D, Pons A, Montreuil J, Zanetta JP.** 2003. Diversity of the human erythrocyte membrane sialic acids in relation with blood groups. FEBS letters **534:**185-189.

37. **B SB, Orenstein JM, Fox LM, Randell SH, Rowley AH, Baker SC.** 2009. Human airway epithelial cell culture to identify new respiratory viruses: coronavirus NL63 as a model. Journal of virological methods **156:**19-26.

38. **Mossel EC, Wang J, Jeffers S, Edeen KE, Wang S, Cosgrove GP, Funk CJ, Manzer R, Miura TA, Pearson LD, Holmes KV, Mason RJ.** 2008. SARS-CoV replicates in primary human alveolar type II cell cultures but not in type I-like cells. Virology **372:**127-135.

39. **Scobey T, Yount BL, Sims AC, Donaldson EF, Agnihothram SS, Menachery VD, Graham RL, Swanstrom J, Bove PF, Kim JD, Grego S, Randell SH, Baric RS.** 2013. Reverse genetics with a full-length infectious cDNA of the Middle East respiratory syndrome coronavirus. Proceedings of the National Academy of Sciences of the United States of America **110:**16157-16162.

40. **Schauer R, Schmid H, Pommerencke J, Iwersen M, Kohla G.** 2001. Metabolism and role of O-acetylated sialic acids. Advances in experimental medicine and biology **491:**325-342.

41. **Vijgen L, Keyaerts E, Lemey P, Maes P, Van Reeth K, Nauwynck H, Pensaert M, Van Ranst M.** 2006. Evolutionary history of the closely related group 2 coronaviruses: porcine hemagglutinating encephalomyelitis virus, bovine coronavirus, and human coronavirus OC43. Journal of virology **80:**7270-7274.

42. **Peng G, Sun D, Rajashankar KR, Qian Z, Holmes KV, Li F.** 2011. Crystal structure of mouse coronavirus receptor-binding domain complexed with its murine receptor. Proceedings of the National Academy of Sciences of the United States of America **108:**10696-10701.

730 43. **Langereis MA.** 2011. Viral hemagglutinin-esterases; mediators of dynamic virion-glycan interactions.
731 Ph.D. thesis. University of Utrecht, Utrecht. http://dspace.library.uu.nl/handle/1874/211181.

Figure 1

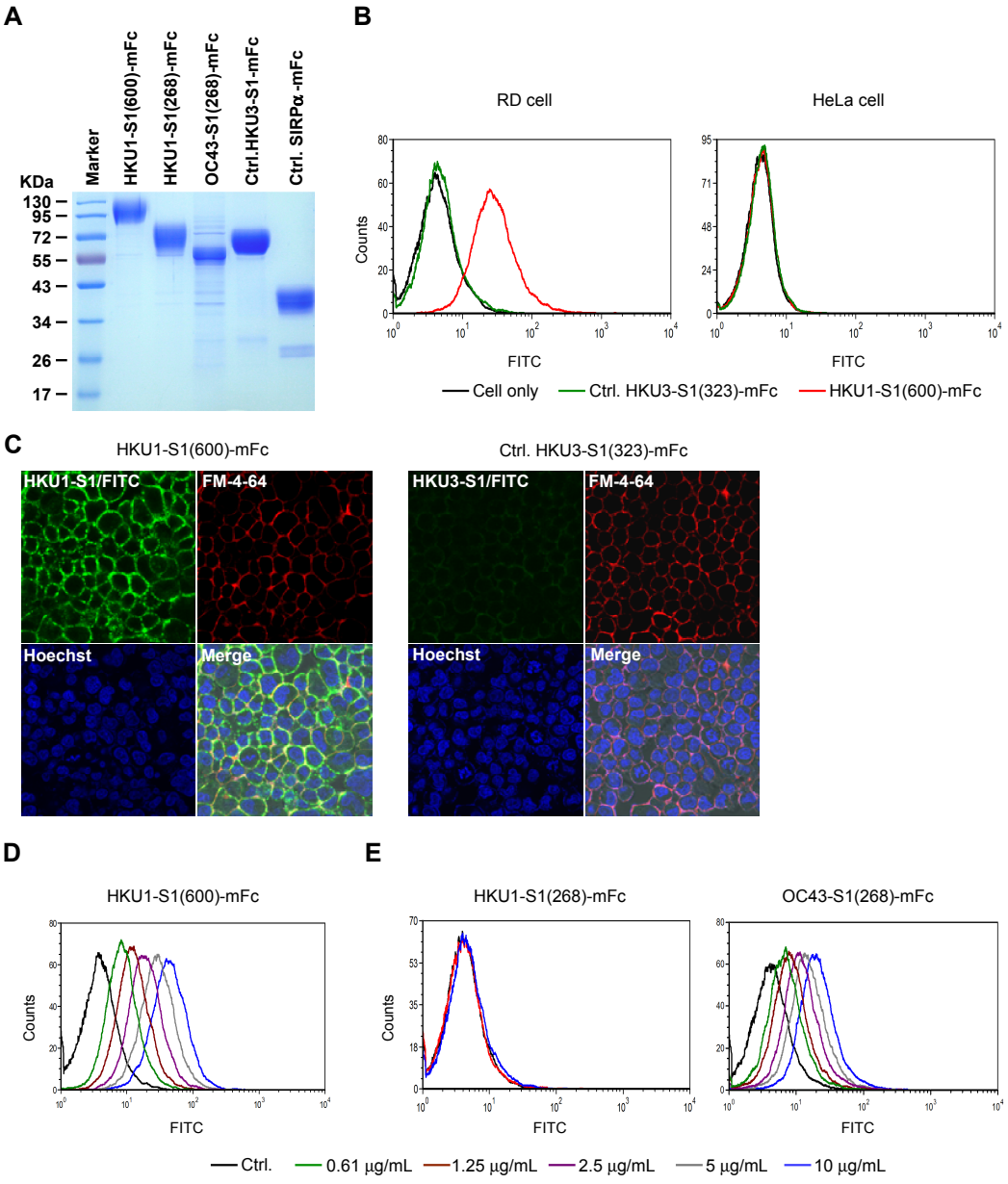

Fig. 1. **Specific binding of HKU1-S1 to RD cells**. **(A)** SDS-PAGE of expressed recombinant S1 or control proteins. All proteins were expressed in 293T cells and purified by protein A sepharose beads. Purified proteins were run on SDS-PAGE and stained by coomassie blue. **(B)** FACS analysis of HKU1-S1(600)-mFc (5 μg/mL) binding to RD and Hela cells. HKU3-323-mFc was used a negative control protein. **(C)** HKU1-S1(600)-mFc binding to a molecule(s) located on RD cell surface. Immunofluorescence microscopy imaging of RD cells stained by HKU1-S1(600)-mFc or control protein, HKU3-323-mFc. Cells membrane was stained with FM-4-64 in red, Hoechst dye 33258 stained the nuclei (blue), and the HKU1-S1 staining was detected by a FITC-labeled anti-mouse Fc antibody. **(D)** HKU1-S1(600)-mFc binding to RD cells in a dose dependent manner in a FACS analysis. **(E)** FACS analysis of the N-terminals of HKU1-S1 and OC43-S1 binding to RD cells. HKU3-323-mFc at 10 μg/mL was used as a control (labeled as Ctrl.) in panels D and E. Graphs shown in panel B-E are reprehensive of at least two independent experiments for each panel.

Figure 2

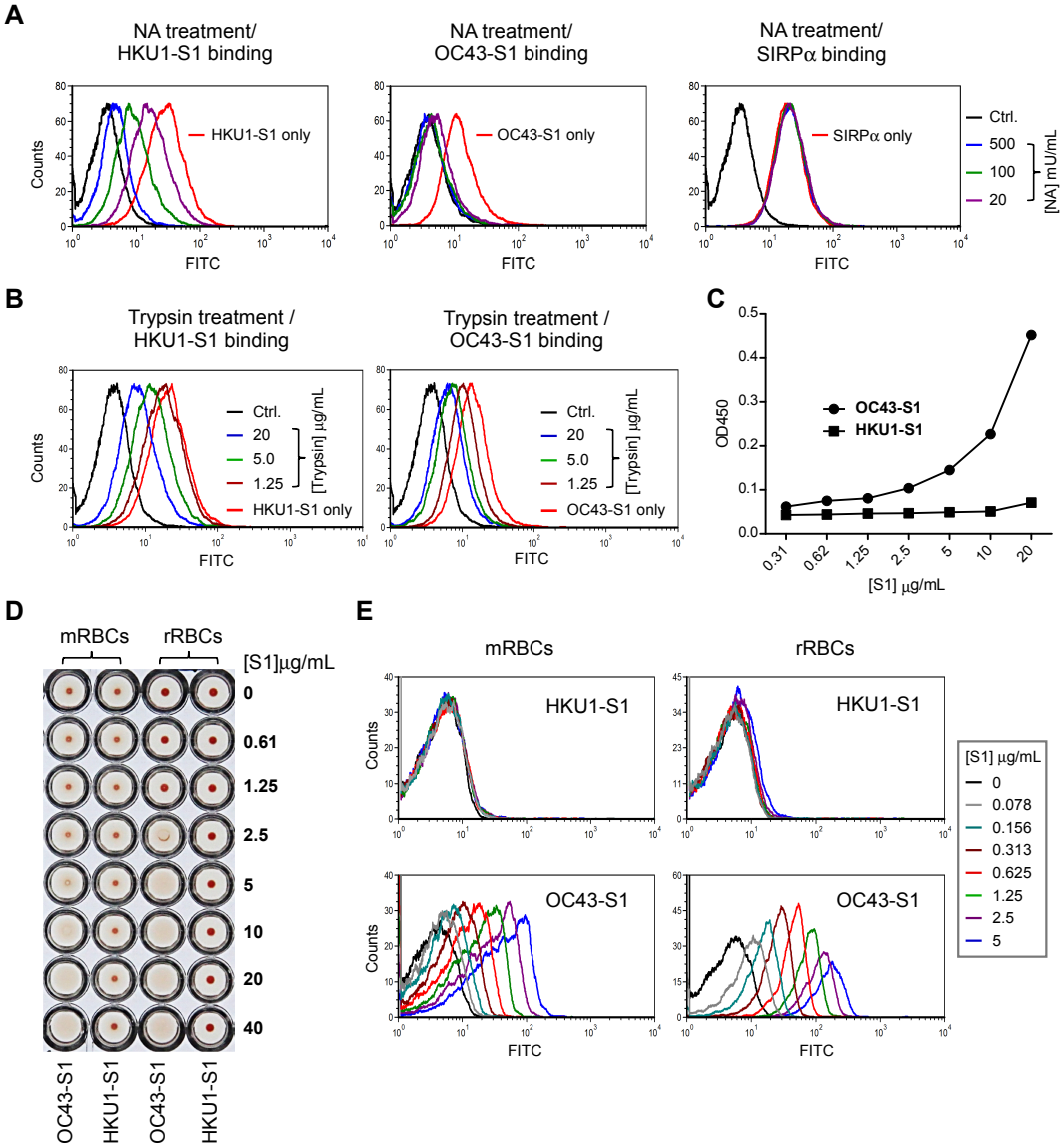

Fig. 2. Characterization of the binding receptor of HKU1-S1 protein on RD cells by FACS. (A) Pretreatment of RD cells with Neuramidase (NA) greatly reduced HKU1-S1 binding in a dose-dependent manner. RD cells were pretreated with NA at different concentrations as indicated and then stained with 5 μg/mL of HKU1-S1(600)-mFc, OC43(268)-mFc or SIRPα-mFc control protein, respectively, then followed by detecting with a FITC-labeled anti-mouse Fc antibody. **(B)** Pretreatment of RD cells with TPCK-treated Trypsin reduced HKU1-S1(600) and OC43-S1(268) binding to RD cells. The FACS assay method was similar as described in panel A. Ctrl. indicates RD cells were stained with HKU1-S1(268)-mFc only in panel A and B. **(C)** HKU1-S1(600)-mFc did not bind to BSM (containing 9-O-Ac-sia) directly coated on ELISA plate. OC43-S1 served as a positive control. **(D)** HKU-S1 did not hemagglutinate mouse or rat RBCs. OC43-S1(268)-mFc served as a positive control and showed HA activity dose-dependently. **(E)** Binding of HKU1-S1 and OC43-S1 to rat and mouse RBCs by FACS analysis. Data shown are reprehensive of at least two independent experiments for each panel.

Figure 3

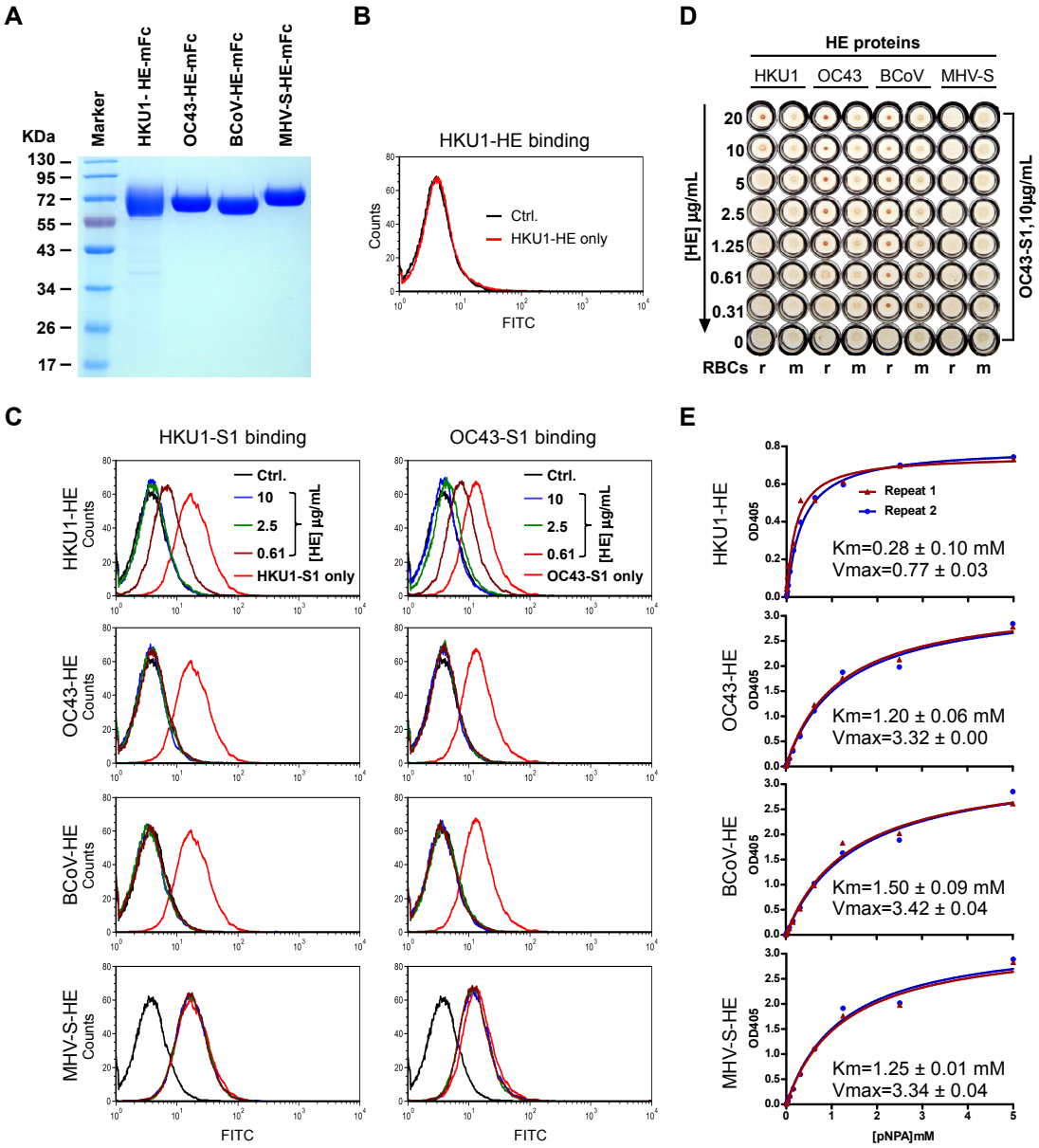

Fig. 3. HKU1-HE is an O-acetylesterase and RDE for HKU1-S1 binding to RD cells. (A) SDS-PAGE of expressed recombinant HE proteins. All proteins were expressed in 293T cells and purified by protein A sepharose beads. Purified proteins were run on SDS-PAGE and stained by coomassie blue. **(B)** HE-mFc protein itself did not bind to RD cells. **(C)** HKU1-HE acted as a RDE on RD cells for HKU1- and OC43-S1 protein. Pretreatment of RD cells with HKU1-HE as well as OC43- and BCoV-HE greatly reduced HKU1-S1 or OC43-S1 (at 5 μg/mL) binding to RD cells in a dose-dependent manner. MHV-S-HE showed no effect. Graphs shown in panel B-C are reprehensive of at least two independent experiments. **(D)** HKU1-HE acted as a RDE on RBCs for OC43-S1 protein. Pretreatment of rat or mouse RBCs with HKU1-, OC43-, BCoV-HE inhibited OC43-S1 mediated hemagglutination activity, whereas MHV-S-HE had no effect. Representative data or images of at least two independent experiments were shown for panels A-D. **(E)** HKU1-HE is an acetylesterase. HEs of HKU1, OC43, BCoV and MHV-S at 2 μg/mL were used to hydrolyze pNPA (2-fold serially diluted) at room temperature for 15 mins. Enzyme activity was assessed by measuring optical density at 405 nm (OD405). The Km and Vmax values were calculated from the Michaelis-Menten Enzyme Kinetics curve fitting of the two independent repeats.

Figure 4

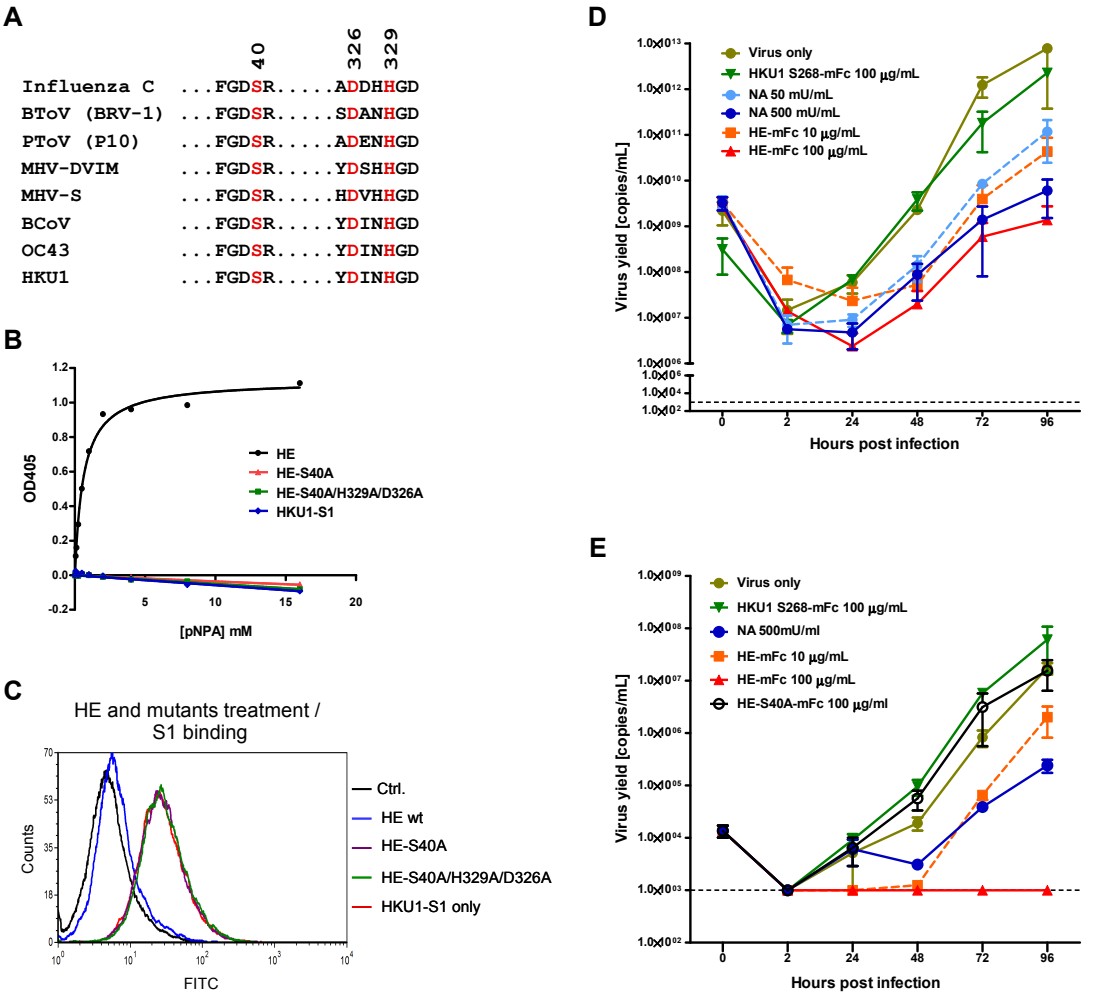

Fig. 4. Treatment of HAE with HE or NA but not the enzymatic inactive HE mutant inhibited HKU1 infection. (A) Esterase catalytic active site residues in HE. Sequence alignment of amino acids around the catalytic active site (in red) was shown. BCoV-HE amino acid numbering scheme was used (22). GenBank accession numbers of HE proteins of BCoV, OC43, HKU1, MHV-DVIM and influenza C virus are AAA92991.1, AAX85668.1, NC_006577.2, AAC63044.1 and AJ872181, respectively. For HE proteins of MHV-S, bovine Torovirus (BToV), and porcine Torovirus (PToV) stain p10, the Uniprot accession numbers are P31614, P0C0V9, and Q70KP1, respectively. **(B)** HKU1-HE proteins with substitutions at catalytic active sites are enzymatic inactive. HKU1-HE or the mutants at 1 µg/mL were incubated with 2-fold serially diluted pNPA at room temperature for 15 mins prior to measuring OD405. Each data points represent OD405 of HE or its mutants subtracted with that of a negative control. **(C)** Esterase inactive mutants of HE did not block HKU1-S1 protein binding to RD cells. RD cells were treated with 10 µg/mL of HE or HE mutant proteins prior to HKU1-S1 staining (5 µg/mL). **(D)** HE and NA pretreatment of HAE inhibited HKU1 infection. Prior to HKU1 virus inoculation, HAE cells were pretreated with HE proteins or NA at different concentrations as indicated for 1 hr, followed by HKU1 virus inoculation at high dose as indicated. Replication kinetics of HKU1 virus was assessed in apical washes from infected HAE cultures by real-time RT-PCR. Virus yield is presented as number of virus RNA copies/mL. **(E)** HE pretreatment blocked HKU1 infection of HAE cells. HAE were pretreated with HE protein or enzymatic inactive HE mutant (S40A) for 1 hour prior to viral challenge at lower dose than used panel D. Replication kinetics of HKU1 virus was assessed as in panel D. Dotted line indicates the detection limit of the assay for panel D-E.