Identification of sialic acid-binding function for the Middle East respiratory syndrome coronavirus spike glycoprotein

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Middle East respiratory syndrome coronavirus (MERS-CoV) targets the epithelial cells of the respiratory tract both in humans and in its natural host, the dromedary camel. Virion attachment to host cells is mediated by 20-nm-long homotrimers of spike envelope protein S. The N-terminal subunit of each S protomer, called S1, folds into four distinct domains designated S1A through S1D. Binding of MERS-CoV to the cell surface entry receptor dipeptidyl peptidase 4 (DPP4) occurs via S1A. We now demonstrate that in addition to DPP4, MERS-CoV binds to sialic acid (Sia). Initially demonstrated by hemagglutination assay with human erythrocytes and intact virus, MERS-CoV Sia-binding activity was assigned to S1 or S1A bound to human erythrocytes and to human mucin in a strictly Sia-dependent fashion. Glycan array analysis revealed a preference for α2,3-linked Sias over α2,6-linked Sias, which correlates with the differential distribution of α2,3-linked Sias and the predominant sites of MERS-CoV replication in the upper and lower respiratory tracts of camels and humans, respectively. Binding is hampered by Sia modifications such as N-glycolylation and O-acetylation. Depletion of cell surface Sia by neuraminidase treatment inhibited MERS-CoV entry of Calu-3 human airway cells, thus providing direct evidence that virus–Sia interactions may aid in virion attachment. The combined observations lead us to propose that high-specificity, low-affinity attachment of MERS-CoV to sialic acids (Sias) serving as attachment factors (27) may form another determinant governing the host range and tissue tropism of this zoonotic pathogen.

Significance

Middle East respiratory syndrome coronavirus (MERS-CoV) recurrently infects humans from its dromedary camel reservoir, causing severe respiratory disease with an ∼35% fatality rate. The virus binds to the dipeptidyl peptidase 4 (DPP4) entry receptor on respiratory epithelial cells via its spike protein. We here report that the MERS-CoV spike protein selectively binds to sialic acid (Sia) and demonstrate that cell-surface sialoglyconjugates can serve as an attachment factor. Our observations warrant further research into the role of Sia binding in the virus’s host and tissue tropism and transmission, which may be influenced by the observed Sia-binding fine specificity and by differences in sialoglycomes among host species.
erythrocytes. Such low-affinity interactions may be augmented by simultaneous low-affinity binding of multiple spike proteins on the virus envelope, thus enabling hemagglutination by MERS-CoV was mediated through multivalency-driven, high-avidity binding (34–50). However, no hemagglutination was seen for MERS-CoV S1-Fc protein, not even when tested at concentrations of up to 500 μg/mL. The Sia-dependent nature of the hemagglutination phenotype was confirmed by testing the virus particles with multiple receptors on the surface of human erythrocytes. Such low-affinity interactions may be augmented through multivalency-driven, high-avidity binding (34–36). Therefore, we opted to design a well-defined, self-assembling nanomaterial for arrayed presentation of S1 and S1 subdomains.

Results

MERS-CoV Can Hemagglutinate Human Erythrocytes. To identify cellular factors, other than DPP4, potentially involved in MERS-CoV cell attachment, we performed classical hemagglutination assays commonly used to detect virus–Sia interactions. Unlike SARS-CoV but similar to influenza A virus (IAV), MERS-CoV virions caused agglutination of human erythrocytes (Fig. 1). Because the spike glycoprotein is the only surface projection of clade C betacoronaviruses, including MERS-CoV, we attempted to validate the observation in a hemagglutination assay using the recombinantly expressed S1 subunit of the MERS-CoV S protein that was transformed into a dimer by C-terminal fusion with the Fc part of human IgG. However, no hemagglutination was seen for MERS-CoV S1-Fc protein, not even when tested at concentrations of up to 500 μg/mL. Therefore, we considered the option that the observed hemagglutination by MERS-CoV was mediated through simultaneous low-affinity binding of multiple spike proteins on the virus particles with multiple receptors on the surface of human erythrocytes. Such low-affinity interactions may be augmented through multivalency-driven, high-avidity binding (34–36). Therefore, we opted to design a well-defined, self-assembling nanomaterial for arrayed presentation of S1 and S1 subdomains.

Design and Expression of Lumazine Synthase Nanoparticles Enabling Multivalent Presentation of Fc-Tagged Viral Receptor-Binding Proteins.

We selected the 154-residue-long lumazine synthase (LS) protein of the hyperthermophile Aquifex aeolicus bacterium that self-assembles into 15-nm-wide 60-meric particles with icosahedral symmetry (37). To allow multivalent presentation of Fc-tagged MERS-CoV S1, the LS protein was extended N-terminally with the 59-residue-long domain B of protein A (pa) of Staphylococcus aureus (Fig. 2A), known for its capacity to bind immunoglobulins via their Fc region (38). The pA-LS protein was provided with an N-terminal signal sequence to allow secretion from mammalian cells, and a C-terminal streptavidin tag was appended for affinity purification (SI Appendix, Fig. S1). The pA-LS protein was affinity-purified from cell culture supernatant of transiently transfected HEK-293T cells and migrated according to its calculated molecular mass of 26 kDa (SI Appendix, Figs. S2 and S3). Analysis of purified pA-LS by electron microscopy revealed spherical particles of ±15 nm in diameter (Fig. 2B), and size exclusion chromatography coupled with multistate light scattering (SEC-MALS) analysis of these pA-LS particles demonstrated that the mass of the main elution peak was close to that of the predicted 60-meric state of the nanoparticle (SI Appendix, Fig. S3). Binding of Fc-tagged S1 subunits (Fig. 2C) to the pA domain on pA-LS nanoparticles was confirmed by ELISA (SI Appendix, Fig. S4), and electron microscopy (Fig. 2B, Right).

MERS-CoV S1 Hemagglutinating Activity Is Sia-Dependent. Nanoparticle-displayed MERS-CoV S1 exhibited hemagglutination activity (2.5 μg of S1-Fc combined with 0.5 μg of pA-LS; HA titer = 128) (Fig. 3A). Since no hemagglutination activity was observed for the (meric) MERS-CoV S1-Fc, multivalent presentation of S1-Fc appeared critical for the hemagglutination phenotype. No hemagglutination was observed with human erythrocytes desialylated by pretreatment with bacterial neuraminidase (NA; Arthrobacter ureafaciens), indicating that the observed MERS-CoV S1-erythrocyte interaction is strictly Sia-dependent (Fig. 3A).

The pA-LS/S1-Fc ratio showing the highest hemagglutination titer was determined to be 1 μg per 2.5 μg (molar ratio = 1:0.6), indicating that ~36 MERS-CoV S1 receptor-binding subunits, presented as 18 S1-Fc dimers, can be accommodated on the 60-meric pA-LS nanoparticle providing optimal hemagglutination efficiency (Fig. 3B and SI Appendix, Table S1). Aside from S1-Fc proteins of MERS-CoV and several previously documented Sia-interacting CoVs (TGEV, porcine epidemic diarrhea virus (PEDV), BCoV, HCoV-OC43, and infectious bronchitis virus (IBV) (39–43)), none of the tested S1 subunits, including those of the MERS-CoV-related Betacoronavirus clade C bat CoVs HKU4 and HKU5, displayed hemagglutination activity in the nanoparticle-based assay (SI Appendix, Table S2).

Sia Binding Site Locates Within Domain A of the MERS-CoV S1 Subunit. To identify the S1 domain of the MERS-CoV spike protein involved in Sia binding, we expressed (SI Appendix, Fig. S2) and assessed the hemagglutination activity of Fc-tagged MERS-CoV S1A and S1B domains, as these are the two domains known to facilitate receptor interactions for Betacoronaviruses. In contrast to the MERS-CoV S1B domain, nanoparticle-displayed MERS-CoV S1A-Fc protein was capable of mediating hemagglutination (Fig. 4). These data demonstrate that the Sia-binding capacity of the MERS-CoV S1 subunit resides in its domain A.

MERS-CoV S1 A Preferentially Binds to Nonmodified α2,3-Linked N-Acetyleneuraminic Acid. Sialylglycans occur in an extraordinary structural variety, which arises from the composition and complexity of the glycan chain, differences in the glycosidic linkage through which the Sia is joined to the adjacent sugar residue, and differential modification of Sia at carbon 5 [either N-acetyl moeity [N-acetyleneuraminic acid (Neu5Ac)] or N-glycoly moeity [N-glycolyleneuraminic (Neu5Gc)]] in combination with modifications of any of the hydroxyl groups at C4, C7, C8, and C9, most often by acetate esters. To assess the specificity of MERS-CoV, glycans array analysis was performed with Fc-tagged MERS-S1A, loaded pA-LS nanoparticles against a library of 135 glycans. We
MERS-CoV S1A did not agglutinate equine erythrocytes that exclusively contain Neu5Gc sialoglycans (47), irrespective of the Sia-binding potential of the MERS-CoV S1 subunit (48), consequently enhancing glycan array sensitivity. A Sia interaction has been shown to be biologically relevant for which it is known that the acetyl moiety at the Sia C9 position prevents recognition of the saccharide by HA (50). No binding of MERS-CoV S1A and IAV HA was observed upon pretreatment of BSM with NA, confirming that the spike–mucin interaction was not a prerequisite for binding. We wondered what effect Sia 9-O-acetylation would have on MERS-CoV spike–Sia interaction, and hence used a bovine submaxillary mucin (BSM)-based ELISA, as BSM is a well-characterized substrate rich in 9-O-acetylated Sias with only a minor population of unmodified Neu5Ac (49). While MERS-CoV S1A was unable to interact with mock-treated mucin of bovine origin (Fig. 6A), pretreatment of BSM with sialate–9-O-acetylersterase [i.e., BCoV hemagglutinin-esterase (HE)] enabled its interaction. Similarly, pretreatment of BSM with sialate–9-O-acetylersterase clearly enhanced binding of IAV HA to bovine mucin (Fig. 6A), for which it is known that the acetyl moiety at the Sia C9 position prevents recognition of the saccharide by HA (50). No binding of MERS-CoV S1A and IAV HA was observed upon pretreatment of BSM with NA, confirming that the spike–mucin interaction was not a prerequisite for binding.
α2,3-Linked Sias Colocalize with Site of MERS-CoV Replication in Vivo.

With the preferential binding of MERS-CoV S1^A to α2,3-linked sialylglycans in mind, we next studied the distribution of these glycans in the upper (nose) and lower (lung) respiratory tissues of humans and the natural reservoir of MERS-CoV: dromedary camels. Lectin histochemistry on these tissues suggests that α2,3-linked sialylglycans are abundant in the camel nasal respiratory epithelium and the alveoli of the human lung (Fig. 6C), which coincides with DPP4 expression and the site of MERS-CoV replication in these mammals (53).

Sia Serves as an Attachment Receptor for MERS-CoV. To study whether the MERS-CoV Sia-binding activity may aid virus cell entry, African green monkey kidney Vero cells and human airway epithelial Calu-3 cells were depleted for cell surface Sias by NA treatment or mock-treated. The cells were then inoculated with MERS-CoV and IAV [multiplicity of infection (MOI) = 0.1], and infection levels were assessed by immunostaining. As expected, NA treatment rendered Vero and Calu-3 cells almost completely resistant to the Sia-dependent IAV. In Vero cells, NA treatment did not affect MERS-CoV entry efficiency. However, Sia depletion of Calu-3 cells reduced MERS-CoV entry by more than 70% compared with control-treated cells (Fig. 7A and B). The difference between Vero and Calu-3 cells may be explained by a difference in the levels of Sia cell surface expression. As shown by flow cytometry, both cell types display similar levels of the entry receptor DPP4 (Fig. 7C), but in Calu-3 cells, those of cell surface MERS-CoV S1^A glycotopes are fivefold higher (Fig. 7D). Our findings provide direct evidence that sialylated proteins or glycolipids on the surface of human airway epithelial cells can be used as an attachment receptor by MERS-CoV, and thereby increase infection efficiency.

Discussion

Our data demonstrate that MERS-CoV carries dual-binding specificity for host molecules and engages both sialglycans and DPP4 via distinct domains of its spike protein. The multivalent presentation of S1 receptor-binding subunits onto 60-meric nanoparticles enabled us to detect a low-affinity interaction of the MERS-CoV spike protein with Sias via its domain A. The existence of CoV spike–Sia interactions has been reported for several CoVs, including clade A betacoronaviruses; the alphacoronaviruses TGEV, PEDV, and FeCV; and Gammacoronavirus IBV (27, 39–43), all of which interact with Sia via their N-terminal spike domains. Although the structural conservation of these domains is not obvious from the primary protein sequence, available
cryoelectron microscopy structures demonstrated that the spike N-terminal domains of the Alphacoronavirus HCoV-NL63 and betacoronaviruses murine hepatitis virus (MHV), HCoV-HKU1 and MERS-CoV display structural similarity (54, 55). Despite the apparent structural conservation of this domain, the observation that genetically distant CoVs comprise Sia-binding activity implies that this feature has independently evolved several times throughout CoV history.

Viral interactions with Sias are typically of a reversible nature so as to prevent nonproductive binding to the abundance of...
sialoglycans on off-target host cells and non-cell-associated structures, as well as for release of viral progeny from infected cells. For instance, clade A betacoronaviruses and ICV encode sialate-O-acetyl-esterases to mediate their reversible binding to O-acetylated Sias (56), while IAVs and IBVs are provided with the sialidase NA to facilitate reversible binding to Sia decoys. By contrast, reversible interaction with Sia by viruses that lack Sia-destroying enzymes, such as several noroviruses, polyoma viruses, and CoVs, including MERS-CoV, is merely governed by the binding equilibrium (57). Because of the inherent low affinity for such receptors by viruses lacking a receptor-destroying enzyme, detection of these weak interactions, particularly by isolated viral lectin domains, may prove difficult. This is illustrated by our observation that only nanoparticle-based assays constituting enhanced avidity through multivalency proved sensitive enough to detect the direct interaction between MERS-CoV S1 and sialoglycans.

Our data indicate that sialoglycans may aid MERS-CoV entry on DPP4-positive cells. Absence of a Sia-dependent effect on Vero cell infection correlated with a low abundance of MERS-CoV S1A receptors. By contrast, infection efficiency of a more relevant cell type, namely, the human airway Calu-3 epithelial cells, was reduced significantly after depletion of cell surface Sias. These cells, derived from bronchial submucosal glands that are targeted by MERS-CoV (58), showed high abundance of MERS-CoV-receptive glycans. These findings provide formal evidence that sialoglycans may serve as an attachment receptor for virus particles, and thereby aid viral entry. Presumably, binding to host cell sialoglycans mediates virion concentration at the cell surface, and thus may increase the likelihood of MERS-CoV spike engagement with the DPP4 entry receptor.

The receptor use of MERS-CoV resembles that of the Alphacoronavirus TGEV and Betacoronavirus MHV, for which binding to cell surface-sialylated glycans was not required for infection of cultured cells but contributes to the efficiency of binding (30, 59–62). In addition to Sias, TGEV is able to interact with porcine APN (29) and MHV engages with CEACAM1a (63), both of which serve as a cellular receptor. High-affinity binding to proteinaceous receptors by MERS-CoV, TGEV, and MHV appears sufficient for entry in cultured cells, and primary attachment to sialylated proteins or lipids on the cell surface may aid the virus to get into contact with its protein receptor. This is in sharp contrast to other Sia-binding CoVs such as BCoV and IBV, for which no protein receptor has been identified and which critically depend on (O-acetylated) Sias during cell entry (45, 48, 64).

Our data indicate that MERS-CoV has evolved a binding site of low affinity but high selectivity for Neu5Ac that excludes Neu5Gc. Likewise, binding to Sias modified by the addition of an acetyl group at the C9 position is not permitted. The selection on Sia linkage types to the penultimate sugar in the glycan chain appears less stringent, although a distinct preference for α2,3-linked sialoglycans (over α2,6 linkages) is observed, the distribution of which correlates with the predominant sites of MERS-CoV replication in the upper and lower respiratory tracts of camels and humans, respectively. In particular, long bi- and triantennary α2,3-linked

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Fig. 6. (A) Sia-binding activity of MERS-CoV S1A nanoparticles is inhibited by the acetyl moiety of 9-O-acetylated Sias. BSM-coated ELISA plates were mock treated, de-9-O-acetylated using BCoV HE, or desialylated using NA before incubation with twofold serially diluted (pA-LS-complexed) viral proteins. Fc-tagged IAV HA and hCoV OC43 S1 were taken along as described above. BSM pretreated with NA served as a negative control. The experiment was performed in triplicate and repeated at least two times. A representative experiment is shown. Error bars represent the corresponding SEM. (B) Binding of MERS-CoV S1A nanoparticles to human mucin. Equimolar amounts of Fc-tagged MERS-CoV S1A, IAV HA, and pAPN were coupled to pA-LS nanoparticles, serially diluted twofold, and incubated on plates coated with human mucin that were mock-pretreated or pretreated with NA. IAV HA-Fc and pAPN-Fc were taken along as controls. Mucin-bound pA-LS nanoparticles were detected as described in A. The experiment was performed in triplicate and repeated at least two times. A representative experiment is shown. Error bars represent the corresponding SEM. (C) Staining with M. amurensis lectin II (specific for α2,3-linked Sias) of camel and human nose and lung tissues. (Magnification: 400×.)
sialylated poly-LacNAc structures are recognized, indicating that multivalency of the ligand is important for binding.

Virus-receptor-binding specificity and variation in receptor distribution can be critical for host and tissue tropism, pathogenesis, and (cross-species) transmissibility of viruses (65, 66). For the zoonotic MERS-CoV, the spike–DPP4 interaction has been a focus of study to understand these aspects of the virus biology and epidemiology. The high sequence identity of spike-interacting residues on dromedary and human DPP4 facilitates cross-species transmission without the need for adaptive mutations (17, 67). The reported differential receptor distribution in the respiratory tract may account for the observed dissimilar MERS-CoV–induced pathology in dromedaries and humans, as well as the apparent inefficient interhuman transmission (53). Although receptor interaction and distribution are key determinants for virus tropism, they cannot solely explain the species’ susceptibility to MERS-CoV infection, as illustrated by the resistance of horses to MERS-CoV infection (32). MERS-CoV–contacting residues on equine and human DPP4 are identical (68), and equine DPP4 supports MERS-CoV infection in cultured cells (69). In addition, DPP4 is highly and widely expressed along the respiratory tract of horses (32). Nevertheless, horses appear to be resistant to experimental infection with MERS-CoV (32), an observation that is supported by the lack of MERS-CoV antibodies in horse sera collected in the Middle East region (70), suggesting that host factors other than DPP4 contribute to viral tropism. For influenza viruses, it has been well established that Sia-binding preference and distribution of Sia species in animals and humans are critical determinants for host and tissue tropism (65, 66, 71, 72). Intriguingly, the epithelial cells of the horse trachea are highly enriched in N-glycolylneuraminic acid (71), and equine nose epithelia are reportedly covered with a particularly thick mucus layer (32). Whether and how the MERS-CoV Sia-binding fine specificity and the differences in sialoglycomes among species affect virus host and tissue tropism clearly warrant further investigation. Detailed structural insight into the binding mode of the S1A domain with Sias would enable generation of MERS-CoV mutants incapable of Sia interaction that could be instrumental in determining the role of Sia interaction in vivo.

Materials and Methods

Protein Design and Expression. Genes encoding the 6,7-dimethyl-8-ribityllumazine synthase (LS; GenBank accession no. WP_010880027.1) of A. aeolicus and domain B of pa (UniProt accession no. P38507; amino acids 212–270) of S. aureus were synthesized using human-preferred codons obtained from GenScript USA, Inc. The cytoine at position 37 and asparagine at position 102 of LS were mutated to alanine and glutamine, respectively. A pA-LS expression vector was generated by ligating the pA-encoding sequence in-frame with the N terminus of the LS-encoding sequence via a Gly-Ser linker. A pA-LS expression vector was generated by ligating the pA-encoding sequence in-frame with the N terminus of the LS-encoding sequence via a Gly-Ser linker and subsequent cloning into the pCAGGS mammalian expression vector. In addition, LS ORFs in the LS and pA-LS expression vectors were provided with an N-terminal version encoding a HA peptide, and a streptomyycin tag purification tag sequence (IBA) (SI Appendix, Materials and Methods). For Sia depletion of Calu-3 cells inhibits MERS-CoV infection. Vero and Calu-3 cells were treated with or without NA and inoculated with MERS-CoV or IAV for 1 h at 37 °C. Cells were then washed once, and fresh medium was added. Following incubation at 37 °C for 8 h, cells were fixed with 4% paraformaldehyde for 10 min, and immunostaining and counting. Data are presented as mean ± SE and statistically analyzed with a t test. (B) Immunofluorescence staining on MERS-CoV–infected Vero and Calu-3 cells with and without NA pretreatment measured by flow cytometry. (C) Surface expression of DPP4 on Vero and Calu-3 cells measured by flow cytometry. (D) Binding of nanoparticle-displayed MERS-CoV spike domain S1 to Vero and Calu-3 cells with and without NA pretreatment measured by flow cytometry. FACS data are presented as the geometric mean fluorescence intensity (gMFI) ratio between samples and isotype controls at a 95% confidence interval. The differences in sialoglycomes among species affect virus host and tissue tropism clearly warrant further investigation. Detailed structural insight into the binding mode of the S1A domain with Sias would enable generation of MERS-CoV mutants incapable of Sia interaction that could be instrumental in determining the role of Sia interaction in vivo.

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proteins were purified from tissue culture supernatants 5-6 d posttransfection by pA affinity chromatography (17-0780-01; GE Healthcare), eluted using acid solution (0.1 M citric acid at pH 3.0), and neutralized immediately using Tris at pH 8.8 (0.2 M final concentration), with the exception of HA-Fc, which was purified via a C-terminal streptomyctyn tag and Streptactin Sepharose beads and eluted using Streptactin elution buffer (100 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 2.5 mM biotin). Purified proteins were analyzed on a 12% SDS/PAGE gel under reducing conditions and stained with GelCodeBlue stain reagent (Thermo Scientific). Purified proteins were stored at 4 °C until further use.

Electron Microscopic Analysis of LS Nanoparticles. Before the application of samples, 400-mesh copper grids with a pure carbon film were exposed to a glow discharge in air for 20 s to make them hydrophilic. Ten microliters of purified LS, pA-LS, or pA-LS + MERS-CoV S1-Fc (premixed at a 1:1.2 ratio) at 0.2-0.3 mg/mL was applied to the grids and incubated for 1 min. Excess sample was blotted with a filter paper, and negative staining, 10 μL of 1% phosphotungstic acid at pH 6.8 was applied. After 1 min, excess stain was blotted and grids were left to dry. The specimens were examined in a JEOL JEM2100 transmission electron microscope at 200 kV, and images were taken at a magnification of 80,000× with a Gatan US4000 4K × 4K camera. At least 50 particles were measured to calculate the mean diameter and SD of the pA-LS particles.

SEC-MALS. For SEC-MALS analysis, purified LS nanoparticles (150 μg in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl) were loaded onto a Superose 6 10/300 GL column (0.4 mL min⁻¹; GE Life Sciences) and passed through a Wyatt DAWN Heleos II 18-angle laser photometer coupled to a Wyatt Optilab TRX differential refractive index detector. Data were analyzed, and weight-averaged molecular masses and mass distributions (polydispersity) for each sample were calculated using Astra 6 software (Wyatt Technology Corp.).

MERS-CoV S1-Fc-EndoELISA. Nunc Maxisorp plates were coated with 100 μL of D-PBS (PBS with Ca²⁺/Mg²⁺) containing 50 ng of MERS-CoV S1-Fc per well and incubated for 4 h at room temperature. Plates were washed three times with washing buffer (PBS + 0.05% Tween-20) and subsequently blocked overnight at 4 °C with 200 μL of blocking buffer (PBS + 0.1% BSA), containing 2% BSA). Plates were put at room temperature and washed three times with washing buffer before use. Streptomyctyn-tagged LS and pA-LS were threefold serially diluted in blocking buffer and incubated for 1 h at 37 °C. Plates were washed three times with washing buffer and once with PBS + 2% BSA, and then incubated for 1 h at 37 °C with 100 μL of a 1:10,000 StreptMAB-classic HRP (BIA) dilution in 2% PBS + 0.3 mg/mL rabbit or goat anti-mouse IgG antibody as a second step (25). The staining was performed as previously described using biotinylated Maackia amurensis lectin II (76). The use of human material was approved by the local ethical medical committee (MEC approval: 2014-414). Human tissues were residual human biomaterials, which are collected, stored, and issued by the Erasmus MC Tissue Bank under ISO 15189:2007 standard operating procedures. Use of these materials for research purposes is regulated according to the Human Tissue and Medical Research code of conduct for responsible use (https://www.federa.org/sites/default/files/digital_version_first_part_code_of_conduct_in_uk_2012_12092012.pdf). Experimen-tal procedures using dromedaries were approved by the local Ethical Committee of the Autonomous University of Barcelona (no. 8003).

Microscopy. The μ2,3-Sia expression on human and dromedary Calu-3 cells and lung tissue samples was assessed by lectin histochemistry. Human and camel tissue samples used in this study were similarly to those described in our previous study (53). The staining was performed as previously described using biotinylated Maackia amurensis lectin II (76). The use of human material was approved by the local medical ethical committee (MEC approval: 2014-414). Human tissues were residual human biomaterials, which are collected, stored, and issued by the Erasmus MC Tissue Bank under ISO 15189:2007 standard operating procedures. Use of these materials for research purposes is regulated according to the Human Tissue and Medical Research code of conduct for responsible use (https://www.federa.org/sites/default/files/digital_version_first_part_code_of_conduct_in_uk_2012_12092012.pdf). Experimental procedures using dromedaries were approved by the local Ethical Committee of the Autonomous University of Barcelona (no. 8003).

Flow Cytometric Analysis of Cell Surface DPP4 and Sia Expression. To measure DPP4 expression, Vero and Calu-3 cells were incubated with 5 μg/mL polyclonal goat anti-DPP4 antibody (clone AF1180; R&D Systems) and stained with rabbit anti-goat IgG antibodies conjugated with Alexa Fluor 488 in a 1:250 dilution (Life Technologies). To measure Sia expression, Vero and Calu-3 cells were incubated with 5 μg/mL sialate-9-O-acetylesterase (BCoV-HE), or treated with 20 mU/mL NA (A. ureafaciens; Roche) (diluted in PBS, 100 μL per well) for 3 h at 37 °C. MERS-CoV S1-Fc, IAV HA-Fc [A/California/04/2009 (H1N1)] and pAPN-Fc were incubated with 20 μg/mL sialate-9-O-acetylesterase (BCoV-HE), or treated with 20 μM NA (A. ureafaciens; Roche) in PBS for 3 h at 37 °C. Fc-tagged proteins were complexed with pA-LS (2.1 molar ratio) for 30 min at 4 °C, and twofold serial dilutions of mixtures were made using ice-cold blocking buffer. Complexed nanoparticles were incubated on mucin-coated plates for 2 h at 4 °C, after which bound LS nanoparticles were detected by ELISA using the HRP-conjugated anti-StrepMAB antibody that recognizes their C-terminally appended streptococm tag.

MERS-CoV Infection Experiments. Confocal layers of Vero or Calu-3 cells were mock-treated or treated with NA (A. ureafaciens) for 2 h at 37 °C, followed by infection with MERS-CoV (EMC isolate) or IAV (PR8 strain). The inoculum was removed after 1 h, and cells were washed once before addition of fresh culture medium. At 8 h postinfection, cells were fixed with formaldehyde, permeabilized with 70% ethanol, and stained using monoclonal antibody against MERS-CoV Nucleoprotein (Immunosource) (32) or a monoclonal antibody against influenza nucleoprotein (lgG2A, Clone Hb65; American Type Culture Collection) according to standard protocols using Alexa Fluor 488-conjugated goat-α-rabbit or goat-α-mouse antibodies as a second step (25). The percentage of infected cells was determined by immunostaining and counting.

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