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Species-Specific Colocalization of Middle East Respiratory Syndrome Coronavirus Attachment and Entry Receptors

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ABSTRACT Middle East respiratory syndrome coronavirus (MERS-CoV) uses the $S1^B$ domain of its spike protein to bind to dipeptidyl peptidase 4 (DPP4), its functional receptor, and its $S1^A$ domain to bind to sialic acids. The tissue localization of DPP4 in humans, bats, camelids, pigs, and rabbits generally correlates with MERS-CoV tropism, highlighting the role of DPP4 in virus pathogenesis and transmission. However, MERS-CoV $S1^A$ does not indiscriminately bind to all α2,3-sialic acids, and the species-specific binding and tissue distribution of these sialic acids in different MERS-CoV-susceptible species have not been investigated. We established a novel method to detect these sialic acids on tissue sections of various organs of different susceptible species by using nanoparticles displaying multivalent MERS-CoV $S1^A$. We found that the nanoparticles specifically bound to the nasal epithelial cells of dromedary camels, type II pneumocytes in human lungs, and the intestinal epithelial cells of common pipistrelle bats. Desialylation by neuraminidase abolished nanoparticle binding and significantly reduced MERS-CoV infection in primary susceptible cells. In contrast, $S1^A$ nanoparticles did not bind to the intestinal epithelium of serotine bats and frugivorous bat species, nor did they bind to the nasal epithelium of pigs and rabbits. Both pigs and rabbits have been shown to shed less infectious virus than dromedary camels and do not transmit the virus via either contact or airborne routes. Our results depict species-specific colocalization of MERS-CoV entry and attachment receptors, which may be relevant in the transmission and pathogenesis of MERS-CoV.

IMPORTANCE MERS-CoV uses the $S1^B$ domain of its spike protein to attach to its host receptor, dipeptidyl peptidase 4 (DPP4). The tissue localization of DPP4 has been mapped in different susceptible species. On the other hand, the $S1^A$ domain, the N-terminal domain of this spike protein, preferentially binds to several glycotopes of α2,3-sialic acids, the attachment factor of MERS-CoV. Here we show, using a novel method, that the $S1^A$ domain specifically binds to the nasal epithelium of dromedary camels, alveolar epithelium of humans, and intestinal epithelium of common pipistrelle bats. In contrast, it does not bind to the nasal epithelium of pigs or rabbits, nor does it bind to the intestinal epithelium of serotine bats and frugivorous bat species. This finding supports the importance of the $S1^A$ domain in MERS-CoV infection and tropism, suggests its role in transmission, and highlights its potential use as a component of novel vaccine candidates.

KEYWORDS Middle East respiratory syndrome coronavirus, $S1^A$ domain, common pipistrelle bats, dromedary camels, humans

Coronaviruses use their spike (S) protein to attach to host cell surface molecules and enter target cells. The N-terminal part of this S protein, known as S1, is responsible for attachment to host cells, while the C-terminal part mediates virus fusion to host cells

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postattachment (1). For Middle East respiratory syndrome coronavirus (MERS-CoV), the S1 protein comprises four individually folded domains, designated $S1^A$ through $S1^D$ (2, 3). Two of these domains, $S1^A$ and $S1^B$, are involved in binding to host cell surface molecules during the attachment phase. The $S1^A$ domain preferentially binds to several glycotopes of α2,3-sialic acids, while the $S1^B$ domain recognizes a host exopeptidase named dipeptidyl peptidase 4 (DPP4), the viral receptor (4, 5). The absence of DPP4 renders cells insusceptible to MERS-CoV (4). Meanwhile, elimination of sialic acids in susceptible cell lines significantly reduces MERS-CoV infection (5). These findings indicated that besides DPP4, the functional entry receptor of MERS-CoV, α2,3-sialic acids act as attachment receptors (4, 5).

DPP4 expression has been mapped in tissues of different susceptible species. It is expressed in the nasal epithelium of camelids, pigs, and rabbits, in which MERS-CoV causes upper respiratory tract infection (6–12). In the human respiratory tract, it is mainly expressed in type II pneumocytes in the lungs (8, 13), in line with clinical data showing that in humans, MERS-CoV mainly replicates in the lower respiratory tract (14–16). In common pipistrelle bats, a potential reservoir for MERS-CoV-like viruses, DPP4 is scarcely detected in the respiratory tract but abundantly present in the intestinal tract (17). Accordingly, MERS-CoV-like viruses are detected mostly in fecal samples of this species as well as in other insectivorous bat species (18–20). Sheep, on the other hand, do not seem to express DPP4 in their respiratory tract and thus hardly shed infectious virus and did not seroconvert upon experimental intranasal MERS-CoV inoculation (7, 21). Epidemiological studies did not reveal MERS-CoV-seropositive sheep in the field, except for one study using sheep sera obtained from Senegal (22–25). Altogether, these data support the role of DPP4 in determining the host range and tissue tropism of MERS-CoV.

The localization of α2,3-sialic acids in the respiratory tract of both humans and dromedary camels has been mapped using lectin histochemistry (5). These molecules are mainly present in the lower respiratory tract epithelium of humans and the upper respiratory tract epithelium of dromedary camels, in line with the localization of DPP4 (5, 26). However, it is important to note that MERS-CoV $S1^A$ does not indiscriminately bind to all α2,3-sialic acids. It does not recognize those with 5-*N*-glycosylation or 9-*O*-acetylation but preferentially binds 5-*N*-acetyl-modified sialic acids (5). Among these α2,3-linked, *N*-acetyl-modified sialic acids, MERS-CoV $S1^A$ predominantly binds to short, sulfated, α2,3-linked monosialosaccharides and to long, branched, di- and triantennary α2,3-linked sialic acids, with a minimum extension of 3 *N*-acetyl-D-lactosamine tandem repeats (5). These glycotopes were previously identified by glycan array analysis using nanoparticles displaying multivalent MERS-CoV $S1^A$ (5). Here, using these nanoparticles, we developed a histochemistry-based technique to map the MERS-CoV-recognized glycotopes in the tissues of different susceptible species. The results of our study offer further insight into the importance of these glycotopes in MERS-CoV host range, tropism, and transmission.

RESULTS

MERS-CoV $S1^A$ binds specifically to the nasal epithelium of dromedary camels. The nasal epithelia of dromedary camels express DPP4 and are susceptible to MERS-CoV upon experimental inoculation (8, 27). Recent studies revealed that these tissues also express α2,3-sialic acids based on *Maackia amurensis* lectin II binding (5). However, this lectin binds to a broad range of modified α2,3-sialic acids and thus may not represent a specific marker for glycotopes recognized by MERS-CoV $S1^A$ (28). In order to map these glycotopes, we displayed MERS-CoV $S1^A$ in a multivalent manner using 60-meric self-assembled nanoparticles generated from the lumazine synthase protein of the bacterium *Aquifex aeolicus* (np-$S1^A$) (5) and subsequently used these nanoparticles to set up a histochemistry assay. We previously showed that both np-$S1^A$ and MERS-CoV virions can agglutinate human erythrocytes, while dimeric MERS-CoV $S1^A$ cannot, indicating that multivalent presentation is necessary for the hemagglutination phenotype of MERS-CoV $S1^A$. Similarly, np-$S1^A$, but not dimeric MERS-CoV $S1^A$, can bind

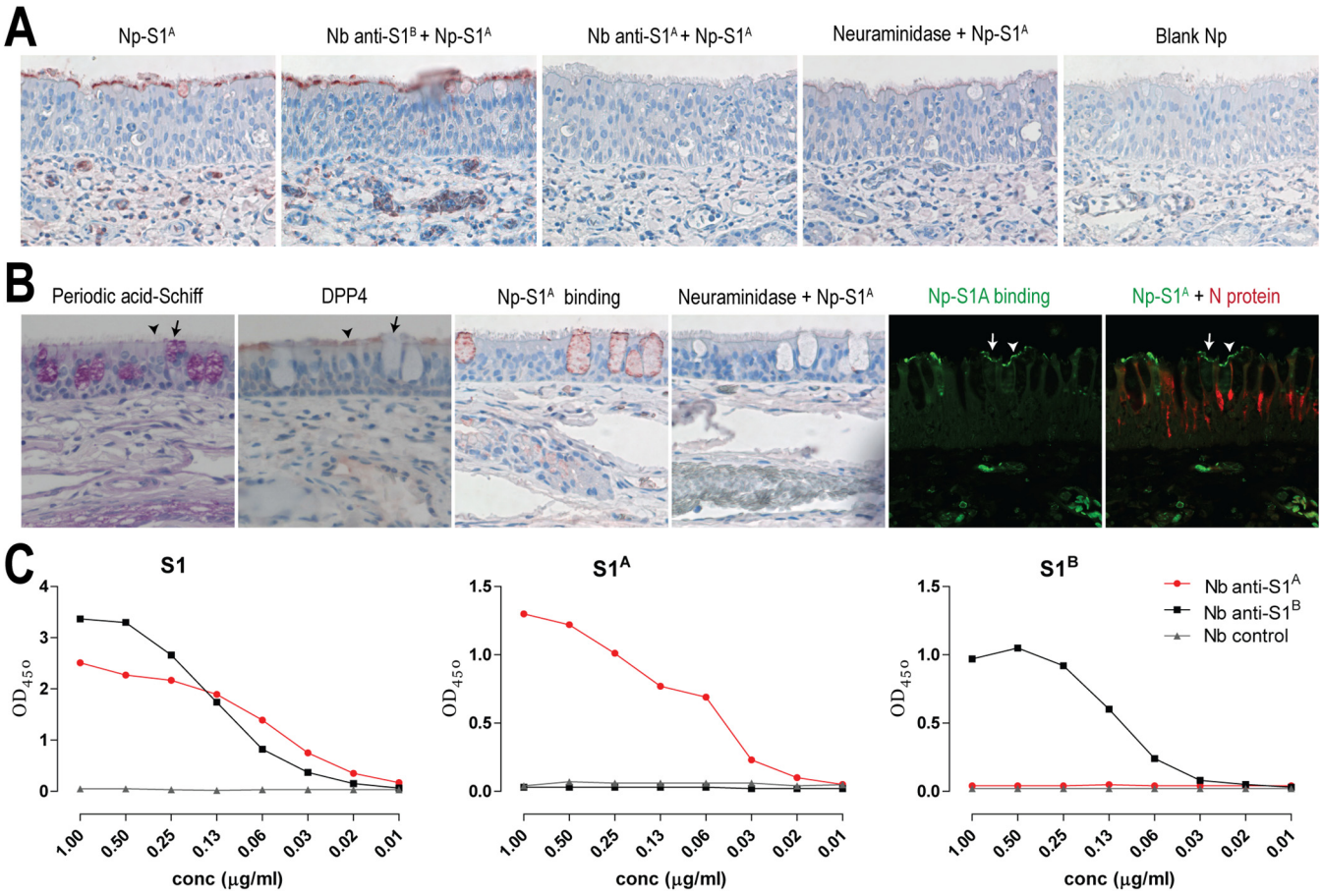

FIG 1 MERS-CoV S1^A binds specifically to the nasal epithelium of dromedary camels. (A) Nanoparticles displaying a multivalent MERS-CoV S1^A domain (np-S1^A) bind to the apical surface of camel nasal ciliated epithelial cells, as revealed by red staining. np-S1^A binding is inhibited by prior neuraminidase treatment of these nasal tissues. Blank nanoparticles also do not bind to these tissues. (B) Goblet cells (arrows), visualized in purple by periodic acid-Schiff stain, are DPP4 negative, unlike nasal ciliated columnar epithelial cells (arrowheads). DPP4 expression is indicated in red. MERS-CoV S1^A binding to these goblet cells (red) can be abrogated by neuraminidase treatment. In MERS-CoV-infected camels, MERS-CoV S1^A (green) binds to both nasal ciliated columnar epithelial cells and goblet cells, while MERS-CoV N protein (red) is detected only in nasal ciliated columnar epithelial cells. np-S1^A binding is abrogated by a nanobody against the S1^A domain (Nb anti-S1^A) but not by one against the S1^B domain (Nb anti-S1^B). The tissues used in these experiments were sequentially cut. All pictures were taken at a ×400 magnification. (C) Nb anti-S1^A and Nb anti-S1^B bind specifically to S1^A and S1^B domains, respectively, and both bind to S1 protein, as revealed by an ELISA. The control nanobody does not bind to S1, S1^A, and S1^B. Nanobody binding is expressed as optical density at 450 nm (OD_{450}) values determined by an ELISA.

to tissues in our assay. As shown in Fig. 1, np-S1^A binds specifically to the nasal epithelium of dromedary camels. We found that these glycotopes are expressed in clusters of ciliated and goblet cells in the nasal epithelium in a random multifocal pattern (Fig. 1A). We determined the binding specificity of np-S1^A by using blank nanoparticles and tissues pretreated with neuraminidase as negative controls (Fig. 1A). Goblet cells, however, are DPP4 negative and thus are not susceptible to MERS-CoV despite expressing these glycotopes (Fig. 1B). The nasal tissues used for the np-S1^A binding experiment, DPP4 detection, and periodic acid-Schiff staining were obtained from noninfected dromedary camels, while those used to visualize both np-S1^A binding and MERS-CoV nucleoprotein were obtained from MERS-CoV-infected dromedary camels. The tissues of these animals were collected during a previous study (27).

In a previous study, we generated a nanobody library from the bone marrow of dromedary camels vaccinated with modified vaccinia virus Ankara (MVA) expressing the MERS-CoV spike protein and identified S1^B-reactive nanobodies (29). We rescreened this library using the S1^A domain and identified an S1^A-reactive nanobody. We confirmed its specific binding to the S1^A domain through S1, S1^B, and S1^A enzyme-linked immunosorbent assays (ELISAs). While the control nanobody (29) was negative in all

three ELISAs, each of the anti-S1^A and anti-S1^B nanobodies reacted specifically to its corresponding domain and to S1 in a dose-dependent manner (Fig. 1B). The identified S1^A nanobody inhibited np-S1^A binding to nasal epithelial cells, whereas the S1^B-reactive nanobody did not, further confirming the np-S1^A binding specificity and the potential role of S1^A-specific antibodies in blocking MERS-CoV attachment (Fig. 1A).

MERS-CoV S1^A does not bind to the nasal epithelium of pigs and rabbits. Similar to dromedary camels, pigs and rabbits also develop upper respiratory tract infection upon MERS-CoV inoculation. However, both pigs and rabbits shed less infectious virus (mainly between 10^2 and 10^3 50% tissue culture infectious doses [$TCID_{50}$]/ml) than do dromedary camels (between 10^4 and 10^5 $TCID_{50}$/ml) postinoculation (6, 7, 9–11, 27, 30). In line with these findings, we found smaller numbers of infected nasal epithelial cells in the nasal tissues of these MERS-CoV-infected pigs and rabbits than in dromedary camels (Fig. 2). Using the nasal tissues obtained from mock-infected dromedary camels, pigs, and rabbits, we show that DPP4 is highly expressed in the nasal epithelium of these three species (Fig. 2), indicating that the differences in infectious virus shedding observed in these three species are not likely due to differences in DPP4 expression. Subsequent screening for the presence of α2,3-sialic acids in these tissues using lectin histochemistry revealed that the nasal epithelium of pigs does not express these sialic acids, in accordance with the results of previous studies (31, 32). In contrast, these sialic acids were detected in the nasal epithelium of dromedary camels and rabbits (Fig. 2). We then used np-S1^A to test for the presence of MERS-CoV-recognized glycotopes in the nasal epithelium of pigs and rabbits and found that unlike dromedary camels, both species do not express these glycotopes (Fig. 2). The absence of these glycotopes in the nasal epithelium of rabbits, despite the abundant presence of α2,3-sialic acids, further supports the fine specificity of A domain binding.

MERS-CoV S1^A binds specifically to the intestinal epithelium of pipistrelle bats. DPP4 has been reported to mediate MERS-CoV infection in various bat cell lines derived from different bat species (33, 34). It has also been shown to mediate entry of pseudotyped viruses expressing spike proteins of two MERS-CoV-like viruses, i.e., HKU4 and *Hypsugo pulveratus* BatCoV HKU25, in target cells (19, 35, 36). DPP4, detected using a previously described immunohistochemistry method (8, 17), has also been shown to be abundantly expressed in the intestinal epithelium of both insectivorous and frugivorous bats (17). Altogether, these studies suggest the susceptibility of various bat species to MERS-CoV-like viruses. However, not all susceptible bat species may act as hosts for these viruses, as they were preferentially detected in insectivorous bats (18–20). One study conducted a large screening of over 5,000 insectivorous bats, from Ghana, Ukraine, Romania, Germany, and The Netherlands, showing that these viruses were mainly detected in *Nycteris* bats and pipistrelle bats (20). Using np-S1^A, we investigated whether MERS-CoV-recognized glycotopes were differentially expressed in the intestinal epithelium of insectivorous bats, i.e., common pipistrelle (*Pipistrellus pipistrellus*) and serotine (*Eptesicus serotinus*) bats, and frugivorous bats, i.e., Gambian epauletted (*Epomophorus gambianus*) and Egyptian fruit (*Rousettus aegyptiacus*) bats. We observed that np-S1^A binds to the apical surface of the intestinal epithelium of common pipistrelle bats in both villi and crypts, while in others, np-S1^A binds only to the intestinal crypts (Fig. 3).

MERS-CoV S1^A binds specifically to cells in the human lower respiratory tract. Since lower respiratory tract samples from MERS human cases have higher levels of virus and the virus was detected by immunohistochemistry in the lungs of two cases, it has been concluded that MERS-CoV mainly replicates in the human lower respiratory tract (14–16, 37). In line with these observations, DPP4 is expressed in the lower airway epithelium but not in the upper airway epithelium. It was detected in bronchiolar and alveolar epithelial cells but primarily in type II pneumocytes (Fig. 4A), consistent with previous studies (8, 13). Our lectin histochemistry staining showed that both bronchiolar epithelial cells and type II pneumocytes express α2,3-sialic acids (26). Both cell types also express MERS-CoV-recognized glycotopes, as indicated by the binding of np-S1^A

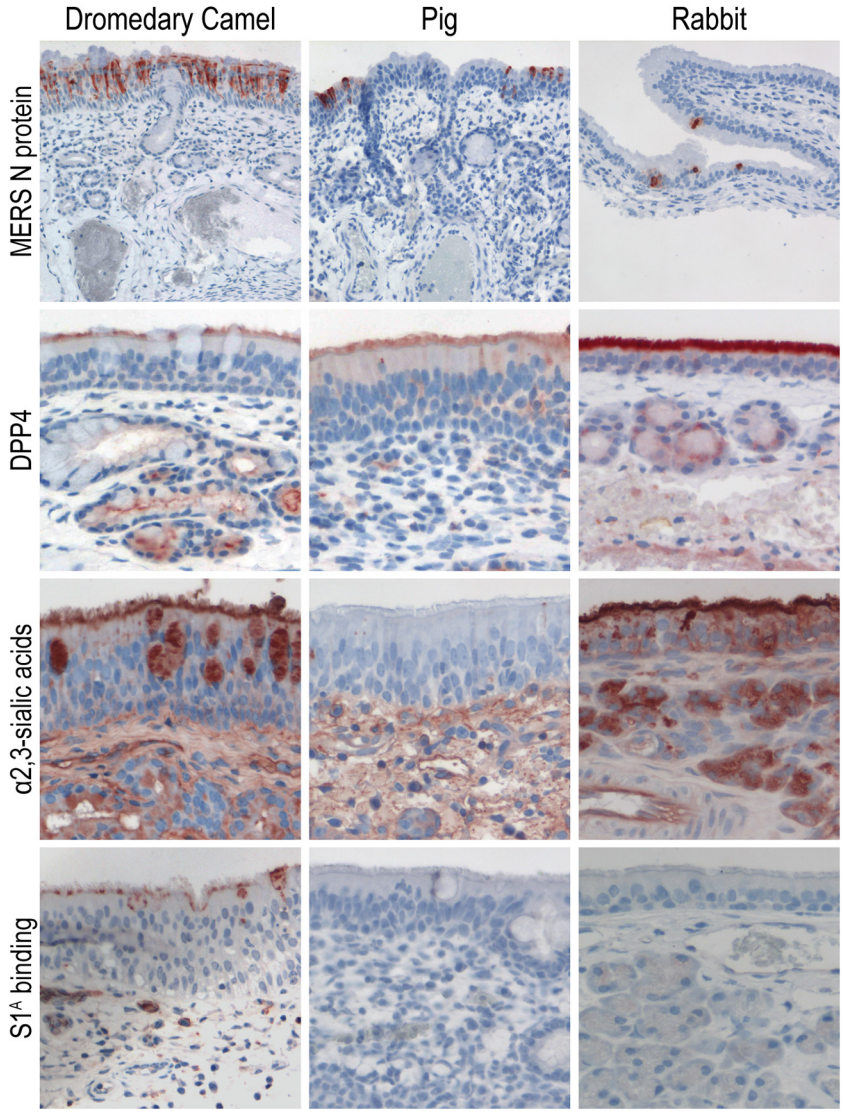

FIG 2 Detection of MERS-CoV N protein, DPP4, α2,3-sialic acids, and MERS-CoV S1^A binding in the nasal epithelium of dromedary camels, pigs, and rabbits. MERS-CoV N protein, DPP4, α2,3-sialic acid, and MERS-CoV S1^A binding are all indicated in red. MERS-CoV N protein is detected in the nasal epithelium tissues of MERS-CoV-infected dromedary camels, pigs, and rabbits. DPP4, α2,3-sialic acid, and MERS-CoV S1^A binding were evaluated on the tissues of noninfected animals. MERS-CoV N protein and DPP4 are detected in the nasal epithelium of dromedary camel, pig, and rabbit. α2,3-Sialic acids are detected in the nasal epithelium of dromedary camel and rabbit but not in that of pig. Meanwhile, MERS-CoV S1^A binds merely to the nasal epithelium of dromedary camel. Magnification, $\times$400.

(Fig. 4B). Using immunofluorescence, we show that DPP4 expression and np-S1^A binding colocalize in the same alveolar epithelial cells in the human lung (Fig. 4C).

The function of MERS-CoV-recognized glycotopes as an attachment factor thus far has been observed only in Calu3 cells, which is a cell line originating from a human lung adenocarcinoma. These cells express both DPP4 and the glycotopes of α2,3-sialic acids recognized by MERS-CoV. Removal of sialic acids on these cells using neuraminidase prior to MERS-CoV infection significantly reduced the number of infected cells (5). In contrast, neuraminidase treatment of Vero cells, which do not express MERS-CoV-recognized glycotopes, had no effect on the number of infected cells (5). Primary well-differentiated normal human bronchiolar epithelial (wd-NHBE) cells also express DPP4 and MERS-CoV-recognized glycotopes (Fig. 5A). These wd-NHBE cells were obtained from healthy human bronchial epithelial cells and cultured at the air-liquid

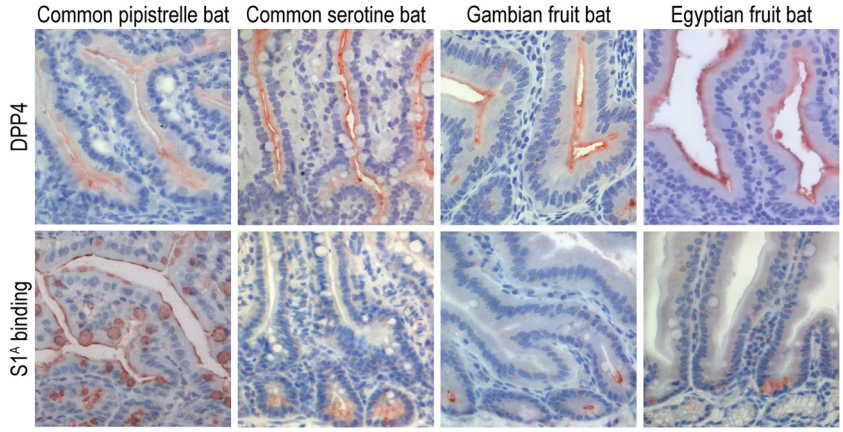

FIG 3 DPP4 expression and MERS-CoV $S1^A$ binding in intestinal tissues of common pipistrelle bat, serotine bat, Gambian epauletted fruit bat, and Egyptian fruit bat. DPP4 expression and MERS-CoV $S1^A$ binding are indicated in red. DPP4 is expressed at the apical surface of the intestinal epithelial cells of these four bat species. MERS-CoV $S1^A$ binds to the apical surface of the intestinal epithelial cells of common pipistrelle bats in both villi and crypts, while in other bat species, it mostly binds to intestinal epithelial cells within the crypts. Magnification, ×400.

interface to mimic the human airway environment. Previous studies have reported that these cells are susceptible to MERS-CoV in a DPP4-dependent manner (38–40). Here we show that neuraminidase treatment of paraffin-embedded wd-NHBE cells reduces np-$S1^A$ binding but not DPP4 expression (Fig. 5A). Neuraminidase treatment prior to MERS-CoV infection of these cells also significantly reduced the number of infected cells (Fig. 5B), similar to our previous findings in Calu3 cells (5). Thus, our results further support the importance of MERS-CoV-recognized glycotopes as an attachment factor during infection of human airway epithelial cells.

DISCUSSION

The S1 protein is an important determinant for the host range and tissue tropism of coronaviruses. This domain initiates infection by binding to host cell surface molecules, either proteinaceous, sialoglycan based, or both (41). The S1 proteins of feline coronavirus, transmissible gastroenterovirus, and MERS-CoV have been demonstrated to have dual-binding specificity, allowing them to engage both sialoglycans and proteinaceous molecules (5, 38, 41–45). We have previously reported that for MERS-CoV, this dual binding is facilitated by distinct domains of its S1 protein, i.e., $S1^A$ and $S1^B$ (4, 5). The $S1^B$ domain binds DPP4, the functional receptor of MERS-CoV (38). DPP4 has been demonstrated to have a major influence on viral host range and tropism since its tissue localization varies between species (7, 8, 13, 17, 33, 38, 46–48). It is detected in the nasal epithelium of camelids, pigs, and rabbits (7, 8). In bats, it is mainly expressed in the small intestinal epithelium of common pipistrelle and serotine bats and in both the respiratory and intestinal epithelia of Gambian epauletted and Egyptian fruit bats (17). Meanwhile, in the human airways, it is present merely in the lower respiratory tract epithelium, particularly in type II pneumocytes (8, 13). Besides DPP4, MERS-CoV preferentially binds α2,3-linked sialic acids via its $S1^A$ domain and uses these sialic acids as an attachment factor (5). Using nanoparticles displaying a multivalent $S1^A$ domain, we show that the tissue localization of these glycotopes varies between various tissues in susceptible species. The $S1^A$ domain bound to the nasal epithelium of dromedary camels and type II pneumocytes in human lungs but not the nasal epithelium of pigs and rabbits. Binding of particles to other molecules besides sialic acids such as mucus and extracellular factors that are removed during the preparation of tissue sections as a result of the boiling procedure, however, cannot be excluded.

Previous studies have shown that MERS-CoV-inoculated pigs and rabbits shed less

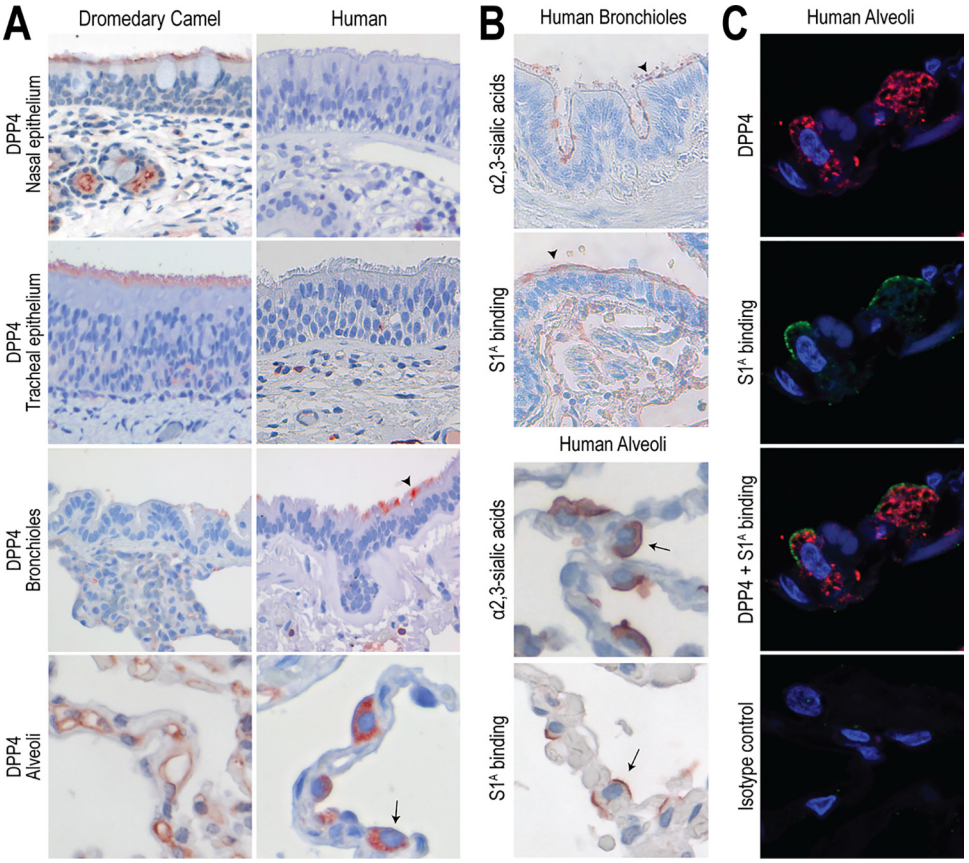

FIG 4 MERS-CoV receptor and attachment factor in the human lower respiratory tract epithelium. (A) The MERS-CoV receptor, DPP4, is expressed in the nasal epithelium of dromedary camels, while in the lungs, it is mainly expressed in endothelial cells. In the human respiratory tract, DPP4 is expressed in bronchiolar epithelial cells (arrowhead) and type II pneumocytes (arrow) in the lungs but not in the nasal epithelium. (B) α2,3-Sialic acid expression and MERS-CoV S1^A binding are also detected in human bronchiolar epithelial cells (arrowheads) and type II pneumocytes (arrows). DPP4 expression, α2,3-sialic acids, and MERS-CoV S1^A binding are indicated in red. (C) In human alveoli, DPP4 expression (red) colocalizes in the same cells where MERS-CoV S1^A binds (green). Pictures of the nasal epithelium were taken at a $\times$400 magnification, and those of the alveoli were taken at a $\times$1,000 magnification.

infectious virus than did dromedary camels (6, 7, 10, 11, 27). In addition, these animals did not transmit the virus via either contact or airborne routes (30, 49). In contrast, MERS-CoV is easily transmitted among dromedary camels (12, 50–53). The absence of MERS-CoV-recognized glycotopes in the nasal epithelium of pigs and rabbits might render them less permissive to MERS-CoV, thus shedding less infectious virus, which subsequently limited virus transmission. This is supported by our finding that the loss of these glycotopes, e.g., through desialylation, could significantly reduce MERS-CoV infection (5). However, it is important to note that the expression of other factors, such as proteases and interferons, may also influence MERS-CoV replication and transmission. MERS-CoV has been reported to use host cell proteases, such as TMPRSS2, furin, and cathepsins, to mediate fusion to host cells (54–56). The virus has been shown to be sensitive to type I interferon, an essential host innate immune cytokine (5, 54–58). Thus, the absence of these proteases and the presence of an inhibiting host innate immune response could effectively limit MERS-CoV replication and transmission, in combination with the absence of MERS-CoV-recognized glycotopes (5, 54–58). The importance of glycotopes in MERS-CoV transmission, either solely or in combination with other factors, remains to be further elucidated. These studies would likely require experimental infection of camelids, particularly dromedary camels (50, 51, 59, 60).

Our data also show that besides their presence in the respiratory tract of dromedary camels and humans, MERS-CoV-recognized glycotopes are expressed at the apical

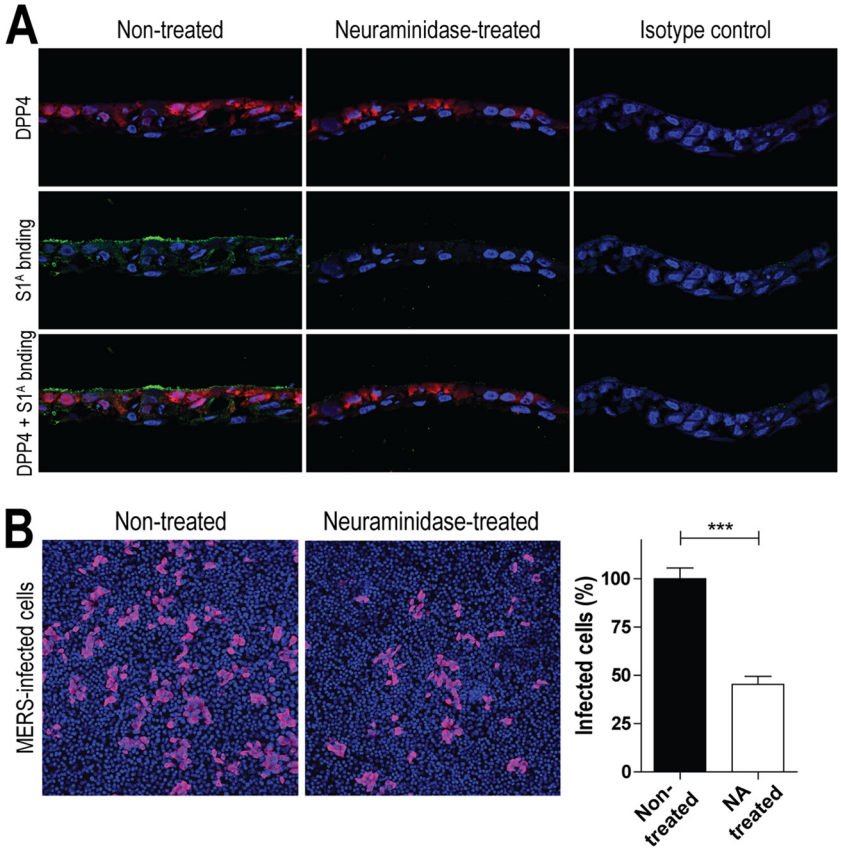

FIG 5 Binding of the MERS-CoV $S1^A$ domain and MERS-CoV infection in primary normal human bronchial epithelial cells are inhibited upon prior neuraminidase treatment. (A) Removal of sialic acids using neuraminidase (NA) treatment diminishes MERS-CoV $S1^A$ binding to primary normal human bronchial epithelial cells but not DPP4 expression in these cells. (B) The same treatment also significantly inhibits MERS-CoV infection in these cells up to 50%. Immunofluorescence images in panel A were taken at a $\times$400 magnification, and those in panel B were taken at a $\times$100 magnification. ***, *P* value of $<$0.0001 by a *t* test.

surface of the villi and crypts of the intestinal epithelium of common pipistrelle bats. Interestingly, in serotine bats and both Gambian epauleted and Egyptian fruit bats, these glycotopes are detected merely in the intestinal crypts. This finding further supports the belief that insectivorous bats are one of the natural hosts of MERS-CoV-like viruses (18–20) and also suggests that not all insectivorous bats express the α2,3-sialic acid glycotopes recognized by MERS-CoV in their intestines. However, how sialic acid abundance and distribution in the intestine relate to infection by MERS-CoV-like viruses in various bat species remains to be investigated. Such studies would likely rely on the availability of primary bat intestinal cell culture or bat intestinal organoids, since experiments in insectivorous bats are difficult to perform, partly due to legal restrictions.

In general, our results showed that the tissue localization of α2,3-sialic acid glycotopes recognized by MERS-CoV $S1^A$ varies between susceptible species. These glycotopes and DPP4 are both expressed in the nasal ciliated epithelial cells of dromedary camels, type II pneumocytes of humans, and intestinal epithelial cells of common pipistrelle bats, providing further evidence that these tissues are the main replication sites of MERS-CoV in the respective species. This study corroborates α2,3-sialic acid glycotopes as an important attachment factor for MERS-CoV (5) and highlights the necessity to further understand their role in MERS-CoV pathogenesis and transmission. Importantly, our results also imply that the MERS-CoV $S1^A$ domain should be considered a target for vaccines (61, 62).

MATERIALS AND METHODS

Tissue samples. Human formalin-fixed paraffin-embedded (FFPE) lung tissues were obtained from the Erasmus MC Tissue Bank and had been used in a previous study (8). These tissue samples were residual human biomaterials taken either from healthy donors or from patients with nonmalignant lung tumors, which were 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 human tissue and medical research under the code of conduct for responsible use (64). Dromedary camel, pig, and rabbit FFPE nasal tissues, both MERS-CoV infected and mock infected, were obtained from previous studies. The dromedary camels and pigs were inoculated via the intranasal route with 10^7 tissue culture infectious doses ($TCID_{50}$), while the rabbits were inoculated with 10^6 $TCID_{50}$ via the intranasal route and 4×10^6 $TCID_{50}$ via the intratracheal route. Mock-infected animals were inoculated with cell culture medium. Infected animals were all sacrificed at day 4 postinoculation (6, 7, 27). Bat FFPE intestinal tissues were also obtained from a previous study (17). Tissues of common pipistrelle and serotine bats were obtained from bats that were stranded or severely wounded and admitted to an official local bat shelter in The Netherlands. The Gambian and Egyptian fruit bats used in this study originated from free-ranging populations in Ghana. The animals were euthanized and necropsied by licensed veterinarians. The bat tissues included in this study were histologically normal as determined by using hematoxylin-eosin staining prior to our experiment.

Histochemistry and immunofluorescence analysis. MERS-CoV nucleoprotein was detected with 5 μg/ml mouse anti-MERS nucleoprotein (Sino-Biological, Beijing, China), while DPP4 expression was detected with either 5 μg/ml goat anti-human DPP4 (R&D, Minneapolis, MN, USA) or 10 μg/ml mouse anti-human DPP4 (clone 11D7; Origene, Rockville, MD, USA). Briefly, the paraffin-embedded tissues were deparaffinized using xylene, hydrated using graded concentrations of alcohol, boiled in 10 mM citric acid buffer (pH 6) for 15 min, and subsequently incubated in 3% H_2O_2 for 10 min and in 5% normal goat serum for 30 min before staining with antibodies. This protocol has been more thoroughly described in previous studies (7, 8, 13, 17). Periodic acid-Schiff staining was performed by deparaffinizing and hydrating the tissue slides, subsequently incubating them in a periodic acid solution for 5 min and Schiff's reagent for 15 min, and counterstaining them with Gill's hematoxylin for 1 min, with water rinsing between steps. This protocol has also been used in our previous study (7). Expression of α2,3-sialic acids was detected using biotinylated *Maackia amurensis* lectin II (Vector Labs, Burlingame, CA, USA) at a 1:800 dilution and streptavidin-horseradish peroxidase (HRP) at a 1:300 dilution, both diluted in 1× Tris-buffered saline containing 0.1 M $MnCl_2$, 1 M $MgCl_2$, and 0.1 M $CaCl_2$, and then subsequently visualized using 3-amino-9-ethylcarbazole and counterstained with hematoxylin. Detection of S1^A binding on the tissues was performed using nanoparticles displaying a multivalent S1^A domain (np-S1^A) and a Strep-tag generated in a previous study (5). The tissues were boiled in 10 mM citric acid buffer (pH 6) for 15 min and blocked with 5% normal goat serum (Dako, Glostrup, Denmark) before staining with 3 μg/ml np-S1^A overnight at 4°C. Tissues that were stained with an equal concentration (3 μg/ml) of blank nanoparticles and those that were pretreated with 800 mU/ml neuraminidase from *Vibrio cholerae* (Sigma-Aldrich, St. Louis, MO, USA) for 4 h at 37°C were used as negative controls. Additional controls were performed by staining tissues with np-S1^A previously incubated for 1 h at 37°C with either anti-S1^A, anti-S1^B, or control nanobodies at a 15-μg/ml concentration. These nanobodies were obtained from a nanobody library generated in a previous study (29). These tissues were subsequently stained with rabbit anti-Strep-tag sera generated in-house and goat anti-rabbit IgG-HRP (Dako, Glostrup, Denmark), each at a 1:100 dilution, for 1 h at room temperature and then visualized with 3-amino-9-ethylcarbazole and counterstained with hematoxylin. For immunofluorescence staining, fluorescence-conjugated secondary antibody was applied in the experiment, i.e., goat anti-rabbit IgG conjugated with Alexa Fluor 488 and goat anti-mouse IgG conjugated with Alexa Fluor 594 (Life Technologies, Carlsbad, CA, USA), both at a 1:250 dilution, with a 1-h incubation at room temperature.

MERS-CoV S1, S1^B, and S1^A ELISAs. The specificity of anti-S1^A, anti-S1^B, and control nanobodies for MERS-CoV S1, S1^A, and S1^B proteins was determined using ELISAs as previously described (29). In brief, 96-well ELISA plates were coated with 1 μg/ml MERS-CoV S1 (amino acids 1 to 751), S1^A (amino acids 1 to 357), or S1^B (amino acids 358 to 588) protein in phosphate-buffered saline (PBS) (pH 7.4) and incubated overnight at 4°C. Wells were then washed with PBS and blocked with 1% bovine serum albumin in PBS–0.5% Tween 20 for 1 h at 37°C. Nanobodies were 2-fold serially diluted in blocking buffer starting at a 1-μg/ml concentration, 100 μl of each dilution was added per well, and plates were incubated at 37°C for 1 h. Next, plates were washed three times in PBS–0.05% Tween 20 (PBST), after which they were incubated with mouse anti-His tag antibodies (1:2,000; Thermo Fisher Scientific) at 37°C for 1 h. Following incubation, the plates were washed and further incubated with goat anti-mouse HRP (1:2,000; Dako) at 37°C for 1 h. After this incubation, plates were washed three times in PBST, a 3,3′,5,5′-tetramethylbenzidine substrate (eBioscience) was added, and the plates were incubated for 10 min. The reaction was stopped with 0.5 N H_2SO_4 (Sigma). The absorbance of each sample was read at 450 nm with an ELISA reader (Tecan Infinite F200).

MERS-CoV infection in well-differentiated primary normal human bronchial epithelial cells. Primary NHBE cells (Lonza, Basel, Switzerland) were cultured on Transwell permeable support (Costar) according to the protocol suggested by the manufacturer (Clonetics airway epithelial cell systems; Lonza, Basel, Switzerland). The cells were differentiated at the air-liquid interface for 6 weeks to promote mucociliary differentiation, resulting in the presence of a multilayered epithelium, ciliated cells, and goblet cells (63). These cells were subsequently either mock treated or pretreated with a mixture of 40 mU of *Arthrobacter ureafaciens* neuraminidase (Sigma-Aldrich, St. Louis, MO, USA) and 50 U of *Clostridium perfringens* neuraminidase (NEB, Ipswich, MA, USA) for 1 h before infection. Each well was inoculated with

the MERS-CoV EMC/2012 strain (6, 7) at 10^6 $TCID_{50}$/ml (multiplicity of infection [MOI] of 5), incubated for 36 h, and fixed in 10% formalin. MERS-CoV-infected cells were visualized with 5 μg/ml mouse anti-MERS nucleoprotein (Sino-Biological, Beijing, China) and a 1:250 dilution of goat anti-mouse IgG conjugated with Alexa Fluor 594 (Life Technologies, Carlsbad, CA, USA). MERS-CoV infection experiments in NHBE cells were performed in triplicate per individual donor. The number of infected cells was counted for each well, and the percentage of infected cells was determined as described in our previous study (5). Statistical analysis was performed using Student's *t* test, and results are presented as means ± standard deviations for each group.

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