Molecular mechanism for antibody-dependent enhancement of coronavirus entry

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Abstract

Antibody-dependent enhancement (ADE) of viral entry has been a major concern for epidemiology, vaccine development and antibody-based drug therapy. However, the molecular mechanism behind ADE is still elusive. Coronavirus spike protein mediates viral entry into cells by first binding to a receptor on host cell surface and then fusing viral and host membranes. Here we investigated how a neutralizing monoclonal antibody (mAb), which targets the receptor-binding domain (RBD) of MERS coronavirus spike, mediates viral entry using pseudovirus entry and biochemical assays. Our results showed that mAb binds to the virus-surface spike, allowing it to undergo conformational changes and become prone to proteolytic activation. Meanwhile, mAb binds to cell-surface IgG Fc receptor, guiding viral entry through canonical viral-receptor-dependent pathways. Our data suggest that the antibody/Fc-receptor complex functionally mimics viral receptor in mediating viral entry. Moreover, we characterized mAb dosages in viral-receptor-dependent, antibody-dependent, and both-receptors-dependent entry pathways, delineating guidelines on mAb usages in treating viral infections. Our study reveals a novel molecular mechanism for antibody-enhanced viral entry and can guide future vaccination and antiviral strategies.

Significance

Antibody-dependent enhancement (ADE) of viral entry has been observed for many viruses. It was shown that antibodies target one serotype of viruses but only sub-neutralize another, leading to ADE of the latter viruses. Here we identify a novel mechanism for ADE: a neutralizing antibody binds to the virus-surface spike protein of coronaviruses like a viral receptor, triggers a conformational change of the spike, and mediates viral entry into IgG-Fc-receptor-expressing cells through canonical viral-receptor-dependent pathways. We further evaluated how antibody dosages impacted viral entry into cells expressing viral receptor, Fc receptor, or both receptors. This study reveals complex roles of antibodies in viral entry and can guide future vaccine design and antibody-based drug therapy.

Introduction

Antibody-dependent enhancement (ADE) occurs when antibodies facilitate viral entry into host cells and enhance viral infection in these cells (1, 2). ADE has been observed for a variety of viruses, most notably in flaviviruses (e.g., dengue virus) (3-6). It has been shown that when patients are infected by one serotype of dengue virus (i.e., primary infection), they produce neutralizing antibodies targeting the same serotype of the virus. However, if they are later infected by another serotype of dengue virus (i.e., secondary infection), the preexisting antibodies cannot fully neutralize the virus. Instead, the antibodies first bind to the virus, then bind to the IgG Fc receptors on immune cells, and mediate viral entry into these cells. Similar mechanism has been observed for HIV and Ebola virus (7-10). Thus, sub-neutralizing antibodies (or non-neutralizing antibodies in some cases) are responsible for ADE of these viruses. Given the critical roles of antibodies in host immunity, ADE causes serious concerns in epidemiology, vaccine design and antibody-based drug therapy. This study reveals a novel mechanism for ADE where fully neutralizing antibodies mimic the function of viral receptor in mediating viral entry into Fc-receptor-expressing cells.

Coronaviruses are a family of large, positive-stranded, and enveloped RNA viruses (11, 12). Two highly pathogenic coronaviruses, SARS coronavirus (SARS-CoV) and MERS coronavirus (MERS-CoV), cause lethal infections in humans (13-16). An envelope-anchored spike protein guides coronavirus entry into host cells (17). As a homo-trimer, the spike contains three receptor-binding S1 subunits and a trimeric membrane-fusion S2 stalk (18-25). This state of the spike on the mature virions is called "pre-fusion". SARS-CoV and MERS-CoV recognize angiotensin-converting enzyme 2

(ACE2) and dipeptidyl peptidase 4 (DPP4), respectively, as their viral receptor (26-28). Their S1 each contains a receptor-binding domain (RBD) that mediates receptor recognition (29, 30) (Fig. 1A). The RBD is located on the tip of the spike trimer and is present in two different states – standing up and lying down (18, 21) (Fig. 1B). Binding to a viral receptor can stabilize the RBD in the standing-up state (20). Receptor binding also triggers the spike to undergo further conformational changes, allowing host proteases to cleave at two sites sequentially – first at the S1/S2 boundary (i.e., S1/S2 site) and then within S2 (i.e., S2' site) (31, 32). Proteolysis of the spike can take place during viral maturation (by proprotein convertases), after viral release (by extracellular proteases), after viral attachment (by cell-surface proteases), or after viral endocytosis (by lysosomal proteases) (33-39). After two protease cleavages, S1 dissociates and S2 undergoes a dramatic structural change to fuse host and viral membranes; this membrane-fusion state of the spike is called "post-fusion" (40, 41). Due to the recent progresses towards understanding the receptor recognition and membrane fusion mechanisms of coronavirus spikes, coronaviruses represent an excellent model system for investigating ADE of viral entry.

ADE has been observed for coronaviruses. Several studies have shown that sera induced by SARS-CoV spike enhance viral entry into Fc-receptor-expressing cells (42-44). Further, one study demonstrated that unlike receptor-dependent viral entry, sera-dependent SARS-CoV entry does not go through the endosome pathway (44). Additionally, it has long been known that immunization of cats with feline coronavirus spike leads to worsened future infection due to the induction of infection-enhancing antibodies (45-47). However, detailed molecular mechanisms for ADE of coronavirus

entry are still unknown. We previously discovered a monoclonal antibody (mAb) (named Mersmab1), which has strong binding affinity for MERS-CoV RBD and efficiently neutralizes MERS-CoV entry by outcompeting DPP4 (48); this discovery allowed us to comparatively study the molecular mechanisms for antibody-dependent and receptor-dependent viral entries.

In this study, we examined how Mersmab1 binds to MERS-CoV spike, triggers the spike to undergo conformational changes, and mediates viral entry into Fc-receptor-expressing cells. We also investigated the pathways and antibody dosages for Mersmab1-dependent and DPP4-dependent viral entries. Our study sheds lights on the mechanisms of ADE and provides insight into vaccine design and antibody-based antiviral drug therapy.

Results

Antibody-dependent enhancement of coronavirus entry

To investigate ADE of coronavirus entry, we first characterized the interactions between Mersmab1 (which is a MERS-CoV-RBD-specific mAb) and MERS-CoV spike using biochemical methods. First, ELISA was performed between Mermab1 and MERS-CoV RBD and between Mersmab1 and MERS-CoV spike ectodomain (S-e) (Fig. 2A). To this end, Mersmab1 (which was in excess) was coated to the ELISA plate, and gradient amounts of recombinant RBD or S-e were added for detection of potential binding to Mersmab1. The result showed that both the RBD and S-e bound to Mersmab1. S-e bound to Mersmab1 more tightly than the RBD did, likely due to the multivalent effects

associated with the trimeric state of S-e. Second, we prepared Fab from Mersmab1 using papain digestion and examined the binding between Fab and S-e using ELISA. Here recombinant S-e (which was in excess) was coated to the ELISA plate, and gradient amounts of Fab or Mersmab1 were added for detection of potential binding to S-e. The result showed that both Fab and Mersmab1 bound to S-e (Fig. 2B). Mersmab1 bound to S-e more tightly than Fab did, also likely due to the multivalent effects associated with the dimeric state of Mersmab1. Third, flow cytometry assay was carried out to detect the binding between S-e and DPP4 receptor and among S-e, Mersmab1 and CD32A (which is an Fc receptor). To this end, DPP4 or CD32A was expressed on the surface of human HEK293T cells (human kidney cells), and recombinant S-e was added for detection of potential binding to one of the two receptors in the absence or presence of Mersmab1. The result showed that without Mersmab1, S-e bound to DPP4 only; in the presence of Mersmab1, S-e bound to CD32A (Fig. 2C). As a negative control, a SARS-CoV RBD-specific mAb (49) did not mediate the binding of S-e to CD32A. The cell-surface expressions of both DPP4 and CD32A were measured and used for calibrating the flow cytometry result (Fig. 2D), demonstrating that the direct binding of S-e to DPP4 is stronger than the indirect binding of S-e to CD32A through Mersmab1. Overall, these biochemical results reveal that Mersmab1 not only directly binds to the RBD region of MERS-CoV S-e, but also mediates the indirect binding interactions between MERS-CoV S-e and the Fc receptor.

Next we investigated whether Mersmab1 mediates MERS-CoV entry into Fc-receptor-expressing cells. To this end, we performed MERS-CoV pseudovirus entry assay, where retroviruses pseudotyped with MERS-CoV spike (i.e., MERS-CoV

153 pseudoviruses) were used to enter human cells expressing CD32A on their surface. The
154 main advantage of pseudovirus entry assay is to focus on the viral entry step (which is
155 mediated by MERS-CoV spike) by separating viral entry from the other steps of viral
156 infection cycles (e.g., replication, packaging and release). We tested three different types
157 of Fc receptors: CD16A, CD32A, and CD64A; each of these Fc receptors was
158 exogenously expressed in HEK293T cells. We also tested macrophage cells where
159 mixtures of Fc receptors were endogenously expressed. Absence of Mersmab1 served as
160 a control for Mersmab1 (a non-neutralizing mAb would be appropriate as another control
161 for Mersmab1, but we do not have access to any non-neutralizing mAb). The result
162 showed that in the absence of Mersmab1, MERS-CoV pseudoviruses could not enter Fc-
163 receptor-expressing cells; in the presence of Mersmab1, MERS-CoV pseudoviruses
164 demonstrated significant efficiency in entering CD32A-expressing HEK293T cells and
165 macrophage cells (Fig. 3A). In comparison, in the absence of Mersmab1, MERS-CoV
166 pseudoviruses entered DPP4-expressing HEK293T cells efficiently, but the entry was
167 blocked effectively by Mersmab1 (Fig. 3A). In control experiments, anti-SARS mAb did
168 not mediate MERS-CoV pseudoviruses entry into Fc-receptor-expressing HEK293T cells
169 or macrophages, and neither did it block MERS-CoV pseudoviruses entry into DPP4-
170 receptor-expressing HEK293T cells (Fig. 3A). In another set of control experiments, we
171 showed that neither the Fc nor the Fab portion of Mersmab1 could mediate MERS-CoV
172 pseudoviruses entry into Fc-receptor-expressing HEK293T cells or macrophages (Fig.
173 3B), suggesting that both the Fc and Fab portions of anti-MERS mAb are required for
174 antibody-mediated viral entry. Here the above DPP4-expressing HEK293T cells were
175 induced to exogenously express high levels of DPP4. To detect background expression

levels of DPP4, we performed qRT-PCR on HEK293T cells. The result showed that HEK293T cells express very low levels of DPP4 (Fig. 3C). In comparison, MRC5 cells (human lung cells) express high levels of DPP4, whereas Hela cells (human cervical cells) do not express DPP4 (Fig. 3C). Because of the comprehensive control experiments that we performed, the very low endogenous expression of DPP4 in HEK293T cells should not affect our conclusions. Nevertheless, we confirmed the above results using Hela cells that do not express DPP4 (Fig. 3D). Overall, our results reveal that Mersmab1 mediates MERS-CoV entry into Fc-receptor-expressing cells, but blocks MERS-CoV entry into DPP4-expressing cells.

To expand the above observations to another coronavirus, we investigated ADE of SARS-CoV entry. We previously identified a SARS-CoV-RBD-specific mAb, named 33G4, which binds to the ACE2-binding region of SARS-CoV RBD (49, 50); this mAb was examined here for its potential capability to mediate ADE of SARS-CoV entry (Fig. 3E). The result showed that 33G4 mediated SARS-CoV pseudovirus entry into CD32A-expressing cells, but blocked SARS-CoV pseudovirus entry into ACE2-expressing cells. Therefore, both the MERS-CoV-RBD-specific mAb and the SARS-CoV-RBD specific mAb can mediate the respective coronavirus to enter Fc-receptor-expressing human cells, while blocking the entry of the respective coronavirus into viral-receptor-expressing human cells. For the remaining of this study, we selected the MERS-CoV-RBD-specific mAb, Mersmab1, for in-depth analysis of ADE.

Molecular mechanism for antibody-dependent enhancement of coronavirus entry

To understand the molecular mechanism of ADE, we investigated whether Mersmab1 triggers any conformational change of MERS-CoV spike. It was shown previously that DPP4 binds to MERS-CoV spike and stabilizes the RBD in the standing-up position (Fig. 1A, 1B), resulting in a weakened spike structure and allowing the S2' site to become exposed to proteases (51). Here we repeated this experiment: MERS-CoV pseudoviruses were incubated with DPP4 and then subjected to trypsin cleavage (Fig. 4A). The result showed that during the viral packaging process, virus-surface-anchored MERS-CoV spike molecules were cleaved at the S1/S2 site by proprotein convertases; in the absence of DPP4, the spike molecules could not be cleaved further at the S2' site by trypsin. These data suggest that only the S1/S2 site, but not the S2' site, was accessible to proteases in the free form of the spike trimer. In the presence of DPP4, a significant amount of MERS-CoV spike molecules were cleaved at the S2' site by trypsin, indicating that DPP4 binding triggered a conformational change of MERS-CoV spike to expose the S2' site. Interestingly, we found that Mersmab1 binding also allowed MERS-CoV spike to be cleaved at the S2' site by trypsin. As a negative control, the SARS-CoV-RBD-specific mAb did not trigger MERS-CoV spike to be cleaved at the S2' site by trypsin. Hence, like DPP4, Mersmab1 triggers a similar conformational change of MERS-CoV spike to expose the S2' site for proteolysis.

We further analyzed the binding between Mersmab1 and MERS-CoV S-e using negative-stain electron microscopy (EM). We previously demonstrated through mutagenesis studies that Mersmab1 binds to the same receptor-binding region on MERS-CoV RBD as DPP4 does (Fig. 1C) (48). Because full-length Mersmab1 (which is a dimer) triggered aggregation of S-e (which is a trimer), we prepared the Fab part (which

220 is a monomer) of Mersmab1, detected the binding between Fab and S-e (Fig. 2B), and
221 used Fab in the negative-stain EM study. The result showed that Fab bound to the tip of
222 the S-e trimer, where the RBD is located (Fig. 4B). Due to the limited resolution of
223 negative-stain EM, we could not clearly see the conformation of the Fab-bound RBD.
224 However, based on previous studies, the receptor-binding site on the RBD in the spike
225 trimer is only accessible when the RBD is in the standing-up position (18, 20, 21). Hence,
226 the fact that the mAb binds to the receptor-binding region of the RBD in the spike trimer
227 suggests that the RBD is in the standing-up state. Thus, the results from negative-stain
228 EM and the proteolysis study are consistent with each other, supporting that like DPP4,
229 Mersmab1 stabilizes the RBD in the standing-up position and triggers a conformational
230 change of the spike. Future study on the high-resolution cryo-EM structure of MERS-
231 CoV S-e trimer complexed with Mersmab1 will be needed to provide detailed structural
232 information for the Mersmab1-triggered conformational changes of MERS-CoV S-e.

233 To understand the pathways of Mersmab1-dependent MERS-CoV entry, we
234 evaluated the potential impact of different proteases on MERS-CoV pseudovirus entry;
235 these proteases are distributed along the viral entry pathway. First, proprotein convertase
236 inhibitor (PPCi) was used for examining the role of proprotein convertases in the
237 maturation of MERS-CoV spike and the impact of proprotein convertases on the ensuing
238 Mersmab1-dependent viral entry (Fig. 5A). The result showed that when MERS-CoV
239 pseudoviruses were produced from HEK293T cells in the presence of PPCi, the cleavage
240 of MERS-CoV spike by proprotein convertases was significantly inhibited (Fig. 5B). In
241 the absence of Mersmab1, MERS-CoV pseudoviruses packaged in the presence of PPCi
242 entered DPP4-expressing human cells more efficiently than those packaged in the

absence of PPCi (Fig. 5A). In the presence of Mersmab1, MERS-CoV pseudoviruses packaged in the presence of PPCi entered CD32A-expressing cells more efficiently than those packaged in the absence of PPCi (Fig. 5A). These data suggest that proprotein convertases play a role (albeit not as drastic as some other proteases; see below) in both DPP4-dependent and Mersmab1-dependent MERS-CoV entry. Second, cell-surface protease TMPRSS2 (transmembrane Serine Protease 2) was introduced to human cells for evaluation of its role in Mersmab1-dependent viral entry (Fig. 5C). The result showed that in the absence of Mersmab1, TMPRSS2 enhanced MERS-CoV pseudovirus entry into DPP4-expressing cells, consistent with previous reports (36). In the presence of Mersmab1, TMPRSS2 also enhanced MERS-CoV pseudovirus entry into CD32A-expressing cells, suggesting that TMPRSS2 activates Mersmab1-dependent MERS-CoV entry. Third, lysosomal protease inhibitors were evaluated for the role of lysosomal proteases in Mersmab1-dependent viral entry (Fig. 5D). Two inhibitors were used, lysosomal acidification inhibitor Baf-A1 and cysteine protease inhibitor E64d. The result showed that lysosomal protease inhibitors blocked the DPP4-dependent viral entry pathway, consistent with previous reports (39). Lysosomal protease inhibitors also blocked the Mersmab1-dependent viral entry pathway, suggesting that lysosomal proteases play important roles in Mersmab1-dependent MERS-CoV entry. Taken together, the DPP4-dependent and Mersmab1-dependent MERS-CoV entries can both be activated by proprotein convertases, cell-surface proteases, and lysosomal proteases; hence the same pathways are shared by DPP4-dependent and Mersmab1-dependent MERS-CoV entries.

Antibody dosages for antibody-dependent enhancement of coronavirus entry

To determine the range of Mersmab1 dosages in ADE, MERS-CoV pseudovirus entry was performed in the presence of different concentrations of Mersmab1. Three types of human HEK293T cells were used: HEK293T cells exogenously expressing DPP4 only, CD32A only, or both DPP4 and CD32A. Accordingly, three different results were obtained. First, as the amount of Mersmab1 increased, viral entry into DPP4-expressing HEK293T cells continuously dropped (Fig. 6A). This result reveals that Mersmab1 blocks the DPP4-dependent viral entry pathway by outcompeting DPP4 for binding to MERS-CoV spike. Second, as the amount of Mersmab1 increased, viral entry into CD32A-expressing HEK293T cells first increased and then decreased (Fig. 6A). The turning point was about 100 ng/ml Mersmab1. A likely explanation for this result is as follows: at low concentrations, more mAb molecules enhance the indirect interactions between MERS-CoV spike and the Fc receptor; at high concentrations, mAb molecules saturate the cell-surface Fc receptor molecules and then further bind to MERS-CoV spike and block the indirect interactions between MERS-CoV spike and the Fc receptor. Third, as the amount of Mersmab1 increased, viral entry into cells expressing both DPP4 and CD32A first dropped, then increased, and finally dropped again (Fig. 6B). This result is the cumulous effect of the previous two results. It reveals that when both DPP4 and CD32A are present on host cell surface, Mersmab1 inhibits viral entry (by blocking the DPP4-dependent entry pathway) at low concentrations, promotes viral entry (by enhancing the CD32A-dependent entry pathway) at intermediate concentrations, and inhibits viral entry (by blocking both the DPP4- and CD32A-dependent entry pathways) at high concentrations. We further confirmed the above results using MRC5 cells, which are human lung cells endogenously expressing DPP4 (Fig. 6C, 6D). Therefore, ADE of

289 MERS-CoV entry depends on the range of Mersmab1 dosages as well as expressions of
290 the viral and Fc receptors on cell surfaces.

291 **Discussions**

292 ADE of viral entry has been observed and studied extensively in flaviviruses,
293 particularly dengue virus (3-6). It has also been observed in HIV and Ebola viruses (7-
294 10). For these viruses, it has been proposed that primary viral infections of hosts led to
295 production of antibodies that are sub-neutralizing or non-neutralizing for secondary viral
296 infections; these antibodies cannot completely neutralize secondary viral infections, but
297 instead guide virus particles to enter Fc-receptor-expressing cells. ADE can lead to
298 worsened symptoms in secondary viral infections, causing major concerns for
299 epidemiology. ADE is also a major concern for vaccine design and antibody-based drugs
300 therapy, since antibodies generated or used in these procedures may lead to ADE. ADE
301 has been observed in coronavirus for decades, but the molecular mechanisms are
302 unknown. Recent advances in understanding the receptor recognition and cell entry
303 mechanisms of coronaviruses have allowed us to use coronaviruses as a model system for
304 studying ADE.

305 In this study we first demonstrated that a MERS-CoV-RBD-specific neutralizing
306 mAb binds to the RBD region of MERS-CoV spike and further showed that the mAb
307 mediates MERS-CoV pseudovirus entry into Fc-receptor-expressing human cells.
308 Moreover, a SARS-CoV-RBD-specific neutralizing mAb mediates ADE of SARS-CoV
309 pseudovirus entry. These results demonstrated that ADE of coronaviruses is mediated by
310 neutralizing mAbs that target the RBD of coronavirus spikes. In addition, the same

311 coronavirus strains that led to the production of fully neutralizing mAbs can be mediated
312 to go through ADE by these neutralizing mAbs. Our results differ from previously
313 observed ADE of flaviviruses where primary infections and secondary infections are
314 caused by two different viral strains and where ADE-mediating mAbs are only sub-
315 neutralizing or non-neutralizing for secondary viral infections (3-6). Therefore, our study
316 expands the concept of ADE of viral entry.

317 We then examined the molecular mechanism for ADE of coronavirus entry. We
318 showed that the mAb binds to the tip of MERS-CoV spike trimer, where the RBD is
319 located. mAb binding likely stabilizes the RBD in the standing-up position, triggers a
320 conformational change of MERS-CoV spike, and exposes the previously inaccessible S2'
321 site to proteases. During the preparation of this manuscript, a newly published study
322 demonstrated that a SARS-CoV-RBD-specific mAb (named S230) bound to the ACE2-
323 binding region in SARS-CoV RBD, stabilized the RBD in the standing-up position, and
324 triggered conformational changes of SARS-CoV spike (Fig. 7A) (52). In contrast, a
325 MERS-CoV-RBD-specific mAb (named LCA60) bound to the side of MERS-CoV RBD,
326 away from the DPP4-binding region, stabilized the RBD in the lying-down position, and
327 did not trigger conformational changes of MERS-CoV spike (Fig. 7B). These published
328 results are consistent with our result on Mersmab1-triggered conformational changes of
329 MERS-CoV spike, together suggesting that in order to trigger conformational changes of
330 coronavirus spikes, mAbs need to bind to the receptor-binding region in their RBD and
331 stabilize the RBD in the standing-up position. Moreover, our study revealed that ADE of
332 MERS-CoV entry follows the same entry pathways of DPP4-dependent MERS-CoV
333 entry. Specifically, proprotein convertases partially activate MERS-CoV spike. If cell-

surface proteases are present, MERS-CoV spike can be further activated and fuse membranes on the cell surface; otherwise, MERS-CoV enters endosomes and lysosomes, where lysosomal proteases activate MERS-CoV spike for membrane fusion. Taken together, RBD-specific neutralizing mAbs bind to the same region on coronavirus spikes as viral receptors do, trigger conformational changes of the spikes as viral receptors do, and mediate ADE through the same pathways as viral-receptor-dependent viral entries. In other words, RBD-specific neutralizing mAbs mediate ADE of coronavirus entry by functionally mimicking viral receptors.

Finally we analyzed ADE of coronavirus entry at different antibody dosages. MERS-CoV entry into cells expressing both viral and Fc receptors demonstrates complex mAb-dosage-dependent patterns. As the concentration of mAb increases, (i) viral entry into DPP4-expressing cells is inhibited more efficiently because mAb binds to the spike and blocks the DPP4-dependent entry pathway, (ii) viral entry into Fc-receptor-expressing cells is first enhanced and then inhibited because mAb binds to the Fc receptor to enhance the ADE pathway until the Fc receptor molecules are saturated, and (iii) viral entry into cells expressing both DPP4 and Fc receptor is first inhibited, then enhanced, and finally inhibited again because of the cumulative effects of the previous two patterns. In other words, for viral entry into cells expressing both DPP4 and Fc receptor, there exist a balance between the DPP4-dependent and antibody-dependent entry pathways that can be shifted and determined by mAb dosages. Importantly, ADE occurs only at intermediate mAb dosages. Our study explains an earlier observation where ADE of dengue viruses only occurs at certain concentrations of mAb (5). While many human tissues express either DPP4 or Fc receptor, a few of them, most notably placenta, express

both of them (53, 54). For other viruses that use viral receptors different from DPP4, there may also be human tissues whether the viral receptor and Fc receptor are both expressed. The expression levels of these two receptors in specific tissue cells likely are determinants of mAb dosages at which ADE would occur in these tissues. Other determinants of ADE-enabling mAb dosages may include the binding affinities of the mAb for the viral and Fc receptors. Overall, our study suggests that ADE of viruses depends on antibody dosages, tissue-specific expressions of viral and Fc receptors, and some intrinsic features of the antibody.

Our findings not only reveal a novel molecular mechanism for ADE of coronaviruses, but also provide general guidelines on viral vaccine design and antibody-based antiviral drug therapy. As we have shown here, RBD-specific neutralizing mAbs may mediate ADE of viruses by mimicking the functions of viral receptors. Neutralizing mAbs targeting other parts of viral spikes would be less likely to mediate ADE if they do not trigger the conformational changes of the spikes. Hence, to reduce the likelihood of ADE, spike-based subunit vaccines lacking the RBD can be designed to prevent viral infections. Based on the same principle, neutralizing mAbs targeting other parts of the spike can be selected to treat viral infections. Moreover, as already discussed, our study stresses on the importance of choosing antibody dosages that do not cause ADE and points out that different tissue cells should be closely monitored for potential ADE at certain antibody dosages.

The *in vitro* systems used in this study provide a model framework for ADE. Future research using *in vivo* systems is needed to further confirm these results. Our previous study showed that a humanized version of Mersmab1 efficiently protected

human DPP4-transgenic mice from live MERS-CoV challenges (48, 55), suggesting that given the antibody dosages used in this previous study as well as the binding affinity of the mAb for human DPP4, the receptor-dependent pathway of MERS-CoV entry dominated over ADE *in vivo*. Thus, future *in vivo* studies may need to screen for a wide range of antibody dosages and also for a variety of tissues with different ratios of DPP4 and Fc receptor expressions. Although ADE has not been observed for MERS-CoV *in vivo*, our study suggests that ADE occurs under some specific conditions *in vivo*, depending on the antibody dosages, binding affinity of the mAb for DPP4, and tissue expressions of DPP4 and Fc receptor. Moreover, the mechanism that we have identified for ADE of MERS-CoV *in vitro* may account for the ADE observed *in vivo* for other coronaviruses such as SARS-CoV and feline coronavirus (42-47). Overall, our study reveals complex roles of antibodies in viral entry and can guide future vaccine design and antibody-based drug therapy.

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Materials and Methods

Cell lines and Plasmids

HEK293T cells and HEK293F cells (human embryonic kidney cells), Hela cells (human cervical cells), and MRC5 cells (human lung cells) were obtained from the ATCC (American Type Culture Collection). HEK293-gamma chain cells (human embryonic kidney cells) were constructed previously (56). These cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. THP-1 cells (human macrophage cells) were obtained from the ATCC and were cultured in Roswell Park Memorial Institute (RPMI) culture medium (Invitrogen) containing 10% of heat inactivated fetal bovine serum and supplemented with 10 mM Hepes, 1 mM pyruvate, 2.5 g/l D-glucose, 50 pM ß-mercaptoethanol, and 100 µg/ml streptomycin.

For induction of macrophages, human monocytic THP-1 cells were treated with 150 nM phorbol 12-myristate 13-acetate for 24 hours, followed by 24 hours incubation in RPMI medium (57) before experiments.

The full-length genes of MERS-CoV spike (GenBank accession number AFS88936.1), SARS-CoV spike (GenBank accession number AFR58742), human DPP4 (GenBank accession number NM_001935.3) and human ACE2 (GenBank accession number NM_021804) were synthesized (GenScript Biotech). Three Fc receptor genes, human CD16A (GenBank accession number NM_000569.7), human CD32A (GenBank accession number NM_001136219.1) and human CD64A (GenBank accession number NM_000566.3), were cloned previously (58, 59). For protein expressions on cell surfaces

421 or pseudovirus surfaces, the above genes were subcloned into the pcDNA3.1(+) vector
422 (Life Technologies) with a C-terminal C9 tag.

423 *Protein purification and antibody preparation*

424 For ELISA and negative-stain electron microscopic study, recombinant MERS-
425 CoV spike ectodomain (S-e) was prepared. The MERS-CoV S-e (residues 1-1294) was
426 subcloned into pCMV vector; it contained a C-terminal GCN4 trimerization tag and a
427 His_6 tag. To stabilize S-e in the pre-fusion conformation, we followed the procedure from
428 a previous study by introducing mutations to the S1/S2 protease cleavage site (RSVR748-
429 751ASVA) and the S2 region (V1060P, L1061P) (21). MERS-CoV S-e was expressed in
430 HEK293F cells using a FreeStyle 293 mammalian cell expression system (Life
431 technologies). Briefly, HEK293F cells were transfected with the plasmid encoding
432 MERS-CoV S-e and cultured for three days. The protein was harvested from the cell
433 culture medium, purified sequentially on Ni-NTA column and Superdex200 gel filtration
434 column (GE Healthcare), and stored in a buffer containing 20 mM Tris pH7.2 and 200
435 mM NaCl. The ectodomain of human DPP4 was expressed and purified as previously
436 described (39). Briefly, DPP4 ectodomain (residues 39-766) containing an N-terminal
437 human CD5 signal peptide and a C-terminal His_6 tag were expressed in insect cells using
438 the Bac-to-Bac expression system (Life Technologies), secreted to cell culture medium
439 and purified in the same way as MERS-CoV S-e.

440 Both the MERS-CoV-RBD-specific mAb (i.e., Mersmab1) and SARS-CoV-RBD-
441 specific mAb (i.e., 33G4) were purified as previously described (48, 49). Briefly,
442 hybridoma cells expressing the mAb were injected into the abdomen of mice. After 7-10
443 days, the mouse ascites containing the mAb were collected. The mAb was then purified

using a Protein A column (GE Healthcare). Fab of Mersmab1 antibody was prepared using Immobilized Papain beads (ThermoFisher Scientific) according to the manufacturer's manual. Briefly, Mersmab1 antibody was incubated with Immobilized Papain beads in digestion buffer (20 mM sodium phosphate, 10 mM EDTA, 20 mM L-cysteine.HCl pH 7.0) in a shaker water bath at 37 ºC overnight. After digestion, the reaction was stopped with 10 mM Tris.HCl pH 7.5, and the supernatant was collected through centrifugation at 12,000g for 15 min. Fab was then separated from undigested IgG and Fc using a Protein A column (GE HealthCare).

ELISA

The binding affinity between mAb and MERS-CoV S-e or RBD was measured using ELISA assay as previously described (60). Briefly, ELISA plates were pre-coated with mAb (350 nM) at 37 °C for 1 hour. After blocking with 1% BSA at 37 °C for 1 hour, MERS-CoV S-e or RBD (300 nM or gradient concentrations as specified in Fig. 2) was added to the plates and incubated with mAb at 37 °C for 1 hour. After washes with PBS buffer, the plates were incubated with anti-His_6 antibody (Santa Cruz) at 37 °C for 1 hour. Then the plates were washed with PBS and incubated with HRP-conjugated goat anti-mouse IgG antibody (1:5,000) at 37 °C for 1 hour. After more washes with PBS, enzymatic reaction was carried out using ELISA substrate (Life Technologies) and stopped with 1 M H_2SO_4. Absorbance at 450 nm (A450) was measured using Tecan Infinite M1000 PRO Microplate Reader (Tecan Group Ltd.). Five replicates were done for each sample. PBS buffer was used as a negative control.

Flow cytometry cell-binding assay

Flow cytometry was performed as previously described (22). Briefly, HEK293T cells exogenously expressing DPP4 or one of the Fc receptors were incubated with MERS-CoV S-e (40 µg/ml) and mAb (50 µg/ml) (both of which contained a C-terminal His_6 tag) at room temperature for 30 min, followed by incubation with fluorescein phycoerythrin (PE)-labeled anti-His_6 probe antibody for another 30 min. The cells then were analyzed using FACS (fluorescence activated cell sorting).

Pseudovirus entry assay

Coronavirus-spike-mediated pseudovirus entry assay was carried out as previously described (61, 62). Briefly, for pseudovirus packaging, HEK293T cells were co-transfected with a plasmid carrying an Env-defective, luciferase-expressing HIV, type 1 genome (pNL4–3.luc.R-E-) and a plasmid encoding MERS-CoV or SARS-CoV spike. Pseudoviruses were harvested and purified using a sucrose gradient ultracentrifugation at 40,000g 72 hours after transfection and then used to enter the target cells. To detect pseudovirus entry, pseudoviruses and cells were incubated for 5 hours at 37°C, and then medium was changed and cells were incubated for an additional 60 hours. Cells were then washed with PBS and lysed. Aliquots of cell lysates were transferred to Optiplate-96 (PerkinElmer), followed by addition of luciferase substrate. Relative light unites (RLUs) were measured using EnSpire plate reader (PerkinElmer). All the measurements were carried out in four replicates. To inhibit proprotein convertases during packaging of MERS-CoV pseudoviruses, 50 nM proprotein convertase inhibitor (PPCi) Dec-RVKR-CMK (Enzo Life Sciences) was added to the cell culture medium 5 hours post transfection, before the packaged pseudoviruses were purified as described above. Inhibition of pseudovirus entry using various protease inhibitors was carried out as

described previously (63). Briefly, cells were pre-treated with 50 nM proprotein convertase inhibitor Dec-RVKR-CMK (Enzo Life Sciences), 100 nM camostat mesylate (Sigma-Aldrich), 100 nM bafilomycin A1 (Baf-A1) (Sigma-Aldrich), 50 nM E64d (Sigma-Aldrich), at 37 °C for 1 hour, or 500 ng/ml antibody for 5 min. The above cells were then used for pseudovirus entry assay.

Isolation and quantification of cell surface receptor proteins

To examine the expression levels of receptor proteins in cell membranes, the cells expressing the receptor were harvested and all membrane-associated proteins were extracted using a membrane protein extraction kit (Thermo Fisher Scientific). Briefly, cells were centrifuged at 300g for 5 min and washed with cell wash solution twice. The cell pellets were resuspended in 0.75 ml permeabilization buffer and incubated at 4°C for 10 min. The supernatant containing cytosolic proteins was removed after centrifugation at 16,000g for 15 min. The pellets containing membrane-associated proteins were resuspended in 0.5 ml solubilization buffer and incubated at 4°C for 30 min. After centrifugation at 16,000g for 15 min, the membrane-associated proteins from the supernatant were transferred to a new tube. The expression level of membrane-associated C9-tagged receptor proteins among all membrane-associated proteins was then measured using Western blot analysis and further used for normalizing the results from flow cytometry cell-binding assays and pseudovirus entry assays.

Extraction of total RNA and qRT-PCR

Total RNAs of cells were extracted using TRIzol reagent according to the manufacturer's manual. Briefly, TRIzol was added to the cell lysate, and then chloroform and phenol-chloroform were added to precipitate RNA. The RNA pellets were washed

using ethanol, solubilized in DEPC-treated water, and then reverse-transcribed using MLV reverse transcriptase (Promega) and oligo dT primers (Promega). Quantitative PCR on DPP4 RNA was performed using DPP4-specific primers and SYBR qPCR kit (Bio-Rad) in CFX qPCR instrument (Bio-Rad). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) RNA was used as a control. The primers are listed below:

DPP4 - forward-5′-AGTGGCGTGTTCAAGTGTGG-3’; reverse-5′-CAAGGTTGTCTTCTGGAGTTGG-3’

GAPDH - forward-5′-GGAAGGTGAAGGTCGGAGTCAACGG-3′; reverse-5′-CTCGCTCCTGGAAGATGGTGATGGG-3′

Proteolysis assay

Purified MERS-CoV pseudoviruses were incubated with 67 µg/ml recombinant DPP4, 67 µg/ml mAb or PBS at 37 ºC for 30 min, and then treated with 10^{-3} mg/ml TPCK-treated-trypsin on ice for 20 min. Samples were subjected to Western blotting analysis. MERS-CoV spike and its cleaved fragments (which contained a C-terminal C9 tag) were detected using an anti-C9 tag monoclonal antibody (Santa Cruz Biotechnology).

Negative-stain electron microscopy

Samples were diluted to a final concentration of 0.02 mg/mL in PBS buffer and loaded onto glow-discharged 400 mesh carbon grids (Electron Microscopy Sciences). The grids were stained with 0.75% uranyl formate. All micrographs were acquired using a Tecnai G2 Spirit BioTWIN at 120 keV (FEI Company) and an Eagle 4 mega pixel CCD camera at 6,000 × nominal magnification at the University of Minnesota.

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Figure Legends:

Figure 1. Structural similarity between DPP4 and mAb in binding MERS-CoV spike. (A) Tertiary structure of MERS-CoV RBD in complex with DPP4 (PDB code: 4KR0) (30). DPP4 is colored yellow. RBD is colored cyan (core structure) and red (receptor-binding motif). DPP4 binds to the receptor-binding motif of the RBD. (B) Modeled structure of MERS-CoV S-e in complex with DPP4. S-e is a trimer (PDB code: 5X5F): one monomeric subunit whose RBD is in the standing-up conformation is colored blue and the other two monomeric subunits whose RBDs are in the lying-down conformation are colored grey (18). To generate the structural model of the S-e in complex with DPP4, the RBD in panel (A) was structurally aligned with the standing-up RBD in the S-e trimer. (C) Tertiary structure of MERS-CoV RBD (PDB 4L3N) (64). Critical mAb-binding residues were identified through mutagenesis studies (48) and are shown as green sticks.

Figure 2. Interactions between coronavirus spike and RBD-specific mAb. (A) ELISA for detection of the binding between MERS-CoV-RBD-specific mAb (i.e., Mersmab1) and MERS-CoV spike ectodomain (S-e). Mersmab1 was pre-coated on the plate, and recombinant S-e or RBD was added subsequently for ELISA. Binding affinities were characterized as ELISA signal at OD 450 nm. PBS was used as a negative control. (B) ELISA for detection of the binding between Fab of Mersmab1 and MERS-CoV S-e. Recombinant S-e was pre-coated on the plate, and Mersmab1 or Fab was added subsequently for ELISA. (C) Flow cytometry for detection of the binding between MERS-CoV S-e and DPP4 receptor and among S-e, Mersmab1 and CD32A (i.e., Fc receptor). Cells expressing DPP4 or CD32A were incubated with S-e alone, S-e plus

802 Mersmab1, or S-e plus a SARS-CoV-RBD-specific mAb (i.e., 33G4). Fluorescence-
803 labeled anti-His_6 antibody was added to target the C-terminal His_6 tag on S-e. Cells were
804 analyzed using FACS (fluorescence-activated cell sorting). (D) The expression levels of
805 cell-membrane-associated DPP4 and CD32A were characterized using Western blot
806 targeting their C-terminal C9 tag, and then used to normalize the binding affinity as
807 measured in panel (C). As an internal control, the expression level of cellular actin was
808 measured using an anti-actin antibody. All of the experiments were repeated at least three
809 times with similar results, and representative results are shown here. Error bars indicate
810 S.D. (n=5). Statistical analyses were performed as one-tailed *t*-test. *** $p < 0.001$.
811 Mersmab1 and its Fab both bind to MERS-CoV RBD and S-e.
812
813 **Figure 3**. Antibody-dependent enhancement of coronavirus entry. (A) Antibody-
814 mediated MERS-CoV pseudovirus entry into human cells. The human cells included
815 HEK293T cells exogenously expressing DPP4, HEK293T cells exogenously expressing
816 one of the Fc receptors (CD16A, CD32A, or CD64A), and macrophage cells (induced
817 from THP-1 monocytes cells) endogenously expressing a mixture of Fc receptors. The
818 antibody was Mersmab1. An anti-SARS mAb (i.e., 33G4) was used as a negative control.
819 Efficiency of pseudovirus entry was characterized by luciferase activities accompanying
820 entry. HEK293T cells not expressing any viral receptor or Fc receptor were used as a
821 mock. (B) Fc- or Fab-mediated MERS-CoV pseudovirus entry into human cells. The Fc
822 or the Fab portion of Mersmab1 was used in MERS-CoV pseudovirus entry performed as
823 in panel (A). (C) Expression levels of DPP4 receptor in different cell lines. Total RNA
824 was extracted from three different cell lines: HEK293T, MRC5 and Hela. Then qRT-PCR

825 was performed on the total RNAs from each cell line. The expression level of DPP4 in
826 each cell line is defined as the ratio between the RNA of DPP4 and the RNA of
827 Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (D) Antibody-mediated MERS-
828 CoV pseudovirus entry into Hela cells that do not express DPP4 receptor. The
829 experiments were performed in the same way as in panel (A), except that Hela cells
830 replaced HEK293T cells. (E) Antibody-mediated SARS-CoV pseudovirus entry into
831 human cells. DPP4 and Mersmab1 were replaced by ACE2 and 33G4, respectively.
832 Mersmab1 was used as a negative control. All of the experiments were repeated at least
833 three times with similar results, and representative results are shown here. Error bars
834 indicate S.D. (n=4). Statistical analyses were performed as one-tailed *t*-test. *** $p <$
835 0.001. RBD-specific mAbs mediate ADE of coronavirus entry, while blocking viral-
836 receptor-dependent coronavirus entry.
837
838 **Figure 4**. Antibody-induced conformational changes of coronavirus spike. (A) Purified
839 MERS-CoV pseudoviruses were incubated with recombinant DPP4, mAb or PBS, and
840 then treated with trypsin. Samples were subjected to Western blotting analysis. MERS-
841 CoV spike and its cleaved fragments (all of which contained a C-terminal C9 tag) were
842 detected using an anti-C9 tag monoclonal antibody. Both DPP4 and Mersmab1 triggered
843 conformational changes of MERS-CoV spike, allowing it to cleaved at the S2' site by
844 trypsin. (B) Negative-stain electron microscopic analysis of MERS-CoV S-e in complex
845 with the Fab of Mersmab1. Both a field of particles and windows of individual particles
846 are shown. Black arrows indicate S-e-bound Fabs. According to previous studies (18, 20,

21), the Fab-binding site on the trimeric S-e is accessible only when the RBD is in the standing-up position.

Figure 5. Pathways for antibody-dependent enhancement of coronavirus entry. (A) Impact of proprotein convertases on ADE of MERS-CoV entry. During packaging of MERS-CoV pseudoviruses, HEK293T cells were treated with proprotein convertase inhibitor (PPCi). The MERS-CoV pseudoviruses packaged in the presence of PPCi were then subjected to MERS-CoV pseudovirus entry into HEK293T cells expressing either DPP4 receptor or CD32A receptor. (B) Western blot of MERS-CoV pseudoviruses packaged in the presence or absence of PPCi. MERS-CoV spike protein was detected using anti-C9 antibody targeting its C-terminal C9 tag. As an internal control, another viral protein, p24, was detected using anti-p24 antibody. (C) Impact of cell-surface proteases on ADE of MERS-CoV entry. HEK293T cells exogenously expressing TMPRSS2 (which is a common cell-surface protease) were subjected to MERS-CoV pseudovirus entry. TMPRSS2 enhanced both the DPP4-dependent and antibody-dependent entry pathways. (D) Impact of lysosomal proteases on ADE of MERS-CoV entry. HEK293T cells exogenously expressing DPP4 or CD32A were pretreated with one of the lysosomal protease inhibitors, E64d and Baf-A1, and then subjected to MERS-CoV pseudovirus entry. Lysosomal proteases blocked both the DPP4-dependent and antibody-dependent entry pathways. Hence DPP4-dependent and Mersmab1-dependent MERS-CoV entries share the same pathways. HEK293T cells not expressing DPP4 or CD32A were used as a negative control. All of the experiments were repeated at least three times with similar results, and representative results are shown here. Error bars indicate S.D.

(n=4). Statistical analyses were performed as one-tailed *t*-test. *** $p < 0.001$. * $p < 0.05$.
Antibody-dependent and DPP4-dependent viral entries share the same pathways.

Figure 6. Antibody dosages for antibody-dependent enhancement of coronavirus entry. (A) Impact of antibody dosages on MERS-CoV pseudovirus entry into HEK293T cells exogenously expressing either DPP4 or CD32A. mAb blocks the DPP4-dependent entry pathway; it enhances the antibody-dependent entry pathway at lower concentrations and blocks it at higher concentrations. (B) Impact of antibody dosages on MERS-CoV pseudovirus entry into HEK293T cells exogenously expressing both DPP4 and CD32A. In the presence of both DPP4 and CD32A, mAb blocks viral entry at low concentrations, enhances viral entry at intermediate concentrations, and blocks viral entry at high concentrations. (C) Same experiment as in panel (A), except that MRC5 cells replaced HEK293T cells. Here MRC5 cells express DPP4 receptor endogenously. (D) Same experiment as in panel (B), except that MRC5 cells replaced HEK293T cells. Here MRC5 cells endogenously express DPP4 and exogenously express CD32A. Please refer to text for more detailed explanations. All of the experiments were repeated at least three times with similar results, and representative results are shown here. Error bars indicate S.D. (n=4).

Figure 7. Two previously published structures of coronavirus spike proteins complexed with antibody. (A) SARS-CoV S-e complexed with S230 mAb (PDB ID: 6NB7). The antibody binds to the side of the RBD, away from the viral-receptor-binding site, stabilizes the RBD in the lying-down state, and hence does not trigger conformational changes of SARS-CoV S-e. (B) MERS-CoV S-e complexed with LCA60 mAb (PDB ID:

6NB4). The antibody binds to the viral-receptor-binding site in the RBD, stabilizes the RBD in the standing-up state, and hence triggers conformational changes of MERS-CoV S-e.