1 **Neutralizing and protective human monoclonal antibodies recognizing the N-**

2 **terminal domain of the SARS-CoV-2 spike protein**

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25 **SUMMARY**

26   Most human monoclonal antibodies (mAbs) neutralizing SARS-CoV-2 recognize the spike

27   (S) protein receptor-binding domain and block virus interactions with the cellular receptor

28   angiotensin-converting enzyme 2. We describe a panel of human mAbs binding to diverse epitopes

29   on the N-terminal domain (NTD) of S protein from SARS-CoV-2 convalescent donors and found

30   a minority of these possessed neutralizing activity. Two mAbs (COV2-2676 and COV2-2489)

31   inhibited infection of authentic SARS-CoV-2 and recombinant VSV/SARS-CoV-2 viruses. We

32   mapped their binding epitopes by alanine-scanning mutagenesis and selection of functional SARS-

33   CoV-2 S neutralization escape variants. Mechanistic studies showed that these antibodies

34   neutralize in part by inhibiting a post-attachment step in the infection cycle. COV2-2676 and

35   COV2-2489 offered protection either as prophylaxis or therapy, and Fc effector functions were

36   required for optimal protection. Thus, natural infection induces a subset of potent NTD-specific

37   mAbs that leverage neutralizing and Fc-mediated activities to protect against SARS-CoV-2

38   infection using multiple functional attributes.

39

42   **INTRODUCTION**

43   Since the emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)

44   as a major threat to global public health, many studies have focused efforts on discovery of potent

45   neutralizing monoclonal antibodies (mAbs) against the spike (S) protein of SARS-CoV-2 (Baum

46   et al., 2020a; Cao et al., 2020; Hansen et al., 2020; Ju et al., 2020; Liu et al., 2020a; Pinto et al.,

47   2020; Robbiani et al., 2020; Zost et al., 2020b). The S protein exists as a trimer on the surface of

48   SARS-CoV-2 and facilitates entry of the virus into cells. The receptor binding domain (RBD)

49   situated in the S1 region of the S protein binds to human angiotensin-converting enzyme 2

50   (hACE2). The S2 region then changes conformation and inserts its fusion peptide into the target

51   cell membrane, thus triggering viral fusion and entry. Previous studies have demonstrated that the

52   RBD region is a key target for potently neutralizing antibodies (Alsoussi et al., 2020; Barnes et al.,

53   2020; Baum et al., 2020a; Hansen et al., 2020; Hassan et al., 2020; Zost et al., 2020a). These

54   studies also defined inhibition of S trimer binding to the cellular receptor ACE2 as a principal

55   mechanism of action of RBD-targeting antibodies against SARS-CoV-2, and showed protection

56   in animals against infection and disease by this class of mAbs (Baum et al., 2020a; Hansen et al.,

57   2020; Hassan et al., 2020; Zost et al., 2020a).

58   Since the start of the outbreak, circulating SARS-CoV-2 field strains have acquired genetic

59   changes that facilitate transmission (Avanzato et al., 2020; Choi et al., 2020; Hou et al., 2020;

60   Kemp et al., 2020; McCarthy et al., 2020; Plante et al., 2020; Rambaut et al., 2020; Tegally et al.,

61   2020). This rapid viral evolution also could affect the protective efficacy of vaccines and mAb-

62   based therapeutics currently in clinical trials or approved under emergency use authorization. Most

63   of the mAbs under evaluation in clinical trials or authorized for the emergency use bind to the

64   RBD (Baum et al., 2020a; Zost et al., 2020a). Several groups have described mAbs targeting non-

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65   RBD regions, but their epitopes, mechanisms of action, and protective activity *in vivo* remain

66   unclear (Chi et al., 2020; Liu et al., 2020a). Here we define the structure-function relationship of

67   potent NTD-reactive antibodies from a panel of 389 human SARS-CoV-2 S protein mAbs we

68   isolated from survivors of natural infection (Zost et al., 2020a; Zost et al., 2020b). We found 43

69   mAbs recognizing the NTD. Three of the 43 NTD-reactive mAbs exhibited neutralizing capacity

70   against authentic SARS-CoV-2 virus (Zost et al., 2020b), with two being potently inhibitory. We

71   mapped the epitopes for the two most potently neutralizing NTD-reactive mAbs and dissected the

72   mechanism by which these mAbs inhibited SARS-CoV-2 infection. These two mAbs conferred

73   protection in hACE2-expressing mice when administered either as prophylaxis or therapy, and

74   intact Fc effector functions were required for optimal activity *in vivo*. Thus, we show that regions

75   of the NTD are recognized by neutralizing and protective antibodies against SARS-CoV-2 and

76   could function as part of antibody cocktails to minimize the selection of escape variants or

77   resistance to natural variants in RBD as they emerge.

78

79 **RESULTS**

80   **Two non-RBD anti-SARS-CoV-2 S-protein-specific mAbs potently neutralize virus.**

81 We previously isolated 389 SARS-CoV-2 reactive mAbs from the B cells of convalescent COVID-

82 19 patients. While most of the neutralizing antibodies mapped to the RBD and blocked ACE2

83 binding to the S protein, multiple neutralizing mAbs that did not bind RBD were also identified

84 (Zost et al., 2020a; Zost et al., 2020b). We selected the two most potently neutralizing S-protein-

85 reactive mAbs that did not bind to RBD, designated COV2-2676 and COV2-2489, for further

86 study. We first assessed binding and neutralizing activities of COV2-2676 and COV2-2489 to

87 determine their potency. Both mAbs bound weakly to a recombinant SARS-CoV-2 S6P$_{ecto}$ protein

88 expressed as a trimer, but failed to react with a recombinant soluble NTD protein. In contrast,

89 additional SARS-CoV-2-specific non-RBD mAbs, COV2-2263 and COV2-2490, which are non-

90 neutralizing(Zost et al., 2020a), reacted to both SARS-CoV-2 S6P$_{ecto}$ protein and recombinant

91 soluble NTD protein, whereas the dengue virus mAb DENV-2D22, a negative control for the

92 assays, reacted to neither (**Fig 1A and B**). We performed two types of virus neutralization assays

93 with COV2-2676 and COV2-2489. The first method used authentic WA1/2020 strain SARS-CoV-

94 2 in a focus reduction neutralization test (FRNT) (Harcourt et al., 2020a; Harcourt et al., 2020b),

95 whereas the second was a real-time cell analysis (RTCA) assay using recombinant replication-

96 competent vesicular stomatitis virus (VSV) expressing the SARS-CoV-2 S protein in place of the

97 endogenous VSV glycoprotein (Case et al., 2020b). Both COV2-2676 and COV2-2489

98 individually neutralized the authentic SARS-CoV-2 in a dose-dependent manner with a half-

99 maximal inhibitory (IC$_{50}$) value of 501 or 199 ng/mL, respectively (**Fig 1C**). In addition, COV2-

100 2676 and COV2-2489 neutralized chimeric VSV-SARS-CoV-2 with IC$_{50}$ values of 38 or 56

101 ng/mL, respectively (**Fig 1D**).

102   Competition-binding analysis using the S6P$_{ecto}$ protein revealed that COV2-2676 and

103   COV2-2489 competed for binding with one another (**Fig S1**). However, these mAbs did not

104   compete for binding with any of the other 33 previously identified non-neutralizing NTD-reactive

105   human mAbs (Zost et al., 2020b) or with mAbs that recognize non-overlapping antigenic sites on

106   the surface of RBD (including COV2-2196, COV2-2130, and a recombinant form of CR3022(ter

107   Meulen et al., 2006) [**Fig 1E**]) . These findings indicate an antigenic site for COV2-2676 and

108   COV2-2489 distinct from those previously identified for neutralizing human mAbs targeting the

109   RBD. Moreover, neither COV2-2676 nor COV2-2489 inhibited the interaction of soluble ACE2

110   with soluble RBD protein (**Fig 1F**).

111   **COV2-2676 and COV2-2489 recognize the NTD of SARS-CoV-2 S protein.** To

112   determine the binding sites for these mAbs, we used negative-stain electron microscopy to image

113   a stabilized trimeric form of the ectodomain of S protein (S6P$_{ecto}$ trimer) in complex with Fab

114   fragment forms of COV2-2676 or COV2-2489 (**Fig 2A**). These antibodies bind to the NTD and

115   recognize the 'closed' conformational state of the S6P$_{ecto}$ trimer. By overlaying the negative stain

116   EM maps of the two Fab/S complexes, we determined that these antibodies bind to a common

117   antigenic site on the NTD of the S6P$_{ecto}$ trimer. These findings are consistent with the competition-

118   binding data between COV2-2676 and COV2-2489. Sequence alignment of the variable gene

119   sequences of these mAbs with previously published anti-NTD neutralizing mAbs 4A8 and 4-8

120   showed that COV2-2676 or COV2-2489 are independent clonotypes. COV2-2489 is encoded by

121   heavy chain variable gene segment *IGHV-4-39*. COV2-2676 mAb is encoded by *IGHV1-69*, as is

122   mAb 4-8, but the HCDR3 regions of the two mAbs differ completely, and mAb 4A8 is encoded

123   by *IGHV1-24* (**Table S1**). We superimposed the COV2-2676 negative stain-EM Fab complex with

124   the cryo-EM structure of mAb 4-8 and found that the binding interfaces of both mAbs are similar.

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125   The heavy chain of the antibodies interact with the N3 and N5 loops of NTD (**Fig S2**). This

126   revealed a distinct site of vulnerability on NTD region of spike protein for human neutralizing

127   mAbs and suggested a convergent responses in SARS-CoV-2 immune individuals.

128   We next defined the antibody epitopes at the amino acid level using two complementary

129   methods: alanine-scanning loss-of-binding experiments in cell-surface antigen display and

130   selection of virus escape mutants followed by sequence analysis. Screening of the NTD Ala-scan

131   library identified residues A123, G142, Y144, F157 and N164 as important for binding of COV2-

132   2489, and Y144 for binding of mAb COV2-2676. None of these single-residue alanine mutants

133   affected binding of the control NTD-reactive mAb COV2-2305, likely due to the location of key

134   contact residues in the N3 and N5 loops of NTD (**Fig 2B and S3A**).

135   To identify neutralization escape mutations, we used a high-throughput RTCA assay as

136   previously described (Gilchuk et al., 2020a; Gilchuk et al., 2020b). We selected viral variants that

137   escape antibody neutralization at a single saturating concentration of 5 µg/mL (COV2-2676) or 50

138   µg/mL (COV2-2489) and identified point mutations. The mutations were F140S (orange) for

139   COV2-2676, and G142D and R158S (green) for COV2-2489 (**Fig 2C**). We confirmed that the

140   mutations in the viral variants we selected in the RTCA assay experiments above conferred

141   resistance to 10 or 100 µg/mL of the rescpetive mAbs (**Fig S3B**). The location of the escape

142   mutations was in the same region identified by the alanine-scanning method, although the specific

143   amino acids differed between the two methods. Thus, the two neutralizing NTD antibodies bind

144   to a common antigenic site, but the fine specificity of the epitopes differ.

145   **COV2-2676 and COV2-2489 bind avidly to S protein on the surface of infected cells,**

146   **show cell-type specific neutralization, and inhibit at a post-attachment step.** We evaluated the

147   ability of mAbs targeting the NTD to bind to the surface of SARS-CoV-2-infected cells since this

148   property could contribute to immune cell-mediated clearance *in vivo*. Following SARS-CoV-2

149   infection, cells were incubated with serial dilutions of COV2-2489, COV2-2676, the neutralizing

150   RBD-targeting mAb COV2-2381(Zost et al., 2020a), or the dengue virus mAb 2D22 isotype

151   control, prior to analysis of staining intensity by flow cytometry. COV2-2489 and COV2-2676

152   exhibited similar avidity for cell-surface-associated S protein, with EC_{50} values for staining of

153   infected cells of 896 ng/mL or 1,438 ng/mL, respectively (**Fig 3A**, left panel). Whereas the RBD-

154   targeting mAb COV2-2381 had a more potent EC_{50} value for binding to infected cells (45 ng/mL),

155   the NTD-targeting mAbs had greater staining intensity of infected cells, as the peak integrated

156   mean fluorescence intensity (iMFI) was higher for COV2-2489 and COV2-2676 than for COV2-

157   2381 (**Fig 3A**, left and right panels, and **Fig S4A**). These data suggest that NTD-targeting mAbs

158   can bind efficiently and at high density to S protein on the surface of SARS-CoV-2 infected cells.

159   We next assessed the neutralizing activity of COV2-2489 and COV2-2676 by FRNT on

160   additional cell lines: MA104 cells, HEK-293T cells ectopically expressing ACE2 (293T+ACE2),

161   and Vero cells ectopically expressing TMPRSS2 (Vero+TMPRSS2), in comparison to wild-type

162   Vero cells. For both mAbs, neutralization potency was greater on Vero cells than MA104 cells or

163   293T+ACE2 cells; both mAbs had weak neutralization potency when assessed using the latter two

164   cell types (**Fig 3B**). TMPRSS2 over-expression did not alter the potency of the mAbs, as

165   neutralization assays using Vero+TMPRSS2 cells had similar potency to the parental wild-type

166   cell (**Fig 3B**). These data indicate that mAbs against the NTD exhibit cell type-dependent

167   neutralization, which may be due to variable expression of entry factors and receptors on the

168   surface of different cell types.

169   To probe further the mechanism of neutralization of mAbs targeting the NTD, we

170   performed modified FRNTs, in which SARS2-CoV-2 was incubated with mAb before (pre-

171   attachment FRNT) or after (post-attachment FRNT) virus was absorbed to the surface of Vero

172   cells. Both COV2-2489 and COV2-2676 efficiently neutralized SARS-CoV-2 when added

173   following virus absorption to the surface of cells (**Fig 3C**). These data suggest that mAbs targeting

174   the NTD can neutralize SARS-CoV-2 at a post-attachment step of the viral life cycle in Vero cells.

175   We also performed experiments that directly assessed the ability of NTD mAbs to block

176   virus attachment to Vero E6 cells and Vero overexpressing hACE2 and TMPRSS2 cells. When

177   hACE2 and TMPRSS2 were expressed in cells ectopically, greater SARS-CoV-2 attachment was

178   observed (**Fig 3D**, left panel). Neither anti-NTD (COV2-2489 or COV2-2676) nor anti-RBD

179   neutralizing mAbs (COV2-2381 or COV2-2196) blocked attachment to Vero E6 cells (**Fig 3D,**

180   middle panels). However, in hACE2 and TMPRSS2 over expressing cells binding was inhibited

181   by anti-RBD but not anti-NTD mAbs (**Fig 3D**, right panels).

182   **COV2-2676 and COV2-2489 require intact IgG or F(ab′)$_2$ for neutralizing activity.**

183   Next, to determine the valency of binding required for activity, we created recombinant IgG,

184   F(ab′)$_2$, and Fab forms of COV2-2676 and COV2-2489 and investigated their neutralizing potency

185   on Vero cells. IgG or F(ab′)$_2$ versions of COV2-2676 or COV2-2489 exhibited neutralizing

186   activity in the RTCA assay, with IC$_{50}$ values of 0.17 or 0.32 nM (COV2-2676) or 0.28 or 1.20 nM

187   (COV2-2489), respectively (**Fig 3E**). Neutralizing activity of the F(ab′)$_2$ molecules was

188   comparable to that of the IgG versions. However, Fab fragments of COV2-2676 or COV2-2489

189   did not neutralize infection, whereas a potently neutralizing RBD antibody did inhibit infection as

190   a Fab (**Fig 3E**). The reason for loss of neutralization activity by Fab fragments of COV2-2676 and

191   COV2-2489 is unclear. The larger IgG or F(ab′)$_2$ might sterically hinder functional interactions of

192   the S protein in entry. Alternatively, the monovalent Fab molecule may bind with too low an

193   affinity to the NTD to inhibit entry.

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194   **COV2-2676 and COV2-2489 protect against SARS-CoV-2 challenge in mice**. We

195   tested the protective efficacy of COV2-2676 or COV2-2489 monotherapy in a K18-hACE2-

196   transgenic mouse model of SARS-CoV-2 infection (Golden et al., 2020; Oladunni et al., 2020;

197   Winkler et al., 2020). Mice treated with 200 μg (10 mg/kg) of the isotype control mAb DENV-

198   2D22 one day before intranasal inoculation with 10^3 plaque-forming units (PFU) of SARS-CoV-

199   2 (strain 2019n-CoV/USA_WA1/2020) experienced weight loss between 4 and 7 days after

200   inoculation (**Fig 4A**). In contrast, prophylaxis with 200 μg of COV2-2676 or COV2-2489

201   prevented weight loss in all mice, in a manner similar to that mediated by COV2-2381, a

202   neutralizing RBD-specific human mAb known to protect *in vivo* against SARS-CoV-2 infection

203   (Zost et al., 2020a). Pre-treatment with COV2-2676 or COV2-2489 also significantly decreased

204   viral burden at 7 days post-infection (dpi) in the upper and lower respiratory tracts and at a distal

205   site, the heart, compared to DENV-2D22, in a manner similar to COV2-2381 (**Fig 4B**).

206   Prophylaxis with 200 μg of COV2-2676, COV2-2489, or COV2-2381 was associated with serum

207   IgG1 concentrations of ~ 4 μg/mL (**Fig 4C**). Pre-treatment with COV2-2676 or COV2-2489

208   protected against weight loss and viral burden at a 25-fold lower dose, whereas pre-treatment with

209   COV2-2381 protected at a 125-fold lower dose. These treatments were associated with serum IgG1

210   concentrations of ~200 ng/mL or ~10 ng/mL, respectively (**Fig 4D-F**). Since SARS-CoV-2

211   infection causes substantial lung inflammation in K18-hACE2 mice and in humans (Golden et al.,

212   2020; Oladunni et al., 2020; Winkler et al., 2020), we evaluated the effects of mAb treatment on

213   cytokine and chemokine production in lung tissues at 7 dpi. Prophylaxis with 200 μg of COV2-

214   2676 reduced multiple cytokine and chemokine levels in a manner similar to COV2-2381, in

215   contrast with results from the isotype control mAb DENV-2D22 group (**Fig 4G and S5A**).

216   Consistent with these results, analysis of hematoxylin and eosin-stained lung sections showed a

217   reduction in perivascular and parenchymal immune cell infiltration, and alveolar space

218   consolidation in the lungs of COV2-2676 or COV2-2381-treated mice compared to DENV-2D22-

219   treated animals (**Fig 4H**).

220   We next assessed the therapeutic efficacy of monotherapy with COV2-2676 or COV-2489

221   in a post-exposure setting. Mice treated with 200 μg of COV2-2676 or COV2-2489 one day after

222   SARS-CoV-2 inoculation maintained weight, in a manner similar to COV2-2381 treatment and in

223   contrast to the isotype control mAb, DENV-2D22 (**Fig 5A**). Therapy with COV2-2676 or COV2-

224   2489 also decreased viral burden at 7 dpi in the upper and lower respiratory tract and heart,

225   compared to DENV-2D22, in a manner similar to COV2-2381 (**Fig 5B**). Animals treated with

226   COV2-2676 also had lower levels of multiple cytokines and chemokines in lung homogenates,

227   similar to COV2-2381 and in contrast to DENV-2D22 (**Fig 5C and S5B**). Pathological analysis

228   also showed less immune cell infiltration and airspace consolidation after COV2-2676 or COV2-

229   2381 therapy compared to DENV-2D22 treatment (**Fig 5D**).

230   **Fc effector functions contribute to optimal protection by COV2-2676 or COV-2489.**

231   Because the NTD mAbs bound avidly to the surface of SARS-CoV-2-infected cells (**Fig 3A and**

232   **S4A**), we speculated that part of their protective activity might be mediated by effector functions

233   through Fc engagement of C1q or FcγRs that could promote clearance. To test this hypothesis, we

234   engineered LALA-PG mutations into the Fc region of these human IgG1 mAbs to abrogate

235   interaction of the Fc region with FcγRs and complement proteins (Lund et al., 1991; Wines et al.,

236   2000). We confirmed that the LALA-PG mutations in COV2-2676 and COV2-2489 did not affect

237   S protein binding (**Fig S4B**) or neutralizing activity (**Fig S4C**).

238   We then evaluated if the LALA-PG Fc variants lost protective activity *in vivo*. Despite

239   similar concentrations in serum at the time of SARS-CoV-2 infection (**Fig 4C**), increased weight

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240   loss and viral burden, as well as greater lung inflammation and pathology were observed in animals

241   pre-treated with COV2-2676 LALA-PG Fc or COV2-2489 LALA-PG Fc variant IgGs compared

242   to the intact parental mAbs (**Fig 4A, B, E, G, and H**). This pattern also was seen with therapy

243   initiated one day after SARS-CoV-2 infection using COV2-2676 LALA-PG Fc or COV2-2489

244   LALA-PG Fc variants (**Fig 5A-D**). Overall, these findings suggest that Fc effector function

245   activities contribute to the protection conferred by each of the NTD mAbs.

246   The combination of an RBD-specific neutralizing mAb and an NTD mAb in a cocktail

247   would confer equivalent or better levels of protection, since binding to distinct antigenic sites

248   might mitigate the risk of selection of antibody escape variants. Thus, mAb cocktails that include

249   components binding to different epitopes on S protein offer higher resistance to escape mutations

250   (Baum et al., 2020b; Greaney et al., 2021) and protect animals from SARS-CoV-2 challenge

251   (Baum et al., 2020a; Zost et al., 2020a). Initially, to test this approach, we used VSV expressing

252   escape variants of the SARS-CoV-2 S protein that were resistant to neutralization by RBD-specific

253   antibodies (COV-2479 or COV2-2130) (Greaney et al., 2021) and the NTD-specific antibodies

254   described here (COV2-2676 and COV2-2489). As expected, NTD-reactive mAbs neutralized the

255   RBD mAb escape mutant viruses, and RBD-reactive mAbs neutralized the NTD mAb escape

256   mutant viruses (**Fig 6A**).

257   We next treated mice two days after inoculation with SARS-CoV-2 with individual mAbs

258   or a combination of COV2-2381 (RBD-specific) and COV2-2676 (NTD-specific) mAbs. Mice

259   treated with 200 μg of COV2-2676, COV2-2489 or COV2-2831 two days after SARS-CoV-2

260   inoculation showed weight loss, viral burden, and inflammatory mediator profiles that were similar

261   to DENV-2D22-treated mice (**Fig 6B-D**), consistent with a limited therapeutic window for

262   effective treatment in this stringent transgenic hACE2 mouse model. While mice were not

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263   protected from the initial phase of weight loss, viral infection in the lower respiratory tract or heart,

264   or lung inflammation (**Fig 6B-D, S5C),** treatment with mAb monotherapy or the cocktail resulted

265   in weight gain beginning approximately one week after infection and survival through two weeks

266   after infection, and this recovery was associated with decreased nasal wash titers and reduced lung

267   pathology (**Fig 6B-D**). In comparison, all mice treated with the isotype control mAb DENV-2D22

268   had severe lung pathology and succumbed to infection between eight and nine days after

269   inoculation. These results suggested that diversification of neutralizing antibody responses after

270   natural infection or vaccination (*e.g.*, targeting of vulnerable RBD and NTD sites of the spike)

271   could be beneficial for protective immunity, with a potential implication for therapeutic cocktail

272   design.

DISCUSSION

Several research groups have identified RBD-specific mAbs using B cells from SARS-CoV-2 convalescent individuals, with a number of these mAbs now in Phase III clinical trials (Baum et al., 2020a; Chen et al., 2020a; Jones et al., 2020) and two mAb products having obtained Emergency Use Authorization from the U.S. Food and Drug Administration. Due to the large number of natural variant viruses with polymorphisms in the RBD that are emerging, identification of neutralizing and protective human mAbs that bind to other antigenic sites on the S protein is warranted. In a large (n =389) panel of mAbs we isolated with reactivity to the SARS-CoV-2 S protein, the majority of mAbs bound to S protein but not to the RBD (Zost et al., 2020b). We found that a small subset of non-RBD antibodies that recognized the NTD could neutralize SARS-CoV-2 infection and confer protection in a stringent hACE2 transgenic mouse model of disease.

Our studies highlight the promising functional activities of NTD-specific mAbs. Although these mAbs did not bind as avidly to recombinant S6P$_{ecto}$ protein as did the most potent RBD-reactive mAbs we isolated, they neutralized SARS-CoV-2 with potencies comparable to many RBD-targeting antibodies (Zost et al., 2020a; Zost et al., 2020b). Very few NTD-targeting neutralizing antibodies have been reported to date. Sequence comparison of recently published NTD-reactive SARS-CoV-2 mAbs 4A8 (Chi et al., 2020) and 4-8 (Liu et al., 2020a) revealed that our mAbs are genetically distinct and not members of a public clonotype matching those mAbs (**Table S1**).

Most neutralizing SARS-CoV-2 mAbs block RBD interactions with ACE2 (Brouwer et al., 2020; Cao et al., 2020; Ju et al., 2020; Liu et al., 2020a; Rogers et al., 2020; Seydoux et al., 2020; Wec et al., 2020; Wu et al., 2020; Zost et al., 2020a; Zost et al., 2020b). However, the mechanism of neutralization of NTD-reactive antibodies appears complex. Our data suggest that

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296   the anti-NTD antibodies COV2-2676 and COV2-2489 inhibit at a post-attachment phase of

297   infection and may block subsequent virus entry or fusion steps. In cells in which anti-RBD and

298   anti-NTD antibodies do not appear to block virus attachment (*i.e.*, conventional Vero cells), both

299   groups inhibited at a post-attachment step. Virus attachment to cells can differ in diverse cells,

300   likely because of the capacity of SARS-CoV-2 to use multiple attachment factors including

301   heparan sulfates, hACE2, TAM receptors, and possibly other molecules (Cantuti-Castelvetri et al.,

302   2020; Chen et al., 2020b; Chiodo et al., 2020; Clausen et al., 2020; Gao and Zhang, 2020). The

303   mechanism of attachment in Vero (monkey kidney) cells without human ACE2 transfection is

304   uncertain; attachment might be mediated by monkey ACE2 although this has not been formally

305   demonstrated. It is possible that the NTD antibodies block entry by indirectly interfering with

306   ACE2 binding through steric interference of the Fc region. In analogous experiments with MERS-

307   CoV, structural studies presented showed that the MERS-CoV mAb 7D10 could bind to the NTD

308   of S protein of MERS-CoV and inhibit RBD-DPP4 interaction through its light chain, which was

309   close to the RBD (Zhou et al., 2019). We tested that model here but found that neither COV2-2676

310   nor COV2-2489 blocked ACE2 interaction with RBD in soluble protein or in cell-surface-display

311   assays. Antibody fragment studies using Fab, F(ab′)$_2$ and IgG forms of COV2-2676 and COV2-

312   2489 showed that the Fab forms lost neutralizing activity, possibly suggesting some blocking

313   occurs indirectly by steric effects of the projecting Fc region into the direction of the RBD as in

314   the case for the MERS-CoV mAB 7D10 (Zhou et al., 2019). However, we did not obtain direct

315   evidence for this mechanism. In cells expressing high hACE2 levels, anti-RBD antibodies partially

316   inhibited attachment of virus, but anti-NTD antibodies did not.

317   To define the fine specificity of this protective epitope, we performed saturation alanine-

318   scanning mutagenesis studies and identified critical residues for binding of COV2-2676 or COV2-

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319   2489 mAbs in the NTD. We showed that SARS-CoV-2 S protein variants with mutations at F140S

320   and G142D, or R158S in the NTD conferred resistance to COV2-2676 or COV2-2489,

321   respectively, using a chimeric replication-competent VSV expressing the SARS-CoV-2 S protein.

322   Consistent with these data, Weisblum *et al.* identified an NTD epitope (residues 148 to 151), and

323   mutations in this epitope enabled escape from neutralizing antibody activity in a donor plasma

324   specimen (Weisblum et al., 2020).

325   We first tested the prophylactic efficacy of COV2-2676 or COV2-2489 in a well-defined

326   model of SARS-CoV-2 infection in hACE2-expressing transgenic mice (Winkler *et al.*, 2020).

327   Mice pre-treated with COV2-2676 or COV2-2489 mAbs exhibited substantially lower viral titers

328   than mice treated with an isotype-control mAb and offered complete protection against death in

329   this model. We also assessed the therapeutic potential of these mAbs in the same mouse model.

330   Each of the mAbs mediated a therapeutic effect when administered after infection. Although

331   several protection studies of anti-SARS-CoV-2 mAbs have been published, all of these

332   experiments used mAbs directed against the RBD (Baum et al., 2020a; Chen et al., 2020a; Hassan

333   et al., 2020; Wang et al., 2020; Zost et al., 2020a). There is only limited precedence for *in vivo*

334   protection against SARS-CoV-2 infection or disease mediated by mAbs that react to S protein

335   regions outside the RBD (McCallum et al., 2021; Voss et al., 2020; Zhang et al., 2020).

336   IgG interactions with Fcγ receptors and complement contribute to antibody-dependent viral

337   clearance during many infections, including HIV-1, Ebola, influenza, and chikungunya viruses

338   (DiLillo et al., 2014; Fox et al., 2019; Halper-Stromberg et al., 2014; Lu et al., 2016). MAbs against

339   SARS-CoV-1 and SARS-CoV-2 also can confer protective effects in part through through Fc-

340   mediated effector functions (Atyeo et al., 2020; Pinto et al., 2020; Schafer et al., 2021). To test

341   whether this was the case with our mAbs, we generated COV2-2676 and 2489 LALA-PG variant

16

342 Fc versions. Indeed, Fc-mediated activity for NTD-specific mAbs did contribute to protection in

343 the models we tested. Animals in the LALA-PG mAb-treated mice groups had increased viral

344 burden and lost weight, but still survived viral challenge, suggesting involvement of Fc-effector

345 function dependent and independent mechanisms in the protection *in vivo* conferred by anti-NTD

346 mAbs.

347   SARS-CoV-2 continues to evolve, and its capacity to escape the activity of neutralizing

348 antibodies in current clinical development is not fully understood. The emergence of mutations at

349 E484K in South African strains is concerning, because this change can impact the neutralizing

350 activity of many RBD-specific mAbs and immune serum generated from convalescent subjects

351 (Avanzato et al., 2020; Greaney et al., 2021; Liu et al., 2020b; Oude Munnink et al., 2021; Piccoli

352 et al., 2020; Rambaut et al., 2020; Starr et al., 2020). Moreover, the administration of convalescent

353 plasma with suboptimal levels of neutralizing antibodies might increase resistance in circulating

354 SARS-CoV-2 populations (Bloch, 2020). Furthermore, candidate vaccines that include only RBD

355 antigens lack the ability to induce NTD-reactive neutralizing antibodies (Laczko et al., 2020;

356 Mulligan et al., 2020; Smith et al., 2020; Yu et al., 2020). Antibody therapy or vaccine approaches

357 that target additional antigenic sites may limit escape and prevent compromising of vaccine- or

358 natural infection-induced immunity. A combination of RBD- and NTD-reactive neutralizing mAbs

359 may offer an attractive alternative approach to treatments that target only RBD. The data presented

360 here describing neutralization escape viruses suggests the potential utility of using mAb cocktails

361 to avoid selection of neutralization resistant viruses. Moreover, we establish that a combination of

362 RBD- and NTD-reactive neutralizing mAbs can be used as an effective therapeutic cocktail *in*

363 *vivo*. Overall, the results presented here provide compelling evidence that some NTD-targeting

364   mAbs can inhibit SARS-CoV-2 infection efficiently *in vitro* and *in vivo*, using both neutralizing

365   and Fc-mediated effector function activities.

366

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AUTHOR CONTRIBUTIONS

Conceived of the project: N.S., P.G., S.J.Z., R.H.C., L.B.T., M.S.D., J.E.C.; Obtained funding: M.S.D. and J.E.C. Performed laboratory experiments: N.S., S.S., P.G., E.S.W., E.B., S.J.Z., L.V., R.S.N., R.E.S., S.P.K., M.E.F., L.B.T. Performed computational work: E.C.C. Supervised research: R.H.C., L.B.T., E.D., B.D., M.S.D., J.E.C. Wrote the first draft of the paper: N.S., P.G., S.S., L.B.T., M.S.D., J.E.C.; All authors reviewed and approved the final manuscript.

DECLARATION OF INTERESTS

M.E.F., E.D., and B.J.D. are employees of Integral Molecular. B.J.D. is a shareholder of Integral Molecular. M.S.D. is a consultant for Inbios, Vir Biotechnology, NGM Biopharmaceuticals, and Carnival Corporation, is on the Scientific Advisory Boards of Moderna

390   and Immunome, and the Diamond laboratory at Washington University School of Medicine has

391   received sponsored research agreements from Emergent BioSolutions, Moderna, and Vir

392   Biotechnology. J.E.C. has served as a consultant for Eli Lilly, GlaxoSmithKline and Luna

393   Biologics, is a member of the Scientific Advisory Boards of CompuVax and Meissa Vaccines and

394   is Founder of IDBiologics. The Crowe laboratory at Vanderbilt University Medical Center has

395   received unrelated sponsored research agreements from IDBiologics and AstraZeneca.

396

397 **FIGURE LEGENDS**

398

399 **Figure 1. Two non-RBD-reactive mAbs specific to SARS-CoV-2 S protein neutralize the**

400 **virus.**

401 **A.** ELISA binding of COV2-2196, COV2-2263, COV2-2489, COV2-2490, COV2-2676 or

402 rDENV-2D22 to trimeric S-6P$_{ecto}$. Data are mean ± standard deviatioms (S.D.) of technical

403 triplicates from a representative experiment repeated twice.

404 **B.** ELISA binding of COV2-2196, COV2-2263, COV2-2489, COV2-2490, COV2-2676 or

405 rDENV-2D22 to rNTD protein. Data are mean ± S.D. of technical triplicates from a representative

406 experiment repeated twice.

407 **C.** Neutralization curves for COV2-2196, COV2-2489, COV2-2676 or rDENV-2D22 using wild-

408 type SARS-CoV-2 in a FRNT assay. Error bars indicate S.D.; data represent at least two

409 independent experiments performed in technical duplicate.

410 **D.** Neutralization curves for COV2-2196, COV2-2489, COV2-2490, COV2-2676 or rDENV-

411 2D22 in a SARS-CoV-2-rVSV neutralization assay using RTCA. Error bars indicate S.D.; data

412 are representative of at least two independent experiments performed in technical duplicate.

413 **E.** Competition binding of the panel of neutralizing mAbs with reference mAbs COV2-2130,

414 COV2-2196, COV2-2263, COV2-2489, COV2-2676 or rCR3022. Binding of reference mAbs to

415 trimeric S-6P$_{ecto}$ protein was measured in the presence of saturating concentration of competitor

416 mAb in a competition ELISA and normalized to binding in the presence of rDENV-2D22. Black

417 indicates full competition (<25% binding of reference antibody); grey indicates partial competition

418 (25 to 60% binding of reference antibody); white indicates no competition (>60% binding of

419 reference antibody).

21

420   **F.** Human-ACE2-blocking curves for COV2-2196, COV2-2263, COV2-2489, COV2-2490,

421   COV2-2676 and rDENV-2D22 in a human-ACE2-blocking ELISA. Data are mean ± S.D. of

422   technical triplicates from a representative experiment repeated twice.

423

424   **Figure 2. COV2-2676 and COV2-2489 bindng map to the NTD of SARS-COV-2-S protein.**

425   **A.** Top row (side view), bottom row (top view) of Fab–S6Pecto closed trimer (S protein model

426   PDB:7JJI) complexes visualized by negative-stain electron microscopy for COV2-2676 Fab model

427   in pink, COV2-2489 Fab model in blue and superimpose 3D volume of CoV2-S-Fab 2676 complex

428   in grey and CoV2-S-Fab 2489 in mesh. The S-NTD is shown in yellow and electron density in

429   grey. Representative two-dimensional (2D) class averages for each complex are shown at the

430   bottom (box size is 128 pixels, with 4.36 Å per pixel). Data are from a single experiment; detailed

431   collection statistics are provided in **Supplementary Table 2**.

432   **B.** Identification of critical contact residues by alanine-scanning mutagenesis. Top (side view) with

433   loss of binding residues (cyan) for COV2-2489 or COV2-2676 to mutant S-NTD constructs,

434   normalized to the wild-type. Bottom, escape mutations mapped to the NTD region for COV2-2489

435   (green G142D, R158S) or COV2-2676 (orange F140S). **C.** Results of viral selections with COV2-

436   2489 or COV2-2676 individual mAbs. The number of replicates in which escape variants were

437   selected is indicated. Mutations present in the NTD of the selected escape variants are indicated.

438

439   **Figure 3. Virus neutralization and binding of infected cells by anti-SARS-CoV-2 mAbs**

440   **targeting the NTD. A.** SARS-CoV-2-infected Vero cells were stained with serial dilutions of

441   COV2-2489, COV2-2676, COV2-2381, or DENV2-2D22 (isotype control) prior to analysis of

442   staining intensity by flow cytometry. The positively stained cells were gated using uninfected and

22

443   isotype control mAb-stained infected cells, and the integrated mean fluorescence intensity (iMFI)

444   was determined by MFI of positive cells multiplied by the percent of total positive cells. The left

445   panel shows representative dose response curves for the staining intensity of infected cells by each

446   mAb. The right panel shows the mean EC_{50} values for infected cell staining, determined from three

447   independent experiments. Error bars represent SEM.

448   **B-D.** The neutralization potency of COV2-2489 and COV2-2676 against SARS-CoV-2 was

449   assessed by FRNT using (**B**) Vero, MA104, 293T+ACE2, and Vero+TMPRSS2 cells. Results are

450   representative of three independent experiments performed in duplicate. (**C**) COV2-2489 and

451   COV2-2676 were assayed for neutralization potency by modified FRNT in which mAb was added

452   to SARS-CoV-2 before (pre-attachment, filled cirlces) or after (post-attachment, open circles)

453   virus was absorbed to Vero E6 cells. (**C**) Error bars represent the range from two technical

454   replicates. Data shown are representative of three independent experiments. **D**. Attachment

455   inhibition. (*Left panel*) Vero or Vero+hACE2+TMPRSS2 cells were incubated with SARS-CoV-

456   2 at 4°C for 1 h. Afer extensive washing, cell-bound viral RNA was measured by qRT-PCR. (*Right*

457   *4 panels*) SARS-CoV-2 was pre-incubated with 5 or 50 μg/mL of indicated anti-RBD or anti-NTD

458   mAbs for 1 h prior to addition to Vero or Vero+hACE2+TMPRSS2 cells. Cell-bound viral RNA

459   was measured by qRT-PCR. Data are pooled from three independent experiments. (*Left*) t-test:

460   ****$p<0.0001$; (*Right*) One-way ANOVA with Dunnett's multiple comparisons test compared to

461   isotype control mAb treatment: *$p<0.05$; **$p<0.01$; ***$p<0.001$. **E**. Neutralization curves for

462   COV2-2196 IgG, F(ab')$_2$, F(ab) or rDENV-2D22; COV2-2489 IgG, F(ab')$_2$, F(ab); or COV2-2676

463   IgG, F(ab')$_2$, F(ab) in a SARS-CoV2-rVSV assay using RTCA. Error bars indicate S.D.; data

464   represent at least two independent experiments performed in technical triplicates.

465

466   **Figure 4. Prophylaxis with COV2-2676 or COV2-2489 confers protection against SARS-**

467   **CoV-2 in mice**.

468   Eight to nine week-old male and female K18 hACE2 transgenic mice were administered by

469   intraperitoneal (i.p.) injection 200, 40, 8 or 1.6 µg of COV2-2676, COV2-2489, COV2-2676

470   LALA-PG, COV2-2489 LALA-PG, COV2-2381 (a positive control) or DENV-2D22, an isotype

471   control, mAb a day before virus inoculation (D-1). One day later, mice were inoculated intranasally

472   (i.n.) with 10^3 PFU of SARS-CoV-2.

473   **A.** Body weight change of mice over time. Data shows the mean ± S.E.M. compared to the isotype

474   control mAb for two independent experiments (n = 6 to 11 for each experimental group; one-

475   way ANOVA with Dunnett's post hoc test of area under the curve from 4 to 7 dpi: ns, not

476   significant, **$p < 0.01$,****$p < 0.000\ 1$).

477   **B.** Tissues were harvested at 7 dpi from a subset of mice in **A**. Viral burden in the lung, nasal

478   wash, and heart was assessed by qRT-PCR of the N gene. Data shows the mean ± S.E.M.

479   compared between all groups for two independent experiments (n = 6 for each experimental

480   group; one-way ANOVA with Tukey's post hoc test: ns, not significant, *$p < 0.05$, **$p < 0.01$,

481   ***$p < 0.001$, ****$p < 0.0001$). Dashed line represents limit of detection of assay.

482   **C.** Serum concentration (ng/ml) of human mAbs at the time of SARS-CoV-2 infection of the mice

483   in **B**. Data shows the mean ± S.E.M. compared between all groups for two independent

484   experiments (n = 6 for each experimental group; one-way ANOVA with Tukey's post hoc test:

485   ns, not significant, ****$p < 0.0001$).

486   **D.** Body weight change of mice over time. Data shows the mean ± S.E.M. compared to isotype

487   control mAb for two independent experiments (n = 4 to 8 for each experimental group; one-

24

488 way ANOVA with Dunnett's post hoc test of area under the curve from 4 to 7 dpi: ns, not

489 significant, ***$p < 0.001$, ****$p < 0.0001$).

490 **E.** Tissues were harvested at 7 dpi from mice in **D**. Viral burden in the lung, nasal wash, and heart

491 was assessed by qRT-PCR of the N gene. Data shows the mean ± S.E.M. compared between

492 all groups for two independent experiments (n = 4 to 8 for each experimental group; one-way

493 ANOVA with Tukey's post hoc test: ns, not significant, *$p < 0.05$, **$p < 0.01$, ***$p <$

494 0.001, ****$p < 0.0001$).

495 **F.** Serum concentration (ng/mL) of human mAbs at the time of SARS-CoV-2 infection of the

496 mice in **D**. Data shows the mean ± S.E.M. compared to isotype control mAb at various doses

497 of mAb for two independent experiments (n = 6; one-way ANOVA with Dunnett's post hoc

498 test: ns, not significant).

499 **G.** Heat map of cytokine and chemokine levels in lung tissue homogenates harvested in **B** as

500 measured by a multiplex platform. Log_2 fold change compared to lungs from mock-infected

501 animals was plotted in the corresponding heat map (associated statistics are reported in **Fig**

502 **S6A**).

503 **H.** Hematoxylin and eosin staining of lung sections harvested at 7 dpi from mice in **B**. Images are

504 low (top; scale bars, 500 µm) and high power (bottom; scale bars, 50 µm). Representative

505 images are shown from two independent experiments (n = 3).

506

507 **Figure 5. Therapeutic activity of COV2-2676 or COV-2489 after SARS-CoV-2 challenge.**

508 Eight to nine week-old male and female K18 hACE2 transgenic mice were inoculated with 10^3

509 PFU of SARS-COV2. One day later (D+1), mice were given an i.p. administration of 200 µg of

25

510   COV2-2676, COV2-2489, COV2-2676 LALA PG, COV2-2489 LALA PG, COV2-2381 (a

511   positive control) or DENV-2D22, an isotype control, mAb.

512   **A.** Body weight change of mice over time. Data shows the mean ± S.E.M. compared to isotype

513   control mAb for two independent experiments (n = 6 to 11 for each experimental group; one-

514   way ANOVA with Dunnett's post hoc test of area under the curve from 4 to 7 dpi: ns, not

515   significant, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$).

516   **B.** Tissues were harvested at 7 dpi from a subset of mice in **A**. Viral burden in the lung, nasal

517   wash, and heart was assessed by qRT-PCR of the N gene. Data shows the mean ± S.E.M.

518   compared between all groups for two independent experiments (n = 6 to 7 for each

519   experimental group; one-way ANOVA with Tukey's post hoc test: ns, not significant, $*p <$

520   0.05, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$).

521   **C.** Heat map of cytokine and chemokine levels in lung homogenates harvested in **B** as measured

522   by a multiplex platform. Log_2 fold change compared to lungs from mock-infected animals was

523   plotted in the corresponding heat map (associated statistics are reported in **Fig S6B**).

524   **D.** Hematoxylin and eosin staining of lung sections harvested at 7 dpi from mice in **B**. Images are

525   low (top; scale bars, 500 μm) and high power (bottom; scale bars, 50 μm). Representative

526   images are shown from two independent experiments (n = 3).

527

528   **Figure 6. Rationale for design of an antibody cocktail targeting both RBD and NTD**

529   **A.** Neutralization matrix of RBD mAbs (COV2-2479 and COV2-2130 shown in purple) and NTD

530   mAb (COV2-2676 in orange and COV2-2489 in green) escape viruses. Black = full neutralization,

531   grey = partial neutralization, white = no neutralization & * indicates escape viruses that were

532   isolated in previously published study. Eight to nine week-old male or female K18 hACE2

26

533   transgenic mice were inoculated intranasally with 10^3 PFU of SARS-CoV2. Two days later (D+2),

534   mice were given an i.p. administration of 200 μg of COV2-2676, COV2-2489, COV2-2676

535   LALA-PG, COV2-2489 LALA-PG, COV2-2381 (a positive control) or DENV-2D22, an isotype

536   control, mAb for monotherapy or 100 μg each of COV2-2381 and COV2-2676 for combination

537   therapy.

538   **B**. (Left panel) Body weight change of mice over time. Data shows the mean ± S.E.M. compared

539   to isotype control mAb for two independent experiments (n = 6 to 14 for each experimental group:

540   one-way ANOVA with Dunnett's post hoc test of area under the curve from 4 to 7 dpi. Significant

541   differences were not detected. (Right panel) Percent survival of mice over time. Survival was

542   compared to isotype control for two independent experiments (n = 6 to 14 for each experimental

543   group; log-rank Mantel-Cox test; ****p < 0.0001).

544   **C.** Tissues were harvested at 7 dpi mice from a subset of mice in **B**. Viral burden in the lung, nasal

545   wash, and heart was assessed by qRT-PCR of the N gene. Data shows the mean ± S.E.M. compared

546   between all groups for two independent experiments: n = 6 to 8 for each experimental group; one-

547   way ANOVA with Tukey's post hoc test: ns, not significant, *p < 0.05, ***p <0.001).

548   **D.** Heat map of cytokine and chemokine levels in lung homogenates harvested in **C** as measured

549   by a multiplex platform. Log_2 fold change compared to lungs from mock-infected animals was

550   plotted in the corresponding heat map (associated statistics are reported in **Fig S6C**.)

551   **E.** Hematoxylin and eosin staining of lung sections harvested at 7 dpi from mice in **B**. Images are

552   low (top; scale bars, 500 μm) and high power (bottom; scale bars, 50 μm). Representative images

553   are shown from two independent experiments (n = 3).

554

27

STAR METHODS

RESOURCE AVAILABILITY

     LEAD CONTACT. Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, James E. Crowe, Jr. (james.crowe@vumc.org).

     MATERIALS AVAILABILITY. Materials described in this paper are available for distribution for nonprofit use using templated documents from Association of University Technology Managers "Toolkit MTAs", available at: https://autm.net/surveys-and-tools/agreements/material-transfer-agreements/mta-toolkit.

     DATA AND CODE AVAILABILITY. All data needed to evaluate the conclusions in the paper are present in the paper or the Supplemental Information. The antibodies in this study are available by Material Transfer Agreement with Vanderbilt University Medical Center.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Antibodies. The human antibodies studied in this paper were isolated from blood samples from two individuals in North America with previous laboratory-confirmed symptomatic SARS-CoV-2 infection that was acquired in China. The original clinical studies to obtain specimens after written informed consent were previously described (Zost et al., 2020b) and approved by the Institutional Review Board of Vanderbilt University Medical Center, the Institutional Review Board of the University of Washington and the Research Ethics Board of the University of Toronto. The individuals (a 56-year-old male and a 56-year-old female) are a married couple and residents of Wuhan, China who travelled to Toronto, Canada, where PBMCs were obtained by

28

578   leukopheresis 50 days after symptom onset. The antibodies were isolated using diverse tools for

579   isolation and cloning of single antigen-specific B cells and the antibody variable genes that encode

580   mAbs (Zost et al., 2020b).

581

582   **Cell lines.** Vero E6 (ATCC, CRL-1586), Vero (ATCC, CCL-81), HEK293 (ATCC, CRL-1573) and

583   HEK293T (ATCC, CRL-3216) cells were maintained at 37°C in 5% CO_2 in Dulbecco's minimal

584   essential medium (DMEM) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS), 10 mM

585   HEPES pH 7.3, 1 mM sodium pyruvate, 1× non-essential amino acids and 100 U/mL of penicillin–

586   streptomycin. Vero-furin cells were obtained from T. Pierson (NIAID, NIH) and have been described

587   previously (Mukherjee et al., 2016). Vero-hACE2-TMPRSS2 cells were a gift of A. Creanga and B.

588   Graham (Vaccine Research Center, NIH). FreeStyle 293F cells (Thermo Fisher Scientific, R79007)

589   were maintained at 37°C in 8% CO_2. Expi293F cells (Thermo Fisher Scientific, A1452) were

590   maintained at 37°C in 8% CO_2 in Expi293F Expression Medium (Thermo Fisher Scientific,

591   A1435102). ExpiCHO cells (Thermo Fisher Scientific, A29127) were maintained at 37°C in 8%

592   CO_2 in ExpiCHO Expression Medium (Thermo Fisher Scientific, A2910002). Authentication analysis

593   was not performed for the cell lines used. Mycoplasma testing of Expi293F and ExpiCHO cultures

594   was performed on a monthly basis using a PCR-based mycoplasma detection kit (ATCC, 30-1012K).

595

596   **Viruses.** SARS-CoV-2 strain 2019 n-CoV/USA_WA1/2020 was obtained from the Centers for

597   Disease Control and Prevention (a gift from N. Thornburg). Virus was passaged in Vero CCL81 cells

598   and titrated by plaque assay on Vero E6 cell culture monolayers as previously described (Case et al.,

599   2020a). The generation of a replication-competent VSV expressing SARS-CoV-2 S protein with a 21

600   amino-acid C-terminal deletion that replaces the VSV G protein (VSV-SARS-CoV-2) was described

29

601 previously (Case et al., 2020b). The S protein-expressing VSV virus was propagated in MA104 cell

602 culture monolayers (African green monkey, ATCC CRL-2378.1) as described previously (Case *et al.*,

603 2020), and viral stocks were titrated on Vero E6 cell monolayer cultures. VSV plaques were visualized

604 using neutral red staining. All work with infectious SARS-CoV-2 was performed in Institutional

605 Biosafety Committee approved BSL3 and A-BSL3 facilities at Washington University School of

606 Medicine using appropriate positive pressure air respirators and protective equipment.

607

608 **Mouse models.** Animal studies were carried out in accordance with the recommendations in the Guide

609 for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols were

610 approved by the Institutional Animal Care and Use Committee at the Washington University School

611 of Medicine (assurance number A3381–01). Virus inoculations were performed under anesthesia that

612 was induced and maintained with ketamine hydrochloride and xylazine, and all efforts were made to

613 minimize animal suffering. Heterozygous K18-hACE c57BL/6J mice (strain: 2B6.Cg-Tg(K18-

614 ACE2)2Prlmn/J) were obtained from Jackson Laboratory (034860). Eight to nine week-old mice of

615 both sexes were inoculated with 10^3 PFU of SARS-CoV-2 by an intranasal route.

616

617 **METHOD DETAILS**

618

619 **Recombinant antigens and proteins**. A gene encoding the ectodomain of a pre-fusion

620 conformation-stabilized SARS-CoV-2 S protein ectodomain (S6P$_{ecto}$) (Hsieh et al., 2020) was

621 synthesized and cloned into a DNA plasmid expression vector for mammalian cells. A similarly

622 designed S protein antigen with two prolines and removal of the furin cleavage site for stabilization

623 of the prefusion form of S (S2P$_{ecto}$) was reported previously (Wrapp et al., 2020). In brief, this

624   gene includes the ectodomain of SARS-CoV-2 (to residue 1,208), a T4 fibritin trimerization

625   domain, an AviTag site-specific biotinylation sequence and a C-terminal 8× His tag. To stabilize

626   the construct in the pre-fusion conformation, we included substitutions F817P, A892P, A899P,

627   A942P, K986P and V987P and mutated the furin cleavage site at residues 682–685 from RRAR

628   to ASVG. The recombinant S6P$_{ecto}$ protein was isolated by metal affinity chromatography on

629   HisTrap Excel columns (Cytiva), and protein preparations were purified further by size-exclusion

630   chromatography on a Superose 6 Increase 10/300 column (Cytiva). The presence of trimeric, pre-

631   fusion conformation S protein was verified by negative-stain electron microscopy (Zost et al.,

632   2020b). For electron microscopy with S protein and Fabs, we expressed a variant of S6P$_{ecto}$ lacking

633   an AviTag but containing a C-terminal Twin-Strep-tag, similar to that described previously (Zost

634   et al., 2020b). Expressed protein was isolated by metal affinity chromatography on HisTrap Excel

635   columns (Cytiva), followed by further purification on a StrepTrap HP column (Cytiva) and size-

636   exclusion chromatography on TSKgel G4000SWXL (TOSOH). To express the RBD subdomain

637   of the SARS-CoV-2 S protein, a synthetic DNA (Twist Bioscience) encoding residues 319–541

638   was cloned into a mammalian expression vector downstream of an IL-2 signal peptide and

639   upstream of a thrombin cleavage site, an AviTag and a 6× His tag. Recombinant SARS-CoV-2 S

640   NTD protein was kindly provided by P. McTamney, K. Ren and A. Barnes (AstraZeneca). Purified

641   proteins were analyzed by SDS–PAGE to ensure purity and appropriate molecular weights.

642

643   **MAb production and purification**. Sequences of mAbs that had been synthesized (Twist

644   Bioscience) and cloned into an IgG1 monocistronic expression vector (designated as pTwist-

645   mCis_G1) or Fab expression vector (designated as pTwist-mCis_FAB) were used for production

646   in mammalian cell culture. This vector contains an enhanced 2A sequence and GSG linker that

31

647   allows the simultaneous expression of mAb heavy and light chain genes from a single construct

648   upon transfection (Chng et al., 2015). For antibody production, we performed transfection of

649   ExpiCHO cell cultures using the Gibco ExpiCHO Expression System as described by the vendor.

650   IgG molecules were purified from culture supernatants using HiTrap MabSelect SuRe (Cytiva) on

651   a 24-column parallel protein chromatography system (Protein BioSolutions). Fab proteins were

652   purified using CaptureSelect column (Thermo Fisher Scientific). Purified antibodies were buffer-

653   exchanged into PBS, concentrated using Amicon Ultra-4 50-kDa (IgG) or 30 kDa (Fab) centrifugal

654   filter units (Millipore Sigma) and stored at 4°C until use. F(ab′)$_2$ fragments were generated after

655   cleavage of IgG with IdeS protease (Promega) and then purified using TALON metal affinity resin

656   (Takara) to remove the enzyme and protein A agarose (Pierce) to remove the Fc fragment. Purified

657   mAbs were tested routinely for endotoxin levels (found to be less than 30 EU per mg IgG).

658   Endotoxin testing was performed using the PTS201F cartridge (Charles River), with a sensitivity

659   range from 10 to 0.1 EU per mL, and an Endosafe Nexgen-MCS instrument (Charles River).

660

661   **ELISA binding assays**. Wells of 96-well microtiter plates were coated with purified recombinant

662   SARS-CoV-2 S6P$_{ecto}$, SARS-CoV-2 S NTD, or SARS-CoV-2 RBS protein at 4 °C overnight.

663   Plates were blocked with 2% non-fat dry milk and 2% normal goat serum in Dulbecco's phosphate-

664   buffered saline (DPBS) containing 0.05% Tween-20 (DPBS-T) for 1 h. The bound antibodies were

665   detected using goat anti-human IgG conjugated with horseradish peroxidase (HRP) (Southern

666   Biotech, cat. 2040-05, lot B3919-XD29, 1:5,000 dilution) and a 3,3′,5,5′-tetramethylbenzidine

667   (TMB) substrate (Thermo Fisher Scientific). Color development was monitored, 1M HCl was

668   added to stop the reaction, and the absorbance was measured at 450 nm using a spectrophotometer

669   (Biotek). For dose–response assays, serial dilutions of purified mAbs were applied to the wells in

670 triplicate, and antibody binding was detected as detailed above. EC_{50} values for binding were

671 determined using Prism v.8.0 software (GraphPad) after log transformation of the mAb

672 concentration using sigmoidal dose–response nonlinear regression analysis.

673

674 **Focus reduction neutralization test (FRNT)**. Serial dilutions of mAbs were incubated with

675 10^2 FFU of SARS-CoV-2 for 1 h at 37 °C. The antibody-virus complexes were added to Vero E6

676 cell-culture monolayers in 96-well plates for 1 h at 37 °C. Cells then were overlaid with 1% (w/v)

677 methylcellulose in minimum essential medium (MEM) supplemented to contain 2% heat-

678 inactivated FBS. Plates were fixed 30 h later by removing overlays and fixed with 4%

679 paraformaldehyde (PFA) in PBS for 20 min at room temperature. The plates were incubated

680 sequentially with 1 μg/mL of rCR3022 anti-S antibody or an murine anti-SARS-COV-2 mAb,

681 SARS2-16 (hybridoma supernatant diluted 1:6,000 to a final concentration of ~20 ng/mL) and

682 then HRP-conjugated goat anti-human IgG (Sigma-Aldrich, A6029) in PBS supplemented with

683 0.1% (w/v) saponin (Sigma) and 0.1% BSA. SARS-CoV-2-infected cell foci were visualized using

684 TrueBlue peroxidase substrate (KPL) and quantitated on an ImmunoSpot 5.0.37 Macro Analyzer

685 (Cellular Technologies). IC_{50} values were determined by nonlinear regression analysis (with a

686 variable slope) using Prism software.

687

688 **Real-time cell analysis (RTCA) neutralization assay**. To determine neutralizing activity of IgG,

689 Fab, or F(ab′)$_2$ proteins, we used real-time cell analysis (RTCA) assay on an xCELLigence RTCA

690 MP Analyzer (ACEA Biosciences Inc.) that measures virus-induced cytopathic effect (CPE)

691 (Gilchuk et al., 2020a; Zost et al., 2020b). Briefly, 50 μL of cell culture medium (DMEM

692 supplemented with 2% FBS) was added to each well of a 96-well E-plate using a ViaFlo384 liquid

33

693   handler (Integra Biosciences) to obtain background reading. A suspension of 18,000 Vero-E6 cells

694   in 50 μL of cell culture medium was seeded in each well, and the plate was placed on the analyzer.

695   Measurements were taken automatically every 15 min, and the sensograms were visualized using

696   RTCA software version 2.1.0 (ACEA Biosciences Inc). VSV-SARS-CoV-2 (0.01 MOI, ~120 PFU

697   per well) was mixed 1:1 with a dilution of mAb in a total volume of 100 μL using DMEM

698   supplemented with 2% FBS as a diluent and incubated for 1 h at 37°C in 5% CO_2. At 16 h after

699   seeding the cells, the virus-mAb mixtures were added in replicates to the cells in 96-well E-plates.

700   Triplicate wells containing virus only (maximal CPE in the absence of mAb) and wells containing

701   only Vero cells in medium (no-CPE wells) were included as controls. Plates were measured

702   continuously (every 15 min) for 48 h to assess virus neutralization. Normalized cellular index (CI)

703   values at the endpoint (48 h after incubation with the virus) were determined using the RTCA

704   software version 2.1.0 (ACEA Biosciences Inc.). Results are expressed as percent neutralization

705   in a presence of respective mAb relative to control wells with no CPE minus CI values from control

706   wells with maximum CPE. RTCA IC_{50} values were determined by nonlinear regression analysis

707   using Prism software.

708

709   **Pre- and post-attachment neutralization assays.** For pre-attachment assays, serial dilutions of

710   mAbs were prepared at 4°C in Dulbecco's modified Eagle medium (DMEM) with 2% FBS and

711   preincubated with 10^2 FFU of SARS-CoV-2 for 1 h at 4°C. MAb-virus complexes were added to

712   a monolayer of Vero cells for 1 h at 4°C. Virus was allowed to internalize during a 37°C incubation

713   for 30 min. Cells were overlaid with 1% (wt/vol) methylcellulose in MEM. For post-attachment

714   assays, 2 x 10^2 FFU of SARS-CoV-2 was adsorbed onto a monolayer of Vero cells for 1 h at 4°C.

715   After removal of unbound virus, cells were washed twice with cold DMEM, followed by the

34

716   addition of serial dilutions of MAbs in cold DMEM. Virus-adsorbed cells were incubated with

717   mAd dilutions for 1 h at 4°C. Virus then was allowed to internalize for 30 min at 37°C, and

718   subsequently cells were overlaid with methylcellulose as described above. Thirty hours later, the

719   plates were fixed with 4% PFA and analyzed for antigen-specific foci as described above for

720   FRNTs.

721

722   **Attachment inhibition assay.** SARS-COV-2 was incubated with mAbs at the specified

723   concentration for 1 h at 4°C. The mixture was added to pre-chilled Vero or

724   Vero+ACE2+TMPRSS2 cells at an MOI of 0.005 and incubated at 4°C for 1 h. Cells were washed

725   six times with chilled PBS before addition of lysis buffer and extraction of RNA using MagMax

726   viral RNA isolation kit (Thermo Fisher Scientific) and a Kingfisher Flex 96-well extraction

727   machine (Thermo Fisher Scientific). SARS-CoV-2 RNA was quantified by qRT-PCR using the

728   N-specific primer/ probe set described below. Viral RNA levels were normalized to GAPDH, and

729   the fold change was compared with isotype control mAb.

730

731   **Human ACE2 binding inhibition analysis**. Wells of 384-well microtiter plates were coated

732   with 1 μg/mL purified recombinant SARS-CoV-2 S2P$_{ecto}$ protein at 4°C overnight. Plates were

733   blocked with 2% non-fat dry milk and 2% normal goat serum in DPBS-T for 1 h. For screening

734   assays, purified mAbs from microscale expression were diluted two-fold in blocking buffer

735   starting from 10 μg/mL in triplicate, added to the wells (20 μL per well) and incubated for 1 h at

736   ambient temperature. Recombinant human ACE2 with a C-terminal Flag tag peptide was added to

737   wells at 2 μg/mL in a 5 μL per well volume (final 0.4 μg/mL concentration of human ACE2)

738   without washing of antibody and then incubated for 40 min at ambient temperature. Plates were

35

739   washed and bound human ACE2 was detected using HRP-conjugated anti-Flag antibody (Sigma-

740   Aldrich, cat. A8592, lot SLBV3799, 1:5,000 dilution) and TMB substrate. ACE2 binding without

741   antibody served as a control. The signal obtained for binding of the human ACE2 in the presence

742   of each dilution of tested antibody was expressed as a percentage of the human ACE2 binding

743   without antibody after subtracting the background signal. For dose–response assays, serial

744   dilutions of purified mAbs were applied to the wells in triplicate, and mAb binding was detected

745   as detailed above. IC_{50} values for inhibition by mAb of $S2P_{ecto}$ protein binding to human ACE2

746   was determined after log transformation of antibody concentration using sigmoidal dose–response

747   nonlinear regression analysis.

748

749   **Electron microscopy negative stain grid preparation, imaging and processing of $S6P_{ecto}$–Fab**

750   **complexes**. To perform electron microscopy imaging, Fabs were recombinantly expressed and

751   purified or produced by digesting recombinant chromatography-purified IgGs using resin-

752   immobilized cysteine protease enzyme (FabALACTICA, Genovis). The digestion occurred in 100

753   mM sodium phosphate and 150 mM NaCl pH 7.2 (PBS) for around 16 h at ambient temperature.

754   To remove cleaved Fc from intact IgG, the digestion mix was incubated with CaptureSelect Fc

755   resin (Genovis) for 30 min at ambient temperature in PBS buffer. If needed, the Fab was buffer-

756   exchanged into Tris buffer by centrifugation with a Zeba spin column (Thermo Fisher Scientific).

757

758   For screening and imaging of negatively-stained SARS-CoV-2 $S6P_{ecto}$ protein in complex with

759   human Fabs, the proteins were incubated at an Fab:spike monomer molar ratio of 4:3 for about 1

760   hour at ambient temperature, and approximately 3 µL of the sample at concentrations of about 10–

761   15 µg/mL was applied to a glow-discharged grid with continuous carbon film on 400 square mesh

762   copper electron microscopy grids (Electron Microscopy Sciences). The grids were stained with

763   0.75% uranyl formate (Ohi et al., 2004) . Images were recorded on a Gatan US4000 4k × 4k CCD

764   camera using an FEI TF20 (TFS) transmission electron microscope operated at 200 keV and

765   control with Serial EM. All images were taken at 50,000× magnification with a pixel size of 2.18

766   Å per pixel in low-dose mode at a defocus of 1.5 to 1.8 μm. The total dose for the micrographs

767   was around 30e− per Å2. Image processing was performed using the cryoSPARC software

768   package. Images were imported, CTF-estimated and particles were picked. The particles were

769   extracted with a box size of 256 pixels and binned to 128 pixels. 2D class averages were performed

770   and good classes selected for *ab initio* model and refinement without symmetry. Model docking

771   to the EM map was done in Chimera (Pettersen et al., 2004). For SARS-CoV-2 S6P$_{ecto}$ protein, the

772   closed model (PDB:7JJI) was used and PDB:12E8 was used for the Fab (see also **Table S2** for

773   details).

774

775   **Epitope mapping of antibodies by alanine-scanning mutagenesis.** Epitope mapping was

776   performed essentially as described previously (Davidson and Doranz, 2014) using a SARS-CoV-

777   2 (strain Wuhan-Hu-1) spike protein NTD shotgun mutagenesis mutation library, made using a

778   full-length expression construct for spike protein, where 215 residues of the NTD (between spike

779   residues 9 and 310) were mutated individually to alanine, and alanine residues to serine. Mutations

780   were confirmed by DNA sequencing, and clones arrayed in a 384-well plate, one mutant per well.

781   Binding of mAbs to each mutant clone in the alanine scanning library was determined, in duplicate,

782   by high-throughput flow cytometry. A plasmid encoding cDNA for each spike protein mutant was

783   transfected into HEK-293T cells and allowed to express for 22 h. Cells were fixed in 4% (v/v)

784   paraformaldehyde (Electron Microscopy Sciences), and permeabilized with 0.1% (w/v) saponin

785   (Sigma-Aldrich) in PBS plus calcium and magnesium (PBS++) before incubation with mAbs

786   diluted in PBS++, 10% normal goat serum (Sigma), and 0.1% saponin. MAb screening

787   concentrations were determined using an independent immunofluorescence titration curve against

788   cells expressing wild-type S protein to ensure that signals were within the linear range of detection.

789   Antibodies were detected using 3.75 μg/mL of Alexa-Fluor-488-conjugated secondary antibodies

790   (Jackson ImmunoResearch Laboratories) in 10% normal goat serum with 0.1% saponin. Cells

791   were washed three times with PBS++/0.1% saponin followed by two washes in PBS, and mean

792   cellular fluorescence was detected using a high-throughput Intellicyte iQue flow cytometer

793   (Sartorius). Antibody reactivity against each mutant S protein clone was calculated relative to

794   wild-type S protein reactivity by subtracting the signal from mock-transfected controls and

795   normalizing to the signal from wild-type S-transfected controls. Mutations within clones were

796   identified as critical to the mAb epitope if they did not support reactivity of the test MAb, but

797   supported reactivity of other SARS-CoV-2 antibodies. This counter-screen strategy facilitates the

798   exclusion of S protein mutants that are locally misfolded or have an expression defect.

799

800   **Selection of virus escape mutants using the S protein-expressing VSV**. To screen for escape

801   mutations selected in the presence of individual mAbs, we used a modification of the RTCA assay

802   as recently described (Greaney et al., 2021). Fifty μL of cell culture medium (DMEM

803   supplemented with 2% FBS) was added to each well of a 96-well E-plate to obtain a background

804   reading. A suspension of 18,000 Vero E6 cells in 50 μL of cell culture medium was seeded per

805   each well, and plates were placed on the analyzer. Measurements were taken automatically every

806   15 min and the sensograms were visualized using RTCA software version 2.1.0 (ACEA

807   Biosciences Inc). VSV-SARS-CoV-2 virus (5,000 PFU per well, ~0.3 MOI) was mixed with a

38

808   saturating neutralizing concentration of COV2-2676 (5 μg/mL) and COV2-2489 (50 μg/mL) in a

809   total volume of 100 mL and incubated for 1 h at 37°C. At 16 to 20 h after seeding the cells, the

810   virus-antibody mixtures were added into 1 to 88 replicate wells of 96-well E-plates with cell

811   monolayers. Wells containing only virus in the absence of antibody and wells containing only

812   Vero E6 cells in medium were included on each plate as controls. Plates were measured

813   continuously (every 15 min) for 72 h. The escape mutants were identified by unexpectedly high

814   CPE in wells containing neutralizing antibody. To verify escape from antibody selection, isolated

815   viruses were assessed in a subsequent RTCA experiment in the presence of 10 μg/mL (COV2-

816   2676) and 100 μg/mL (COV2-2489) of mAb as used for the escape virus selection.

817

818   **Sequence analysis of the gene encoding S protein from S protein-expressing VSV escape**

819   **mutants.** To identify escape mutations present in S protein-expressing VSV mAb-selected escape

820   variants, the escape viruses isolated after RTCA escape screening were propagated in 6-well

821   culture plates with confluent Vero E6 cells in the presence of 10 μg/mL of the corresponding

822   antibody. Viral RNA was isolated using a QiAmp Viral RNA extraction kit (QIAGEN) from

823   aliquots of supernatant containing a suspension of the selected virus population. The S protein

824   gene cDNA was amplified with a SuperScript IV One-Step RT-PCR kit (Thermo Fisher Scientific)

825   using primers flanking the S gene. The amplified PCR product (4,000 bp) was purified using SPRI

826   magnetic beads (Beckman Coulter) at a 1:1 ratio and sequenced by the Sanger sequence technique

827   using primers giving forward and reverse reads of the NTD.

828

829   **MAb binding to the surface of SARS-CoV-2 infected cells.** Vero E6 cells were inoculated with

830   SARS-CoV-2 at an MOI of 0.01. At 48 h post-infection, cells were trypsinized and resuspended

831   in a staining buffer composed of DPBS with 5% FBS, 5 mM EDTA, 0.05% NaN$_3$. mAbs were

832   diluted in the staining buffer and incubated with ~3 x 10^4 cells for 30 min at 4°C. Cells were

833   washed twice and incubated with Alexa Fluor 647-conjugated secondary antibody (Invitrogen)

834   diluted 1:1,000 in staining buffer for 30 min at 4°C. Cells were washed twice and fixed with 4%

835   PFA prior to detection of fluorescence signal by flow cytometry (MacsQuant) and analysis using

836   FlowJo software.

837

838   **Protection against wild-type SARS-CoV-2 in mice**. Male and female heterozygous K18-hACE

839   C57BL/6J mice were housed in groups of up to 5 mice per cage at 18 to 24°C ambient temperatures

840   and 40 to 60% humidity. Mice were fed a 20% protein diet (PicoLab 5053, Purina) and maintained on

841   a 12-h light–dark cycle (06:00 to 18:00). Food and water were available *ad libitum*. Mice (8 to 9 weeks

842   old) were inoculated with 1×10^3 PFU of SARS-CoV-2 via the intranasal route. Anti-SARS-CoV-2

843   human mAbs or isotype control mABS were administered 24 h before (prophylaxis) or 24 h or 48 h

844   after (therapy) SARS-CoV-2 inoculation. Weights and lethality were monitored on a daily basis for

845   up to 14 days after inoculation and a subset of mice were euthanized at 7 dpi and tissues were collected.

846

847   **Measurement of viral burden**. For RT–qPCR, mouse tissues were weighed and homogenized

848   with zirconia beads in a MagNA Lyser instrument (Roche Life Science) in 1 mL of DMEM

849   medium supplemented with 2% heat-inactivated FBS. Tissue homogenates were clarified by

850   centrifugation at 10,000 rpm for 5 min and stored at −80°C. RNA was extracted using a MagMax

851   mirVana Total RNA isolation kit (Thermo Fisher Scientific) and a Kingfisher Flex 96-well

852   extraction machine (Thermo Fisher Scientific). RNA was reverse transcribed and amplified using

853   the TaqMan RNA-to-CT 1-Step Kit (ThermoFisher). RNA levels were measured by one-step

854   quantitative reverse transcriptase PCR (qRT-PCR) assay as previously described (Hassan *et al.,*

855   2020). A TaqMan assay was designed to target the N gene, as previously described (Case; PMID

856   32838945). Specific primers and probe were used: Forward primer:

857   ATGCTGCAATCGTGCTACAA; Reverse primer: GACTGCCGCCTCTGCTC; Probe: /56-

858   FAM/TCAAGGAAC/ZEN/AACATTGCCAA/3IABkFQ/. N gene copy numbers were

859   determined down to 10 copies per reaction.

860

861   **ELISA to detect recombinant human mAbs**

862   Goat anti-human kappa cross-absorbed against mouse IgG (Southern Biotech catalog # 2061-01) and

863   goat anti-human lambda cross-absorbed against mouse IgG (Southern Biotech catalog # 2071-01)

864   were coated onto 96-well Nunc Maxisorp flat-bottomed plates at 2 µg/mL in coating buffer (0.1 M

865   sodium carbonate, 0.1 M sodium bicarbonate, 0.02% NaN_3, pH 9.6) overnight at 4°C. Coating buffers

866   were aspirated, and wells were blocked with 2% BSA (blocking buffer) (Fisher Bioreagents catalog #

867   BP1600-100), for 1 h at 37°C. Heat-inactivated serum samples were diluted in blocking buffer in a

868   separate polypropylene plate. The plates then were washed 4× with 1× PBS + 0.05% Tween-20

869   (PBST) (Fisher Bioreagents catalog # BP337-100), followed by addition of 50 µL of respective serum

870   dilutions and was incubated for 1 h at 4°C. The ELISA plates were again washed 4× in PBST, followed

871   by addition of 50 µL of 1:2,000 Goat Anti-Human IgG Fc, Multi-Species (Southern Biotech catalog

872   # 2014-05). Plates were incubated for 1 h at 4°C. Plates were washed with 4× PBST, followed by 100

873   µL of TMB-ELISA substrate (Thermo Fisher Scientific catalog # 34028) and incubated at room

874   temperature for 3 to 5 min. Color development was observed and reactions were stopped with 50 µL

875   of 2N sulfuric acid. Optical density (450 nm) measurements were determined using a microplate reader

876   (Bio-Rad).

877

878   **Cytokine and chemokine protein measurements**. Lung homogenates were incubated with Triton-

879   X-100 (1% final concentration) for 1 h at room temperature to inactivate SARS-CoV-2. The samples

880   were then analyzed by Eve Technologies Corporation (Calgary, AB, Canada). Cytokine and

881   chemokine protein expression were determined using the Mouse Cytokine Array / Chemokine Array

882   31-Plex (MD31) platform. Fold-change was calculated by comparing anti-SARS-CoV-2-specific or

883   isotype-control mAb-treated mice to naive control mice.

884

885   **Lung histology.** Mice were euthanized and tissues were harvested before lung was inflation and

886   fixation. The left lung was first tied off at the left main bronchus and collected for viral RNA analysis.

887   The right lung was inflated with approximately with 1.2 mL of 10% neutral buffered formalin using a

888   3-mL syringe and catheter inserted into the trachea. The inflated lung was then kept in 40 mL neutral

889   buffered formalin for 7 days. Tissues were embedded in paraffin, and sections were stained with

890   hematoxylin and eosin. Tissue sections were then scanned using Hamamatsu NanoZoomer slide

891   scanning system. Scanned image was then viewed by using the NDP view software (ver.1.2.46).

892

893   **QUANTIFICATION AND STATISTICAL ANALYSIS**

894   Mean ± S.E.M. or mean ± S.D. were determined for continuous variables as noted. Technical and

895   biological replicates are described in the figure legends. For analysis of mouse studies, the

896   comparison of weight-change curves was performed using a one-way ANOVA with Dunnett's

897   post hoc test using Prism v.9.0 (GraphPad). Viral burden and gene-expression measurements were

898   compared to each other or the isotype control using a one-way ANOVA with Tukey's or Dunnett's

899   post hoc test, respectively, using Prism v.9.0 (GraphPad). Survival curves were estimated using

900   the Kaplan-Meier method and differences assessed using the log-rank Mantel-Cox test and a

901   Bonferroni correction for multiple comparisons using Prism v.9.0 (GraphPad).

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Figure 1

ELISA binding

A. S6P_ecto protein

B. rNTD protein

Neutralization

C. Authentic virus

D. rVSV-SARS-CoV-2

COV2-2196
COV2-2263
COV2-2489
COV2-2490
COV2-2676
DENV-2D22
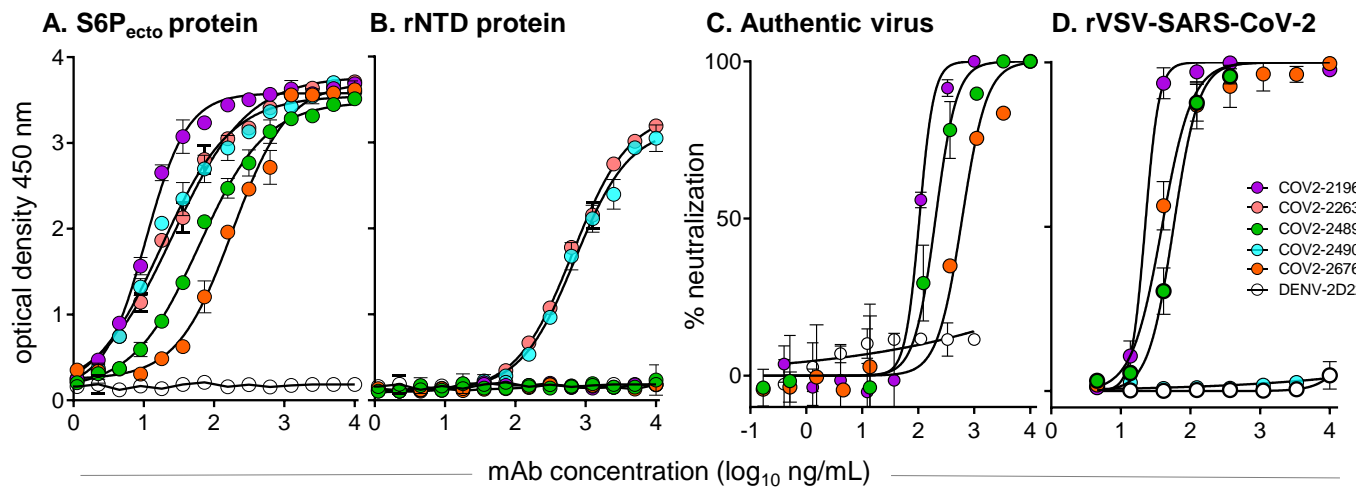
E. Competition-binding ELISA

1st mAb added (unlabeled)

F. ACE2 blocking ELISA

COV2-2196
COV2-2263
COV2-2489
COV2-2490
COV2-2676
DENV-2D22

Figure 2

A

Spike-COV2-2489 Fab complex   **Spike-COV2-2676 Fab complex**   **Superimposed (Spike + COV2-2489 and -2676 Fabs)**

Top view

Side view

B

Loss of binding residues - alanine mutagenesis
A123S
G142A
Y144A
F157A
N164A

Side view

Top view

VSV escape mutations
F140S
G142D
R158S
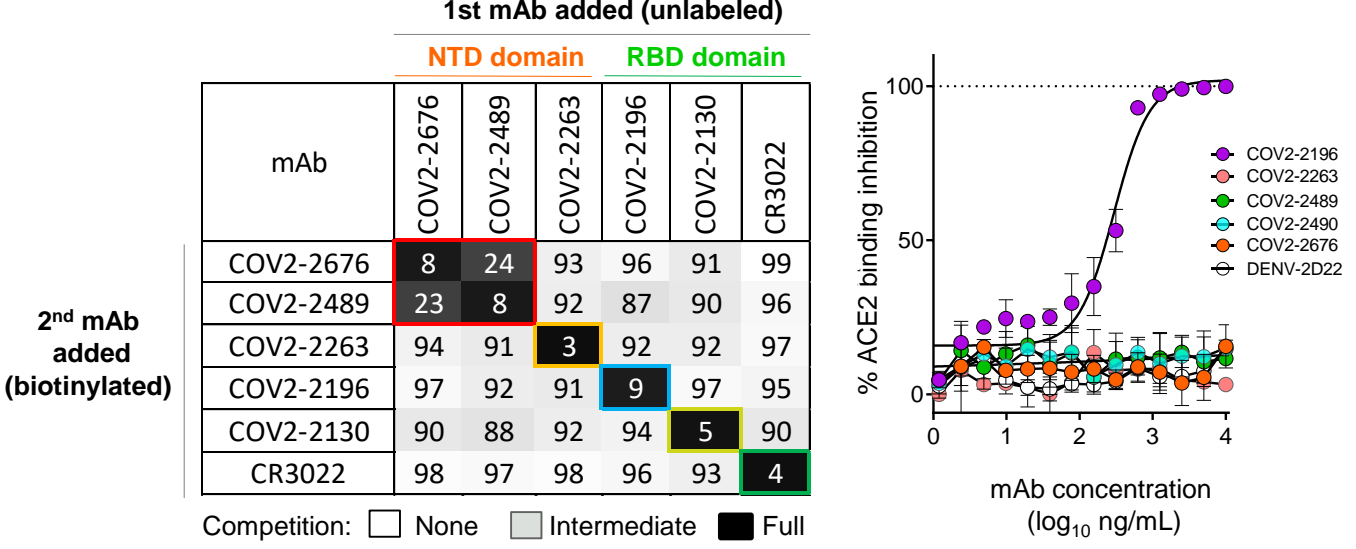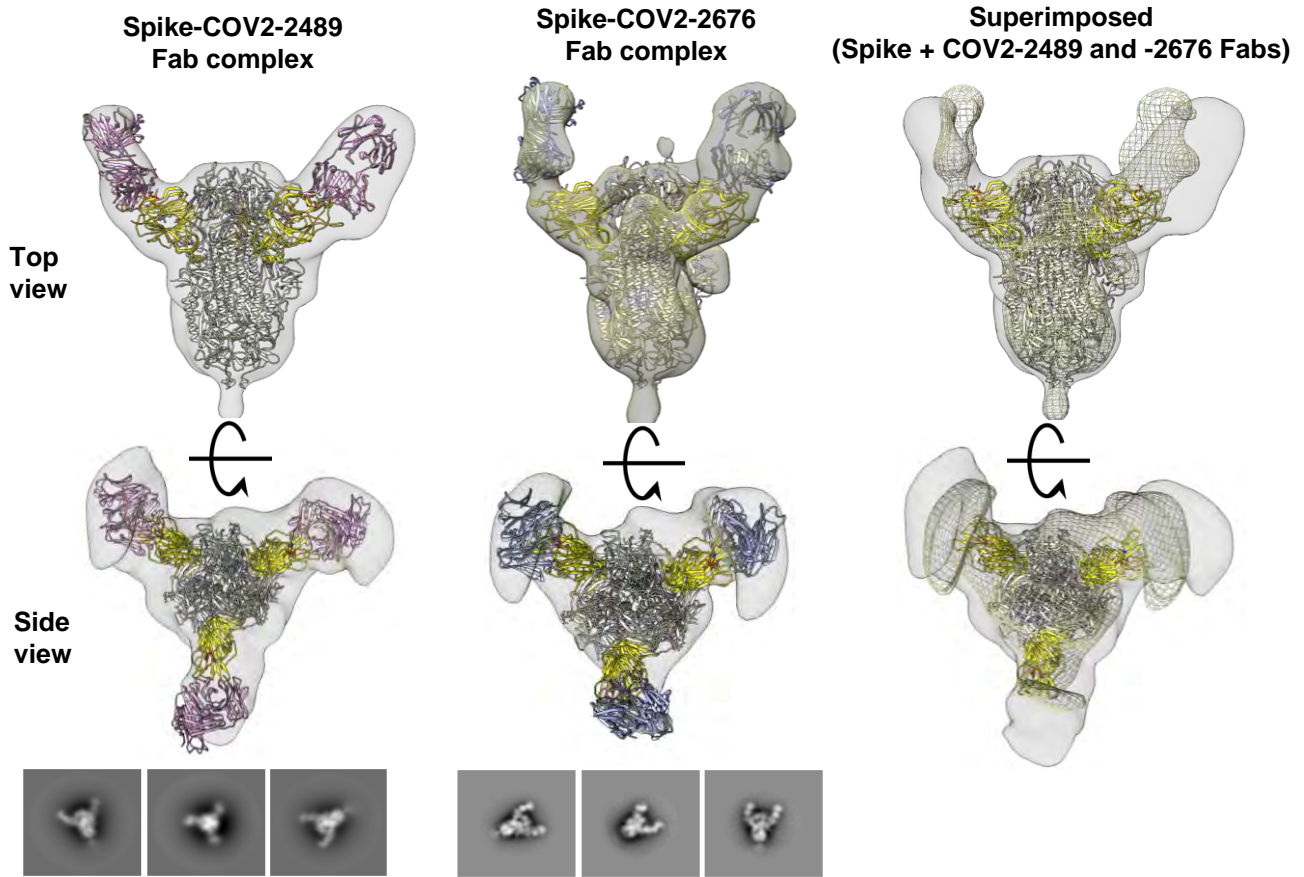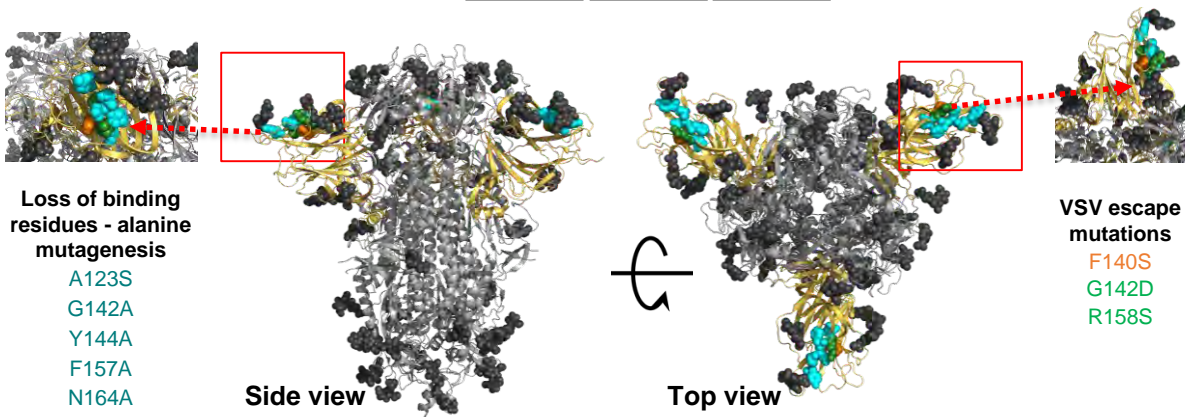
C

Features of escape mutant virus selection	MAb used for escape selection	
	COV2-2489	COV2-2676
Number of wells escaped of wells tested with mAb	5 of 88 (5.6 %)	1 of 88 (1.1 %)
Mutations selected (number of times)	G142D (twice) R158S (3 times)	F140S (once)

Figure 3

A Cell-surface binding

iMFI

integrated mean fluorescence intensity vs mAb (log$_{10}$ ng/mL)
- COV2-2381
- COV2-2489
- COV2-2676

EC$_{50}$ iMFI

log$_{10}$ EC$_{50}$ (ng/mL)
- COV2-2381
- COV2-2489
- COV2-2676

B Cell type dependent binding

COV2-2489 | COV2-2676

% relative infectivity vs mAb (log$_{10}$ ng/mL)
- Vero
- MA104
- 293T-ACE2
- Vero + TMPRSS2

C NTD mAbs neutralize the virus at post-attachment step

COV2-2489 | COV2-2676 | SARS2-38(RBD)

% relative infectivity vs mAb (log$_{10}$ ng/mL)
- Pre-attachment
- Post-attachment

Fold change

Fold change in EC$_{50}$ pre/post-attachment
- COV2-2676
- COV2-2489
- SARS2-38 (RBD)

D Attachment inhibition

Fold change (viral RNA)
- Vero
- Vero+ACE2+TMPRSS2

****

Vero cells

50 µg/mL mAb | 5 µg/mL mAb

Fold change (viral RNA)

Vero+ACE2+TMPRSS2 cells

50 µg/mL mAb | 5 µg/mL mAb

*** *** *** ns ns | *** ** *** ns ns

- DENV-2D22
- 4x lower MOI
- COV2-2381
- COV2-2196
- COV2-2676
- COV2-2489

E VSV-SARS-CoV-2 neutraliztion

MAb COV2-2196 | MAb COV2-2489 | MAb COV2-2676

% neutralization vs [mAb] (log$_{10}$ nM)
- IgG
- F(ab')2
- Fab
- r2D22

Figure 4

Figure 5

Figure 6

A

Antigenic site	MAb used to select escape virus	Mutation identified in escape virus	MAb tested for neutralization of escape virus			
			COV2-2479	COV2-2130	COV2-2489	COV2-2676
RBD	COV2-2479 *	E484K	4	97	96	99
	COV2-2130 *	K444R	100	8	101	99
NTD	COV2-2489-1	G142D	92	97	-3	7
	COV2-2489-2	R158S	90	97	-3	-1
	COV2-2676	F140S	93	98	13	18

-5   % neutralization   105
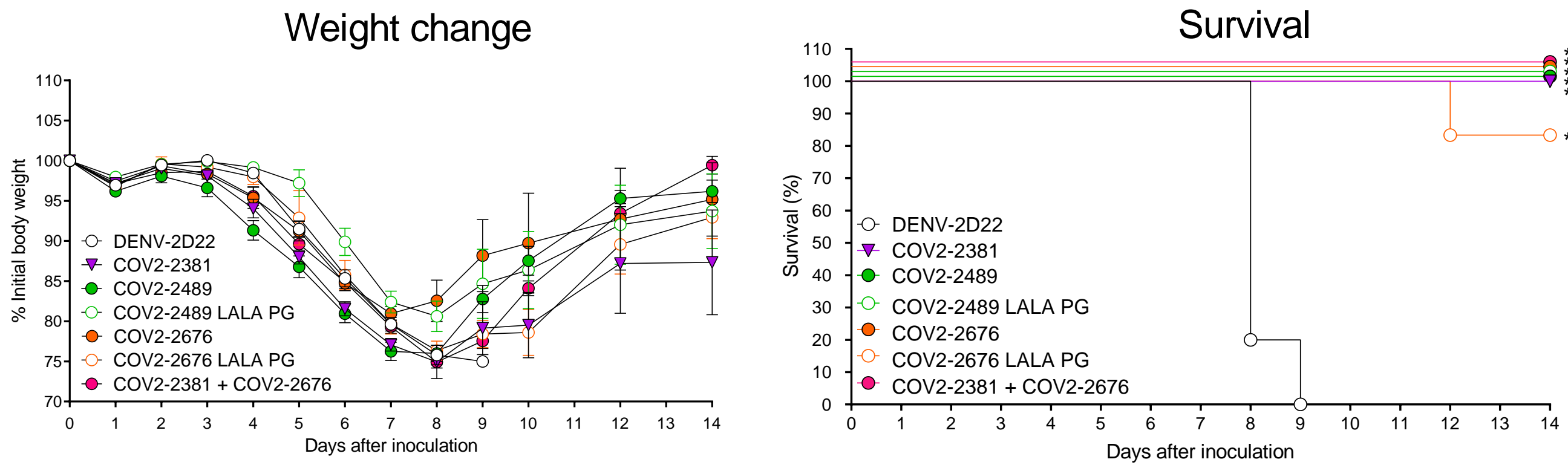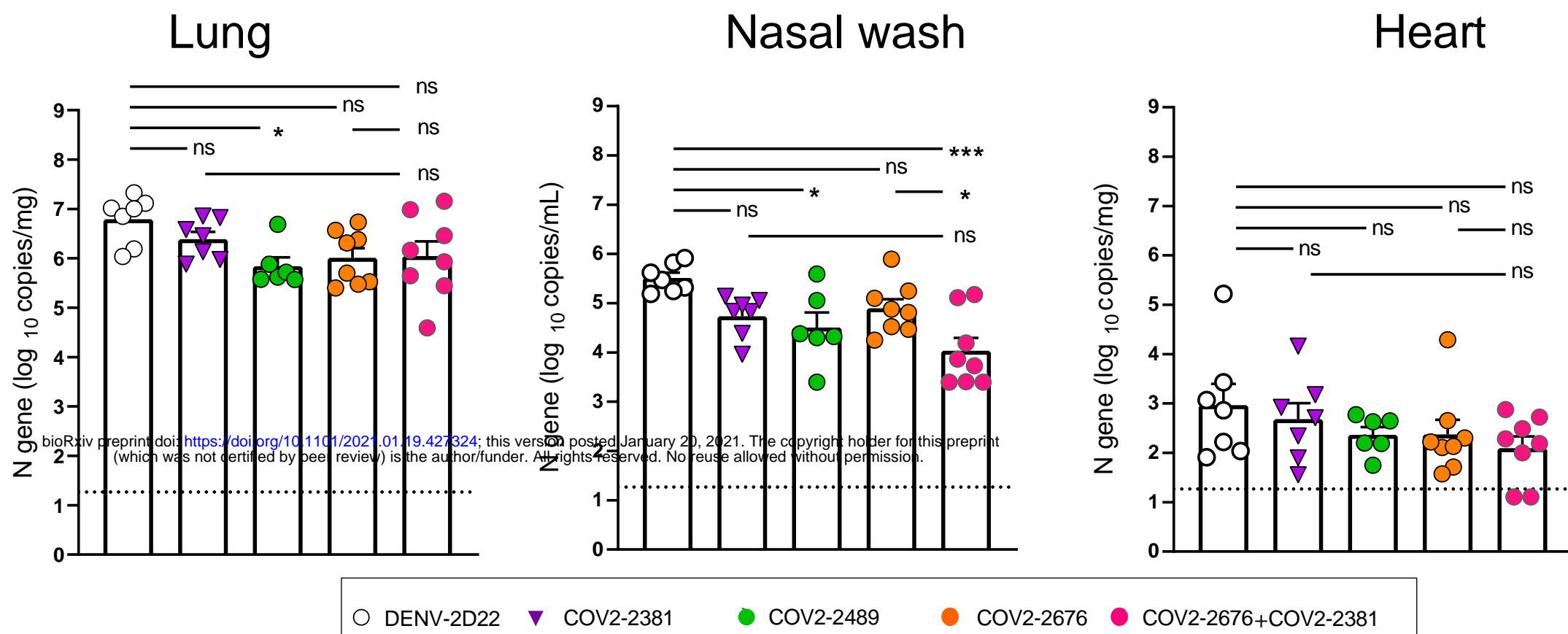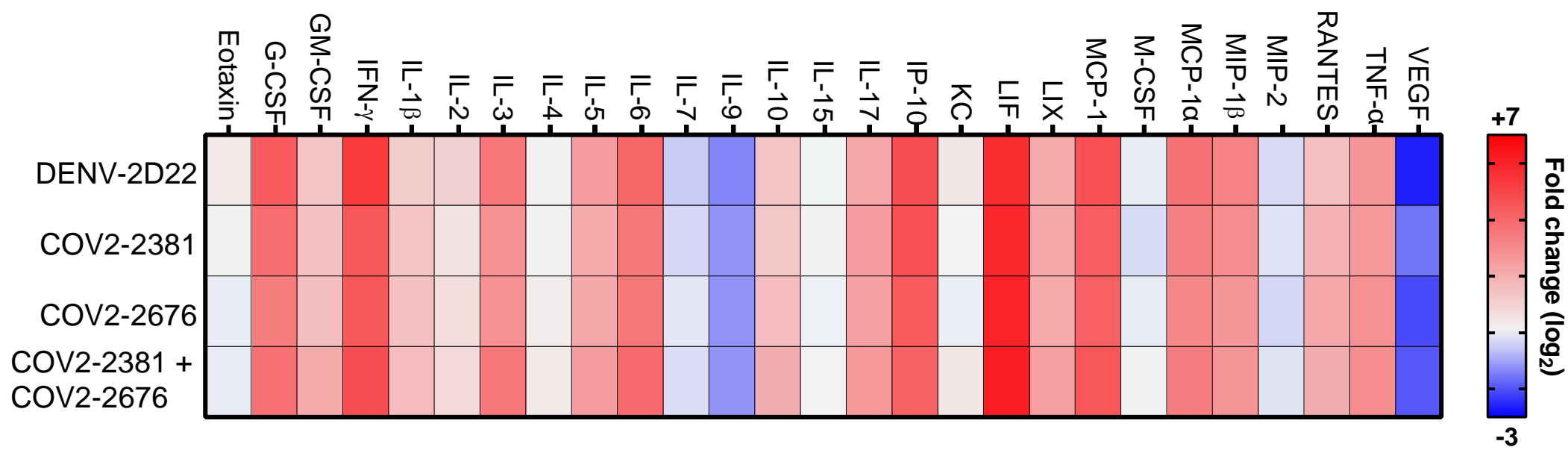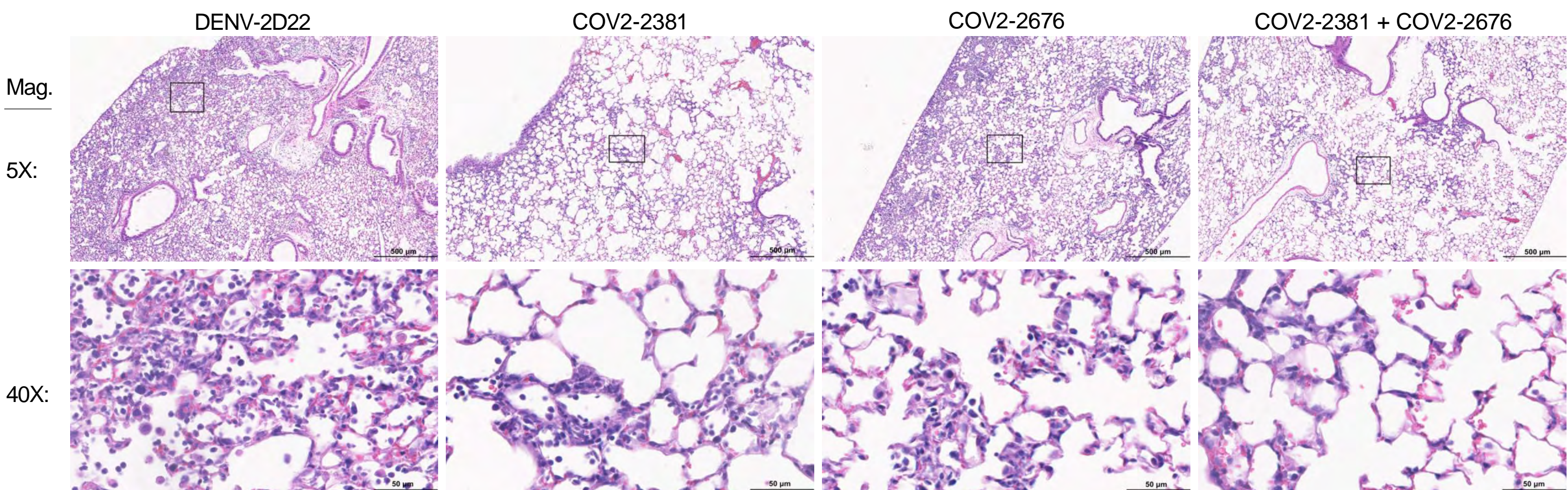
Competition binding

Second mAb added

1st mAb added:	mAb name	COV2-2016	COV2-2021	COV2-2026	COV2-2130	COV2-2196	COV2-2251	COV2-2257	COV2-2260	COV2-2263	COV2-2293	COV2-2305	COV2-2335	COV2-2346	COV2-2384	COV2-2394	COV2-2405	COV2-2438	COV2-2444	COV2-2455	COV2-2459	COV2-2466	COV2-2489	COV2-2490	COV2-2495	COV2-2525	COV2-2526	COV2-2533	COV2-2558	COV2-2563	COV2-2571	COV2-2622	COV2-2673	COV2-2676	COV2-2769	COV2-2816	COV2-2830	CR3022
	COV2-2263	97	88	91	92	92	80	94	91	13	90	82	82	93	84	88	98	90	91	84	79	86	91	93	92	89	89	98	91	86	53	75	88	96	92	95	95	97
	COV2-2676	45	60	79	91	96	95	85	92	93	95	96	89	92	91	94	95	89	89	93	91	87	23	72	73	86	67	90	87	92	96	97	94	10	66	96	90	99

Figure S1. Competition binding of NTD-reactive MAbs

Competition of the panel of neutralizing mAbs with reference mAbs COV2-2676 and COV2-2263. Binding of reference mAbs to trimeric S-6P$_{ecto}$ was measured in the presence of a saturating concentration of competitor mAb in a competition ELISA and normalized to binding in the presence of rDENV-2D22. Black indicates full competition (<25% binding of reference antibody); grey indicates partial competition (25 to 60% binding of reference antibody); white indicates no competition (>60% binding of reference antibody).
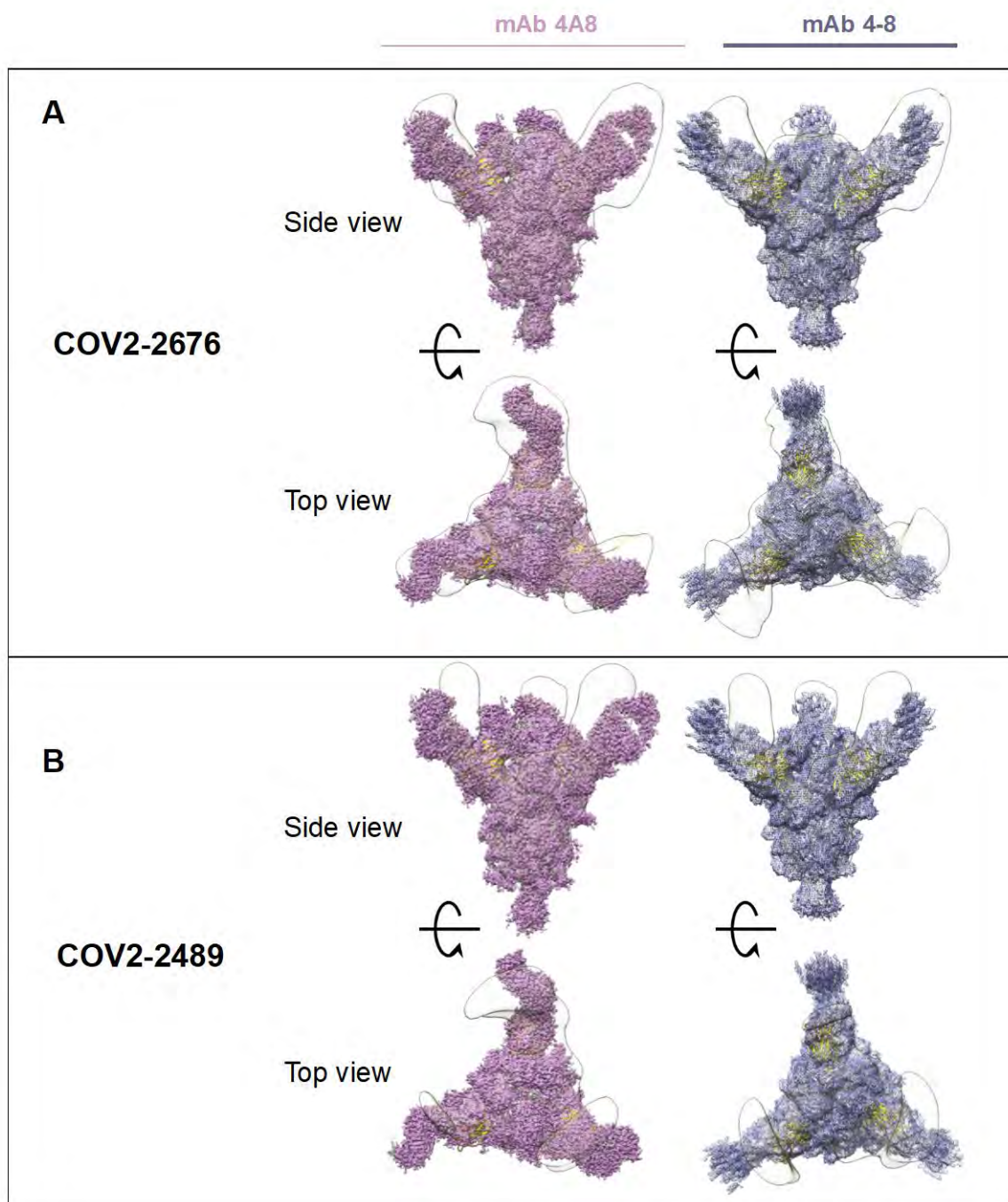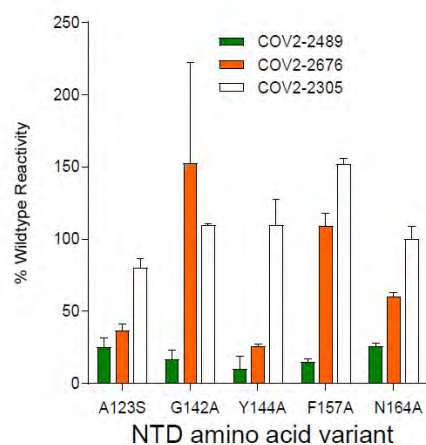
Figure S2. Superimposed Fab-Spike negative stain EM

A) COV2-2676 with mAb on the top is side view 4A8 (left), mAb 4-8 (right) and bottom is top view of the same.
B) COV2-2489 with mAb on the top is side view 4A8 (left), mAb 4-8 (right) and bottom is top view of the same.
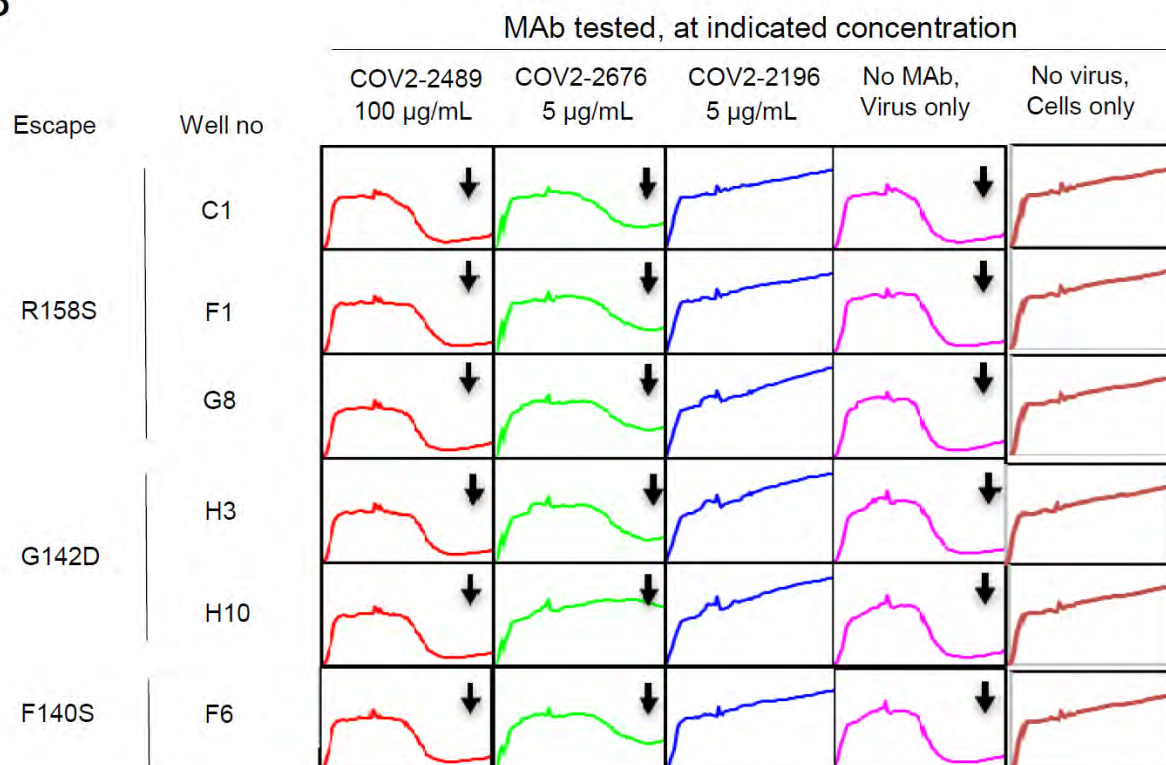
Figure S3. A) Identification of critical contact residues by alanine mutagenesis. Binding values for mAbs COV2-2489, -2676, and -2305. The binding values are shown as a percentage of mAb binding to wild-type (WT) SARS-CoV-2 spike protein and are plotted with the range (highest-minus lowest binding value) of at least two measurements. **B) Real-time cell analysis (RTCA) to select for spike-protein-expressing VSV viruses that escape antibody neutralization, related to Figure 2 B & C.** Example sensograms from individual wells of 96-well E-plate analysis showing viruses that escaped neutralization (noted with arrow) by indicated antibodies. Cytopathic effect (CPE) was monitored kinetically in Vero E6 cells inoculated with virus in the presence of a saturating concentration of antibody COV2-2489 at 100 μg/mL (red), COV2-2676 at 5 μg/mL (green) or lack of escape using RBD-specific mAb COV2-2196-blue at 5 μg/mL (blue) are shown. Uninfected cells (brown) or cells inoculated with virus without antibody (magenta) serve as controls. All curves represented show a mean of technical duplicates.

A. **Gating strategy**
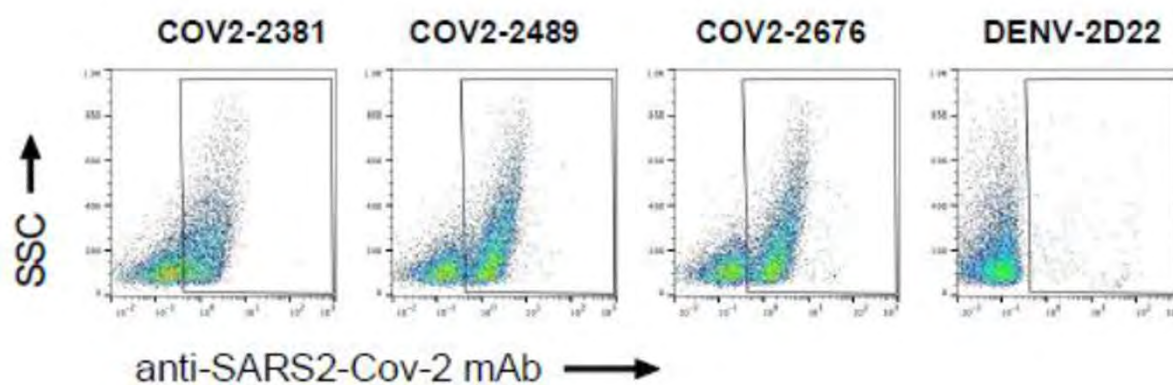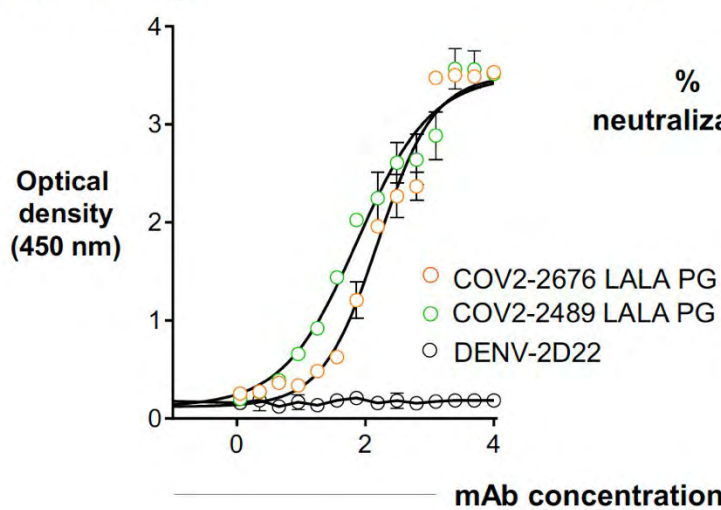
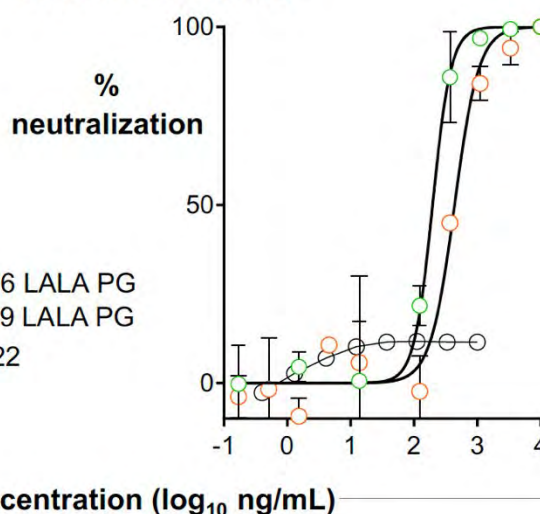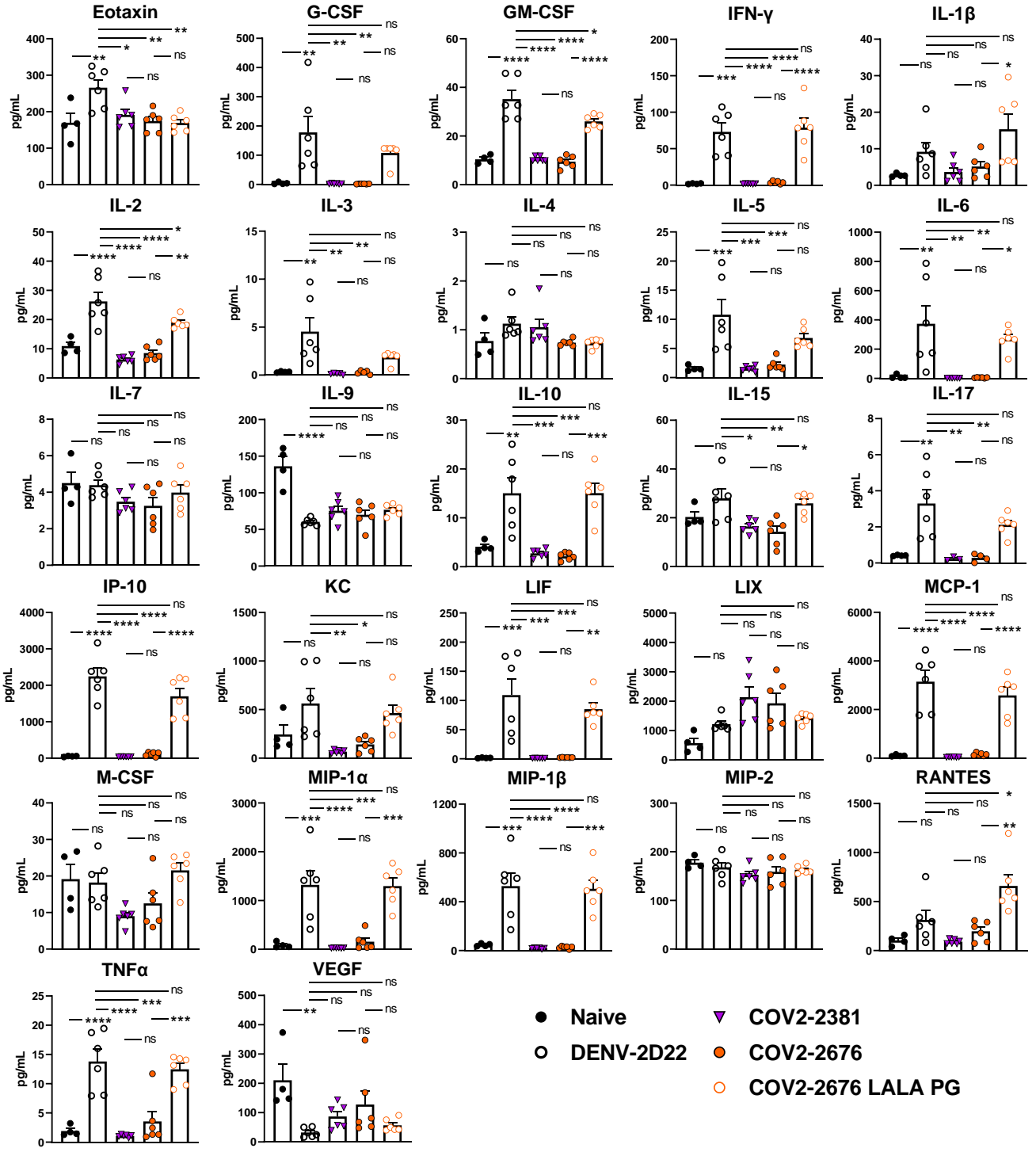B. Binding C. Neutralization

Figure S4. Gating strategy of Vero-E6 cells infected with SARS-CoV-2 for cell surface-displayed spike protein binding assay and ELISA and FRNT for COV2-2676 and COV2-2489 mAbs made with LALA-PG Fc variants.

A. A representative gating strategy illustrating stained with primary COV2-2381, COV2-2676, COV2-2489 or DENV-2D22 MAb.
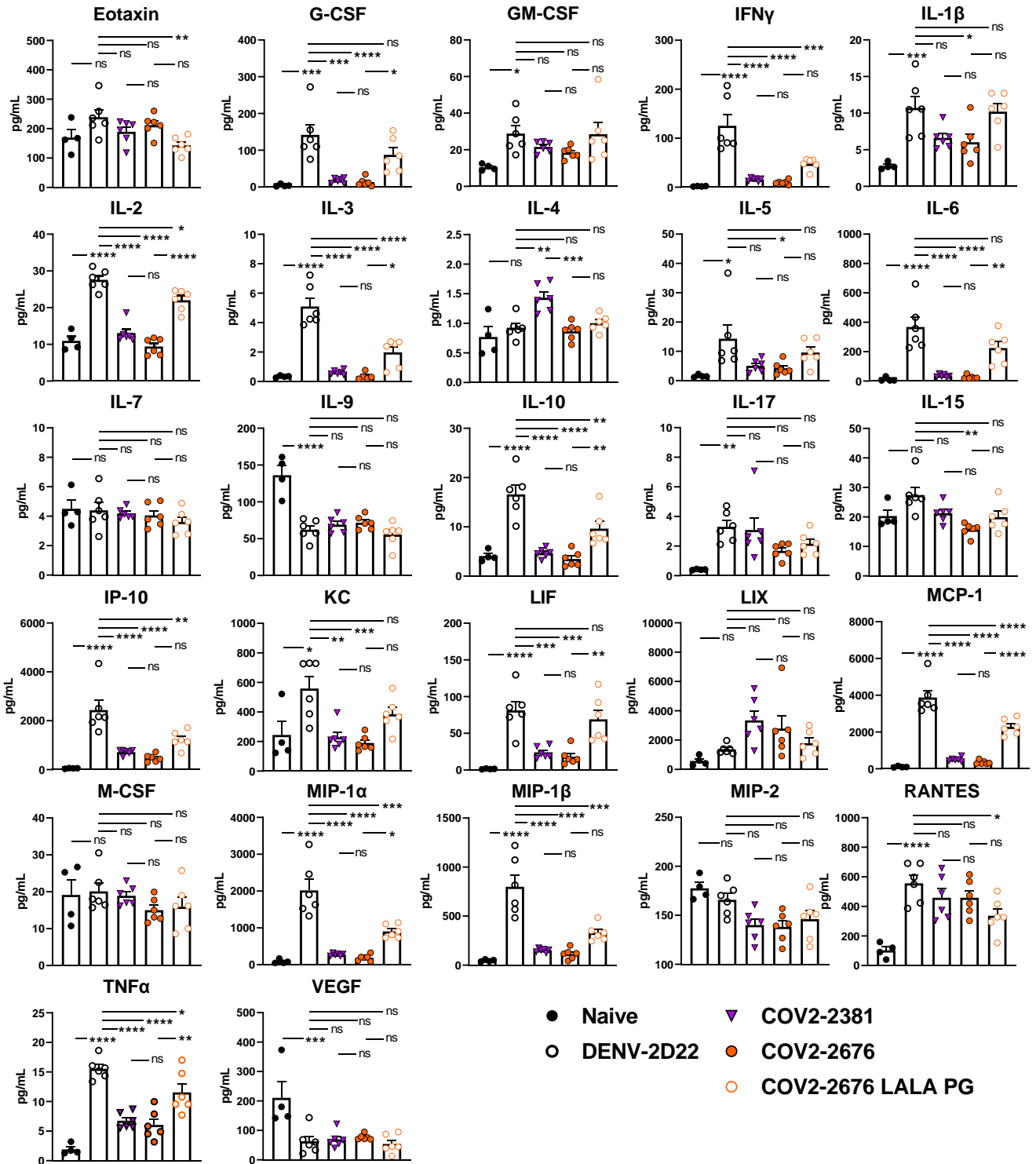
B. ELISA binding of COV2-2676-LALA-PG, COV2-2489-LALA-PG or DENV-2D22 to trimeric S-6P$_{ecto}$. Data are mean ± S.D. of technical triplicates from a representative experiment repeated twice.

C. Neutralization curves for COV2-2676-LALA-PG, 2489-LALA-PG or DENV-2D22 using wild-type authentic SARS-CoV-2 in a FRNT assay. Error bars indicate S.D.; data represent at least two independent experiments performed in technical duplicate.
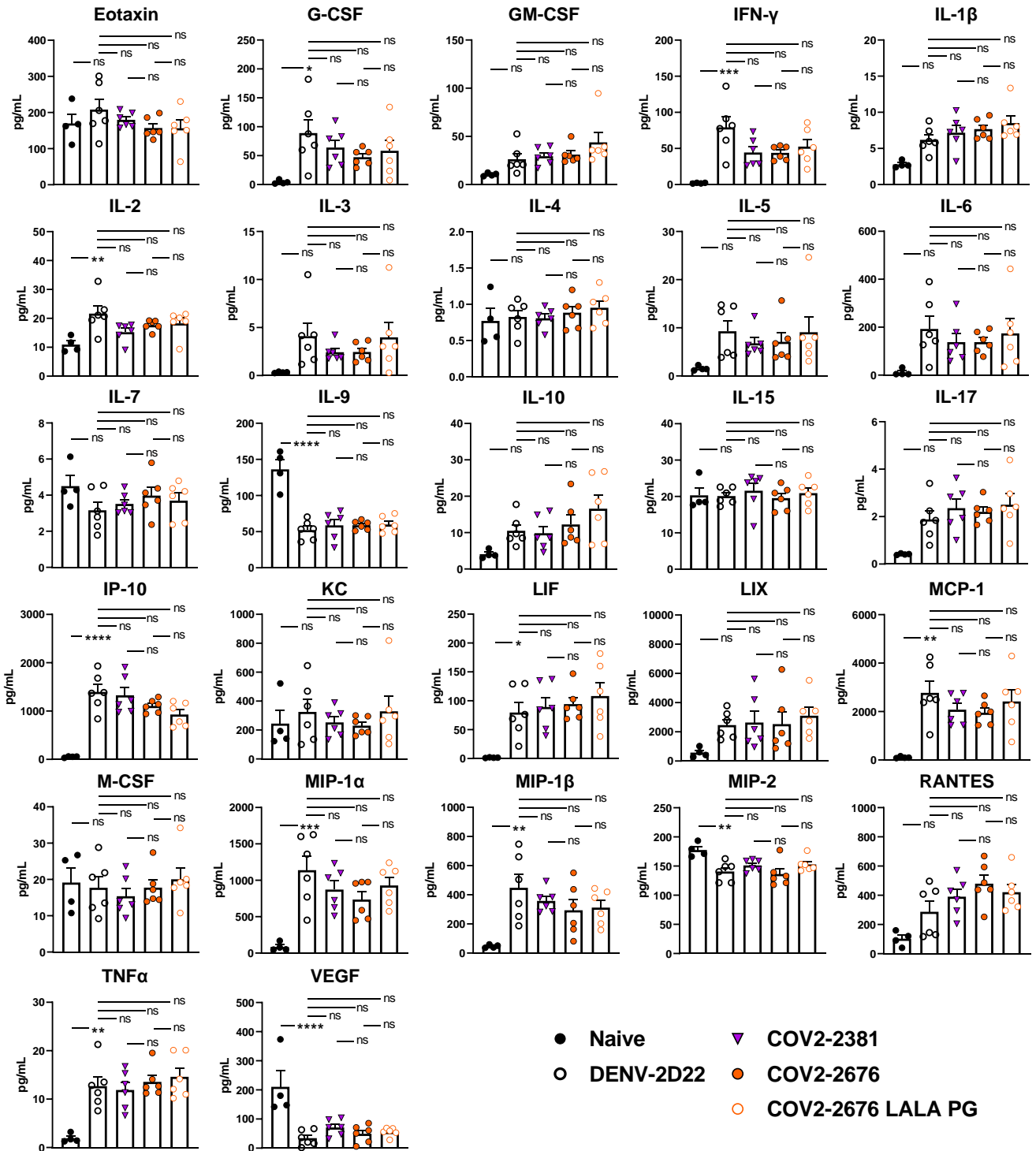
D-1

D+1

Eotaxin, G-CSF, GM-CSF, IFNγ, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-17, IL-15, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIP-1α, MIP-1β, MIP-2, RANTES, TNFα, VEGF

Legend:
- ● Naive
- ○ DENV-2D22
- ▼ COV2-2381
- ● COV2-2676
- ○ COV2-2676 LALA PG

Figure. S5A-C. Cytokine and chemokine levels in the lungs of SARS-CoV-2 infected mice .

A. Cytokine and chemokine levels in the lungs of SARS-CoV-2 infected mice at 7 dpi following d-1 treatment with isotype, COV2-2381, COV2-2676, and COV2-2676 LALA PG as measured by a multiplex platform (two independent experiments, n = 6 per group. One-way ANOVA with Tukey's post hoc test: ns, not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.)

B. Cytokine and chemokine levels in the lungs of SARS-CoV-2 infected mice at 7 dpi following d+1 treatment with isotype, COV2-2381, COV2-2676, and COV2-2676 LALA PG as measured by a multiplex platform (two independent experiments, n = 6 per group. One-way ANOVA with Tukey's post hoc test: ns, not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.)

C. Cytokine and chemokine levels in the lungs of SARS-CoV-2 infected mice at 7 dpi following d+1 treatment with isotype, COV2-2381, COV2-2676, and COV2-2676 LALA PG as measured by a multiplex platform (two independent experiments, n = 6 per group. One-way ANOVA with Tukey's post hoc test: ns, not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.)