

1 **Novel SARS-CoV-2 specific antibody and neutralization assays reveal**  
2 **wide range of humoral immune response during COVID-19**

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## 26    **Abstract**

27    Development of antibody protection during SARS-CoV-2 infection is a pressing question for public  
28    health and for vaccine development. We developed highly sensitive SARS-CoV-2-specific  
29    antibody and neutralization assays. SARS-CoV-2 Spike protein or Nucleocapsid protein specific  
30    IgG antibodies at titers more than 1:100,000 were detectable in all PCR+ subjects (n=115) and  
31    were absent in the negative controls. Other isotype antibodies (IgA, IgG1-4) were also detected.  
32    SARS-CoV-2 neutralization was determined in COVID-19 and convalescent plasma at up to  
33    10,000-fold dilution, using Spike protein pseudotyped lentiviruses, which were also blocked by  
34    neutralizing antibodies (NAbs). Hospitalized patients had up to 3000-fold higher antibody and  
35    neutralization titers compared to outpatients or convalescent plasma donors. Interestingly, some  
36    COVID-19 patients also possessed NAbs against SARS-CoV Spike protein pseudovirus.  
37    Together these results demonstrate the high specificity and sensitivity of our assays, which may  
38    impact understanding the quality or duration of the antibody response during COVID-19 and in  
39    determining the effectiveness of potential vaccines.

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## 44 Introduction

45 Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), which has caused the  
46 COVID-19 pandemic, enters target cells through the interaction of its envelope Spike protein with  
47 the primary host cell receptor Angiotensin Converting Enzyme-2 (ACE2), which is then cleaved  
48 by a serine protease (TMPRSS2) to allow viral fusion and entry across the cell membrane<sup>1</sup>.  
49 Antibodies that can bind to the Spike protein have the potential to neutralize viral entry into cells  
50 and are thought to play an important role in the protective immune response to SARS-CoV-2  
51 infection<sup>2-11</sup>

52

53 To predict protection against SARS-CoV-2, it is critical to understand the quantity, quality and  
54 duration of the antibody responses during different stages of COVID-19 and in the convalescent  
55 period. In this regard, assessing the level of neutralizing antibodies (Nabs) that block viral entry  
56 into cells could be a critical parameter in determining protection from SARS-CoV-2 and  
57 management of convalescent plasma therapies, which are being tested as a COVID-19 treatment  
58 option<sup>12-15</sup>. Defining the relationship between disease severity, other individual-specific co-  
59 morbidities and the neutralizing antibody responses will be critical in our understanding of COVID-  
60 19 and in tailoring effective therapies.

61

62 Currently available SARS-CoV-2 antibody tests mostly lack sufficient dynamic range and  
63 sensitivity to allow for accurate detection or determination of the magnitude of the antibody  
64 response<sup>16</sup>. Furthermore, potential cross-reactivity among SARS-CoV-2 specific antibodies to  
65 other endemic coronaviruses could also be confounders in these tests<sup>17-20</sup>, thus making them  
66 less reliable. Determining neutralization activity in patient plasma also has challenges, as these  
67 assays generally rely on live virus replication, requiring a high-level biohazard security BSL-3 level  
68 laboratory. Therefore, there is an unmet need to develop sensitive antibody and virus

69 neutralization assays that are sufficiently robust for screening and monitoring large numbers of  
70 SARS-CoV-2 infected or convalescent subjects.

71

72 To overcome these experimental challenges, here we developed: 1) Highly sensitive bead-based  
73 fluorescent immunoassay for measuring SARS-CoV-2 specific antibody levels and isotypes, and  
74 2) Robust SARS-CoV-2 Spike protein pseudovirus to measure NAb levels in COVID-19 patient  
75 plasma. We found striking differences in total antibody levels and neutralization titers between  
76 hospitalized or severe COVID-19 patients relative to outpatient or convalescent plasma donors,  
77 which were obtained with the purpose of transfer to and treatment of patients. Significant  
78 correlations between antibody levels and neutralization titers, age and NAbs to SARS-CoV were  
79 also observed. These assays and findings have important implications for assessing the breadth  
80 and depth of the humoral immune response during SARS-CoV-2 infection and for the  
81 development of effective antibody-based therapies or vaccines.

82

## 83 **Results**

### 84 Development of SARS-CoV-2 specific antibody assay

85 Determining antibody responses in SARS-CoV-2 infected subjects remains challenging, due to  
86 lack of sufficient dynamic range to determine precise antibody titers with antibody isotypes  
87 simultaneously. To overcome these obstacles, we developed a fluorescent bead-based  
88 immunoassay that takes advantage of the high dynamic range of fluorescent molecules using  
89 flow cytometry (Fig. 1a). In this assay, we immobilized biotinylated SARS-CoV-2 Spike protein  
90 receptor binding domain (RBD) or the Nucleoprotein (N) on streptavidin beads to detect specific  
91 antibodies from patient plasma (Fig. 1a). Different antibody isotypes were measured using anti-  
92 Ig (IgG, IgA, IgM, IgG1-4) specific secondary antibodies conjugated to a fluorescent tag (Fig. 1a).  
93 Using either anti-S-RBD antibody or soluble ACE2-Fc, we show very high sensitivity in detecting

94 Spike protein binding, down to picogram ranges (Fig. 1b). Similarly, S-RBD-specific antibodies  
95 were detectable in serial dilutions up to 100,000-fold of plasma samples from SARS-CoV-2 PCR+  
96 subjects at high specificity and sensitivity (Fig. 1c). We then used the titration curves from COVID-  
97 19 convalescent and healthy control plasma to normalize the area under the curve (AUC) values  
98 to quantitate the antibody levels (Supplementary Fig. 1a). Negative threshold values were set  
99 using healthy control AUC levels plus one standard deviation of the mean.

100

101 In addition to S-RBD and Nucleocapsid protein, we also attached different viral components such  
102 as S1 subunit of Spike protein, S1 subunit N Terminal Domain (NTD) and S2 Extracellular Domain  
103 (ECD) onto the magnetic beads and tested IgG levels specific to those viral proteins to compare  
104 the antibody levels they detect. Interestingly, S-RBD captured significantly more antibodies  
105 compared to S1 which is the subunit of Spike protein that contains S-RBD (Fig. 1d).

106

107 We also evaluated the dynamic range of our assay by screening some of the plasma samples  
108 with a commercial ELISA-based antibody assay next to our bead-based assay and comparing  
109 the detected antibody levels. Antibody levels from the two antibody assays showed a high  
110 correlation ( $r_s = 0.86$ ), confirming our assay's precision, and the bead-based antibody assay  
111 showed a wider dynamic range compared to the ELISA-based assay. (Fig. 1e)

112

113 Using bead-based assay, we screened COVID-19 patient or convalescent plasma samples  
114 (Table-1; n=115) for total S-RBD and Nucleocapsid specific IgG AUC values of COVID-19 positive  
115 subjects, which varied 3-logs from  $\sim 10^4$  to  $\sim 10^7$  (Fig. 2a). S-RBD specific IgM (40/40) and IgA  
116 (115/115) were also detectable and above the negative control threshold in all subjects (Fig. 2a).  
117 Statistical sensitivity and specificity estimates of our bead-based antibody assays were 100% and  
118 99.34% for S-RBD IgG; 100% and 90.9% for S-RBD IgM, 94.26% and 87.87% for S-RBD IgA and

119 99.13% and 94.93% for Nucleocapsid IgG, respectively. Furthermore; S1 subunit, S1 N Terminal  
120 Domain (NTD), S2 Extracellular Domain (ECD) and Nucleocapsid protein-specific IgG and S-RBD  
121 specific IgA levels positively correlated with S-RBD IgG antibodies (Supplementary Fig. 1b, c)  
122 with the highest correlation with S1 IgG ( $r_s = 0.987$ ). Notably, IgG1 subclass antibody levels were  
123 comparable to total IgG levels whereas the other subtypes were relatively lower (Fig. 2b). There  
124 were significant differences in S-RBD or Nucleocapsid antibody levels between outpatient,  
125 hospitalized, and ICU/deceased subjects, with the highest levels observed in the most severe  
126 cases (Fig. 2c, d, e). Importantly, subjects who had recovered from COVID-19 and were also  
127 potential donors for convalescent plasma therapy (hereafter referred to as plasma donors), also  
128 had significantly lower antibody titers than hospitalized, intensive care unit (ICU) or deceased  
129 patients (Fig. 2c, d, e). Overall, individual S-RBD and Nucleocapsid IgG levels appeared to  
130 correlate with their IgA and IgG subclass (IgG1-4) responses to S-RBD (Supplementary Fig. 1d).  
131 Subdividing the subjects by sex did not reveal any statistical difference in IgG levels at any of the  
132 disease stages (Fig. 2f).

133

134 **Table 1. Characteristics of SARS-CoV-2 infected and control subjects**

Demographics		Healthy Controls (n=56)	Negative (n=94)	Outpatient (n=39)	Hospitalized (n=19)	ICU/Deceased (n=24)	Plasma Donors (n=33)
Sex	Male	14	33	11	7	10	18
	Female	42	61	28	12	14	15
Age	Mean (+-SEM)	45.5 (+-1.78)	54.1 (+-1.97)	46.0 (+-2.20)	62.2 (+-3.41)	68.0 (+-1.87)	45.5 (+-1.99)
	Median	47.0	59.0	47.0	63.0	70.0	48.0
Days between PCR/Blood	Mean (+-SEM)	N/A	N/A	40.7 (+-2.79)	21.0 (+-3.28)	25.8 (+-3.17)	65.4 (+-1.68)
	Median	N/A	N/A	43.0	28.0	24.5	66.0

135

136

137 Development of SARS-CoV-2 Spike protein pseudovirus

138 Next, we sought to develop a sensitive and high throughput SARS-CoV-2 neutralization assay by  
139 incorporating SARS-CoV-2 Spike protein into lentiviruses to assess specific inhibition of viral  
140 entry. To produce Spike protein pseudotyped lentiviral particles, we first ensured expression of  
141 the Spike protein on the cell membrane of transfected 293 cells, from which it would incorporate  
142 into the lentiviruses. Human codon optimized SARS-CoV-2 Spike protein sequences with and  
143 without endoplasmic reticulum retention signal (ERRS), which would be predicted to be more  
144 efficiently expressed on the cell surface membranes, were cloned into an expression vector and  
145 transfected into 293 cells. To evaluate membrane expression of Spike protein, cells were stained  
146 with recombinant soluble ACE2-Fc fusion protein followed by a secondary staining with an anti-  
147 Fc antibody (Fig 3a). The percentage of Spike protein over-expressing cells was similar in the  
148 presence or absence of ERRS, but cells expressing Spike protein without ERRS showed a higher  
149 geometric mean of expression (Fig. 3b). As such, we used Spike protein lacking the ERRS for  
150 lentiviral pseudotyping to ensure its higher incorporation onto viral membranes.

151

152 We then co-transfected 293 cells with replication defective lentivectors encoding GFP or RFP  
153 reporter genes and the Spike protein encoding plasmid and harvested the supernatant at 24  
154 hours, which was then used to infect cells expressing ACE2 (Fig. 3c). Bald particles were  
155 generated by transfecting lentivirus plasmids without any envelope and used as a negative  
156 control. Next, we tested the transduction efficiency of the viruses on wild type 293 cells, given  
157 they express low levels of endogenous ACE2 (Supplementary Fig. 2a, b). While we found clearly  
158 defined infection of 293 cells with Spike-protein pseudovirus compared to bald virions, infection  
159 rate determined by GFP or RFP expression was relatively low (Fig. 3d). We therefore generated  
160 human-ACE2 over-expressing 293 cells with a GFP reporter (ACE2-IRES-GFP) or fused to  
161 fluorescent mKO2 protein (ACE2-mKO2). ACE2 overexpression of ACE2-IRES-GFP or ACE2-

162 mKO2 was confirmed by staining with SARS-CoV-2 Spike-protein S1 subunit fused with mouse  
163 Fc (mFc) and anti-mFc secondary antibody (Supplementary Fig. 2a, b). Indeed, these ACE2 over-  
164 expressing 293 cells (293-ACE2) were efficiently transduced with Spike protein pseudoviruses  
165 encoding either GFP or RFP (Fig. 3e). The efficiency of Spike-protein pseudovirus infection was  
166 comparable in ACE2-IRES-GFP or ACE2-mKO2 fusion protein (Fig. 3e), and therefore both were  
167 used in subsequent neutralization experiments. In addition, we developed SARS-CoV Spike  
168 protein pseudotyped lentivirus, which similarly infected 293-ACE2 cells at almost 100% efficiency  
169 at higher virus supernatant volumes (Fig. 3f). We also tested the stabilities of SARS-CoV-2 and  
170 SARS-CoV Spike protein pseudotyped lentiviruses after serial freeze/thaw cycles and found that  
171 their infectivity remained mostly similar with little loss of activity after 3 cycles (Fig. 3f).

172

### 173 Neutralization of SARS-CoV-2 Spike protein pseudovirus with soluble ACE2, 174 NAbs and COVID-19 plasma

175 We next investigated whether Spike protein pseudoviruses could be neutralized by soluble ACE2  
176 (sACE) or Spike protein specific NAbs (Fig. 4a). For this experiment, Spike protein pseudotyped  
177 SARS-CoV-2 and SARS-CoV pseudoviruses were pre-cultured with different concentrations of  
178 sACE2 or NAbs, then added to 293-ACE2 cells. Subsequently, infection was determined 3 days  
179 post-infection based on GFP or RFP expression as described above. sACE2 neutralized both  
180 SARS-CoV-2 and SARS-CoV pseudovirus infections in a dose dependent manner (Fig. 4b, c),  
181 although neutralization of SARS-CoV-2 was slightly better than that of SARS-CoV pseudoviruses  
182 (Fig. 4b, c, and Supplementary Fig. 3a). Furthermore, Spike-RBD specific NAb neutralized SARS-  
183 CoV-2 pseudovirus entry much more efficiently than sACE2 but had no effect on SARS-CoV  
184 pseudovirus (Fig. 4c). One of the SARS-CoV-2 S-RBD specific antibodies (non-NAb) did not show  
185 any neutralization of SARS-CoV-2, however very low level neutralization of SARS-CoV  
186 pseudovirus was detected (Fig. 4c). We also observed measurable differences in the neutralizing

187 activity of four different NAbs and two different soluble ACE2 proteins from different sources (Fig.  
188 4d), showing the utility of this assay for such screening. Taken together, these experiments  
189 demonstrate that the combination of pseudotyped viruses and 293-ACE2 cells can be used to  
190 generate highly sensitive SARS-CoV-2 and SARS-CoV neutralization assays.

191

192 Using this approach, we then tested neutralization titers from COVID-19 patients or seropositive  
193 donors with serial dilution of their plasma. Accordingly, plasma samples in 3-fold serial dilutions  
194 were incubated with Spike pseudovirus and added to 293-ACE2 cells and infection was  
195 determined as described in Figure 4. Healthy control plasma samples were used as negative  
196 controls whereas anti-S-RBD NAb served as a positive control (Fig. 5a). None of the control  
197 plasma (n=34, 1 shown in Fig. 5a) tested showed any neutralization activity, whereas patient  
198 plasma efficiently neutralized the virus at up to 10,000-fold serial dilution (Fig. 5a). The 50%  
199 neutralization titer (NT50), was determined using the half-maximal inhibitory concentration values  
200 of plasma samples, normalized to control infections, from their serial dilutions. Importantly, the  
201 NT50 values of the subjects were much higher in hospitalized patients than in outpatients (Fig.  
202 5b). NT50 values for hospitalized and ICU/deceased subjects were also up to 1000-fold higher  
203 than convalescent plasma donors (Fig. 5b). Hospitalized males and females, separately, also  
204 remained higher in their NT50 levels and no difference was observed within each group (Fig. 5c).

205

206 We also tested whether SARS-CoV-2 PCR+ plasma could neutralize the SARS-CoV pseudovirus.  
207 104 plasma samples from all groups were tested for their ability to neutralize SARS-CoV-2 and  
208 SARS-CoV pseudoviruses. Remarkably, most of the plasma samples also neutralized SARS-CoV  
209 pseudovirus, although less efficiently than SARS-CoV-2 pseudovirus (Fig. 5d). Interestingly,  
210 NT50 levels of plasma samples for SARS-CoV-2 and SARS-CoV significantly correlated in  
211 hospitalized subjects (Fig. 5e), whereas there were no correlations in outpatients and plasma

212 donors or when all groups were combined (Supplementary Fig. 5a). Additionally, when we  
213 compared the severity groups for their SARS-CoV NT50 values, we did not observe any  
214 significant difference between the groups (Supplementary Fig. 5b).

215

216 Correlations of SARS-CoV-2 neutralization, antibody levels and COVID-19  
217 subject characteristics

218 To better understand the associations between patient characteristics and the humoral immune  
219 response in COVID-19, we next determined correlations between antibody AUC levels, NT50  
220 values and demographics of the study subjects. First, we assessed the correlation between NT50  
221 values with S-RBD or Nucleocapsid antibody titers or their subclasses. All IgGs including S-RBD  
222 IgG ( $r_s = 0.81$ ), Nucleocapsid IgG ( $r_s = 0.689$ ), S-RBD IgA ( $r_s = 0.60$ ) and S-RBD IgM ( $r_s = 0.47$ )  
223 showed significant correlation with NT50 values of each subject (Fig. 6a). Among IgG subclasses  
224 specific to S-RBD; IgG1 ( $r_s = 0.80$ ), IgG3 ( $r_s = 0.69$ ) and IgG2 ( $r_s = 0.67$ ) and, to a lesser degree,  
225 IgG4 ( $r_s = 0.51$ ) also correlated with NT50 values (Fig. 6b). Total S-RBD IgG also correlated in a  
226 similar fashion with other IgG isotypes, with IgG1 showing the highest positive correlation ( $r_s =$   
227 0.96) (Supplementary Fig. 4a).

228

229 Next, we correlated the antibody AUC levels and NT50 values of the subjects with their age.  
230 Subjects had significantly higher S-RBD IgG ( $r_s = 0.53$ ), Nucleocapsid IgG ( $r_s = 0.43$ ), S-RBD IgA  
231 ( $r_s = 0.41$ ) and NT50 ( $r_s = 0.579$ ) values, as their age increased (Fig. 6c).

232

233 We also explored the relationship of the number of days between PCR test result and blood draw  
234 with antibody levels or NT50 values, excluding the subjects that had 15 days or fewer between  
235 those dates to ensure that antibody levels had already reached their peak. Of note, there was no

236 correlation between the number of days and the IgG to S-RBD or the NT50 (Supplementary Fig.  
237 4b), suggesting a potential persistence in antibody titers at least for 3 month duration in this cohort.

238

## 239 Discussion

240 The COVID-19 pandemic is continuing to spread globally unabated, including within the United  
241 States. There is an urgent need to better understand the immune response to the virus so that  
242 effective immune-based treatments and vaccines can be developed<sup>21,22</sup>. Neutralization of the  
243 virus by antibodies (NAbs) is one of the goals to achieve protection against SARS-CoV-2<sup>23</sup>.  
244 Despite rapid development of many serological tests, important questions about the quality and  
245 quantity of seroprevalence in individuals remains still unclear<sup>24,25</sup>. Here, we developed highly  
246 sensitive and specific humoral assays that measure both the magnitude and neutralization  
247 capacity of antibody responses in COVID-19 patients. Every SARS-CoV-2 infected subject we  
248 tested (n=115) had detectable antibodies and all subjects except one exhibited neutralization;  
249 both of these qualities were completely absent in non-infected controls. However, there was a  
250 profound difference in antibody and neutralization titers among subjects, ranging in more than  
251 1000-fold differences. Furthermore, we found that almost all COVID-19 patients also had  
252 neutralizing antibodies for SARS-CoV, suggesting a high degree of cross-reactivity between these  
253 two virus Spike proteins.

254

255 One of our key findings was clustering of antibody responses based on severity of the disease;  
256 as hospitalized patients showed much higher antibody levels and neutralization capacity than  
257 outpatient subjects or convalescent plasma donors. This finding is consistent with recent reports  
258 suggesting that patients with more severe disease contain relatively higher levels of antibodies  
259 for SARS-CoV-2 infection<sup>2,26-30</sup>. Interestingly, most of the convalescent plasma donors had much  
260 lower levels of neutralizing antibodies (by at least an order of magnitude) than hospitalized

261 patients, who would be the suitable recipients for such plasma transfer therapy. This finding raises  
262 the question of whether convalescent plasma transfers may actually provide benefit to severe  
263 COVID-19 patients by providing neutralizing antibodies. It may perhaps be more beneficial to  
264 identify donors with much higher neutralizing antibody titers for the plasma donation. As such, our  
265 findings point to the importance of having access to assays that have a large dynamic range to  
266 detect antibody responses in COVID-19 patients or seropositive individuals. This neutralization  
267 assay also revealed differences in commercial antibodies to SARS-CoV-2 in their capacity to  
268 block virus entry, and as such can be used for rapid identification or generation of synthetic NAbs.  
269 In addition to measuring neutralization titers, the pseudoviruses can be used to probe cells that  
270 have the potential to be infected with SARS-CoV-2, given lentiviruses can infect most cell types  
271 and do not require cell division to integrate into the genome. This infection assay may also be  
272 used to screen small molecules that may impact virus cell entry.

273  
274 Along with SARS-CoV-2, we also developed a pseudotyped lentivirus with SARS-CoV Spike  
275 protein, which was equally efficient at infecting ACE2 overexpressing cells. This finding is  
276 consistent with results that SARS-CoV-2 Spike protein in complex binding with human ACE2  
277 (hACE2) is similar overall to that observed for SARS-CoV<sup>31</sup>. There was however slightly better  
278 neutralization of SARS-CoV-2 cell entry than SARS-CoV with soluble ACE2, which could be due  
279 to key residue substitutions in SARS-CoV-2, creating a slightly stronger interaction and thus  
280 higher affinity for receptor binding than SARS-CoV Spike protein<sup>31</sup>. Accordingly, we also tested  
281 the ability of COVID-19 patient plasma for SARS-CoV neutralizing capacity and found significant  
282 SARS-CoV specific neutralization in COVID-19 patients. However, the neutralization titers for  
283 SARS-CoV were significantly lower and there was no correlation with the neutralization activity  
284 against SARS-CoV-2 when all severity groups were combined. Indeed, some donors even had  
285 relatively higher SARS-CoV neutralization (Fig. 5e). Although there was significant correlation

286 between SARS-CoV-2 and SARS-CoV NT50 levels when hospitalized and ICU/Deceased  
287 subjects were analyzed separately. Given that the two viruses share ~75% identical amino acid  
288 sequences in their Spike proteins and there are conserved epitopes between them<sup>32</sup>, it is  
289 conceivable that some of the SARS-CoV-2 NAbs have cross-neutralizing activity<sup>33,34</sup>. It is  
290 noteworthy that a recent study showed a NAb developed for SARS-CoV was highly effective at  
291 neutralizing SARS-CoV-2<sup>35</sup>. It is also tempting to speculate that presence of SARS-CoV specific  
292 NAbs could be more potent perhaps by targeting highly conserved regions of the Spike protein,  
293 making it more difficult for the virus to select for escape mutants.

294

295 Other antibody and neutralization assays have been developed during the submission of our  
296 manuscript<sup>2,3,9,36-40</sup>. Although direct comparison is difficult due to differences in the assay  
297 methodologies and different sample sets, we believe our assays embody distinctive features that  
298 further enhance this critically important immune response to SARS-CoV-2. The use of the flow  
299 cytometry bead based fluorescent system that detects Spike or Nucleocapsid protein bound  
300 antibodies provides a high-throughput assay with a very high dynamic range and sensitivity, as it  
301 could detect antibodies from some subjects at up to a million-fold dilution of the plasma. This  
302 assay is also scalable and can be easily adaptable to other viral antigens. Using a flow cytometry  
303 platform is also important in that the assay can be further developed in a single panel to identify  
304 all antibody isotypes simultaneously and to complement flow cytometric immune phenotyping of  
305 COVID-19 patients. The high sensitivity and specificity of our assay has allowed us to correlate  
306 the Spike protein RBD-specific antibody levels with neutralization titers, which showed very high  
307 concordance, and thus can be utilized as a proxy for neutralization in a clinical setting.  
308 Furthermore, the bead-based immunoassay can also be further developed to screen for  
309 antibodies reacting to other SARS-CoV-2 antigens simultaneously and can be useful in identifying  
310 antibodies that cross-react between different species of coronavirus proteins. Determining other

311 isotypes such as IgA and IgG subclasses may also help in future mechanistic studies. It is clear  
312 that the dominant antibody response in almost all donors was IgG1, but some also show high IgA  
313 and IgG2-4, at varying levels. For example, given the importance of IgA antibodies in providing  
314 immunity on mucosal surfaces within the respiratory system, SARS-CoV-2 RBD-spike protein  
315 specific IgA levels may also play an important role in the upper and lower respiratory system, or  
316 perhaps also in the gut, of COVID-19 patients<sup>41,42</sup>.

317

318 There are also several potential practical implications to our findings. First, the patient population  
319 with the greatest risk factors for severe outcomes from infection such as age and co-morbid  
320 conditions had the highest antibody titers as well as neutralization of the virus. This is also the  
321 case for those patients who had lethal disease. It is therefore possible that surviving COVID-19  
322 may require non-antibody dependent factors or that producing too much antibody may even have  
323 deleterious effects<sup>43</sup>, such as potential antibody-dependent enhancement phenomenon by  
324 triggering Fc receptors on macrophages<sup>44</sup>. In this regard, it is interesting to note that a Bruton  
325 tyrosine kinase (BTK) inhibitor that targets Fc-receptor signaling in macrophages is being tested  
326 in a randomized clinical trial <sup>45</sup>. Thus, understanding the mechanism of survival from COVID-19  
327 and immune response dynamics will be critical in the better prediction of outcomes as well as in  
328 assaying for a protective response to potential vaccines.

329

330 In conclusion, the assays developed herein can have utility in uncovering dynamic changes in the  
331 antibody levels in SARS-CoV-2 infected subjects over time, in responses to vaccines and as  
332 potential clinical determinants for plasma or antibody therapies for COVID-19 patients.

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334

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336

337 **Materials and Methods**

338 **Participants**

339 COVID-19 subjects (**n=115**) were recruited at SUNY Downstate Medical Center, New York, NY,  
340 Cedars Sinai, Los Angeles, CA, or the University of Connecticut, School of Medicine, Farmington  
341 CT following testing and/or admission for COVID-19 infection. Written informed consent was  
342 obtained from all participants in this study and was approved by the following IRBs: 1) IRB#  
343 SUNY:269846. The patients were recruited at SUNY Downstate, NY and processed and  
344 biobanked at Amerimmune, Fairfax VA; 2) IRB# STUDY00000640. Convalescent plasma was  
345 collected at Cedars Sinai Medical Center according to FDA protocol  
346 (<https://www.fda.gov/vaccines-blood-biologics/investigational-new-drug-ind-or-device-exemption-ide-process-cber/recommendations-investigational-covid-19-convalescent-plasma#Collection%20of%20COVID-19>). The source of the convalescent plasma was volunteer  
347 blood donors who were recovered from COVID-19. Donors met routine blood donor eligibility  
349 requirements established by the FDA and had a prior SARS-CoV-2 infection documented by a  
350 laboratory test for the virus during illness, or antibodies to the virus after recovery of suspected  
351 disease. All donors were at least 28 days from either resolution of COVID-19 symptoms or  
352 diagnostic clearance, whichever was longer; 3) IRB# 20-186-1. UConn Healthcare workers who  
354 tested positive for the virus by PCR were recruited and samples banked for future testing. 4) IRB#:  
355 17-JGM-13-JGM or 16-JGM-06-JGM. De-identified control subjects (**n=56**) with previously frozen  
356 (more than a year ago) samples obtained from healthy controls or determined to be SARS-CoV-  
357 2 PCR negative (IRB SUNY:269846). All antibody assays were performed at the Jackson  
358 Laboratory for Genomic Medicine, Farmington, CT. Subject characteristics are shown in Table 1.  
359 All plasma samples were aliquoted and stored at -80°C. Prior to experiments, aliquots of plasma  
360 samples were heat-inactivated at 56°C for 30 minutes.

361

362 Over-expression of SARS-CoV-2 Spike protein and cell culture

363 Human codon optimized SARS-CoV-2 Spike protein sequence was synthesized by  
364 MolecularCloud (MC\_0101081). 5'-ACGACGGAATTCATGTTCGTCTTCCTGGTCCTG-3' and 5'-  
365 ACGACGGAATTCTAACAGCAGGAGGCCACAGC-3' primers were used to amplify the SARS-  
366 CoV-2 Spike protein sequence without Endoplasmic Reticulum Retention Signal that is the last  
367 19 amino acids<sup>33</sup>. Full length and truncated SARS-CoV-2 spike protein sequences were then  
368 cloned into pLP/VSVG plasmid from Thermo Fisher under CMV promoter after removing the  
369 VSVG sequence via EcoRI-EcoRI restriction digestion. HEK-293T cells (ATCC; mycoplasma-free  
370 low passage stock) were transfected with the expression plasmids using Lipofectamine 3000  
371 (Invitrogen) according to the manufacturer's protocol as previously described<sup>46</sup>. The cells were  
372 cultured in complete RPMI 1640 medium (RPMI 1640 supplemented with 10% FBS; Atlanta  
373 Biologicals, Lawrenceville, GA), 8% GlutaMAX (Life Technologies), 8% sodium pyruvate, 8%  
374 MEM vitamins, 8% MEM nonessential amino acid, and 1% penicillin/streptomycin (all from  
375 Corning Cellgro) for 72 hours, collected using %0.05 Trypsin-0.53 mM EDTA (Corning Cellgro)  
376 and stained with Biotinylated Human ACE2 / ACEH Protein, Fc, Avitag (Acro Biosystems) then  
377 stained with APC anti-human IgG Fc Antibody clone HP6017 (Biolegend). Samples were acquired  
378 on a BD FACSymphony A5 analyzer and data were analyzed using FlowJo (Tree Star).

379

380 Pseudotyped lentivirus production and titer measurement

381 Lentivector plasmids containing RFP or GFP reporter genes were co-transfected with either  
382 SARS-CoV-2 Spike protein or SARS-CoV Spike protein (Human SARS coronavirus (SARS-CoV)  
383 Spike glycoprotein Gene ORF cDNA clone expression plasmid (Codon Optimized) from  
384 SinoBiological) plasmids into HEK-293T cells using Lipofectamine TM 3000 (Invitrogen)  
385 according to the manufacturer's protocol. Viral supernatants were collected 24-48 hours post-

386 transfection, filtered through a 0.45 µm syringe filter (Millipore) to remove cellular debris, and  
387 concentrated with Lenti-X (Invitrogen) according to the manufacturers protocol. Lentivirus  
388 supernatant stocks were aliquoted and stored at -80°C. To measure viral titers, virus preps were  
389 serially diluted on ACE2 over-expressing 293 cells. 72 hours after infection, GFP or RFP positive  
390 cells were counted using flow cytometry and the number of cells transduced with virus  
391 supernatant was calculated as infectious units/per mL.

392

### 393 Generating human ACE2 over-expressing cells

394 Wildtype ACE2 sequence was obtained from Ensembl Gene Browser (Transcript ID:  
395 ENST00000252519.8) and codon optimized with SnapGene by removing restriction enzyme  
396 recognition sites that are necessary for subsequent molecular cloning steps, preserving the amino  
397 acid sequence. The sequence of mKO2 (monomeric Kusabira-Orange-2<sup>47</sup>) obtained from  
398 Addgene (#54625)<sup>48</sup>, and was added onto the C terminal end of ACE2 before the stop codon with  
399 a small linker peptide (ccggtcgccacc) encoding the amino acids "PVAT". The fusion constructs  
400 were synthesized via GenScript and cloned into a lentiviral vector lacking a fluorescent reporter.  
401 The full length human ACE2 sequence without fusion fluorescent proteins was amplified from the  
402 ACE2-mKO2 fusion construct using 5'-ACGACGGCGGCCGCATGTCAAGCTTCCCTGGC-3'  
403 and 5'- ACGACGGAATTCTAAAAGGAGGTCTGAACATCATCAG-3' primers, generating a stop  
404 codon at the C-terminus, and then cloned into a lentiviral vector encoding GFP reporter separated  
405 from multiple cloning site via an internal ribosome entry site (IRES) sequence. To determine the  
406 virus titers, HEK-293T cells were transduced with full length ACE2-IRES-GFP, ACE2-mKO2  
407 fusion construct lentiviruses and analyzed via flow cytometry for their reporter gene expression  
408 72 hours after infection. WT and ACE2 over-expressing HEK-293T were also stained with SARS-  
409 CoV-2 S1 protein, Mouse IgG2a Fc Tag (Acro Biosystems) followed with APC Goat anti-mouse

410 IgG2a Fc Antibody (Invitrogen). Samples were acquired on a BD FACSymphony A5 analyzer and  
411 data were analyzed using FlowJo (BD Biosciences).

412

### 413 SARS-CoV-2 antibody detection using Flow immunoassay

414 To screen for antibody binding to SARS-CoV-2 proteins, The DevScreen SAv Bead kit (Essen  
415 BioScience, MI) was used. Biotinylated 2019-nCoV (COVID-19) Spike protein RBD, His, Avitag,  
416 Biotinylated CoV-2 (COVID-19) Nucleocapsid protein, His,Avitag, Biotinylated CoV-2 (COVID-19)  
417 S1 protein, His,Avitag (Acro Biosystems, DE), Biotinylated SARS-CoV-2 (2019-nCoV) Spike S1  
418 NTD-His & AVI recombinant protein and Biotinylated SARS-CoV-2 (2019-nCoV) Spike S2 ECD-  
419 His recombinant protein (Sino Biological Inc.) were coated to SAv Beads according to  
420 manufacturer's instructions. Confirmation of successful bead conjugation was determined by  
421 staining with anti-His Tag (Biolegend) and flow cytometry analysis. S-RBD, N, S1, S1-NTD and  
422 S2-ECD conjugated beads were then used as capture beads in flow immunoassay where they  
423 were incubated with anti-S-RBD human IgG positive control (provided in GenScript SARS-CoV-  
424 2 Spike S1-RBD IgG & IgM ELISA Detection Kit as positive control), recombinant Human ACE2-  
425 Fc (Acro Biosystems) or plasma and serum samples for 1 hour at room temperature. Plasma  
426 samples were assayed at a 1:100 starting dilution and 3 additional 10-fold serial dilutions. Anti-S-  
427 RBD antibody and ACE2-Fc were both tested at a 5 µg/mL starting concentration and in additional  
428 5-fold serial dilutions. Detection reagent was prepared using Phycoerythrin-conjugated anti-  
429 human IgG Fc clone HP6017, anti-human IgM clone MHM-88 (Biolegend), anti-human IgA clone  
430 IS11-8E10, anti-human IgG1 clone IS11-12E4.23.20 (Miltenyi Biotec), anti-human IgG2 Fc clone  
431 HP6002, anti-human IgG3 Hinge clone HP6050 and anti-human IgG4 pFc clone HP6023  
432 (Southern Biotech), added to the wells and incubated for another hour at room temperature.  
433 Plates were then washed twice with PBS and analyzed by flow cytometry using iQue Screener  
434 Plus (IntelliCyt, MI). Flow cytometry data were analyzed using FlowJo (BD biosciences).

435 Geometric means of PE fluorescence in different titrations were used to generate the titration  
436 curve and 20 healthy control plasma were used to normalize the area under the curve (AUC).  
437 Statistical analyses were performed using GraphPad Prism 8.0 software (GraphPad Software).

438

439 **SARS-CoV-2 antibody detection using ELISA**

440 To evaluate antibodies binding to CoV-2 S-RBD protein, SARS-CoV-2 Spike S1-RBD IgG and  
441 IgM ELISA Detection Kit from GenScript was used according to the manufacturer's instructions.  
442 Absorbance was measured at 450 nm using an Epoch 2 microplate spectrophotometer (BioTek).

443

444 **Pseudotyped virus neutralization assay**

445 Three-fold serially diluted monoclonal antibodies including anti-SARS-CoV-2 Neutralizing human  
446 IgG1 Antibody from Acro Biosystems, NAb#1 (Fig 4c, d), GenScript clone ID 6D11F2, NAb#2 (Fig  
447 4d) and GenScript clone ID 10G6H5, NAb#3 (Fig 4d), Invitrogen clone ID MA5-35939 Nab#4 (Fig  
448 4d), recombinant human ACE2-Fc (Acro Biosystems, sACE2#1 and GenScript, sACE2#2 (Fig  
449 4d) or plasma from COVID-19 convalescent individuals and healthy donors were incubated with  
450 RFP-encoding SARS-CoV-2 or GFP-encoding SARS-CoV pseudotyped virus with 0.2 multiplicity  
451 of infection (MOI) for 1 hour at 37°C degrees. The mixture was subsequently incubated with 293-  
452 ACE2 cells for 72h hours after which cells were collected, washed with FACS buffer (1xPBS+2%  
453 FBS) and analyzed by flow cytometry using BD FACSymphony A5 analyzer. Percent infection  
454 obtained was normalized for samples derived from cells infected with SARS-CoV-2 or SARS-CoV  
455 pseudotyped virus in the absence of plasma, ACE2-Fc or monoclonal antibodies. The half-  
456 maximal inhibitory concentration for plasma (NT50), ACE2-Fc or monoclonal antibodies (IC50)  
457 was determined using 4-parameter nonlinear regression (GraphPad Prism 8.0).

458

459

460 **Statistical Analyses**

461  
462 All statistical analyses were performed using GraphPad Prism V8 software. Continuous variable  
463 datasets were analyzed by Mann-Whitney U test for non-parametric datasets when comparing  
464 clinical groups, and exact P values are reported. Spearman p analysis was used to determine the  
465 relationship existing between two sets of non-parametric data, where a value of 0 indicated no  
466 relationship, values between 0 and +/-0.3 indicated a weak relationship, values between +/-0.3  
467 and +/-0.7 indicated a moderate relationship, values between +/-0.7 and +/-1.0 indicated a strong  
468 relationship, and a value of +/-1.0 indicated a perfect relationship between sets of data.<sup>49</sup>

469

470

471 **Data availability**

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472 The source data for the Figures along with the Supplementary Figures presented in this paper  
473 are available upon request.

474

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478

479

480 **Author contributions**

481 M.D., L.K. and D.U. conceived and designed the experiments. M.D., L.K., L.P., M.Y. and R.H.  
482 carried out the experiments. B.T.L. designed the clinical research study on UConn Healthcare

483 workers and M.K. recruited participants and executed clinical protocols. R.G. and O.A designed  
484 the clinical research study at SUNY State Medical Center, Z.B., M.B, P.C and K.L, recruited,  
485 processed, coordinated and executed the clinical protocols. M.A., E.K., N.M. and C.H, designed,  
486 recruited and stored convalescent plasma samples at Cedars Sinai hospital. M.D., L.K, C.L.G.  
487 and D.U. analyzed the data, performed statistics, drew illustrations and prepared the figures. M.D.  
488 and D.U. wrote the manuscript.

489

490

## 491 **Figure Legends**

492

### 493 **Figure 1: SARS-CoV-2 specific antibody detection assay.**

494 **a.** Illustration of antibody detection assay. Biotinylated S-RBD or Nucleocapsid proteins are  
495 captured by streptavidin coated beads, then incubated with plasma samples and stained with PE  
496 conjugated anti-IgG, IgA, IgM, IgG1, IgG2, IgG3, IgG4 antibodies. Fluorescence intensity  
497 analyzed by flow cytometry. **b.** Histogram overlays demonstrating the detection of anti-S-RBD  
498 human IgG antibody (left) and soluble ACE2-Fc (right) as positive controls for plasma antibody  
499 assay. **c.** Representative patient plasma titration. Healthy control plasma at 1:100 dilution was  
500 used as a negative control. Serial dilutions were used in the flow cytometry overlay. **d.**

501 Comparison of IgG antibody levels captured by S-RBD, S1 Subunit of Spike, S1 N Terminal  
502 Domain (NTD), S2 Extracellular Domain (ECD) and Nucleocapsid Protein coated beads. **e.**

503 Correlation and comparison of bead-based assay S-RBD IgG antibody levels with ELISA-based  
504 assay. Two-tailed Mann-Whitney U test was used to determine the statistical significance in figure  
505 **d** and two-tailed Spearman's was used for correlation significance in figure **e**. Horizontal bars in  
506 **d** and **e** indicate mean values.

507

508 **Figure 2: SARS-CoV-2 specific antibody detection in COVID-19 and convalescent plasma  
509 samples.**

510 **a.** Measurement of Spike protein and Nucleocapsid protein specific IgG and Spike protein specific  
511 IgM and IgA antibodies as described in Figure 1. Area under the curve (AUC) values of plasma  
512 antibodies were calculated from reciprocal dilution curves in antibody detection assay. Dotted  
513 lines indicate the negative threshold calculated by adding 1 standard deviation to the mean AUC  
514 values of healthy controls' plasma. Horizontal bars indicate the mean. **b.** S-RBD specific IgG  
515 subclass AUC levels. **c.** S-RBD IgG AUC values of subject plasma grouped by outpatient,  
516 hospitalized, ICU or deceased and plasma donors. **d.** Nucleocapsid protein IgG AUC values of  
517 subject plasma grouped by outpatient, hospitalized, ICU or deceased and convalescent plasma  
518 donors. **e.** S-RBD IgA AUC values of subject plasma grouped by outpatient, hospitalized, ICU or  
519 deceased and plasma donors. **f.** S-RBD IgG AUC values of severity groups and plasma donors  
520 subdivided into males and females. Statistical significances were determined using two-tailed  
521 Mann-Whitney U test.

522

523 **Figure 3: Development of SARS-CoV-2 and SARS-CoV Spike protein pseudotyped  
524 lentiviruses.**

525 **a.** Schematic illustration of Spike protein expression on the cell surface and soluble ACE2-Fc  
526 staining followed by an anti-Fc antibody staining. **b.** 293 cells transfected with Spike protein with  
527 or without endoplasmic reticulum retention signal (ERRS) or with VSV-G as a negative control.  
528 The cells were stained with ACE2-Fc and anti-Fc-APC secondary antibody, flow cytometry data  
529 overlays are shown. **c.** Schematic representation of Spike protein pseudovirus generation and  
530 subsequent infection of ACE2-expressing host cells. A lentivector plasmid and a Spike protein  
531 over-expressing envelope plasmid are used to co-transfect 293 cells to generate Spike  
532 pseudovirus that in turn infect engineered cells over-expressing wild type ACE2 or ACE2-mKO2

533 fusion. **d.** Infection of wild type 293 cells with either bald lentiviruses generated without envelope  
534 plasmid or Spike protein pseudovirus. **e.** Infection of 293-ACE2 cells with bald and Spike  
535 lentiviruses. GFP and mKO2 markers are used to determine ACE2 over-expressing cells in ACE2-  
536 IRES-GFP and ACE2-mKO2, respectively. **f.** The titrations of SARS-CoV-2 and SARS-CoV Spike  
537 protein pseudoviruses encoding RFP. ACE2-IRES-GFP expressing 293 cells were incubated with  
538 3-fold serial dilutions of virus supernatant, stored for several hours at 4°C or serially frozen and  
539 thawed for 1, 2 and 3 cycles, and analyzed for RFP expression by flow cytometry on day 3 post-  
540 infection. Percent infection is % RFP+ cells after gating on GFP+ cells (i.e. ACE2+). Titration  
541 experiments were replicated twice except for the “1 freeze/thaw cycle” for which titrations were  
542 replicated 4 times. Error bars represent 1 standard deviation of mean values.

543

544 **Figure 4: Neutralization of SARS-CoV-2 and SARS-CoV pseudoviruses with soluble ACE2**  
545 **and NAbs**

546 **a.** Illustration of Spike protein pseudovirus blocked by soluble ACE2 or neutralizing antibodies. **B.**  
547 SARS-CoV-2 and SARS-CoV pseudovirus neutralization with soluble ACE2. SARS-CoV-2 RFP  
548 and SARS-CoV GFP pseudoviruses were pre-incubated with soluble ACE2 for 1 hour and added  
549 to 293 cells expressing ACE2-IRES-GFP or ACE2-mKO2 fusion, respectively. **c.** Neutralization  
550 of SARS-CoV-2 and SARS-CoV with S-RBD specific antibodies and soluble ACE2 (sACE2).  
551 Viruses were pre-incubated with antibodies (NAb#1 and SARS-CoV-2 S-RBD non-Nab) or soluble  
552 ACE2 (sACE2) proteins for 1 hour at the concentrations shown and subsequently added to target  
553 cells. Expression of RFP was determined at day 3 post-infection. Infection percentages were  
554 normalized to negative controls which are the infection conditions with no blocking agent. **d.**  
555 Neutralization of SARS-CoV-2 pseudoviruses using 4 different S-RBD NAbs and 2 different  
556 soluble ACE2 proteins. NAb #1 and #4 were human antibodies whereas NAb #2 and #3 were

557 mouse. Graphs in figures **c** and **d** represent 3 replicates of the experiments. Error bars indicate  
558 one standard deviation of mean values.  
559

560 **Figure 5: Neutralizing titers for SARS-CoV-2 and SARS-CoV in COVID-19 subject plasma**

561 **a.** Neutralization assay with S-RBD specific NAb, healthy control plasma, and a COVID-19 patient  
562 plasma. 3-fold serial dilutions of NAb from 10 µg/ml to 1 ng/ml or the plasma from 1:10 to 1:10,000  
563 were pre-incubated with Spike protein pseudovirus and added to 293-ACE2 cells. GFP  
564 expression was analyzed by flow cytometry 3 days post infection. **b.** SARS-CoV-2 neutralization  
565 titers (NT50) of COVID-19 plasma grouped as outpatient, hospitalized, ICU or deceased and  
566 convalescent plasma donor groups. **c.** NT50 of COVID-19 patient and plasma donor groups  
567 subdivided into males and females. **d.** Comparison of NT50 of COVID-19 plasma for SARS-CoV-  
568 2 and SARS-CoV neutralization. SARS-CoV-2 or SARS-CoV pseudoviruses were pre-incubated  
569 with COVID-19 plasma from all severity groups (n=104), 293-ACE2 cells were infected and RFP  
570 expression was determined at day 3 using flow cytometry. **e.** Graph of SARS-CoV-2 NT50 values  
571 from hospitalized subjects plotted against SARS-CoV. Two-tailed Mann-Whitney U test was used  
572 to determine the statistical significances in figures **b**, **c** and **d** and two-tailed Spearman's was  
573 used for figure **e**. Horizontal bars in **b**, **c** and **d** indicate mean values.

574

575 **Figure 6: Correlations of antibody, neutralization levels and COVID-19 subject  
576 characteristics**

577 **a.** Neutralization (NT50) of COVID-19 plasma correlated with S-RBD IgG, S-RBD IgA, S-RBD  
578 IgM and Nucleocapsid IgG. **b.** Correlation of NT50 with S-RBD specific IgG subclasses; IgG1,  
579 IgG2, IgG3 and IgG4. **c.** Correlation of S-RBD IgG, Nucleocapsid IgG, S-RBD IgA and NT50 with  
580 Age. Two-tailed Spearman's was used to determine statistical significances.

581

582

583 **Supplementary Figure legends**

584

585 **Supplementary Figure 1: SARS-CoV-2 S-RBD antibody detection**

586 **a.** Antibody assay measuring the plasma reactivity to S-RBD. Flow cytometry analysis of the PE  
587 fluorescence conjugated to anti-human IgG recognizing antibodies, present in the patient plasma  
588 and bound to S-RBD protein on the beads. Means of PE values in reciprocal dilutions were used  
589 to generate a curve for each positive plasma. Subject plasma with high and low antibody levels  
590 and a healthy control plasma were color-coded. **b.** Correlation of S1 subunit IgG, S1 N Terminal  
591 Domain (NTD) IgG, S2 Extracellular Domain (ECD) IgG and Nucleocapsid IgG with S-RBD IgG.  
592 **c.** Correlation of S-RBD IgA with S-RBD IgG. Two-tailed Spearman's was used to determine  
593 statistical significances. **d.** Heat map represents AUC values of Nucleocapsid (N) protein IgG, S-  
594 RBD IgG, S-RBD IgG subclasses and S-RBD IgA antibodies from individual subjects clustered  
595 as outpatients, hospitalized and ICU or deceased.

596

597 **Supplementary Figure 2: ACE2 detection on cell surface membrane**

598 **a.** Wild type or ACE2-IRES-GFP over-expressing 293 cells were stained with SARS-CoV-2 S1  
599 protein fused to mouse Fc, and anti-mouse Fc secondary antibody. **B.** ACE2 expression, detected  
600 as in **a**, in wild type and ACE2 overexpressing 293 cells compared in an overlay of flow data.

601

602 **Supplementary Figure 3: Neutralization of SARS-CoV-2 and SARS-CoV  
603 pseudoviruses**

604 **a.** Normalized percent infection levels of SARS-CoV-2 and SARS-CoV pseudoviruses in  
605 neutralization assay using soluble ACE2 at 1 µg/mL, 3 µg/mL and 10 µg/mL concentrations.  
606 Significance was determined using two-tailed Mann-Whitney U test.

607

608

609 **Supplementary Figure 4: S-RBD IgG subclasses correlation with total S-RBD IgG**

610 **a.** Correlation of AUC levels of S-RBD specific IgG subclasses (IgG 1-4) with S-RBD specific total

611 IgG. **b.** Correlation graphs of S-RBD IgG and NT50 with the number of days between PCR

612 confirmation and the blood draw. Two-tailed Spearman's was used to determine the statistical

613 significance.

614

615 **Supplementary Figure 5: SARS-CoV-2 NT50 correlation with SARS-CoV NT50**

616 **a.** Correlation of SARS-CoV-2 NT50 with SARS-CoV when all severity groups were combined, or

617 only outpatient and plasma donor subjects were combined. **b.** SARS-CoV neutralization titers

618 (NT50) of COVID-19 plasma grouped as outpatient, hospitalized, ICU or deceased and

619 convalescent plasma donor groups. Two-tailed Spearman's was used for statistical analysis.

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629 **References**

630

- 631 1 Hoffmann, M. *et al.* SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is  
632 Blocked by a Clinically Proven Protease Inhibitor. *Cell* **181**, 271-280 e278,  
633 doi:10.1016/j.cell.2020.02.052 (2020).
- 634 2 Robbiani, D. F. *et al.* Convergent antibody responses to SARS-CoV-2 in convalescent  
635 individuals. *Nature*, doi:10.1038/s41586-020-2456-9 (2020).
- 636 3 Rogers, T. F. *et al.* Isolation of potent SARS-CoV-2 neutralizing antibodies and  
637 protection from disease in a small animal model. *Science*, doi:10.1126/science.abc7520  
638 (2020).
- 639 4 Wu, Y. *et al.* Identification of Human Single-Domain Antibodies against SARS-CoV-2.  
640 *Cell Host Microbe* **27**, 891-898 e895, doi:10.1016/j.chom.2020.04.023 (2020).
- 641 5 Wang, C. *et al.* A human monoclonal antibody blocking SARS-CoV-2 infection. *Nat  
642 Commun* **11**, 2251, doi:10.1038/s41467-020-16256-y (2020).
- 643 6 Seydoux, E. *et al.* Analysis of a SARS-CoV-2-Infected Individual Reveals Development  
644 of Potent Neutralizing Antibodies with Limited Somatic Mutation. *Immunity*,  
645 doi:10.1016/j.jimmuni.2020.06.001 (2020).
- 646 7 Cao, Y. *et al.* Potent neutralizing antibodies against SARS-CoV-2 identified by high-  
647 throughput single-cell sequencing of convalescent patients' B cells. *Cell*,  
648 doi:10.1016/j.cell.2020.05.025 (2020).
- 649 8 Hassan, A. O. *et al.* A SARS-CoV-2 Infection Model in Mice Demonstrates Protection by  
650 Neutralizing Antibodies. *Cell*, doi:10.1016/j.cell.2020.06.011 (2020).
- 651 9 Zost, S. J. *et al.* Potently neutralizing and protective human antibodies against SARS-  
652 CoV-2. *Nature* **584**, 443-449, doi:10.1038/s41586-020-2548-6 (2020).

- 653 10 Wu, Y. *et al.* A noncompeting pair of human neutralizing antibodies block COVID-19  
654 virus binding to its receptor ACE2. *Science* **368**, 1274-1278,  
655 doi:10.1126/science.abc2241 (2020).
- 656 11 Liu, L. *et al.* Potent neutralizing antibodies against multiple epitopes on SARS-CoV-2  
657 spike. *Nature* **584**, 450-456, doi:10.1038/s41586-020-2571-7 (2020).
- 658 12 Duan, K. *et al.* Effectiveness of convalescent plasma therapy in severe COVID-19  
659 patients. *Proc Natl Acad Sci U S A* **117**, 9490-9496, doi:10.1073/pnas.2004168117  
660 (2020).
- 661 13 Barone, P. & DeSimone, R. A. Convalescent plasma to treat coronavirus disease 2019  
662 (COVID-19): considerations for clinical trial design. *Transfusion* **60**, 1123-1127,  
663 doi:10.1111/trf.15843 (2020).
- 664 14 Bloch, E. M. *et al.* Deployment of convalescent plasma for the prevention and treatment  
665 of COVID-19. *J Clin Invest* **130**, 2757-2765, doi:10.1172/JCI138745 (2020).
- 666 15 Chen, B. & Xia, R. Early experience with convalescent plasma as immunotherapy for  
667 COVID-19 in China: Knowns and unknowns. *Vox Sang*, doi:10.1111/vox.12968 (2020).
- 668 16 Lisboa Bastos, M. *et al.* Diagnostic accuracy of serological tests for covid-19: systematic  
669 review and meta-analysis. *Bmj*, doi:10.1136/bmj.m2516 (2020).
- 670 17 Huang, A. T. *et al.* A systematic review of antibody mediated immunity to coronaviruses:  
671 antibody kinetics, correlates of protection, and association of antibody responses with  
672 severity of disease. *medRxiv*, doi:10.1101/2020.04.14.20065771 (2020).
- 673 18 Hicks, J. *et al.* Serologic cross-reactivity of SARS-CoV-2 with endemic and seasonal  
674 Betacoronaviruses. *medRxiv*, doi:10.1101/2020.06.22.20137695 (2020).
- 675 19 Nagappa, B. & Marimuthu, Y. Seroconversion rate and diagnostic accuracy of  
676 serological tests for COVID-19. *Clin Infect Dis*, doi:10.1093/cid/ciaa676 (2020).

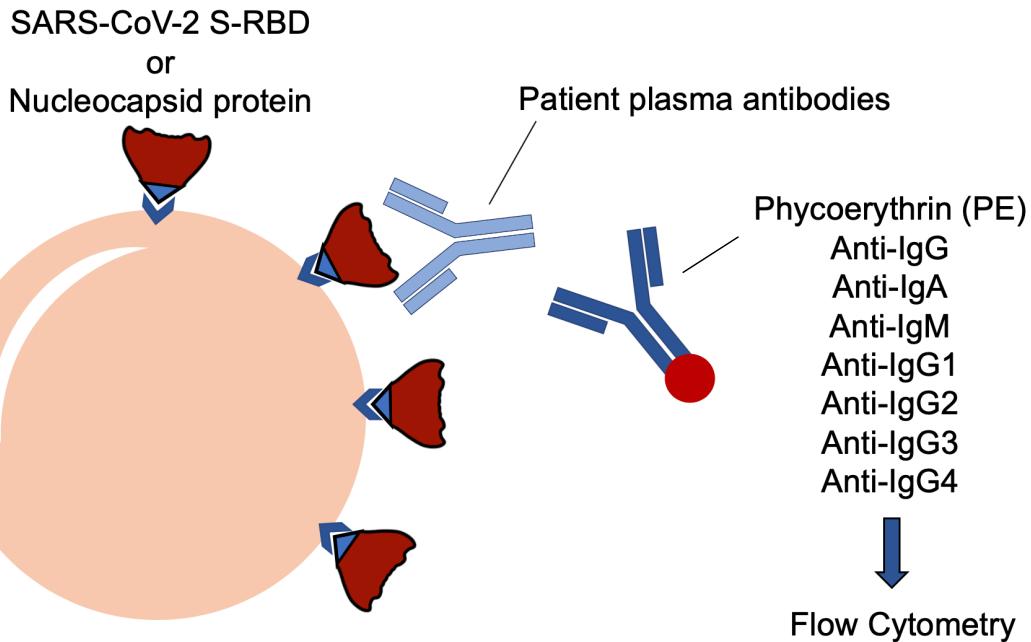
- 677 20 Che, X. Y. et al. Antigenic cross-reactivity between severe acute respiratory syndrome-  
678 associated coronavirus and human coronaviruses 229E and OC43. *J Infect Dis* **191**,  
679 2033-2037, doi:10.1086/430355 (2005).
- 680 21 St John, A. L. & Rathore, A. P. S. Early Insights into Immune Responses during COVID-  
681 19. *J Immunol*, doi:10.4049/jimmunol.2000526 (2020).
- 682 22 Yazdanpanah, F., Hamblin, M. R. & Rezaei, N. The immune system and COVID-19:  
683 Friend or foe? *Life Sci* **256**, 117900, doi:10.1016/j.lfs.2020.117900 (2020).
- 684 23 Tong, P. B., Lin, L. Y. & Tran, T. H. Coronaviruses pandemics: Can neutralizing  
685 antibodies help? *Life Sci* **255**, 117836, doi:10.1016/j.lfs.2020.117836 (2020).
- 686 24 Deeks, J. J. et al. Antibody tests for identification of current and past infection with  
687 SARS-CoV-2. *Cochrane Database Syst Rev* **6**, CD013652,  
688 doi:10.1002/14651858.CD013652 (2020).
- 689 25 Siracusano, G., Pastori, C. & Lopalco, L. Humoral Immune Responses in COVID-19  
690 Patients: A Window on the State of the Art. *Front Immunol* **11**, 1049,  
691 doi:10.3389/fimmu.2020.01049 (2020).
- 692 26 Ma, H. et al. Serum IgA, IgM, and IgG responses in COVID-19. *Cell Mol Immunol*,  
693 doi:10.1038/s41423-020-0474-z (2020).
- 694 27 Zhao, J. et al. Antibody responses to SARS-CoV-2 in patients of novel coronavirus  
695 disease 2019. *Clin Infect Dis*, doi:10.1093/cid/ciaa344 (2020).
- 696 28 Wang, Y. et al. Kinetics of viral load and antibody response in relation to COVID-19  
697 severity. *J Clin Invest*, doi:10.1172/JCI138759 (2020).
- 698 29 Long, Q. X. et al. Clinical and immunological assessment of asymptomatic SARS-CoV-2  
699 infections. *Nat Med*, doi:10.1038/s41591-020-0965-6 (2020).
- 700 30 Long, Q. X. et al. Antibody responses to SARS-CoV-2 in patients with COVID-19. *Nat  
701 Med* **26**, 845-848, doi:10.1038/s41591-020-0897-1 (2020).

- 702 31 Wang, Q. *et al.* Structural and Functional Basis of SARS-CoV-2 Entry by Using Human  
703 ACE2. *Cell* **181**, 894-904 e899, doi:10.1016/j.cell.2020.03.045 (2020).
- 704 32 Walls, A. C. *et al.* Structure, Function, and Antigenicity of the SARS-CoV-2 Spike  
705 Glycoprotein. *Cell* **181**, 281-292 e286, doi:10.1016/j.cell.2020.02.058 (2020).
- 706 33 Ou, X. *et al.* Characterization of spike glycoprotein of SARS-CoV-2 on virus entry and its  
707 immune cross-reactivity with SARS-CoV. *Nat Commun* **11**, 1620, doi:10.1038/s41467-  
708 020-15562-9 (2020).
- 709 34 Lv, H. *et al.* Cross-reactive antibody response between SARS-CoV-2 and SARS-CoV  
710 infections. *bioRxiv*, doi:10.1101/2020.03.15.993097 (2020).
- 711 35 Pinto, D. *et al.* Cross-neutralization of SARS-CoV-2 by a human monoclonal SARS-CoV  
712 antibody. *Nature*, doi:10.1038/s41586-020-2349-y (2020).
- 713 36 Zost, S. J. *et al.* Rapid isolation and profiling of a diverse panel of human monoclonal  
714 antibodies targeting the SARS-CoV-2 spike protein. *Nat Med* **26**, 1422-1427,  
715 doi:10.1038/s41591-020-0998-x (2020).
- 716 37 Wec, A. Z. *et al.* Broad neutralization of SARS-related viruses by human monoclonal  
717 antibodies. *Science* **369**, 731-736, doi:10.1126/science.abc7424 (2020).
- 718 38 Schmidt, F. *et al.* Measuring SARS-CoV-2 neutralizing antibody activity using  
719 pseudotyped and chimeric viruses. *J Exp Med* **217**, doi:10.1084/jem.20201181 (2020).
- 720 39 Crawford, K. H. D. *et al.* Protocol and Reagents for Pseudotyping Lentiviral Particles with  
721 SARS-CoV-2 Spike Protein for Neutralization Assays. *Viruses* **12**,  
722 doi:10.3390/v12050513 (2020).
- 723 40 Nie, J. *et al.* Establishment and validation of a pseudovirus neutralization assay for  
724 SARS-CoV-2. *Emerg Microbes Infect* **9**, 680-686, doi:10.1080/22221751.2020.1743767  
725 (2020).

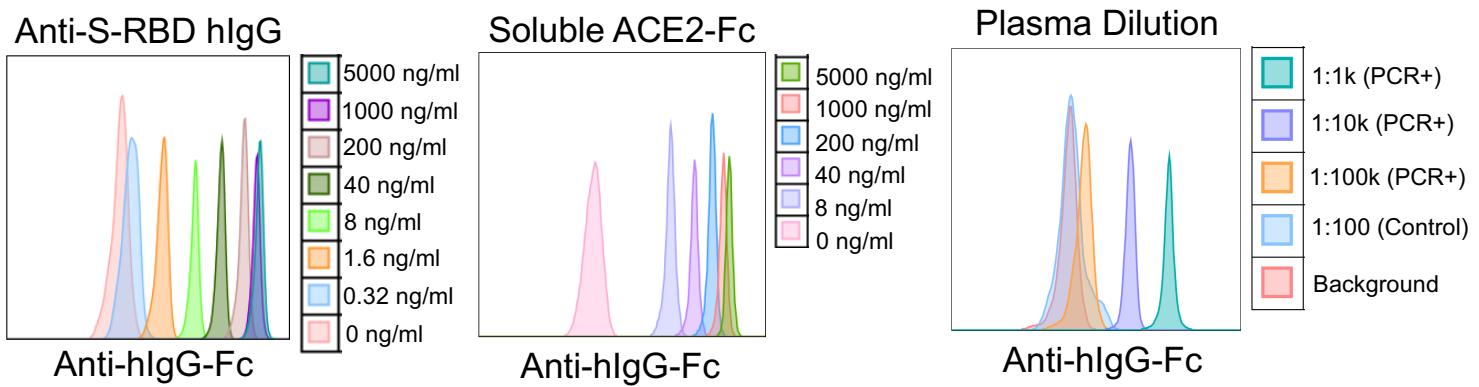
- 726 41 Padoan, A. et al. IgA-Ab response to spike glycoprotein of SARS-CoV-2 in patients with  
727 COVID-19: A longitudinal study. *Clin Chim Acta* **507**, 164-166,  
728 doi:10.1016/j.cca.2020.04.026 (2020).
- 729 42 Ejemel, M. et al. IgA MAb blocks SARS-CoV-2 Spike-ACE2 interaction providing  
730 mucosal immunity. *bioRxiv*, doi:10.1101/2020.05.15.096719 (2020).
- 731 43 Woodruff, M. C. et al. Extrafollicular B cell responses correlate with neutralizing  
732 antibodies and morbidity in COVID-19. *Nat Immunol*, doi:10.1038/s41590-020-00814-z  
733 (2020).
- 734 44 Ulrich, H., Pillat, M. M. & Tarnok, A. Dengue Fever, COVID-19 (SARS-CoV-2), and  
735 Antibody-Dependent Enhancement (ADE): A Perspective. *Cytometry A*,  
736 doi:10.1002/cyto.a.24047 (2020).
- 737 45 Roschewski, M. et al. Inhibition of Bruton tyrosine kinase in patients with severe COVID-  
738 19. *Sci Immunol* **5**, doi:10.1126/sciimmunol.abd0110 (2020).
- 739 46 Chen, X. et al. Functional Interrogation of Primary Human T Cells via CRISPR Genetic  
740 Editing. *J Immunol* **201**, 1586-1598, doi:10.4049/jimmunol.1701616 (2018).
- 741 47 Karasawa, S., Araki, T., Nagai, T., Mizuno, H. & Miyawaki, A. Cyan-emitting and orange-  
742 emitting fluorescent proteins as a donor/acceptor pair for fluorescence resonance energy  
743 transfer. *Biochem J* **381**, 307-312, doi:10.1042/BJ20040321 (2004).
- 744 48 Sakaue-Sawano, A. et al. Visualizing spatiotemporal dynamics of multicellular cell-cycle  
745 progression. *Cell* **132**, 487-498, doi:10.1016/j.cell.2007.12.033 (2008).
- 746 49 Ratner, B. The correlation coefficient: Its values range between +1/-1, or do they?  
747 *Journal of Targeting, Measurement and Analysis for Marketing* **17**, 139-142,  
748 doi:10.1057/jt.2009.5 (2009).
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# Figure 1

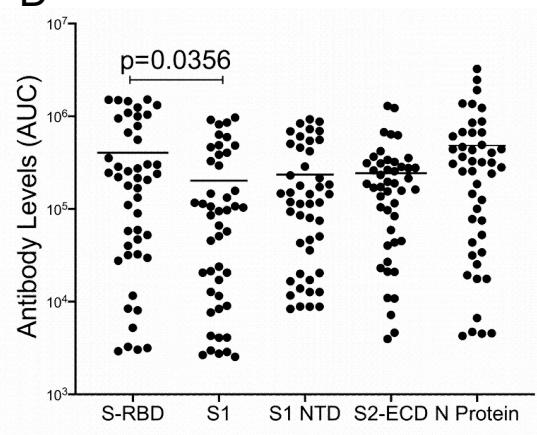
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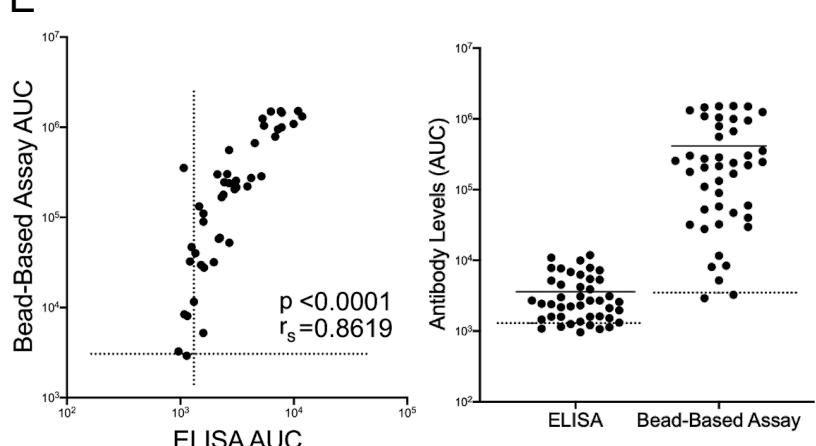
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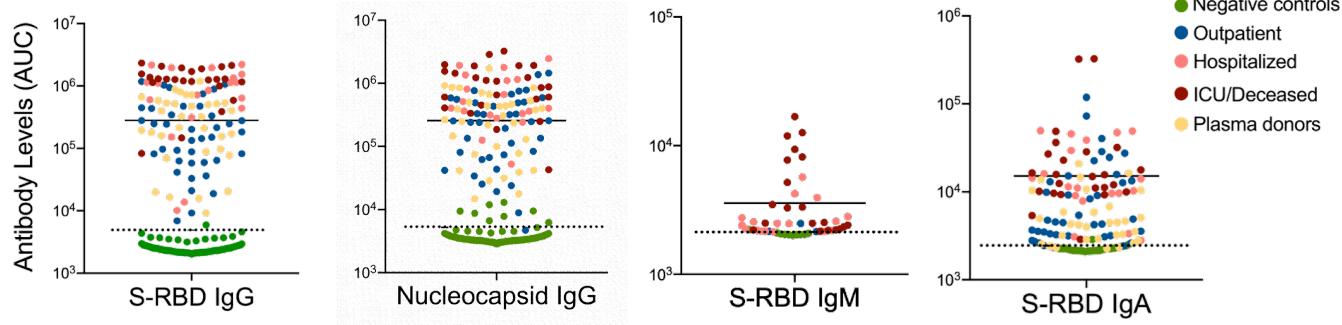


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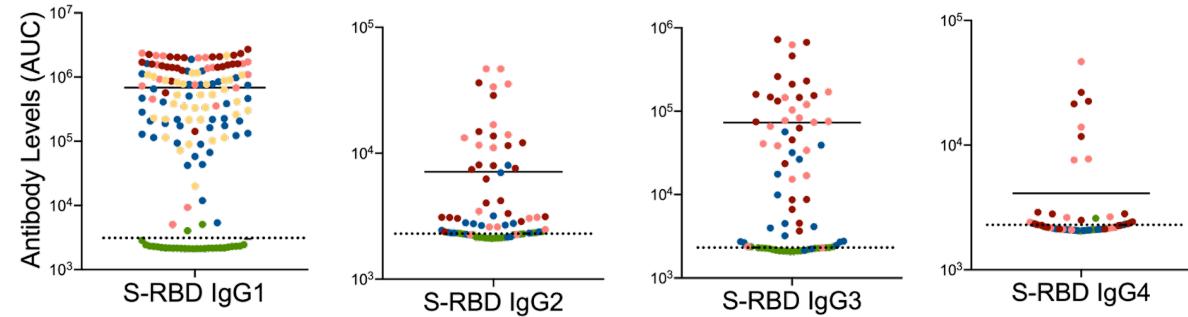


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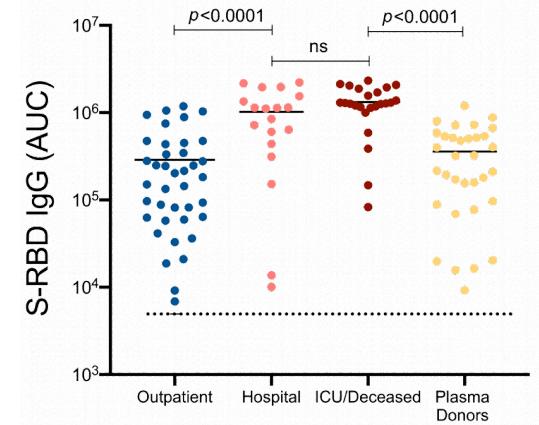
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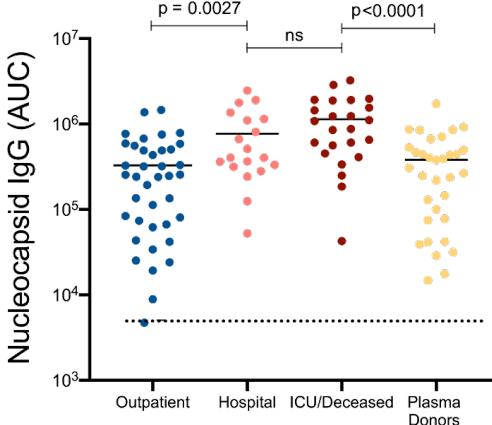
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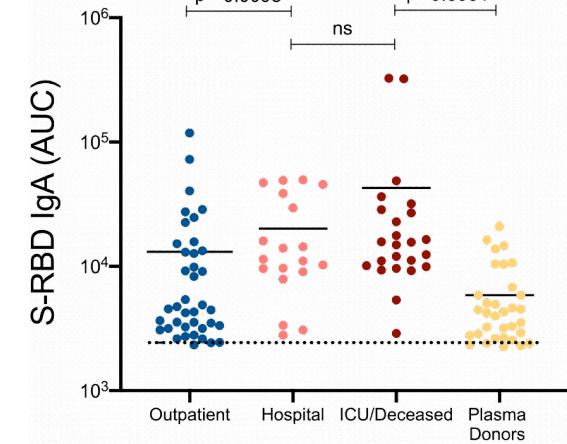
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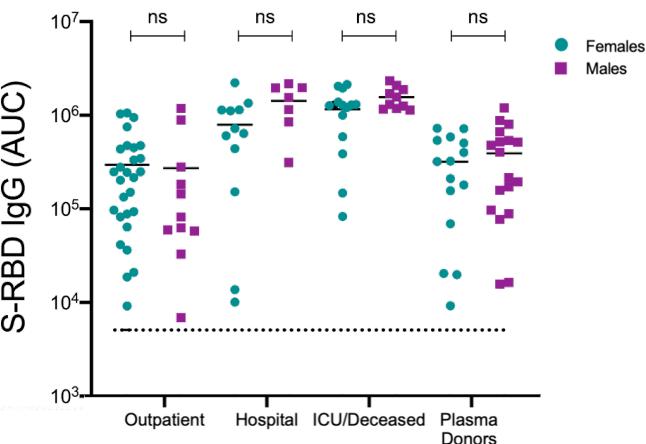
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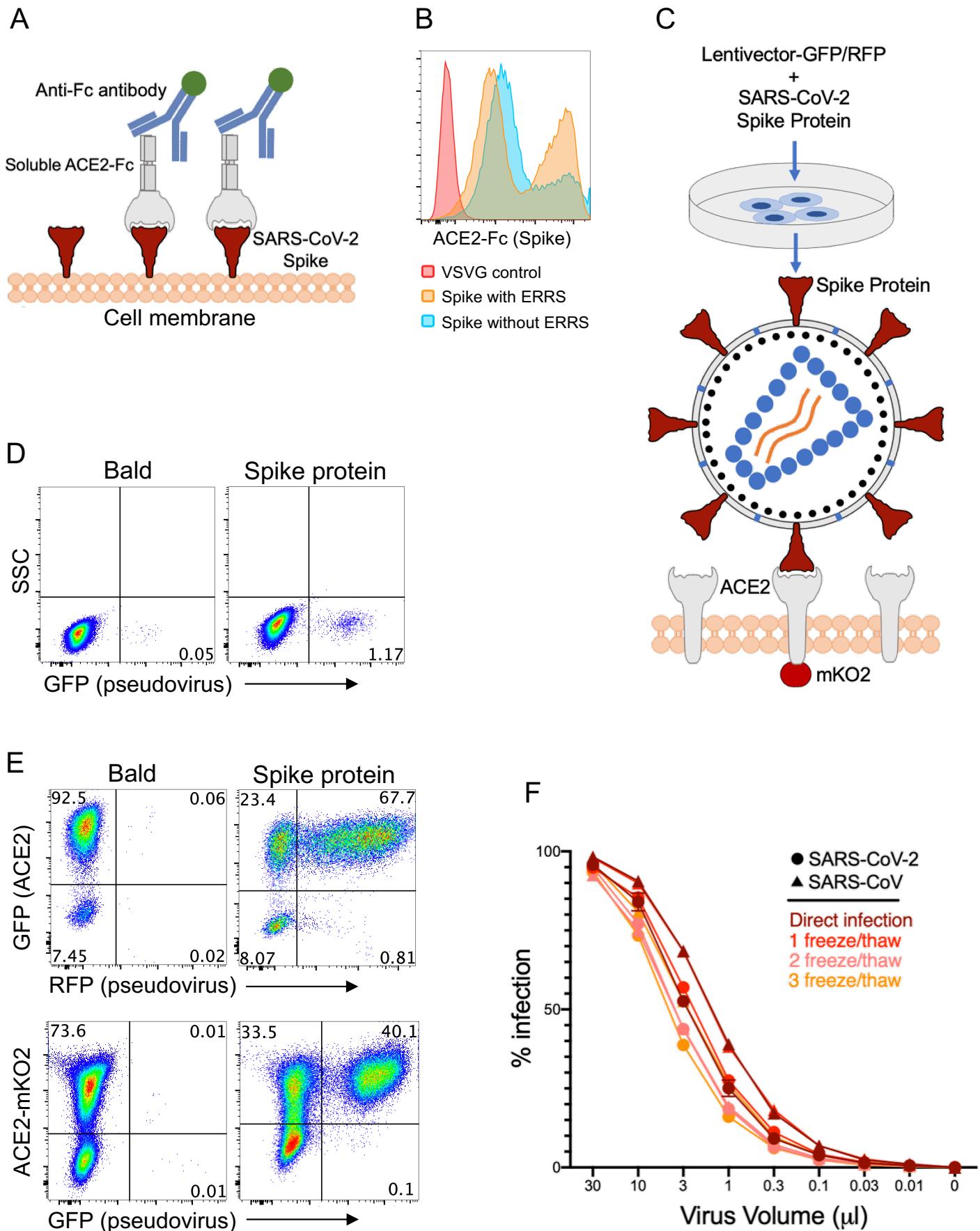
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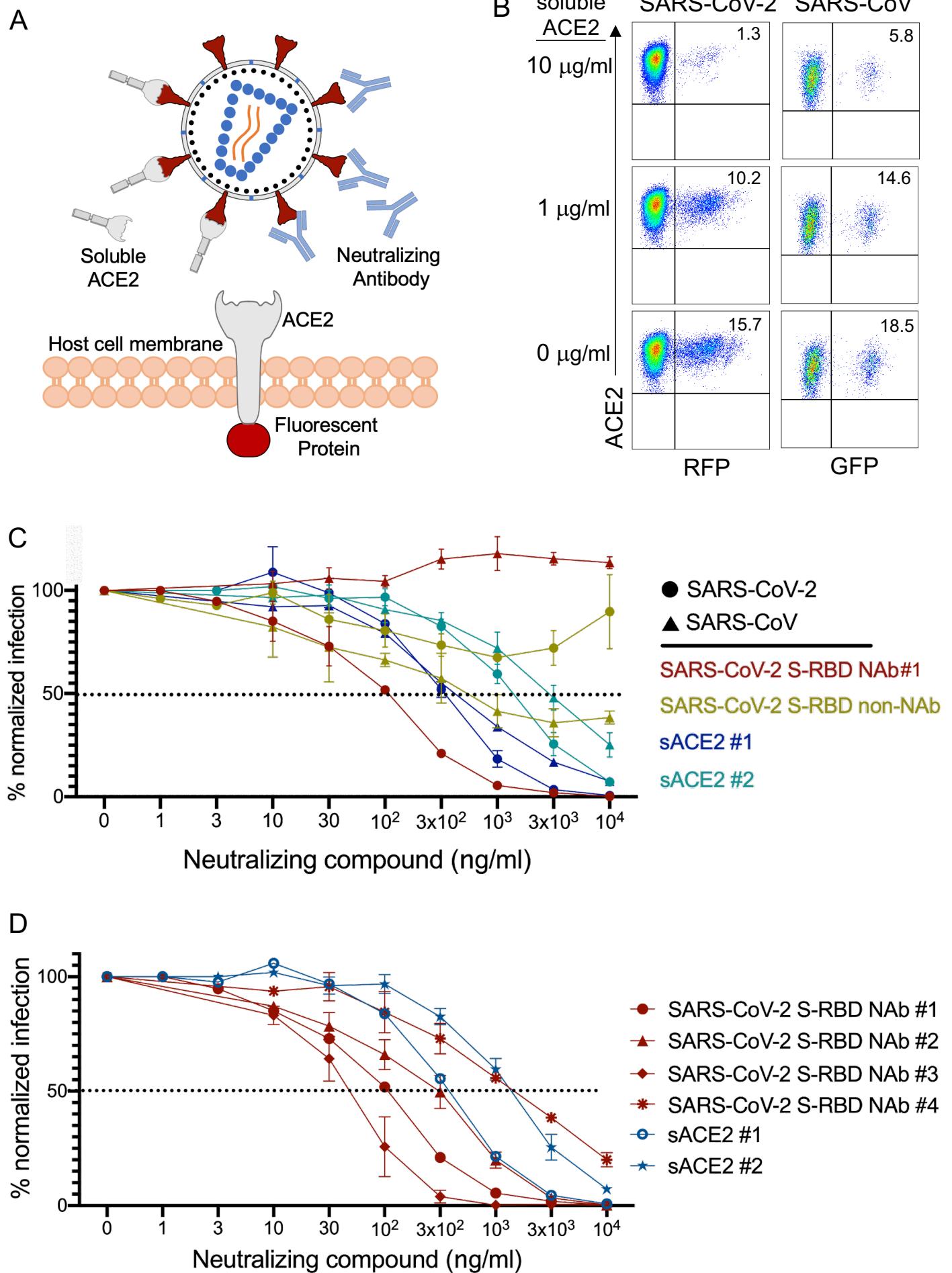
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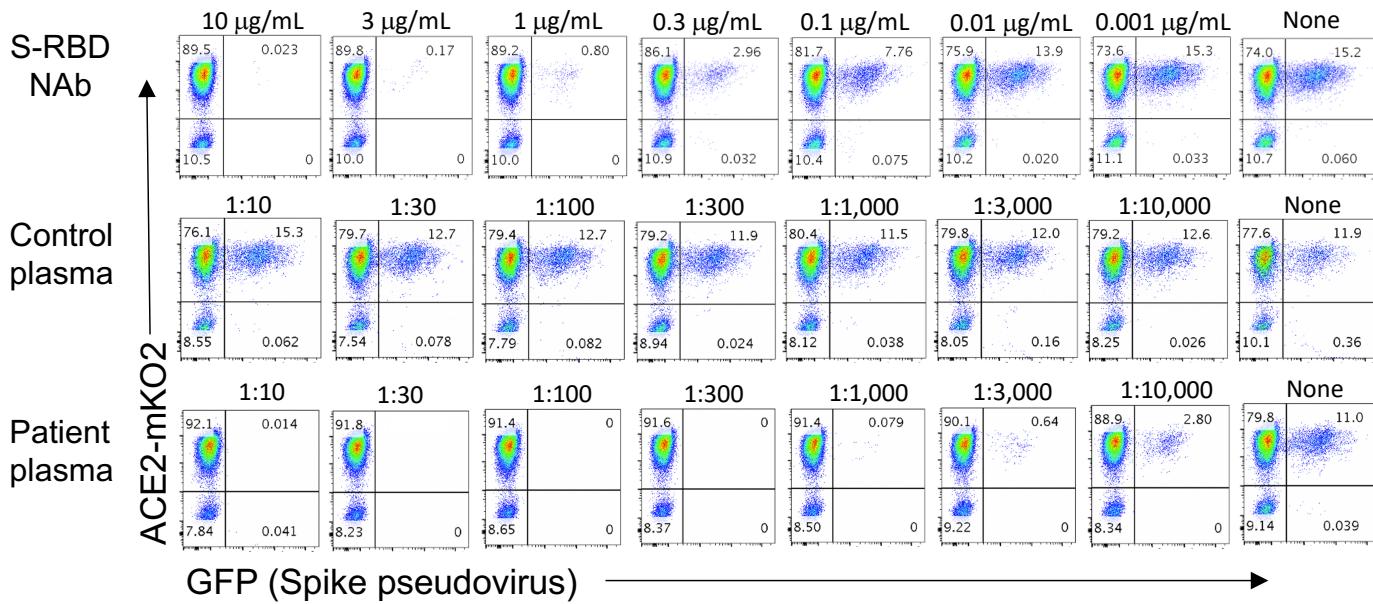


# Figure 4

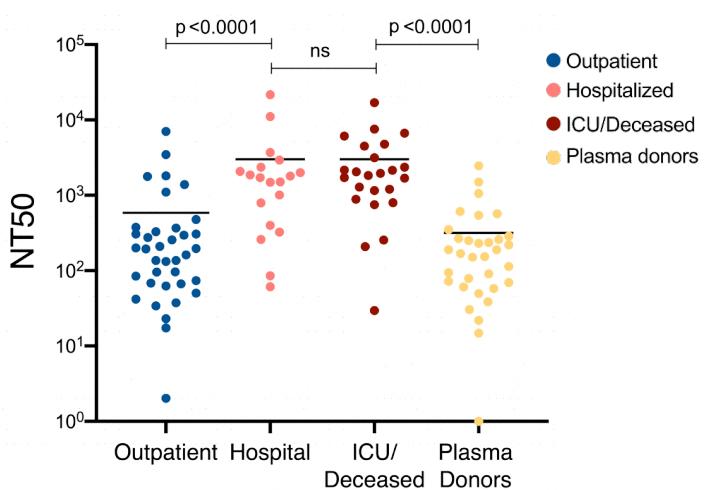


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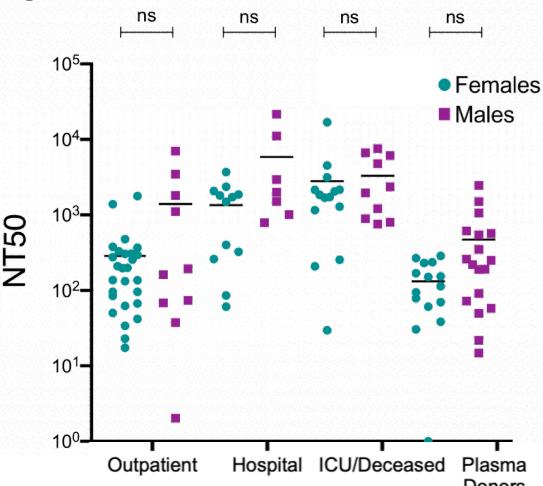
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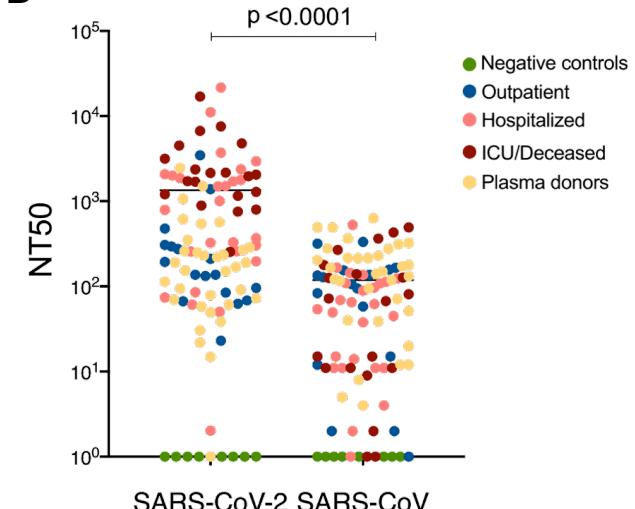
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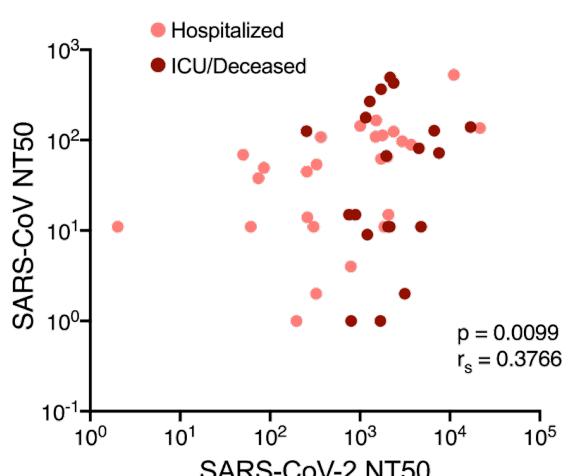
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D



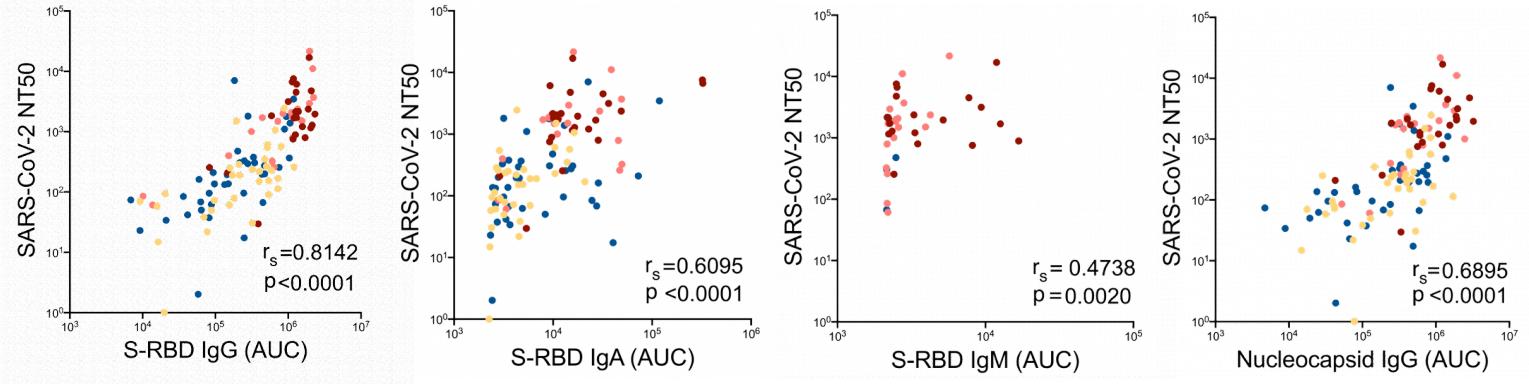
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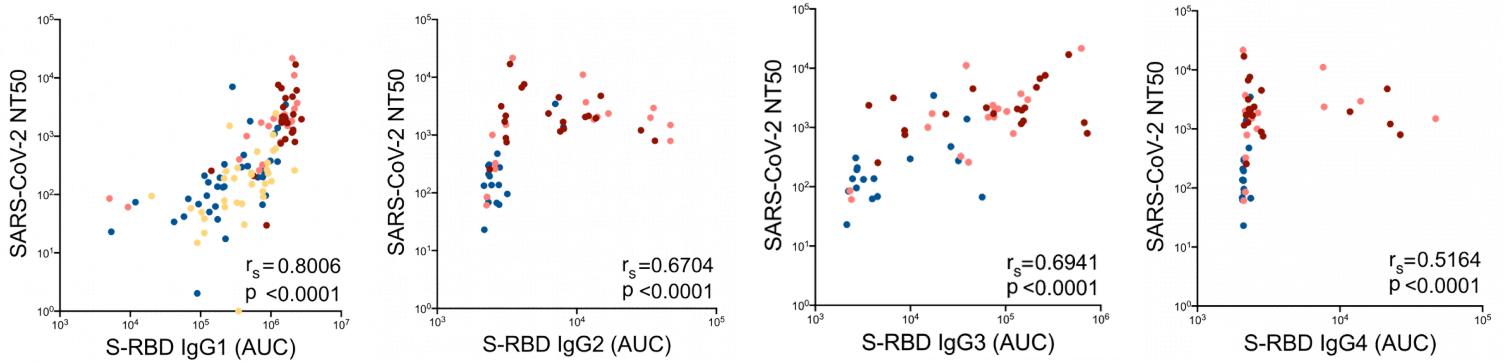
## Figure 6

A

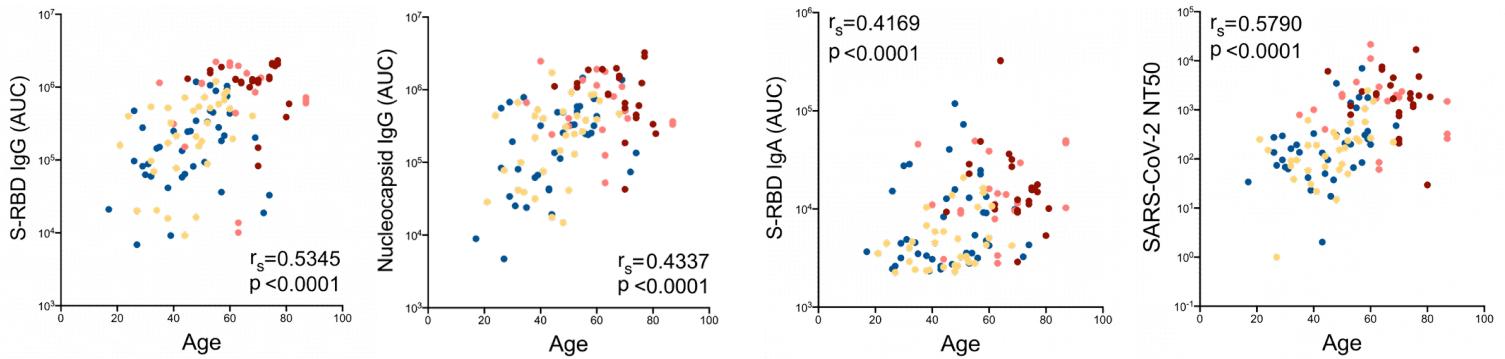
● Outpatient    ● Plasma Donors    ● Hospital    ● ICU/Deceased



B

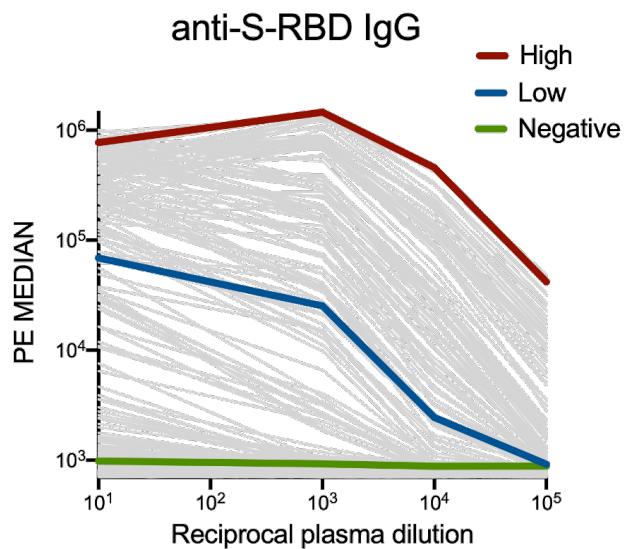


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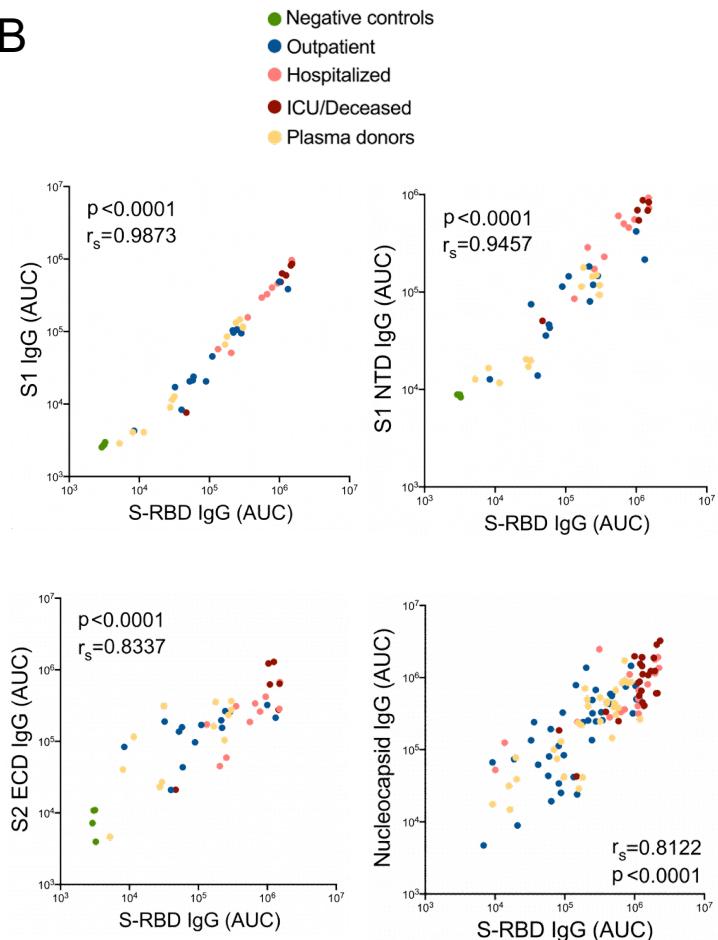


# Supplementary Figure 1

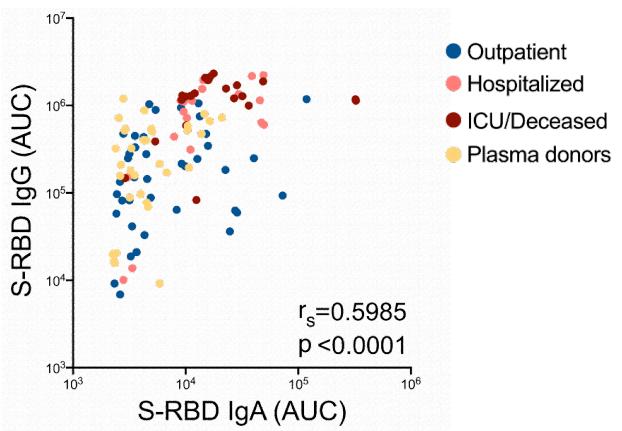
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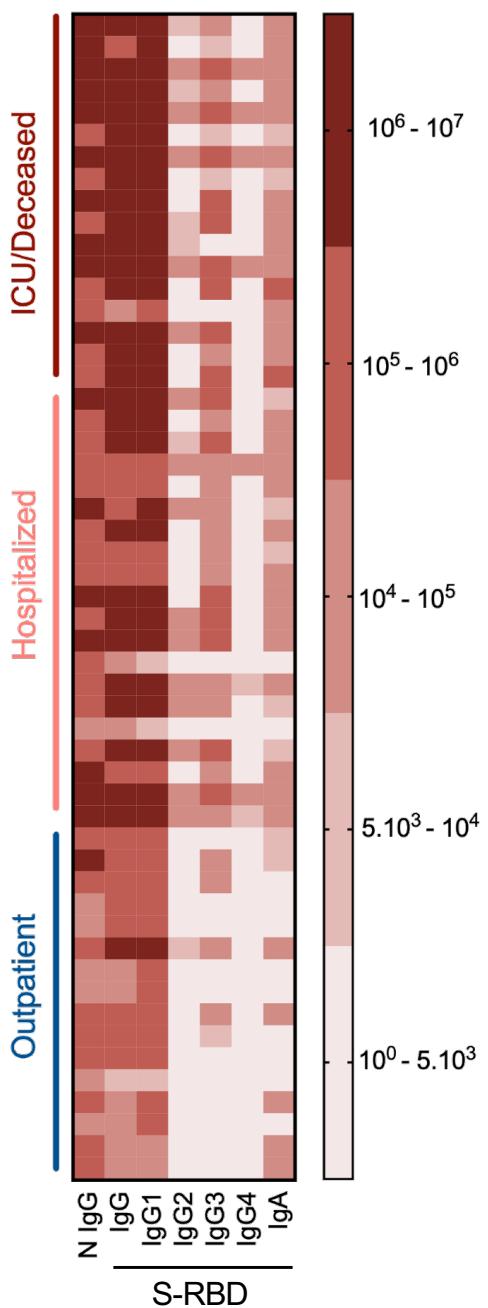
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C

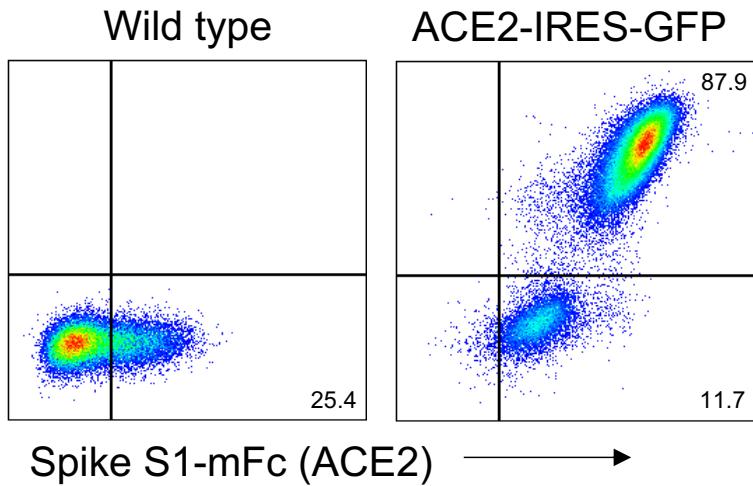


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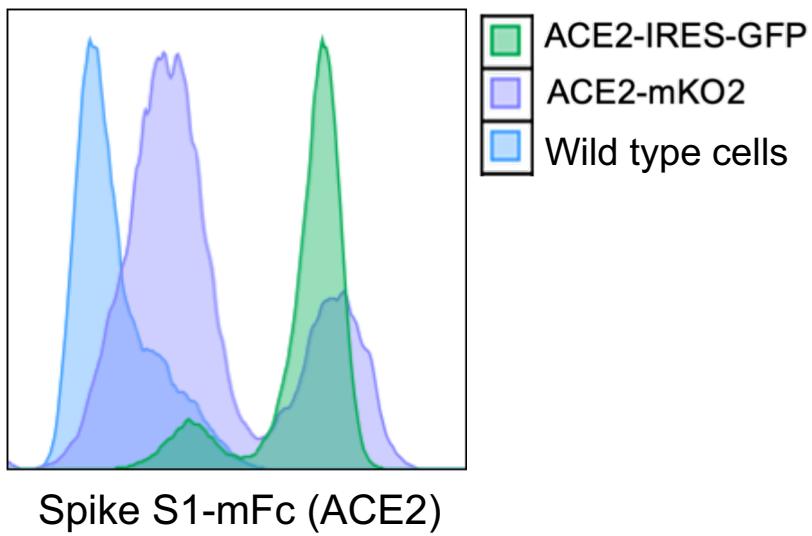


## Supplementary Figure 2

A

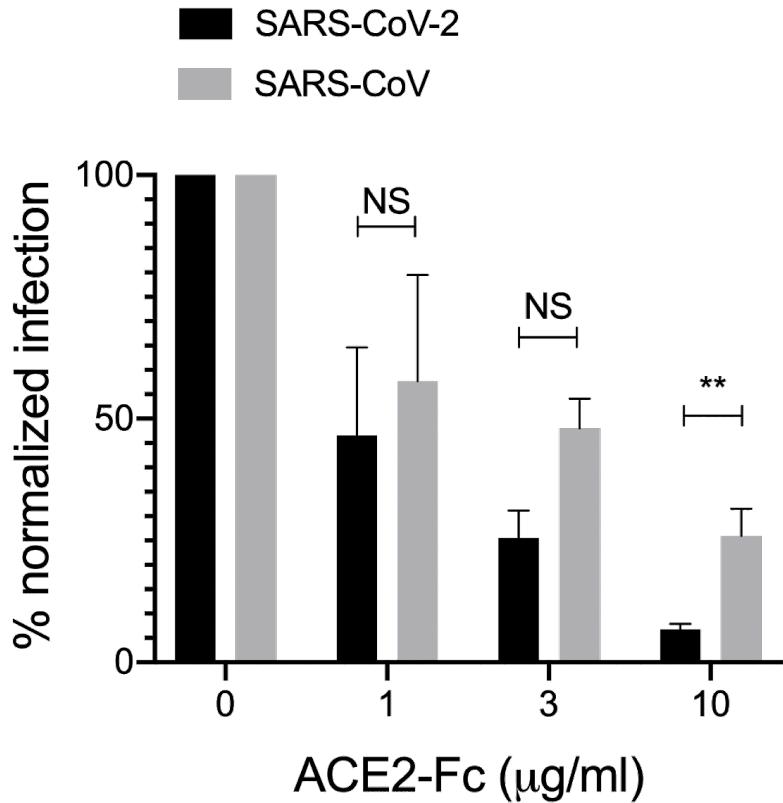


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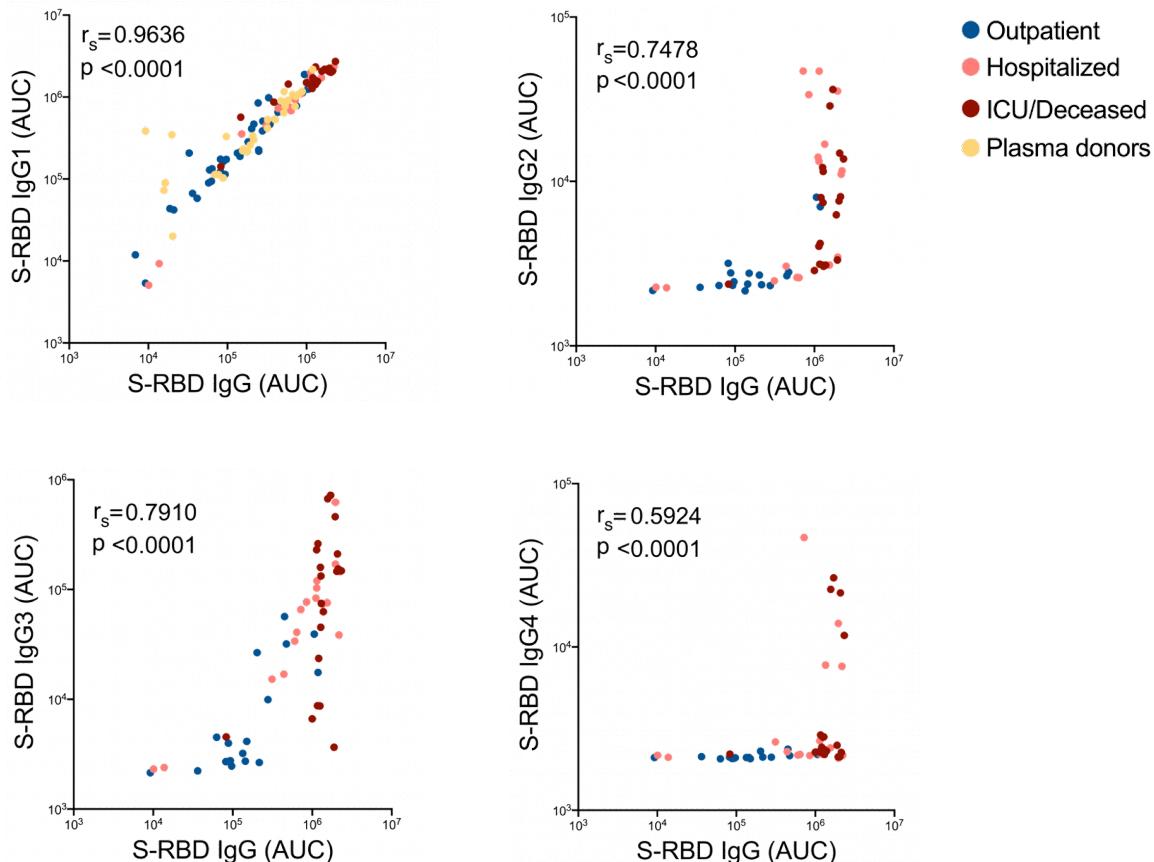
## Supplementary Figure 3

A

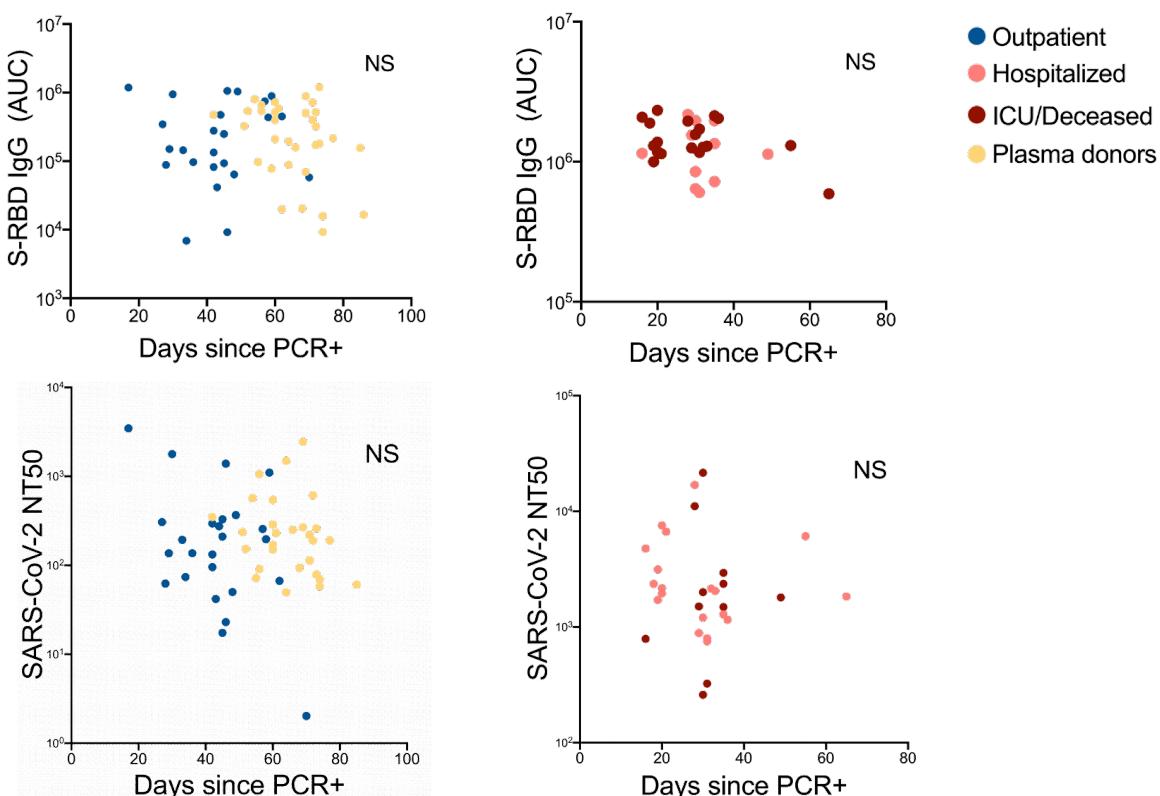


## Supplementary Figure 4

A

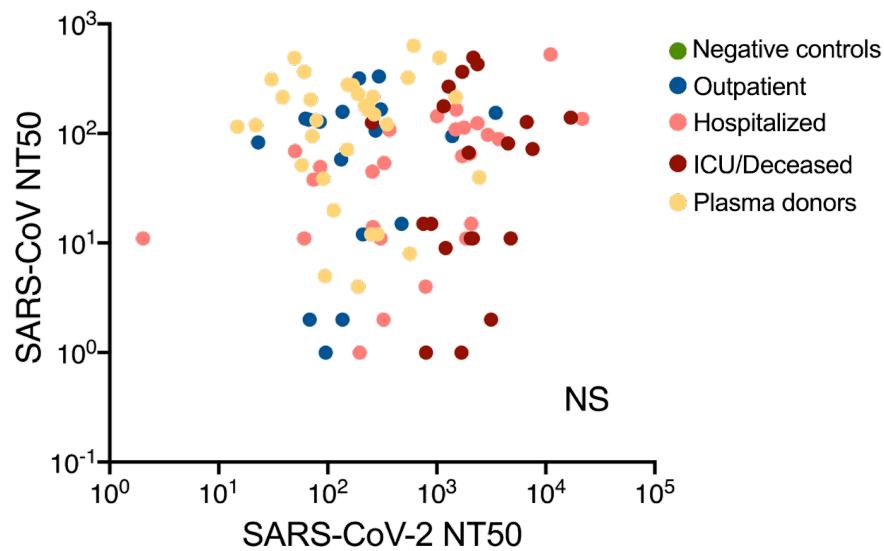
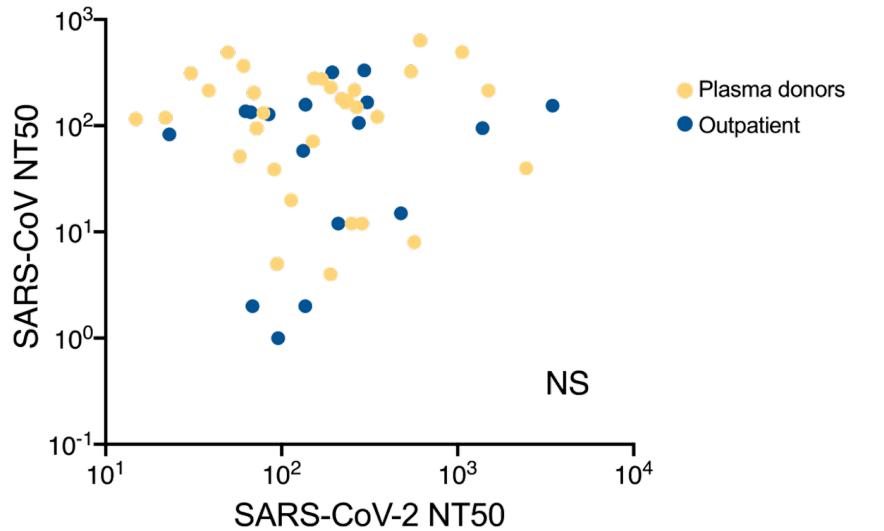


B



## Supplementary Figure 5

A



B

