Expansion of SARS-CoV-2-specific Antibody-secreting Cells and Generation of Neutralizing Antibodies in Hospitalized COVID-19 Patients

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Summary

COVID-19, caused by SARS-CoV-2, emerged in late 2019 and has since become a global pandemic. Pathogen-specific antibodies are typically a major predictor of protective immunity, yet B cell and antibody responses during COVID-19 are not fully understood. Here, we analyzed antibody-secreting cell (ASC) and antibody responses in twenty hospitalized COVID-19 patients. We observed a significant expansion of SARS-CoV-2 nucleocapsid protein-specific ASCs in all twenty COVID-19 patients using a multicolor FluoroSpot assay. Out of the 20 patients, 16 had developed SARS-CoV-2-neutralizing antibodies by the time of sampling. Additionally, we found that SARS-CoV-2-specific IgA, IgG and IgM antibody levels positively correlated with SARS-CoV-2-neutralizing antibody titers. This study constitutes a detailed description of B cell and antibody responses to SARS-CoV-2 in COVID-19, and provides tools to study immune responses to SARS-CoV-2 infection and vaccination.

Introduction

Characterizing immune responses to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of coronavirus disease 2019 (COVID-19), is an important step towards understanding correlates of protection (Zhu et al. 2020). In the majority of viral infections, pathogen-specific antibodies are one of the main contributors to protective immunity, yet B cell and antibody responses during COVID-19 are currently not fully understood. Early during acute infections, activated B cells differentiate into antibody-secreting cells (ASCs), including plasmablasts and plasma cells, which produce large quantities of pathogen-specific antibodies (Nutt et al. 2015). For example, during acute human dengue virus infection, ASCs expand to constitute an average of 47% of all circulating B cells (Wrammert et al. 2012). In the context of COVID-19, a recent report on

51 longitudinal immune responses in a single COVID-19 patient with mild disease showed a
52 detectable ASC expansion peaking at day 8 after symptom onset and representing 7% of all
53 circulating B cells (Thevarajan et al. 2020). Here, we investigated early B cell and antibody
54 responses to SARS-CoV-2 infection in a cohort of hospitalized COVID-19 patients.
55
56 **Results and Discussion**
57 Twenty COVID-19 patients were enrolled in this study during hospitalization at Karolinska
58 University Hospital, Sweden. All twenty patients exhibited typical COVID-19 symptoms of
59 fever, cough and breathing difficulties, while a few also experienced chest pain, myalgia and
60 diarrhea (Table 1). Seven patients also presented with co-morbidities such as hypertension,
61 asthma, cardiovascular disease and diabetes mellitus type II. Peripheral blood samples from
62 these patients were collected at median 15 days after the onset of COVID-19 symptoms. In
63 parallel, seven donors without an ongoing respiratory disease or signs of inflammation were
64 included in the study as healthy controls.
65
66 **Significant Increase of Antibody-secreting Cells in COVID-19 Patients**
67 To assess the presence of ASC expansion in COVID-19 patients, we analyzed freshly
68 isolated peripheral blood mononuclear cells (PBMCs) by flow cytometry. As expected, low
69 ASC frequencies and numbers in peripheral blood were observed in healthy controls (Figure
70 1A-C). However, a significant increase in ASC frequencies (defined here as $CD19^{+}CD20^{low/-}$
71 $IgD^{-}CD38^{high}CD27^{high}$) was observed in COVID-19 patients, constituting up to 31% of all B
72 cells in peripheral blood (Figure 1A-B). ASC numbers in blood were also increased in
73 COVID-19 patients, as compared to healthy controls (Figure 1C). Noteworthy, a substantial
74 ASC expansion was detected as early as 7 days, and as late as 19 days after the onset of
75 symptoms (Supplemental Figure 1A).

76

77 IgA^+ ASCs have previously been shown to be the dominant subset at steady state (Mei et al.
78 2009). In agreement with this, the majority of the ASCs in healthy controls were IgA^+,
79 whereas the IgG^+ ASC subset dominated in COVID-19 patients (Figure 1D). Moreover, IgA^+,
80 IgG^+ and IgM^+ ASC numbers in peripheral blood were significantly increased in COVID-19
81 patients, as compared to healthy controls (Figure 1E).

82

83 **Detection of SARS-CoV-2-specific Antibody-Secreting Cells in COVID-19 Patients**

84 The expansion of ASCs is usually characterized by high specificity towards the infectious
85 agent (Lee et al. 2011). To evaluate SARS-CoV-2-specific ASC response in COVID-19
86 patients, we developed a FluoroSpot assay allowing for the detection of total and SARS-
87 CoV-2 nucleocapsid (N) protein-specific IgA-, IgG-, and IgM-ASCs. The FluoroSpot assay
88 confirmed the ASC expansion detected by flow cytometry, as the total numbers of IgA-, IgG-
89 , and IgM-ASCs in COVID-19 patients were significantly higher than in healthy controls
90 (Figure 1F and H). Moreover, the total ASC frequencies and numbers detected by flow
91 cytometry positively correlated with total numbers of ASCs measured by FluoroSpot (r_s =
92 0.537, P = 0.01, and r_s =0.636, P = 0.003, respectively). Consistent with flow cytometry data,
93 IgG-ASCs constituted the predominant subset of all ASCs in patients, followed by IgA-ASCs
94 (Figure 1H).

95

96 Importantly, we detected SARS-CoV-2 N protein-specific ASCs in all twenty COVID-19
97 patients, but not in controls, suggesting an active SARS-CoV-2-specific B cell response in
98 acute COVID-19 (Figure 1G and I). Total ASC frequencies determined by flow cytometry
99 positively correlated with N protein-specific ASC numbers (r_s = 0.574, P = 0.008), suggesting

100 that the total ASC expansion detected by flow cytometry may reflect the magnitude of
101 SARS-CoV-2 N protein-specific ASC response.
102
103 **Characterization of SARS-CoV-2-specific Antibody Responses in COVID-19 Patients**
104 Seroconversion in COVID-19 patients, measured by detectable SARS-CoV-2-specific IgG
105 levels, has been recently shown to take place within nineteen days after the onset of
106 symptoms (Long et al. 2020). The expansion of SARS-CoV-2-specific ASCs in all of the
107 twenty COVID-19 patients in our cohort, but in none of the healthy controls, suggested that
108 the patients had developed SARS-CoV-2-specific antibodies in response to the infection. To
109 investigate this in detail, we next analyzed SARS-CoV-2-specific antibody responses.
110
111 First, we measured SARS-CoV-2 spike S1-specific IgA and IgG, as well as N-protein-
112 specific IgM antibody levels using ELISAs. We found detectable SARS-CoV-2-specific IgA
113 (15/20 patients), IgG (15/20 patients) and IgM (16/20 patients) antibody levels in most of the
114 COVID-19 patients (Figure 2A and B). Next, we determined total anti-SARS-CoV-2 IgG
115 antibody levels measured towards SARS-CoV-2-infected cells using an immunofluorescence
116 assay (IFA) (Figure 2C). We found that 16 out of the 20 patients were positive in this assay,
117 with titers ranging from 40 to 5120 (Figure 2C). None of the healthy controls were positive in
118 any of the antibody assays (Figure 2A). Higher IgG levels were detected in patients who were
119 sampled later compared to early after the onset of symptoms, total and spike S1-specific
120 SARS-CoV-2 IgG antibody levels positively correlated with the number of days since
121 symptom onset ($r_s = 0.577$, $P = 0.01$ and $r_s = 0.603$, $P = 0.005$, respectively) (Supplemental
122 Figure 1B and C).
123

124 To measure neutralizing antibody titers against SARS-CoV-2, we utilized a micro-
125 neutralization assay (Manenti et al. 2020). Neutralizing antibodies were detected in most of
126 the patients (16/20), with titers ranging from 10 to 1920 (Figure 2D). Three of the four
127 patients with undetectable levels of SARS-CoV-2-neutralizing antibodies were also below
128 the level of detection for SARS-CoV-2-specific antibodies in IFA and all ELISA's (Figure
129 2A).
130
131 Total SARS-CoV-2-specific IgG antibody levels positively correlated with neutralizing
132 antibody titers ($r_s = 0.865$, $P < 0.001$) (Figure 2E). Both S1-specific IgA and IgG, as well as
133 N-specific IgM levels also correlated with SARS-CoV-2-neutralizing antibody titers ($r_s =$
134 0.876, $P < 0.001$; $r_s = 0.809$, $P < 0.001$ and $r_s = 0.62$, $P = 0.004$, respectively) suggesting that
135 antibody titers in general may reflect the levels of neutralizing antibodies during the acute
136 phase of COVID-19 (Supplemental Figure 1D-F).
137
138 **Detection of SARS-CoV-2 RNA in Serum of COVID-19 Patients**
139 An important aspect of neutralizing antibodies is to limit viral spread. To analyze for possible
140 SARS-CoV-2 viremia, we screened all serum samples by real time RT-PCR (Corman et al.
141 2020). Three COVID-19 patients were positive for SARS-CoV-2 RNA in serum (Figure 2A).
142 Notably, 2 out of the 3 SARS-CoV-2 RT-PCR-positive patients lacked detectable levels of
143 neutralizing antibodies (Fig 2A), which might allow for a more efficient viral spread in those
144 patients. However, our attempts to isolate live SARS-CoV-2 from patient serum on Vero E6
145 cells were unsuccessful (data not shown), suggesting absence or low levels of live SARS-
146 CoV-2 in serum of COVID-19 patients.
147
148

149 **Increased T cell Activation and Inflammatory Response in COVID-19 Patients**

150 According to the latest reports, COVID-19 patients generally present with decreased
151 lymphocyte numbers in peripheral blood (Wang et al. 2020; Qin et al. 2020; Zhang et al.
152 2020). In line with this, we observed decreased absolute numbers of lymphocytes ($CD45^+$)
153 and T cells (total $CD3^+$ and $CD3^+CD8^+$ cells) as compared to controls, but no significant
154 decrease of B cells ($CD19^+$) or $CD4^+$ T cells (Supplemental Figure 1G). Furthermore,
155 COVID-19 patients showed significantly higher frequencies of activated $CD8^+$ and $CD4^+$ T
156 cells compared to the controls (Supplemental Figure 1H). The frequencies of activated $CD8^+$
157 T cells positively correlated with activated $CD4^+$ T cell frequencies in COVID-19 patients (r_s
158 $= 0.699$, $P < 0.001$). Interestingly, $CD8^+$ T cell activation level, but not $CD4^+$ T cell
159 activation level, positively correlated with neutralizing antibody titers ($r_s = 0.544$, $P = 0.01$; r_s
160 $= 0.271$, $P = 0.25$) (Supplemental Figure 1O and P).

161

162 The pro-inflammatory cytokine IL-6 and C-reactive protein (CRP) serum levels have been
163 shown to correlate with disease severity in COVID-19 patients (Zhang et al. 2020; Liu et al.
164 2020). As expected, we observed increased serum levels of IL-6 and CRP in this COVID-19
165 patient cohort (Supplemental Figure 1I and Supplemental Table 1). Moreover, serum levels
166 of both IL-6 and of CRP correlated with the duration of hospitalization, and the number of
167 days between symptom onset and discharge from hospital (Supplemental Figure 1J – M).
168 Interestingly, IL-6 levels negatively correlated with neutralizing antibody titers in COVID-19
169 patients ($r_s = -0.583$, $P = 0.007$), indicating a possible link between inflammation and
170 humoral responses in COVID-19. Further research is needed to describe this relationship in
171 detail (Supplemental Figure 1N).

172

173

174 **Conclusion**

175 In this study, we demonstrated that COVID-19 patients elicit a significant SARS-CoV-2-
176 specific B cell response, indicated by the expansion of SARS-CoV-2-specific ASCs.
177 Although not all patients in this cohort had detectable levels of SARS-CoV-2-specific
178 antibodies at the time of sampling, SARS-CoV-2 N protein-specific ASCs could be detected
179 in all patients using the FluoroSpot assay. In addition, we showed a clear relationship
180 between the levels of SARS-CoV-2-specific antibodies and total SARS-CoV-2-neutralizing
181 antibodies. This suggests that standard serological assays may reflect the ability of COVID-
182 19 patients to neutralize SARS-CoV-2, which may offer protection from a re-infection.
183 Additionally, tools employed in this study may be of relevance in the assessment of long-
184 lasting immunity after SARS-CoV-2 infection and vaccination.
185

186 **Materials and Methods**

187

188 **Ethics statement**

189 The study was approved by the Regional Ethical Review Board in Stockholm, Sweden and
190 by the Swedish Ethical Review Authority. All COVID-19 patients and healthy controls
191 included in this study provided a written informed consent for participation.

192

193 **Study subjects and sampling of peripheral blood**

194 Peripheral blood samples were collected from 20 adult COVID-19 patients hospitalized in
195 April 2020 at the Karolinska University Hospital in Stockholm, Sweden (5 females and 15
196 males; age range between 34 and 67 years; median age 53 years) (Table 1). Patients were
197 diagnosed with COVID-19 by RT-PCR(Corman et al. 2020) for SARS-CoV-2 in either
198 nasopharyngeal swabs (18/20 patients) or sputum (2/20 patients). Diagnostics were
199 performed at the diagnostic laboratory at the Karolinska University Hospital, Stockholm,
200 Sweden. Peripheral blood samples from patients were taken at median 15 days after self-
201 reported onset of symptoms (range 7-19 days). Peripheral blood samples of 7 healthy controls
202 were collected in parallel (2 females and 5 males; age range between 26 and 53 years; median
203 age 31 years). Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized
204 anti-coagulated blood using density gradient Lymphoprep medium (Stemcell Technologies)
205 following the manufacturer's instructions, and immediately used for flow cytometry and
206 FluoroSpot assays. Serum was collected from COVID-19 patients and healthy controls in BD
207 Vacutainer serum tubes with spray-coated silica (BD Biosciences). After coagulation for up
208 to 2 hours at RT, serum was isolated by centrifugation at 2000 *g* for 10 min and immediately
209 stored at -80 ºC for later analysis.

210

211 **Absolute counts of leukocytes in peripheral blood**

212 Absolute numbers of $CD45^+$, $CD3^+$, $CD4^+$, $CD8^+$, and $CD19^+$ cells in peripheral blood were
213 measured using BD Trucount Tubes (BD Biosciences). 50 µL of anti-coagulated whole blood
214 were added into Trucount Tubes within 3 hours after blood extraction and stained with either
215 anti-CD45-PerCP (2D1), anti-CD3-FITC (SK7), anti-CD4-APC (SK3) and anti-CD8-PE
216 (SK1), or anti-CD45-PerCP (2D1) and anti-CD19-AF488 (HIB19) (all from BioLegend).
217 After 15 minutes of incubation at RT, stained whole blood was fixed and red blood cells
218 lysed with 2X BD FACS Lysing Solution (BD Biosciences). Samples were acquired on a BD
219 Accuri C6 Plus flow cytometer. Bead number recorded was used to quantify absolute $CD45^+$,
220 $CD3^+$, $CD4^+$, $CD8^+$, and $CD19^+$ cell counts per microliter of blood. ASC numbers per
221 microliter of blood were calculated based on $CD19^+$ B cell numbers measured by absolute
222 cell counting and on frequencies of ASCs within $CD19^+$ cells measured by flow cytometry.
223

224 **Flow cytometry**

225 Staining with fluorescently-labelled antibodies was performed on freshly isolated PBMCs.
226 Briefly, cells were incubated with surface staining antibodies diluted in PBS for 30 min at
227 4°C in the dark, followed by 3 washes with flow cytometry buffer (2% FCS and 2 mM
228 EDTA in PBS). Cells were then fixed and permeabilized using eBioscience
229 Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific) and later
230 incubated with antibodies diluted in PBS for intracellular and intranuclear staining for 30 min
231 at 4°C in the dark. Finally, samples were incubated in a 2% formaldehyde solution
232 (Polysciences) for 2 h, washed and resuspended in flow cytometry buffer, and data
233 subsequently acquired on a BD LSRFortessa flow cytometer equipped with 355, 405, 488,
234 561, and 639 nm lasers and BD FACSDiva Software (BD Biosciences). For a detailed gating
235 strategy see Supplemental Figure 2.

236

237 The following monoclonal antibody conjugates were used for cell surface staining: anti-CD8-
238 Qdot605 (3B5) (Thermo Fisher Scientific), anti-CD19-BUV395 (SJ25C1), anti-CD14-V500
239 (MφP9), anti-CD4 -BUV737 (RPA-T4) (all from BD Biosciences), anti-CD123-BV510
240 (6H6), anti-CD27-BV650 (O323), anti-CD20-FITC (2H7), anti-CD38-BV421 (HB-7), anti-
241 IgD-PE-Cy7 (IA6-2), anti-IgM-BV785 (MHM-88) (all from BioLegend), anti-CD3-PE-Cy5
242 (UCHT1), anti-CD56-ECD (N901) (all from Beckman Coulter), and anti-IgA-APC
243 (REA1014) (Miltenyi). LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Thermo Fisher
244 Scientific) was used as a viability marker. The following monoclonal antibody conjugates
245 were used for intracellular and intranuclear staining: anti-IgG-PE (HP6017) (BioLegend) and
246 anti-Ki67-AF700 (B56) (BD Biosciences).

247

248 **FluoroSpot assay for antibody-secreting cells**

249 The number of SARS-CoV-2 nucleocapsid (N) protein-specific IgA, IgG and IgM antibody-
250 secreting cells (ASCs), as well as the total number of IgA-, IgG- and IgM-ASCs in freshly
251 isolated PBMCs were measured using a multicolor B cell FluoroSpot kit with modifications
252 (Mabtech). Briefly, ethanol-activated IPFL membrane plates were coated overnight with
253 either: (i) anti-IgG, anti-IgA, and anti-IgM capture antibodies (15µg/mL of each) for the
254 detection of all ASCs, or (ii) SARS-CoV-2 N protein (10 µg/mL) for the detection of SARS-
255 CoV-2-specific ASCs. The plates were washed with PBS and blocked with R10 media
256 (RPMI-1640 with 10% FCS, 1% Pen/Strep, 2mM L-Glutamine (all from Thermo Fisher
257 Scientific)) for 30 minutes at RT before the addition of freshly isolated PBMCs. Plates were
258 then incubated at 37°C in 5% CO_2 for 20 hours and then developed with anti-human IgG-550
259 (yellow fluorescence), anti-human IgA-490 (green fluorescence) and anti-human IgM-640
260 (red fluorescence) secondary detection antibodies (diluted 1:500 each) (all antibodies from

261 Mabtech). Fluorescent spots indicating a single ASC were detected with an IRIS FluoroSpot
262 reader and counted with Apex software (Mabtech).
263
264 **Recombinant SARS-CoV-2 nucleocapsid protein**
265 A full-length nucleocapsid (N) phosphoprotein nucleotide sequence (1293 base-pairs) of the
266 SARS-CoV-2 virus was optimized and synthesized (Genscript). The synthesized sequence
267 was cloned into a PET-30a(+) vector with a carboxyterminal His tag for detection of protein
268 expression in *E.coli*. The *E. coli* strain BL21 Star (DE3) was transformed with the
269 recombinant plasmid and a single colony was inoculated into TB medium containing
270 antibiotic and cultured at 37°C at 200 rpm and then induced with IPTG. Protein purity and
271 molecular weight were determined by SDS-PAGE and Western blot according to standard
272 procedures (Genscript).
273
274 **SARS-CoV-2 isolation from serum**
275 50 µL of serum were mixed with 150 µL of EMEM (Gibco) and added to confluent Vero E6
276 cells seeded in 24-well plates. Cells were incubated with diluted serum for 1 hour, and 1 mL
277 of Vero E6 medium was added after the incubation. Cells were subsequently incubated
278 further for 10 days at 37 °C and 5% CO_2 and monitored for cytopathic effect (CPE) by
279 optical microscopy.
280
281 **Immunofluorescence assay for IgG against SARS-CoV-2**
282 Vero E6 cells were infected with SARS-CoV-2 (isolate SARS-CoV-2/human/SWE/01/2020,
283 accession number MT093571) for 24 hours, trypsinized and mixed with uninfected Vero E6
284 cells, and then seeded on microscope slides. Twelve hours later, slides were fixed in acetone
285 and stored at -80˚C until further use. Serum samples were heat-inactivated at 56˚C for 30

286 minutes prior to analysis. For analysis of total SARS-CoV-2 IgG antibody titers, serum
287 samples were serially diluted from 1:20 to 1:5120. 25 µL of diluted serum was then added to
288 fixed cells and incubated at 37˚C for 30 min, after which the slides were washed in NaCl for
289 30 min. Bound SARS-CoV-2 IgG antibodies were then detected by incubating for 30 min at
290 37˚C with a secondary AF488-conjugated AffiniPure goat anti-human IgG antibody (Jackson
291 Immunoresearch), diluted 1:200 in 0.1% Evan's Blue. SARS-CoV-2 IgG positive cells were
292 visualized using a Nikon Eclipse Ni fluorescence microscope (x40 magnification). The titer
293 of IgG in each serum sample was determined by the inverted dilution factor value for the
294 highest dilution with positive staining.
295
296 **ELISAs**
297 SARS-CoV-2 specific IgG and IgA antibodies in serum were detected using anti-SARS-
298 CoV-2 ELISA kits (both from Euroimmun), according to the manufacturer's instructions.
299 SARS-CoV-2 specific IgM antibodies were detected using EDI Novel Coronavirus COVID-
300 19 IgM ELISA kit (Epitope Diagnostics), according to the manufacturer's instructions. Serum
301 samples were heat-inactivated at 56˚C for 30 minutes prior to analysis.
302
303 IL-6 levels in serum from patients and healthy controls were measured in freshly thawed
304 serum using human IL-6 ELISA development kit (Mabtech), according to the manufacturer's
305 instructions. Serum samples were diluted 1:2 in ready-to-use ELISA diluent (Mabtech) prior
306 to performing the IL-6 ELISA assay.
307
308 **Micro-neutralization assay**
309 Two-fold dilution series from 1:10 to 1:10240 in EMEM (Gibco) + 5% FCS (Thermo Fisher
310 Scientific) were performed on the serum samples, which were previously heat inactivated at

311 56˚C for 30 minutes. Each dilution was subsequently mixed with equal volume of 4000
312 $TCID_{50}$/ml SARS-CoV-2 (50 µl serum plus 50 µl virus) and incubated for 1 hour at 37 °C
313 and 5% CO_2. Each sample was prepared in duplicates. After incubation, the mixtures were
314 added on confluent Vero E6 cells seeded on 96-well plates and incubated at 37 °C 5% CO_2.
315 Four days later the cells were inspected for signs of cytopathic effect (CPE) by optical
316 microscopy. Each well was scored as either 'neutralizing' if less than 50% of the cell layer
317 showed signs of CPE, or 'non-neutralizing' if ≥50% CPE was observed. Results are shown as
318 the arithmetic mean of the reciprocals of the highest neutralizing dilutions from the two
319 duplicates for each sample.
320
321 **Real-time RT-PCR**
322 RNA was extracted from freshly thawed serum samples using the MagDEA Dx SV reagent
323 kit and the magLEAD instrument (Precision System Science). The assay used to detect
324 SARS-CoV-2 RNA was modified from (Corman et al. 2020): forward primer 5'-
325 CATGTGTGGCGGTTCACTATATGT-3', reverse primer 5'-
326 TGTTAAARACACTATTAGCATAWGCAGT-3', and RdRp_SARSr-P2 probe. The assay
327 was carried out in 25 µL reaction mixtures containing 5µL RNA template, TaqMan Fast
328 Virus 1-Step Master Mix, 0.6 µM forward primer, 0.8 µM reverse primer, and 0.2 µM probe
329 (Applied Biosystems). Thermal cycling was performed at 50°C for 5 min, 95°C for 20 sec,
330 followed by 40 cycles of 95°C for 3 sec, and 60°C for 30 sec in a StepOne Plus real-time
331 PCR (Applied Biosystems).
332
333 **Statistics and data analysis**
334 Statistical analyses were performed using GraphPad Prism software 7.0 for MacOSX
335 (GraphPad Software). Correlation analyses were performed using Spearman's correlation

336 test. Statistical significance for differences between COVID-19 patients and healthy controls
337 was determined by two-sided Mann-Whitney *U* test. P values of < 0.05 were considered
338 statistically significant. FlowJo software version 10.5.3 (Tree Star) was used to analyze all
339 flow cytometry data. FluoroSpot data was analyzed with Apex software (Mabtech).

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Author Contributions

R.V., M.G., J.T.S., K.B., J.K. and S.G.R. designed and led the study.

R.V., M.G., J.T.S., K.T.M., J.T., G.A., L.F. designed experiments and optimized assays.

H.G., H.A., S.G.R. included patients and summarized clinical information.

R.V., M.G., K.T.M., J.T.S., J.T., W.C., N.L., J.K. performed experiments.

R.V., M.G., H.G., K.T.M., J.T.S., J.T., W.C., N.L., H.A., H.G.L., G.A., L.F., M.S., K.B., J.K., S.G.R. contributed to conceptualization of the study, discussed data analysis and interpreted the results.

R.V. wrote the paper, with input provided by all co-authors.

Competing Interests

Authors declare no competing interests.

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391

392

393 **Abbreviations**

394 ASC – antibody-secreting cell

395 CPE – cytopathic effect

396 COVID-19 – coronavirus disease 2019

397 N protein – nucleocapsid protein

398 SARS-CoV-2 – severe acute respiratory syndrome coronavirus 2

399 S1 protein – subunit 1 of SARS-CoV-2 spike protein

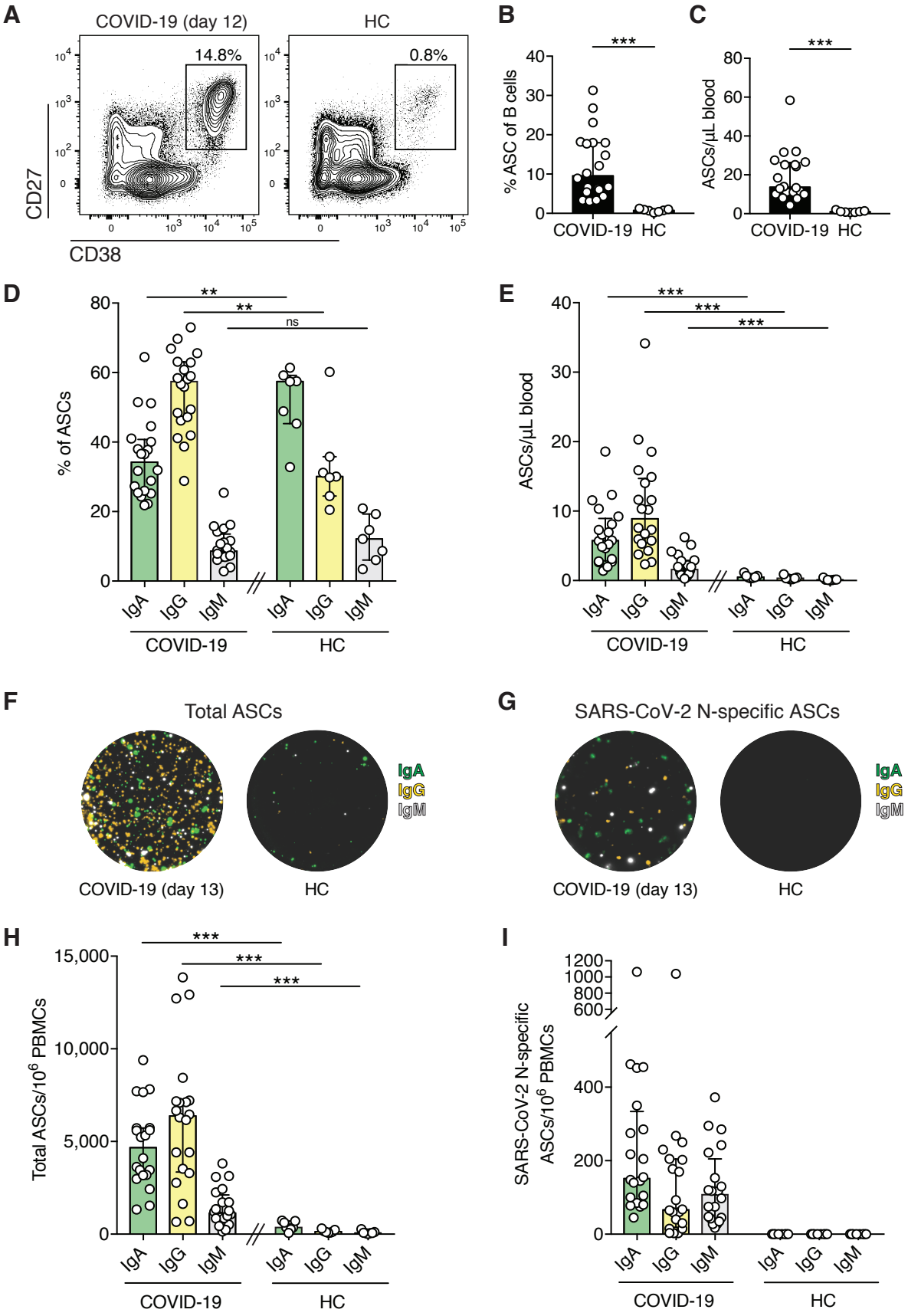


Figure 1. Detection of SARS-CoV-2 nucleocapsid protein-specific antibody-secreting cells (ASCs) in COVID-19 patients. (A) Representative flow cytometry plots of ASCs from one COVID-19 patient (12 days after symptom onset) and from one healthy control (HC). ASCs are marked with rectangle and gated on all $CD19^{+}$ B cells. (B) Frequencies of ASCs within all B cells in COVID-19 patients and in HCs. ASCs were defined as $CD19^{+}CD20^{low/-}IgD^{-}CD38^{high}CD27^{high}$. (C) Numbers of ASCs per microliter of whole blood, calculated using absolute B cell numbers and frequencies of ASCs measured by flow cytometry. (D) Frequencies of IgA-, IgG-, and IgM-ASCs within the total ASC population measured by flow cytometry. (E) Numbers of IgA-, IgG-, and IgM-ASCs per microliter of whole blood. Calculated using B cell numbers and frequencies of Ig subsets within ASCs measured by flow cytometry. (F and G) Representative images of wells from a FluoroSpot assay showing total IgA-, IgG-, and IgM-ASCs (F), and SARS-CoV-2 nucleocapsid protein-specific ASCs (G) from one COVID-19 patient (13 days after symptom onset) and one healthy control. IgM fluorescence is originally red, but replaced with white in this figure for visualization purpose. (H) Numbers of total IgA-, IgG-, and IgM-ASCs per million PBMCs, as measured by FluoroSpot assay. (I) Numbers of SARS-CoV-2 nucleocapsid protein-specific IgA-, IgG-, and IgM-ASCs per million PBMCs. Experiments were performed on all COVID-19 patients (n = 20) and healthy controls (n = 7). Statistical significance was determined using Mann-Whitney *U* test (B-E and H). Bar graphs display median and IQR. **, $P < 0.01$; ***, $P < 0.001$; ns – not significant.

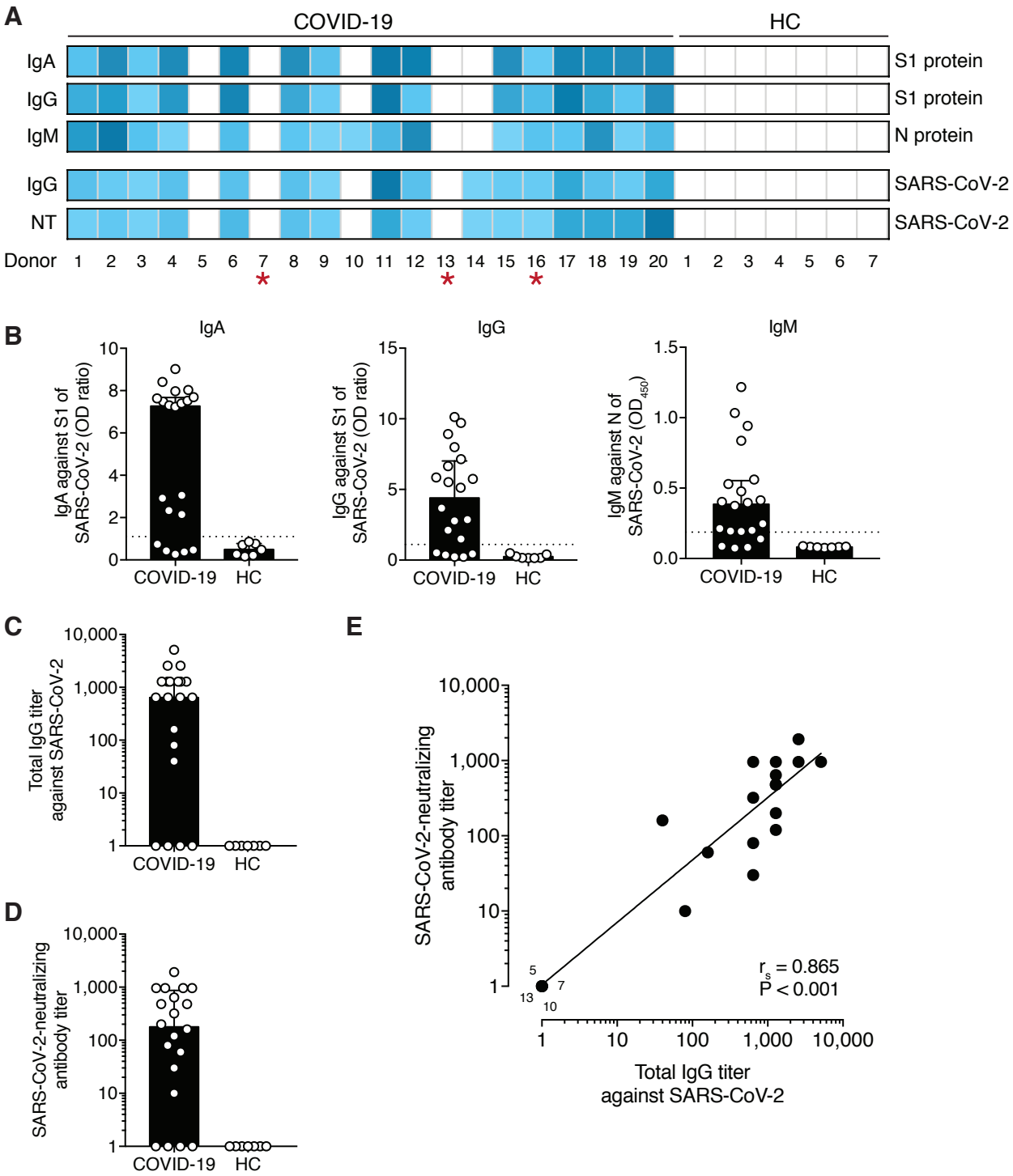


Figure 2. Detection of SARS-CoV-2-specific and neutralizing antibodies in COVID-19 patients
(A) Individual antibody responses to SARS-CoV-2 in COVID-19 patients (n = 20) and healthy controls (HC) (n = 7). Positivity for IgA against S1-protein, IgG against S1-protein, IgM against N protein, total IgG antibodies against whole SARS-CoV-2, and SARS-CoV-2-neutralizing antibody titers (NT) are presented in the heatmap. Blue color indicates a positive response and the color scale is adjusted for the minimum positive assay value and the highest value recorded within the patient cohort for each assay. White boxes indicate values below positive threshold or below detection level for each assay. Red asterisks represent patients with detectable levels of SARS-CoV-2 RNA in serum. (B) IgA, IgG, and IgM antibody levels in COVID-19 patients and controls, analyzed by ELISAs. Dotted horizontal line indicates the threshold for positive result. OD, optical density. OD ratio = OD of the sample divided by OD of the calibrator. (C) Total SARS-CoV-2 IgG antibody titers determined by immunofluorescence assay. Patients with titers < 20 were assigned a value of 1. (D) SARS-CoV-2-neutralizing antibody titers determined by microneutralization assay. Patients with titers < 10 were assigned a value of 1. (E) Correlation between total SARS-CoV-2-specific IgG titers and SARS-CoV-2-neutralizing antibody titers, examined by Spearman's correlation test. r_s: Spearman's rank correlation coefficient. $P < 0.05$ was considered statistically significant. Four COVID-19 patients with undetectable antibody levels in both assays are highlighted (5, 7, 10 and 13). Bar graphs display median and IQR.

Table 1. Clinical characteristics of 20 hospitalized COVID-19 patients.

Cohort characteristics	
Age, years, median (range)	53 (34-67)
Male, n (%)	15 (75)
Symptom onset to sampling, days, median (range)	15 (7-19)
Symptom onset to discharge, days, median (range)	19.5 (9-30)
Duration of hospitalization, days, median (range)	7.5 (3-25)
Intensive care unit (ICU) treatment[a], n (%)	3 (15)
Fatal outcome, n (%)	1 (5)

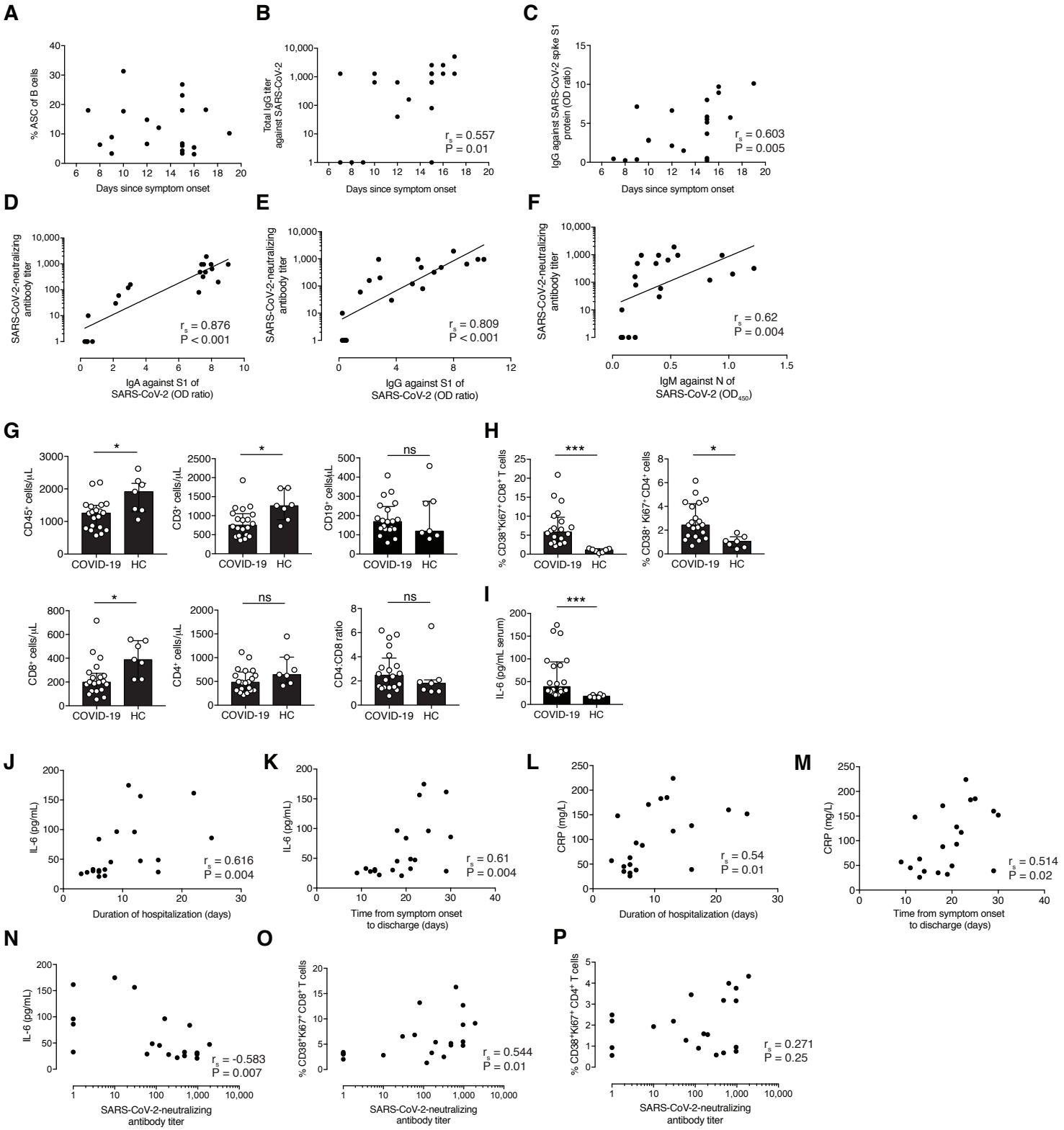
Comorbidities	**n (%)**
Total	7 (35)
Diabetes mellitus type II	4 (20)
Hypertension	3 (15)
Asthma	2 (10)
Cardiovascular disease	1 (5)

Symptoms	**n (%)**
Fever (>38°C)	20 (100)
Cough	20 (100)
Respiratory difficulties	20 (100)
Myalgia	9 (45)
Chest pain	6 (30)
Diarrhea	4 (20)

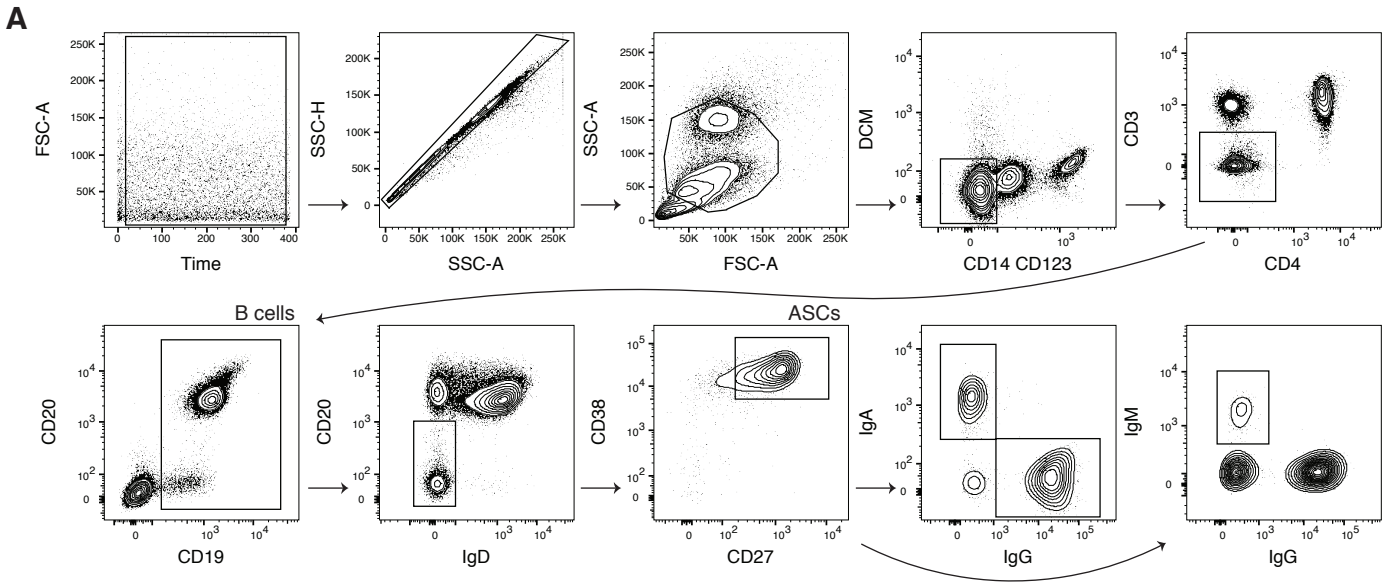
Treatment	**n (%)**
Supplemental oxygen[b]	20 (100)
High flow nasal oxygen	4 (20)
Inhaled bronchodilators	2 (10)
Low molecular weight heparin	17 (85)
Antibiotics	14 (70)
Immunomodulatory drugs[c] (given before sampling)	3 (15)

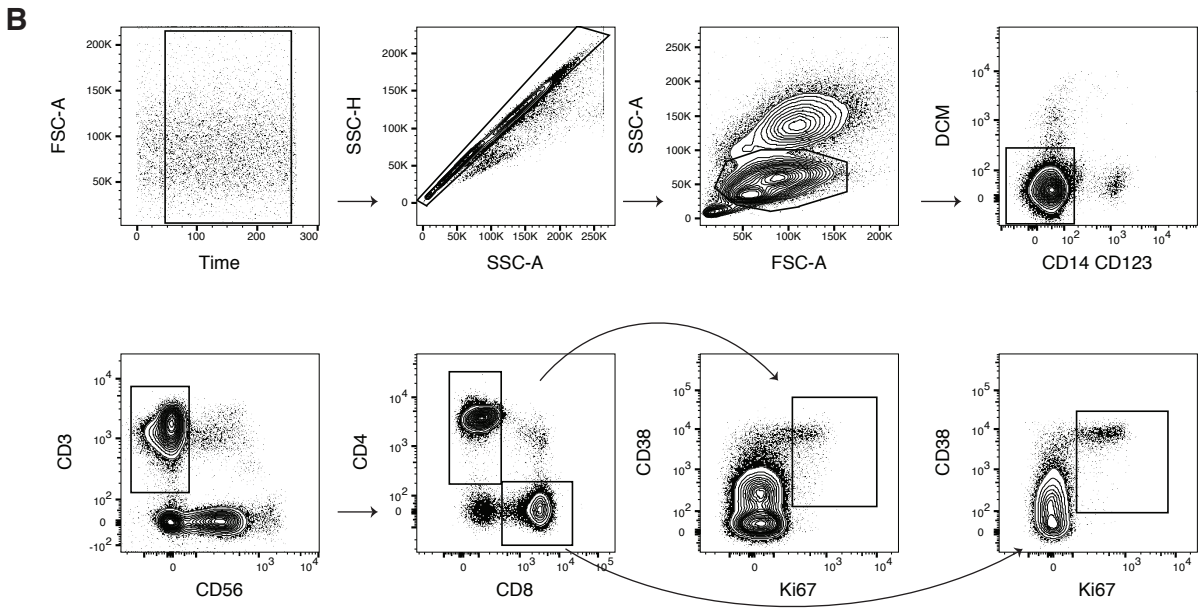
Abbreviations: n: number of patients.

[a.] ICU treatment before study sampling (n=2), ICU treatment after study sampling (n=1)
[b.] Oxygen flow rate – minimum 2.5 L/min. 1 patient in mechanical ventilation.
[c.] Chloroquine phosphate (n=2) or Anakinra (n=1).



Supplemental Figure 1. (A) Graph displaying the antibody secreting cell (ASC) frequencies within the total pool of $CD19^+$ B cells and the number of days since symptom onset of COVID-19. (B) Correlation between total IgG titers against SARS-CoV-2 (measured by IFA) and the number of days since symptom onset of COVID-19. (C) Correlation between levels of SARS-CoV-2 S1 protein-specific IgG levels and the number of days since symptom onset of COVID-19. (D-F) Correlations between SARS-CoV-2-neutralizing antibody titers and SARS-CoV-2 S1 protein-specific IgA levels (D), SARS-CoV-2 S1 protein-specific IgG levels (E), and SARS-CoV-2 N protein-specific IgM levels (F). (G) Absolute counts of lymphocytes ($CD45^+$), T cells ($CD3^+$, $CD4^+$, and $CD8^+$) and B cells ($CD19^+$) in peripheral blood of COVID-19 patients and HCs. (H) Frequencies of CD38 and Ki67 co-expressing $CD8^+$ T cells (left graph) and $CD4^+$ T cells (right graph) in COVID-19 patients and HCs. (I) IL-6 serum levels in COVID-19 patients and HCs. (J – M), Correlations between IL-6 and CRP levels in serum in relation to duration of hospitalization (J and L) and the number of days from symptom onset to discharge from hospital (K and M). (N-P) Correlations of IL-6 (N), $CD8^+$ T cell activation (O) and $CD4^+$ T cell activation (P) with SARS-CoV-2 neutralizing antibody titers. Bars graphs display medians and IQR. Statistical significance was determined using Mann-Whitney U test (G, H and I). Correlations were examined by Spearman's correlation test. r_s: Spearman's rank correlation coefficient. $P < 0.05$ was considered statistically significant.





Supplemental Figure 2. (A) Flow cytometry gating strategy for antibody secreting cells (ASCs) and the IgA, IgG and IgM subsets. ASC frequencies are expressed as percentage of all CD19⁺ cells (B cells) in Figure 1. (B) Flow cytometry gating strategy for CD4⁺ and CD8⁺ T cell activation, defined as the co-expression of CD38 and Ki67 markers.

Supplemental Table 1. Analysis of basic clinical chemistry parameters in peripheral blood[a] of COVID-19 patients.

Blood cell subset	$x10^9$/L, median (IQR)	Reference values	n
Leukocytes	5.85 (5.30-6.48)	3.5-8.8	20
Neutrophils	3.85 (3.35-4.38)	1.6-5.9	20
Monocytes	0.45 (0.28-0.60)	0.2-0.8	20
Lymphocytes	1.20 (1.00-1.73)	1.1-3.5	20
Platelets	299 (255-359)	145-348	20

Parameter	Median (IQR)	Reference values	n
HB (g/L)	132 (126-140)	134-179 (M), 117-153 (F)	20
CRP (mg/L)	90 (43-154)	<3	20
PCT (µg/L)	0.16 (0.08-0.31)	<0.5	16
D-dimer (mg/L)	0.65 (0.53-0.91)	<0.50	13
Myoglobin (µg/L)	30.5 (29-35)	<73	4
Ferritin (µg/L)	1209 (760-1852)	30-350	18
ASAT (µkat/L)	0.88 (0.68-1.31)	<0.76	20
ALAT (µkat/L)	1.07 (0.56-1.64)	<1.1	20
LD (µkat/L)	6.7 (5.48-7.63)	<3.5	20
Troponin T (ng/L)	7 (7-10.25)	<15	17

[a]Data are obtained from peripheral blood taken on the same day (+/- 24 hours) as the study samples were taken.

Abbreviations: n: number of patients for whom data was available; IQR: interquartile range; M: male; F: female; HB: haemoglobin; CRP: C-reactive protein; PCT: procalcitonin; ASAT: aspartate transaminase; ALAT: alanine transaminase; LD: lactate dehydrogenase.