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

Authors

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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
longitudinal immune responses in a single COVID-19 patient with mild disease showed a
detectable ASC expansion peaking at day 8 after symptom onset and representing 7% of all
circulating B cells (Thevarajan et al. 2020). Here, we investigated early B cell and antibody
responses to SARS-CoV-2 infection in a cohort of hospitalized COVID-19 patients.

Results and Discussion
Twenty COVID-19 patients were enrolled in this study during hospitalization at Karolinka
University Hospital, Sweden. All twenty patients exhibited typical COVID-19 symptoms of
fever, cough and breathing difficulties, while a few also experienced chest pain, myalgia and
diarrhea (Table 1). Seven patients also presented with co-morbidities such as hypertension,
esthma, cardiovascular disease and diabetes mellitus type II. Peripheral blood samples from
these patients were collected at median 15 days after the onset of COVID-19 symptoms. In
parallel, seven donors without an ongoing respiratory disease or signs of inflammation were
included in the study as healthy controls.

Significant Increase of Antibody-secreting Cells in COVID-19 Patients
To assess the presence of ASC expansion in COVID-19 patients, we analyzed freshly
isolated peripheral blood mononuclear cells (PBMCs) by flow cytometry. As expected, low
ASC frequencies and numbers in peripheral blood were observed in healthy controls (Figure
1A-C). However, a significant increase in ASC frequencies (defined here as CD19+CD20low/-
IgD-CD38highCD27high) was observed in COVID-19 patients, constituting up to 31% of all B
cells in peripheral blood (Figure 1A-B). ASC numbers in blood were also increased in
COVID-19 patients, as compared to healthy controls (Figure 1C). Noteworthy, a substantial
ASC expansion was detected as early as 7 days, and as late as 19 days after the onset of
symptoms (Supplemental Figure 1A).
IgA+ ASCs have previously been shown to be the dominant subset at steady state (Mei et al. 2009). In agreement with this, the majority of the ASCs in healthy controls were IgA+, whereas the IgG+ ASC subset dominated in COVID-19 patients (Figure 1D). Moreover, IgA+, IgG+ and IgM+ ASC numbers in peripheral blood were significantly increased in COVID-19 patients, as compared to healthy controls (Figure 1E).

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

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

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

**Characterization of SARS-CoV-2-specific Antibody Responses in COVID-19 Patients**

Seroconversion in COVID-19 patients, measured by detectable SARS-CoV-2-specific IgG levels, has been recently shown to take place within nineteen days after the onset of symptoms (Long et al. 2020). The expansion of SARS-CoV-2-specific ASCs in all of the twenty COVID-19 patients in our cohort, but in none of the healthy controls, suggested that the patients had developed SARS-CoV-2-specific antibodies in response to the infection. To investigate this in detail, we next analyzed SARS-CoV-2-specific antibody responses.

First, we measured SARS-CoV-2 spike S1-specific IgA and IgG, as well as N-protein-specific IgM antibody levels using ELISAs. We found detectable SARS-CoV-2-specific IgA (15/20 patients), IgG (15/20 patients) and IgM (16/20 patients) antibody levels in most of the COVID-19 patients (Figure 2A and B). Next, we determined total anti-SARS-CoV-2 IgG antibody levels measured towards SARS-CoV-2-infected cells using an immunofluorescence assay (IFA) (Figure 2C). We found that 16 out of the 20 patients were positive in this assay, with titers ranging from 40 to 5120 (Figure 2C). None of the healthy controls were positive in any of the antibody assays (Figure 2A). Higher IgG levels were detected in patients who were sampled later compared to early after the onset of symptoms, total and spike S1-specific SARS-CoV-2 IgG antibody levels positively correlated with the number of days since symptom onset ($r_s = 0.577$, $P = 0.01$ and $r_s = 0.603$, $P = 0.005$, respectively) (Supplemental Figure 1B and C).
To measure neutralizing antibody titers against SARS-CoV-2, we utilized a micro-
neutralization assay (Manenti et al. 2020). Neutralizing antibodies were detected in most of
the patients (16/20), with titers ranging from 10 to 1920 (Figure 2D). Three of the four
patients with undetectable levels of SARS-CoV-2-neutralizing antibodies were also below
the level of detection for SARS-CoV-2-specific antibodies in IFA and all ELISA’s (Figure
2A).

Total SARS-CoV-2-specific IgG antibody levels positively correlated with neutralizing
antibody titers ($r_s = 0.865$, $P < 0.001$) (Figure 2E). Both S1-specific IgA and IgG, as well as
N-specific IgM levels also correlated with SARS-CoV-2-neutralizing antibody titers ($r_s =
0.876$, $P < 0.001$; $r_s = 0.809$, $P < 0.001$ and $r_s = 0.62$, $P = 0.004$, respectively) suggesting that
antibody titers in general may reflect the levels of neutralizing antibodies during the acute
phase of COVID-19 (Supplemental Figure 1D-F).

Detection of SARS-CoV-2 RNA in Serum of COVID-19 Patients

An important aspect of neutralizing antibodies is to limit viral spread. To analyze for possible
SARS-CoV-2 viremia, we screened all serum samples by real time RT-PCR (Corman et al.
2020). Three COVID-19 patients were positive for SARS-CoV-2 RNA in serum (Figure 2A).
Notably, 2 out of the 3 SARS-CoV-2 RT-PCR-positive patients lacked detectable levels of
neutralizing antibodies (Fig 2A), which might allow for a more efficient viral spread in those
patients. However, our attempts to isolate live SARS-CoV-2 from patient serum on Vero E6
cells were unsuccessful (data not shown), suggesting absence or low levels of live SARS-
CoV-2 in serum of COVID-19 patients.
Increased T cell Activation and Inflammatory Response in COVID-19 Patients

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

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

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

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

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

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

**Flow cytometry**

Staining with fluorescently-labelled antibodies was performed on freshly isolated PBMCs. Briefly, cells were incubated with surface staining antibodies diluted in PBS for 30 min at 4°C in the dark, followed by 3 washes with flow cytometry buffer (2% FCS and 2 mM EDTA in PBS). Cells were then fixed and permeabilized using eBioscience Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific) and later incubated with antibodies diluted in PBS for intracellular and intranuclear staining for 30 min at 4°C in the dark. Finally, samples were incubated in a 2% formaldehyde solution (Polysciences) for 2 h, washed and resuspended in flow cytometry buffer, and data subsequently acquired on a BD LSRFortessa flow cytometer equipped with 355, 405, 488, 561, and 639 nm lasers and BD FACSDiva Software (BD Biosciences). For a detailed gating strategy see Supplemental Figure 2.
The following monoclonal antibody conjugates were used for cell surface staining: anti-CD8-Qdot605 (3B5) (Thermo Fisher Scientific), anti-CD19-BUV395 (SJ25C1), anti-CD14-V500 (MφP9), anti-CD4- BUV737 (RPA-T4) (all from BD Biosciences), anti-CD123-BV510 (6H6), anti-CD27-BV650 (O323), anti-CD20-FITC (2H7), anti-IgD-PE-Cy7 (IA6-2), anti-IgM-BV785 (MHM-88) (all from BioLegend), anti-CD38-BV421 (HB-7), anti-IgG-PE (HP6017) (BioLegend), anti-CD3-PE-Cy5 (UCHT1), anti-CD56-ECD (N901) (all from Beckman Coulter), and anti-IgA-APC (REA1014) (Miltenyi). LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Thermo Fisher Scientific) was used as a viability marker. The following monoclonal antibody conjugates were used for intracellular and intranuclear staining: anti-IgG-PE (HP6017) (BioLegend) and anti-Ki67-AF700 (B56) (BD Biosciences).

**FluoroSpot assay for antibody-secreting cells**

The number of SARS-CoV-2 nucleocapsid (N) protein-specific IgA, IgG and IgM antibody-secreting cells (ASCs), as well as the total number of IgA-, IgG- and IgM-ASCs in freshly isolated PBMCs were measured using a multicolor B cell FluoroSpot kit with modifications (Mabtech). Briefly, ethanol-activated IPFL membrane plates were coated overnight with either: (i) anti-IgG, anti-IgA, and anti-IgM capture antibodies (15µg/mL of each) for the detection of all ASCs, or (ii) SARS-CoV-2 N protein (10 µg/mL) for the detection of SARS-CoV-2-specific ASCs. The plates were washed with PBS and blocked with R10 media (RPMI-1640 with 10% FCS, 1% Pen/Strep, 2mM L-Glutamine (all from Thermo Fisher Scientific)) for 30 minutes at RT before the addition of freshly isolated PBMCs. Plates were then incubated at 37°C in 5% CO₂ for 20 hours and then developed with anti-human IgG-550 (yellow fluorescence), anti-human IgA-490 (green fluorescence) and anti-human IgM-640 (red fluorescence) secondary detection antibodies (diluted 1:500 each) (all antibodies from
Mabtech). Fluorescent spots indicating a single ASC were detected with an IRIS FluoroSpot reader and counted with Apex software (Mabtech).

Recombinant SARS-CoV-2 nucleocapsid protein

A full-length nucleocapsid (N) phosphoprotein nucleotide sequence (1293 base-pairs) of the SARS-CoV-2 virus was optimized and synthesized (Genscript). The synthesized sequence was cloned into a PET-30a(+) vector with a carboxyterminal His tag for detection of protein expression in E.coli. The E. coli strain BL21 Star (DE3) was transformed with the recombinant plasmid and a single colony was inoculated into TB medium containing antibiotic and cultured at 37°C at 200 rpm and then induced with IPTG. Protein purity and molecular weight were determined by SDS-PAGE and Western blot according to standard procedures (Genscript).

SARS-CoV-2 isolation from serum

50 µL of serum were mixed with 150 µL of EMEM (Gibco) and added to confluent Vero E6 cells seeded in 24-well plates. Cells were incubated with diluted serum for 1 hour, and 1 mL of Vero E6 medium was added after the incubation. Cells were subsequently incubated further for 10 days at 37 °C and 5% CO₂ and monitored for cytopathic effect (CPE) by optical microscopy.

Immunofluorescence assay for IgG against SARS-CoV-2

Vero E6 cells were infected with SARS-CoV-2 (isolate SARS-CoV-2/human/SWE/01/2020, accession number MT093571) for 24 hours, trypsinized and mixed with uninfected Vero E6 cells, and then seeded on microscope slides. Twelve hours later, slides were fixed in acetone and stored at -80°C until further use. Serum samples were heat-inactivated at 56°C for 30
For analysis of total SARS-CoV-2 IgG antibody titers, serum samples were serially diluted from 1:20 to 1:5120. 25 µL of diluted serum was then added to fixed cells and incubated at 37°C for 30 min, after which the slides were washed in NaCl for 30 min. Bound SARS-CoV-2 IgG antibodies were then detected by incubating for 30 min at 37°C with a secondary AF488-conjugated AffiniPure goat anti-human IgG antibody (Jackson Immunoresearch), diluted 1:200 in 0.1% Evan’s Blue. SARS-CoV-2 IgG positive cells were visualized using a Nikon Eclipse Ni fluorescence microscope (x40 magnification). The titer of IgG in each serum sample was determined by the inverted dilution factor value for the highest dilution with positive staining.

ELISAs

SARS-CoV-2 specific IgG and IgA antibodies in serum were detected using anti-SARS-CoV-2 ELISA kits (both from Euroimmun), according to the manufacturer's instructions. SARS-CoV-2 specific IgM antibodies were detected using EDI Novel Coronavirus COVID-19 IgM ELISA kit (Epitope Diagnostics), according to the manufacturer's instructions. Serum samples were heat-inactivated at 56°C for 30 minutes prior to analysis.

IL-6 levels in serum from patients and healthy controls were measured in freshly thawed serum using human IL-6 ELISA development kit (Mabtech), according to the manufacturer's instructions. Serum samples were diluted 1:2 in ready-to-use ELISA diluent (Mabtech) prior to performing the IL-6 ELISA assay.

Micro-neutralization assay

Two-fold dilution series from 1:10 to 1:10240 in EMEM (Gibco) + 5% FCS (Thermo Fisher Scientific) were performed on the serum samples, which were previously heat inactivated at
56°C for 30 minutes. Each dilution was subsequently mixed with equal volume of 4000 TCID₅₀/ml SARS-CoV-2 (50 µl serum plus 50 µl virus) and incubated for 1 hour at 37 °C and 5% CO₂. Each sample was prepared in duplicates. After incubation, the mixtures were added on confluent Vero E6 cells seeded on 96-well plates and incubated at 37 °C 5% CO₂. Four days later the cells were inspected for signs of cytopathic effect (CPE) by optical microscopy. Each well was scored as either 'neutralizing' if less than 50% of the cell layer showed signs of CPE, or 'non-neutralizing' if ≥50% CPE was observed. Results are shown as the arithmetic mean of the reciprocals of the highest neutralizing dilutions from the two duplicates for each sample.

Real-time RT-PCR

RNA was extracted from freshly thawed serum samples using the MagDEA Dx SV reagent kit and the magLEAD instrument (Precision System Science). The assay used to detect SARS-CoV-2 RNA was modified from (Corman et al. 2020): forward primer 5'-CATGTGTGGCGGTTCACTATATGT-3', reverse primer 5'-TGTTAAARACACTATTAGCATAWGCAGT-3', and RdRp_SARSr-P2 probe. The assay was carried out in 25 µL reaction mixtures containing 5µL RNA template, TaqMan Fast Virus 1-Step Master Mix, 0.6 µM forward primer, 0.8 µM reverse primer, and 0.2 µM probe (Applied Biosystems). Thermal cycling was performed at 50°C for 5 min, 95°C for 20 sec, followed by 40 cycles of 95°C for 3 sec, and 60°C for 30 sec in a StepOne Plus real-time PCR (Applied Biosystems).

Statistics and data analysis

Statistical analyses were performed using GraphPad Prism software 7.0 for MacOSX (GraphPad Software). Correlation analyses were performed using Spearman’s correlation.
test. Statistical significance for differences between COVID-19 patients and healthy controls was determined by two-sided Mann-Whitney $U$ test. $P$ values of $<0.05$ were considered statistically significant. FlowJo software version 10.5.3 (Tree Star) was used to analyze all flow cytometry data. FluoroSpot data was analyzed with Apex software (Mabtech).
Acknowledgments

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Author Contributions


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

Competing Interests

Authors declare no competing interests.
References


Abbreviations

ASC – antibody-secreting cell
CPE – cytopathic effect
COVID-19 – coronavirus disease 2019
N protein – nucleocapsid protein
SARS-CoV-2 – severe acute respiratory syndrome coronavirus 2
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+CD20low/-IgD-CD38highCD27high. (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.

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<tr>
<th>Cohort characteristics</th>
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<tr>
<td>Age, years, median (range)</td>
<td>53 (34-67)</td>
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<td>Male, n (%)</td>
<td>15 (75)</td>
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<td>Symptom onset to sampling, days, median (range)</td>
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<td>Symptom onset to discharge, days, median (range)</td>
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<td>Duration of hospitalization, days, median (range)</td>
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<td>Intensive care unit (ICU) treatment(^a), n (%)</td>
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<td>Fatal outcome, n (%)</td>
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<tr>
<td>Total</td>
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<tr>
<td>Diabetes mellitus type II</td>
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<td>Hypertension</td>
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<td>Asthma</td>
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<td>Cardiovascular disease</td>
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<table>
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<tr>
<th>Symptoms</th>
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<td>Fever (&gt;38°C)</td>
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<td>Cough</td>
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<tr>
<td>Respiratory difficulties</td>
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<td>Myalgia</td>
<td>9 (45)</td>
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<tr>
<td>Chest pain</td>
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<tr>
<td>Diarrhea</td>
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<th>Treatment</th>
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<td>Supplemental oxygen(^b)</td>
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<td>Inhaled bronchodilators</td>
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<td>Antibiotics</td>
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<tr>
<td>Immunomodulatory drugs(^c) (given before sampling)</td>
<td>3 (15)</td>
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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 SARS-CoV-2-neutralizing antibody titers and the number of days from symptom onset to discharge from hospital. (J and K) Correlations between IL-6 and CRP levels in 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 peripheral blood of 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 COVID-19 patients and HCs. (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. (F) Correlations between SARS-CoV-2-neutralizing antibody titers and the number of days since symptom onset of COVID-19. (D) Correlation between levels of SARS-CoV-2 S1 protein-specific IgG levels and the number of days since symptom onset of COVID-19. (E) Correlation between SARS-CoV-2 S1 protein-specific IgA levels 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. (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 of COVID-19 patients.

<table>
<thead>
<tr>
<th>Blood cell subset</th>
<th>x10⁹/L, median (IQR)</th>
<th>Reference values</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes</td>
<td>5.85 (5.30-6.48)</td>
<td>3.5-8.8</td>
<td>20</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>3.85 (3.35-4.38)</td>
<td>1.6-5.9</td>
<td>20</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.45 (0.28-0.60)</td>
<td>0.2-0.8</td>
<td>20</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>1.20 (1.00-1.73)</td>
<td>1.1-3.5</td>
<td>20</td>
</tr>
<tr>
<td>Platelets</td>
<td>299 (255-359)</td>
<td>145-348</td>
<td>20</td>
</tr>
</tbody>
</table>

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

*aData 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.