Detection of SARS-CoV-2 Specific Memory B cells to Delineate Long-Term COVID-19 Immunity

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January 15, 2021

Abstract

Background: The ongoing COVID-19 pandemic, caused by the novel coronavirus SARS-CoV-2, represents a serious worldwide health concern. A deeper understanding of the immune response to SARS-CoV-2 will be required to refine vaccine development and efficacy as well as to evaluate long-term immunity in convalescent patients. With this in mind, we investigated the formation of SARS-CoV-2 specific BMEMORY cells from patient blood samples. Methods: A standard flow cytometry-based protocol for the detection of SARS-CoV-2 specific B cells was applied using fluorochrome-coupled SARS-CoV-2 spike (S) full-length protein. Cohorts of 26 central European convalescent mild/moderate COVID-19 patients and 14 healthy donors were assessed for the levels of SARS-CoV-2 S- specific BMEMORY cells. Results: Overall B cell composition was not affected by SARS-CoV-2 infection in convalescent patients. Our analysis of SARS-CoV-2 specific BMEMORY cells in samples collected at different time points revealed that S-protein specific B cells remain in peripheral blood at least up to 6 months after COVID-19 diagnosis. Conclusions: Detection of SARS-CoV-2 specific BMEMORY cells may improve our understanding of the long-term adaptive immunity in response to SARS-CoV-2, allowing for an improved public health response and vaccine development during the COVID-19 pandemic. Further validation of the study in larger and more diverse populations and a more extended observation period will be required.

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Word count (main text) : 2501

Acknowledgments: We are grateful to the patients who donated their blood samples and clinical data for this project. We would like to acknowledge the excellent technical assistance as well as the expertise of the immune diagnostic laboratory of the Center for Translational Medicine at Marien Hospital Herne. N.B. received funding from the Mercator Foundation, the German Federal Ministry of Education and Research (BMBF) e:KID (01ZX1612A), and BMBF NoChro (FKZ 13GW0338B). MA received funding from the Arab German Young Academy (AGYA), a project by the BMBF.

Abstract

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Methods: A standard flow cytometry-based protocol for the detection of SARS-CoV-2 specific B cells was applied using fluorochrome-coupled SARS-CoV-2 spike (S) full-length protein. Cohorts of 26 central European convalescent mild/moderate COVID-19 patients and 14 healthy donors were assessed for the levels of SARS-CoV-2 S- specific B_{MEMORY} cells.

Results: Overall B cell composition was not affected by SARS-CoV-2 infection in convalescent patients. Our analysis of SARS-CoV-2 specific B_{MEMORY} cells in samples collected at different time points revealed that S-protein specific B cells remain in peripheral blood at least up to 6 months after COVID-19 diagnosis.

Conclusions: Detection of SARS-CoV-2 specific B_{MEMORY} cells may improve our understanding of the long-term adaptive immunity in response to SARS-CoV-2, allowing for an improved public health response and vaccine development during the COVID-19 pandemic. Further validation of the study in larger and more diverse populations and a more extended observation period will be required.

Keywords : COVID-19, Long-term immunity, Memory B cells, SARS-CoV-2

Introduction

The COVID-19 pandemic has led to devastating health outcomes with the death toll exceeding one million cases by the end of 2020. Furthermore, the global lockdowns impeded social and commercial interactions, with psychological and economic impacts on the public[1]. Vaccines are currently in the final stages of approval or have already been authorized under emergency use authorization[2]. However, there is still limited data on long-term immunity against SARS-CoV-2[3]. Studying long term immunity is essential to aid decision making for reopening and easing social distancing, assess the need for booster doses of vaccines, and estimate the potential of reinfections[4,5]. Focusing mainly on antibody-based assays as readouts for long-term immunity

may be strongly impeded due to lack of consistency in circulating antibody levels regardless of disease severity. Indeed, there are conflicting results about the longevity of antibody response in SARS-CoV-2 infected individuals[6–10], suggesting that long-lived plasma cells may not have been efficiently established in a proportion of COVID-19 patients. Another study found that about 20 % of German SARS-CoV-2 PCR-positive patients with mild to moderate symptoms did not have detectable spike-specific antibodies when tested 3 weeks or longer after infection[11]. In line with these findings, Long et al. reported that 40 % of non-symptomatic patients, as well as 12.9% with symptoms, became seronegative with no detectable antibodies in the blood eight weeks after discharge from hospital[12]. Therefore, it is of uttermost importance to develop and test other diagnostic assays to study the long-term immunity against SARS-CoV-2.

For diagnostic assays, the right target, the test accuracy, and minimizing false-negative and false-positive outcomes are the major challenges[13]. One potential approach that can help to improve the detection of long-term immunity is the analysis of SARS-CoV-2-specific memory T and B cell formation. We and others have reported on the detection of a strong SARS-CoV-2 specific memory T cell response in immune-competent and immunosuppressed patients[14–18]. Similar studies of B cell-related immunity have so far focused mainly on characterizing antibody specificities[19–24]. Other studies identified and isolated SARS-COV-2 B cells to generate neutralizing antibodies or to characterize the B cell receptor usage[20–22]. B_{MEMORY} cells can remain for decades or potentially lifelong, located within lymph nodes, spleen, bone marrow, and the lung, or circulate in the blood[25–28]. Upon reinfection, they become re-activated by encountering the same antigen and immediately start to proliferate and differentiate into plasma cells secreting neutralizing antibodies. Thus, quantifying B_{MEMORY} cell levels by a reliable assay may be used as an indicator of long-term immunity in convalescent patients. Although the formation of B_{MEMORY} cells was described, controlled studies comparing SARS-COV-2 specific memory B cell formation in unexposed individuals and COVID 19 patients are limited. To our knowledge, only studies with Australian and US-American cohorts assessed the long-term immunity through characterization of the B_{MEMORY} cells for more than 6 months[29,30]. Since the reports on duration of S1-specific antibody detection upon SARS-CoV-2 resolution are conflicting, several studies on antibody-producing B_{MEMORY} cells performed within different ethnic origin populations are required. In this work, we focus on detecting SARS-CoV2 spike (S)-protein specific memory B cells (B_{MEMORY}) in a well characterized cohort of central European COVID-19 patients as compared to unexposed controls. Furthermore, we analysed the sustainability for several months after infection as a specific long-term marker of adaptive immunity.

Methods

Protocol development

Well established and tested approaches were adapted for ease and simplicity[31–33]. A schematic presentation of the protocol is depicted in Figure 1.

In brief, blood was collected using EDTA coated Vacuette® tubes. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation technique with BioColl separating solution permitting cell separation based on polysucrose polymers. Short-term cell culture maintenance was performed by Roswell Parm Memorial Institute (RPMI) 1640 medium supplemented with GlutaMAX™, 1% Penicillin/ Streptomycin, and 10% fetal bovine serum (FBS). For flow cytometric screening of human samples, SARS-CoV-2 spike protein was labeled with fluorescein (FITC) or Cy5 fluorochromes using Lightning-Link® rapid labeling kits. This allows direct linkage of SARS-CoV-2 specific S- protein by robust covalent binding and fast processing of the assay as corresponding conjugates are ready-to-use in less than twenty minutes. Labeling the protein of interest independently with two different fluorochromes before incubation of the sample and analyzing the double-positive cells greatly enhances the specificity[31,32]. Subsequently, only double-positive B cells binding S-FITC and S-Cy5 were subjected to further evaluations. Another control measure to ensure specificity is to demonstrate that the response can be blocked by excess unlabelled SARS-CoV-2 S-protein, which blocks the S-protein specific B-cell receptors, thereby inhibiting the binding of the S-FITC and S-Cy5 to the B cells[33].

A detailed list of all materials and reagents, as well as devices used, is provided in Table 1.

We designed a staining panel to enable the detection of SARS-CoV-2 specific B cells by applying a set of fluorophore-coupled antigens characteristic to different B cell subsets such as naïve, switched, and unswitched B_{MEMORY} cells. Detailed information on the panel is provided in Table 2.

Study participants / Validation cohorts

Between April and October 2020, a cohort of 26 convalescent COVID-19 patients and 14 healthy donors were included in the study in 3 German centres (Berlin, Bochum, Essen). Symptomatic patients with mild to moderate symptoms were eligible to participate at a median time of 48 days after onset of COVID-19 symptoms (during which they become convalescent) according to the WHO management guidance (27 May 2020) and having a confirmed SARS-CoV-2 IgG ELISA at the time of study inclusion. All patients gave written informed consent. The ethical committee of the Ruhr-University Bochum approved the study (20-6886).

Measurement of SARS-CoV-2 specific B cells

Briefly, SARS-CoV-2 S1/S2-protein (further referred to as S-protein) (Sino Biological Inc.) was divided into three samples. Sample 1 was left unlabelled for blocking, sample 2 was coupled to fluorescein isothiocyanate (FITC), and sample 3 was linked to Cy5 fluorochrome. Lightning-Link® Rapid Conjugation System (Abcam PLC) was used for labelling according to the manufacturers' protocol. Labelling was performed with 100 µg of protein diluted to a concentration of 1 µg/µL. After the labelling procedure, the protein integrity was checked by a gel electrophoresis. PBMC were divided into the respective samples (blocked and unblocked samples in concentrations of 1:500 and 1:1000 of fluorochrome-labelled S-protein) with $5x10^6$ PBMC each (Fig. 1). Blocking was performed by using 10 the excess unlabelled protein. After blocking, PBMC were surface stained with fluorochrome-labelled antibodies, as indicated in Table 2. Finally, mixed FITC and Cy5 labelled protein was added in concentrations of 1:500 and 1:1000. Cells were stained for 10 min at 4 °C. After washing with phosphate-buffered saline (PBS), samples were stored at 4 °C until measurement on a Cytoflex flow cytometer. Directly before analysis, the samples were stained with DAPI to differentiate live from dead cells. Further information on applied materials and equipment is provided in Table 1.

Measurement of anti-S IgG antibodies

Analysis of SARS-CoV-2 specific antibodies was performed using the SARS-Cov-2 IgG kit (EUROIMMUN AG).

Data analysis and statistical evaluation

Flow cytometry data were analysed using FlowJo version 10.6.2 (BD Biosciences). Gating was performed according to single stains and fluorescent-minus-one controls. The corresponding gating strategy is presented in Figure 2. Lymphocyte identification and doublet exclusion were performed by forward-scatter (FSC) versus sideward parameters (SSC) and FSC/SSC-height (H) versus FSC/SSC-area (A) plotting, respectively. Cells positive for DAPI (dead cells), CD14 (mainly monocytes), or CD3 (T cells) were excluded from further analyses. CD19 expressing cells were further characterized for IgD, CD27, and CD38 expression. Frequencies of SARS-CoV-2-protein specific B cells in recovered COVID-19 patients and healthy donors were compared using two-way repeated measurements ANOVA and Sidak's multiple comparisons test. Patient and healthy donor age was compared using unpaired two-tailed t-test, and gender was compared using two-tailed Fisher's exact test. Correlations were calculated using Spearman's rank coefficient. Statistical analysis and graphical representation were realized with GraphPad Prism v7 and R v3.6.2.

Results

Baseline characteristics of the validation cohort

The cohort comprised 26 convalescent COVID-19 patients and 14 healthy donors as a control group. The median age of the COVID-19 patients at the time of study inclusion was 59 years, with participants ranging from 27 to 88 years of age. Subjects were of mixed gender with a higher percentage of males (58 %) than females (42 %). COVID-19 patients were included at a median time of 48 days after diagnosis or onset of

symptoms (range 15-214). Eighteen patients had mild COVID-19 that did not require hospitalization, and they did not receive specific COVID-19 treatment. Five patients had moderate disease, of whom two were hospitalized and one received treatment with antibiotics and remdesivir. Three patients had severe disease, were hospitalized and received antibiotic treatment. All included patients were confirmed to be SARS-CoV-2 positive by RT-PCR and/or serological testing. Healthy volunteers (median age 48, range 26-60) were significantly younger than COVID-19 patients (median 59, range 27-88) (P =0.0323, two-tailed unpaired t-test). There were no significant differences regarding gender between COVID-19 patients and the control group (P =0.5024, Fisher's exact test). Baseline characteristics are provided in Table 3.

Composition of B cell subsets in convalescent COVID-19 patients and healthy controls

SARS-CoV-2 specific B cell response was analysed in COVID-19 patients and healthy donors by characterizing levels of $IgD^{-}CD27^{+}$ B_{MEMORY}, $IgD^{+}CD27^{+}$ unswitched B_{MEMORY}, IgD^{+} $CD27^{-}$ B_{NAIVE},and $CD27^{++}$ $CD38^{++}$ plasmablasts (gating strategy shown in Figure 2).

Overall frequencies for all B cell subsets were similar between convalescent COVID-19 patients and healthy donors. IgD^{+} $CD27^{-}$ B_{NAIVE}cells represented most B cells at around 50% of the population, while IgD^{-} $CD27^{+}$ B_{MEMORY}cells and IgD^{+} $CD27^{+}$ unswitched B_{MEMORY} cells demonstrated levels ranging between 15 – 20 % of the entire B cell population. $CD27^{++}CD38^{++}$ plasmablasts constituted on average less than 1 % of the B cells (Figure 3a).

Presence of SARS-CoV-2 S-protein specific B_{MEMORY}cells in convalescent COVID-19 patients

Unlabelled SARS-CoV-2 S-protein in class-switched B_{MEMORY} cells could significantly block binding in COVID-19 patients, but not healthy donor samples (Figure 2b, Figure 3c). SARS-CoV-2 specific B_{MEMORY} cells correlated moderately with anti-S-protein IgG antibodies (Figure 3b). Overall, the frequency of S-protein specific B_{MEMORY} cells was significantly higher in the COVID-19 disease cohort compared to healthy individuals (Figure 3c). The difference between COVID-19 patients and healthy individuals was restricted to B_{MEMORY} cells and was not observed for other B cell subsets such as naïve or unswitched B_{MEMORY} cells (Figure 3d-f). This has two important implications: First, it demonstrates the specificity of the protocol for the detection of SARS-CoV-2 specific B_{MEMORY} cells, since it is expected that only COVID-19 convalescent, but not unexposed individuals generate SARS-CoV-2 specific B cell memory. Second, it demonstrates that the humoral immune response in the analysed patient cohort could be mediated by long-lived memory B cells. We did not observe substantial amounts of SARS-CoV-2 specific plasmablasts (Figure 3e).

The longevity of B cell memory can also be demonstrated by analysing the frequency of SARS-CoV-2 specific B_{MEMORY} cells concerning the time of sample collection after COVID-19 diagnosis (Fig. 3g). While there was a tendency of lower frequencies in samples collected at later time points, SARS-CoV-2 specific B_{MEMORY} cells remained detectable also 6 months after COVID-19 diagnosis.

Discussion

In this study, we investigated the formation of S- protein-specific B_{MEMORY} cells in convalescent COVID-19 patients. We employed a control group to rule out significant cross-reactivity. Our findings indicated that there were minimal cross-reactive responses in healthy donors. Studying immunological memory and protection after SARS-CoV-2 infection is of particular importance for understanding the formation of B cell memory and, subsequently, the effectiveness of vaccination approaches as well as the need for further booster dosing. Thus, this study represents a step toward understanding COVID-19 long-term immunity.

Serological testing offers the potential for screening, contact tracing, evaluating the viral reservoir, and supporting epidemiologic assessments by detecting SARS-Cov-2 specific antibodies. Another challenge for a broad and robust application of antibody-based SARS-CoV-2 detection assays, especially in the long-term, is the currently questionable durability and high variability of the SARS-CoV-2 induced antibody responses, where neutralizing antibody titers decrease over time in SARS-CoV-2 infected individuals in some studies[6–10]. Furthermore, recent studies provided evidence that a proportion of SARS-CoV-2 infected individuals did not develop specific antibodies[11,34,35]. Thus, failed antibody formation or their rapid wane in circulation

may limit our ability to verify COVID-19 immunity in the long-term, especially for mild or asymptomatic disease.

We, therefore, aimed at exploring immune responses to SARS-CoV-2 by studying and characterizing the B cell response. Such an approach enables us to understand long-term immunity more thoroughly. We were able to detect B_{MEMORY} cells specific to SARS-CoV-2 S-Protein, which also persisted in patients for more than 200 days. B cells can provide serological memory for decades or potentially the entire lifespan of individuals[25–27]. Thus, although the antibodies that B cells produce during initial exposure may disappear within a few weeks, the generated B_{MEMORY}cells can persist for much longer. For instance, previous studies were able to identify IgG memory B cells specific to the receptor-binding domain (RBD) in the blood of COVID-19 subjects[22,24,29,36]. Moreover, Zhang et al. demonstrated protective immunity formation with detectable levels of B_{MEMORY} cells in a pediatric population[37]. Similarly, a recent study addressing SARS-CoV-2 infection in rhesus macaques showed that two recovered subjects were resistant to reinfection one month later[38]. Our analysis of the S-protein SARS-CoV-2 specific B_{MEMORY} cells in samples collected at different time points revealed that these cells remain in peripheral blood at least up to 6 months after COVID-19 diagnosis. Thus, our study performed on a central European cohort of patients is in line with the data on the recently published US American and Australian cohorts and thus, confirms and extends the knowledge on the B cell response against SARS-CoV-2[29,30]. Therefore, our results demonstrating the longevity of SARS-CoV-2-specific B cell response can improve the general acceptance towards immunization in the broad community.

We acknowledge the limitations of our studies. First, only convalescent patients were studied, hence studying the B cell in patients with acute SARS-CoV-2 infection should be considered in further studies. Subsequent studies should also enroll larger patient cohorts with greater demographic variability and longer follow-up periods to decipher potential intra- and inter variability and to validate the finding that COVID-19 B_{MEMORY} cells reflect a long-term immunity. Furthermore, our control cohort was significantly younger than the COVID-19 cohort, with a median difference of 10 years. However, this difference does not negatively affect the differences observed in SARS-CoV-2-specific B_{MEMORY} in the two cohorts, since younger patients are supposed to have a stronger immune response. Nevertheless, we did not observe specific staining in the healthy donor cohort as evidenced by the lack of significant blocking of the staining.

In conclusion, our study evaluated the long-term immunity through the characterization of SARS-COV-2 specific B_{MEMORY} cells in a cohort of mild/moderate symptomatic COVID-19 patients versus healthy control subjects. We were able to detect S-specific B cells in the COVID-19 cohort even in mild cases. Overall, our study demonstrating the formation of SARS-CoV-2-specific long-term immunity may enable further opportunities for improving general vaccination acceptance thus supporting efforts to implement effective public health care measures during the COVID-19 pandemic.

Conflict of interests

All authors declare no competing interests.

Authors' contributions

C.J.T., M.Ab., T.R. and N.Ba. designed the study. CJT, M.An., S.S., K.P. performed the experiments. A.D., K.P., F.S.S., O.W., T.H.W. recruited patients. C.J.T., M.Ab, T.R. and N.Ba. analysed the data. C.J.T., M.Ab., E.F. and N.Ba. wrote the manuscript. M.An., N.Br., U.S., G.H., M.E reviewed and edited the manuscript. N.Br. supervised the project. All authors discussed the results and commented on the manuscript.

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Tables

Table 1: List of essential protocol equipment

Materials	**Manufacturer**	**Country**	**Cat. Nr.**
Vacutainer EDTA 10ml (K2E K2EDTA)	Greiner Bio-One	Austria	455046
BioColl separating solution	Bio&Sell	Germany	L 6115
Dulbecco's Phosphate Buffered Saline	Gibco	USA	14190-094
Transfer pipette 3.5ml	Sarstedt	Germany	861171001
50 ml sterile conical tubes	Greiner Bio-One	Austria	227261

Materials	**Manufacturer**	**Country**	**Cat. Nr.**
Trypan Blue	Biochrome	Germany	L6323
Roswell Parm Memorial Institute 1640 + GlutaMax	Gibco	USA	61870-036
Penicillin Streptomycin	Gibco	USA	15140-122
Fetal bovine serum	Biochrome	Germany	S0115
5ml Polystyrene Round-Bottom Tube	Falcon	USA	352052
Brilliant stain buffer	BD Horizon	USA	566349
SARS-CoV-2 (2019-nCoV) Spike S1+S2 ECD-His recombinant Protein	Sinobiological	China	40589-V08B1
Lightning-Link Rapid Fluorescein Labeling Kit	Novus Biologicals	USA	310-0010
Lightning-Link Rapid Cy5 Labeling Kit	Novus Biologicals	USA	342-0010
SafeSeal tube 0.5ml	Sarstedt	Germany	72.704
Devices	**Manufacturer**	**Country**	
Standard laboratory equipment			
CytoFLEX LX	Beckman Coulter	USA	
Software	**Manufacturer**	**Country**	
FlowJo version 10.6.2	BD Biosciences	USA	
Prism version 7	GraphPad software	USA	
R, version 3.6.2	R-Core-Team	Austria	

Table 2: B-cell staining panel

B cell panel

Antigen	**Fluorophore**	**Clone**	**Manufacturer**	**Cat. Nr.**
CD27	PE	O323	BioLegend	302808
CD20	BV510	2H7	BioLegend	302340
IgD	VioBlue	IgD26	Miltenyi Biotec	130-123-319
CD19	BV605	HIB19	BioLegend	302244
CD38	BV650	HB-7	BioLegend	356620
CD3	BV785	OKT3	BioLegend	317330
CD14	APC-Cyanine 7	M5E2	BioLegend	301820
DAPI			ThermoScientific	62248

Table 3: Baseline characteristics and protocol parameters applied for COVID-19 patient and healthy donor validation cohort.

COVID-19 patient cohort

ID	**Severity [mild, severe]**	**Age [years]**
P01	mild	30
P02	mild	27
P03	mild	53
P04	mild	51
P05	mild	74
P06	mild	55
P07	mild	77
P08	mild	81
P09	mild	61
P10	mild	41
P11	mild	63

P12	mild	57
P13	mild	35
P14	mild	47
P15	moderate	n.a.
P16	mild	27
P17	mild	66
P18	mild	59
P19	mild	71
P20	moderate	79
P21	severe	43
P22	severe	66
P23	moderate	51
P24	moderate	60
P25	severe	88
P26	moderate	82
Median	Median	59
Min	Min	27
Max	Max	88
Healthy donor cohort	**Healthy donor cohort**	**Healthy donor cohort**
H01	NA	45
H02	NA	58
H03	NA	48
H04	NA	28
H05	NA	27
H06	NA	49
H07	NA	26
H08	NA	48
H09	NA	60
H10	NA	60
H11	NA	40
H12	NA	49
H13	NA	60
H14	NA	46
Median	Median	48
Min	Min	26
Max	Max	60
COVID-19 vs. Healthy donor cohort	**COVID-19 vs. Healthy donor cohort**	**COVID-19 vs. Healthy donor**
P=	P=	0.0323

Figure legends

Figure 1: Conceptual overview of the protocol for SARS-CoV-2 spike specific Memory B-cell detection . *Detailed B-cell staining panel is provided in Table 2. Abbreviations: EDTA = Ethylenediaminetetraacetic acid, PBMC = Peripheral blood mononuclear cell.

Figure 2: Detection of SARS-CoV-2 specific B cell subsets. (a) Gating strategy for the detection of B cell subsets. (b) Representative example for the detection of dual-labelled SARS-CoV-2 S-protein binding B-cells and quantification of antigen-specific B cell subsets. Comparison of samples without fluorochrome-coupled SARS-CoV-2 protein ("No S-protein") and SARS-CoV-2 S-protein in concentrations of 1:500 and 1:1000 with and without excess unlabelled protein to block B cell receptors.

Figure 3: Detection of COVID-19 specific BMEMORY cells using spike (S)-protein. Periph-

eral blood mononuclear cells of convalescent COVID-19 patients (n=26) and healthy donors (n=14) were incubated with fluorochrome labeled SARS-CoV-2 S-protein with or without excess unlabelled protein to block labeling. (a) Percentage of IgD- CD27+ B_{MEMORY}, IgD+ CD27+ unswitched B_{MEMORY}, IgD+ CD27- B_{NAIVE} and CD27++ CD38++ plasmablasts within the entire B-cell population analysed for COVID-19 disease and healthy cohorts. Bars show median with interquartile range. (b) Correlation of fluorochrome labelled SARS-CoV-2 S-protein binding B cells and anti-S1/S2-IgG. n=21 samples of 19 COVID-19 patients (two patients with two samples collected at different time points) and 14 healthy donors. Analysis was performed with Spearman's rank coefficient, rho=0.42, P=0.011. (c)-(f) Frequencies of S-protein binding B_{MEMORY} (c), B_{NAIVE} (d), plasmablasts (e) and unswitched B_{MEMORY} cells (f) after staining with preincubation of unlabelled Covid-19 antigen (blocked, left) and without preincubation (not blocked staining, right) samples. n=26 COVID-19 patients and n=14 healthy donors. Statistical comparison was done with two-way repeated measurements ANOVA and Sidak's multiple comparisons test. (g) Correlation of fluorochrome labelled SARS-CoV-2 S-protein binding B cells and days after COVID-19 diagnosis. n=25 samples of 23 COVID-19 patients (two patients with two samples collected at different time points). The analysis was performed with Spearman's rank coefficient, rho= -0.34, P=0.095.