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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Detection of SARS-CoV-2-specific memory B cells to delineate long-term COVID-19 immunity

To the Editor,

The COVID-19 pandemic has led to devastating health outcomes with the death toll exceeding two million cases as of February 2021. However, there is still limited data on long-term immunity against SARS-CoV-2. Long-term immunity can be analysed by SARS-CoV-2-specific memory T and B cell formation. We and others have reported on SARS-CoV-2-specific memory T-cell responses in acute infection and long-term follow up.[1,2] To our knowledge, however, only studies with Australian and US-American cohorts assessed long-term B_{MEMORY}-cells for more than 6 months.[2,3] Memory B (B_{MEMORY})-cells can persist lifelong, and upon reinfection can be triggered to immediately start forming plasma cells secreting neutralizing antibodies.[4] Thus, quantifying B_{MEMORY}-cell levels may be used as an indicator of long-term immunity in convalescent patients. Therefore, this study aimed to delineate SARS-CoV-2 spike (S)-protein-specific B_{MEMORY}-cells in a well-characterized cohort of central-European COVID-19-patients up to several months after infection.

Between April and October 2020, a cohort of 27 convalescent COVID-19 patients and 14 healthy donors were included in the study in three German centers (Berlin, Bochum, Essen). Baseline characteristics are provided in Table S1. All patients gave written informed consent. The ethical committee of the Ruhr-University Bochum approved the study (20–6886). A schematic presentation of the protocol is depicted in Figure S1, and materials are listed in Table S2–S3.

COVID-19 patients were included at a median time of 53 days after diagnosis or onset of symptoms (range 15–214). SARS-CoV-2-specific B cell response was analysed by characterizing levels of IgD^-CD27^+ B_{MEMORY}, IgD^+CD27^+unswitched B_{MEMORY}, IgD^+CD27^- B_{NAIVE}, and $CD27^{++}CD38^{++}$plasmablasts (Figure 1). Overall B cell composition was not affected by SARS-CoV-2-infection in convalescent patients (Figure 2A). We did not observe T-cell lymphopenia in reconvalescent COVID-19 patients (Figure 2B).

For flow-cytometric analysis of specific B cells, SARS-CoV-2-S-protein was labelled with two different fluorochromes, and double-positive B cells were determined. Blocking of specific staining by excess unlabelled S protein demonstrates specificity of labeling.[5,6] Unlabelled S-protein in class-switched B_{MEMORY}-cells could significantly block binding in COVID-19 patients, but not in healthy donor samples, indicating only minimal cross-reactivity in healthy donors (Figures 1B, 2D). Accordingly, the frequency of S-protein-specific B_{MEMORY}-cells was significantly higher in the COVID-19-cohort compared to healthy individuals (Figure 2D). SARS-CoV-2-specific B_{MEMORY}-cells were also detectable 200 days

Thieme, Abou-el-Enein, Heine, Roch and Babel equal contribution

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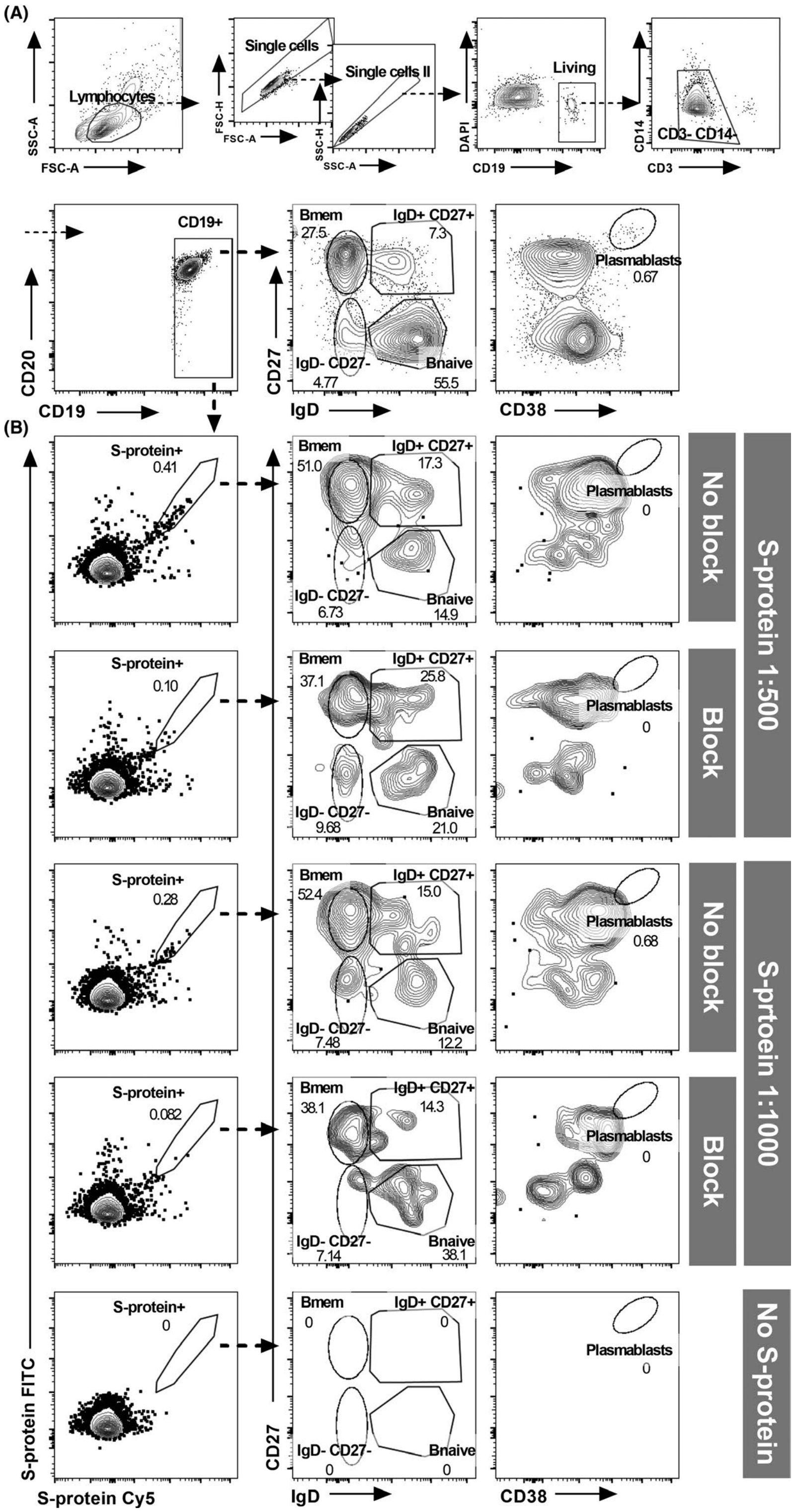

FIGURE 1 Detection of SARS-CoV-2-specific <>B cell subsets. (A) Gating strategy for the detection of B cell subsets. (B) Representative example for the detection of dual-labelled SARS-CoV-2 S-protein-binding B cells and quantification of antigen-specific B cell subsets. Comparison of samples without fluorochrome-coupled SARS-CoV-2 protein ("No S-protein") and SARS-CoV-2 S-protein in concentrations of 1:500 (200 ng of each labelled protein in 100 µl PBS) and 1:1000 (100 ng of each labelled protein in 100 µl PBS) with and without excess unlabelled protein to block SARS-CoV-2-reactive B cell receptors. Gates were set according to staining controls. To increase specificity and exclude unspecific fluorochrome-binding cells, only B cells double-positive for FITC and Cy5 labelled S-protein were considered as S-protein binding. In median, nearly 1 million lymphocytes were recorded per sample (minimum of three samples per patient, blocked and unblocked S-protein samples and staining controls). Of these, B cells contributed about 5% (median 48,611 cells, IQR 31,141–74,189 cells). The numbers of recorded antigen-specific cells varied greatly in the individuals. In the recovered COVID-19 patients, we recorded in median 73 S-protein binding cells in unblocked samples (IQR 25–163 cells)

after COVID-19 diagnosis with a tendency of lower frequencies in samples collected at later time points (Figure 2C). Specific labelling with SARS-CoV-2 S-protein and difference between COVID-19 patients and healthy individuals was restricted to B_{MEMORY}-cells and not observed for other B cell subsets (Figure 2D–G). S-protein-specific B_{MEMORY}-cells have been described to be pivotal for effective antibody responses.[4] SARS-CoV-2-specific B_{MEMORY}-cells correlated moderately with anti-S-protein IgG-antibodies, but correlation with neutralizing antibodies did not achieve statistical significance (Figure 2H–I). Thus, we demonstrate specific detection of SARS-CoV-2-S-protein-binding B_{MEMORY}-cells over 6 months post-infection.

We acknowledge limitations of our study. Subsequent studies should enrol larger patient cohorts with longer follow-up periods. Using bifluorescent tetramer-based staining may increase sensitivity to detect SARS-Cov2-specific B cells.[6] Control patients were

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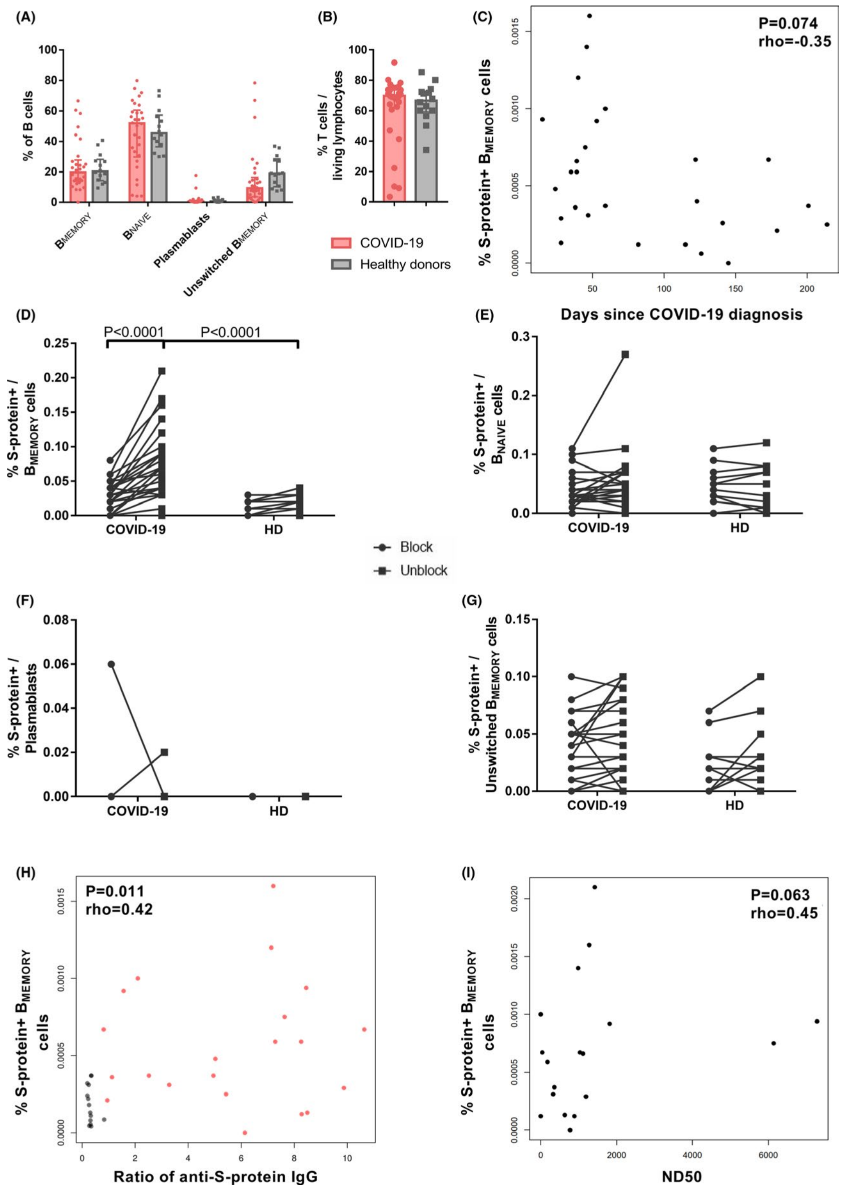

FIGURE 2 Detection of COVID-19-specific B_{MEMORY} cells using spike (S)-protein. Peripheral blood mononuclear cells of convalescent COVID-19 patients (n = 26) and healthy donors (n = 14) were incubated with 200 ng dissolved in 100 µl PBS (1:500) Cy5 as well as FITC fluorochrome-labelled SARS-CoV-2 S-protein with or without excess unlabelled protein to block labelling. For three COVID-19 patients, follow-up samples from two different time points were included. All but six samples of COVID-19 patients were directly processed without prior freezing and storage. (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. Repeated measurements two-way ANOVA did not detect significant differences between COVID-19 patients and healthy donors. (B) Percentage of $CD3^+$ T cells within living lymphocytes of COVID-19 and healthy donors. Bars show median with interquartile range. Parametric distribution was assessed with Shapiro–Wilk normality test and two-tailed Mann–Whitney test used for statistical comparison. (C) Correlation of fluorochrome labelled SARS-CoV-2 S-protein binding B cells and days after COVID-19 diagnosis. N = 27 samples of 24 COVID-19 patients (three patients with two samples collected at different time points). The analysis was performed with Spearman's rank coefficient, rho = −0.34, p = .095. (D)–(G) Frequencies of S-protein binding B_{MEMORY} (D), B_{NAIVE} (E), plasmablasts (F), and unswitched B_{MEMORY} cells (G) 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. Only the first sample of patients with multiple samples was included. Statistical comparison was done with two-way repeated measurements ANOVA and Sidak's multiple comparisons test. (H) Correlation of fluorochrome labelled SARS-CoV-2 S-protein binding B_{MEMORY}-cells and anti-S1/S2-IgG. Red dots: 21 samples of 19 COVID-19 patients (two patients with two samples collected at different time points). Grey dots: 14 healthy donors. Analysis was performed with Spearman's rank coefficient, rho = .42, p = .011. (I) Correlation of fluorochrome labelled SARS-CoV-2 S-protein binding B_{MEMORY}-cells and serum 50% neutralization dose titre (ND50). Virus neutralization was assessed using a propagation incompetent vesicular stomatitis pseudovirus system bearing SARS-CoV-2 S-protein. N = 18 samples of 17 patients (one patient with two samples collected at different time points). Analysis was performed with Spearman's rank coefficient, rho = .45, p = .063

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significantly younger than the COVID-19-cohort. Nevertheless, we did not observe specific staining in the control cohort as evidenced by the lack of significant blocking of the staining.

In conclusion, evaluating the long-term immunity in a cohort of convalescent COVID-19 patients, we demonstrated SARS-COV-2-specific B_{MEMORY}-cells in individuals both early as well as over 6 months after infection. Thus, our study performed on a central-European cohort is in line with the data on the recently published US-American and Australian cohorts and accordingly, confirms and extends the knowledge on the B cell response against SARS-CoV-2.[2,3] Demonstrating the persistence of SARS-CoV-2-specific B cell response, our results point towards an additional hallmark of immunisation beyond specific serum antibodies.

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KEYWORDS

COVID-19, long-term immunity, memory B cells, SARS-CoV-2

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CONFLICT OF INTERESTS

All authors declare no competing interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Higher risk of allergies at 4–6 years of age after systemic antibiotics in the first week of life

To the Editor,

In humans, the first 100 days appear to be a "critical window" of colonization during which microbial communities shape immune maturation.[1,2] The use of antibiotics early in life may disrupt the normal maturation process leading to adverse health outcomes such as atopic disorders.[1,3-5] The effects of antibiotic exposure immediately

TABLE 1 Baseline characteristics

	AB− N = 227	AB+ N = 114	AB2 N = 32	AB7 N = 82
Age median (IQR)[a]	5 (4.6–5.9)	4.7 (4.4–5.0)	4.7 (4.4–5.3)	4.7 (4.4–5.0)
Sex (male n %)	122 (54)	65 (57)	14 (44)	51 (62)
BMI mean (SD)	15.6 (1.5)	15.6 (1.4)	15.7 (1.4)	15.5 (1.4)
Delivery mode[a] *n* (%)				
Vaginal	146 (64)	86 (75)	22 (69)	64 (78)
C-Section	81 (36)	28 (25)	10 (31)	18 (22)
Breastfeeding				
Median duration (IQR)	4 (1–8)	2.5 (1–7)	2 (0–7)	3.5 (1–6)
Median duration exclusive (IQR)	2 (0–5)	0 (0–4)	0 (0–6)	0.5 (0–4)
Pets *n* (%)				
No	84 (37)	45 (39)	11 (34)	34 (42)
Cat	59 (26)	34 (30)	9 (28)	25 (31)
Dog	37 (16)	18 (16)	6 (19)	12 (15)
Cat +dog	23 (10)	7 (6)	3 (9)	4 (5)
Other	24 (11)	10 (9)	3 (9)	7 (9)

(contintues)

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