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Title:
SARS-CoV-2 cross-reactive B and T cell responses in kidney-transplant patients
The manuscript is submitted as an original work.

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**Tables:** 4
**Figures:** 4 (1 & 2 in colour, figure 3 & 4 black and white)

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**Highlights**
- Similar frequencies of preexisting SARS-CoV-2 T and B cell subsets among Tx and non-Tx individuals
- Detection of cytokine producing preexisting CD4+ T cells among the transplant individuals
- Transplant adults can generate preexisting CD4+CXCR5+ cells

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**Abstract**
Background: Immune responses to seasonal endemic coronaviruses might have a pivotal role in protection against SARS-CoV-2. Those SARS-CoV-2-crossreactive T cells were recently described in immunocompetent individuals. Still, data on cross-reactive humoral and cellular immunity in kidney transplant recipients is currently lacking.

Methods: The preexisting, crossreactive antibody, B, and T cell immune responses against SARS-CoV-2 in unexposed adults with kidney transplantation (Tx, n=14) and without (non-Tx, n=12) sampled before the pandemic were compared with 22 convalescent COVID-19 patients (Cp) applying ELISA and flow cytometry.

Results: In both unexposed groups SARS-CoV-2 IgG antibodies were not detectable. Memory B cells binding spike (S) protein SARS-CoV-2 were detected in unexposed individuals (64% Tx, 50% non-Tx) and higher frequencies after infection (80% Cp). The numbers of SARS-CoV-2-reactive T cells were comparable between Tx and non-Tx. Of note, SARS-CoV-2-reactive follicular T helper (Tfh) cells were present in 61% of the unexposed cohort in both, Tx and non-Tx.

Conclusions: Cross-reactive memory B and T cells against SARS-CoV-2 exist also in transplanted adults suggesting a primed adaptive immunity. The impact on disease course may depend on the concomitant immunosuppressive drugs.

Statements and Declarations
The authors have no relevant financial or non-financial interests to disclose.

Introduction

Preexisting immunity to SARS-CoV-2, most likely resulting from cross-reactivity against seasonal corona viruses has been addressed early in the pandemic as a potential immunomodulatory factor affecting the immune response after SARS-CoV-2 infection or vaccination [1-5]. Grifoni et al. and Nelde et al. found wild-type S-reactive CD4+ T cells in 40%–60% and 80% of unexposed individuals, respectively, suggesting a SARS-T cell immunity that is cross-reactive with HCoV [6-7]. Despite the weak evidence of pre-existing SARS-CoV-2 cross-reactive serum antibodies in prepandemic donors [8-9], independent study groups have detected cross-reactive preexisting SARS-CoV-2 B cell memory cells in healthy individuals unexposed to SARS-CoV-2 [8-10]. Preexisting cross-reactive T memory cells are recalled and expanded upon SARS-CoV-2 infection, reinforcing but not preventing a robust and persistent primary response to new epitopes of SARS-CoV-2 [8, 11-12].
However, still data on preexisting SARS-CoV-2 immunity among immunosuppressed patients, such as transplant recipients, are currently scarce. In this study we aimed to characterize SARS-CoV-2-reactive preexisting SARS-CoV-2-reactive T and B cells in a cohort of renal transplant patients under immunosuppressive therapy that were unexposed to SARS-CoV-2 in comparison to unexposed immunocompetent blood donors.

Methods

Study participants
Peripheral blood mononuclear cells (PBMCs) from 26 individuals unexposed to SARS-CoV-2 were collected and cryopreserved between 2017 and January 2020, and from a control group of 22 Cp patients. The unexposed cohort consisted of two populations, the immunocompetent study control group of healthy donors (non-Tx, n=12) and immunocompromised kidney transplant recipients (Tx, n=14). Samples from the unexposed individuals were collected between 2017 and January 2020 before the spread of SARS-COV-2 in Europe, excluding any possibility of SARS-CoV-2 infection. Of note, the first SARS-CoV-2 infection in Germany was documented on 27.01.2020 [13], followed by documentation of the first case in the state of North Rhine-Westphalia on 26.02.2020. The study was approved by the Ethics Committee of the Ruhr University Bochum (20-6886) and University Hospital Essen (20-9214-BO). Written informed consent was obtained from all participants. Demographic characteristics are provided in Tables 1 and 2.

Preparation of PBMCs
Peripheral blood was collected in S-Monovette K3 EDTA blood collection tubes (Sarstedt). Collected blood was prediluted in phosphate-buffered saline PBS/BSA (Gibco) at a 1:1 ratio and underlaid with 15 mL of Ficoll-Paque Plus (GE Healthcare). Tubes were centrifuged at 800 g for 20 min at room temperature. Isolated PBMCs were washed twice with PBS/BSA and stored at -80 °C. The cryopreserved PBMCs were thawed by incubating cryovials for 2-3 minutes at 37 °C in a bead bath, washed twice in 37 °C RPMI 1640 medium (Life Technologies) supplemented with 1% penicillin-streptomycin-glutamine (Sigma–Aldrich) and 10% fetal calf serum (FCS) (PAN-Biotech) and incubated overnight at 37 °C.

Flow cytometry
Measurement of SARS-CoV-2-reactive T cells

In brief, as previously described [14], PBMCs were plated in 96-U-Well plates in RPMI 1640 medium (Life Technologies) and stimulated with SARS-CoV-2 S-peptide (Miltenyi Biotec) or left untreated as a control for 16 h. As a positive control, cells were stimulated with staphylococcal enterotoxin B (1 μg/mL, Sigma–Aldrich). After 2 h, brefeldin A was added. A detailed list of the antibody panel for general phenotyping and T cell activation ex vivo is shown in Table 3. After stimulation overnight, the PBMCs were stained with optimal concentrations of antibodies for 10 min at room temperature in the dark. Stained cells were washed twice with PBS/BSA before preparation for intracellular staining using the Intracellular Fixation & Permeabilization Buffer Set (Thermo Fisher Scientific) according to the manufacturer’s instructions. Fixed and permeabilized cells were stained for 30 min at room temperature in the dark with an optimal dilution of antibodies against the intracellular antigen. All samples were immediately acquired on a CytoFLEX flow cytometer (Beckman Coulter). Quality control was performed daily using the recommended CytoFLEX daily QC fluorospheres (Beckman Coulter). No modification to the compensation matrices was required throughout the study. Antigen-reactive responses were considered positive after the nonreactive background was subtracted and greater than 0.01% was detectable. Negative values were set to zero. In one exception to the abovementioned minimum limit of 0.01%, we evaluated all positive frequencies of CD4+CD154+CD137+CXCR5+ cells after the background was subtracted, as no large populations of Tfh cells were expected to be found in circulation.

Measurement of SARS-CoV-2-reactive B cells

As previously described [15], SARS-CoV-2 S1/S2-protein (henceforth referred to as S-protein) (Sino Biological Inc.) was aliquoted into three samples. Sample 1 was left unlabeled for blocking, and samples 2 and 3 were coupled to fluorescein isothiocyanate (FITC) and Cy5 fluorochromes, respectively. PBMCs were divided into three samples (blocked, unblocked, and negative control samples). Blocking was performed by using a 10 times excess of unlabeled protein. After blocking, PBMCs were surface-stained with fluorochrome-labeled antibodies, as described in Table 4. Finally, mixed FITC- and Cy5-labeled protein was added. Cells were stained for 10 min at 4 °C. After washing with PBS, the 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. Antigen-reactive responses
were considered positive after the blocked background was subtracted and greater than 0.001% was detectable. Negative values were set to zero.

**SARS-CoV-2 IgG Antibody Titers**

Peripheral blood was collected in S-Monovette Z-Gel (Sarstedt). SARS-CoV-2 IgG titers were analyzed in purified serum using a SARS-CoV-2 IgG kit (Euroimmun, Lübeck, Germany). The test was performed according to the manufacturer’s instructions. Briefly, serum samples were diluted 1:100 and added to plates coated with recombinant SARS-CoV-2 antigen. Bound SARS-CoV-2 S1 protein-reactive IgG was detected by horseradish peroxidase-conjugated anti-human IgG. The absorbance was assessed on a microplate reader at 450 nm with a reference at 620 nm and evaluated as the ratio of the absorbance of the sample to the absorbance of the internal standard.

**Statistical Analysis**

Flow cytometry data were analyzed using FlowJo version 10.6.2 (BD Biosciences); gating strategies are presented in figures 1 and 2. For the analysis of anti-SARS-CoV-2 T and B cells, a threshold of 0.01% and 0.001% was employed respectively, to define a detectable response. Single stains and fluorescence-minus-one controls were used for gating. Gates for each individual were adjusted according to the negative control. CD4+ T cells expressing CD154 and CD137 and CD8+ T cells expressing CD137 were defined as reactive T cells. Statistical analysis was performed using GraphPad Prism v7. Categorical variables are summarized as numbers and frequencies; quantitative variables are reported as medians and interquartile ranges. Normality tests were performed with D’Agostino & Pearson, Shapiro–Wilk and Anderson–Darling tests. All applied statistical tests were two-sided. The frequencies of SARS-CoV-2-reactive B and T cells in recovered COVID-19 patients and immunocompetent donors were compared using an exact two-tailed Mann–Whitney test, and for grouped data, the Mann–Whitney test was used. Unexposed and exposed patient age was compared using an unpaired two-tailed t-test, and sex was compared using a two-tailed Fisher’s exact test. p values below 0.050 were considered significant; only significant p values are reported in the figures. p values were not corrected for multiple testing, as this study was of an exploratory nature.

**Results**
1. Baseline characteristics of the study cohort
We analyzed 26 unexposed individuals unexposed to SARS-CoV-2, of which 14 were Tx and 12 non-Tx, and 22 Cp (Table 1). The median age of the unexposed individuals at the time of study inclusion was 69 years, with participant ages ranging from 37 to 91 years, with 58% males and 42% females. Tx patients (12 kidney transplant, 1 liver transplant, 1 combined kidney/liver transplant recipient) were significantly younger, with a median age of 55 years (range of 37-75 years, p=0.0069), compared to the non-Tx patients with a median age of 73 years (range of 49-91 years). We compared the Tx patients to immunocompetent Cp patients at a median time of 110 days after diagnosis or onset of symptoms (range of 22-198 days). All included Cp patients were confirmed to be SARS-CoV-2-positive by PCR. The median age of the Cp group was 54.5 years (range of 28-89 years) and not significantly different from that of the unexposed group (median age 69 years, range of 37-91 years) (p=0.5182, two-tailed unpaired t-test). There were no significant differences regarding sex between the unexposed and COVID-19 patients (p=0.5626, two-tailed Fisher’s exact test). Demographic characteristics are provided in Tables 1 and 2.

2. Presence of preexisting SARS-CoV-2-reactive T cells in unexposed study participants
As applied in previous studies [14, 16], antigen-reactive T cell responses were considered positive after the background was subtracted and greater than 0.01% was detectable. The exception to this rule was the detection of circulating follicular CD4+ T helper cells, for which no minimum numerical limit was set, due to the extremely low number of this cell population normally in circulation. We found detectable SARS-CoV-2 S-protein-reactive CD4+ and CD8+ T cells in 94% and 22% of unexposed individuals, respectively. The frequencies of SARS-CoV-2-reactive CD4+ T cells in the exposed cohort were higher, without statistical significance regarding SARS-CoV-2-reactive CD4+ T cells (p=0.19, Mann–Whitney test) (Fig. 3A). However, the frequencies of SARS-CoV-2-reactive CD8+ cells were significantly higher in the Cp cohort (p=0.0002, Mann–Whitney test) (Fig. 3B). Interferon γ (IFNγ)-producing S-protein-reactive CD4+ T cells showed significantly higher frequencies in Cp patients than in unexposed individuals (p=0.006). For all other cytokines determined in our study, no significant differences were observed regarding the frequencies of cytokine-producing CD4+ T cells between the Cp and unexposed study participants (Fig. 3C).

4. Positive frequencies of SARS-CoV-2-reactive follicular CD4+ T cells in the unexposed cohort
Tfh cells directly interact with B cells, indicate maturation of the humoral immune response and are crucial for the establishment of antigen-reactive B memory cells, which provide long-term immunity [17]. We characterized circulating SARS-CoV-2 S-protein-reactive Tfh cells in the unexposed cohort by the expression of CXCR5 (Fig. 3D). In one exception to the abovementioned minimum limit of 0.01%, we evaluated all positive frequencies of CD4+CD154+CD137+CXCR5+ cells after the background was subtracted, as no large populations of Tfh cells were expected to be found in circulation. Among the total population of unexposed individuals, 61% (n=11) showed positive frequencies for CD4⁺CXCR5⁻ T cells.

3. Detection of preexisting SARS-CoV-2 S-protein-reactive B cells in 58% of unexposed individuals

To explore whether B cells reactive against SARS-CoV-2 S-protein were detectable in unexposed individuals, we analyzed the frequencies of SARS-CoV-2 S-protein-reactive B cells by flow cytometry using FITC- and Cy5-labeled S-protein as previously described [15]. Specificity was controlled by blocking with excess unlabeled SARS-CoV-2 S-protein (Fig. 2) [18-19]. Double-positive S-protein-FITC- and S-protein-Cy5-reactive B cells were considered to specifically bind to the S-protein when the frequency was above 0.001% after the frequency of the blocked sample was subtracted.

We observed detectable S-reactive B cells in 80% of the Cp control group and in 58% of the unexposed individuals. The control group of Cp patients showed significantly higher frequencies of SARS-CoV-2-reactive B cells compared to unexposed individuals (p=0.0047, exact two-tailed Mann–Whitney Test) (Fig. 3E). Out of the 18 unexposed individuals with characterized T and B cell responses, 11 individuals presented preexisting SARS-CoV-2-specific Tfh cells, 7 of whom had a detectable SARS-CoV-2-specific B cell response.

5. Similar frequencies of preexisting T and B cell subsets among Tx and non-Tx individuals

The frequencies of SARS-CoV-2-reactive and cytokine-producing CD4⁺ T cells were similar among unexposed Tx patients and non-Tx individuals (Fig. 4A). Similarly, CD4⁺CXCR5⁻ cells among Tx patients and non-Tx participants showed no significant difference (Fig. 4C). The frequencies of SARS-CoV-2-reactive B cells in the Tx group were not significantly different compared to the immunocompetent participants (Fig. 4D, p=0.1588). Of note, SARS-CoV-2-reactive B cells were found more frequently among Tx patients, as 64% (n=9) Tx patients
showed SARS-CoV-2-reactive B cells compared to 50% (n=6) of non-Tx participants demonstrating SARS-CoV-2-reactive B cell immunity.

Discussion
Here, we report cross-reactive and preexisting B and T cell immunity to SARS-CoV-2 in a cohort of unexposed individuals, including immunocompetent individuals and renal transplant recipients. Our study suggests that renal transplant patients are able to generate a preexisting SARS-CoV-2 response that is comparable to immunocompetent adults.

Low frequencies of circulating preexisting Tfh cells could be detected in Tx and non-Tx to a comparable extent. Accumulating data show that SARS-CoV-2-reactive circulating Tfh cells play a key role in effective immunity and the generation of B cell memory and are consistent with the observation that the frequency of total circulating Tfh cells increases at the time of SARS-CoV-2 clearance and the detection of robust Tfh cell responses [20-23]. Lipsitch et al. imply in a theoretical model the potential contribution of SARS-CoV-2 preexisting Tfh cells in accelerating antibody production in the case of SARS-CoV-2 infection [24]. The presence of cross-reactive Tfh cells in the unexposed cohort, including immunosuppressed adults, may be of particular clinical importance and could in theory boost the protective role of preexisting SARS-CoV-2 immunity. In line, a detailed recent study shows robust vaccination-induced immune responses in NTX patients, depending on the concomitant medication, especially the absence of Rituximab [25].

We detected SARS-CoV-2-specific B cells among the unexposed individuals, and surprisingly, we observed a slight predominance in the Tx patients in the formation of preexisting B cells compared to non-Tx individuals. The predominance of Tx patients in the formation of preexisting B cells might be explained by the higher incidence of different viral, bacterial or fungal coinfections, as transplant patients demonstrate a broader antigenic experience with a higher chance of generating cross-reactive cellular immunity than the immunocompetent population. Bacher et al. and others have suggested that preexisting SARS-CoV-2 memory is the result of a diverse memory pool that may not be of viral origin at all, which accumulates in humans throughout life and might contain T cell receptors specific for neoantigens similar to the naive T cell pool with a broad range of affinities [12, 26-27].
A limitation of our study was the small number of patients, which makes robust assumptions challenging. Subsequent studies should enroll larger patient cohorts with a greater demographic variability to include individuals of all ages from different social levels and environments. Also multi-center design should be performed to exclude a local bias (ethnicity, environmental/seasonal corona viruses, treatment). The significant age gap between the Tx and non-Tx cohorts should also be taken into consideration. Overall, our study demonstrates preexisting SARS-CoV-2 immunity among the transplant cohort, which is comparable to the immunocompetent study group. Independent working groups demonstrate the poor immune response and waning of antibodies after SARS-CoV-2 infection or vaccination among transplant recipients [28-29]. Taking also into consideration the emerging SARS-CoV-2 variants of concern understanding the influence of preexisting cross-reactive immunity to SARS-CoV-2 on the adaptive immune response is of critical importance.

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Compliance with ethical Standarts
The study was approved by the Ethics Committee of the Ruhr University Bochum (20-6886) and University Hospital Essen (20-9214-BO). Written informed consent was obtained from all participants. The authors have no relevant financial or non-financial interests to disclose.

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Table 1: Demographic characteristics of the exposed and unexposed study participants

| SARS-CoV-2 unexposed patients |  |
|-------------------------------|--|
| Numbe r | Age-years (transplant cohort) | Female(%) transplant cohort(%) | Sample collection dates | Transplant (%) | Characterization of T Cells (Number of Tx Participants) | Characterization of B cells - number of participants |
|-------------------------------|--|--|----------------|----------------|----------------|----------------|
| 26 | N/A | 11(42%)-5(36%) | 2017-01/2020 | 14(54%) | 18(7) | 26 |
| Median | N/A | 69(55) | N/A | N/A | N/A | N/A |
| Min | N/A | 37(37) | N/A | N/A | N/A | N/A |
| Max | N/A | 91(75) | N/A | N/A | N/A | N/A |

| Convalescent COVID-19 patients (Cp) |  |
|-------------------------------|--|
| Age (years) | Gender | SARS-CoV-2 PCR | Sample collection day (Day 1=1st Positive PCR) | Disease Severity | Disease Outcome | Characterization of T Cells | Characterization of B cells |
|-------------------------------|--|--|----------------|----------------|----------------|----------------|----------------|
| CoV1 | 64 | W | positive | 110 | severe | Recover | x |
| CoV2 | 65 | W | positive | 121 | mild | Recover | y |
| CoV3 | 60 | M | positive | 121 | mild | Recover | y |
| CoV4 | 60 | M | positive | 121 | mild | Recover | y |
| CoV5 | 73 | M | positive | 79 | severe | Recover | y |
| CoV6 | 45 | W | positive | 45 | moderate | Recover | y |
| CoV7 | 67 | M | positive | 62 | severe | Recover | y |
| CoV8 | 89 | W | positive | 36 | severe | Recover | y |
| CoV9 | 52 | W | positive | 178 | mild | Recover | y |
| CoV10 | 75 | M | positive | 178 | mild | Recover | y |
| Patient | Age (years) | Gender | Transplanted organs | Year of transplantation | No. Previous transplants | Donor type (living/deceased) | IS (target concentration, ng/ml) | Viral co/infections (>1000 IU/ml) |
|---------|-------------|--------|---------------------|------------------------|-------------------------|----------------------------|--------------------------------|---------------------------------|
| Tx1     | 57          | M      | kidney              | 03.08.2019             | 0                       | deceased                   | Tacrolimus (3-5)                | BK-Virus (BKV)+CM V               |

Table 2: Clinical characteristics of transplant unexposed participants
| # | Tx   | Age | Sex | Organ   | Date     | Days | Status | Immunosuppression |
|---|------|-----|-----|---------|----------|------|--------|------------------|
| 1 | Tx2  | 55  | M   | kidney  | 19.12.2019 | 0    | deceased | Tacrolimus (4-7) |
|   |      |     |     |         |           |      |         | MMF 360mg        |
|   |      |     |     |         |           |      |         | Prednisolon 7.5mg|
| 2 | Tx3  | 51  | M   | kidney  | 06.12.2017 | 1    | deceased | Cyclosporin (80-120) |
|   |      |     |     |         |           |      |         | MMF 1080mg       |
|   |      |     |     |         |           |      |         | Prednisolon 5mg   |
| 3 | Tx4  | 72  | M   | kidney  | 04.02.2017 | 0    | deceased | Tacrolimus (3-5) |
|   |      |     |     |         |           |      |         | Everolimus (3-5) |
|   |      |     |     |         |           |      |         | Prednisolon 5mg   |
|   |      |     |     |         |           |      |         | EBV+CMV+BKV      |
| 4 | Tx5  | 55  | W   | kidney  | 07.05.2019 | 0    | deceased | Tacrolimus (4-6) |
|   |      |     |     |         |           |      |         | Everolimus (4-6) |
|   |      |     |     |         |           |      |         | Prednisolon 7.5mg|
| 5 | Tx6  | 73  | M   | kidney  | 29.12.2016 | 0    | deceased | Tacrolimus (4-6) |
|   |      |     |     |         |           |      |         | MMF 500mg        |
|   |      |     |     |         |           |      |         | Prednisolon 10mg |
| 6 | Tx7  | 51  | M   | kidney  | 08.01.2020 | 1    | deceased | Cyclosporin (80-120) |
|   |      |     |     |         |           |      |         | MMF 720mg        |
|   |      |     |     |         |           |      |         | Prednisolon 7.5mg|
| 7 | Tx8  | 69  | W   | kidney  | 10.12.2016 | 0    | deceased | Tacrolimus (4-6) |
|   |      |     |     |         |           |      |         | MMF 1440mg       |
|   |      |     |     |         |           |      |         | Prednisolon 5mg   |
| Tx  | Age | Sex | Organ  | Date of Transplant | Days | Status   | Immunosuppression | Pathogen |
|-----|-----|-----|--------|-------------------|------|----------|-------------------|-----------|
| Tx9 | 68  | W   | Kidney | 11.04.2019        | 0    | deceased | Tacrolimus (6-8)  | EBV       |
|     |     |     |        |                   |      |          | Prednisolon 7,5mg |          |
|     |     |     |        |                   |      |          | MMF 720mg       |          |
| Tx10| 52  | M   | Kidney | 04.01.2020        | 0    | living   | Tacrolimus (5-8)  | BKV       |
|     |     |     |        |                   |      |          | MMF 1440mg      |          |
|     |     |     |        |                   |      |          | Prednisolon 7,5mg |          |
| Tx11| 53  | M   | Kidney | 06.12.2015        | 0    | deceased | Tacrolimus (2-4)  | EBV       |
|     |     |     |        |                   |      |          | Everolimus (4-6)  |          |
|     |     |     |        |                   |      |          | Prednisolon 4mg   |          |
| Tx12| 37  | W   | Kidney | 19.02.2009        | 0    | deceased | Tacrolimus (4-6)  | EBV       |
|     |     |     |        | 11.12.2001        | 0    |          | Belatacept        |          |
|     |     |     | Liver  |                   | 0    |          | Prednisolon 7,5mg |          |
| Tx13| 75  | W   | Kidney | 20.02.2006        | 0    | deceased | Tacrolimus (5-7)  | EBV       |
|     |     |     |        |                   |      |          | MMF 1440mg       |          |
|     |     |     |        |                   |      |          | Prednisolon 5mg   |          |
| Tx14| 44  | M   | Liver  | 25.07.1998        | 0    | unknown  | Tacrolimus (5-7ng/ml)| EBV     |
|     |     |     |        |                   |      |          | Prednisolon 5mg   |          |

**Table 3:** Fluorochrome coupled antibodies and fluorescent dye for analysis of SARS-CoV-2 reactive T cells

| Antibodies or fluorescent dye | Fluorochrome | Source  | Cat. Nr. |
|------------------------------|--------------|---------|----------|
| Fixable Viability-Dye        | eFluor780    | eBioscience | 65-0865-14 |
| anti CCR7 (clone G043H7)     | PerCP-Cy5.5  | BioLegend | 353220   |
| Antibodies or fluorescent dye | Fluorochrome | Source      | Cat. Nr.   |
|-------------------------------|--------------|-------------|------------|
| anti CD20 (clone 2H7)         | BV510        | BioLegend   | 302340     |
| IgD (clone IgD26)             | VioBlue      | Miltenyi Biotec | 130-123-319 |
| anti CD19 (clone HIB19)       | BV605        | BioLegend   | 302244     |
| anti CD3 (clone OKT3)         | BV785        | BioLegend   | 317330     |
| anti CD14 (clone M5E2)        | APC-Cyanine 7| BioLegend   | 301820     |
| anti CD27 (clone O232)        | PE           | BioLegend   | 302808     |
| DAPI                          | N/A          | ThermoScientific | 62248     |

Table 4: Fluorochrome coupled antibodies and fluorescent dye for analysis of SARS-CoV-2 reactive B cells.

Figures
Figure 1. Flow cytometry gating strategy for identification and quantification of SARS-CoV-2 reactive T cells. PBMCs were stimulated for 16h with SARS-CoV-2 S-peptide. After 2h Brefeldin A was added to the culture to block secretion of cytokines and effector molecules. Living single lymphocytes were analyzed for expression of CD3, CD4, and CD8. CD4+ T cells (oranges boxes) were analyzed for the expression of CD154 and CD137. CD8+ T cells (green boxes) were analyzed for expression of CD137. Both CD4+ and CD8+ T cells were further analyzed for production of cytokines IFNγ, TNFα, IL-2 and GrB. The grey box includes untreated samples. Furthermore CD4+CD154+CD137+ and CD8+CD137+ cells were analyzed for the expression of CXCR5. Representative example of 26 unexposed and 14 convalescent patients. Plots of an unexposed study subject are being presented.
Figure 2. Flow cytometry gating strategy for identification and quantification of SARS-CoV-2 reactive B cells. Representative example for the detection of dual-labeled SARS-CoV-2 S-protein binding B-cells and quantification of antigen-reactive B cell subsets. Comparison of samples without fluorochrome-coupled SARS-CoV-2 protein (untreated) and SARS-CoV-2 S-protein in and without excess unlabeled protein to block B cell. Representative example of 26 unexposed and 14 convalescent patients. Plots of a convalescent COVID-19 patient are being presented.
Figure 3. Characterization of SARS-CoV-2 S-reactive T and B specific cells in unexposed and exposed subjects. Blood samples of 18 unexposed patients (7 out of 18 Tx) and 9 control Cp patients were stimulated with SARS-CoV-2 S-protein and analyzed by flow cytometry. (A) Frequencies of CD4+CD154+CD137+ and (B) CD8+CD137+. (C) Frequencies of mono- and bifunctional SARS-CoV-2 reactive CD4+ T cells expressing granzyme B (GrB), IFNγ, interleukin 2 (IL2) or tumor necrosis factor α (TNFα). (D) SARS-CoV-2 reactive CD4+CD154+CD137+ cells of unexposed donors were analyzed for CXCR5 positivity. (E) Correlation of fluorochrome labelled SARS-CoV-2 S-protein binding B cells in 26 unexposed and 14 Cp patients. Analysis was performed exact two-tailed Mann-Whitney Test.

As reactive SARS-CoV-2 T cells are defined the CD4+CD154+CD137+ and CD8+CD137+ cells. Negative controls were subtracted from reactive stimulated samples to exclude unreactive activation. Statistical comparison was done with Mann-Whitney-test. P<0.05 was considered significant, only significant p values are documented in the figures.
Figure 4. Characterization of SARS-CoV-2 reactive T and B cells in Tx and non-Tx donors. (A) CD4+CD154+CD137+ showed no significant statistical difference among Tx and non-Tx. (B) Analysis of the monofunctional CD4+CD154+CD137+ cells. (C) Correlation of CD4+CD154+CD137+CXCR5+ among Tx and non-Tx (D) Analysis of fluorochrome labelled SARS-CoV-2 S-protein binding B cells among the unexposed cohort Tx vs immunocompetent patients (p=0.1588).

Statistical comparison was done with Mann-Whitney-test. P<0.05 was considered significant, only significant p values are documented in the figures.