Detection of Humoral and Cellular Responses to SARS-CoV-2 in COVID-19 Convalescents

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Introduction

The emergence of the SARS-CoV-2 virus, causing the COVID-19 pandemic, is a major global health hazard. To combat COVID-19, comprehensive characterization of humoral and cellular immune responses to this virus is essential. Our goal was to develop reliable methods and tools for analyzing humoral and cellular B and T cell responses. These studies will facilitate scientific research on the prediction of disease progression, long-term immunity and will support evaluation of the efficacy of new vaccines with the ultimate goal to induce protective immune responses.

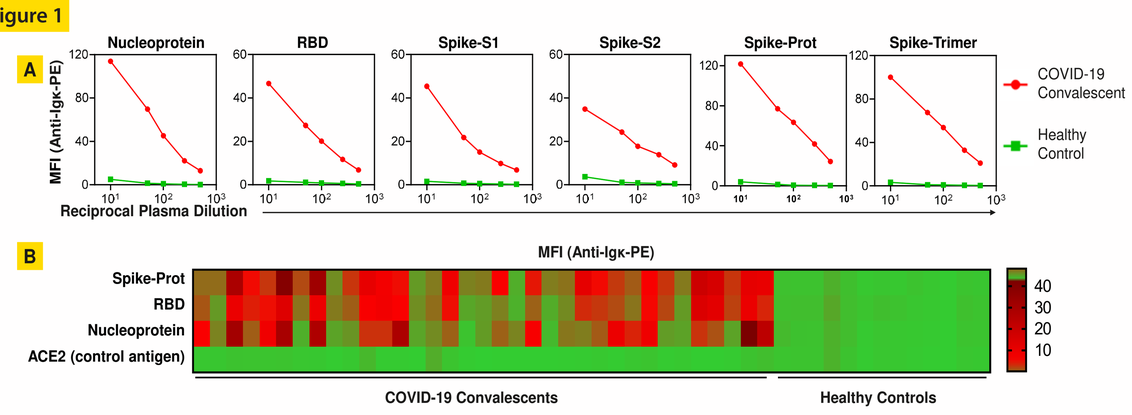
Methods

Plasma samples and PBMCs from COVID-19 convalescent and healthy / uninfected donors were obtained. Label-free B cells were obtained with REAlease® CD19 MicroBead Kit, human. Cells were used fresh or cryopreserved in STEMCELL™ Cryo-Brew. To enable characterization of humoral- and B cell- responses to SARS-CoV-2, recombinant protein variants of the crucial structural proteins of the virus were produced. The humoral response to SARS-CoV-2 infection was analyzed in a bead-based flow cytometric assay, where biotinylated recombinant SARS-CoV-2 proteins (Nucleoprotein, RBD, Spike-S1, Spike-S2, Spike-Prot and Spike-Trimer) and biotinylated control antigen (ACE2) were coupled to streptavidin-coated PMMA beads (PolyAn). After incubation with plasma samples from recovered COVID-19 patients or healthy donors, captured antibodies were detected with PE-conjugated secondary antibodies (e.g. anti-Igκ-PE, anti-IgG-PE, anti-IgM-PE). For flow-cytometric analysis, magnetic isolation and MACSQuant® Tyto®-sorting of SARS-CoV-2–specific B cells, antigen-tetramers were prepared by mixing Recombinant SARS-CoV-2 Spike-Protein (HEK)-Biotin or Recombinant SARS-CoV-2 RBD (HEK)-Biotin and fluorescently-labeled Streptavidin (PE or PE-Vio® 770) at a protein:streptavidin molar ratio of 4:1. For the magnetic separation, antigen-specific B cells that bound the antigen-tetramer-PE were subsequently labeled with anti-PE MicroBeads Ultrapure and magnetically separated over one MS column. Whenever further characterization of B cells was required, staining of cells using CD19-APC-Vio®770 (clone LT19), CD27 FITC (clone LG.3A10), anti-IgG VioBlue® (clone IS11-3B2.2.3), anti-IgA VioGreen® (clone IS11-8E10), anti-IgM APC (clone PJ2-22H3) and 7-AAD was performed. To identify antigen-reactive T cells, SARS-CoV-2 PepTivator® Peptide Pools were synthetized for the spike protein (S), the nucleoprotein (N) and the membrane protein (M) and used for stimulation. PepTivator Peptide Pools consist mainly of 15-mer peptides with 11-amino-acid overlaps, covering the sequence of the respective antigen. Samples were acquired with the MACSQuant Analyzer 10, MACSQuant X or MACSQuant Analyzer 16. Flow cytometric data were analyzed with the MACSQuantify™ or the Flowlogic™ Software. Data quantification and statistical analysis was performed using GraphPad Prism.

Results

1 Detection of SARS-CoV-2–specific antibodies in plasma of COVID-19 convalescents

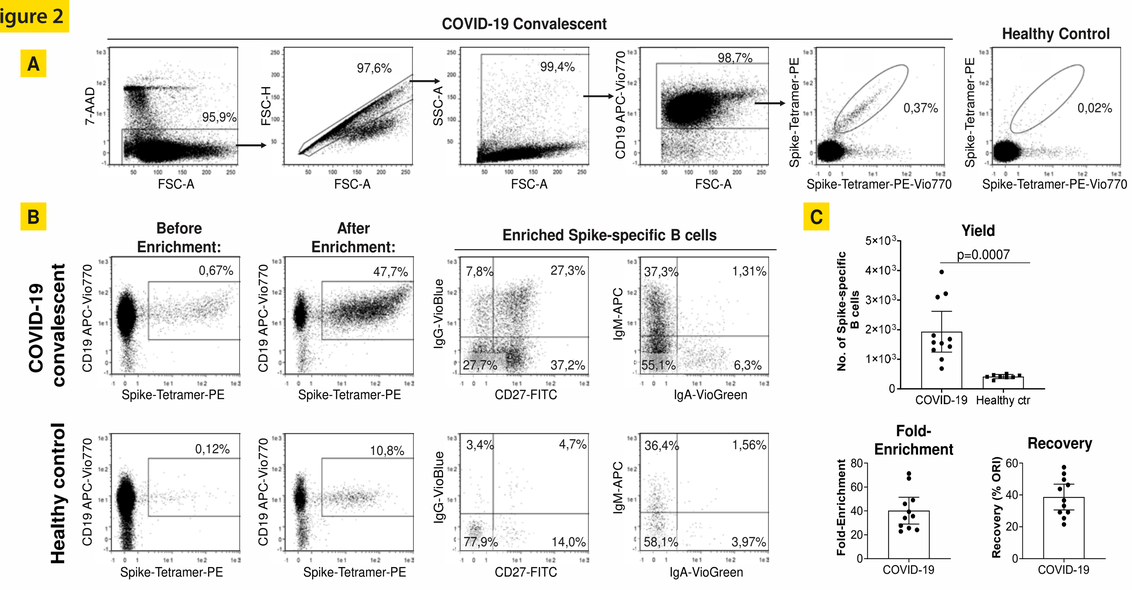
Figure 1



To enable detection of patient-derived antibodies against SARS-CoV-2 antigens, a bead-based flow cytometric immunoassay was established. First, the respective biotinylated recombinant SARS-CoV-2 proteins and the control antigen (ACE2) were coupled to streptavidin-coated PMMA beads. After incubation with plasma samples from recovered COVID-19 patients or healthy donors, captured antibodies were detected with anti-Igκ-PE. A) Quantification of median fluorescence intensity (MFI) for captured anti-SARS-CoV-2 antibodies at different plasma dilutions. B) Heat map for the quantified anti-Igκ-PE MFI values depicting plasma reactivity to SARS-CoV-2 antigens in recovered COVID-19 patients and healthy controls (1:50 dilution of plasma). ACE2 was used as a control antigen to determine the background immunoglobulin-binding level. Data demonstrates that our recombinant antigens are perfectly suited for highly sensitive characterization and quantification of humoral responses to SARS-CoV-2. Similar results could be obtained by using anti-IgG-PE and anti-IgM-PE (data not shown).

2 Detection and isolation of SARS-CoV-2 spike-specific B cells

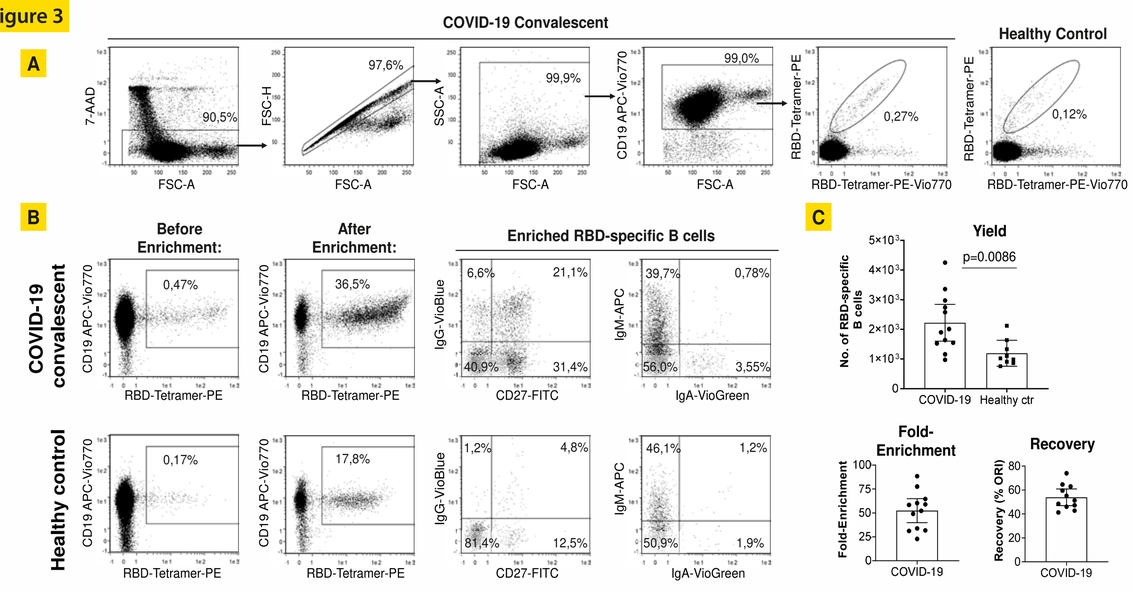
Figure 2



A) To detect antigen-specific B cells, freshly-isolated label-free B cells from recovered COVID-19 patients and healthy controls were stained with Recombinant SARS-CoV-2 Spike-Prot (HEK)-Biotin tetramerized on Streptavidin-PE and Streptavidin-PE-Vio770 (abbreviated as Spike-Tetramer-PE and Spike-Tetramer-PE-Vio770, respectively). An exemplary flow cytometric staining and gating strategy is displayed to identify spike-specific B cells using double discrimination of cells after labeling with two fluorescent tetramers for the same antigen. B) To isolate antigen-specific B cells, label-free B cells were labeled with Spike-Tetramer-PE, afterwards cells were incubated with anti-PE MicroBeads Ultrapure and magnetically separated over one MS column. Exemplary dotplots illustrate the outcome of spike-specific B cell isolation and phenotype of enriched cells. Spike-specific B cells from COVID-19 convalescents show predominantly memory phenotype (CD27$^+$) and express mostly IgG or IgM. C) Analysis of all samples processed illustrates the high specificity of our Recombinant SARS-CoV-2 Spike-Prot (HEK) and high-fold enrichment, yield and recovery of spike-specific B cells from COVID-19 convalescents, with significantly higher numbers of spike-specific B cells isolated from recovered COVID-19 patients than from uninfected controls (p=0.0007). Yield analysis shows numbers of isolated antigen-specific B cells per 1x10^6 label-free B cells. Each dot corresponds to one donor and means with 95% CI are shown.

3 Detection and isolation of SARS-CoV-2 RBD-specific B cells

Figure 3



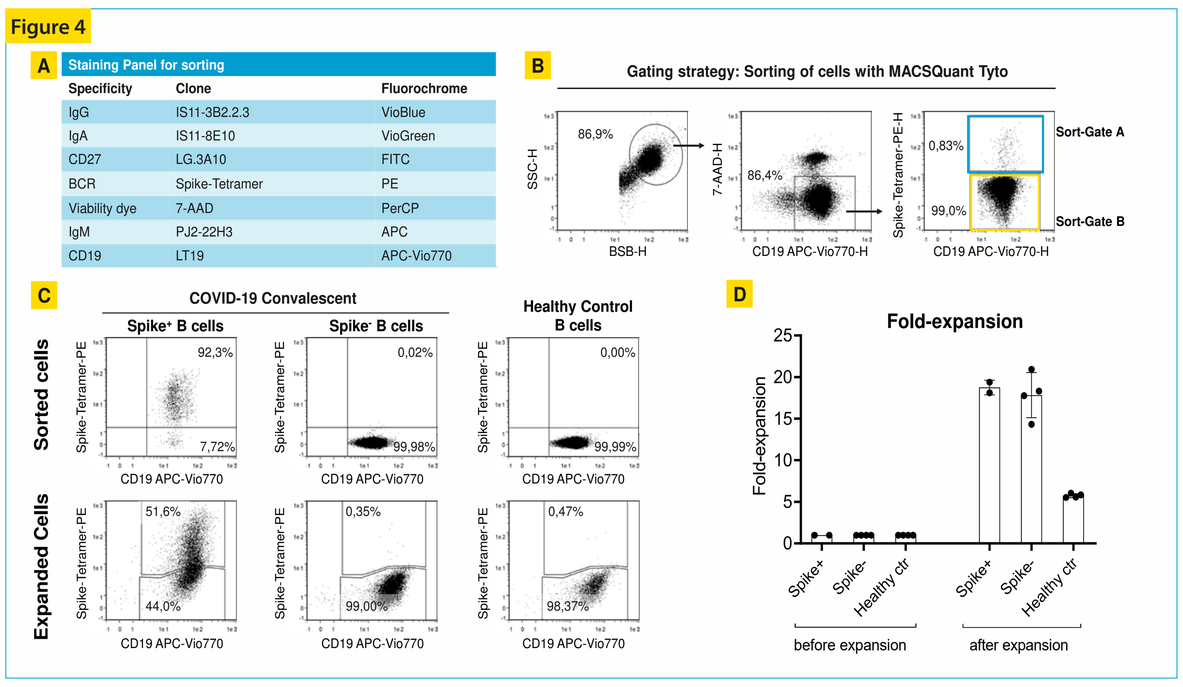
A) To analyze SARS-CoV-2-reactive T cells, PBMCs from COVID-19 convalescent patients were analyzed according to the procedure provided in the SARS-CoV-2 T Cell Analysis Kit (PBMC), human. PBMCs were stimulated with PepTivator Peptide Pools covering the protein sequences of the nucleoprotein (N), the membrane protein (M) and the spike protein (Mix S, containing S, S+ and S1 peptide pools) or combination of all mentioned peptide pools (Mix all). As the negative control (unstim.) cells were left untreated. Exemplary pseudo-color dotplots of the indicated conditions are shown. Gatings in the upper and lower row indicate the frequencies of CD154$^+$ within CD4$^+$ cells and TNFa$^+$ within CD8$^+$ T cells, respectively. B) Quantitative analysis of the frequencies of CD154$^+$ within CD4$^+$ T cells (left) and TNFa$^+$ within CD8$^+$ T cells (right). Each dot corresponds to one donor, the mean values with 95% CI are shown. C) Heat map of the frequencies of CD154$^+$ within CD4$^+$ T cells (upper) and TNFa$^+$ within CD8$^+$ T cells (lower) are shown. Data illustrates donor-dependent stimulation of SARS-CoV-2–specific T cells, while the strongest T cell immune response was observed with PepTivators covering the spike protein (Mix S) and the combination of all SARS-CoV-2 PepTivators (Mix all).

4 Sorting of antigen-specific B cells with the MACSQuant® Tyto®

Figure 4

A

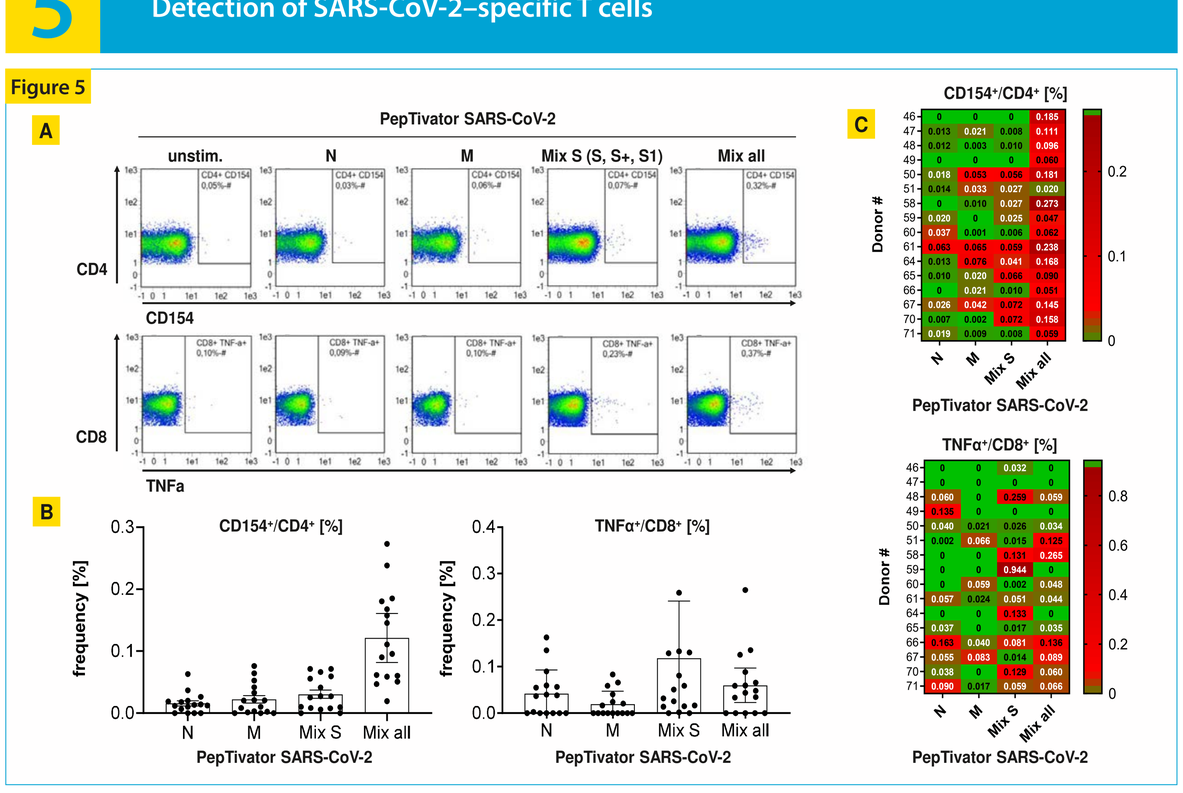
Staining Panel for sorting		
Specificity	Clone	Fluorochrome
IgG	IS11-3B2.2.3	VioBlue
IgA	IS11-8E10	VioGreen
CD27	LG.3A10	FITC
BCR	Spike-Tetramer	PE
Viability dye	7-AAD	PerCP
IgM	PJ2-22H3	APC
CD19	LT19	APC-Vio770



To demonstrate the feasibility of sorting of antigen-specific B cells in a fully closed cartridge system, previously cryopreserved label-free B cells isolated from recovered COVID-19 patient and healthy control were used. A) Staining panel composed of Recombinant SARS-CoV-2 Spike-Protein (HEK)-Biotin tetramerized on Streptavidin-PE and fluorochrome-conjugated antibodies to facilitate sorting and phenotype analysis of antigen-specific B cells with MACSQuant Tyto. B) Exemplary gating strategy for sorting of antigen-specific B cells (Sort-Gate A) or B cells lacking the SARS-CoV-2 specificity (Sort-Gate B). C) The purity analysis in the upper panel shows that highly pure fractions of spike-specific (Gate A, 92.3%) and non-specific (Gate B, >99.9%) B cells can be obtained. Recovery of spike-specific B cells was 81.4% (data not shown). Sorted B cells were expanded further using the B Cell Expansion Kit, human and phenotyped after 14 days of cultivation, as depicted in the lower panel. D) An exemplary quantification of B cell numbers expanded for 14 days using the B Cell Expansion Kit, human, demonstrating up to 20-fold-expansion of cells sorted with the MACSQuant Tyto. Expanded B cells showed secretion of spike-specific antibodies to the culture medium (data not shown). Each dot corresponds to the fold-expansion of cells harvested from one well in the experiment and mean value of the fold-expansion ± SD is shown.

5 Detection of SARS-CoV-2–specific T cells

Figure 5



Conclusions

We have developed effective tools and assays which ensure a high standard and reproducible results to support research activities for identification and characterization of SARS-CoV-2 reactive humoral and cellular B and T cell responses.
Our workflow supports:

- Sensitive and specific quantification of humoral responses to SARS-CoV-2
- Detection of SARS-CoV-2–specific B cells
- Enrichment and expansion of SARS-CoV-2–specific B cells for possible downstream applications (e.g. BCR cloning, sequencing)
- Detection of SARS-CoV-2–specific T cells

Together, these solutions ensure successful and reliable research, enabling valuable contributions into the investigation of healthy and dysfunctional immune reactions of SARS-CoV-2 infections.

Figure 6