Spike-specific immune response induced by BNT162b2 mRNA vaccine in former COVID-19 patients and high responsive subjects

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Abstract

Background: The worldwide escalation of Coronavirus Disease 2019 (COVID-19) has urgently required the development of safe and effective vaccines against the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is the causative agent of disease. The BNT162b2 (Pfizer–BioNTech) RNA-based vaccine confers 95% protection against COVID-19 by encoding a mutated isoform of SARS-CoV-2 full-length spike (S) protein.

Objective: Here, we report the antigen-specific immune profile against SARS-CoV-2 S protein after vaccination with a single dose of BNT162b2 in order to define the immunological landscape required for an efficient response to the SARS-CoV-2 vaccine.

Methods: We determined the levels of antibodies and antigen-specific B, T and NK-T cells against a recombinant GFP tagged SARS-CoV-2 S protein in subjects up to 20 days after injection of a single dose of BNT162b2 vaccine using a combined approach involving serological assays and flow cytometry analyses. Former COVID-19 patients have been also included in this study to evaluate the effect of vaccine after exposition to SARS-CoV-2.

Results: The level of antigen-specific helper T-cells against SARS-CoV-2 S protein was reduced in subjects, low responsive or unresponsive to vaccination with respect to the highly responsive individuals, while the numbers of antigen-specific regulatory and cytotoxic T-cells were comparable. Of interest, in former COVID-19 patients, a single dose of BNT162b2 vaccine induced a significant increase of antibody production simultaneous with an antigen-specific B and NK-T cell response.

Conclusion: Taken together, these results suggest that favorable immune profiles support the progression and an effective reaction to BNT162b2 vaccination.

Introduction

Coronavirus disease 2019 (COVID-19) is a new viral infection, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (1, 2) and declared pandemic by the World Health Organization (WHO) on March 2020. To date, COVID-19 has affected over 130,000,000 people and caused more than 2,800,000 deaths (WHO Coronavirus (COVID-19) Dashboard). Therefore, the development of safe and effective vaccines against SARS-CoV-2COVID-19 has urgently been needed (3-5).

The BNT162b2 (Pfizer–BioNTech) RNA-based vaccine was the first to obtain a conditional marketing authorization by the European Medicines Agency (EMA) for COVID-19 prevention (6). This consists of nucleoside-modified mRNA encapsulated in lipid nanoparticles, encoding the SARS-CoV-2 full-length spike (S) with two proline mutations to block pre- and post-fusion conformations (3, 7). The receptor-binding domain (RBD) of SARS-CoV-2 S protein plays an important role in the viral infection process fostering the binding of virus to host cells (8). This is the main target of cellular and humoral immune response inducing the production of antigen-specific antibodies (9). Moreover, recent studies have reported that a single dose of BNT162b2 vaccine induces anti-SARS-CoV-2 neutralizing antibodies with a highly detectable titer (4, 10-12) and enforces pre-existing T cell components, including T_H1 type CD4+ and IFNg+ CD8+ T cells (6, 13). Taken together, these results support the efficacy of BNT162b2 vaccine against SARS-CoV-2.

Here, we report the levels of antibodies and antigen-specific B, T and NK-T cells against a recombinant GFP tagged SARS-CoV-2 S protein in a cohort of healthy volunteers and former COVID-19 patients up to 20 days after injection of a single dose of BNT162b2 vaccine in order to determine the immunophenotypic cellular changes in response to COVID-19 vaccination.

Materials and Methods

Human samples and count blood cells. Blood samples of subjects, exposed at a single dose of BNT162b2 vaccine or controls were obtained at 20 days after injection with appropriate institutional approvals (CSS Research Ethics Boards), and informed consent under guidelines established by the Declaration of Helsinki. The complete blood count (CBC), including white blood cells (WBCs), neutrophiles, full lymphocytes, monocytes, eosinophiles and basophiles, was performed for each sample at the "Casa Sollievo della Sofferenza" Hospital by the Sysmex XT-4000i automated hematology analyzer following standard procedures (14).

SARS-CoV-2 IgG Assay. Blood serum samples were collected from a cohort of healthcare workers after Pfizer–BioNTech vaccination at our medical center in Southern Italy. The Siemens Healthineers SARS-CoV-2 IgG (COV2G) assay was employed for qualitative and semi-quantitative detection of IgG antibodies against SARS-CoV-2 using the Atellica® IM Analyzer and following manufacturer's procedures. Antibody levels were measured before vaccine for control samples and at day 20 after dose 1. Seropositive status due to prior SARS-CoV-2 exposure was determined on a previously established cutoff (15).

Cell culture and transfection. HEK-293T cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (ThermoFisher). The pcDNA3-SARS-CoV-2-S-RBD-sfGFP plasmid encoding the receptor binding domain (RBD) of SARS-CoV-2 protein S (spike) fused with sfGFP fluorescent marker was derived from Addgene (cod. # 141184)(8). An empty vector, expressing only the sfGFP was used as control in independent experiments. HEK-293T cells were transiently transfected using Polyethylenimine, Linear, MW 25000, Transfection Grade (PEI 25K) (Polysciences, Inc) as described previously (16). After transfection HEK-293T cells were cultured for 2 days and subsequently assessed for GFP expression by flow cytometry before protein purification.

Protein purification and Western Blot assay. HEK-293T cells, transiently transfected with pcDNA3-SARS-CoV-2-S-RBD-sfGFP plasmid or control were collected, washed in ice-cold phosphate-buffered saline and then lysed in ice-cold 50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM sodium chloride, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 2.5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, and protease inhibitor cocktail (cat #539134, Calbiochem). Whole cell lysates were incubated at 95°C for 10 minutes, loaded on SDS-PAGE gels and then transferred to Hybond-ECL membranes (Amersham). The membranes were blocked with 5% milk/0.3% TBS-Tween20 at 4°C for 1 hour and then probed with primary antibodies against GFP (1:1,000 dilution; cat. ab290, AbCam) or β-Actin (1:6,000 dilution; cat. A1978, Sigma). HRP-conjugated secondary antibodies (Cat. NEF812001EA, Perkin Elmer) were used at 1:10000 dilution. The chemiluminescent signal was detected with enhanced chemiluminescence (ECL) (cat. 32106, Pierce) and subsequently autoradiography.

Flow Cytometry. 30 μl of fresh blood from control or vaccinated individuals was treated with 300 μl of ammonium chloride solution (Cat. #07850, Stemcell Technologies). After red blood cell (RBC) lysis, cells were washed with DPBS and resuspended in 200 μl of DPBS with 35 μg of protein extracts, containing the recombinant sfGFP-tagged SARS-CoV-2 S/RBD protein or sfGFP only as control. Cells were incubated at 4°C for 20 min, washed with DPBS and then stained for surface markers (**Table S1**) in DPBS with BD Horizon Brilliant Stain Buffer (Becton Dickinson) for 20min at room temperature. The DRAQ7 fluorescent DNA dye (1:1,000 dilution; cat. D15106, ThermoFisher) was used to identify alive cells. We performed FACS assays on FACS Calibur, Canto2 (Becton Dickinson) and MoFlo Astrios cell sorter (Beckman Coulter) and used FlowJo software (Becton Dickinson) for the analysis of flow cytometry data and visualization of multiparameter data through the tSNE algorithm (17).

Statistics. We used GraphPad Prism 8.4.3 software for the visualization and statistical analyses of quantitative data, including Pearson and Spearman correlations and Welch's t-test.

Results and Discussion

Emerging studies including the serological level of SARS-CoV-2-specific antibodies in response to BNT162b2 (Pfizer–BioNTech) mRNA vaccine, have suggested that individuals previously infected with SARS-CoV-2 have naturally acquired an immunity and thus, proposed alternative approaches for the vaccination of former COVID-19 patients (10, 18-20). In order to explore the pre-existing and different immune profiles after anti-SARS-CoV-2 vaccination in low versus high responsive individuals as well as subjects previously infected with SARS-CoV-2, we analyzed blood samples, collected from 160 healthcare workers at a large medical center in Southern Italy up to 20 days after injection of a single dose of BNT162b2 mRNA vaccine. The enrolled cohort included Caucasian from the same geographic area and 52% female. The median age was 50 years, and 23% of participants were older than 60 years of age. Furthermore, 23% of subjects were former COVID-19 patients, completely negative to SARS-CoV-2 molecular testing, at least for two months (**Table 1**).

It has been recently reported that SARS-CoV-2-specific antibodies are detectable in the majority of seronegative individuals at day 20 after the first dose of BNT162b2 mRNA vaccine (18, 19). To assess the serological level of anti-SARS-CoV-2 spike (S) IgG antibody in our sample cohort, we performed a S-binding chemiluminescent immunoassay (21). We determined the antigen-specific antibody response to vaccine for all participants at day 20 and distinguished the low responsive individuals in the lower 10th percentile of antibody titer distributions (**Table 1**). Of interest, the levels of anti-SARS-CoV-2 spike-specific IgG were significantly increased in individuals previously exposed to SARS-CoV-2 as recently reported (10, 20) (**Figure S1A**). We also found a significant slight reduction in people older than 60 years of age (p-value=0.010, two-tailed Mann–Whitney test) (**Figure S1B**) and no difference between male and female at the considered time point (p-value>0.05, two-tailed Mann–Whitney test) (**Figure S1C**).

To assess the immunophenotypic cellular changes up to day 20 after the first dose of vaccine, we designed a multiparameter flow cytometry panel, including fluorophore-conjugated antibodies against surface lineage markers for the detection of discrete subsets of B, T and

natural killer (NK) cells. Furthermore, in this flow cytometry assay, the peripheral blood mononuclear cells (PBMCs) of all participants interacted with protein extracts including the recombinant protein constituted with the receptor binding domain (RBD) of SARS-CoV-2 S glycoprotein, fused to the superfolder green fluorescent protein (sfGFP) (8), in order to identify the S/RBD-binding cells within each population (**Figure 1**). Subsequently, t-SNE dimensional reduction (17) was performed for visualization of cell subsets in two dimensional plots using standard parameters (perplexity = 30, theta = 0.5). In this assay, the samples were divided in two main groups depending whether they were originated from seronegative (NO ex-COVID-19) or seropositive (ex-COVID-19) participants at the time (d0) of injection of the first dose of BNT162b2 vaccine. The "NO ex-COVID-19" subjects were further subdivided in low and high responsive individuals, based on the level of antibody titer (**Figure S1A**). Samples derived from not vaccinated seronegative (NO ex-COVID-19) or seropositive (ex-COVID-19) people were also analyzed and included as control. We observed that the proportions of all major lineages, such as total lymphocytes, B and T cells, were highly similar in all participants (**Figure 2**) and confirmed these observations by complete blood cell counts, including also neutrophiles, monocytes, eosinophiles and basophiles (**Figure S2**). Nonetheless, the frequencies of plasma B cells and helper and cytotoxic T cells were increased in seronegative highly responsive individuals with respect to the other cohorts (**Figure 2**). Of interest, we also observed an expansion of regulatory T cells and NK-T cell subsets specifically in seropositive (ex-COVID-19) subjects after exposure to BNT162b2 vaccine (**Figure 2**).

To assess antigen-specific cell subsets binding the recombinant GFP-tagged S/RBD protein, we determined the fraction of GFP positive cells within each cluster. As expected, we observed an increase of total and plasma B cells, interacting with S/RBD protein in all subjects at day 20 after vaccine. Of interest, the level of B cell subsets was higher in seropositive (ex-COVID-19) individuals with respect to the other cohorts (**Figure 3**). Furthermore, the level of CD3+ CD4+ CD8- helper T cells significantly boosted in the seronegative high versus low responsive participants (p-value=0.0004, two-tailed Welch's t-test), but was similar to seropositive subjects after vaccine, suggesting that distinct T cell subpopulations can stimulate the production of

antibodies against SARS-CoV-2 (**Figure 3**). We also observed an increase of antigen-specific CD3+ CD8+ CD4- cytotoxic T cells between responsive individuals and controls in the seronegative cohort, but not in the former COVID-19 patients. Nonetheless, the level of S/RBD-interacting regulatory T cells augmented only in the ex-COVID-19 participants after vaccine, but not in the seronegative cohort. We also found a statistically significant enrichment of S/RBD-interacting NK-T cells after exposure to BNT162b2 vaccine. Notably, the increase of S/RBD specific NK-T cells was higher in former COVID-19 patients than highly responsive seronegative subjects (p-value<0.0001, two-tailed Welch's t-test) (**Figure 3**). Taken together, these data suggest that the efficacy of immune response to vaccine involves cellular changes that might be enforced by pre-existing immunological states in former COVID-19 patients.

To assess any direct correlation between antibody levels and cellular changes, we determined the Pearson (r) and Spearman (ρ) correlation coefficients between S/RBD-interacting cells and anti-SARS-CoV-2 IgG levels at day20 after vaccine. As expected, the antibody titer was directly correlated with the levels of total and plasma B cell fractions in the considered cohort (**Figure 4**). Of interest, natural killer T cells were also significantly correlated with the anti-SARS-CoV-2 IgG level, suggesting that this cell subset can enhance the antibody production and B cell response to BNT162b2 vaccine as reported previously (22, 23). Finally, we did not found correlations between anti-SARS-CoV-2 IgG levels and T cell subsets, including helper, cytotoxic or regulatory T cells.

Previously described clinical trials reported that BNT162b2 mRNA vaccine is safe and 95% effective against COVID-19 (4, 24). Despite not being designed to assess the efficacy of a single-dose regimen, a divergence between placebo and vaccine recipients starts at 12 days after the first dose, an indication of an early onset of a partially protective immunization, with an efficacy of 52% in the interval between the first and second dose (4, 13, 25). In this work, we aimed to understand the immunological landscape and response after the first dose of the BNT162b2 mRNA vaccine, focusing on the antigen-specific immune profile against SARS-CoV-2 S. In addition to the expected increase in total and plasma B cells interacting with the antigen, we

noted that ex-COVID-19 participants had a higher level of B cells, while helper T cells were significantly higher in the high respondents' group and similar to seropositive subjects. Surprisingly, an increase of antigen specific NK-T cells characterized ex-COVID-19 participants, after injection of the first dose. Overall, our data suggest that there are changes in the cellular immunological landscape between participants that were exposed to SARS-CoV-2 infection and naïve participants, mainly characterized by an increased response in the previously exposed group, after the first dose of the BNT162b2 mRNA vaccine. Moreover, differences in naïve high and low participants' immune response also point towards a different pre-existing immunological landscape that correlates with a positive response to the first dose of the vaccine. Future studies could help in elucidating the underlying causes of these different responses that could arise from a history of previous similar infections, or genetic, individual, and environmental differences.

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Disclosure

The authors declare no competing financial interests.

Data availability statement

The data sets generated in the current study are available from the corresponding author on reasonable request.

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		DEMOGRAPHIC AND CLINICAL CHARACTERISTICS OF PARTECIPANTS									
		Sampling Distribution						COV-IgG			
		Counts	% of tot.	M	F	Low Responsive (COV-IgG < 3.05 AU/ml)	High Responsive (COV-IgG ≥ 3.05 AU/ml)	Min (AU/ml)	Max (AU/ml)	Mean	SD
INDIVIDUALS	NO ex-COVID-19	123	77%	57	66	30	93	0	> 750	77.8	196.6
	ex-COVID-19	37	23%	20	17	2	35	0.6	> 750	306.9	275.6
	Tot.	160	100%	77	83	32	128	0	> 750	130.8	237.2
		Counts	% of tot.	Average age	ex-COV. (%)	Low Responsive (COV-IgG < 3.05 AU/ml)	High Responsive (COV-IgG ≥ 3.05 AU/ml)	Min (AU/ml)	Max (AU/ml)	Mean	SD
GENDER	M	77	48%	50	26%	17	60	0	> 750	140.65	240.5
	F	83	52%	49	20.5%	15	68	0	> 750	121.61	235.0
		Counts	% of tot.	Gender	ex-COV. (%)	Low Responsive (COV-IgG < 3.05 AU/ml)	High Responsive (COV-IgG ≥ 3.05 AU/ml)	Min (AU/ml)	Max (AU/ml)	Mean	SD
AGE CLASS	21-30	16	10%	9M-7F	25%	1	15	1.7	> 750	126.84	217.9
	31-40	22	13.7%	8M-14F	27.3%	3	19	0.3	> 750	136.36	251.9
	41-50	34	21.3%	18M-16F	20.6%	5	29	0.5	673	136.98	238.2
	51-60	52	32.5%	20M-32F	21.2%	11	41	0	> 750	125.52	238
	60+	36	22.5%	22M-14F	25%	12	24	0	> 750	130.83	246.9

Table 1. Demographic distribution and anti-SARS-CoV-2 IgG plasma levels of participants. Whole blood samples were collected from 160 healthcare workers at a large medical center in Southern Italy. All participants are Caucasian from the same geographic area and 52% female. The median age was 50 years, and 23% of individuals (N=37) were former COVID-19 patients (ex-COVID-19), completely negative to SARS-CoV-2 molecular testing, at least for two months at the time (day 0) of BNT162b2 mRNA vaccine administration. Among the seronegative participants at day 0 (NO ex-COVID-19), we distinguished low (N=30) and high (N=93) responsive individuals based on the lower 10^{th} percentile of anti-SARS-CoV-2 IgG sampling distribution which set up the cut-off point at 3.05 AU/ml as determined by the SARS-CoV-2 IgG ADVIA Centaur® immunological assay (Siemens Healthineers).

Figure Legends

Figure 1. Immunophenotypic characterization of peripheral blood mononuclear cells (PBMCs) in response to a single dose of BNT162b2 vaccine.

A) Schematic overview of experimental approach for the identification of S/RBD-specific B, T and NK cell subsets. Specifically, HEK-293T cells were transiently transfected with pcDNA3.1-SARS-CoV-2-S-RBD-sfGFP vector, encoding the receptor binding domain (RBD) of SARS-CoV-2 protein S (spike) fused with sfGFP fluorescent marker. Afterwards, the recombinant GFP-tagged S/RBD protein was purified after 2 days from transfection and employed for the immunostaining of mononuclear cells (PBMCs) after red blood cell (RBC) lysis. Initially, cells interacted with the recombinant sfGFP-tagged SARS-CoV-2 S/RBD protein or sfGFP only as control. Subsequently after washing, cells were labelled with a panel of 8 fluorophore-conjugated antibodies against lineage-specific cell surface markers in order to identify the B, T and NK cell subset and GFP positive fraction in each cell population.

B) Gating strategy for classifying the different subpopulations of lymphocyte with the reported panel (Table S1). Fluorescence Minus One (FMO) controls were used to set up all gates. Singlets were initially discriminated on SSC-H and SSC-A, followed by the exclusion of non-viable cells with DRAQ7 far-red fluorescent DNA dye. Subsequent gating identifies CD3 + cells, followed by the identification of CD4+CD8- helper T cells and CD4-CD8+ cytotoxic T cells. Within CD4+ cell fraction, regulatory T cells were discriminated as CD127- $CD25^{high}$ cells. Natural killer T-cells were also identified as CD56+ cells in the CD3+ fraction. B cells were identified as CD3-CD19+ and plasma B cells were differentiated as $CD38^{high}$ within B cell subset. Finally, NK cells were discriminated as CD3- CD56+ CD19-.

C) Table summarizing the gating strategy described in B.

Figure 2. Distribution of lymphocyte populations at day 20 after BNT162b2 vaccination.

A) tSNE map based on the flow cytometry data from participants, subdivided in low (N=30) and high (N=93) responsive "NO ex-COVID-19" individuals at day 20 and control (N=30) at day 0. Flow cytometry data from seropositive (ex-COVID-19) individuals at days 0 and 20 were also analyzed and reported as response (N=37) and control (N=27) respectively. 100,000 live lymphocytes for each sample were considered for concatenation and downsampling. t-SNE dimensional reduction was performed for visualization of cell subsets for each cohort in two dimensional plots using standard parameters (perplexity = 30, theta = 0.5).

B) Heat map analysis of different immune cell subsets in the considered cohorts of participants. In the plot, the distribution of each cell population is indicated as frequency of total alive cells.

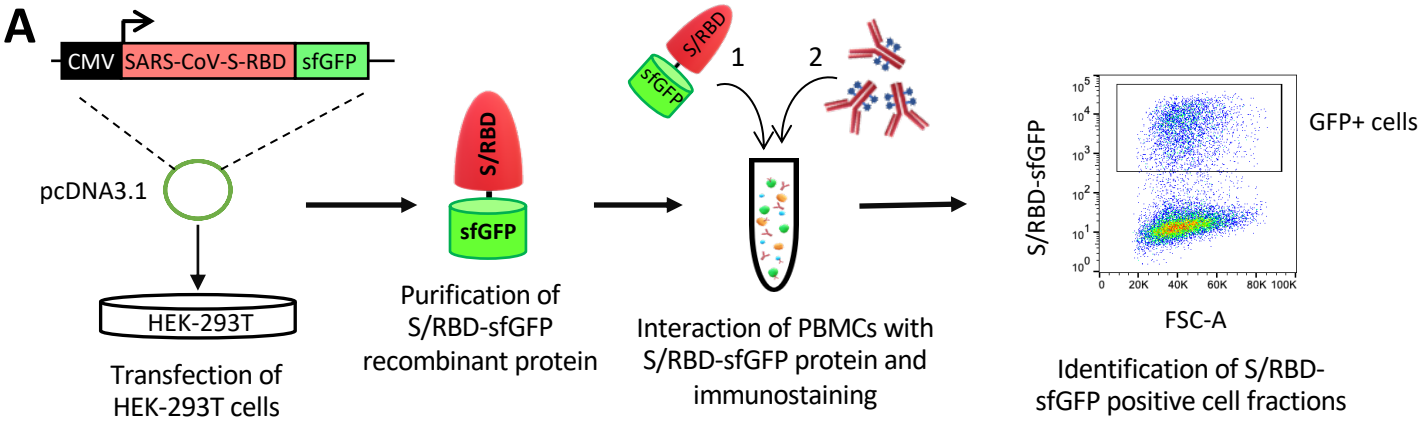
Figure 3. Distribution of spike/RBD-specific lymphocyte subpopulations in response to a single dose of BNT162b2 vaccine.

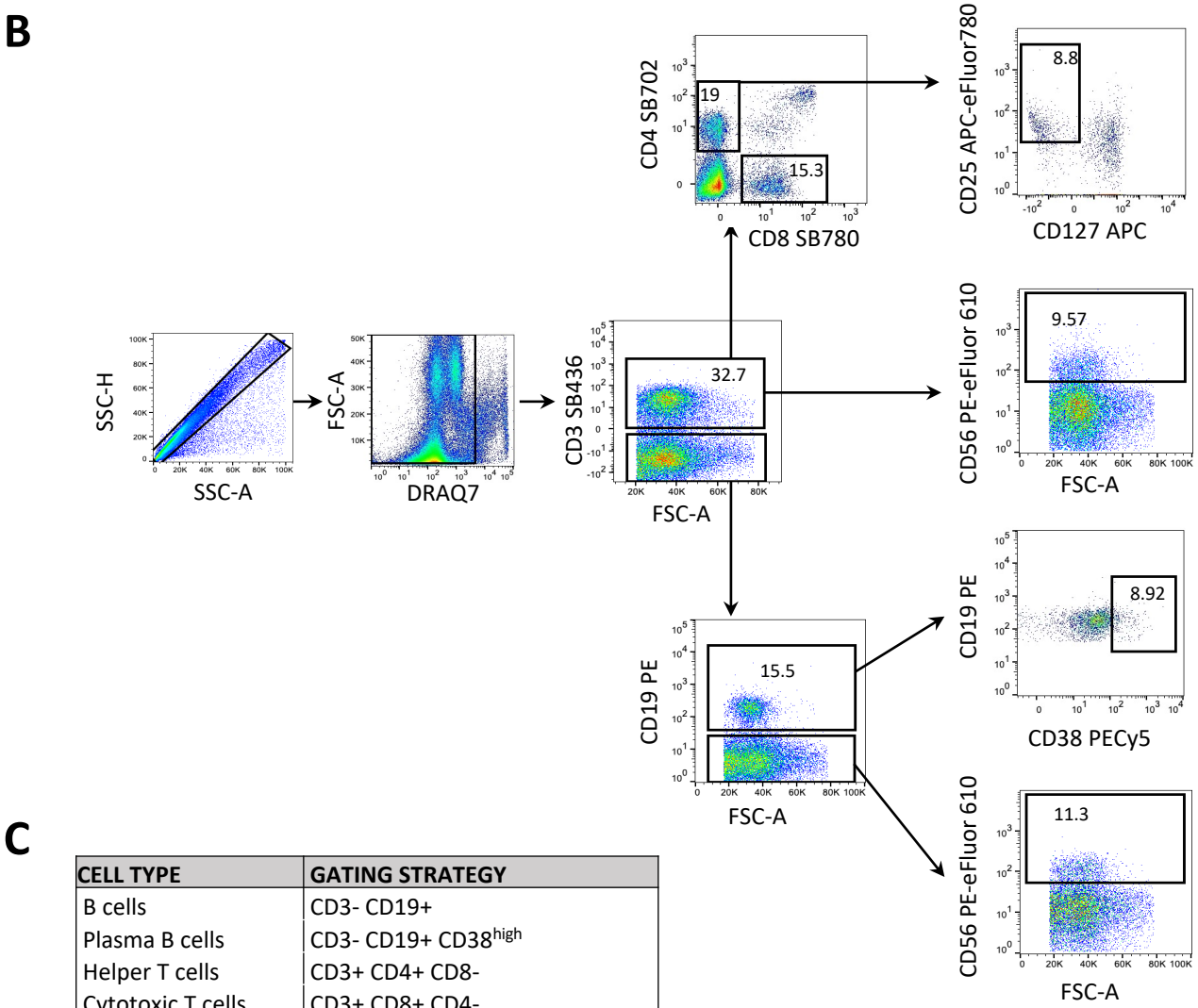
A) GFP+ cell fraction of indicated B, T and NK cell subsets as result of interaction with the recombinant GFP-tagged SARS-CoV-2 Spike RBD protein (S/GFP+). Seronegative participants at day 0 are reported as "NO ex-COV." and subdivided in low (in blue, N=30) and high (in red, N=93) responsive individuals based on the lower 10^{th} percentile of anti-SARS-CoV-2 IgG sampling distribution. Former COVID-19 patients at day 20 after vaccine are indicated as "ex-COV" (in green, N=37). The S/RBD-specific cellular response of not-vaccinated ex-COVID (N=27) and no ex COVID (N=28) samples are reported in black as control. Welch's t-test statistical analysis was performed on the percentage values of different GFP+ cell fractions.

B) In the table Welch's t-test p-values are indicated for each comparison reported in (A). *ns, not significant*; ***, p<0.05; **, p<0.01; ***, p<0.001, **** p<0.0001 (two-tailed unpaired Welch's t-test).*

Figure 4. The levels of S/RBD-binding B and NK-T cells are directly correlated with anti-SARS-CoV-2 IgG titer.

Correlation analysis between the abundance of indicated cell subsets, interacting with SARS-CoV-2 S RBD protein (S/GFP+ cells) and the anti-SARS-CoV-2 IgG levels in all vaccinated participants (N=160). Pearson correlation coefficient (r), Spearman's rank correlation coefficient (ρ) and their statistical significance (P-value) are reported in the graphs. Linear correlation was evaluated trough the linear regression model. The linear regression line in black and R squared (R^2) are also shown in the graphs.

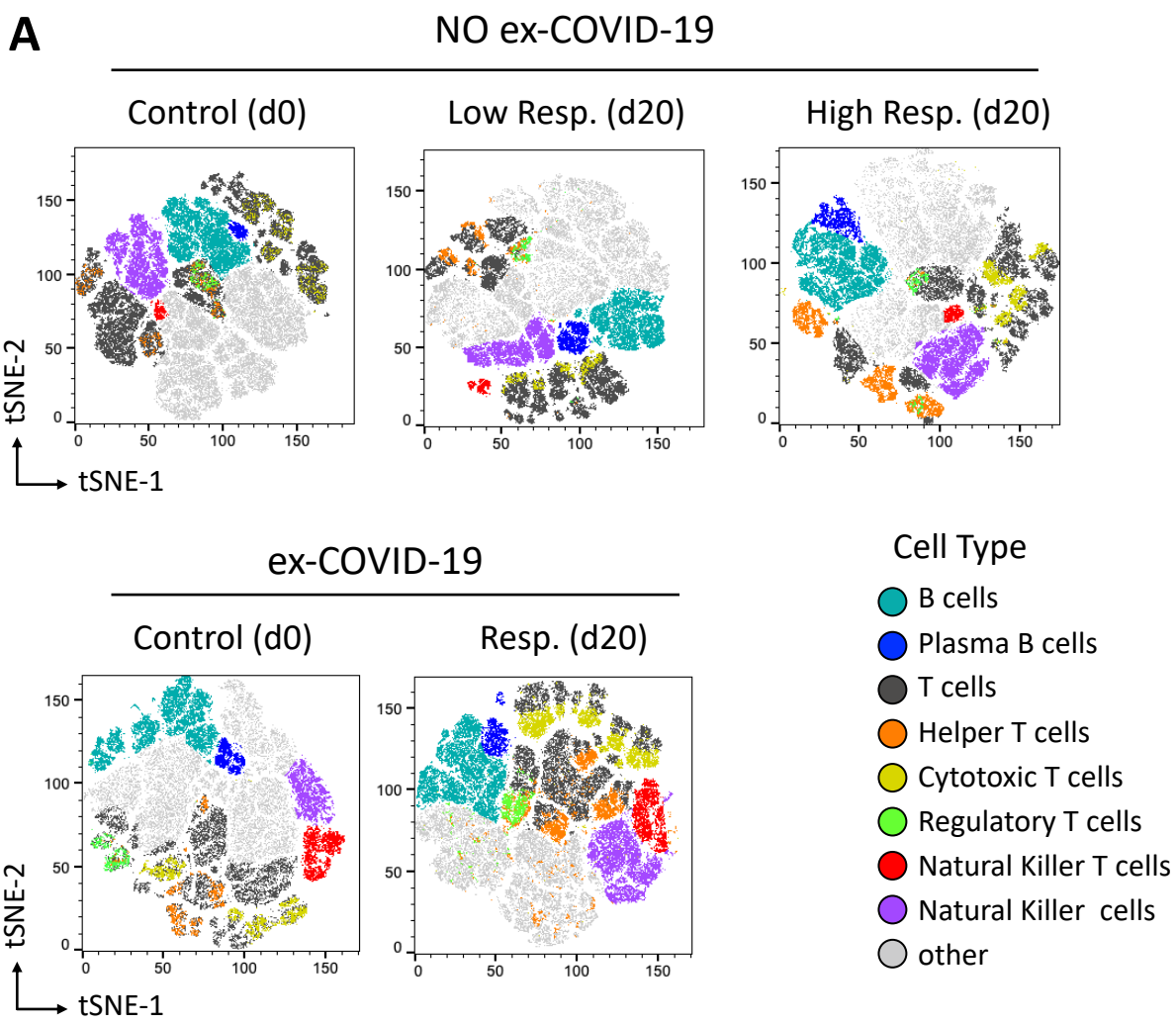




C

CELL TYPE	GATING STRATEGY
B cells	CD3- CD19+
Plasma B cells	CD3- CD19+ $CD38^{high}$
Helper T cells	CD3+ CD4+ CD8-
Cytotoxic T cells	CD3+ CD8+ CD4-
Regulatory T cells	CD3+ CD4+ CD8- CD127- $CD25^{high}$
Natural Killer T cells	CD3+ CD56+
Natural Killer cells	CD3- CD56+ CD19-

Figure 1


A
NO ex-COVID-19
Control (d0)
Low Resp. (d20)
High Resp. (d20)
tSNE-2
tSNE-1
ex-COVID-19
Control (d0)
Resp. (d20)
tSNE-2
tSNE-1
Cell Type
B cells
Plasma B cells
T cells
Helper T cells
Cytotoxic T cells
Regulatory T cells
Natural Killer T cells
Natural Killer cells
other

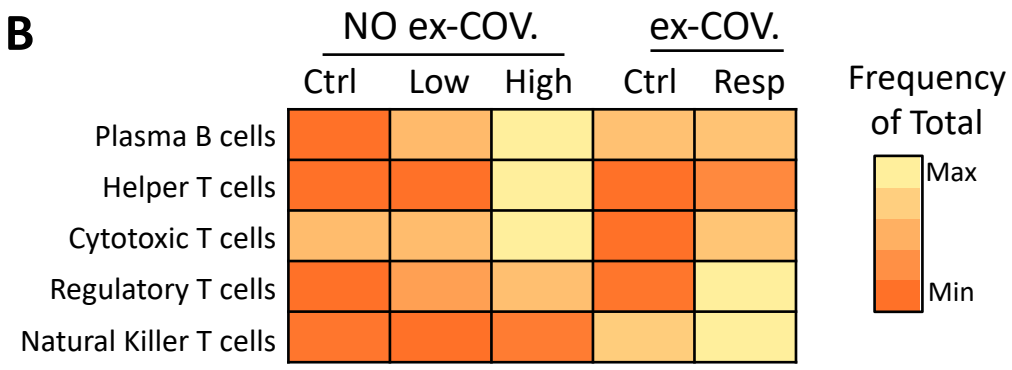
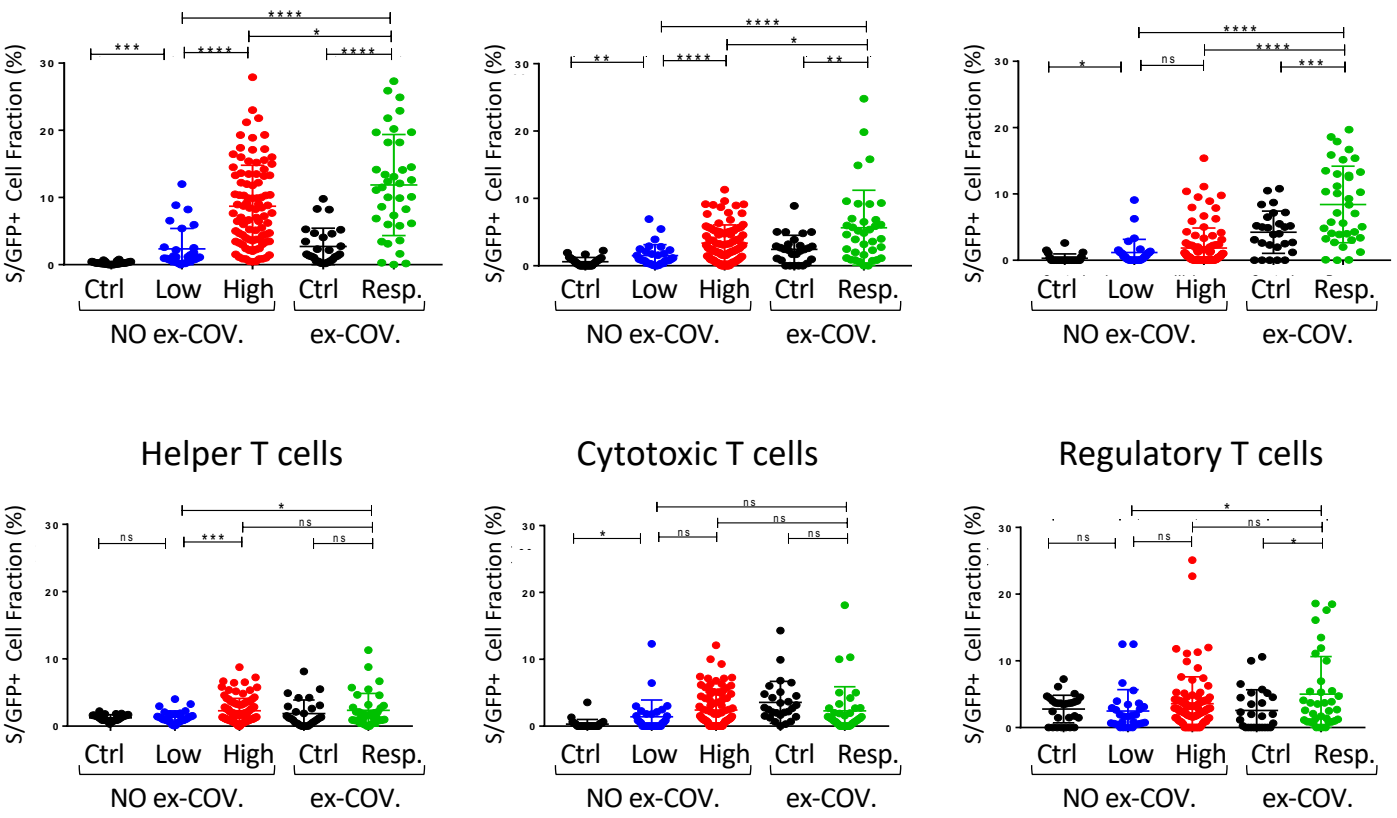

B
NO ex-COV.
ex-COV.
Ctrl
Low
High
Ctrl
Resp
Plasma B cells
Helper T cells
Cytotoxic T cells
Regulatory T cells
Natural Killer T cells
Frequency of Total
Max
Min

Figure 2

A

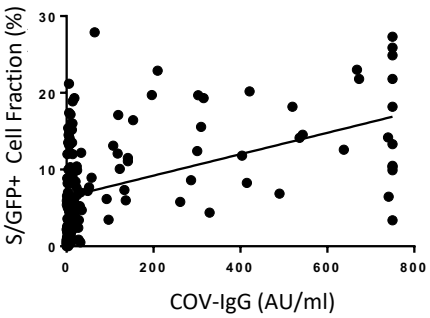


B

Cell Type	Control vs. Low	Low vs. High	Control vs. ex-COV resp.	Low vs. ex-COV resp.	High vs. ex-COV resp.
S/GFP+ B cells	0.001	1.83E-11	3.14E-08	1.32E-08	0.031
S/GFP+ Plasma B cells	0.007	1.61E-05	0.003	0.0001	0.026
S/GFP+ Natural Killer T cells	0.030	0.163	0.0005	5.80E-09	4.83E-08
S/GFP+ Helper T cells	0.493	0.0004	0.386	0.033	0.856
S/GFP+ Cytotoxic T cells	0.026	0.067	0.152	0.233	0.898
S/GFP+ Regulatory T cells	0.671	0.136	0.033	0.026	0.166

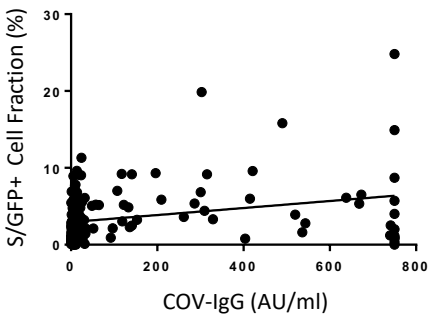
Figure 3

B cells



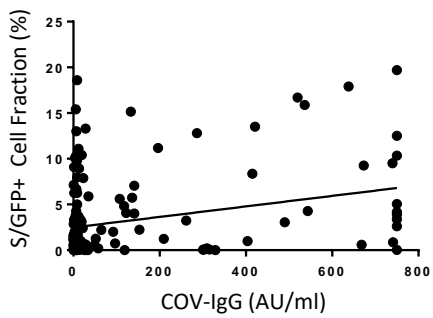
R^2=0.240
Pearson r=0.490 (p<0.0001)
Spearman ρ=0.591 (p<0.0001)

Plasma B cells



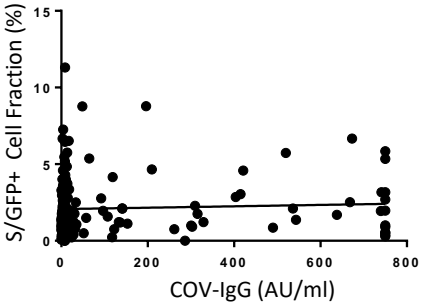
R^2=0.087
Pearson r=0.295 (p=0.0002)
Spearman ρ=0.425 (p<0.0001)

Natural Killer T cells



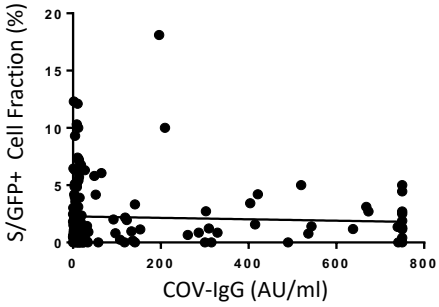
R^2=0.086
Pearson r=0.293 (p=0.0002)
Spearman ρ=0.241 (p=0.002)

Helper T cells



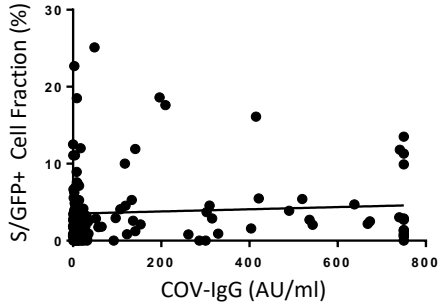
R^2=0.003
Pearson r=0.057 (p=0. 477)
Spearman ρ=0.086 (p=0.279)

Cytotoxic T cells



R^2=0.002
Pearson r=0.051 (p=0.523)
Spearman ρ=0.098 (p=0.217)

Regulatory T cells



R^2=0.006
Pearson r=0.077 (p=0.333)
Spearman ρ=0.091 (p=0.253)

Figure 4