Supplementay methods

Patients

22 healthcare workers who received BNT162b2 mRNA COVID-19 vaccine were recruited at the Careggi University Hospital, Florence, Tuscany, Italy, by the Infectious and Tropical Diseases Unit. Among them, 11 had a previous history of symptomatic SARS-CoV-2 infection (COVID-19 recovered). Confirmation of SARS-CoV-2 infection was obtained by PCR analysis of nasopharyngeal swab. Absence of infection in naïve individuals was based on absence of symptoms, absence of anti-N and anti-S IgG before vaccination and routine monitoring by nasopharyngeal swab PCR testing). Among COVID-19 recovered individuals, disease severity was defined according to WHO guidelines (1). Main demographic and clinical characteristic are summarized in Supplementary Table S1. Following immunization schedule approved by the European Medicines Agency, each subject received two vaccine injections, 21 days apart. Blood samples were collected before the first dose (basal time, T0) and every 7 days (T7, T14, T21) until T28 (28 days after the first dose, 7 days after the second one) (Supplementary Figure S1). 75 additional individuals (57 naïve and 18 COVID-19 recovered) were also recruited in addition to the 22 previously described subjects to expand serological data on a larger cohort. These subjects (97 in total, 68 naïve and 29 recovered COVID-19) were serologically tested at day 0, 21 and 50 following vaccine administration. Recruited subjects were not affected by chronic medical conditions that may affect vaccine response, with the exception of only one individual in the COVID-19 recovered group who was under immune suppressive treatment following solid organ transplantation. Main demographic and clinical characteristic of the expanded cohort are summarized in Supplementary Table S2.

PBMNCs were obtained following density gradient centrifugation of blood samples using Lymphoprep (Axis Shield Poc As™) and were frozen in FCS plus 10% DMSO to be stored in liquid nitrogen. For each subject, longitudinal samples were defrost and analyzed together for B cells and T cells evaluation. Serum was frozen and stored for Ig levels evaluation.

Evaluation of SARS-CoV-2-Spike-reactive T cells

For T cells stimulation in vitro, 1.5 million PBMNCs were cultured in complete RPMI plus 5% human AB serum in 96 well flat bottom plates in presence of medium alone (background, negative control) or of a pool of Spike SARS-CoV-2 peptide pools (Prot_S1, Prot_S+ and Prot_S to achieve a complete sequence coverage of the Spike protein) at 0.6 μM/peptide, accordingly to manufacturer's instructions (Miltenyi Biotech). Staphylococcal enterotoxin B SEB 1 μg/ml (Sigma Aldrich) was used as a positive control. After 2 hours of incubation at 37°C, 5% CO2, Brefeldin A (5 μg/mL) was added, followed by additional 4 hours incubation. Finally, cells were fixed and stained using fluorochrome-conjugated antibodies listed in Table S3 and Table S4. Samples were acquired on a BD LSR II flow cytometer (BD Biosciences). Flow cytometry experiments were performed using published guidelines (2).

Evaluation of SARS-CoV-2-Spike-specific IgM, IgA, IgG

For B cells evaluation, 2 million PBMNCs were stained for 30 minutes at 4°C with fluorochrome-conjugated antibodies listed in Table S5, then washed with PBS/EDTA buffer (PEB), and incubated 5 minutes with 7-AAD for viability evaluation. Samples were acquired on a BD LSR II flow cytometer (BD Biosciences). Recombinant biotinylated SARS-Cov2 Spike protein (Miltenyi Biotech) was conjugated separately with streptavidin PE and PE-Cy7 for 15 minutes at room temperature and pooled in 1:2 ratio, before being added to final staining mix.

Evaluation of SARS-CoV-2-specific IgM and IgG

Evaluation of SARS-CoV-2-Spike specific IgG (Diasorin), IgM (Abbott) and IgA (Euroimmun); Nucleoprotein-specific IgG (Abbott); RBD-specific IgG (Abbott); neutralizing Ab (Dia.Pro Diagnostic Bioprobes) was performed following manufacturer's instructions. The antibody reactivity of each specimen was expressed by the ratio between optical density and cut-off value (index) or as arbitrary units/ml.

Statistics

Unpaired Mann-Whitney test was used to compare COVID-19 recovered subjects versus naïve subjects; Wilcoxon-Signed Rank test was used to compare different time points in each study group. In all cases, p values ≤0.05 were considered significant.

Supplementary References

1. World Health Organization. Clinical management of COVID-19 interim guidance. WHO, Geneva. 27 May 2020. Available at: https://apps.who.int/iris/bitstream/handle/10665/332196/WHO-2019-nCoV-clinical-2020.5-eng.pdf?sequence=1&isAllowed=y
2. Cossarizza A, et al. Guidelines for the use of flow cytometry and cell sorting in immunological studies (second edition). Eur J Immunol. 2019 Oct;49(10):1457-1973. doi: 10.1002/eji.201970107. PMID: 31633216; PMCID: PMC7350392.

Supplementary Table S1. Demographic and clinical features of subjects enrolled for the study of early kinetic of immune response following vaccination.

Case ID	Gender	Age	COVID-19	Time from COVID-19 diagnosis° to I dose vaccine (days)	Comorbidities
Recovered COVID-19					
EC1	F	51	severe	294	HBP, DYSL
EC2	F	52	mild	286	
EC3	M	60	moderate	302	CKD
EC4	M	65	moderate	312	
EC5	F	53	moderate	289	Migraine
EC6	M	37	moderate	297	
EC7	F	54	mild	293	DYSL
EC8	M	61	critical	288	HBP
EC9	M	64	critical	292	HBP, DYSL
EC10	F	51	mild	222	
EC11	F	44	moderate	306	
Median (years)	-	53	-	-	
IQR	-	51-61	-	-	
Non-COVID-19					
NC1	F	35	no	na	
NC2	F	61	no	na	HBP
NC3	F	44	no	na	
NC4	F	41	no	na	DM, DYSL
NC5	M	41	no	na	
NC6	M	32	no	na	
NC7	F	37	no	na	
NC8	F	29	no	na	
NC9	F	46	no	na	
NC10	F	24	no	na	
NC11	M	53	no	na	
Median (years)	-	41	-	-	
IQR	-	32-46	-	-	

Legend: HBP: high blood pressure; DM: diabetes mellitus; CKD: chronic kidney disease; DYSL: Dyslipidaemia

na denotes not applicable

Supplementary Table S2. Demographic and clinical features of subjects enrolled for serological analysis up to 50 days post vaccination.

	Recovered COVID-19 N=29	Non-COVID-19 N=68
Gender		
- Male	8 (28%)	22 (32%)
- Female	21 (72%)	46 (68%)
Age		
- Median (IQR)	53 (45-60)	52 (36-60)
- Range	28-66	23-70
Comorbidities		
- HBP	6	10
- COPD	0	2
- Dyslipidaemia	6	4
- DM	1	5
- CKD	1	1
- CHD	1	0
- SOT	0	1
- Asthma	1	3
- Thyroid disorder	2	8
- Other*	1	3
COVID-19 severity[#]		
- asymptomatic/mild	21	na
- moderate	2	
- severe	2	
- critical	4	
Time from COVID-19 diagnosis° to I dose vaccine (days)		
- Median (IQR)	287 (270-293)	na
- Range	222-308	

Legend: HBP: high blood pressure; COPD: Chronic obstructive pulmonary disease; DM: diabetes mellitus; CKD: chronic kidney disease; CHD: coronary heart disease; SOT: solid organ transplantation

*including: MGUS (1), migraine (n=1), urticarial (n=1), glaucoma (n=1)

°first positive test on nasopharyngeal swab

Supplementary Table S3. List of all fluorochrome mAbs used for flow cytometric analysis of antigen specific T cells.

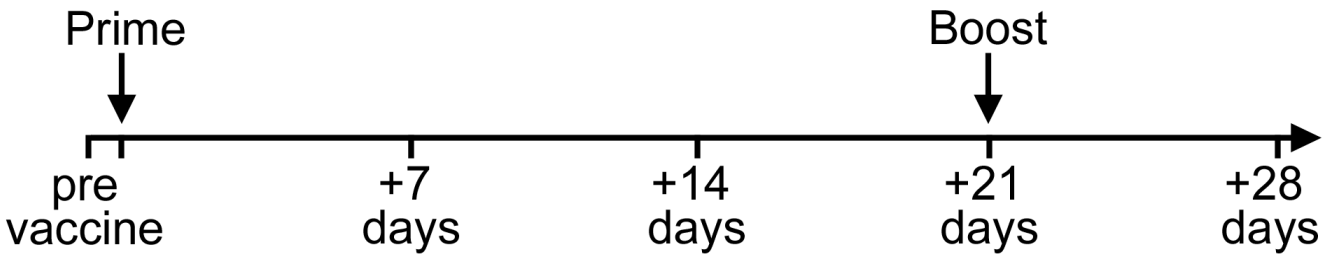
Antigen	Flurochrome	Clone	Company
TNF-α	FITC	6401.1111	BDBioscience
CD154	PE	TRAP1	BDBioscience
CD3	PerCP	SK7	BDBioscience
CD4	PE-Cy7	SK3	Invitrogen
CD8	Super Bright 600	SK1	eBioscience™
IL-2	APC	MQ1-17H12	BDBioscience
IFN-γ	Pacific Blue	B27	BioLegend
L/D	Fixable Viability Stain 780		BDBioscience

Supplementary Table S4. List of all fluorochrome mAbs used for flow cytometric analysis of immune checkpoint expression by antigen specific T cells.

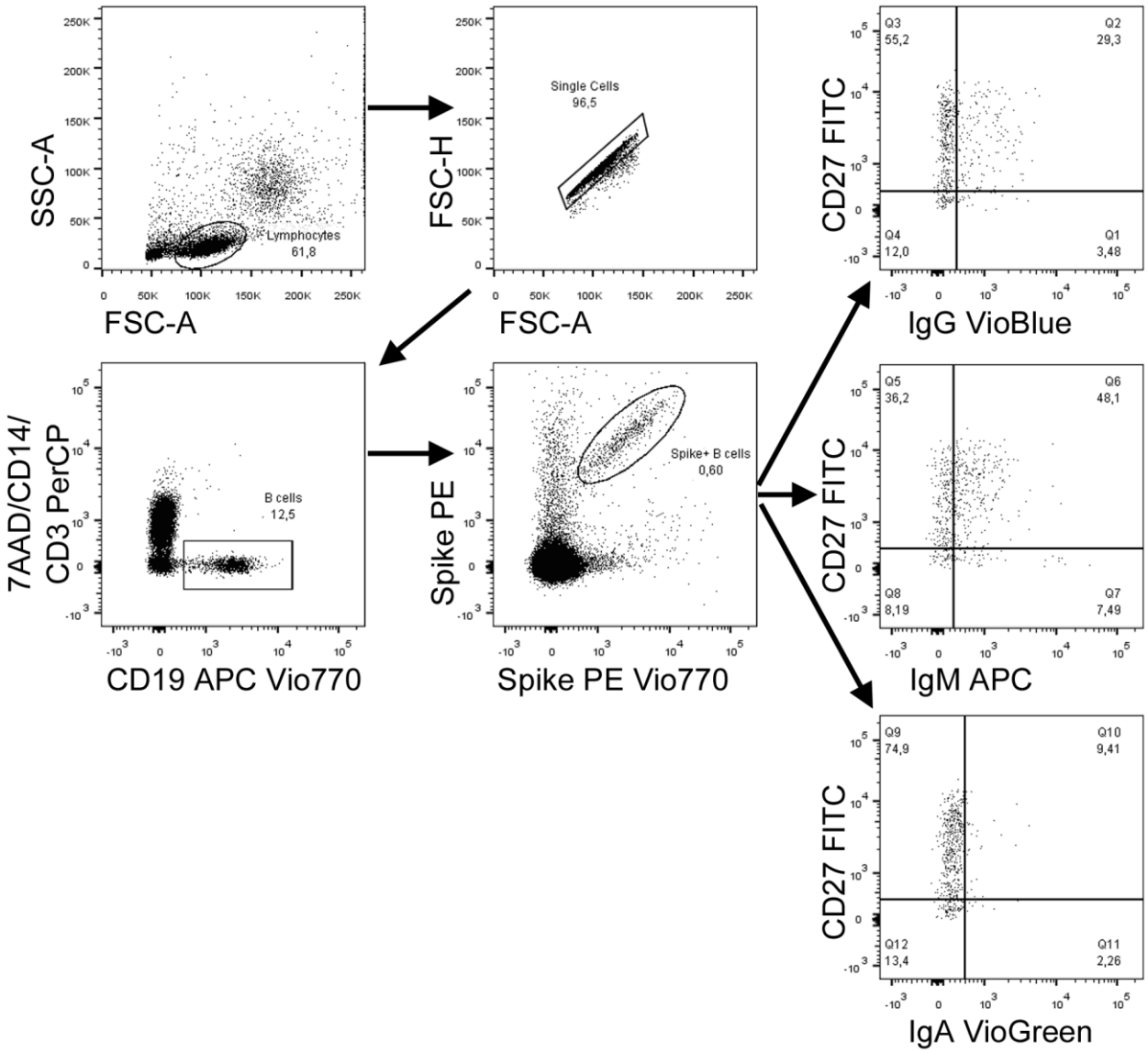
Antigen	Flurochrome	Clone	Company
TNF-α	FITC	MAb11	BDBioscience
IFN-γ	FITC	25723.11	BDBioscience
IL-2	FITC	5344.111	BDBioscience
CD8	PerCP	SK1	BDBioscience
CD4	eFluor506	RTA-T4	Invitrogen
CD3	Pacific Blue	UCHT1	BDBioscience
PD1 (CD279)	PE-Cy7	EH12.2H7	BioLegend
TIGIT	APC	MBSA43	Invitrogen
CD154	PE	TRAP1	BDBioscience
L/D	Fixable Viability Stain 780		BDBioscience

Supplementary Table S5. List of all fluorochrome mAbs used for flow cytometric analysis of antigen specific B cells.

Antigen	Flurochrome	Clone	Company
CD19	APC-Vio770	LT19	Miltenyi
CD27	VioBright FITC	M-T271	Miltenyi
IgA	VioGreen	IS11-8E10	Miltenyi
IgG	VioBlue	IS11-3B2.2.3	Miltenyi
CD14	PerCP	TÜK4	Miltenyi
IgM	APC	PJ2-22H3	Miltenyi
CD3	PerCP	BW264/56	Miltenyi
7AAD			Miltenyi
Spike	PE		Miltenyi
Spike	PE-Vio770		Miltenyi

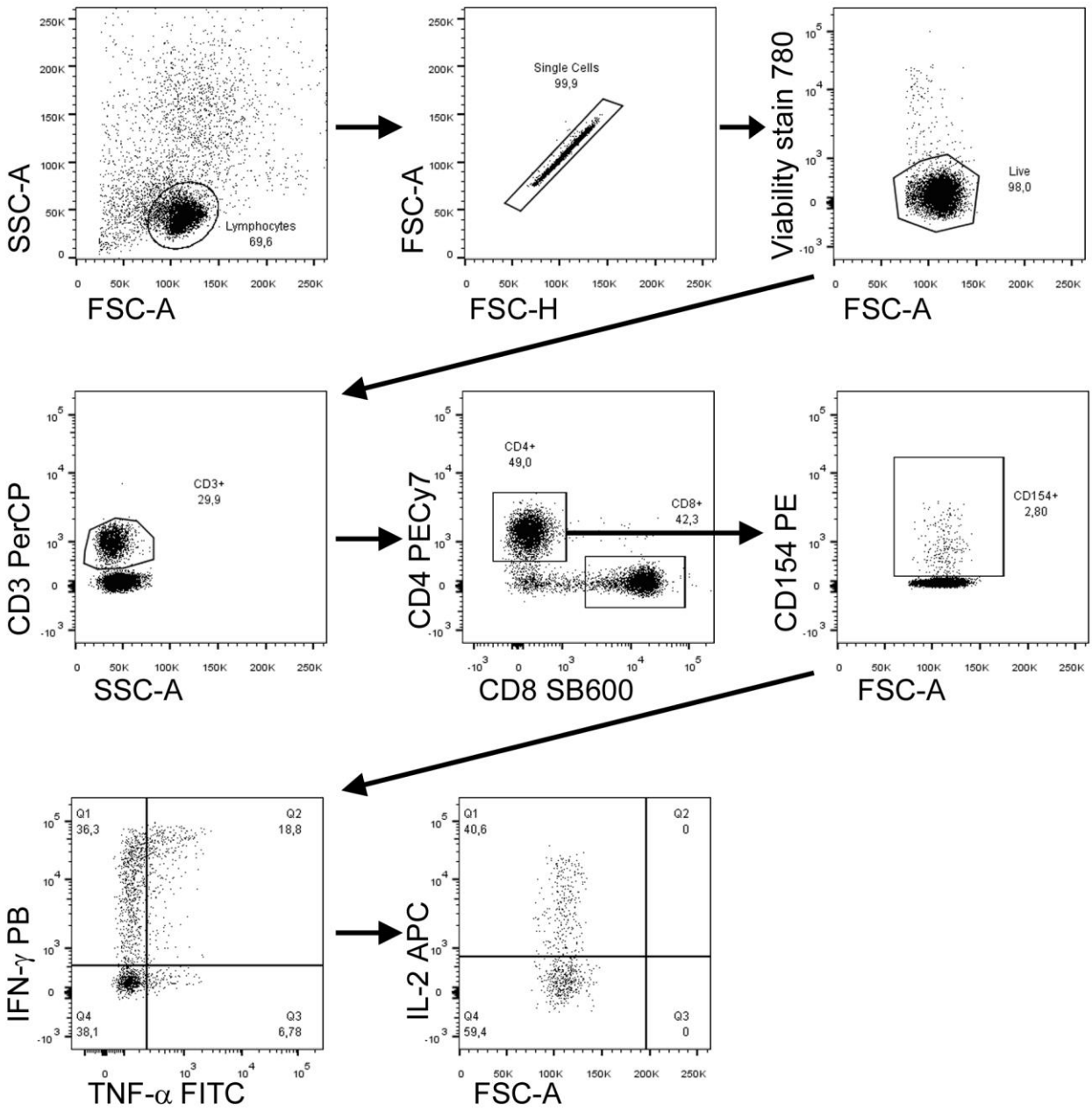


Supplementary Figure S1. Vaccination schedule and time points of analysis



Supplementary Figure 2: Gating strategy for the identification of spike-specific B cells

Lymphocytes were gated based on physical parameters (FSC-SSC), then doublets were removed using FSC-A and FSC-A parameters. PerCP was used as dump channel for the exclusion of dead cells (7AAD), CD3+ T cells and CD14 monocytes. B cells were identified as CD19+. B cells binding PE- and PE Vio770-conjugated spike protein were then identified as spike-specific. Among spike-specific B cells we evaluated CD27 expression associated to IgG, IgM and IgA.



Supplementary Figure 3: Gating strategy for the identification of spike-specific T cells

Lymphocytes were gated based on physical parameters (FSC-SSC), then doublets were removed using FSC-A and FSC-A parameters. Dead cells were excluded using viability stain 780. T cells were identified as CD3+. We then identified CD4+ T cells. Among these, we identified CD154 expressing cells. CD4+CD154+ T cells were then evaluated for IFN-γ, TNF-α and IL-2 expression.

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COVID-19 Research group

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