



Communication

A Rapid and Simple Multiparameter Assay to Quantify Spike-Specific CD4 and CD8 T Cells after SARS-CoV-2 Vaccination: A Preliminary Report

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Abstract: mRNA and Adenovirus vaccines for COVID-19 are used to induce humoral and cell-mediated immunity, with the aim to generate both SARS-CoV-2 B and T memory cells. In present study, we described a simple assay to detect and quantify Spike-specific CD4⁺ and CD8⁺ T cell responses induced by vaccination in healthy donors and in subjects with B cell compartment impairment, in which antibody response is absent due to primary immunodeficiencies or CD20 depleting therapy. We detect and quantified memory T cell immune responses against SARS-CoV-2 evoked by vaccination in both groups, irrespective to the humoral response. Furthermore, we identified TNF- α as the main cytokine produced by T memory cells, after antigen-specific stimulation in vitro, that could be considered, other than IFN- γ , an additional biomarker of induction of T memory cells upon vaccination. Further studies on the vaccine-induced T cell responses could be crucial, not only in healthy people but also in immunocompromised subjects, where antigen specific T cells responses play a protective role against SARS-CoV-2.

Keywords: Pfizer/BioNTech; memory T cell; CD4⁺; CD8⁺; SARS-CoV-2; cytokines

1. Introduction

After more than 2 years, a syndrome case by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused more than 4 million deaths worldwide, and it still is a prevalent outbreak [1]. To date, different licensed vaccines for COVID-19 with different techniques are available, and all presumably induce both humoral and cell-mediated immunity, both of which are required for recovery following SARS-CoV-2 infection. In addition, patients with more severe diseases develop neutralizing antibodies (NAb) that correlate with viral load [2,3]. Still, on the other hand, SARS-CoV-2 can spread from cell to cell without exposure to the extracellular environment [4], limiting the role of NAb in reducing viral spread within the host. For this reason, T cells could be important mediators of the protective host response to SARS-CoV-2 infection. Hence, there is an urgent need to consider the role of SARS-CoV-2-specific T cell immune response after vaccination, analyzing either CD4⁺ T cells, which help B cells for antibody production, or CD8⁺ T cells that kill virus-infected cells [5,6]. Therefore, a simple and rapid assessment of T cell immune response after vaccination remains a challenge. Using flow cytometry, Riou and colleagues described a rapid assay to qualitatively and quantitatively measure SARS-CoV-2-specific CD4⁺ T cell responses in 31 healthcare workers [7]. Despite several studies demonstrating the feasibility of detecting SARS-CoV-2-specific CD4⁺ and CD8⁺ T cells, most of them

have been performed in SARS-CoV-2-infected individuals and using different techniques. Still, there is very limited knowledge on similar assays performed after vaccination. Here, we report a rapid (~18 h) method to monitor SARS-CoV-2-specific T cell responses after vaccination independently from NAb blood concentration, which is also usable in patients with primary or secondary B cell defects.

2. Methods

This assay relies on simultaneous expression of three cytokines (IL-2, IFN- γ and TNF- α) after 18 h of stimulation *in vitro* with a pool of lyophilized peptides, consisting mainly of 15-mer sequences with an 11 amino acid overlap, covering the immunodominant sequence domains of the spike glycoprotein of SARS-CoV-2 (PepTivator[®] SARS-CoV-2 Prot_S, Miltenyi Biotec, Surrey, UK). The study was approved by the Ethical Committee of the University Hospital. Peripheral blood mononuclear cells (PBMCs) were obtained from $n = 41$ fully vaccinated (mRNA vaccine BNT162b2, Pfizer/BioNTech, Mangonza, Germany) individuals, 15 to 21 days after receiving the second dose vaccine. Asymptomatic subjects negative for SARS-CoV-2 with a real-time PCR test were enrolled in the study.

The enrolled subjects ($n = 40$), were divided into four groups; the first three groups were composed of healthy donors ($n = 28$) with different titers of SARS-CoV-2 NAb (SARS-CoV-2 Trimeric S IgG) at the time of venipuncture: G1 with a level of NAb < 390 BAU/mL, G2 with $390 < \text{NAb} < 1040$ BAU/mL, G3 with NAb > 1040 BAU/mL. The fourth group (G4) was composed of patients ($n = 12$) without functional B cells and no NAb in peripheral blood (six patients with multiple sclerosis in therapy with ocrelizumab and six patients with primitive B cell deficiencies). The demographic characteristics of the groups are reported in Table 1 while the specific clinical and therapeutic characteristics of G4 patients are reported in Table 2.

Table 1. Characteristics of enrolled subjects.

	G1 HD, Nab < 390 BAU/mL		G2 HD, $390 < \text{Nab} < 1040$ BAU/mL		G3 HD, Nab > 1040 BAU/mL		G4 Patients Ocrelizumab or Primitive B Cell Deficiencies		Total	
Enrolled Subjects (%)	9	(22.5%)	8	(20%)	11	(27.5%)	12	(30%)	40	(100%)
Mean Age	50		44		45		41		44	
Range	27–66		27–59		22–55		21–64		22–66	
Male Gender (%)	5	(55.7%)	3	(37.5%)	3	(77.5%)	3	(27.3%)	14	(35%)

PBMCs were stimulated for 18 h at 37 °C 5% CO₂ in RPMI1640 complete medium, with spike-specific peptide pool (1 $\mu\text{g}/\text{mL}$) at 1×10^6 cells/mL. RPMI or ionomycin/PMA were included in each sample as negative or positive controls, respectively. Brefeldin-A (10 $\mu\text{g}/\text{mL}$) was added after 2 h.

After 18 h of stimulation, cells were harvested and stained, first with live/dead marker (Zombie dye, Biolegend San Diego, CA, USA) then with mAb anti-human CD3PerCP-Vio[®] 700, mAb anti-human CD4 PE-Vio[®] 770 and mAb anti-human CD8 APC. After surface staining, cells were fixed, permeabilized and stained at room temperature for 30 min with mAb to anti-human IL-2 APC-Vio[®] 770, mAb anti-human IFN- γ FITC and mAb anti-human TNF- α PE. Samples were acquired on a FACSARIA II flow cytometer (BD Bioscience San Jose CA, USA) and analyzed using FlowJo v10 (BD Bioscience San Jose CA, USA).

The gating strategy is shown in Figure 1A. The threshold for positivity for spike-specific CD4⁺ T cell responses ($>0.02\%$) and antigen-specific CD8⁺ T cell responses ($>0.05\%$) was set according to Dan et al. [7–9] and calculated using the median two-fold standard deviation of all negative controls measured. GraphPad software was used to perform statistical analysis, and the groups were analyzed by using a Kruskal–Wallis test with Dunn’s correction and a Pearson correlation test.

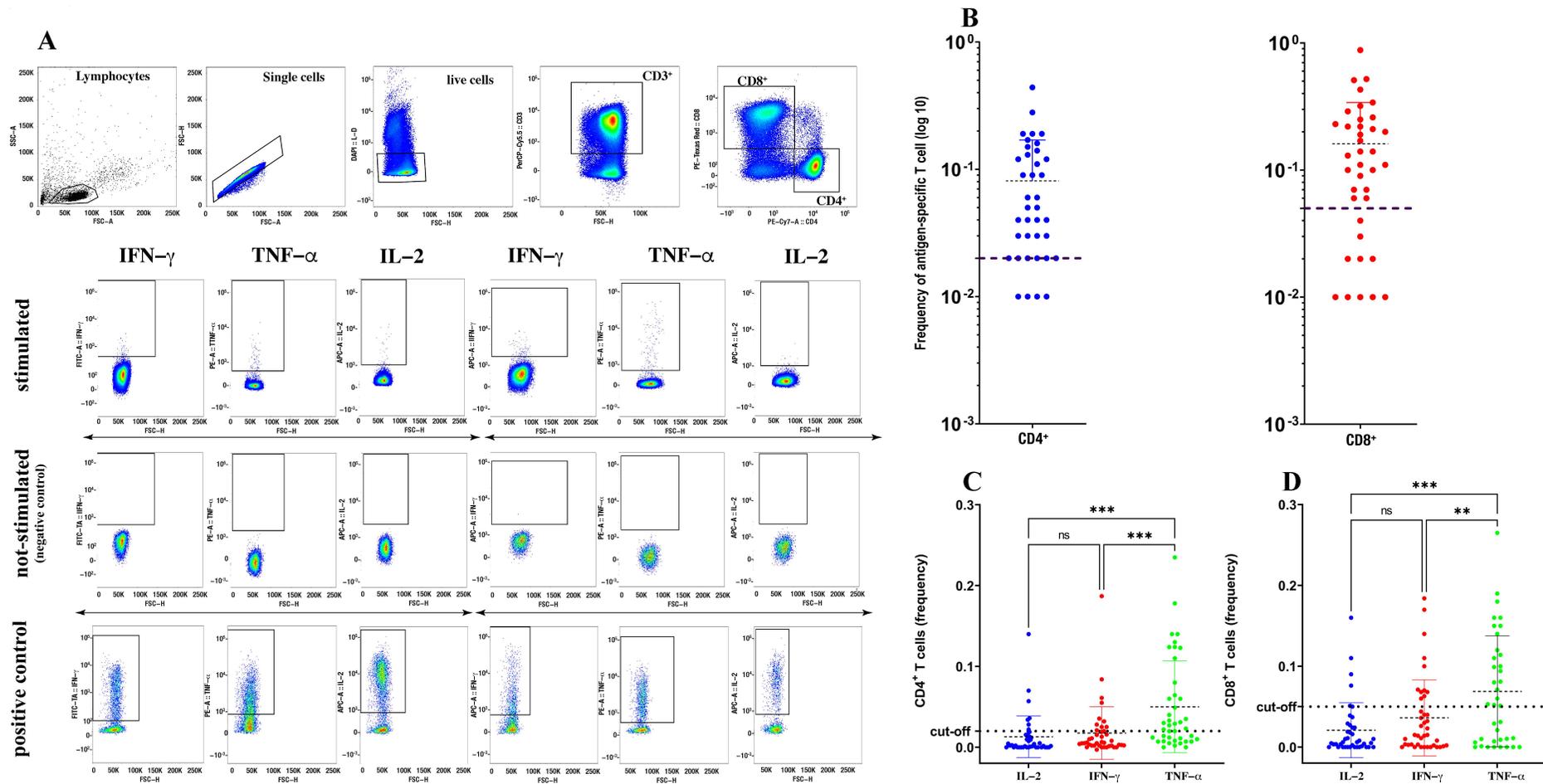


Figure 1. Quantification of spike-specific CD4⁺ and CD8⁺ T cells after SARS-CoV-2 vaccination. Gating strategy used to identify spike-specific CD4⁺ and CD8⁺ T cells and to detect their cytokine expression in response to spike-derived peptides. (A) Cumulative frequency and distribution of spike-specific CD4⁺ and CD8⁺ T cells (B) in SARS-CoV-2-vaccinated individuals ($n = 40$). Cut-off for positivity was set at <0.02 for CD4⁺ T cells and <0.05 for CD8⁺ T cells. Analysis of distinct cytokine expression by spike-specific CD4⁺ (C) and CD8⁺ (D) T cells using a Kruskal–Wallis test with Dunn’s correction. ** $p < 0.01$; *** $p < 0.001$; ns: not significant.

Table 2. Clinical and therapeutic characteristics of G4 enrolled patients.

G4 Enrolled Patients	Pathology	CCI	Therapy	N Days	IgG mg/dL
ID20	RRMS	0	Ocrelizumab	180	-
ID30	RRMS	0	Ocrelizumab	180	-
ID31	RRMS	0	Ocrelizumab	180	-
ID39	RRMS	1	Ocrelizumab	160	-
ID40	SPMS	2	Ocrelizumab	180	-
ID41	RRMS	0	Ocrelizumab	180	-
ID25	CVID	-	IgG Vein Kendron	28	>600
ID38	CVID	-	Subcutaneous IgG Hyqvia Takeda	28	>600
ID45	XLA	-	Subcutaneous IgG Hyqvia Takeda	28	>600
ID46	CVID	-	CSL Bhering Hizentra	15	>600
ID47	XLA	-	IgG Vein Kendron	28	>600
ID48	PNH	-	CSL Bhering Hizentra	15	>600

RRMS = Relapsing–remitting multiple sclerosis; SPMS = Secondary progressive multiple sclerosis; CVID = Common variable immunodeficiency; XLA = Bruton's agammaglobulinemia; PNH = Paroxysmal nocturnal hemoglobinuria; CCI = Charlson comorbidity index; N days = Days from the last infusion of monoclonal Ab or human IgG with respect to the sampling day.

3. Results

The proportion of vaccinated subjects positive for spike-specific memory CD4⁺ and CD8⁺ T cells, measured as the frequency of CD4⁺ and CD8⁺ T cells simultaneously expressing IL-2, IFN- γ and TNF- α , was 90% (36/40) and 70% (28/40), respectively (Figure 1B). Indeed, both the spike-specific CD4⁺ and CD8⁺ responses were characterized by very faint (if any) IL-2 expression and very low IFN- γ expression, but were enriched in cells expressing TNF- α (Figure 1C,D). Thus, and also in agreement with the study of Riou and colleagues [7], in our assay, TNF- α was the predominant cytokine produced, either by spike-specific CD4⁺ or CD8⁺ T cells, suggesting that TNF- α could be a more reliable biomarker than any other cytokine to detect spike-specific T cells in response to vaccination.

To study the correlation between the spike-specific T cell response and the NAb response, we stratified vaccinated healthy donors into three groups according to the titer of SARS-CoV-2NAbs: G1, <390 BAU/mL; G2, 390–1040 BAU/mL; G3, >1040 BAU/mL.

As shown in Figure 2A,B, the spike-specific CD4⁺ and CD8⁺ responses were of similar magnitude irrespective of the NAb titers in the G1, G2 and G3 groups. Accordingly, we did not find significant correlation between frequencies of spike-specific CD4⁺ and CD8⁺ T cells with NAb titers (Figure 2C,D), at least limited to those vaccinated subjects for whom precise antibody titers were available.

Finally, we analyzed the spike-specific T cell response in a group of subjects affected by primary or secondary B cell deficiencies, that had been vaccinated with the Pfizer/BioNTech BNT162b2 mRNA vaccine. Despite failing to produce spike-specific antibodies (Table 3), the B cell-deficient subjects showed a measurable spike-specific T cell response, comparable with that of the other groups.

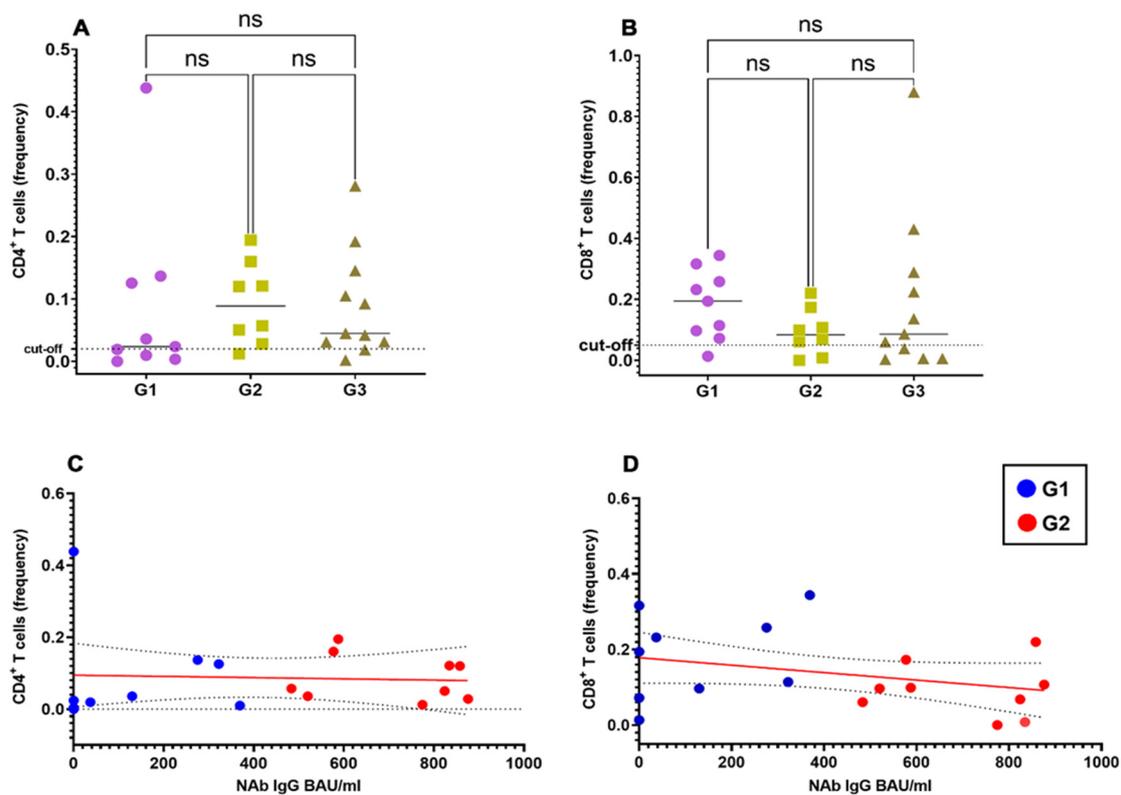


Figure 2. Correlation of spike-specific CD4⁺ and CD8⁺ T cells with spike-specific NAb titers. Frequencies of spike-specific CD4⁺ (A) and CD8⁺ (B) T cells upon stratification of vaccinated individuals in 3 different groups according to their SARS-CoV-2 NAb titers (groups G1 to G3). Pearson correlation coefficient with one-tailed P-value and 90% confidence interval between titers of SARS-CoV-2 NABs and the frequency of spike-specific CD4⁺ (C) and CD8⁺ (D) T cells.

Table 3. NAb titer and frequency of responder T cells in patients with B cell deficiencies.

Enrolled Patients	NAb BAU/mL	Frequency (%) of CD4 ⁺ T Cells	Frequency (%) of CD8 ⁺ T Cells
ID20	0	0.0558	0.0174
ID25	0	0.1933	0.2069
ID30	0	0.0271	0.0136
ID31	0	0.0600	0.5232
ID38	0	0.0350	0.0256
ID39	0	0.0021	0.1285
ID40	0	0.0390	0.0230
ID41	0	0.0867	0.0631
ID45	0	0.0359	0.0969
ID46	0	0.0120	0.5109
ID47	0	0.1700	0.1440
ID48	0	0.0120	0.2530

Values above the cut-off are indicated in bold.

4. Discussion

Much scientific evidence suggests that the cell-mediated response assumes relevant importance for an effective immune response against SARS-CoV-2 virus [10]. One study, in particular, showed that in cancer patients, the establishment of the cell-mediated response induced by the vaccine, even in the absence of a corresponding antibody response, was sufficient to confer protection against infection [11]. Another study has highlighted the establishment of the cellular immune response against SARS-CoV-2 in subjects vaccinated with BNT162b2Pfizer/BioNTech in the absence of an antibody response at the time of the assessment of adaptive immunity, after the second injection [12]. Furthermore, many

studies have shown that mRNA vaccines induced a reduced humoral response in patients with acquired immunodeficiencies due to hematological diseases [13] or immunosuppressive therapies, including the use of biological drugs such as rituximab [14,15]. Finally, the Pfizer vaccine also provoked a cell-mediated immune response in subjects with primary immunodeficiencies affecting the B compartment [16]. This literature shows the potential usefulness of a test to evaluate the efficacy of vaccination by measuring the cellular immune response.

Some attempts have been made in this direction, also considering evaluating IFN- γ using IGRA tests [17]. However, the T cells' response is expressed through different activation pathways, and the evaluation of a single cytokine may not fully reflect the state of activation of this compartment. A multiparametric flow cytometry test, such as the one proposed in this communication, through the evaluation of the simultaneous expression of TNF- α , IL2 and IFN- γ can identify more completely the presence of a cell-mediated immune response induced by vaccination regardless of the evaluation of the humoral response.

Our study shows that vaccination with Pfizer gives a robust cell-mediated immunological memory against spike protein antigens, independently from the titer of NAb, meaning that the T cell-mediated specific immune response against SARS-CoV-2 caused by vaccination can develop independently from B cell response. This finding highlights the importance of the cell-mediated immunity against SARS-CoV-2 induced by vaccination and identifies TNF- α as the main product of T cells of vaccinated subjects after specific stimulation in vitro, making this cytokine a correlate of successful vaccination. In conclusion, we have reported a proof-of-concept study describing a simple and easy assay to detect and quantify spike-specific CD4⁺ and CD8⁺ T cell responses induced by vaccination. Although our study has many limitations (small number of tested individuals, optimization and validation), it provides a tool to monitor the immunogenicity of SARS-CoV-2 vaccines and study the correlation between the quantity and quality of B and T cell-mediated responses and protection. Finally, studying vaccine-induced T cell responses may be of value in those subjects with B cell depletion following ocrelizumab or due to primary immunodeficiencies, in which serological responses are impaired, but T cell responses are preserved.

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Informed Consent Statement: Informed consent was obtained from all subjects enrolled for this study.

Data Availability Statement: The Data will be available upon reasonable request to the corresponding author.

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References

1. Cucinotta, D.; Vanelli, M. WHO declares COVID-19 a pandemic. *Acta Bio Med. Atenei Parm.* **2020**, *91*, 157.
2. Robbiani, D.F.; Gaebler, C.; Muecksch, F.; Lorenzi, J.C.C.; Wang, Z.; Cho, A.; Agudelo, M.; Barnes, C.O.; Gazumyan, A.; Finkin, S. Convergent antibody responses to SARS-CoV-2 in convalescent individuals. *Nature* **2020**, *584*, 437–442. [[CrossRef](#)] [[PubMed](#)]
3. Lee, W.T.; Girardin, R.C.; Dupuis, A.P.; Kulas, K.E.; Payne, A.F.; Wong, S.J.; Arinsburg, S.; Nguyen, F.T.; Mendu, D.R.; Firpo-Betancourt, A. Neutralizing antibody responses in COVID-19 convalescent sera. *J. Infect. Dis.* **2021**, *223*, 47–55. [[CrossRef](#)] [[PubMed](#)]

4. Fenrich, M.; Mrdenovic, S.; Balog, M.; Tomic, S.; Zjalic, M.; Roncevic, A.; Mandic, D.; Debeljak, Z.; Heffer, M. SARS-CoV-2 dissemination through peripheral nerves explains multiple organ injury. *Front. Cell. Neurosci.* **2020**, *14*, 229. [[CrossRef](#)] [[PubMed](#)]
5. Dan, J.M.; Mateus, J.; Kato, Y.; Hastie, K.M.; Yu, E.D.; Faliti, C.E.; Grifoni, A.; Ramirez, S.I.; Haupt, S.; Frazier, A.; et al. Immunological memory to SARS-CoV-2 assessed for up to 8 months after infection. *Science* **2021**, *371*, eabf4063. [[CrossRef](#)] [[PubMed](#)]
6. Sattler, A.; Angermair, S.; Stockmann, H.; Heim, K.M.; Khadzhynov, D.; Treskatsch, S.; Halleck, F.; Kreis, M.E.; Kotsch, K. SARS-CoV-2-specific T cell responses and correlations with COVID-19 patient predisposition. *J. Clin. Investig.* **2020**, *130*, 6477–6489. [[CrossRef](#)] [[PubMed](#)]
7. Riou, C.; Schäfer, G.; du Bruyn, E.; Goliath, R.T.; Stek, C.; Mou, H.; Hung, D.; Wilkinson, K.A.; Wilkinson, R.J. Rapid, simplified whole blood-based multiparameter assay to quantify and phenotype SARS-CoV-2 specific T cells. *medRxiv* **2020**. [[CrossRef](#)]
8. Cossarizza, A.; Chang, H.-D.; Radbruch, A.; Acs, A.; Adam, D.; Adam-Klages, S.; Agace, W.W.; Aghaepour, N.; Akdis, M.; Allez, M.; et al. Guidelines for the use of flow cytometry and cell sorting in immunological studies (second edition). *Eur. J. Immunol.* **2019**, *49*, 1457–1973. [[CrossRef](#)] [[PubMed](#)]
9. Smith, S.G.; Smits, K.; Joosten, S.A.; van Meijgaarden, K.E.; Satti, I.; Fletcher, H.A.; Caccamo, N.; Dieli, F.; Mascart, F.; McShane, H.; et al. Intracellular Cytokine Staining and Flow Cytometry: Considerations for Application in Clinical Trials of Novel Tuberculosis Vaccines. *PLoS ONE* **2015**, *10*, e0138042. [[CrossRef](#)] [[PubMed](#)]
10. Schwarzkopf, S.; Krawczyk, A.; Knop, D.; Klump, H.; Heinold, A.; Heinemann, F.M.; Thümmel, L.; Temme, C.; Breyer, M.; Witzke, O.; et al. Cellular Immunity in COVID-19 Convalescents with PCR-Confirmed Infection but with Undetectable SARS-CoV-2-Specific IgG. *Emerg. Infect. Dis.* **2021**, *27*. [[CrossRef](#)]
11. Kalimuddin, S.; Tham, C.Y.L.; Qui, M.; de Alwis, R.; Sim, J.X.Y.; Lim, J.M.E.; Tan, H.-C.; Syenina, A.; Zhang, S.L.; Le Bert, N.; et al. Early T cell and binding antibody responses are associated with COVID-19 RNA vaccine efficacy onset. *Med* **2021**, *2*, 682–688.e4. [[CrossRef](#)] [[PubMed](#)]
12. Bange, E.M.; Han, N.A.; Wileyto, P.; Kim, J.Y.; Gouma, S.; Robinson, J.; Greenplate, A.R.; Hwee, M.A.; Porterfield, F.; Owoyemi, O.; et al. CD8+ T cells contribute to survival in patients with COVID-19 and hematologic cancer. *Nat. Med.* **2021**, *27*, 1280–1289. [[CrossRef](#)] [[PubMed](#)]
13. Herishanu, Y.; Avivi, I.; Aharon, A.; Shefer, G.; Levi, S.; Bronstein, Y.; Morales, M.; Ziv, T.; Shorer Arbel, Y.; Scarfò, L.; et al. Efficacy of the BNT162b2 mRNA COVID-19 vaccine in patients with chronic lymphocytic leukemia. *Blood* **2021**, *137*, 3165–3173. [[CrossRef](#)] [[PubMed](#)]
14. Connolly, C.M.; Boyarsky, B.J.; Ruddy, J.A.; Werbel, W.A.; Christopher-Stine, L.; Garonzik-Wang, J.M.; Segev, D.L.; Paik, J.J. Absence of Humoral Response After Two-Dose SARS-CoV-2 Messenger RNA Vaccination in Patients with Rheumatic and Musculoskeletal Diseases: A Case Series. *Ann. Intern. Med.* **2021**, *174*, 1332–1334. [[CrossRef](#)] [[PubMed](#)]
15. Spiera, R.; Jinich, S.; Jannat-Khah, D. Rituximab, but not other antirheumatic therapies, is associated with impaired serological response to SARS-CoV-2 vaccination in patients with rheumatic diseases. *Ann. Rheum. Dis.* **2021**, *80*, 1357. [[CrossRef](#)] [[PubMed](#)]
16. Hagin, D.; Freund, T.; Navon, M.; Halperin, T.; Adir, D.; Marom, R.; Levi, I.; Benor, S.; Alcalay, Y.; Freund, N.T. Immunogenicity of Pfizer-BioNTech COVID-19 vaccine in patients with inborn errors of immunity. *J. Allergy Clin. Immunol.* **2021**, *148*, 739–749. [[CrossRef](#)] [[PubMed](#)]
17. Malipiero, G.; Moratto, A.; Infantino, M.; D'Agaro, P.; Piscianz, E.; Manfredi, M.; Grossi, V.; Benvenuti, E.; Bulgaresi, M.; Benucci, M.; et al. Assessment of humoral and cellular immunity induced by the BNT162b2 SARS-CoV-2 vaccine in healthcare workers, elderly people, and immunosuppressed patients with autoimmune disease. *Immunol. Res.* **2021**, 1–8. [[CrossRef](#)]