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Accuracy of QuantiFERON SARS-CoV-2 research use only assay and characterization of the CD4$^+$ and CD8$^+$ T cell-SARS-CoV-2 response: comparison with a homemade interferon-γ release assay

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ABSTRACT

Objectives: In this study, we aimed to characterize the SARS-CoV-2-specific T cell response detected by the QuantiFERON SARS-CoV-2 research use only assay in terms of accuracy and T cell subsets involved compared with a homemade interferon (IFN)-γ release assay (IGRA).

Methods: We evaluated T cell response by the standardized QuantiFERON SARS-CoV-2 tubes (antigen [Ag]1 and Ag2) and a homemade IGRA quantifying IFN-γ response to SARS-CoV-2 spike peptides (homemade-IGRA-SPIKE test). We evaluated the T cell subsets mediating the specific response using flow cytometry.

Results: We prospectively enrolled 66 individuals: COVID-19 or post-COVID-19 subjects and NO-COVID-19-vaccinated subjects, including healthy donors and immunocompromised subjects. The standardized kit detected 62.1% (41/66) of T cell responders. Ag2 tube showed a higher IFN-γ quantitative and qualitative response. Ag1 tube response was mainly mediated by CD4$^+$ T cells; Ag2 tube response was mediated by CD4$^+$ and CD8$^+$ T cells. The homemade-IGRA-SPIKE test detected a higher number of responders (52/66, 78.8%) than the QuantiFERON SARS-CoV-2 assay ($P = 0.056$). The response was found in both T cell subsets, although a higher magnitude and response rate was observed in the CD4$^+$ T cell subset.

Conclusion: The QuantiFERON SARS-CoV-2 response is mediated by CD4$^+$ and CD8$^+$ T cells. A lower number of responders is found compared with the homemade-IGRA-SPIKE test, likely because of the different peptide composition.

Introduction

Humoral and cell-mediated responses are both necessary to control SARS-CoV-2 infection (Sette and Crotty, 2021) and to monitor the immune protection induced by the ongoing SARS-CoV-2 vaccination in the population (Agrati *et al.*, 2021; Aiello *et al.*, 2021; Farroni *et al.*, 2022; Goletti *et al.*, 2021; Petrone *et al.*, 2021b; Picchianti-Diamanti *et al.*, 2021; Tortorella *et al.*, 2022).

The antibody (Ab) evaluation is the common method used to screen on a large scale the population for a current/previous infection or vaccination. However, Abs wane over time, and they may be absent in the mildest forms of COVID-19 (Agrati *et al.*, 2022; Farroni *et al.*, 2022; Petrone *et al.*, 2022a, 2022b; Seow *et al.*, 2020; Yamayoshi *et al.*, 2021).

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T cell-mediated immunity is pivotal for viral clearance and the subsequent induction of a B cell response. Unlike the humoral response, T cell response is more enduring and detectable even in the absence of seroconversion (Ferraccioli *et al.*, 2022; Grifoni *et al.*, 2020; Sekine *et al.*, 2020) and takes on greater importance in the context of the emerging variants that escape the antibody response (Geers *et al.*, 2021; Liu *et al.*, 2022; Tarke *et al.*, 2022).

Although many commercial tests are available for Ab evaluation, reliable tests for detecting the SARS-CoV-2 T cell response are still needed. Currently, various experimental procedures based on using different spike peptides and read-out have been employed. In this context, a whole-blood approach based on interferon (IFN)-γ release assay (IGRA) was set up to monitor the specific immune response either in vaccinated individuals or in subjects with current or previous infection (Aiello *et al.*, 2021; Echeverría *et al.*, 2021; Murugesan *et al.*, 2022, 2021; Petrone *et al.*, 2021b, 2021a). In addition, other experimental settings involving peripheral blood mononuclear cells (PBMCs), such as intracellular cytokine-based assays or the human IFN-γ enzyme-linked immunospot assay, have been developed to detect SARS-CoV-2 T cell responses (Chu *et al.*, 2022; Grifoni *et al.*, 2020; Ogbe *et al.*, 2021; Thieme *et al.*, 2020; Tormo *et al.*, 2022; Yu *et al.*, 2022). However, using a whole-blood platform to evaluate T cell response may offer several advantages compared with other experimental settings, starting from the ease and speed of execution, responses in short times, the lack of necessary specialized facilities, and the possibility of a routine application.

In this study, we evaluated the accuracy of the QuantiFERON SARS-CoV-2 assay, a commercially available kit for T cell response detection. To our knowledge, we characterized for the first time the T cell subsets involved in the specific response by flow cytometry analysis. The results were compared with a homemade IGRA quantifying IFN-γ response to SARS-CoV-2 spike peptides (homemade-IGRA-SPIKE) test that we set up to detect the IFN-γ T cell response to SARS-CoV-2 in patients with COVID-19 and vaccinated individuals (Agrati *et al.*, 2021; Aiello *et al.*, 2021; Farroni *et al.*, 2022; Petrone *et al.*, 2022a, 2021b; Picchianti-Diamanti *et al.*, 2021; Tortorella *et al.*, 2022).

Materials and methods

Study population

In this prospective study, we enrolled subjects with a current (patients with COVID-19) or previous SARS-CoV-2 infection (post-COVID-19), vaccinated healthy donors (HDs), or immunocompromised vaccinated NO-COVID-19 subjects, including patients with immune-mediated inflammatory diseases (IMID) or multiple sclerosis (MS). Individuals were enrolled from the National Institute for Infectious Diseases Lazzaro Spallanzani-IRCCS, Nuovo Regina Margherita Hospital and MS Center of the Department of Neurosciences of San Camillo Forlanini Hospital (Rome, Italy) (Approval numbers 59/2020, 72/2015, 297/2021, 318/2021, and 319/2021). Inclusion criteria for patients with COVID-19 were a SARS-CoV-2 positive nasopharyngeal swab and/or clinical characteristics of COVID-19 (Nicastri *et al.*, 2020). Subjects hospitalized with COVID-19 had moderate or severe disease, according to the World Health Organization (World Health Organization, 2021). The inclusion criteria for post-COVID-19 were: a SARS-CoV-2 infection in the previous 1-16 months and receiving at least two vaccine doses. Inclusion criteria for immunocompromised patients were: a diagnosis of MS according to the 2017 revisions of the McDonald criteria (Thompson *et al.*, 2018) or a diagnosis of IMID based on objective criteria. Regarding the NO-COVID-19 subjects (HDs, IMID, and MS), the inclusion criteria were to have completed at least the first vaccination schedule, whereas the exclusion criterion was a previous SARS-CoV-2 infection.

Enrollment exclusion criteria for all the groups were: HIV infection, inability to sign the consent, and age <18 years. Enrolled subjects signed a written informed consent and clinical and demographic information were collected at enrollment.

QuantiFERON SARS-CoV-2 research use only tubes

QuantiFERON SARS-CoV-2 research use only (RUO) assay was performed according to the manufacturer's recommendations (QIAGEN, Hilden, Germany). QuantiFERON Starter Set Blood Collection Tubes consist of two antigen (Ag) tubes: an Ag1 tube that contains $CD4^+$ T cell epitopes from the S1 subunit of the spike protein and an Ag2 tube with both $CD4^+$ and $CD8^+$ T cell epitopes derived from the S1 and S2 subunits of the spike protein. The control set tubes include negative (Nil) and positive (Mitogen [MIT]) controls. Values were subtracted from the Nil value. The cutoff for a positive response was set at 0.15 IU/ml according to the performance of the kit evaluated in cohorts of vaccinated subjects (Krüttgen *et al.*, 2021; Martínez-Gallo *et al.*, 2021; Stieber *et al.*, 2022; Tychala *et al.*, 2021).

Homemade-IGRA-SPIKE test

The SARS-CoV-2 specific T cell response was evaluated using a homemade IFN-γ release assay. Briefly, 600 µL of heparinized whole blood were stimulated using the PepTivator® SARS-CoV-2 peptide pools (Prot_S, Prot_S1, and Prot_S+; Bergisch Gladbach, Germany) and incubated overnight at 37°C. PepTivator® SARS-CoV-2 are peptide pools consisting mainly of 15-mer sequences with 11 amino acids overlap, covering the entire sequence of the SARS-CoV-2 Wuhan spike glycoprotein. PepTivator® Prot_S covers the predicted immunodominant domains of the spike glycoprotein, Prot_S1 covers the N-terminal S1 domain, and Prot_S+ covers a part of the C-terminal S2 domain. Lyophilized peptides were resuspended in deionized water. For the stimulation, the three pools were grouped into a unique pool (spike), including equal amounts of each at a final concentration of 0.1 µg/ml (Aiello *et al.*, 2021). As a positive control, staphylococcal enterotoxin B (SEB) antigen (Sigma-Aldrich, Milan, Italy) was used at 200 ng/ml, whereas the unstimulated whole blood was used as a negative control. After overnight stimulation, plasma was harvested and stored at -80°C. IFN-γ levels were measured by enzyme-linked immunosorbent assay (ELISA) per the manufacturer's instructions (www.quantiFERON.com) and subtracted from the negative control value. The test has a detection limit of 0.065 IU/ml. Cut-off for the positive response was set at 0.13 IU/ml according to receiver operating characteristic analysis performed comparing patients with COVID-19 and NO-COVID-19 subjects in the previous study (Aiello *et al.*, 2021).

SARS-CoV-2 serology

SARS-CoV-2-specific immunoglobulin G (IgG) was evaluated by ELISA using a commercial kit per the manufacturer's instructions (DIESSE Diagnostica Senese S.p.A., Monteriggioni, Italy). Values are expressed as index (sample [S]/cut-off), and indicated as positive (index >1.1), doubtful (0.9 > index < 1.1), or negative (index <0.9) per the kit's indications.

IFN-γ intracellular staining and flow cytometry analysis

To evaluate $CD4^+$ and $CD8^+$ T cell IFN-γ intracellular production, isolated PBMCs (1×10^6) were placed in each QuantiFERON SARS-CoV-2 tube (Nil, Ag1, Ag2, and MIT) and incubated

at 37°C for 1 hour. Afterward, PBMCs were placed into a new fluorescence-activated cell sorting tube, co-stimulated with anti-CD28 and CD49b monoclonal Abs (mAbs) (2 mg/ml), and GolgiPlug (BD Biosciences, San Jose, US) was added to inhibit cytokine secretion. To characterize the IFN-γ production in response to spike, PBMCs (1×10^6) were stimulated overnight with spike (1 µg/ml) or SEB (200 ng/ml), used as a positive control. Co-stimulatory mAbs (2 mg/ml) and GolgiPlug were added as previously reported (Aiello *et al.*, 2021; Farroni *et al.*, 2022). After overnight incubation, PBMCs were stained with the following Abs: anti-CD3-PerCP, anti-CD4-PE, and anti-CD8-Pacific Blue (all from BD Biosciences, San Jose, US). Cells were then fixed, permeabilized using Cytofix/Cytoperm, and incubated with anti-IFN-γ-APC (BD Biosciences, San Jose, US). Finally, samples were acquired with a DxFlex cytometer (Beckman Coulter) and analyzed with FlowJo software (v10, Tree Star) (Supplementary Figure 1 for gating strategy). Cytokine background in negative controls was subtracted from the stimulated conditions. The IFN-γ-spike-specific T cell response was considered positive when the following conditions were satisfied: i) the percentage of the stimulated cells was at least 2-fold higher than that of the unstimulated control, and ii) a minimum of 10 events were present in the cytokine gate (Farroni *et al.*, 2022; Roederer, 2008). The threshold value was set at 0.005%, which is the lower frequency of response observed among positive responders.

Statistical analysis

Data were analyzed using GraphPad software (GraphPad Prism 9 XML Project, San Diego, California, US). We used chi-square test for categorical variables, Kruskal-Wallis and Friedman tests adjusted with Dunn's multiple comparisons test for comparisons among groups (for unpaired and paired data, respectively), and the Wilcoxon signed-rank test for pairwise comparisons. Nonparametric Spearman's rank test was performed for correlations. Spearman's $r_{ho} > 0.7$ was considered high correlation, $0.7 > r_{ho} > 0.5$ moderate correlation, and $r_{ho} < 0.5$ low correlation. Cohen's kappa was used to assess the agreement between the two assays. Two-tailed *P*-values <0.05 were considered significant.

Results

Description of the enrolled population

We prospectively enrolled 66 individuals: 19 patients with COVID-19, seven post-COVID-19, 13 healthy donors, and 27 immunocompromised patients, including 20 patients with MS and seven with IMID. Within the IMID group, three individuals (42.9%) had rheumatoid arthritis, two had ankylosing spondylitis, and two had psoriatic arthritis or pemphigus. The immunocompromised patients received treatment for their disease as listed in Table 1.

All enrolled individuals completed at least the first SARS-CoV-2 vaccination cycle, except 8/19 (42.1%) patients with COVID-19 who were unvaccinated. Most vaccinated individuals (51/58, 87.9%) received an mRNA vaccine (BNT162b2 or mRNA-1273), and two individuals received a viral vector-based vaccine (ChAdOX1-S or Johnson & Johnson); information was not available for five individuals.

HDs were enrolled 1-4 months after receiving the booster dose, and the MS group consisted of 13 individuals recruited 1 month after the booster dose and seven having completed the vaccination schedule within 6 months after the first dose. Patients with IMID were recruited 6 months after the first dose. A significant age difference was observed among the five groups ($P = 0.0016$).

Ab response

The Ab response was evaluated in 64 available samples, and most subjects scored IgG-positive (49/62, 79%) (Supplementary Figure 2). However, no Ab response was found in eight subjects with MS, of whom five were receiving treatment with ocrelizumab and two with alemtuzumab or fingolimod; the information was not available for one subject. In addition, five patients with COVID-19, including two vaccinated, scored IgG-negative. Regarding the quantitative response, significantly higher IgG titers were observed in HDs compared with patients with COVID-19 ($P = 0.038$) or subjects with MS ($P = 0.005$).

SARS-CoV-2-specific T cell response was detected by the QuantiFERON SARS-CoV-2 RUO and homemade-IGRA-SPIKE tests

We evaluated the accuracy of the QuantiFERON SARS-CoV-2 RUO kit to detect the IFN-γ response to spike protein using both SARS-CoV-2 Ag1 and Ag2 tubes. As depicted in Figure 1, the total number of responders was comparable between Ag1 and Ag2 tubes in all the enrolled cohorts. Only patients with MS presented a higher response rate to the Ag2 tube (12/20, 60%) than Ag1 (5/20, 45%). Stratifying the data according to the response to Ag1 and/or Ag2 tubes, we found a positive response to at least one Ag tube in most individuals (41/66, 62.1%), regardless of the clinical status (Table 2). However, significant differences were observed in the number of responders to only one or both Ag tubes ($P < 0.0001$). In particular, most individuals responded to either Ag1 or Ag2 tubes (32/66, 48.5%), whereas only one subject showed a T cell response to the single Ag1 tube and eight subjects only to Ag2. Most non-responders were observed among HDs (6/13, 46.2%), followed by COVID-19 (8/19, 42.1%) and patients with MS (8/20, 40%) (Table 2). Among COVID-19 subjects, five received cortisone therapy, whereas within the MS cohort, four received fingolimod, and three received cladribine, IFN-β, or alemtuzumab, and the therapy was unavailable for one subject. Regarding the quantitative response, although IFN-γ levels in response to Ag1 and Ag2 tubes strongly correlated with each other ($r_{ho} = 0.908$, $P < 0.0001$), the IFN-γ high median value for the Ag2 tube was significantly different compared with that of the Ag1 tube (median Ag2: 0.25, interquartile range [IQR]: 0.037-0.73 vs Ag1 median: 0.15, IQR: 0.030-0.44, $P < 0.0001$).

Subsequently, we compared the rate of detectable SARS-CoV-2-spike-specific IFN-γ T cell response obtained using the homemade-IGRA-SPIKE test. A positive response was detected in 78.8% (52/66) of the enrolled subjects (Table 3). Most non-responders were observed among patients with MS (6/20, 30%) or COVID-19 (6/19, 31.6%), likely because of the ongoing immunosuppressive therapy. Indeed, within the COVID-19 group, the non-responders were all patients receiving cortisone. In the MS cohort, three subjects were receiving fingolimod and two receiving ocrelizumab; information was unavailable for one subject.

Overall, the QuantiFERON SARS-CoV-2 RUO assay showed a modest concordance with the homemade-IGRA-SPIKE test (71.2%, $k = 0.331$) and a lower rate of positive responders. However, this difference was not significant ($P = 0.056$) (Table 3).

Regarding the quantitative response, significant different IFN-γ levels were observed between spike and Ag tubes in all the cohorts analyzed, except for patients with COVID-19, that presented comparable responses between the two assays (Figure 1). In particular, a higher IFN-γ-specific response to spike was detected in post-COVID-19 (spike vs Ag1: $P = 0.023$), HDs (spike vs Ag1: $P = 0.0008$; spike vs Ag2: $P = 0.018$), MS (spike vs Ag1: $P = 0.0003$), and IMID subjects (spike vs Ag1: $P = 0.033$). No significant differences were observed in the T cell response detected by each assay among the cohorts analyzed (Figure 2). All subjects

Table 1
Demographical and clinical characteristics of the 66 enrolled subjects.

Characteristics		COVID-19		NO-COVID-19			Total	
					Immunocompromised			
		COVID-19	Post-COVID-19[a]	HDs	MS	IMID		*P*-value
N (%)		19 (28.8)	7 (10.6)	13 (19.7)	20 (30.3)	7 (10.6)	66 (100)	
Age median (IQR)		60 (51-72)	27 (22-44)	43 (32-48)	49 (35-58)	63 (34-70)	50 (36-62)	0.0016[c]
Male N (%)		11 (57.9)	1 (14.3)	4 (30.8)	6 (30)	2 (28.6)	24 (36.4)	0.370[d]
Origin N (%)	**West Europe**	13 (68.4)	7 (100)	12 (92.3)	19 (95)	4 (57.1)	55 (83.3)	0.211[d]
	East Europe	3 (15.8)	-	-	1 (5)	2 (28.6)	6 (9.1)	
	Asia	2 (10.5)	-	1 (7.7)	-	-	3 (4.5)	
	South America	1 (5.3)	-	-	-	1 (14.3)	2 (3.1)	
Swab positive results at the time of enrollment N (%)		18 (94.7) [b]	0 (0)	0 (0)	0 (0)	0 (0)	18 (27.3)	
Vaccination status	**No**	8 (42.1)	0 (0)	0 (0)	0 (0)	0 (0)	8 (12.1)	
	Yes	11 (57.9)	7 (100)	13 (100)	20 (100)	7 (100)	58 (87.9)	
Days from swab positive median (IQR)		8 (2-10)	90 (30-180)	-	-	-	-	
Lymphocytes count N (%)		18 (94.7)	-	-	6 (30) 1.86	-	-	
Median (IQR)		1.09 (0.97-1.52)			(1.58-2.24)			
Severity N (%)[e]	**Available**	18 (94.7)	-	-	-	-	-	
	Moderate	7 (38.9)	-	-	-	-	-	
	Severe	11 (61.1)	-	-	-	-	-	
Therapies N (%)	**Available**	11 (57.9)	-	-	18 (90)	6 (85.7)	35 (53)	
	Ocrelizumab	-	-	-	7 (38.9)	-	7 (20)	
	Fingolimod	-	-	-	4 (22.2)	-	4 (11.4)	
	Cladribine	-	-	-	2 (11.1)	-	2 (5.7)	
	Interferon Beta	-	-	-	4 (22.2)	-	4 (11.4)	
	Rituximab/Alemtuzumab	-	-	-	1 (5.6)	2 (33.3)	3 (8.6)	
	Anti-TNF	-	-	-	-	2 (33.3)	2 (5.7)	
	DMARDs	-	-	-	-	1 (16.7)	1 (2.9)	
	Anti-JAK	-	-	-	-	1 (16.7)	1 (2.9)	
	Cortisone	11 (100)	-	-	-	-	11 (31.4)	

[a] Post-COVID-19: individuals that had COVID-19 between 1-16 months before the enrollment.
[b] One individual had COVID-19 interstitial pneumonia.
[c] Kruskal-Wallis statistic test.
[d] Chi-squared test.
[e] World Health Organization criteria (reference World Health Organization).DMARD = disease modifying antirheumatic drug; HDs = healthy donors; JAK = Janus kinase; IQR = interquartile range; MS = multiple sclerosis; IMID = immune-mediated inflammatory disease; N = number; TNF = tumor necrosis factor.

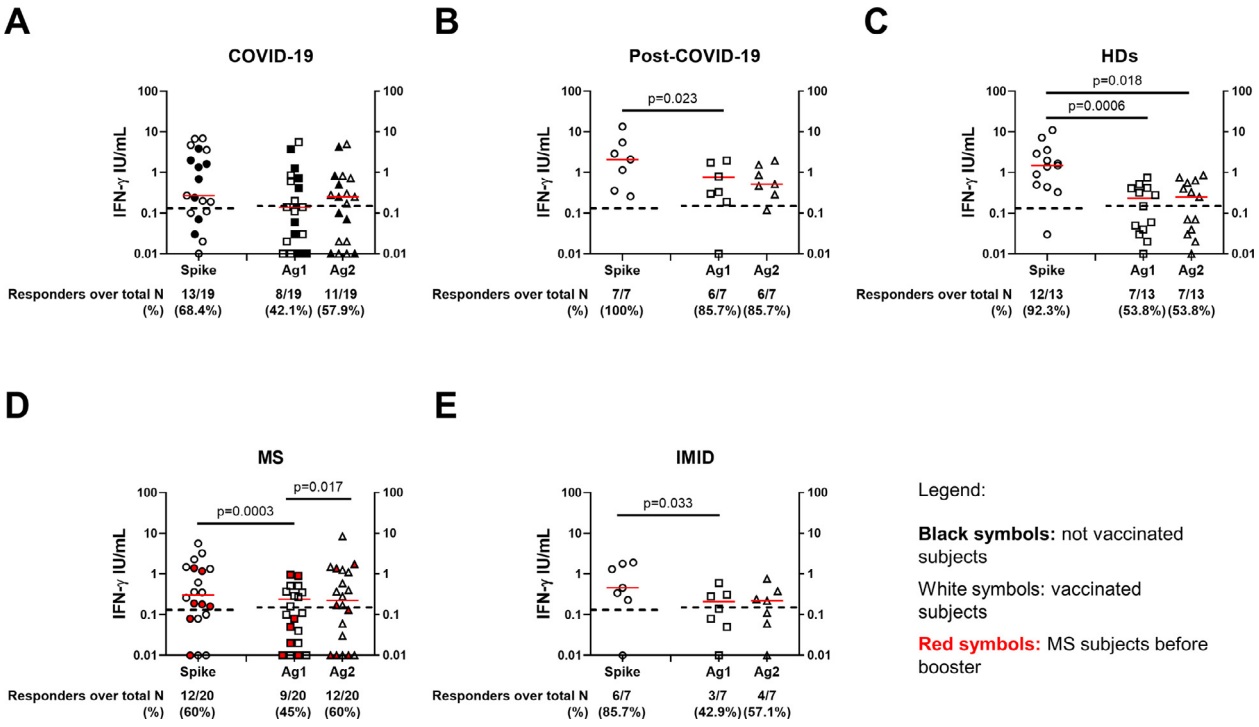

Figure 1. T cell response to QuantiFERON SARS-CoV-2 RUO tubes compared with homemade-IGRA-SPIKE test. Evaluation of the IFN-γ-specific T cell response using SARS-CoV-2 spike and Ag tubes in the enrolled population (n = 66) stratified as follows: (A) COVID-19 (n = 19), (B) post-COVID-19 (n = 7), (C) HDs (n = 13), (D) MS (n = 20) and (E) patients with IMID (n = 7). IFN-γ levels were assessed in plasma harvested from tubes (Ag1 and Ag2) or stimulated samples (spike) and reported by subtracting the background. Dashed lines represent the cut-offs (Ag1 and Ag2 tubes: 0.15 IU/ml; spike: 0.13 IU/ml). Black horizontal lines indicate medians. Black symbols indicate unvaccinated subjects, white symbols indicate vaccinated subjects, and red symbols indicate subjects with MS before a booster dose, as reported in the legend. The Kruskal-Wallis test adjusted with Dunn's multiple comparisons test was performed. A P <0.05 was considered significant. Ag = antigen; HDs = healthy donors; IFN = interferon; IGRA = interferon-gamma release assay; MS = multiple sclerosis; IMID = immune-mediated inflammatory diseases; RUO = research use only; N = number.

Table 2
Responders to Antigen 1 and/or Antigen 2.

Subjects	Ag1$^+$/Ag2$^-$ N (%)	Ag1$^-$/Ag2$^+$ N (%)	Ag1$^+$/Ag2$^+$ N (%)	Ag1$^-$/Ag2$^-$ N (%)	Responders over total N (%)	*P*-value among responders
COVID-19	0/19 (0)	3/19 (15.8)	8/19 (42.1)	8/19 (42.1)	11/19 (57.9)	0.004
Post-COVID-19	1/7 (14.3)	1/7 (14.3)	5/7 (71.4)	0/7 (0)	7/7 (100)	0.032
HDs	0/13 (0)	0/13 (0)	7/13 (53.8)	6/13 (46.2)	7/13 (53.8)	0.0002
MS	0/20 (0)	3/20 (15)	9/20 (45)	8/20 (40)	12/20 (60)	0.001
IMID	0/7 (0)	1/7 (14.3)	3/7 (42.9)	3/7 (42.9)	4/7 (57.1)	0.115
Responders over total	1/66 (1.5)	8/66 (12.1)	32/66 (48.5)	25/66 (37.9)	41/66 (62.1)	<0.0001

Chi-square test was performed for statistical analysis.
Ag = antigen; HDs = healthy donors; MS = multiple sclerosis; IMID = immune-mediated inflammatory diseases; N = number.

responded to the SEB or MIT stimulus used as positive controls. Significant differences were observed in the COVID-19 and IMID cohorts (Supplementary Figure 3).

IFN-γ intracellular responses induced by spike in both assays are mediated by either CD4$^+$ or CD8$^+$ T cells

To assess which T cell subset mediated the IFN-γ response, we characterized the CD4$^+$ and CD8$^+$ T cell response by flow cytometry in PBMCs isolated from 11 subjects (six HDs and five post-COVID-19 subjects) and stimulated either in the QuantiFERON SARS-CoV-2 RUO tubes or the homemade-IGRA-SPIKE test (Figure 3).

Evaluating the total number of responders to the single Ag1 and Ag2 tube in the whole cohort analyzed, we found that the response to the Ag1 tube was mainly mediated by CD4$^+$ T cells, as indicated by the higher response rate (CD4$^+$: 7/11 [63.6%] vs CD8$^+$: 2/11 [18.2%]) (Figure 3A-B). In contrast, the response to the Ag2 tube was mediated by both CD4$^+$ and CD8$^+$ T cell subsets (CD4$^+$: 5/11 [45.4%] vs CD8$^+$: 4/11 [36.4%) (Figure 3A-B). Similar results were also found by stratifying the data according to the clinical status of the subjects (Supplementary Figure 4). No significant differences were observed in the number of responders to the Ag1 and/or Ag2 tubes (Supplementary Table 1).

Using the homemade-IGRA-SPIKE test, we found that the IFN-γ response to spike was mediated by CD4$^+$ and CD8$^+$ T cells in most

Table 3
Responders to QuantiFERON SARS-CoV-2 RUO tubes and homemade-IGRA-SPIKE.

Subjects	Response to any SARS-CoV-2 tubes N (%)	Response to homemade-IGRA-SPIKE N (%)	K Cohen	*P*-value
COVID-19	11/19 (57.9)	13/19 (68.4)	0.329 (68.4%)	0.737
Post-COVID-19	7/7 (100)	7/7 (100)	1.000 (100%)	>0.999
HDs	7/13 (53.8)	12/13 (92.3)	0.177 (61.5%)	0.073
MS	12/20 (60)	14/20 (70)	0.348 (70%)	0.741
IMID	4/7 (57.1)	6/7 (85.7)	0.364 (71.4%)	0.559
Responders over total	41/66 (62.1)	52/66 (78.8)	0.331 (71.2%)	0.056

HDs = healthy donors; IGRA = interferon-gamma release assay; MS = multiple sclerosis; IMID = immune-mediated inflammatory diseases; RUO = research use only; N = number.

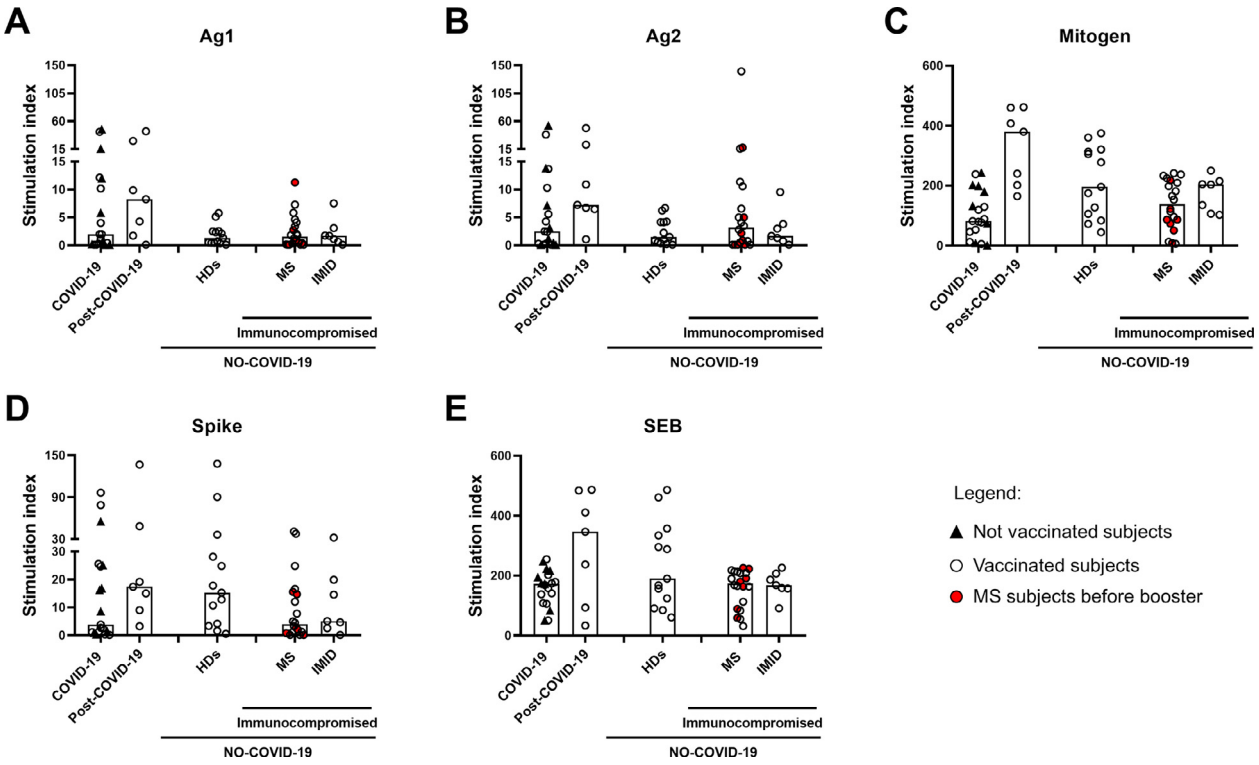

Figure 2. SARS-CoV-2-specific T cell response was detected by both QuantiFERON SARS-CoV-2 RUO and homemade-IGRA-SPIKE test. The enrolled subjects (n = 66) were stratified as follows: COVID-19 (n = 19), post-COVID-19 (n = 7), HDs (n = 13), MS (n = 20) and patients with IMID (n = 7). (A-C) Evaluation of the IFN-γ-specific T cell response using SARS-CoV-2 (A) Ag1, (B) Ag2, and (C) Mitogen tubes. (D) Evaluation of the IFN-γ-specific T cell response using the homemade-IGRA-SPIKE test based on whole-blood stimulation with spike (0.1 µg/ml) or (E) SEB (200 ng/ml), used as a positive control. IFN-γ levels were assessed in plasma harvested from tubes or stimulated samples. Values were reported as stimulation index (signal of stimulated samples divided by negative control signal). Black triangles indicate unvaccinated subjects and white dots vaccinated subjects. Red dots indicate patients before booster dose within the MS cohort. The Kruskal-Wallis test adjusted with Dunn's multiple comparisons test was performed. A P <0.05 was considered significant. Ag = antigen; HDs = healthy donors; IFN = interferon; IGRA = interferon-gamma release assay; MS = multiple sclerosis; IMID = immune-mediated inflammatory diseases; RUO = research use only; SEB = staphylococcal enterotoxin B.

of the subjects analyzed. However, a higher number of responders was found in the CD4$^+$ T cell subset (10/11, 90.9%) compared with the CD8$^+$ subset (6/11, 54.5%) (Figure 3A-B), independently of the clinical status (Supplementary Figure 4).

Overall, minor nonsignificant differences were observed in the frequency of the antigen-specific T cell subsets between the two assays (Figure 3). Importantly, all subjects had a CD4$^+$ and CD8$^+$ T cell response to the SEB or MIT tube, used as positive controls (Figure 3).

Discussion

Several studies have highlighted the importance of the T cell-mediated response to natural SARS-CoV-2 infection and COVID-19 vaccination (Sette and Crotty, 2021). Unlike the humoral response, T cell immunity represents a more sensitive indicator of SARS-CoV-2 exposure because of its early and longer persistence after infection or vaccination (Tarke *et al.*, 2022). Moreover, cell-mediated immunity is less affected by SARS-CoV-2 variants able to partially

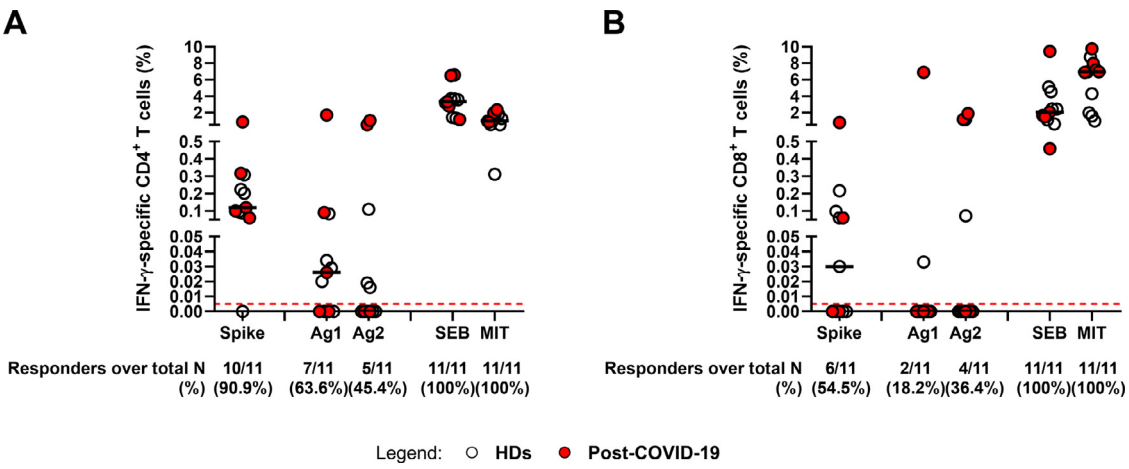

Figure 3. IFN-γ-intracellular T cell response in both assays is mediated by either CD4$^+$ or CD8$^+$ T cells. PBMCs from HDs (n = 6) and patients post-COVID-19 (n = 5) were stimulated using QuantiFERON SARS-CoV-2 RUO tubes and spike of the homemade-IGRA-SPIKE test, and the frequency of IFN-γ-specific T cells was evaluated by flow cytometry. (A) Frequency of CD4$^+$ T cells in all subjects tested. (B) Frequency of CD8$^+$ T cells in all subjects tested. Each dot represents an individual. Red dots indicate post-COVID-19 subjects. Black lines represent medians. Dashed lines indicate the threshold value set at 0.005% (i.e., the lower frequency of response observed among positive responders). White dots indicate HDs and red dots post-COVID-19 subjects, as reported in the legend. The Friedman test adjusted with Dunn's multiple comparisons test was performed to compare paired data of Ag tubes and spike. A P <0.05 was considered significant. IFN = interferon; Ag = antigen; HDs = healthy donors; IGRA = interferon-gamma release assay; MIT = mitogen; PBMCs = peripheral blood mononuclear cells; RUO = research use only; SEB = staphylococcal enterotoxin B; N = number.

evade the Ab response (Geers *et al.*, 2021; Liu *et al.*, 2022; Tarke *et al.*, 2022).

Diagnostic laboratories have numerous validated kits available for Ab detection, whereas valid tests for cellular immunity are lacking. To date, the standardized QuantiFERON SARS-CoV-2 RUO test is a commercially available assay measuring the immune response in different cohorts of vaccinated or SARS-CoV-2 infected individuals (Barreiro *et al.*, 2022; Jaganathan *et al.*, 2021; Krüttgen *et al.*, 2021; Martínez-Gallo *et al.*, 2021; Tormo *et al.*, 2022; Tychala *et al.*, 2021). To our knowledge, we characterized here for the first time the T cell response to QuantiFERON SARS-CoV-2 RUO tubes by flow cytometry. The peptide mix contained in the Ag1 tube induced a response mainly mediated by CD4$^+$ T cells. In contrast, the IFN-γ response to the Ag2 tube was mediated by CD4$^+$ and CD8$^+$ T cells. In addition, the response to the Ag2 tube containing CD4$^+$ and CD8$^+$ T cell epitopes derived from S1 and S2 subunits of the spike protein showed a higher quantitative and qualitative response than that found with the Ag1 tube containing only CD4$^+$ T cell epitopes. These results are in agreement with recent literature (Krüttgen *et al.*, 2021; Martínez-Gallo *et al.*, 2021; Tormo *et al.*, 2022). Unexpectedly, in the present study, we also found a CD8$^+$ T cell response in a few subjects after Ag1 tube stimulation. This is likely because of the antigen-presenting cells that internalize and process the peptides, which are in turn presented by major histocompatibility complex (MHC) class I molecules to CD8$^+$ T cells. This is similar to the findings shown with tests detecting the immune response to other pathogens, such as an assay for the diagnosis of latent tuberculosis infection (Petruccioli *et al.*, 2016).

Using the QuantiFERON SARS-CoV-2 RUO test, most nonresponders were observed among HDs (46.2%), followed by patients with COVID-19 (42.1%) and MS (40%). The epitopes contained in the two tubes induced a higher response in post-COVID-19 subjects, as expected (Grifoni *et al.*, 2020; Yu *et al.*, 2022).

The accuracy for detecting SARS-CoV-2 infection of the standardized kit is lower than the homemade-IGRA-SPIKE test (62.1% vs 78.8%). Moreover, we confirm that the IFN-γ-specific T cell response to spike is mediated by CD4$^+$ and CD8$^+$ T cells in either HDs or post-COVID-19 subjects (Aiello *et al.*, 2021; Farroni *et al.*, 2022; Picchianti-Diamanti *et al.*, 2021; Tortorella *et al.*, 2022). Notably, the magnitude of the response is higher within the CD4$^+$ T cell subset.

The discrepancy in the percentage rate of responders between the QuantiFERON SARS-CoV-2 RUO assay and the homemade-IGRA-SPIKE test might be partly because of the different nature of the S Ag and the concentrations employed in the two assays. In this regard, PepTivator® SARS-CoV-2 peptide pools used in the homemade-IGRA-SPIKE test consist of 15-mer sequences with 11 amino acids overlap, covering the entire sequence of the SARS-CoV-2 Wuhan spike glycoprotein (see Materials and methods section). Because of the great diversity of MHC haplotypes, Ag1 and Ag2 peptide pools might be less fitted to bind to all human leukocyte antigens present in the analyzed cohort. This hypothesis would be supported by the robust spike-specific IFN-γ production found using the homemade-IGRA-SPIKE test. Indeed, significantly higher IFN-γ levels were observed in response to spike than those detected in Ag tubes in all the cohorts analyzed, except for patients with COVID-19 that presented comparable levels between the two assays.

Some limitations of the study need to be considered. Firstly, the small sample size (n = 66) could have limited the robustness of the study. Nevertheless, the cohort of enrolled subjects is heterogeneous and allowed us to test the standardized kit either on subjects with current or previous SARS-CoV-2 infection or vaccinated individuals at different time points and different immune deregulated conditions. Secondly, this study lacks a negative control group consisting of unvaccinated subjects without SARS-CoV-2 infection to evaluate the specificity of the standardized kit. This is because of the large-scale vaccination and COVID-19 cases in Italy (Istituto Superiore di Sanità, 2022) that made it difficult to find unvaccinated and COVID-19-free individuals.

In conclusion, to our knowledge, for the first time, we characterized the CD4$^+$ and CD8$^+$ T cell responses to the QuantiFERON SARS-CoV-2 RUO assay that measures SARS-CoV-2-specific T cell response. Standardized routine tests to measure SARS-CoV-2-specific T cell response accurately are needed. The availability of a complementary test besides the serology may be important, particularly for immunocompromised subjects that, after vaccination or infection, may fail to mount an efficient immune response.

Larger studies are needed to validate the clinical relevance of these findings.

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Author contributions

AA, AC, and DG analyzed, interpreted data and wrote the manuscript; VV, AS, and AMGA processed blood samples, performed the IFN-γ ELISA and SARS-CoV-2 serology; GC, CT, GG, PS, AB, and RL enrolled individuals and collected clinical data; DG conceived and designed the study. All the authors critically revised the article and approved the final version of the manuscript.

Ethical approval statement

The ethics committees of INMI Lazzaro Spallanzani-IRCCS (Approval numbers 59/2020, 72/2015, 297/2021), Nuovo Regina Margherita Hospital (Approval number 318/2021) and MS Center of the Department of Neurosciences of San Camillo Forlanini Hospital (Rome, Italy) (Approval number 319/2021) approved the study.

Conflict of Interest

CT and CG received honoraria for speaking, manuscript writing, or educational events from Merck, Biogen, Roche, Novartis Sanofi, Celgene, and Almiral. EN participates on a data safety monitoring board or advisory board and receives fees for educational training from Gilead, Eli Lilly, GS, SOBI, and Roche. EN has a patent pending for raloxifene use in COVID-19 with Dompè Pharmaceutical. DG is a member of the advisory board of Biomerieux and Eli Lilly and received fees for educational training or consultancy from Almiral, Biogen, Cellgene, Diasorin, Janssen, Qiagen, and Quidel. All the other authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijid.2022.07.049.

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