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Letter to the Editor

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Specific detection of memory T-cells in COVID-19 patients using standardized whole-blood Interferon gammarelease assay

As our understanding of the immune response against SARS-CoV-2 improves, it becomes clear that virus-specific T-cells are key players in the control of infection and that suboptimal T-cells response contribute to Covid-19 severity in many individuals [1]. SARS-CoV-2 specific T-cell responses often target epitopes conserved in different virus clades, including variants of concern [2], and even in other seasonal human coronaviruses (HCoV) [3,4]. Thus, memory T-cells may be more cross-protective than antibodies against different coronavirus infections. In addition, specific SARS-CoV-2 T-cell responses have been detected in convalescent donors without antibody responses suggesting that, at least in some individuals, the measurement of T-cell responses, mainly investigated using ELISpot assay and/or intracellular cytokine staining (ICS) [3,4], may complement serological measurements to uncover and characterize past infections. Thus, novel immunodiagnostics, more suitable for clinical routine than the two methods mentioned above and measuring cellular immune response to SARS-CoV-2, are needed to assess individual immune status and evaluate emerging vaccines in a robust and standardized manner. For this purpose, we designed a semi-automated whole-blood Interferon gamma release assay (WB IGRA) to detect and quantify SARS-CoV-2-specific T-cells immunity. WB assays are rapid and simple assays that preserve all interactions between circulating immune cells reflecting the, in vivo, situation. IFN-ɣ secretion measurement following stimulation with SARS-CoV-2 peptides was used to reveal SARS-CoV-2 specific T-cell response, notably in whole blood and Peripheral blood mononuclear cells (PBMC) assays as previously reported [5–8]. We monitored T-cell immunity directed against different SARS-CoV-2 proteins including nucleocapsid (N), membrane glycoprotein (M) and the spike receptor-binding domain (RBD) in a cohort of 129 Covid-19 convalescent healthcare workers (HCWs) infected 6 months before with SARS-CoV-2. We correlated these measurements with serological levels of antibodies (Abs) against SARS-CoV-2. We also assessed the specificity of the WB IGRA for SARS-CoV-2 by performing measurements in 25 SARS-CoV-2 seronegative healthy volunteers (HVs) and in three HCWs with previously documented infection by HCoV.

We constituted a cohort of HCWs previously infected by SARS-CoV-2 ($n = 129$) or HCoV ($n = 3$) at 6-month post-symptom period. Among patients, 20 of 129 (16%) SARS-CoV-2 positive HCWs were male (sex ratio, 0.18), the median age was 41 years (21-62 years); one of three (33%) HKU-1 (n = 1)/NL-63 (n = 2) positive HCWs were male (sex ratio, 0.5) with a median age of 22 years (21-23 years). We also included HVs (n = 25) of which 13 of 25 (52%) were male (sex ratio, 1.08) with a median age of 42 years (19-68 years). We first monitored SARS-CoV-2 specific Abs using the Wantai total Ab kit in the three different groups of participants. These Abs were undetectable in the sera from the three HCoV positive HCWs and from the 25 HVs, confirming the absence of previous SARS-CoV-2 infection. In contrast, they were detected in all 129 HCWs with documented SARS-CoV-2 infection 6 months before. These sera were further characterized, 82.9% ($n = 107$) had IgG against the SARS-CoV-2 RBD domain (bioMérieux VIDAS®) [8–10] and only 48.8% ($n = 63$) scored positive in a virus neutralization test (VNT) (Table 1), confirming previous findings regarding the relative short half-life of neutralizing antibodies (nAbs) in subjects with mild disease [11,12].

We then monitored the T-cell response against M, RBD, and N peptides using the WB IGRA for the different groups. A detectable IFN-ɣ release was observed for 89.1%, 62.4%, and 97.9% of SARS-CoV-2 positive HCWs (detailed in Table 1) after stimulation with M (Figure 1A), RBD (Figure 1B), and N (Figure 1C) SARS-CoV-2 peptides, respectively. These data suggest that a detectable T-cell response after SARS-CoV-2 peptide stimulation can be observed at 6 months post symptoms, in accordance with recent results [8], and extending previous findings by *Murugesan et al.* [7] who used a similar approach, with different peptides pools, in recently-SARS-CoV-2-infected patients. No or very low IFN-ɣ release (<0.12 IU/mL) was detected for HVs and HCoV upon stimulation with M (Figure 1A) or RBD (Figure 1B). Interestingly, two of the three HCoV positive HCWs (Mean 0.45

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Table 1. Association of SARS-CoV-2 humoral and cellular response

	Anti-SARS-CoV-2-RBD IgG titer	Virus neutralization test
Assay type	ELFA	/
Positive, n (%)	107 (82.9)	63 (48.8)
Negative, n (%)	22 (17.1)	66 (51.2)
Overall, Negative and Positive Percent Agreement with WB IGRA M; T-cell responders 115/129 (89.1%) (IFN-γ titer: median 0.51 [0.24-1.49] IU/mL)		
OPA [95%CI]	78.3 [70.4-84.5]	56.6 [48.0-64.8]
PPA [95%CI]	90.7 [83.7-94.8]	96.8 [89.1-99.1]
NPA [95%CI]	18.2 [7.3-38.5]	18.2 [10.7-29.1]
Overall, Negative and Positive Percent Agreement with WB IGRA RBD; T-cell responders 58/93 (62.4%) (IFN-γ titer: median 0.12 [0.17-0.34] IU/mL)		
OPA [95%CI]	71.0 [61.1-79.2]	62.4 [52.2-71.5]
PPA [95%CI]	69.6 [58.8-78.7]	72.6 [59.1-82.9]
NPA [95%CI]	78.6 [52.4-92.4]	50.0 [35.5-64.5]
Overall, Negative and Positive Percent Agreement with WB IGRA N; T-cell responders 92/94 (97.9%) (IFN-γ titer: median 1.20 [0.60-2.48] IU/mL)		
OPA [95%CI]	81.9 [72.9-88.4]	51.1 [41.1-60.9]
PPA [95%CI]	97.5 [91.2-99.3]	100 [92.3-100]
NPA [95%CI]	0 [0-20.4]	4.2 [1.2-14.0]

[0.10-0.79] IU/mL), as well as 44% (n = 11/25, Mean 0.20 [0.13-0.32] IU/mL) of the HVs showed a low detectable IFN-γ response upon stimulation with N (Figure 1C). These data suggest that N peptides are able to elicit not only SARS-CoV-2 specific T-cells but also cross-reactive HCoV-specific T-cells, which reinforces the existence of potential pre-existing immunity in HVs, as previously noted [4,13,14,15].

The overall percent agreement between the T-cell responses against N, M, or RBD WB IGRA and positive anti-SARS-CoV-2-RBD IgG (bioMérieux IgG assay) was of 81.9%, 78.3%, and 71.0%, respectively. Among the 22 COVID-19 convalescent HCWs with undetectable anti-SARS-CoV-2-RBD IgG, only 4 did not have M-specific T-cells, which resulted in a low negative percent agreement (NPA) between cellular and humoral responses (18.2%), interestingly this NPA was null concerning N-specific T-cells i.e. all patients with undetectable anti-SARS-CoV-2-RBD IgG have detectable N-specific T-cells (detailed in Table 1). Moreover, among the convalescent HCWs who lacked neutralizing Abs at 6 months post-infection, 81.8% (n = 54/66) and 95.8% (n = 46/48) had M- and N-specific T-cells, respectively. These results highlight that the T-cell response persists longer than neutralizing antibodies, suggesting that the measurement of T-cell responses is important to define the history of pastinfections.

Finally, we assessed the production of IFN-γ by $CD4^+$ T-cells measured by

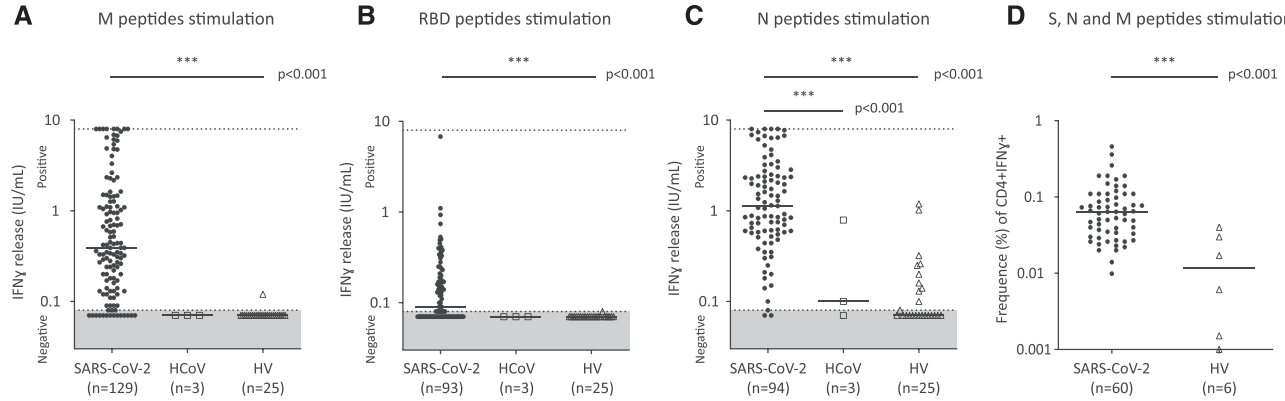

Figure 1. IFN-γ release after in vitro WB stimulation using SARS-CoV-2 peptide pools. IFN-γ levels measurement in WB of samples from SARS-CoV-2 convalescent HCWs (dot), three HCoV convalescent HCWs (square) and 25 controls (HV) (triangle). IFN-γ secretion was measured on VIDAS automated platform (VIDAS® IFNγ RUO, bioMérieux) after a 22-h stimulation using (A) M peptides, (B) RBD peptides, and (C) N peptides. The median and individual secretion levels are shown. (D) Percentage of IFN-γ positive $CD4^+$ T-cells measured by intracellular cytokine staining (ICS) following stimulation of PBMCs from a subset of patients (dot, n = 60) and new controls (HV) (triangle, n = 6) with a pool of peptides (PepTivator, Miltenyi Biotec) derived from Spike, N, and M proteins as detailed in the methods. Statistical differences were inferred using non-parametric Kruskal-Wallis test and Dunn's multiple comparisons test (A-C) or Mann-Whitney test (D) (***$p < 0.001$).

intracellular cytokine staining (ICS) following stimulation of PBMCs from a subset of patients ($n = 60$), with a pool of peptides (PepTivator, Miltenyi Biotec) derived from Spike, N, and M proteins (Figure 1D). We observed a 5.3-fold increase of $CD4^+IFN\gamma^+$ in SARS-CoV-2 convalescent HCWs compared to HV ($p<0.001$), suggesting that IFN-γ is mainly produced by SARS-CoV-2 specific $CD4^+$ T-cells and confirming the use of IFN-γ release as a relevant marker of T-cell immune response as previously described [5–7].

However, we acknowledge that our study has some limitations: (i) the WB IGRA was not able to differentiate between $CD8^+$ and $CD4^+$ T-cells responses, (ii) it does not determine whether memory T-cells are protective against SARS-CoV-2 re-infection, (iii) it was not evaluated in vaccinated subjects, and (iv) it is necessary to assess whole Spike protein epitopes including other non-RBD spike epitopes in order to improve detection of vaccine-induced memory T-cells. Nevertheless, a validated [semi-]automatized SARS-CoV-2 WB IGRA would be of interest to assess T-cell memory induced by natural infection or vaccination in the clinical routine.

Here, we present a WB IGRA that is highly efficient in detecting SARS-CoV-2 specific T-cell responses, confirming the reliability of such tests [5–7]. Our results highlight that peptides derived from distinct viral proteins show a different capacity to trigger a T-cell response in patients having recovered from SARS-CoV-2 infection. Stimulation with RBD-peptides was less efficient in inducing IFN-γ secretion than stimulation with M and N peptides in convalescent patients, however this assay could be helpful to assess T-cell memory after SARS-CoV-2 vaccination targeting the Spike RBD domain. Indeed, the induction of protective immune responses composed of Spike- or RBD-directed nAbs associated with Th1-type cellular responses, is recommended by WHO. N- and M-peptides both induced the production of high levels of IFN-γ, the stimulation by N could even induce low levels of IFN-γ secretion by HCoV-convalescent HCWs and HVs T-cells indicating that the pool of N-peptides contains some cross reactive epitopes able to stimulate HCoV induced T-cells. Hence, stimulation with M appears to be more specific and could be used to measure T-cell memory after SARS-CoV-2 natural infection. Alternatively, N peptides located in regions that are conserved between HCoV and SARS-CoV-2 could be removed from the N pool of peptides [3,4] or a higher positivity threshold, more suitable than detection threshold defined by method protocol, could be set using the pool described in this study.

In summary, WB IGRA could thus be proposed as a suitable and rapid option for assessing the presence of a long-term specific T-cell response against SARS-CoV-2.

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Abbreviations: **Abs**: antibodies · **HVs**: healthy volunteers · **HCoV** : seasonal human coronaviruses · **HCWs**: healthcare workers · **ICS**: intracytoplasmic staining · **IGRA**: IFN gamma release assay · **IU**: international unit · **M**: membrane glycoprotein · **nAbs**: neutralizing antibodies · **N**: nucleocapsid · **RBD**: receptor-binding domain · **WB**: Whole-blood

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