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Coordinated innate and T-cell immune responses in mild COVID-19 patients from household contacts of COVID-19 cases during the first pandemic wave

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Objective: To better define the immunopathogenesis of COVID-19, the present study aims to characterize the early immune responses to SARS-CoV-2 infection in household contacts of COVID-19 cases. In particular, innate, T- and B-cell specific responses were evaluated over time.

Methods: Household contacts of COVID-19 cases screened for SARS-CoV-2 infection by nasopharyngeal swab for surveillance purposes were enrolled (T0, n=42). Of these, 28 subjects returned for a follow-up test (T1). The innate response was assessed by detecting a panel of soluble factors by multiplex-technology in plasma samples. Cell-mediated response was evaluated by measuring interferon (IFN)- γ levels by ELISA in plasma harvested from whole-blood stimulated with SARS-CoV-2 peptide pools, including spike (S), nucleocapsid (N) and membrane (M) proteins. The serological response was assessed by quantifying anti-Receptor-Binding-Domain (RBD), anti-Nucleocapsid (N), whole virus indirect immunofluorescence, and neutralizing antibodies.

Results: At T0, higher levels of plasmatic IFN- α , IL-1ra, MCP-1 and IP-10, and lower levels of IL-1 β , IL-9, MIP-1 β and RANTES were observed in subjects with positive swab compared to individuals with a negative one ($p < 0.05$). Plasmatic IFN- α was the only cytokine detectable in subjects with positive SARS-CoV-2 swabs with high accuracy for swab score positivity (0.93, $p < 0.0001$). Among subjects with positive swabs, significant negative correlations were found among the RT-PCR cycle threshold values reported for genes S and N and IFN- α or IP-10 levels. At T0, the IFN- γ T-cell specific response was detected in 50% (5/10) of subjects with positive swab, while anti-RBD/anti-N antibodies showed a positivity rate of 10% (1/10). At T1, the IFN- γ T-cell specific response was detected in most of the confirmed-infection subjects (77.8%, 7/9), whereas the serological response was still observed in a minority of them (44.4%, 4/9). Overall, the swab test showed a moderate concordance with the T-cell response (78.6%, $k = 0.467$), and a scarce concordance with the serological one (72.9%, $k = 0.194$).

Conclusions: Plasmatic IFN- α and the IFN- γ T-cell specific response appear early even in the absence of seroconversion, and show a greater positivity rate than the serological response in household contacts with positive swab.

KEYWORDS

household contacts, COVID-19, SARS-CoV-2, T-cell response, Interferon-alpha (IFN- α), Interferon-gamma (IFN- γ) release assay (IGRA), whole blood, spike protein

Introduction

The COroNaVIrus Disease (COVID-19) caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is a new zoonosis that has spread since December 2019. SARS-CoV-2 infection occurs with a variety of clinical syndromes; most people present a less severe disease and remain asymptomatic or paucisymptomatic, while approximately 20% of people (at least with the ancestral strain) develop severe respiratory symptoms, which may lead to hospital admission and eventually to death (1–4). The host itself seems to be the major factor explaining disease severity, infection rates (5, 6), and long-term medical consequences (7).

The host response to SARS-CoV-2 infection described during the first epidemic wave, before vaccination, is crucial to understand the mechanisms of effective host-defence in naïve populations. Evidence indicates that a coordinated innate and adaptive immune response, that includes both T and B cells, is necessary to mount an appropriate immune protection that counteracts SARS-CoV-2 infection (8, 9). The T-cell response is also crucial against the variants of concerns (10).

Soluble factors including cytokines, chemokines and growth factors act, both locally and systemically, influencing the release of innate immune cells from the bone marrow into

circulation, as well as their recruitment to inflamed and infected tissues (11).

The variability in innate immune system components has been correlated to the heterogeneous disease courses observed in COVID-19 patients (12). Increased pro-inflammatory or anti-inflammatory cytokines, including T helper type-1 and type-2 cytokines and chemokines, were reported (13–16). Interleukin (IL)-1 β , IL-6, IL-8, and Interferon (IFN)- γ -inducible protein (IP-10) were found to be correlated with severe or fatal outcome (17–19). Strong evidence has also shown that innate immunity mediated by type I IFN responses contributes to protection against critical illness (20–22).

Many studies evaluating immune correlates of protection against SARS-CoV-2 focused on the detection of neutralizing antibodies (23–26). However, antibodies are absent in the early stages of the disease or not detectable in patients with less severe forms of COVID-19 (27–29). Levels of neutralizing antibodies are highly variable (1) and antibody titers wane over time (30, 31). Moreover, there is no agreement on the cut off and, so far, no correlates of protection are available.

Conversely, T-cell response is detectable during acute disease and in recovery and is more durable (32–35). Different works validated a whole-blood test based on IFN- γ release for the detection of a SARS-CoV-2 specific T-cell response to

discriminate COVID-19 patients from uninfected individuals, and to monitor the immune response in vaccinated individuals (13, 36–40).

Both innate and adaptive immune responses are involved in virus clearance, inhibition of virus replication and promotion of tissue repair. Lack of coordination among the immune responses has been associated with poor outcome as in the elderly (41). Accordingly, it is of great importance to evaluate the combination, as well as the timing (kinetics) of both immune responses against COVID-19 disease, starting from the earliest stages. This acquired knowledge can be useful for new diagnostic tools or therapy interventions. However, there is a lack of longitudinal studies on the combined analysis of innate immunity, serological and T-cell specific responses against SARS-CoV-2 in the same patient population during the first epidemic wave (42). Therefore, in this study, we characterized the innate and adaptive immune responses in individuals early exposed to SARS-CoV-2, who have presented an asymptomatic or mild COVID-19, correlating the results with the outcome of the nasopharyngeal swab.

Materials and methods

Study population

The prospective study was conducted between February 10th and June 17th 2021. The Ethical Committee of the National Institute for Infectious Diseases (INMI) L. Spallanzani approved the study (approval number 247/2021).

The study population was selected based on the probability of having or not a recent SARS-CoV-2 infection compared to a control population. Household contacts of confirmed COVID-19 cases were enrolled among the subjects screened for surveillance purposes at the drive in at the ASL Roma 1 Santa Maria della Pietà (Rome, Italy). These subjects were screened for SARS-CoV-2 infection by nasopharyngeal swab to detect early household's positivity. The enrolled subjects were tested at two time points: at the execution of the first swab (T0) and for a follow-up test after 7–20 days (T1), end of the quarantine period.

In addition to these individuals, 53 COVID-19 patients with acute disease were recruited as positive controls. Inclusion criteria for COVID-19 patients were a diagnosis based on a positive nasopharyngeal swab for SARS-CoV-2 and a disease with the clinical characteristics already described (43). The COVID-19 group included patients with a moderate, severe or critical disease according to WHO (44). These patients were classified based on the highest severity score of the disease occurring during the hospitalization as described (36, 45).

“NO-COVID-19”-individuals were enrolled among healthy blood donors (HD) from Transfusional Medicine and Stem Cells Unit at the San Camillo Forlanini hospital (Rome, Italy). Inclusion criteria for the “NO-COVID-19” group were:

negative SARS-CoV-2 serology and/or negative swab and no symptoms of COVID-19.

Exclusion criteria for the enrollment were: HIV infection, having received vaccination against SARS-CoV-2, communication of a previous SARS-CoV-2 infection, incapability to sign an informed consent, and age ≤ 18 years.

All the enrolled patients and controls signed a written informed consent. Demographic and clinical information were obtained at the time of enrollment.

SARS-CoV-2 molecular testing

The molecular research of SARS-CoV-2 in nasopharyngeal swabs was performed using Seegene automated instrumentation (Seegene Inc., Seoul, Republic of Korea). The procedure involves the extraction of RNA using the NIMBUS/STARlet system and STARMag Universal Cartridge kit. The instrumentation automatically sets microplates, which are then processed on the real-time PCR Biorad CFX96 (Bio-Rad Laboratories, CA, USA). Seegene's Allplex SARS-CoV-2 Assay was used as real-time PCR method; it detects, in a single tube, RdRP, S and N genes for SARS-CoV-2. Allplex SARS-CoV-2 Assay was the evolution of the technology developed by Seegene in 2020, based on the Corman protocol (46).

SARS-CoV-2 peptide pools

Stimulations were performed with peptide pools of 15 amino acid length with an 11 amino acid overlap encompassing the sequence of the SARS-CoV-2 spike protein (PepTivator[®] SARS-CoV-2 Prot_S1, Prot_S, and Prot_S+), nucleocapsid phosphoprotein (PepTivator[®] SARS-CoV-2 Prot_N), and membrane glycoprotein (PepTivator[®] SARS-CoV-2 Prot_M). A final concentration of 0.1 $\mu\text{g}/\text{mL}$ was used for S and M peptide pools, whereas a concentration of 1 $\mu\text{g}/\text{mL}$ was used for N peptide pool according to a previous study (36). All peptides were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany).

IFN- γ whole-blood assay

Cell-mediated immune response was evaluated using an IFN- γ release test based on the stimulation of whole-blood. Briefly, heparinized whole-blood (600 μL) was stimulated or not with SARS-CoV-2 peptide pools in a 48-well flat-bottom plate and incubated at 37°C (5% CO₂) for 20–24h. After overnight stimulation, plasma was harvested and stored at -80°C until further analysis. IFN- γ levels were quantified by ELISA, according to manufacturer's instructions (www.quantiferon.com) and reported after subtracting the unstimulated control. The detection

limit of the test was 0.065 IU/mL. For pools S and N, IFN- γ levels \geq 0.13 IU/mL indicated a positive response, whereas for pool M the cut-off was \geq 0.19 IU/mL according to the receiver operating characteristic (ROC) analysis performed comparing COVID-19 patients and “NO-COVID-19” subjects (36).

SARS-CoV-2 antibody testing

The serological response was evaluated by measuring anti-Receptor Binding Domain (RBD) (ARCHITECT SARS-CoV-2 IgG II Quantitative, Abbott Laboratories, Wiesbaden, Germany), anti-Nucleocapsid (N) (ARCHITECT SARS-CoV-2 IgG, Abbott Laboratories) and neutralizing antibodies. Anti-N IgG are expressed as index values, i.e., Sample/Cutoff (S/CO), and values \geq 1.4 indicate positive samples. Anti-RBD IgG are expressed as Binding Antibody Units (BAU/mL) and values \geq 7.1 are considered positive.

The micro-neutralization assay (MNA) was performed as previously described (47), using the SARS-CoV-2/Human/ITA/PAVIA10734/2020 (isolated in March and provided by Fausto Baldanti, Pavia, Italy). The test is based on the evaluation of the cytopathic effect (CPE) at 48h after the infection of Vero E6 cells with 7 two-fold serial dilutions of the virus-serum mixture. The neutralization titer was expressed as the reciprocal of serum dilution (MNA₉₀), i.e., the highest serum dilution inhibiting at least 90% of the CPE. The positivity threshold was set at 1:10.

Indirect immunofluorescence assay (IFA)

To verify the specificity of cell-mediated and serological responses in subjects with negative swab, an indirect immunofluorescence assay was performed using home-made slides prepared with SARS-CoV-2-infected Vero E6 cells, as described elsewhere (48).

Cytokines/chemokines evaluation

To evaluate the cytokine/chemokine profile, blood was collected in heparinized tubes and processed within 2 hours from collection. Briefly, plasma was separated by centrifuging the blood at 500 x g for 10 minutes, aliquoted and stored at -80°C until use. Unstimulated plasma samples were analysed using the Bio-Plex Pro Human Cytokine 27-plex Assay and the MagPix system, according to manufacturer’s instructions (all from Bio-Rad, Hercules, CA, USA). The multiplex allowed the detection of the following cytokines, chemokines and growth factors: interleukin (IL)-1 β , IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17A, eotaxin, basic fibroblast growth factor (FGF), granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN- γ , IP-10,

monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1 α , MIP-1 β , platelet-derived growth factor (PDGF), RANTES (regulated on activation, normal T-cell expressed and secreted), tumour necrosis factor-alpha (TNF- α), vascular endothelial growth factor (VEGF). Data were generated using the Bio-Plex Manager software. Concentrations below the detection range were considered zero and samples acquired with a bead count $<$ 50 were excluded from the analysis.

In addition, unstimulated plasma samples were tested for IFN- α and - β by an automatic ELISA (ELLA, protein simple, R&D System, Minneapolis, MN, USA). The limit of detection of IFN- α was 0.51 pg/mL, whereas for IFN- β was 1.03 pg/mL.

Statistical analysis

The Graph Pad software (GraphPad Prism 8 XML Project, San Diego, CA, USA) was used for the statistical analysis. IFN- γ levels and anti-RBD, anti-N and neutralizing antibody titers were reported as median and interquartile range (IQR), whereas categorical variables were reported as count and proportion. The following non-parametric statistical inference tests were used: Chi-square or Fisher’s exact test for categorical variables with Bonferroni correction when appropriate, Mann-Whitney U-test and Wilcoxon signed rank test for pairwise comparisons (for unpaired and paired data, respectively), the Kruskal-Wallis test and the Dunn’s multiple comparisons test for comparisons among groups. Correlations between assays were assessed by non-parametric Spearman’s Rank test. 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. ROC analysis was used for evaluating the area under the curve (AUC) and the diagnostic performance. Cohen’s kappa was used to assess the agreement between two assays. Two-tailed p-values $<$ 0.05 were considered significant.

Results

Description of the studied population

The study cohort consisted of 111 individuals. In particular, we prospectively enrolled 42 household contacts of laboratory-confirmed COVID-19 cases, and 53 COVID-19 patients and 16 “NO-COVID-19” individuals as control groups (Table 1).

The three groups showed significant difference with respect to age ($p > 0.0001$). Among household contacts, 10/42 (23.8%) scored positive for the swab on the day of sample collection (T0) (Figure 1). After 7-20 days (T1) from the first swab, 28 subjects returned to the follow-up. Among them, 19/20 of the subjects remained swab negative, whereas one individual, who scored negative in the first swab, became positive in the second one. All subjects scored swab positive at baseline remained positive at the follow-up ($n=8$) and had a mild COVID-19.

TABLE 1 Demographical and clinical characteristics of the 111 enrolled subjects.

Characteristics	Household contacts	COVID-19 hospitalized patients	NO-COVID-19 subjects (blood donors)	P value
N (%)	42 (37.8)	53 (47.7)	16 (14.4)	
Age median (IQR)	48 (29-55)	58 (52-71)	42 (38-54)	<0.0001*
Male N (%)	20 (47.6)	34 (64.1)	12 (75.0)	0.104 [§]
Origin N (%)				
	West Europe	50 (94.3)	16 (100)	0.434 [§]
	East Europe	2 (3.8)	–	
	Asia	1 (1.9)	–	
	South America	–	–	
Swab positive results at the time of enrolment N (%)	10 (23.8)	53 (100)	0 (0)	
Days from symptom onset N (%)				
	1-7	22 (41.5)	–	
	8-14	21 (39.6)	–	
	15-30	8 (15.1)	–	
	> 30	2 (3.8)	–	
Cycle threshold (Ct) values				
	gene S	24.2 (19.6-32.3)	–	
	gene N	22.9 (19.2-31.1)	–	
Days of exposure median (IQR)	4 (4-5)	–	–	
Time of follow-up N (%)				
	Available	28 (66.7)	–	
	≤ 7 ^a	13 (46.5)	–	
	8-14	9 (32.1)	–	
	15-20	6 (21.4)	–	
Severity N (%) [†]				
	Moderate	14 (26.4)	–	
	Severe	30 (56.6)	–	
	Critical	9 (17.0)	–	
Cortisone therapy N (%)				
	Available	40 (75.5)	–	
		26 (65)	–	
Other diseases ^b				
	Available	7 (16.7)	0 (0)	
	Metabolic disease	1 (14.3)	–	
	Cardiovascular disease	3 (42.8)	–	
	Cancer in therapy	1 (14.3)	–	
	Thyroid disease	2 (28.6)	–	
	Neurological disease	1 (14.3)	–	

COVID-19, coronavirus disease 19; N, number. *Kruskal–Wallis statistic test. [§]Chi-square test. [†]WHO criteria (ref WHO). The information regarding the hospitalized COVID-19 patients receiving or not cortisone was available only for 40 subjects (75.5%). Among these 40 subjects, only 26 (65%) were under cortisone therapy at the time of enrolment.

^aOnly one subject returned after 6 days.

^bOf them, 2 subjects scored positive for the swab but they were able to mount both T-cell and antibody response.

A specific plasma cytokine/chemokine profile was found in household contacts with positive swab compared to those with a negative swab

To characterize the cytokine/chemokine profile of early SARS-CoV-2 infections, the levels of several cytokines, chemokines and growth factors were assessed in the plasma of the enrolled household contacts at baseline (T0) and at the follow-up (T1). At T0, a different cytokine/chemokine profile

was found in subjects with a swab positive result. In particular, significant higher levels of IFN- α , IL-1ra, MCP-1 and IP-10 were detected in the plasma of swab positive subjects compared to individuals with negative ones ($p < 0.0001$, $p = 0.007$, $p = 0.046$ and $p < 0.0001$, respectively) (Figures 2A–D). By contrast, lower levels of IL-1 β , IL-9, MIP-1 β and RANTES were found compared to swab negative subjects ($p = 0.036$, $p = 0.005$, $p = 0.003$ and $p = 0.026$) (Figures 2E–H). No significant differences were observed at the follow-up for these cytokines (Supplementary Figure S1) neither at both time points for the



FIGURE 1

Flow chart of the enrolled household contacts of COVID-19 cases. Household contacts of COVID-19 cases ($n=42$) were enrolled and analyzed at the execution of the first nasopharyngeal swab (T0) and after 7-20 days (T1), at the end of the quarantine period. Twenty-eight of these subjects returned to follow-up. Footnote: COVID-19, COronaVirus Disease 2019.

other soluble factors tested (**Supplementary Figures S2, S3**). ROC curve analysis showed that the highest AUC was associated with IFN- α (AUC: 0.93, 95% CI: 0.81-1.00, $p<0.0001$) followed by IP-10 (AUC: 0.92, 95% CI: 0.82-1.00, $p<0.0001$) (**Table 2**).

Moreover, within the cohort of swab positive subjects, significant negative correlations were found between the RT-PCR Cycle threshold (Ct) values reported for genes S and N and IFN- α (IFN- α vs gene S: $\rho=-0.635$, $p=0.009$ and IFN- α vs gene N: $\rho=-0.591$, $p=0.022$), or IP-10 levels (IP-10 vs gene S: $\rho=-0.677$, $p=0.004$ and IP-10 vs gene N: $\rho=-0.629$, $p=0.010$) (**Table 3 and Supplementary Figure S4**).

In addition, no significant modulations were observed comparing the two time points, except for the chemokine MIP-1 α that showed a significant increase at T1 in swab negative subjects ($p=0.008$) (**Supplementary Table S1**).

Interestingly, at T0 the IFN- α was the only cytokine specifically detectable in subjects with positive swab and no longer detectable in most of the subjects at follow-up.

The IFN- γ -specific T-cell response to SARS-CoV-2 peptide pools was early detected in household contacts

The adaptive immune response includes both cell-mediated and humoral response. Regarding the cell-mediated response, the positivity rate was evaluated considering any positive T-cell response regardless of the peptides used. In the household

contacts tested at T0, we found a specific T-cell response in 50% (5/10) of swab positive subjects. All these individuals responded to pool S, 3 of them scored positive also to N-specific stimulation, while only 1 individual tested positive to N-, M- and S-specific stimulations (**Figures 3A-D**). Among subjects with negative swab ($n=32$), 4 individuals (12.5%) showed a specific T-cell response. In particular, all subjects responded to pool S, whereas 2/4 to pools N or M (**Figure 3D, right panel**).

At T1, the specific T-cell response to SARS-CoV-2 peptide pools was detected in most of the swab positive subjects (77.8%, 7/9) (**Figures 3A-C**), of whom all subjects responded to pool S, two individuals to both pools S and N and four subjects to all three peptide pools (**Figure 3E, left panel**). Among subjects with negative swab ($n=19$), five individuals (26.3%) showed a specific T-cell response (**Figure 3E, right panel**). In particular, three subjects responded to pool S, 1/5 to pool N and 4/5 to pool M.

In the "NO COVID-19" group the response to all three peptide pools was absent in most of the subjects (14/16, 87.5%), indicating a good accuracy of this test to discriminate "NO COVID-19" subjects from COVID-19 patients (pool S: $p=0.0062$; pool N: $p=0.0043$; pool M: $p=0.034$) (**Figures 3A-C**). In the COVID-19 cohort, the specific T-cell response to SARS-CoV-2 peptide pools was detected in 75.5% (40/53) of the individuals. Half of them responded to all three peptide pools regardless of the number of days elapsed since the onset of symptoms (**Figure 3F**). To note, the percentage of positive T-cell responders among COVID-19 patients was similar to

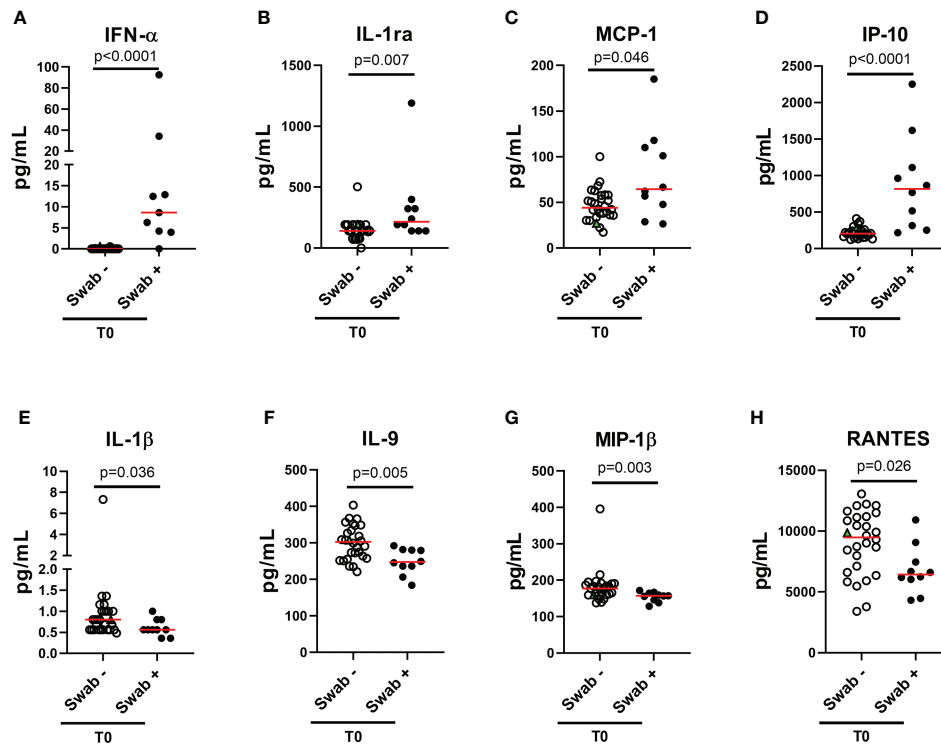


FIGURE 2

Plasmatic cytokines/chemokines modulated in household contacts at baseline. (A–H) Household contacts at T0 ($n = 38$) were stratified according to the swab result: positive ($n = 10$) and negative ($n = 28$). Plasma harvested from unstimulated blood samples were tested for the detection of 27 cytokines/chemokines using the Bio-Plex Pro Human Cytokine 27-plex Assay and for the detection of IFN- α and - β by means of an automatic ELISA. Red horizontal lines indicate medians. The green triangle identifies the subject with a positive swab only at T1. Statistical analysis was performed using Mann-Whitney U test to compare swab positive and negative subjects. A $p < 0.05$ was considered significant. IL, interleukin; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; IP, Interferon-gamma induced protein; RANTES, regulated on activation IFN, interferon.

the percentage observed in swab positive household contacts at T1 (7–20 days from the first swab) (Figure 3E left panel). Differently, at T0 most of the responders scored positive for both pools S and N, but not for pool M (Figure 3D left panel).

Regarding the quantitative response, significant differences were observed between IFN- γ levels of COVID-19 patients and

those detected at T0 in the swab negative cohort of household contacts in response to all three peptide pools ($p < 0.0001$ for both pools S and N, $p = 0.0058$ for pool M). These differences persisted also at T1 for pools S and N ($p = 0.014$ and $p = 0.013$, respectively) (Figures 3A–C). On the contrary, the magnitude of the IFN- γ -specific response to all peptide pools was not significantly

TABLE 2 Accuracy of the eight plasmatic cytokines/chemokines significantly modulated between swab-positive and swab-negative household contacts.

Cytokines/Chemokines	ROC AUC	95% CI	p
IFN- α	0.93	0.81–1.00	<0.0001
IL-1 β	0.73	0.55–0.90	0.033
IL-1ra	0.77	0.60–0.95	0.011
IL-9	0.76	0.61–0.92	0.013
IP-10	0.92	0.82–1.00	<0.0001
MCP-1	0.70	0.48–0.93	0.057
MIP-1 β	0.78	0.64–0.93	0.007
RANTES	0.71	0.54–0.88	0.048

AUC, area under the curve; CI, confidence interval.

TABLE 3 Correlations between plasmatic cytokines/chemokines and RT-PCR cycle threshold (CT) values in swab-positive subjects.

Cytokines/Chemokines	CT gene S		CT gene N	
	rho	p	rho	p
IFN- α	-0.635	0.009	-0.591	0.022
IL-1 β	-0.069	0.790	0.138	0.607
IL-1ra	-0.476	0.055	-0.376	0.151
IL-9	0.024	0.926	0.143	0.595
IP-10	-0.677	0.004	-0.629	0.010
MCP-1	-0.374	0.139	-0.302	0.254
MIP-1 β	0.095	0.713	0.326	0.215
RANTES	-0.212	0.411	-0.072	0.790

S, spike; N, nucleoprotein. In bold are indicated the significant correlations.

different between COVID-19 patients and swab positive household contacts ($p>0.05$).

The serological response was detected in a minority of household contacts

Regarding the serological response, the positivity rate was evaluated considering any antibody response detected regardless of the antibody type considered. Only a minority of household contacts with positive swab showed anti-RBD/anti-N/neutralizing antibodies at baseline (1/10, 10%) (Figures 4A-C). Among individuals scored negative for the swab, 4 were positive for the serology (4/32, 12.5%): 3 subjects scored positive to both serological and cell-mediated responses, whereas one individual had only anti-RBD antibodies (Figure 4D). Among the total 5 subjects with detectable RBD/anti-N antibodies at T0, the neutralizing antibodies were detected in 3/5, of whom 2 were swab negative (Figure 4C). At T1, the serological response was still found only in a minority of swab positive subjects (44.4%, 4/9) (Figure 4E). No significant different proportions of antibody responders were found between swab positive subjects and negative ones (Figure 4).

Samples of the three seroconverted patients with positive swab were collected after 7 days, 14 days and 20 days from T0. For the other patients that did not seroconvert, samples were collected at 8 days (for 2 subjects), 10 days (for 2 subjects) and 18 days (for one individual) from T0. Due to the similarity of the time range, we cannot correlate the seroconversion score to the collection time. In the subjects analysed, the seroconversion score probably depends on the individual variability.

In particular, 3 subjects had both anti-RBD and anti-N antibodies, whereas one showed only anti-N antibodies. Neutralizing antibodies were detected in all 4 subjects with anti-RBD antibodies at T1, of whom one was also swab negative (Figure 4C).

To verify the SARS-CoV-2 specificity of the T-cell and serological responses observed in swab negative subjects, an

indirect immunofluorescence assay was performed. The immunofluorescence IgG, IgM or IgA data revealed that subjects scored positive for the T-cell response but not for the serology (T0: $n=1$ and T1: $n=4$, see Figures 4D, E), were confirmed negative for antibody response (Supplementary Figure S5). Therefore, the T-cell response detected in these swab negative subjects is probably due to a cross-reactivity with other cold coronaviruses. By contrast, in subjects scored positive for both T-cell response and serology (T0: $n=3$ and T1: $n=1$, see Figures 4D, E), the immunofluorescence resulted positive. To note, plasmatic IFN- α was undetectable in these individuals. Therefore, the results suggest that these subjects may have had a previous SARS-CoV-2 infection.

Kinetics of the immune responses in household contacts

To evaluate the kinetics of humoral- and cell-mediated immune responses to SARS-CoV-2 in the household contacts of COVID-19 cases, we compared the immune responses at T0 and T1 in 28 subjects longitudinally sampled. We observed that the number of responders to SARS-CoV-2 peptide pools, in particular to pools S and N, increased from T0 (4/8, 50%) to T1 (7/9, 77.8%) among swab positive subjects, although the difference was not significant ($p=0.335$) (Table 4). The same trend was also observed for the antibody response, although the total number of positive responders was still a minority (T0: 1/8, 12.5% vs T1: 4/9, 44.4%) compared to the T-cell response. A significant increase of the magnitude of the SARS-CoV-2 IFN- γ -N-specific response was observed at T1 compared to T0 (T0 median: 0.01 IU/mL, IQR: 0.01-0.02 vs T1 median: 0.035 IU/mL, IQR: 0.01-0.262, $p=0.042$) (Supplementary Figure S6). The same trend was also observed for the IFN- γ response to pool S (T0 median: 0.01 IU/mL, IQR: 0.01-0.047 vs T1 median: 0.035 IU/mL, IQR: 0.01-1.103, $p=0.053$), although not significant. Neither the quantitative IFN- γ -M-response nor the humoral one (anti-RBD or anti-N) showed significant differences

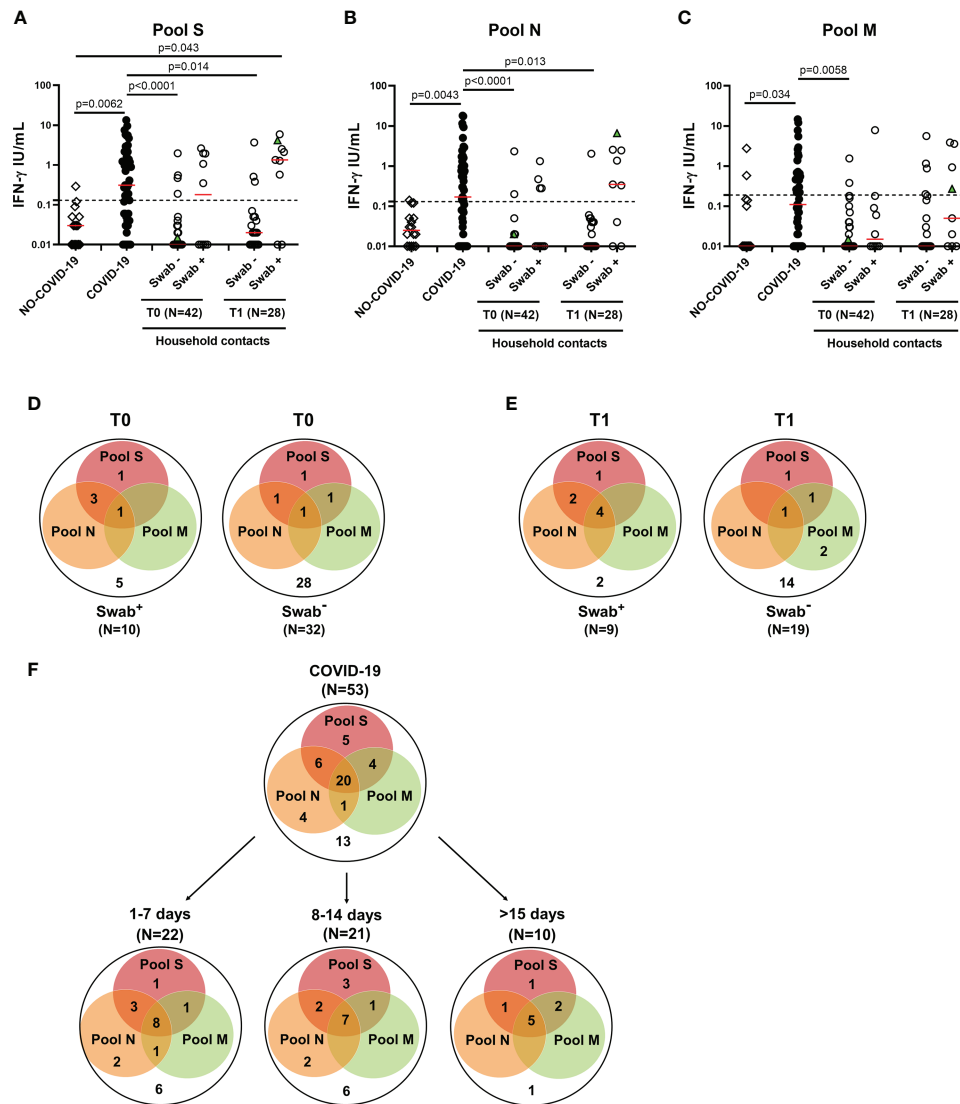


FIGURE 3
 T-cell response in household contacts of COVID-19 subjects. **(A–C)** Evaluation of IFN- γ levels in response to SARS-CoV-2 peptides in household contacts at T0 (n = 42) and T1 (n = 28) after whole-blood stimulation with 0.1 μ g/mL of pools S **(A)** and M **(C)**, and 1 μ g/mL of pool N **(B)**. Healthy donors (n=16) and COVID-19 patients (n = 53) were used as negative and positive control groups, respectively. The household contacts were stratified according to the swab result. The IFN- γ levels were assessed in plasma from stimulated whole-blood samples and reported by subtracting the background. The cut-off for each peptide pool was represented by a dashed line (pools S and N: 0.13 IU/mL; pool M: 0.19 IU/mL). Green triangle indicates the subject who scored positive only at T1. The red horizontal lines indicate the median. **(D, E)** Venn diagrams show the number of household contacts of COVID-19 cases at T0 and T1 with a positive response to the different SARS-CoV-2 peptides pools. **(F)** Venn diagrams show the number of confirmed hospitalized COVID-19 patients with a positive response to the different SARS-CoV-2 peptides pools, stratifying the results also with respect to days from symptom onset. The statistical comparison was done with the Kruskal-Wallis test and the Dunn’s multiple comparisons test, and p<0.05 was considered significant. IFN, interferon; COVID-19, COroNaVirus Disease 2019; S, spike; N, nucleocapsid; M, membrane.

comparing T0 and T1 (p=0.318, p=0.426 and p=0.407, respectively) (**Supplementary Figures S6C–E**).

Overall, the swab result showed a moderate concordance with the T-cell response (78.6%, k=0.467; with at T0: 78.6%, k=0.388 and at T1: 78.6%, k=0.536), and a scarce concordance with the serological response (72.9%, k=0.194 with at T0: 69%, k<1 and at T1: 78.6%, k=0.444).

Discussion

A better understanding of the host immune responses to natural SARS-CoV-2 infection is critical to understand in depth the mechanisms of an effective host-defence in naïve unvaccinated populations. The results of these investigations

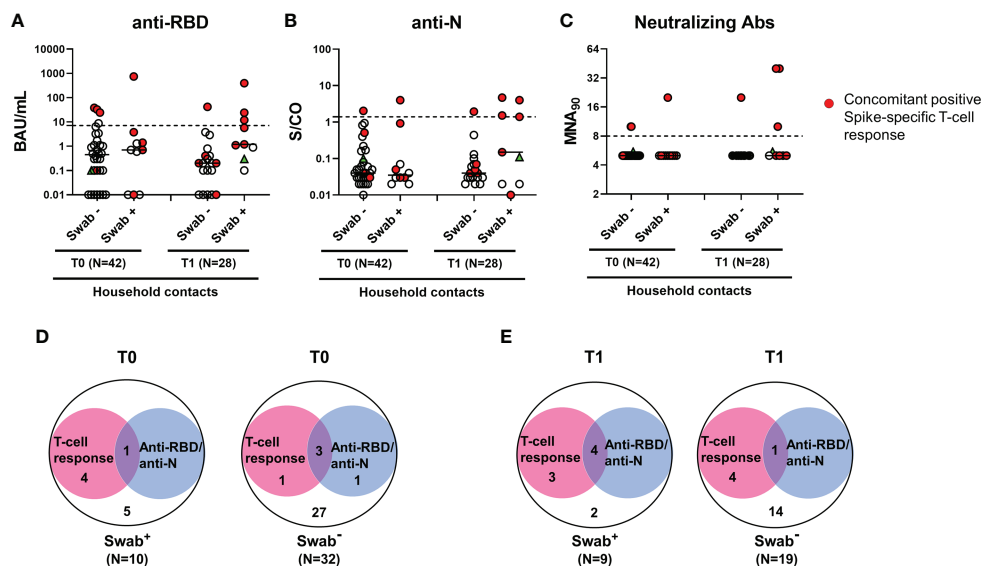


FIGURE 4

Antibody response in household contacts of COVID-19 cases. Evaluation of SARS-CoV-2-specific anti-RBD (A), anti-N (B) and neutralizing (C) antibodies in household contacts at T0 (n=42) and T1 (n=28). Anti-RBD, anti-N and neutralizing antibodies were evaluated in sera samples and reported as Binding Antibody Units (BAU)/mL (A), Sample/Cutoff (S/CO) (B), and reciprocal of dilution (MNA_{90}) (C), respectively. Red dots indicate subjects with also a concomitant IFN- γ specific response as shown in the figure legend. Green triangle indicates the subject with positive swab only at T1. Dashed lines indicate the cut-off (anti-RBD: 7.1 BAU/mL; anti-N: 1.4 (S/CO); MNA_{90} : 8). The black horizontal lines indicate the median. (D, E) Venn diagrams show the number of household contacts of COVID-19 cases at T0 and T1 with an IFN- γ response and/or the serological one (anti-RBD and anti-N). Statistical analysis was performed using Fisher's exact test with Bonferroni correction and $p < 0.01$ was considered significant. IFN, interferon; COVID-19, COroNaVIrus Disease 2019; RBD, receptor binding domain; N, nucleocapsid.

can be useful to find new strategies for diagnosis and therapy. In this study, we investigated both the innate and adaptive immune responses in household contacts of COVID-19 cases followed over time to characterize the early immune responses to SARS-CoV-2 infection and their kinetics. By studying the innate immune response and the two compartments of adaptive immunity, T and B cells, we observed that each component of the SARS-CoV-2 immune response exhibited a distinct kinetic.

One primary function of the innate immune system during viral infection is limiting viral replication by inducing an inflammatory response. Type I IFNs, mainly consisting of IFN- α and IFN- β , represent the first rapid defensive line against invading pathogens, being important regulators of the adaptive immune response.

In the present study, we showed that the innate factor IFN- α is rapidly induced in all SARS-CoV-2-infected subjects at early stage of infection (T0). These patients were characterized by an asymptomatic or mild COVID-19. After 7-20 days (T1), IFN- α quickly disappeared. This data agrees with the type I IFN response detected by a blood transcriptome analysis in a cohort of subjects recently exposed to SARS-CoV-2 (42). We extended the analysis to a large panel of soluble factors identifying other cytokines/chemokines upregulated (IP-10, IL-1ra, MCP-1) or downregulated (IL-9, IL-1 β , RANTES, MIP-1 β)

at the earliest stage of infection in household contacts with a positive swab. Among the cytokines/chemokines significantly modulated, IFN- α discriminated infected individuals from non-infected subjects with the highest accuracy.

Innate immunity mediated by type I IFN responses contributes to the protection against critical illness in COVID-19 (20–22). Indeed, SARS-CoV-2 has evolved several strategies to evade antiviral innate immune responses by reducing type I IFN levels acting at post-transcriptional level (49). In this regard, low serum levels of IFN- α have been reported in severe COVID-19 patients (50, 51) and associated with older age (52). Ineffective IFN-mediated innate immunity, due to neutralizing autoantibodies to type I IFNs and genetic polymorphisms causing a reduced expression of type I IFN receptor or inducible genes, has been strongly associated with inability to control the primary SARS-CoV-2 infection and a high risk of fatal COVID-19. In addition, the innate cell immunopathology and a plasma cytokine signature characterized by elevated IP-10, IL-6, and IL-8 levels have been also reported (18, 19, 53–57).

The up-regulated levels of IP-10, IL-1ra and MCP-1 observed in swab positive subjects are supported also by other findings showing that the levels of these cytokines/chemokines are prominent during the second week after disease onset and are even more pronounced in severe patients (19, 58, 59). The

TABLE 4 T-cell and antibody responses in household contacts of COVID-19 patients evaluated at both time points (T0 and T1).

	T0			T1			P value*
	Positive swab N = 8	Negative swab N = 20	Tot N =28	Positive swab N = 9	Negative swab N = 19	Tot N = 28	
Positive T-cell responders N over total	4 (50)	2 (10)	6 (21.4)	7 (77.8)	5 (26.3)	12 (42.8)	0.335
Pool S responders [N (% among positive T-cell responders)]	4 (100)	2 (100)	6 (100)	7 (100)	3 (60)	10 (83.3)	0.335
Pool N responders [N (% among positive T-cell responders)]	3 (75)	1 (50)	4 (66.7)	6(85.7)	1 (20)	7 (58.3)	0.345
Pool M responders [N (% among positive T-cell responders)]	1 (25)	2 (100)	3(50)	4 (57.1)	4 (80)	8 (66.7)	0.294
Positive antibody responders N over total	1 (12.5)	1(5)	2 (7.1)	4 (44.4)	1 (10.5)	5 (41.7)	0.606
anti-RBD responders [N (% among positive T-cell responders)]	1 (100)	1 (100)	2 (100)	3 (75)	1 (100)	4 (80)	0.576
anti-N responders [N (% among positive T-cell responders)]	1 (100)	1 (100)	2 (100)	4 (100)	1 (100)	5 (100)	0.606

S, spike; N, nucleocapsid; M, membrane; RBD, receptor binding domain. *Chi-square test calculated among swab positive responders at the two time points.

pro-inflammatory chemokine IP-10 and the monocyte chemoattractant factor MCP-1 contribute to the excessive inflammatory and immune response, favoring the recruitment of monocytes, macrophages, and T cells to the infection sites. The higher levels of IL-1ra found in the swab positive cohort are probably the consequence of the inflammatory process in progress. In this context, IL-1ra, as an early inhibitory immune factor, acts by controlling the inflammatory response. Serum concentrations of IL-1ra associated with COVID-19 severity. In particular, much higher levels of IL-1ra were observed in severe cases, indicating the presence of an overactive immune response (60, 61). The lower levels of the pro-inflammatory factors IL-1 β , RANTES and MIP-1 β , and of IL-9, a cytokine with direct and indirect effects on multiple cell types that affect the development of immunity and inflammation, may be indicative in our study cohort of a controlled inflammatory process, in which the IL-1ra exerts an effective action. Indeed, a higher production of IL-1 β , RANTES, MIP-1 β and IL-9 have been found in the severe disease (18, 59, 62). The differences, in terms of cytokine amount, observed between swab positive and negative subjects in our cohort are highly significant (at least $p=0.007$) for certain immune factors (IFN- α , IL-1ra, IP-10, IL-9 and MIP-1 β). Moreover, the cytokine levels detected are comparable with what was reported in mild COVID-19 (61, 63). The cytokine profile observed might be indicative of the ongoing immune response

to SARS-CoV-2 infection that distinguishes swab positive subjects from negative ones. Certainly, what we detect in the plasma is only a partial mirror of what happens in the respiratory tract, which is the target tissue of the virus. Further longitudinal studies on a larger cohort of subjects early exposed to COVID-19 and with different disease outcomes would be important to learn more about.

Regarding the adaptive immune response, it has been reported that CD4⁺ and CD8⁺ T-cell responses appear early after infection (32, 36) or vaccination (64–67), cross-recognize viral variants (10, 68), are over time stable and persist in vulnerable populations, albeit with a low amount (37, 38). In this manuscript, we showed that the T-cell response evaluated by a simple IGRA method based on the stimulation of whole blood with SARS-CoV-2 peptides from the S-, N-, or M-region, appears simultaneously or later compared to the innate immunity. It also increases over time becoming detectable in the majority of infected subjects (77.8%) after 7–20 days from the first swab. Moreover, the percentage of T-cell responders and the magnitude of the response in swab positive household contacts at T1 was similar to what was observed in the cohort of COVID-19 patients (75.5%).

Among the peptides tested, the best stimuli were those for the S- and N-region that detected the greater number of responders among the infected subjects. On the contrary, other works identified M- and N-related immunodominant

peptides as the most effective in detecting the T-cell response. Discrepancies with our findings could be explained by the different methodologies (IGRA method vs flow cytometry analysis), populations analysed (household contacts early exposed vs COVID-19 convalescent and not convalescent patients), as well as the peptide format used (32, 69).

It is known that plasmatic type I IFN levels can be also detectable in response to acute respiratory infections different from SARS-CoV-2 (70). Differently, the IFN- γ response detected in the present study was specifically induced *in vitro* in response to SARS-CoV-2 peptides. However, the detection of a T-cell response also in some subjects with a negative swab and serology (5 five subjects in our household cohort) could be ascribed: i) to a cross-reactivity probably arising from previous seasonal coronavirus exposure, ii) or to previous exposure to SARS-CoV-2 without seroconversion or subsided antibody titers. The presence of SARS-CoV-2-specific T-cell response, whether due to SARS-CoV-2 infection or cross-reactivity, might explain the mild symptoms in infected household contacts and the resistance of other contacts to symptomatic SARS-CoV-2 infection (71).

By contrast, the humoral response was delayed by 1-2 weeks compared to the T-cell response, and it was detectable only in a minority (44%) of household contacts with confirmed infection. To note, the antibody response was further assessed for its ability to neutralize the virus. In this context, neutralizing antibodies were detectable in the few subjects scored positive for the serological response.

SARS-CoV-2 antibodies are detected later or can be absent in patients with less severe forms of COVID-19 (27–29) and their titers are not constant over time (31, 38, 72). Therefore, the IFN- γ T-cell specific response is important for viral containment (8) and potentially useful for the detection of infected subjects, even more in the context of the emerging variants that escape the antibody response (73, 74).

The present study is unique in terms of clinical cohort studied. In literature, the immune response in household contacts of COVID-19 cases was studied at only one time point (42, 71, 75, 76). Differently, our enrolled subjects were analysed at two time points (i.e., at the first nasopharyngeal swab and after 7-20 days), and with an easy-to-use assay to detect the T-cell specific response discriminating among the responses to N, M and S peptides. Moreover, both innate and adaptive immunity were evaluated and correlated to the SARS-CoV-2 viral load detected in swab specimens. In this respect, we showed that both plasma IFN- α and IP-10 levels were strongly associated with the viral load in swab specimens. Moreover, the swab test showed a moderate concordance with the T-cell response (78.6%, $k=0.467$), whereas a scarce concordance with the serological response (72.9%, $k=0.194$).

Some limitations of the study need to be considered. Firstly, the number of the enrolled household contacts was relatively small (42 subjects). This is due to the increasing uptake of the

vaccination campaign that made more difficult the enrolment of unvaccinated individuals. However, the results are robust and in agreement with the recent findings generated in a larger cohort (42). Secondly, SARS-CoV-2 infections included in this study were likely caused by the Alpha variant, dominant between February and June 2021. Therefore, innate and adaptive immune responses may differ in timing and magnitude for the current and future variants.

Another important consideration needs to be made regarding the vaccination. In countries with high vaccination coverage, the T-cell specific response against pool S or anti-RBD antibodies cannot be longer used to discriminate infected and not-infected subjects, therefore the T-cell response to pool N might be a supporting approach for the diagnosis (9, 77).

In conclusion, we showed that household contacts with positive swab for SARS-CoV-2 present detectable plasmatic IFN- α and a viral-specific T-cell response, even in the absence of seroconversion, thus representing better indicators of SARS-CoV-2 exposure than antibodies. The results of our exploratory study underline the role of plasmatic IFN- α and viral-specific T-cell response for a better understanding of the early immunological kinetic and for epidemiological studies.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The Ethical Committee of the National Institute for Infectious Diseases (INMI) L. Spallanzani approved the study (approval number 247/2021). The patients/participants provided their written informed consent to participate in this study.

Author contributions

DG wrote the project to be submitted to the Ethical Committee. DG and EDR conceived and designed the study. Experiments for evaluating Innate and T cell response were performed by AA, VV, RC, AS, AMGA. Experiments for evaluating antibody response were performed by SM. AA performed the statistical analysis. AG, GC, LPi, MM, TAB, GG, and FP enrolled subjects and collected clinical data. AA and DG drafted the article and AG, MM, LPe, LPi, GG, CC, CA, EG, FP, EN, and EDR revised it critically. All authors critically analysed, discussed and interpreted data, and contributed to the article and approved the submitted version.

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Conflict of interest

EG has received grants from Gilead and Mylan. EN is a member of the advisory board by Gilead, Lilly and Roche and

received fees for educational training by Gilead, Lilly and Roche. DG is a member of the advisory board by Biomerieux and Eli-Lilly, and received fees for educational training or consultancy by Biogen, Cellgene, Diasorin, Janssen, Qiagen, Quidel.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2022.920227/full#supplementary-material>

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