The Journal of Infectious Diseases

MAJOR ARTICLE

Rapid Cytokine Release Assays for Analysis of Severe Acute Respiratory Syndrome Coronavirus 2–Specific T Cells in Whole Blood

Andreas Törnell,[1,a] Hanna Grauers Wiktorin,[1,a] Johan Ringlander,[1,2,a] Mohammad Arabpour,[1,2,a] Malin R. Nilsson,[1] Staffan Nilsson,[3] Roberta Kiffin,[1] Magnus Lindh,[1,2] Martin Lagging,[1,2] Kristoffer Hellstrand,[1,2] and Anna Martner[1]

[1]Department of Infectious Diseases, Institute of Biomedicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden, [2]Region Västra Götaland, Sahlgrenska University Hospital, Department of Clinical Microbiology, Gothenburg, Sweden, and [3]Department of Pathology and Genetics, Institute of Biomedicine, University of Gothenburg, Gothenburg, Sweden

Background. Waning of immunoglobulin G (IgG) antibodies against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) complicates the diagnosis of past infection. The durability of T-cell memory against SARS-CoV-2 remains unclear, and most current T-cell protocols are unsuited for large-scale automation.

Methods. Whole-blood samples from 31 patients with verified past coronavirus disease 2019 (COVID-19) and 46 controls, of whom 40 received COVID-19 vaccine, were stimulated with peptides spanning the nucleocapsid (NC) or spike 1 (S1) regions of SARS-CoV-2 and analyzed for interferon γ in supernatant plasma. Diagnostic accuracy of these assays was evaluated against serum anti-NC and anti–receptor-binding domain S1-IgG.

Results. Induction of interferon γ in whole blood by NC or S1 peptides diagnosed past COVID-19 with high accuracy (area under the receiver operating characteristic curve, 0.93 and 0.95, respectively). In accordance with previous studies, NC-IgG levels rapidly waned with only 5 of 17 patients (29%) remaining seropositive >180 days after infection. By contrast, NC peptide–induced T-cell memory responses remained in 13 of 17 study participants (76%) >180 days after infection ($P = .01$ for comparison with NC-IgG; McNemar test). After 2 vaccine doses, all 18 donors exhibited S1-specific T-cell memory.

Conclusions. Cytokine release assays for the monitoring of T-cell memory in whole blood may be useful for evaluating complications following unverified past COVID-19 and for long-term assessment of vaccine-induced T-cell immunity.

Clinical Trials Registration. EudraCT 2021-000349-42.

Keywords. COVID-19; SARS-CoV-2; Cytokine release assay; T cells; nucleocapsid; spike.

The detection of immunoglobulin G (IgG) antibodies in serum is the mainstay of diagnosis for past coronavirus disease 2019 (COVID-19). Previous studies showed that IgG antibodies commonly become detectable within 2–3 weeks after onset of symptoms, albeit with interindividual variation [1–3]. In routine diagnostics, most laboratories use automated, platform-based immunoassays that detect IgG antibodies against nucleocapsid (NC) [4] and/or spike proteins of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). A survey of the performance of platform-based IgG antibody tests, applied to serum samples derived from unvaccinated individuals after a previously verified infection, reported consistently high specificity but lower sensitivity [5]. The sensitivity of these assays is even lower in patients with previous mild or asymptomatic COVID-19 [6–8].

The diagnostic accuracy of serum IgG against SARS-CoV-2 is further limited by diminished antibody titers over time [9, 10]. Lau et al [11] estimated that neutralizing antibodies remain detectable for approximately 14 months in patients with symptomatic COVID-19 and approximately 6 months in those with asymptomatic infection. The waning of serum antibodies against SARS-CoV-2 has been noted also in studies evaluating the durability of serum IgG analyzed using platform-based assays. Levels of antibodies against NC decline more rapidly than those of antibodies against the spike protein [12], which may complicate the diagnosis of past natural COVID-19 in vaccinated persons.

The shortcomings of antibody tests have spurred the development of tests that reflect SARS-CoV-2–specific T-cell immunity. A T-cell assay may thus detect an immunological memory that is not captured by serum IgG. Protocols for detection of SARS-CoV-2–specific T cells typically comprise the isolation of

Received 12 October 2021; editorial decision 6 January 2022; accepted 7 January 2022; published online 12 January 2022.

Presented in part: 18th Smögen Summer Symposium on Virology, Smögen, Sweden, August 2021.

[a]A. T., H. G. W, J. R., and M. A. contributed equally to this work.

Correspondence: Anna Martner, Department of Infectious Diseases, Institute of Biomedicine, Sahlgrenska Academy, University of Gothenburg, Medicinaregatan 1F, 41390 Gothenburg, Sweden (anna.martner@gu.se).

The Journal of Infectious Diseases® 2022;226:208–16

https://doi.org/10.1093/infdis/jiac005

Table 1. Patient Characteristics

Characteristic	Controls (n = 46)	Patients With Past COVID-19 (n = 31)	All Participants (n = 77)
Vaccinated, No.	23	17	40
Female sex, %	78	74	77
Age, median (range), y	43 (19–70)	39 (25–66)	43 (19–70)

Abbreviation: COVID-19, coronavirus disease 2019.

peripheral blood mononuclear cells (PBMCs), followed by analysis of cell subsets using flow cytometry and are thus unsuited for large-scale routine diagnostics. Rapid cytokine-release assays, based on the exposure of whole-blood samples to antigens, are useful in the diagnosis of tuberculosis and other infections [13], and similar tests have been applied to detect specific T-cell reactivity against SARS-CoV-2 antigens. Earlier studies imply that results achieved in cytokine-release assays for T-cell reactivity are correlated with seropositivity for IgG and that these assays capture T-cell memory responses after vaccination [14, 15].

We have developed rapid and refined cytokine-release based T-cell assays in which whole-blood samples were exposed ex vivo to antigens derived from the NC or the spike 1 (S1) portion of the spike protein, followed by analysis of interferon (IFN) γ in supernatant plasma. The assays demonstrated high accuracy in detecting previously verified COVID-19 as well as S1-specific T-cell responses after vaccination. Our results underscore that rapid T-cell assays may be of value for more accurate diagnosis of past natural SARS-CoV-2 infection and for determining the durability of T-cell reactivity after infection or vaccination.

Table 2. Sample and Vaccine Details

No. and Timing of Samples and Vaccine Types	Controls, No.	Patients With Past COVID-19, No.
Three samples		
Before vaccination + after both doses	12	3
Two samples		
Before vaccination + after dose 1	6	8
Before vaccination + after dose 2	1	1
After both doses only	1	2
One sample		
Before vaccination only	23	14
After dose 1 only	1	3
After dose 2 only	2	0
Vaccine types[a]		
Dose 1	15 Pfizer/4 AZ/1 Moderna	7 Pfizer /8 AZ/1 Moderna
Dose 2	11 Pfizer/2 AZ/3 Moderna	4 Pfizer/2 AZ/0 Moderna

Abbreviations: AZ, AstraZeneca; COVID-19, coronavirus disease 2019.

[a]The 3 vaccines types used were BNT162b2 (Pfizer BioNTech), AZD1222 (AstraZeneca), and mRNA-1273 (Moderna).

METHODS

Study Population

This study was conducted between December 2020 and August 2021 at the Sahlgrenska University Hospital in Gothenburg, Sweden. All donors (n = 77) gave written informed consent before enrollment. The DurIRVac study was approved by the Swedish Ethical Review Authority (Etikprövningsmyndigheten; permit nos. 2020-03276, 2021-00374, and 2021-00539) and by the Swedish Medical Products Agency (EudraCT 2021-000349-42; https://www.clinicaltrialsregister.eu/ctr-search/search?query=2021-000349-42). Baseline characteristics of participating blood donors are detailed in Table 1. Peripheral blood samples (8–24 mL) were collected from each participant at up to 4 occasions during the study period (December 2020 to August 2021). Forty-six donors were regarded as naive controls, because they had not had a confirmed COVID-19 infection, while 31 donors had had a reverse-transcription polymerase chain reaction (PCR)–confirmed COVID-19 infection (past COVID), ≥25 days before sampling. One COVID-19 case was classified as severe (defined by hospitalization), and the other 30 cases were classified as mild. Blood samples were collected 3–5 weeks after vaccination from 40 of the 77 study participants. Detailed characteristics of when samples were collected and the type of vaccines received is provided in Table 2. The mean time between vaccine doses 1 and 2 was 39 days (range, 21–113 days), in accordance with the Swedish national guidelines.

IgG Serology

Chemiluminescent microparticle immunoassays were performed on serum using the Alinity system for the quantitation of IgG antibodies against the spike receptor-binding domain (RBD) (SARS-CoV-2 IgG II Quant; Abbott) and the NC (SARS-CoV-2 IgG; Abbott) proteins of SARS-CoV-2. IgG antibody levels against the spike RBD are reported as World Health Organization international standard binding antibody units (BAU) per milliliter, while antibody levels against the NC are reported as arbitrary units [1]. The limits of detection (LOD) for the S1- and NC-IgG tests were 14 BAU/mL and 1.4 arbitrary units/mL, respectively. All values below the LOD were set to 50% of the LOD.

Cytokine Release Assay in Whole Blood

Peripheral venous blood samples were collected in BD Vacutainer lithium-heparin tubes (BD) and stored at room temperature for a maximum of 24 hours. One milliliter of whole blood was stimulated in 10-mL tubes (Sarstedt) with peptide pools from SARS-CoV-2 or no stimuli (negative control). The peptides were dissolved in phosphate-buffered saline (PBS) with 20% dimethyl sulfoxide. Each sample was incubated with 1 μg/ml/peptide of 102 fifteen-mer peptides with 11-amino acid overlap spanning the complete sequence of the SARS-CoV-2 NC phosphoprotein (amino acids 1-419 [130-127-041; Miltenyi Biotec]), 170

fifteen-mer peptides spanning the N-terminal domain of the SARS-CoV-2 surface glycoprotein (amino acids 1-692 [130-127-041; Milentyi Biotec]) or no stimuli. The samples were incubated with these peptides for approximately 48 hours at 37°C and 5% carbon dioxide. Tubes were then centrifuged for 5 minutes at 1500 rpm, and supernatant plasma was recovered and stored at −80°C until the analysis of IFN-γ content.

Whole-blood samples obtained from 10 unvaccinated donors were stimulated with S1 but not NC peptides and were thus analyzed only for antibodies and S1-peptide induced IFN-γ (S1-γ). Samples from 7 of these donors were analyzed for NC-peptide induced IFN-γ (NC-γ) at later time points. In 4 blood samples collected after the second vaccination, hemolysis occurred in the whole-blood sample but not the serum sample. These samples were therefore analyzed only for antibodies and not for peptide-induced IFN-γ. In addition, prevaccination samples were collected before and after infection for 3 individuals. All 3 donors were negative for S1 RBD-IgG, NC-IgG, S1-γ, and NC-γ before infection and became positive in these assays after COVID-19 infection (Supplementary Figure 1*A*–1*D*); the preinfection samples from these individuals were not used for any further analyses.

IFN-γ Enzyme-Linked Immunosorbent Assay

Plasma levels of IFN-γ from unstimulated and NC-stimulated or S1-stimulated whole blood were determined by IFN-γ enzyme-linked immunosorbent assay (DY285B; R&D systems), according to the manufacturer's instructions. Plasma was diluted (1:2) in PBS containing 1% bovine serum albumin and 10% rat or mouse serum (Stemcell Technologies and Invitrogen, respectively) to minimize unspecific reactivity. A reduced concentration of mouse serum in an additional standard curve was used to normalize the data in samples diluted in mouse serum, because this matrix interfered with the standard curve. Optical density was measured at 450 and 570 nm, using a FLUOstar Omega plate reader (BMG). Results are presented as peptide-induced IFN-γ obtained by subtracting levels of IFN-γ in unstimulated samples from those in peptide-stimulated samples. Levels below the LOD (<10 pg/mL) were set to 50% of the LOD.

Isolation of PBMCs

PBMCs were isolated from venous donor blood collected in BD vacutainer lithium-heparin tubes. The blood was diluted 1:2 in PBS and layered onto Ficoll-Paque (Lymphoprep). PBMCs were isolated by means of gradient centrifugation and cryopreserved in Recovery Cell Culture freezing medium (Life Technologies). Samples were stored at −140°C until analysis.

Cytometric Analysis of SARS-CoV-2 Peptide–Stimulated PBMCs

PBMCs were thawed and cultured at 2.5–5 × 10^6 cells/mL in round-bottom 96-well plates in the presence of 1 μg/mL/peptide of SARS-CoV-2 NC- or S1-spanning peptides overnight at 37°C and 5% carbon dioxide. GolgiPlug Protein Transporter Inhibitor (BD Biosciences) was added during the last 4 hours of incubation. Cells were stained with an extracellular panel of antibodies comprising anti–CD3–fluorescein isothiocyanate (clone UCHT1; BD Biosciences), anti–CD4–peridinin chlorophyll protein–cyanine 5.5 (clone RPA-T4; BD Biosciences), anti–CD8-allophycocyanin (clone RPA-T8 [BD Biosciences] and Live/Dead Fixable Near-IR [Life Technologies]). Thereafter, cells were permeabilized and fixated using the BD Cytofix/Cytoperm kit (BD Biosciences), according to the manufacturer's instructions. Cells were subsequently stained with anti–IFN-γ–phycoerythrin–cyanine 7 (clone B27; BD Biosciences) following acquisition on a 5-laser BD LSRFortessa cell analyzer (BD Biosciences). Data were analyzed using FlowJo software, version 10 or later (BD Biosciences).

Statistical Analyses

Statistical analyses were performed using the SPSS statistical software package (version 24) or GraphPad Prism software (version 9 or later) and the logarithmic values of IFN-γ and IgG concentrations. The antibody and T-cell responses shown in Figure 1 and 2 are from unique infected and noninfected study participants and were compared using the Mann-Whitney test. When multiple samples were available from the same donor, the first collected sample was used, except in the longevity analysis, which used the last collected sample within the specified time frame. Individuals for whom only postvaccination samples were available were not included in S1-specific analysis of infection status.

Receiver operating characteristic (ROC) curves were created, and the area under the ROC curve was calculated to evaluate the accuracy of IFN-γ–based tests and IgG antibody levels for detecting infection. Using unique samples from infected donors, correlations were calculated between antibody levels and T-cell memory in whole blood. The McNemar test was used to compare NC-IgG and NC-γ longevity. The induction of serological and cellular responses after vaccination, as well as the difference in vaccine response between previously infected donors and controls, was statistically analyzed based on the empirical distribution of 100 000 permutations of the mean differences. This permutation test allows a combined analysis of paired and unpaired samples. All indicated *P* values are 2 sided.

RESULTS

Induction of IFN-γ by SARS-CoV-2 Peptides in Whole-Blood Samples From Nonvaccinated Patients With Verified Past COVID-19

In a first set of experiments, we aimed to optimize assay conditions and noted that a SARS-CoV-2 peptide concentration of 1 μg/mL/peptide and an incubation time of 48 hours yielded strong IFN-γ formation in whole-blood samples from previously SARS-CoV-2–infected patients but not in uninfected individuals (Supplementary Figure 2*A* and 2*B*). In further experiments,

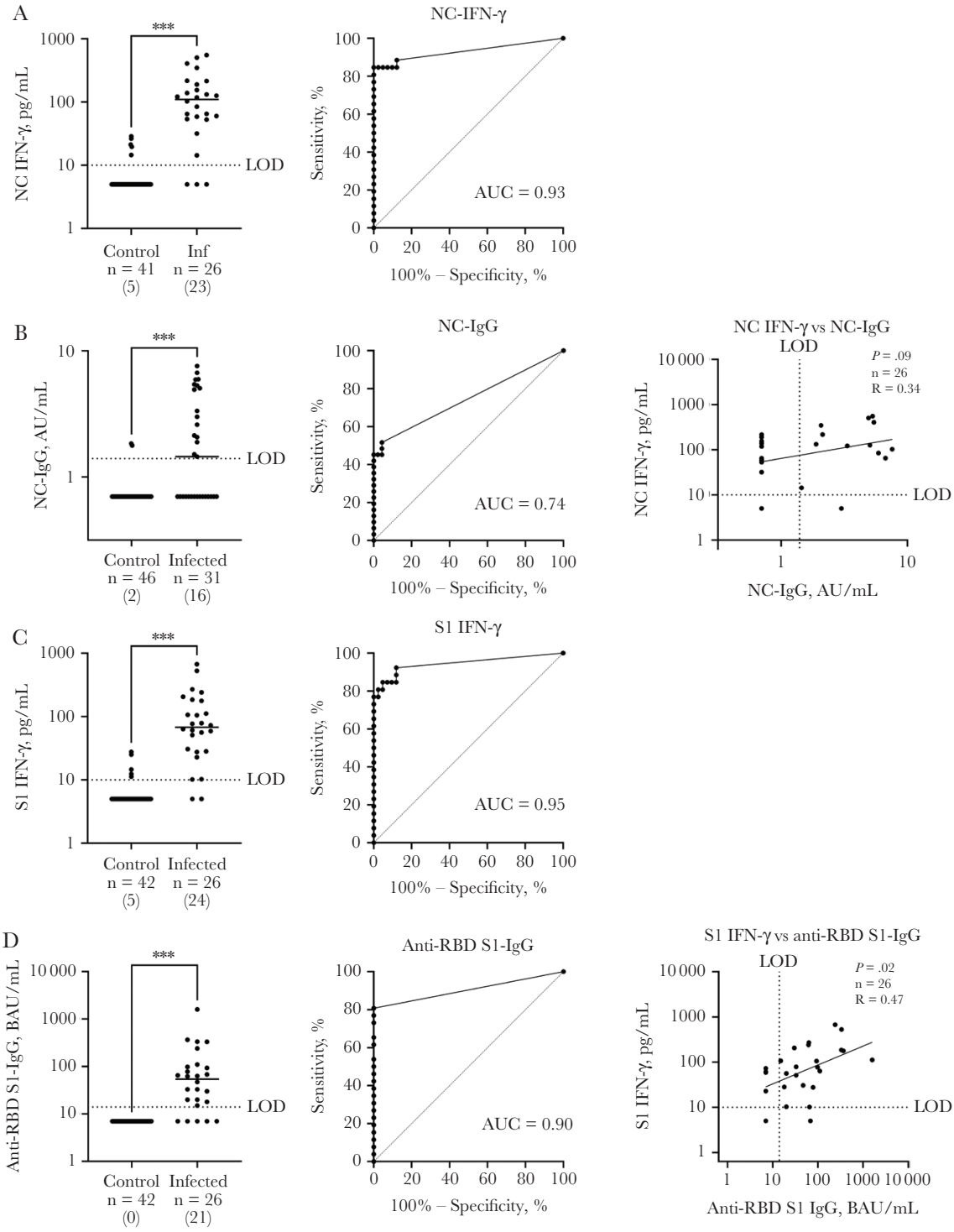

Figure 1. Whole-blood stimulation with nucleocapsid (NC) and spike 1 (S1) peptides triggers a robust induction of interferon (IFN) γ in patients with previous coronavirus disease 2019 (COVID-19) infection. *A, C,* Whole-blood samples from study participants with previous verified severe acute respiratory syndrome coronavirus 2 infection and controls without previous infection were stimulated with peptides spanning the NC (*A*) or the S1 portion of the spike protein (*C*) for 48 hours, and the presence of IFN-γ in supernatant plasma was determined with enzyme-linked immunosorbent assay. *B, D,* Similarly, immunoglobulin G (IgG) antibody levels in serum against the NC (*B*) or the receptor-binding domain (RBD) within S1 (*D*) were determined in participants with or without previous infection. IFN-γ and IgG levels in controls and previously infected patients were compared using the Mann-Whitney test. Receiver operating characteristic (ROC) curves mapping sensitivity versus specificity to detect prior COVID-19 infection based on NC-induced IFN-γ production (*A*) and IgG levels (*B*), as well as S1-induced IFN-γ (*C*) and IgG levels (*D*), with area under the ROC curve (AUC) specified. Correlations between NC-γ and NC-IgG (*B*) and S1-γ and S1-IgG (*D*) among previously infected patients were analyzed using linear regression. Unique samples were chosen from the earliest time point available for each individual. Individuals where samples were only available from after vaccination were not included in S1-specific analyses. For each assay, dotted lines represent the limit of detection (LOD) (IFN-γ, >10 pg/mL; IgG, >1.4 arbitrary units [AU]/mL for NC and >14 binding antibody units [BAU]/mL for S1), and the number of samples above the LOD is shown in parentheses. ***$P < .001$.

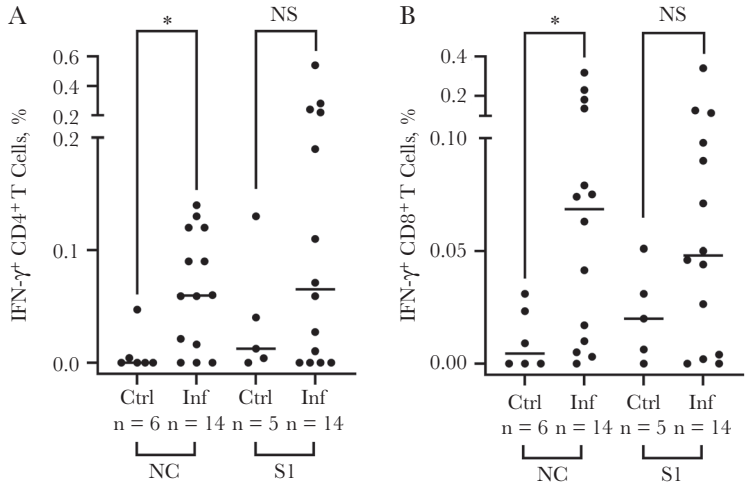

Figure 2. Specific induction of intracellular interferon (IFN) γ in peptide-stimulated T cells following previous coronavirus disease 2019 infection. Peripheral blood mononuclear cells from study participants with previously verified severe acute respiratory syndrome coronavirus infection (Inf) or controls without previous infection (Ctrl) were stimulated with peptides spanning nucleocapsid (NC) or the spike 1 (S1) portion of the spike protein overnight. Intracellular IFN-γ production in $CD4^+$ (*A*) and $CD8^+$ (*B*) T cells was determined using flow cytometry. Unique samples were chosen from the earliest time point available for each individual. Individuals in whom the only available samples were obtained after vaccination were not included in the S1 analysis. $*P < .05$; NS, not significant (Mann-Whitney test).

peptides spanning the NC or S1 regions of SARS-CoV-2 were thus added at 1 μg/mL/peptide to whole-blood specimens from uninfected controls (n = 46) or patients with past COVID-19 previously confirmed via PCR detection of SARS-CoV-2 RNA (n = 31). After 48 hours of incubation, plasma supernatants were harvested and analyzed for content of IFN-γ.

NC peptide–induced formation of IFN-γ in whole blood discriminated patients with previous SARS-CoV-2 infection (median, 194 days since infection; range, 25–395 days) from uninfected controls (area under the ROC curve, 0.93; Figure 1A), whereas the presence of serum antibodies against the NC component of SARS-CoV-2 (NC-IgG) demonstrated greater overlap (area under the ROC curve, 0.74; Figure 1B) between these cohorts. In samples collected from previously infected patients, 23 of 26 (88%) were reactive in the NC-γ test, whereas 16 of 31 (52%) were seropositive for NC-IgG. In addition, 2 of 46 samples (4.3%) from uninfected participants were seropositive for NC-IgG but were negative for NC-γ, whereas 5 of 41 (12%) showed reactivity in the NC-γ test but were seronegative for NC-IgG. While the difference was not significant, the NC-γ test thus tended to show higher reactivity among study participants without a verified COVID-19 infection.

We also performed analyses of S1-induced IFN-γ (S1-γ test) in plasma supernatants from the whole-blood cultures. S1-γ test results as well as the presence of IgG antibodies against S1 (anti-RDB S1-IgG) could be used to diagnose past COVID-19 (median, 179 days from infection; range, 25–303 days) with high accuracy (Figure 1C and 1D). All but 2 patients with previous infection (24 of 26 [92%]), were positive in the S1-γ assay, while 21 of 26 (81%) were seropositive for anti-RBD S1-IgG. Among uninfected participants, 5 of 42 (12%) were positive in the S1-γ assay, while none of the 42 (0%) were seropositive for anti-RDB S1-IgG. However, of the 5 S1-γ–responsive donors without confirmed COVID-19 infection, 2 were also responsive in the NC-γ assay, and 1 had NC-IgG antibodies. Hence, previous asymptomatic infections cannot be excluded. The sensitivity and specificity of each test are listed in Table 3. Among previously infected patients, there was a trend toward correlation between NC-γ and NC-IgG, and there was a significant correlation between levels of S1-γ and anti-RDB S1-IgG in infected donors (Figure 1B and 1D, right panels).

Flow Cytometric Analysis of SARS-CoV-2 Peptide-Induced IFN-γ in $CD4^+$ and $CD8^+$ T Cells

We aimed to determine the T-cell subset producing IFN-γ in parallel experiments where PBMCs were isolated from controls and patients with previously verified COVID-19. The PBMCs were incubated with peptides spanning the NC or S1 regions of SARS-CoV-2, thus mimicking the induction protocol applied for whole-blood specimens. After overnight incubation, intracellular IFN-γ in gated $CD4^+$ or $CD8^+$ T cells was detected by means of flow cytometry. In NC peptide–stimulated PBMCs, the induced IFN-γ in $CD4^+$ and $CD8^+$ T cells significantly discriminated between previously infected patients and controls, with a similar trend for S1 peptide–stimulated PBMCs (Figure 2). For the small subset of infected donors analyzed with both flow cytometry and the whole-blood IFN-γ release assay, there was no significant correlation between intracellular and extracellular IFN-γ production. Concordance between SARS-CoV-2 peptide–induced intracellular IFN-γ in $CD4^+$ and $CD8^+$ T-cell subsets and extracellularly released IFN-γ, however, has been demonstrated previously [16].

Table 3. Sensitivity and Specificity of Analytes for Determining Infection Status

Analyte	Cutoff	Sensitivity, %	Specificity, %
NC-γ	10 pg/mL	89	88
NC-IgG	1.4 AU/mL	52	96
S1-γ	10 pg/mL	92	88
S1-IgG	14 BAU/mL	81	100

Abbreviations: AU, arbitrary units; BAU, binding antibody units; IgG, immunoglobulin G; NC, nucleocapsid; S1, spike 1.

Longevity of T-Cell Memory and Antibodies to NC Protein

We aimed to determine the durability of immune reactivity to SARS-CoV-2 after COVID-19 infection and thus compared seropositivity and results obtained in the whole-blood NC-γ test over time. This analysis was restricted to patients for whom samples for both of these tests were available on the same day. Samples within each interval after confirmed infection are from unique individuals, and the latest collected sample was chosen when multiple samples were available within the same interval. In agreement with earlier findings [12, 17–19], only 5 of 17 patients (29%) remained NC-IgG seropositive when analyzed >180 days after verified COVID-19 (median, 333 days; range 237–408 days). In contrast, 13 of 17 previously infected patients (76%) with samples taken >180 days after PCR-confirmed SARS-CoV-2 infection showed reactivity in the NC-γ whole-blood test ($P = .01$ for comparison with NC-IgG; McNemar test) (Table 4), thus implying that the T-cell response to NC is more long-lasting than antibodies against NC.

T-Cell Reactivity to S1 in Whole-Blood Samples From Vaccinated Study Participants

We obtained samples from 36 study participants after the first dose of vaccine against COVID-19, and from 22 participants after the second dose. Vaccination triggered a strong induction of S1-specific T cells in whole blood along with S1-IgG in serum 4 weeks after vaccination (range, 3–5 weeks) (Figure 3). When comparing T-cell responses after the first dose of vaccine in study participants with or without previous COVID-19, whole blood from vaccinated individuals with previous infection produced significantly higher levels of IFN-γ in the S1-γ assay ($P < .01$, permutation test; Figure 3A), with a similar difference when comparing S1-IgG levels in vaccinated donors with or without previous infection ($P < .001$, permutation test; Figure 3B). As expected, vaccination did not alter levels of NC-induced IFN-γ or NC-IgG (Supplementary Figure 3). In this small cohort, we observed no differences in immune responses among recipients across different vaccines, nor any significant associations between vaccine responsiveness, age, or sex (data not shown).

Table 4. Longevity of Immune Responses to Nucleocapsid Among Patients With Past Coronavirus Disease 2019

Time From Positive SARS-CoV-2 PCR Result	NC-γ Positivity, No. (%)	NC-IgG Positivity, No. (%)	*P* Value[a]
<60 d (n = 8)	8 (100)	7 (88)	1
60–180 d (n = 8)	7 (88)	3 (38)	.125
>180[b] d (n = 17)	13 (76)	5 (29)	.01

Abbreviations: IgG, immunoglobulin G; NC, nucleocapsid; NS, not significant; PCR, polymerase chain reaction; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

[a] *P* values were determined with McNemer test.

[b] Median interval, 333 days; range, 237–408 days.

DISCUSSION

We report that ex vivo stimulation of whole blood from previously SARS-CoV-2–infected patients with peptides spanning the NC and S1 regions of SARS-CoV-2 stalwartly evoked the formation of IFN-γ, likely indicative of antigen-specific T-cell memory. ROC analyses suggested that the sensitivity of these whole-blood assays for identifying previously infected patients is higher than analysis of antigen-specific IgG levels in serum. More controls tested positive in the NC and S1 IFN-γ tests, suggesting lower specificity compared with antibody tests, although previous undiagnosed COVID-19 or cross-reactivity from infection with other coronaviruses cannot formally be excluded in these patients. Our results also imply that SARS-CoV-2–specific T-cell reactivity is significantly more durable than NC-IgG. Thus, NC-γ responses in blood remained detectable for >6 months in >70% of previously infected patients, even though <30% of these patients had detectable antibodies against NC. We observed T-cell responses to S1 in whole-blood samples from vaccinated study participants, responses that coincided with the appearance of anti-RBD S1-IgG in serum. Our results thus confirm and extend results from earlier studies of evoked T-cell memory in whole blood from SARS-CoV-2–infected and vaccinated patients [14, 15].

In vaccinated study participants, assays of anti-RDB S1-IgG or T-cell reactivity against spike proteins obviously cannot be used to diagnose past COVID-19. The NC-γ test displayed superior sensitivity in detecting past COVID-19 compared with the detection of NC-IgG antibodies. We thus propose that the NC-γ test, or similar assays using whole-blood samples, may be valuable in identifying patients with previous infection that was not captured by the detection of virus or antigen during active viral replication. The NC-γ test may thus be instrumental in the evaluation of suspected long-term morbidity from COVID-19 among patients with fading or undetectable NC-IgG, but also in monitoring the durability of T-cell immunity after natural infection in the postvaccine era. Earlier studies suggest that NC-specific T-cell memory, analyzed in PBMCs with flow cytometry, based on OX40/4-1BB expression on $CD4^+$ T cells, persists for ≥8 months after COVID-19 [20], and our results thus support the longevity of T-cell memory although further follow-up from infection is warranted.

In this smaller series of patients, the S1-γ test showed slightly higher sensitivity in diagnosing past COVID-19, compared

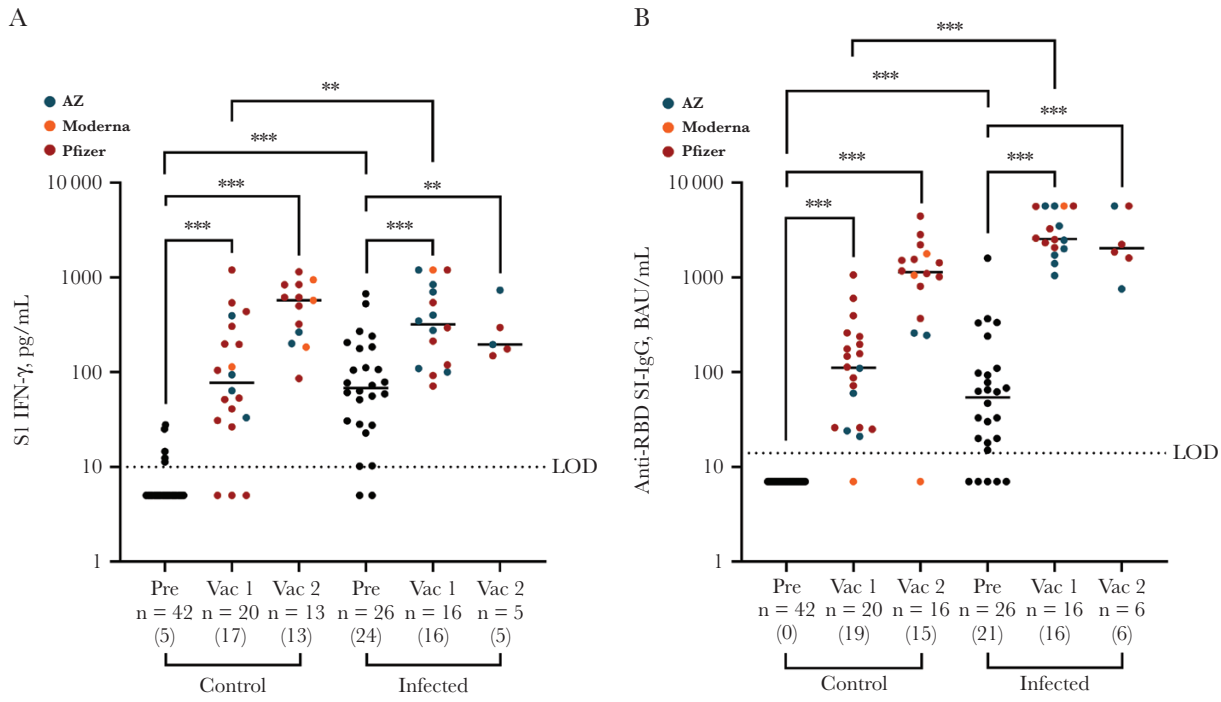

Figure 3. Study participants previously infected with severe acute respiratory syndrome coronavirus had higher spike protein–specific T-cell and antibody responses after 1 vaccine dose than uninfected patients (controls). Samples from study participants were analyzed before vaccination (Pre) and after the first (Vac 1) and second (Vac 2) vaccine doses. *A,* T-cell interferon (IFN) γ responses before and after vaccination, after stimulation of whole blood with the spike 1 (S1) part of the spike protein. *B,* Serum anti–receptor binding domain (RBD) S1–immunoglobulin G (IgG) levels before and after vaccination. Recipients of the AstraZeneca (AZ) , Moderna, and Pfizer vaccines are shown as blue, orange, and red dots, respectively. Statistical analysis was performed using permutation tests. For each assay, dotted lines represent the limit of detection (LOD) (IFN-γ, >10 pg/mL; IgG, >14 binding antibody units [BAU]/mL), and the number of samples above the LOD is shown in parentheses. $^{**}P < .01$; $^{***}P < .001$.

with anti-RBD S1-IgG. The S1-γ test may therefore provide a means to monitor the durability of T-cell memory responses after vaccination. Similar to the NC-IgG test, previous studies also report reduction of S1-IgG over time after natural infection [21]. A 2021 study thus reported waning of S1-IgG also after 2 COVID-19 vaccine doses [9]. Long-term studies are required to clarify whether or not the S1-γ test, or similar whole-blood–based assays, are helpful in monitoring the efficiency of vaccine-induced T-cell immunity and whether the presence of SARS-CoV-2–specific T cells provides clinically meaningful protection against COVID-19 in the absence of S1-specific antibodies.

In addition to whole-blood assays of SARS-CoV-2 peptide–induced IFN-γ, we used a similar induction protocol for the assessment of T-cell activation in Ficoll-separated PBMCs analyzed with flow cytometry. These experiments suggested that $CD4^+$ and $CD8^+$ T cells from infected patients accumulated intracellular IFN-γ after exposure to SARS-CoV-2 peptides, in accordance with previous reports [16, 22], implying that both T-cell subsets contributed to the formation of IFN-γ in whole-blood assays. For the small subset of infected donors analyzed with both flow cytometry and the whole-blood IFN-γ release assay, there was no significant correlation between intracellular and extracellular IFN-γ production. Concordance between SARS-CoV-2 peptide induced intracellular IFN-γ in $CD4^+$ and $CD8^+$ T-cell subsets, and extracellularly released IFN-γ has been demonstrated previously [16].

Limitations of the current study include the small study population, the possibility of undiagnosed asymptomatic infections in the control group and the possibility of false positivity among donors with low IFN-γ or IgG antibody responses. A few participants in the control group showed reactivity in >1 of the NC-γ, S1-γ, and NC-IgG assays, suggesting the possibility of previous undiagnosed SARS-CoV-2 infections. Another possible explanation is that the background levels of IFN-γ observed in S1- or NC-stimulated controls represent cross-reactivity from previous common cold coronavirus infections, as has been suggested to occur in other studies [23, 24]. However, the prevalence of common cold coronaviruses is high [25], while the frequency of reactivity among the controls was low, which speaks against cross-reactivity induced by other coronaviruses. Nevertheless, if the background reactivity in the controls stems from a previous infection, this is congruent with the higher number of controls being reactive in the IFN-γ assays compared with the IgG assays, as the T-cell responses appear more durable.

In conclusion, whole-blood assays for analysis of T-cell memory against SARS-CoV-2 may be useful in determining the duration of T-cell immunity after natural infection or vaccination, particularly in diagnosing past COVID-19 in

patients with waning antibody titers. This study recruited hospital staff members with minimal comorbid conditions, and the results therefore reflect T-cell immunity in a largely healthy population. The potential utility of whole-blood–based assessment of T-cell memory warrants further evaluation in persons at risk for severe COVID-19, including immunosuppressed patients.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Acknowledgments. We are grateful to staff members of the Departments of Virology and Gynecology for their participation in this study.

Financial support. This work was supported by the Swedish Research Council (grant 2020-01437), the AFA Insurance Foundation (grant 20045), the Clas Groschinsky Foundation, the Wilhelm and Martina Lundgren Research Foundation (grant 2021-3819), and the Sahlgrenska Academy at the University of Gothenburg.

Potential conflicts of interest. K. H. is a stockholder in Cytovia and Linnea Pharma and has a planned patent application. All other authors report no potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Kristiansen PA, Page M, Bernasconi V, et al. WHO international standard for anti-SARS-CoV-2 immunoglobulin. Lancet **2021**; 397:1347–8.
2. Wajnberg A, Amanat F, Firpo A, et al. Robust neutralizing antibodies to SARS-CoV-2 infection persist for months. Science **2020**; 370:1227–30.
3. Gudbjartsson DF, Norddahl GL, Melsted P, et al. Humoral immune response to SARS-CoV-2 in Iceland. N Engl J Med **2020**; 383:1724–34.
4. Bryan A, Pepper G, Wener MH, et al. Performance characteristics of the Abbott Architect SARS-CoV-2 IgG assay and seroprevalence in Boise, Idaho. J Clin Microbiol **2020**; 58:e00941-20.
5. Lagerqvist N, Maleki KT, Verner-Carlsson J, et al. Evaluation of 11 SARS-CoV-2 antibody tests by using samples from patients with defined IgG antibody titers. Sci Rep **2021**; 11:7614.
6. Ravichandran S, Lee Y, Grubbs G, et al. Longitudinal antibody repertoire in "mild" versus "severe" COVID-19 patients reveals immune markers associated with disease severity and resolution. Sci Adv **2021**; 7:eabf2467.
7. Garcia-Beltran WF, Lam EC, Astudillo MG, et al. COVID-19-neutralizing antibodies predict disease severity and survival. Cell **2021**; 184:476–488.e11.
8. Li K, Huang B, Wu M, et al. Dynamic changes in anti-SARS-CoV-2 antibodies during SARS-CoV-2 infection and recovery from COVID-19. Nat Commun **2020**; 11:6044.
9. Shrotri M, Navaratnam AMD, Nguyen V, et al. Spike-antibody waning after second dose of BNT162b2 or ChAdOx1. Lancet **2021**; 398:385–7.
10. Crawford KHD, Dingens AS, Eguia R, et al. Dynamics of neutralizing antibody titers in the months after severe acute respiratory syndrome coronavirus 2 infection. J Infect Dis **2021**; 223:197–205.
11. Lau EHY, Tsang OTY, Hui DSC, et al. Neutralizing antibody titres in SARS-CoV-2 infections. Nat Commun **2021**; 12:63.
12. Bolotin S, Tran V, Osman S, et al. SARS-CoV-2 seroprevalence survey estimates are affected by anti-nucleocapsid antibody decline. J Infect Dis **2021**; 223:1334–8.
13. Zhou G, Luo Q, Luo S, et al. Interferon-gamma release assays or tuberculin skin test for detection and management of latent tuberculosis infection: a systematic review and meta-analysis. Lancet Infect Dis **2020**; 20:1457–69.
14. Brand I, Gilberg L, Bruger J, et al. Broad T cell targeting of structural proteins after SARS-CoV-2 infection: high throughput assessment of T cell reactivity using an automated interferon gamma release assay. Front Immunol **2021**; 12:688436.
15. Petrone L, Petruccioli E, Vanini V, et al. A whole blood test to measure SARS-CoV-2-specific response in COVID-19 patients. Clin Microbiol Infect **2021**; 27:286 e7–e13.
16. Vallejo A, Vizcarra P, Quereda C, Moreno A, Casado JL. IFN-γ^+ cell response and IFN-γ release concordance after in vitro SARS-CoV-2 stimulation. Eur J Clin Invest **2021**; 51:e13636.
17. Lumley SF, Wei J, O'Donnell D, et al. The duration, dynamics, and determinants of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) antibody responses in individual healthcare workers. Clin Infect Dis **2021**; 73:e699–e709.
18. Henss L, Scholz T, von Rhein C, et al. Analysis of humoral immune responses in patients with severe acute respiratory syndrome coronavirus 2 infection. J Infect Dis **2021**; 223:56–61.
19. Muecksch F, Wise H, Batchelor B, et al. Longitudinal serological analysis and neutralizing antibody levels in coronavirus disease 2019 convalescent patients. J Infect Dis **2021**; 223:389–98.
20. Kang CK, Kim M, Lee S, et al. Longitudinal analysis of human memory T-cell response according to the severity of illness up to 8 months after severe acute respiratory syndrome coronavirus 2 infection. J Infect Dis **2021**; 224:39–48.

21. Gaebler C, Wang Z, Lorenzi JCC, et al. Evolution of antibody immunity to SARS-CoV-2. Nature **2021**; 591:639–44.
22. Zhao J, Wang L, Schank M, et al. SARS-CoV-2 specific memory T cell epitopes identified in COVID-19-recovered subjects. Virus Res **2021**; 304:198508.
23. Bacher P, Rosati E, Esser D, et al. Low-avidity $CD4^+$ T cell responses to SARS-CoV-2 in unexposed individuals and humans with severe COVID-19. Immunity **2020**; 53:1258–71.e5.
24. Shimizu K, Iyoda T, Sanpei A, et al. Identification of TCR repertoires in functionally competent cytotoxic T cells cross-reactive to SARS-CoV-2. Commun Biol **2021**; 4:1365.
25. Monto AS, DeJonge PM, Callear AP, et al. Coronavirus occurrence and transmission over 8 years in the HIVE cohort of households in Michigan. J Infect Dis **2020**; 222:9–16.