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ORIGINAL ARTICLE

Rapid detection of SARS-CoV-2-specific memory T-cell immunity in recovered COVID-19 cases

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Abstract

Objectives. There is emerging evidence that SARS-CoV-2-specific memory T-cell responses are likely to provide critical long-term protection against COVID-19. Strategies to rapidly assess T-cell responses are therefore likely to be important for assessing immunity in the global population. **Methods.** Here, we have developed a rapid immune-monitoring strategy to assess virus-specific memory T-cell responses in the peripheral blood of COVID-19 convalescent individuals. We validated SARS-CoV-2-specific memory T-cell responses detected in whole blood using *in vitro* expansion with SARS-CoV-2 proteins. **Results.** T-cell immunity characterised by the production of IFN-γ and IL-2 could be consistently detected in the whole blood of recovered participants. T cells predominantly recognised structural SARS-CoV-2 proteins. *In vitro* expansion demonstrated that while $CD8^+$ T cells recognised nucleocapsid protein, spike protein and ORF3a, $CD4^+$ T cells more broadly targeted multiple SARS-CoV-2 proteins. **Conclusion.** These observations provide a timely monitoring approach for identifying SARS-CoV-2 cellular immunity and may serve as a diagnostic for the stratification of risk in immunocompromised and other at-risk individuals.

Keywords: antigen-specific, COVID-19, SARS-CoV-2, T-cell response

INTRODUCTION

As of 20 October 2020, the SARS-CoV-2 virus has infected more than 39 million individuals worldwide and caused more than 1.1 million deaths.[1] Despite optimism that an effective vaccine will be developed in the next 12 months and that the therapeutic administration of antiviral drugs or SARS-CoV-2-specific antibodies will reduce the severity of symptoms associated with COVID-19, it is likely that millions of more individuals will be infected over the coming years.[2–4] Therefore, the need to develop a thorough understanding of all aspects of immunity against SARS-CoV-2 is critical. Antigen-specific T cells, capable of recognising nonstructural viral proteins and proteins involved in viral replication, are critical mediators in the early cellular immune response to all viral pathogens.[5–7] These antigen-experienced T cells serve a myriad of functions, from the direct targeting and cytolytic killing of virally infected cells to the augmentation of other immune

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responses, including the production of antibodies.[8–10]

As a result of their significant role in immunological defence, virus-specific T-cell responses are typically established early after primary infection.[11] However, studies over the past two decades have shown that poor T-cell induction during initial infection can lead to significant T-cell dysfunction and chronic infection.[12–19] Observation of patients during early stage COVID-19 has highlighted that T-cell dysfunction is a driving cause of disease development.[20] Patients with severe symptoms show high expression of checkpoint markers, including PD-1, which suggests poor T-cell priming during the early stages of infection.[21,22] Conversely, patients who resolve infection have recently been shown to generate antigen-specific T-cell responses against proteins encoded by SARS-CoV-2.[23–26] These observations support the contention that T cells play a role in the control of SARS-CoV-2, although it remains to be determined what impact T-cell immunity has upon the risk of re-infection and more importantly disease prevention. This in turn highlights the potential benefit of monitoring these antigen-specific T-cell responses during infection as a means of determining the risk of serious COVID-19 symptoms, and also suggests that immune interventions, such as vaccines, should also target T-cell immunity to reduce disease risk.[27] Here, we have assessed SARS-CoV-2-specific T-cell immunity in whole blood collected from a cohort of 44 individuals convalescing from COVID-19. This whole-blood assay demonstrated consistent T-cell reactivity predominantly directed to viral structural proteins that could be detected by the induction of either interferon gamma (IFN-γ) or interleukin (IL)-2. We further validated *ex vivo* T-cell reactivity by expanding the patients' SARS-CoV-2-specific memory T cells, which displayed broad antigen reactivity towards multiple SARS-CoV-2 antigens.

RESULTS

Cytokine profiling of antigen-specific T cells in the whole blood of study participants

To determine whether antigen-specific T-cell immunity could be rapidly assessed in individuals who have recently recovered from COVID-19, we developed a whole-blood assay using overlapping peptide sets designed from SARS-CoV-2 antigens (Table 1) as stimuli (Figure 1). Using this assay, we analysed whole blood from 44 COVID-19 convalescent patients together with 21 unexposed healthy individuals. Plasma harvested from the overnight stimulation was analysed by CBA, and to demonstrate antigen specificity, we subtracted background cytokine production generated from cultures with no antigen. This identified significantly elevated levels of the T-cell cytokines IFN-γ and IL-2 (Figure 2a and b) in convalescent samples compared to unexposed samples. These responses indicate the successful generation of a T-cell-mediated immune response in convalescent individuals in response to SARS-CoV-2 peptide stimulation. In addition, convalescent patients displayed significantly increased levels of the pro-inflammatory cytokine IL-8 (Figure 2c) compared to the unexposed cohort. Interestingly, we observed minimal differences in the remaining cytokines (TNF, IL-1β, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13 and IL-17a) between COVID-19-recovered and unexposed individuals (Supplementary figure 1). Principal component analysis (PCA) using the combined detection data from all analysed cytokines clearly stratified IL-2, IFN-γ and, to a lesser extent, IL-8 as the top cytokine candidates for differentiating between the SARS-CoV-2-specific responses of convalescent COVID-19 patients and unexposed individuals (Figure 2d, shown in green). This PCA further demonstrates that the remaining cytokines analysed in this assay do not discriminate between convalescent and unexposed groups.

To illustrate the differential levels of cytokine detected from all blood samples (both convalescent and unexposed) in response to SARS-CoV-2 peptide stimulation, we generated individual heat maps (Figure 3). Here, the total range of detection for each individual cytokine was normalised across all samples and displayed on a colour (viridis) scale ranging from 0.0 (purple) to 1.0 (yellow). This analysis distinguished elevated IFN-γ and IL-2 cytokine responses in convalescent patients compared to healthy donors when their blood was stimulated with SARS-CoV-2 peptide pools CoV-3, 4 and 5. Here, statistical analysis confirmed a significant correlation between the production of IL-2 and IFN-γ in convalescent patient responses (Figure 4). In comparison, the heat map illustrates that the remaining cytokines failed to differentiate between convalescent and unexposed individuals (Figure 3). PCA completed on individual cytokine data sets confirmed that detection levels of IL-2 (Supplementary figure 2a), and to a lesser extent IFN-γ (Supplementary figure 2b), could be used to

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Table 1. Antigen composition of the CoV-peptide pools

Pool	Overlapping antigen peptide sets contained in the pool	Total peptides
CoV-1	Protein 3a, nonstructural protein 6, ORF10 protein, ORF9b protein	108
CoV-2	Nonstructural protein 7a, nonstructural protein 7b, nonstructural protein 8, uncharacterised protein 14	80
CoV-3	Nucleoprotein, membrane protein, envelope small membrane protein	171
CoV-4	Spike glycoprotein C terminus region	157
CoV-5	Spike glycoprotein N terminus region	158

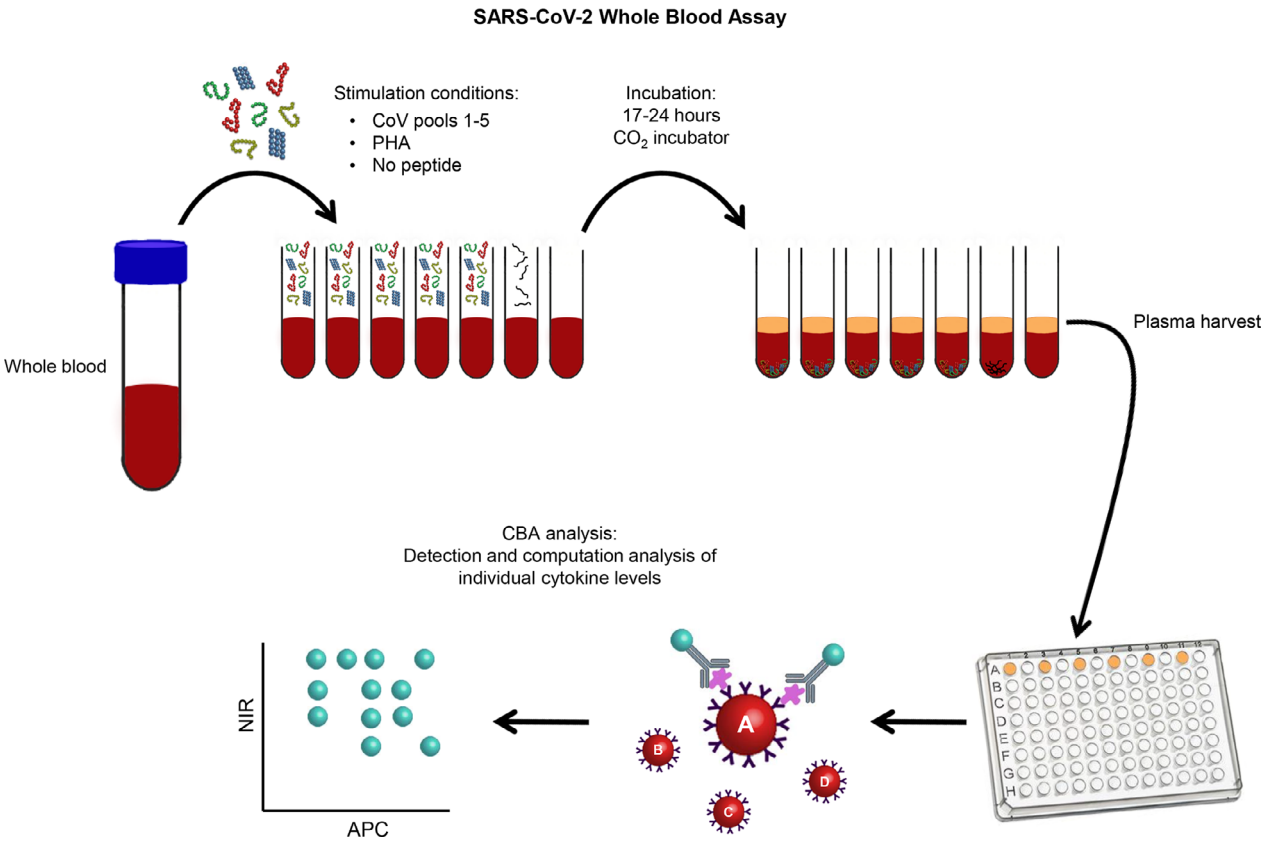

Figure 1. Schematic of the whole-blood assay for the rapid detection of SARS-CoV-2-responsive T-cell cytokine production. Whole blood (200 μL) was added into seven separate wells of a 96-well plate and stimulated under different conditions. Five wells were stimulated with CoV-peptide pools (CoV-1 to 5), the sixth condition was a positive control (mitogen), and the final well received no stimulation. The assay was incubated overnight for an average of 20 h in a CO_2 controlled incubator. Following this, the plate was spun and plasma harvested for cytometric bead array analysis to detect production levels of 12 different cytokines.

group convalescent patients and unexposed individuals into two separate clusters. In contrast, PCA for TNF (Supplementary figure 2c) failed to separate convalescent patient samples from unexposed samples.

Impact of sex and age on the magnitude of the IFN-γ and IL-2 response to SARS-CoV-2

Given that a clear association between sex, age and COVID-19 disease severity has been reported [21,28], we next investigated the impact of these parameters on the magnitude of SARS-CoV-2 T-cell responses in recovered individuals. We observed no significant differences in IFN-γ or IL-2 responses to CoV stimulation between the sexes, suggesting that after the resolution of infection, both women and men generate a similar memory response (Figure 5a and b). However, we did identify a difference in the magnitude of IFN-γ and IL-2 responses based on age. Participants who were >50 years of age at

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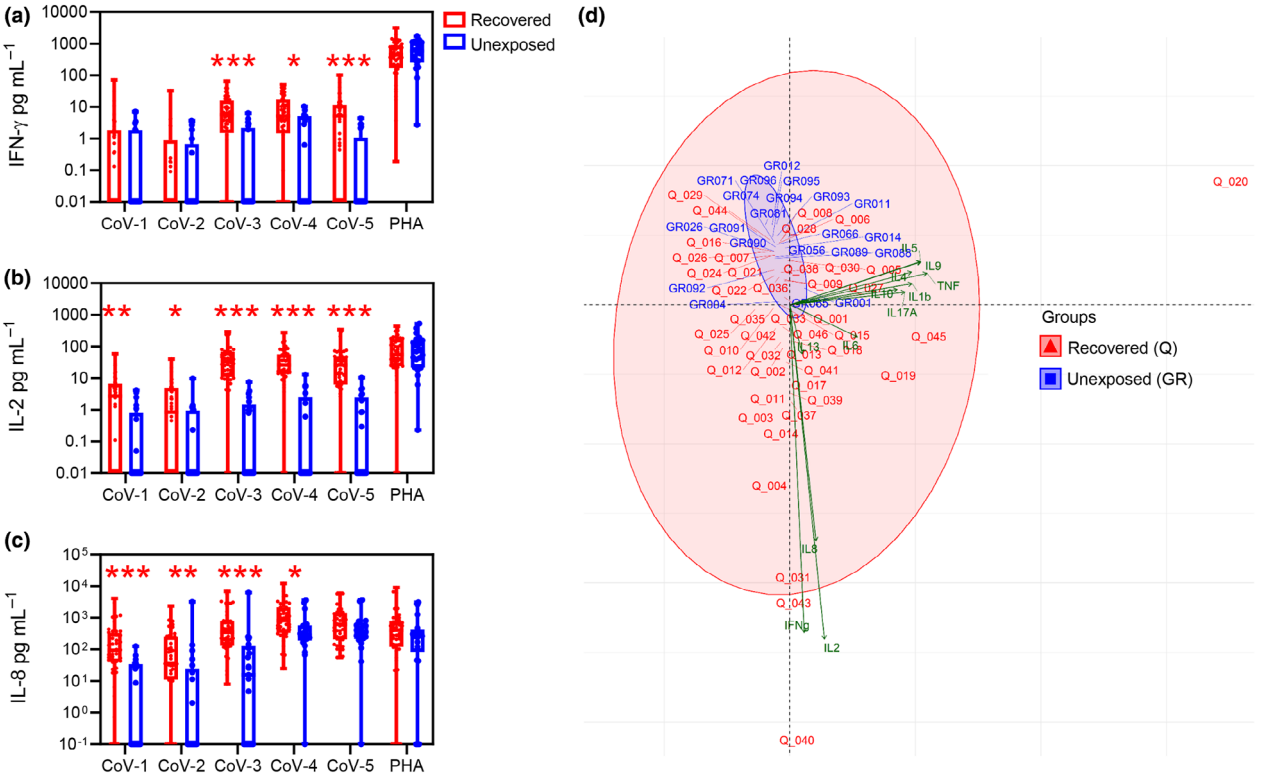

Figure 2. Antigen-specific cytokine detection and principal component analysis demonstrating correlation between cytokine responses in recovered versus unexposed individuals. Box and whisker plots displaying cytokine responses to CoV pools 1-5 and control phytohaemagglutinin (PHA) mitogen stimulation comparing COVID-19-recovered (red) and unexposed (blue) individuals. Data represent values after subtraction of background cytokine levels. Statistical analysis confirmed significantly increased IFN-γ production **(a)** in recovered COVID-19 patients in response to CoV-3 (P = 1.5e-5), CoV-4 (P = 0.0054), CoV-5 (P = 7.0e-5). Significantly increased IL-2 production **(b)** was observed in recovered COVID-19 patients in response to CoV-1 (P = 0.0041), CoV-2 (P = 0.036), CoV-3 (P = 4.4e-09), CoV-4 (P = 9.7e-09), CoV-5 (P = 2.2e-07). Significantly increased IL-8 production **(c)** in recovered COVID-19 patients in response to CoV-1 (P = 4.2e-07), CoV-2 (P = 0.001), CoV-3 (P = 1.1e-06) and CoV-4 (P = 0.0047). Statistical analysis was performed using a Wilcoxon test. (*P = 0.05, **P = 0.005, ***P = 0.0001). Principal component analysis of total cytokine production **(d)** in convalescent COVID-19 (n = 44) patients (red) and unexposed (n = 21) individuals (blue) across 12 cytokines (green). The direction and length of a green line indicates the degree of association between that cytokine and the surrounding patient cohort.

the time of recruitment generated a significantly higher IFN-γ response to the CoV-3 and 5 pools (Figure 5c) and a significantly higher IL-2 response to the CoV-1, 2, 3 and 5 pools (Figure 5d) compared to participants below 50 years of age.

SARS-CoV-2-specific CD4$^+$ and CD8$^+$ memory T-cell immunity

To determine whether there were differences in the global immune composition of recovered and unexposed individuals, we first performed phenotypic analysis on total PBMC and observed no significant differences in lymphocyte populations between these cohorts (Supplementary figure 3). Next, to validate the T-cell reactivity detected in whole blood and to map the antigen specificity of memory T cells, we expanded antigen-specific T cells from PBMC following stimulation with the CoV-peptide pools (Table 1). After 14 days in culture, cytokine production (IFN-γ, TNF, IL-2 and CD107a degranulation) was assessed following recall stimulation with each of the overlapping CoV-peptide pools. Strikingly, while we saw a diverse IFN-γ response from CD4$^+$ T cells, which included recognition of the proteins spike (S), nucleocapsid (N) and membrane (M) together with ORFs 3a, 7a, 8 and 9b (Figure 6a), the predominant memory CD8$^+$ T-cell response was directed against the N protein (Figure 6b). Of the 18 recovered individuals assessed, a majority showed expansion of N protein-specific T cells, with a median frequency of 9.36%, whereas the median response

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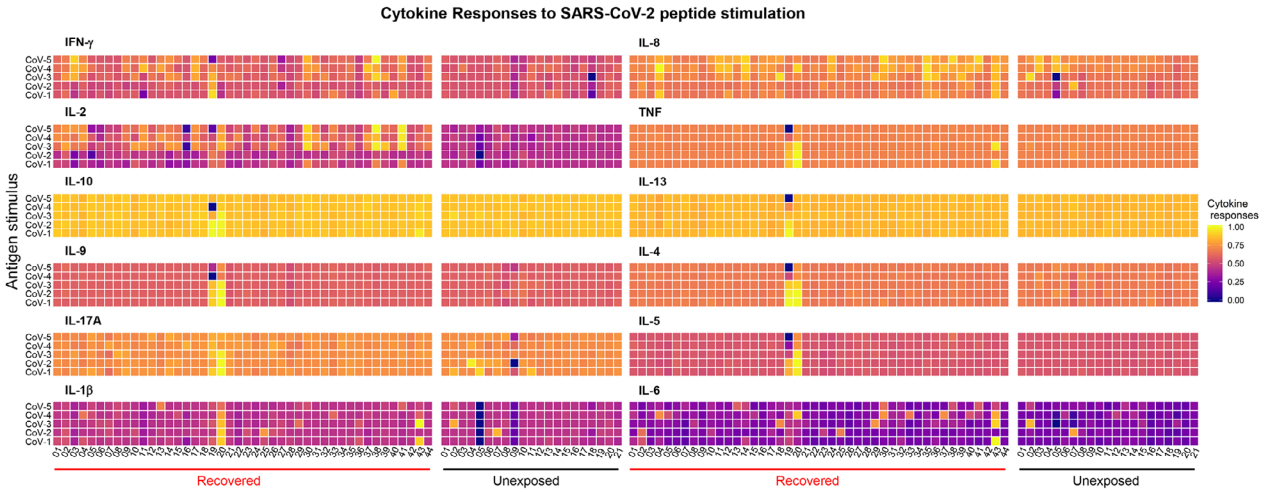

Figure 3. Heat map of cytokine detection in whole blood following stimulation with SARS-CoV-2 peptide sets. Heat maps display cytokine response levels in COVID-19-recovered (Q) and unexposed (GR) individuals. Whole blood (200 μL) from recovered and unexposed individuals was incubated for 16–24 h at 37°C with peptide pools (CoV-1–CoV-5) containing overlapping peptide sets from SARS-CoV-2. Blood was incubated with a global mitogen stimulus (phytohaemagglutinin) as a positive control, or left unstimulated/without peptide to control for spontaneously generated cytokine (background). Blood was then centrifuged and plasma harvested and analysed using a cytometric bead array to detect T-cell cytokines. Data represent the cytokine levels with background values subtracted from each of the peptide-stimulated culture conditions. Cytokine response levels were normalised within each individual cytokine and displayed using a colour (viridis) scale ranging from the lowest detection value of 0.0 (purple) to the highest detection value of 1.0 (yellow).

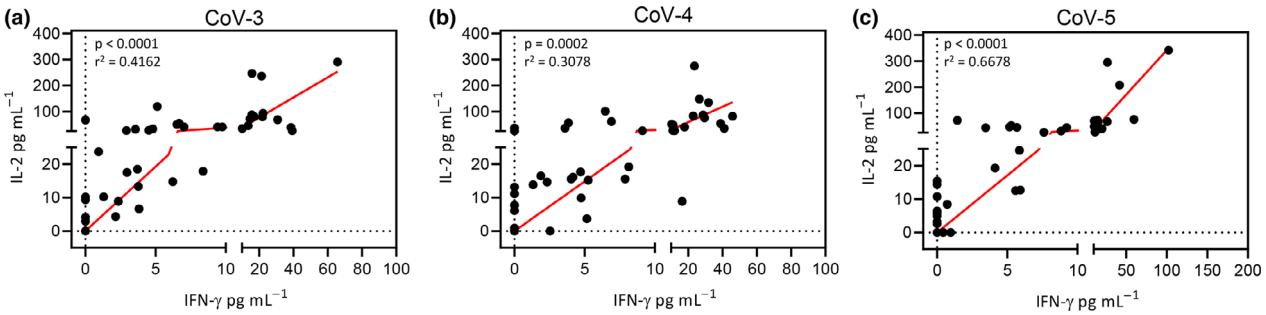

Figure 4. Association of IFN-γ and IL-2 in response to SARS-CoV-2 peptide pools. Data represent a correlation between IFN-γ and IL-2 production in response to **(a)** CoV-3 (r^2 = 0.4162, P < 0.001), **(b)** CoV-4 (r^2 = 0.3078, P = 0.002) and **(c)** CoV-5 (r^2 = 0.6678, P < 0.0001) peptide pools. Statistical analysis was performed to calculate the Pearson's correlation coefficient (r^2) and statistical significance using a two-tailed *t*-test (*P*).

to ORF3a was 1.36%. We also observed consistent low-level recognition of the two spike pools. Polyfunctional analysis on expanded T cells revealed a similar response profile to each dominant antigen, with $CD4^+$ T cells recognising N, S or M characterised by the single production of TNF or co-expression of TNF and IFN-γ (Figure 6c). $CD8^+$ T cells predominantly produced TNF and IFN-γ, with a high proportion also demonstrating degranulation (CD107a). In contrast to our *ex vivo* whole-blood analysis, we saw little IL-2 production, suggesting these memory T cells undergo functional changes following *in vitro* proliferation and differentiation.

DISCUSSION

T cells play an essential role in the control of human viral infections. In the absence of T-cell immunity, chronic infection occurs which typically requires ongoing antiviral treatment. Similarly,

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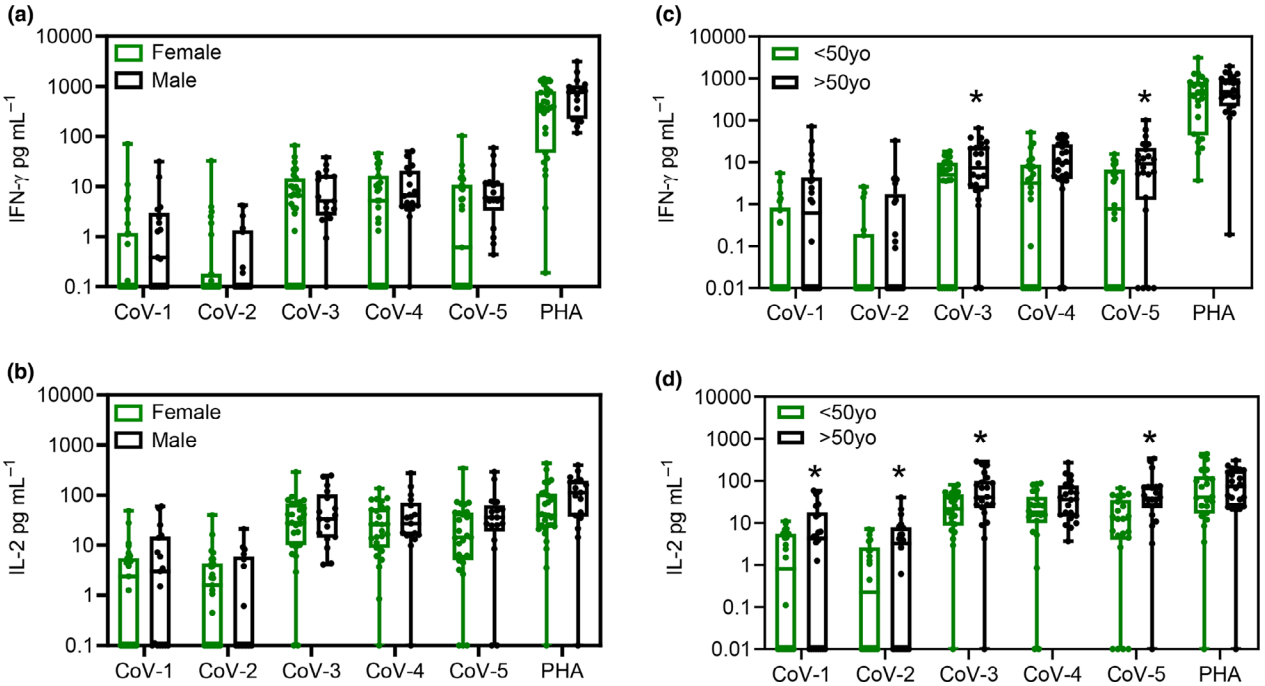

Figure 5. Impact of sex and age on cytokine production in COVID-19 convalescent individuals. Comparison of the IFN-γ **(a)** and IL-2 **(b)** response to peptide pools CoV-1 to 5 and control phytohaemagglutinin (PHA) in females and males. Data represent a comparison of the IFN-γ **(c)** and IL-2 **(d)** response to peptide pools CoV-1 to 5 and control PHA in people aged under 50 years and over 50 years. Statistical significance was determined using multiple *t*-tests in GraphPad Prism software, where the number of *t*-tests = 6 (*$P < 0.05$).

early observations suggest that the induction of a SARS-CoV-2-specific T-cell response is likely important in the control of COVID-19, whereby severe disease is associated with lymphopenia and checkpoint marker expression on T cells.[20,21,27] The capacity to rapidly detect the induction of antigen-specific immunity following diagnosis could provide a means of stratifying patient risk, particularly with regard to the onset of severe disease, and potentially assist in identifying individuals who require earlier intervention with antiviral therapy and other treatment. In this study, we demonstrate a simple whole-blood assay that can rapidly detect cytokine production in response to SARS-CoV-2 antigens. In recovered people, our assay confirmed a predominantly T helper 1 (Th1) cytokine response driven by antigen-specific T cells and identified by detection of IFN-γ and IL-2 in response to both structural and nonstructural SARS-CoV-2 proteins. These cytokines are also known to be important in the effector function of $CD8^+$ T cells.

The results from this study confirm recent observations demonstrating a bias towards T-cell responses against SARS-CoV-2 structural antigens in recently recovered patients.[23,29] While further work is required to delineate and define the key T-cell antigenic determinants in SARS-CoV-2, it is clearly evident in this cohort that most people who successfully resolve infection generate readily detectable T-cell immunity, a majority of which is raised against the N protein. This finding is supported by other recent studies on SARS-CoV-2 T-cell immunity.[23,24,29-31] Based on observations in other coronavirus infections, particularly in animal models, it is also likely that this T-cell response plays a critical role in disease prevention.[32] Ongoing studies in patients with acute infection and symptomatic disease should help to further define the role of T cells in the prevention of COVID-19. Importantly, longitudinal analysis is necessary to provide further insight into the stability of these T-cell responses over time, and their association with COVID-19 disease severity. It also remains to be determined what role measurement of IL-2 has in the analysis of SARS-CoV-2 T-cell memory and whether the quantities detected during this early memory phase are maintained long term. While most current immune-monitoring approaches for viral

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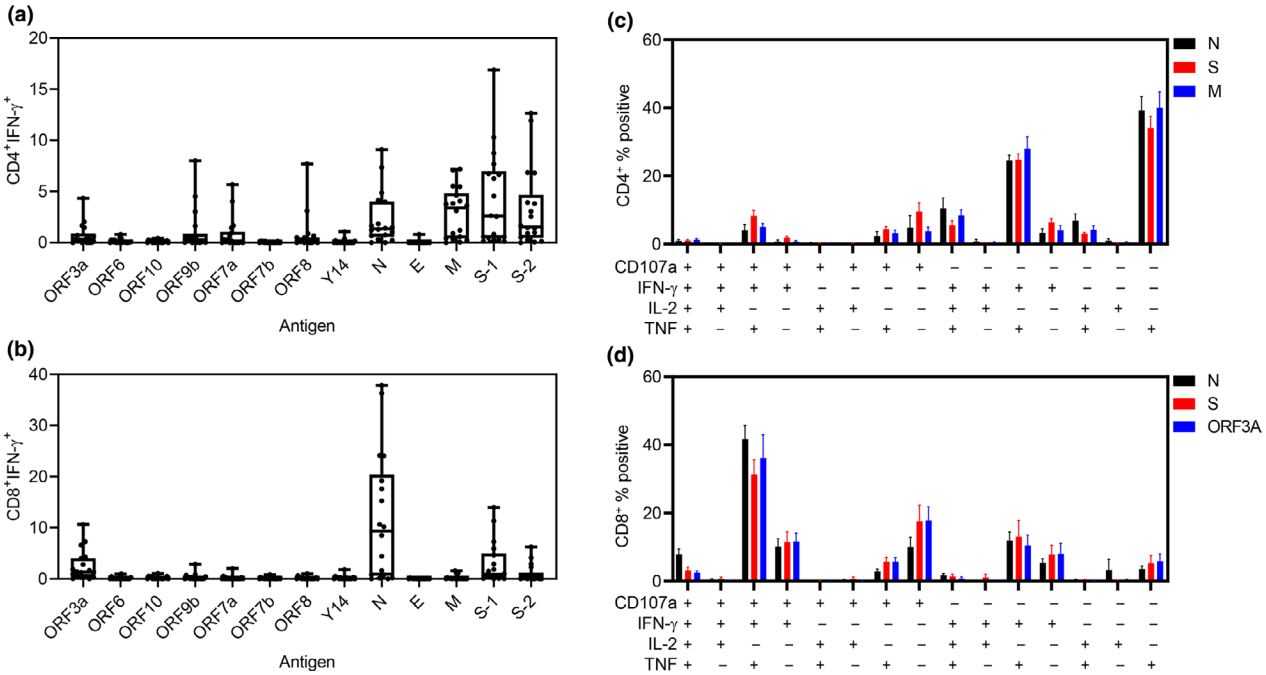

Figure 6. Expanded antigen-specific T-cell responses to SARS-CoV-2 proteins in COVID-19-convalescent individuals. Data represent $CD4^+IFN\text{-}\gamma^+$ **(a)** and $CD8^+IFN\text{-}\gamma^+$ **(b)** responses to individual SARS-CoV-2 proteins. Polyfunctional analysis of dominant $CD4^+$ responses **(c)** to nucleocapsid (N), spike (S) and membrane (M) proteins and dominant $CD8^+$ responses **(d)** to N, S and ORF3a proteins.

infections in humans focus upon IFN-γ, IL-2 production by both $CD4^+$ and $CD8^+$ T cells has been associated with enhanced viral control in other settings and could be a critical mediator of immune defence against SARS-CoV-2.[32–34]

Despite the known importance of T cells in the control of most human viruses, very few clinically approved diagnostic tests are available to study T-cell immunity, with the exception of the QuantiFERON-CMV whole-blood assay, currently employed to assess cytomegalovirus (CMV) infection in solid organ transplant patients. In this context, the QuantiFERON-CMV assay is clinically approved to assess CMV-specific T-cell immunity and is used successfully to stratify patient risk with regard to onset of viral reactivation and disease.[35] This knowledge has allowed for improved implementation of antiviral therapies. The results of our current study demonstrate that a similar rapid whole-blood test can effectively assess SARS-CoV-2-specific T-cell immunity. We anticipate that the ease of application of our SARS-CoV-2 whole-blood assay will provide an effective means to study SARS-CoV-2-specific T-cell immunity in a large number of patients, particularly during the acute stages of infection. We anticipate that this approach will also provide a rapid and early clinical risk assessment for patients that have failed to generate effective immunity, and therefore require additional therapeutic intervention, such as antiviral or immunotherapeutic treatment. This type of approach may also be applicable for monitoring of vaccine-induced T-cell responses.

METHODS

Study participants

This study was performed according to the principles of the Declaration of Helsinki. Ethics approval to undertake the research was obtained from QIMR Berghofer Medical Research Institute Human Research Ethics Committee. Informed consent was obtained from all participants. The inclusion criteria for the study were that participants were over the age of 18, had been clinically diagnosed by PCR with SARS-CoV-2 infection and had subsequently been released from isolation following resolution of symptomatic infection. A total of 44 participants were recruited in May and June 2020 from the south-east region of Queensland, Australia. Participants ranged in age from 20 to 75, 17 were male and 27 were female, and were a median of 58 (46–102) days postinitial diagnosis. The majority of participants were returned overseas travellers. Blood samples were collected from all participants to assess whole-blood SARS-CoV-2 T-cell immunity, and peripheral blood mononuclear cells (PBMCs) were isolated to assess cellular phenotype. A

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cohort of 21 healthy (i.e. non-SARS-CoV-2-exposed) adults was also recruited from south-east Queensland prior to any widespread transmission, to serve as a comparison group.

Whole-blood measurement of SARS-CoV-2-specific T-cell immunity

A small-scale whole-blood assay was used to recall memory T-cell responses to SARS-CoV-2. Heparinised whole blood (200 μL) was aliquoted into seven wells of a 96-well V-bottom plate. SARS-CoV-2 overlapping peptide pools, measuring a final concentration of 1 μg mL^{-1} per peptide, were added to five wells as outlined in Table 1. These peptide pools were supplied by JPT Technologies (Berlin, Germany). Wells containing no peptide (NP), or 20 μg mL^{-1} of the mitogen phytohaemagglutinin (PHA), were used as negative and positive controls. The plate was incubated for an average of 20 h at 37°C in a CO_2-controlled incubator, spun by centrifugation, and then, plasma supernatant was collected and stored at −80°C before use.

T-cell cytokine analysis

A cytometric bead array (CBA) was used to quantify the production of T-cell cytokines: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-13, IL-17A, tumor necrosis factor (TNF) and IFN-γ. The CBA was performed using the BD Biosciences Flex-sets (Franklin Lakes, NJ). Samples were acquired using a BD LSRFortessa with FACSDiva software (BD Biosciences). Postacquisition cytokine analysis was performed using FCAP array (BD Biosciences) software. To generate heat maps of each cytokine, the total range of detection for each individual cytokine was normalised across all samples with the maximum value (in pg mL^{-1}) detected for each cytokine scaled as 1.0. The data are displayed on a colour (viridis) scale ranging from 0.0 (purple) to 1.0 (yellow).

PBMC immunophenotyping

Immune cell phenotyping to assess the frequency and absolute number of T-cell subsets, B-cell subsets, NK cell subsets and monocytes was performed on PBMC isolated from all participants. In a BD TruCount tube (BD Biosciences), 50 μL of PBMC was incubated with the following antibodies: anti-CD3 BV711, anti-CD4 AF700, anti-CD8 SB780, anti-CD16 PE, anti-CD19 PE-Cy5 and live/dead fixable near-IR dead cell stain (Life Technologies). Following incubation, cells were fixed with BD Cytofix (BD Biosciences) and acquired using a BD LSRFortessa with FACSDiva software. TruCount bead counts were used to provide cell counts prior to cryopreservation. Postacquisition analysis was performed using FlowJo version 10 software (FlowJo LLC, Ashland, Oregon).

Expansion of SARS-CoV-2-specific T cells

Peripheral blood mononuclear cells were harvested from peripheral blood within 24 h of collection. PBMCs were incubated with SARS-CoV-2 overlapping peptide pools (Table 1) and were grown in RPMI-1640 culture medium containing 1% penicillin-streptomycin (Gibco) and 10% foetal bovine serum, and supplemented with recombinant IL-2 every 2–3 days thereafter. On day 14, T cells were harvested and assessed for antigen specificity using an intracellular cytokine assay.

Intracellular cytokine assay

Cultured T cells were stimulated with individual SARS-CoV-2 antigen overlapping peptide pools and incubated for 4 h in the presence of GolgiPlug, GolgiStop and anti-CD107a-FITC (BD Biosciences). Following stimulation, cells were washed and stained with anti-CD8-PerCP-Cy5.5 (eBioscience) and anti-CD4-PE-Cy7 or anti-CD4-Pacific Blue (BD Biosciences) for 30 min before being fixed and permeabilised with BD Cytofix/Cytoperm solution (BD Biosciences). After 20 min of fixation, cells were washed in BD Perm/Wash buffer (BD Biosciences) and stained with anti-IFN-γ-Alexa Fluor700, anti-IL-2-PE and anti-TNF-APC (all from BD Biosciences) for a further 30 min. Finally, cells were washed again and acquired using a BD LSRFortessa with FACSDiva software. Postacquisition analysis was performed using FlowJo software (TreeStar). Cytokine detection levels identified in the no-peptide control condition were subtracted from the corresponding test conditions to account for nonspecific, spontaneous cytokine production.

Statistical analysis

GraphPad Prism 8.2.1 (San Diego, CA) was used to perform statistical analysis. Statistical comparisons between participant groups (unexposed and recovered) were made using unpaired Mann-Whitney *U* Wilcoxon rank-sum tests. Correlative analysis was performed using the Pearson correlation coefficient. Box plots were used to represent median (horizontal line), 25th and 75th percentiles (boxes) and minimum and maximum values (whiskers). $P < 0.05$ was considered statistically significant.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Katie Lineburg: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Supervision; Writing-

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original draft; Writing-review & editing. **Sriganesh Srihari:** Data curation; Visualization; Writing-review & editing. **Mohammed Altaf:** Data curation; Writing-review & editing. **Srividhya Swaminathan:** Data curation; Writing-review & editing. **Archana Panikkar:** Data curation; Methodology; Writing-review & editing. **Jyothy Raju:** Data curation; Methodology; Writing-review & editing. **Pauline Crooks:** Data curation; Methodology; Writing-review & editing. **George Ambalathingal:** Conceptualization; Methodology; Writing-review & editing. **Jose Paulo Martins:** Data curation; Writing-review & editing. **Katherine Matthews:** Conceptualization; Project administration; Writing-review & editing. **Michelle Anne Neller:** Conceptualization; Data curation; Project administration; Writing-review & editing. **Rajiv Khanna:** Conceptualization; Data curation; Formal analysis; Writing-review & editing. **Corey Smith:** Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Writing-original draft; Writing-review & editing.

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Supporting Information

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