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ARTICLE

Highly functional virus-specific cellular immune response in asymptomatic SARS-CoV-2 infection

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The efficacy of virus-specific T cells in clearing pathogens involves a fine balance between antiviral and inflammatory features. SARS-CoV-2–specific T cells in individuals who clear SARS-CoV-2 without symptoms could reveal nonpathological yet protective characteristics. We longitudinally studied SARS-CoV-2–specific T cells in a cohort of asymptomatic (*n* = 85) and symptomatic (*n* = 75) COVID-19 patients after seroconversion. We quantified T cells reactive to structural proteins (M, NP, and Spike) using ELISpot and cytokine secretion in whole blood. Frequencies of SARS-CoV-2–specific T cells were similar between asymptomatic and symptomatic individuals, but the former showed an increased IFN-γ and IL-2 production. This was associated with a proportional secretion of IL-10 and proinflammatory cytokines (IL-6, TNF-α, and IL-1β) only in asymptomatic infection, while a disproportionate secretion of inflammatory cytokines was triggered by SARS-CoV-2–specific T cell activation in symptomatic individuals. Thus, asymptomatic SARS-CoV-2–infected individuals are not characterized by weak antiviral immunity; on the contrary, they mount a highly functional virus-specific cellular immune response.

Introduction

Characterization of adaptive immunity mounted against SARS-CoV-2 is crucial for understanding its role in protection or pathogenesis. Antibodies and T cells act together to reduce the spread of virus within the host and to eradicate the pathogen from infected cells. However, the protective immune response can also trigger pathological processes characterized by localized or systemic inflammatory events. Inflammation and tissue damage can result from the direct lysis of infected cells by virus-specific antibodies and T cells, or from the release of inflammatory mediators produced by the infected cells and activated myeloid cells. These scenarios have been reported in the pathogenesis of COVID-19 (Vardhana and Wolchok, 2020). In more severe cases, systemic high levels of inflammatory cytokines (IL-1β and IL-6) and the presence of activated monocytes in the circulatory compartment (Kuri-Cervantes et al., 2020; Silvin et al., 2020) and in the lung (Nienhold et al., 2020) coexist with virus-specific antibodies and T cells (Laing et al., 2020). Thus, the question of whether virus-specific antibodies or T cells are preferentially mediating protection or damage remains open. Antibodies against SARS-CoV-2 Spike (S) protein have protective ability in vitro, but their titers in COVID-19 patients have been reported to be positively correlated with disease severity (Long et al., 2020a; Cervia et al., 2020 *Preprint*). Similarly, a direct relation with disease severity has been reported in studies of SARS-CoV-2–specific T cell frequencies in COVID-19 patients. A broader and quantitatively more robust SARS-CoV-2–specific T cell response has been demonstrated in convalescent cases of severe COVID-19 in comparison to mild cases (Weiskopf et al., 2020). However, the positive relation between SARS-CoV-2–specific T cell quantity and disease severity was not confirmed in recent studies measuring SARS-CoV-2–specific T cells in the early phases of COVID-19. Early induction and more

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robust SARS-CoV-2–specific CD4 and CD8 T cell response has been associated with milder disease development (Rydyznski Moderbacher et al., 2020; Tan et al., 2021a). In addition, a protective role of T cells has been demonstrated in animal models of coronavirus infections, in which virus-specific T cells clear the virus with limited lung pathology (Zhao et al., 2010; 2016).

In this work, we expand our investigation into virus-specific T cells in SARS-CoV-2 infection by studying asymptomatic SARS-CoV-2–infected individuals selected from a cohort of male foreign workers living in a Singapore dormitory (Tan et al., 2021b). Asymptomatic individuals constitute a variable but often large proportion of infected individuals (Lavezzo et al., 2020), and they should hold the key to understanding the immune response capable of controlling the virus without triggering pathological processes. However, current knowledge of their antiviral immunity is limited. SARS-CoV-2–specific antibodies (Long et al., 2020b) and T cells are induced in asymptomatic individuals (Sekine et al., 2020), but the reported lower magnitude of antibodies (Long et al., 2020b) and T cell responses (Sekine et al., 2020; Reynolds et al., 2020) has been interpreted as a sign that asymptomatic individuals are mounting a normal innate immunity (Zhao et al., 2020 *Preprint*), but a weak adaptive antiviral immunity (Long et al., 2020b). Because antibody titers and frequency of SARS-CoV-2–specific T cells in the blood can undergo dynamic changes after viral clearance (Tan et al., 2021a), we reasoned that a proper quantitative comparison between SARS-CoV-2–specific T cells of symptomatic and asymptomatic individuals should be performed at similar time points after infection. The absence of symptoms makes such evaluation difficult. We therefore selected 85 asymptomatic individuals, from a cohort of male workers living in a densely populated dormitory with active spread of SARS-CoV-2 infection (Fig. 1 A), who, based on their kinetics of appearance and disappearance of antibodies against nucleoprotein (NP) and S, were likely exposed to SARS-CoV-2 at different time points. In these individuals, we measured directly ex vivo the quantity of IFN-γ–producing T cells reactive to peptide pools covering different structural proteins (M, NP, and S) and compared it to the T cell magnitude detected in symptomatic patients who were infected with SARS-CoV-2 at similar time points.

We also evaluated, in both symptomatic and asymptomatic individuals, the functional profile of SARS-CoV-2–specific T cells. We designed a test to quantify secretion of IL-2, IFN-γ, IL-4, IL-6, IL-12p70, TNF-α, IL-1β, and IL-10 directly in whole blood after T cell activation with peptide pools covering the different SARS-CoV-2 proteins. This experimental system not only measures the quantity of T cell cytokines (IL-2, IL-4, IFN-γ) directly secreted by SARS-CoV-2–specific T cells, but also can provide a direct evaluation of the T cells' ability to activate inflammatory or regulatory pathways in other circulating immune cells. Our results provide experimental evidence that asymptomatic individuals mount a virus-specific T cell response that is indistinguishable from symptomatic patients in magnitude, but that is functionally more fit, being characterized by an augmented secretion of T helper 1 cell (Th1) cytokines (IFN-γ and IL-2) associated with a proportionate and coordinated production of proinflammatory (IL-6, TNF-α, IL-1β) and antiinflammatory (IL-10) cytokines. The implications of these findings for pathology and vaccine designs are discussed.

Results

Longitudinal serology in asymptomatic SARS-CoV-2 infection

Beginning in April 2020, Singapore experienced large outbreaks of SARS-CoV-2 infections among migrant workers residing in densely populated dormitories (Fig. 1 A; Tan et al., 2021b). To select asymptomatic individuals who were exposed to SARS-CoV-2 at different time points, we followed up on 478 residents of a SARS-CoV-2–affected dormitory who donated blood at recruitment and 2 and 6 wk later for serological testing of anti-NP IgG and neutralizing antibodies (nAbs; Fig. 1 B). Note that at the time of recruitment (June 2020), dormitory residents who were at higher risk of developing COVID-19 (older than 65 yr and with the presence of pathologies such as diabetes and hypertension) were already moved to other facilities. At recruitment, 131 of the 478 participants (27.4%) were seropositive by either assay, with 6 (4.6%) reporting COVID-19–compatible symptoms in the preceding 4 wk (Fig. 2 A). Over the 6-wk follow up, 171 of 347 (49.3%) initially seronegative individuals seroconverted by either assay, demonstrating ongoing exposure to SARS-CoV-2 infection. Only 15 individuals (5.5%) who completed the 6-wk study period displayed symptoms, generally mild, during the follow-up phase (temperature and oxygen saturation were monitored twice a day at a medical post within the dormitory; see Materials and methods for more detailed information). The majority of seropositive individuals (281/302; 93%) were asymptomatic. Individuals with symptoms were excluded from this study (Fig. 1 B).

The kinetics of anti-NP IgG and surrogate virus receptor-binding domain nAbs (sVNT-nAbs; Tan et al., 2020) in the asymptomatic study participants is shown in Fig. 2. Overall, there was a strong correlation between titers of anti-NP IgG and percentage of inhibition by sVNT-nAb (Fig. 2 B). However, at all time points, a higher percentage of asymptomatic donors was positive for sVNT-nAb (30.9, 47.0, and 65.4% at recruitment and weeks 2 and 6, respectively) compared with anti-NP IgG (24.4, 34.6, and 47.5%; Fig. 2 A). Among the 106 individuals with positive anti-NP IgG levels at recruitment, 27 (25.5%) lost them during the 6 wk (Fig. 2, B and C). In contrast, of the 134 participants who were seropositive for sVNT-nAb at baseline, only 12 (9.0%) became negative over the same time period (Fig. S1).

Virus-specific T cell quantification in asymptomatic SARS-CoV-2–exposed donors

To investigate SARS-CoV-2–specific T cells at the 6-wk time point, which corresponds to 3 mo after the first confirmed case in this dormitory, we selected, among the 434 asymptomatic study participants, 85 individuals with distinct antibody profiles (Fig. 2, C and D). 57 were anti-NP IgG positive at recruitment. Of these, 33 individuals remained persistently anti-NP IgG positive during the 6-wk follow up, while in 24, the antibody decreased below the limit of assay detection. We then selected 28 individuals who were seronegative at recruitment. Of these, 15 became anti-NP IgG positive, while 13 were persistently negative.

We first analyzed the frequency of cells reactive to three structural proteins of SARS-CoV-2 by ex vivo IFN-γ-ELISpot

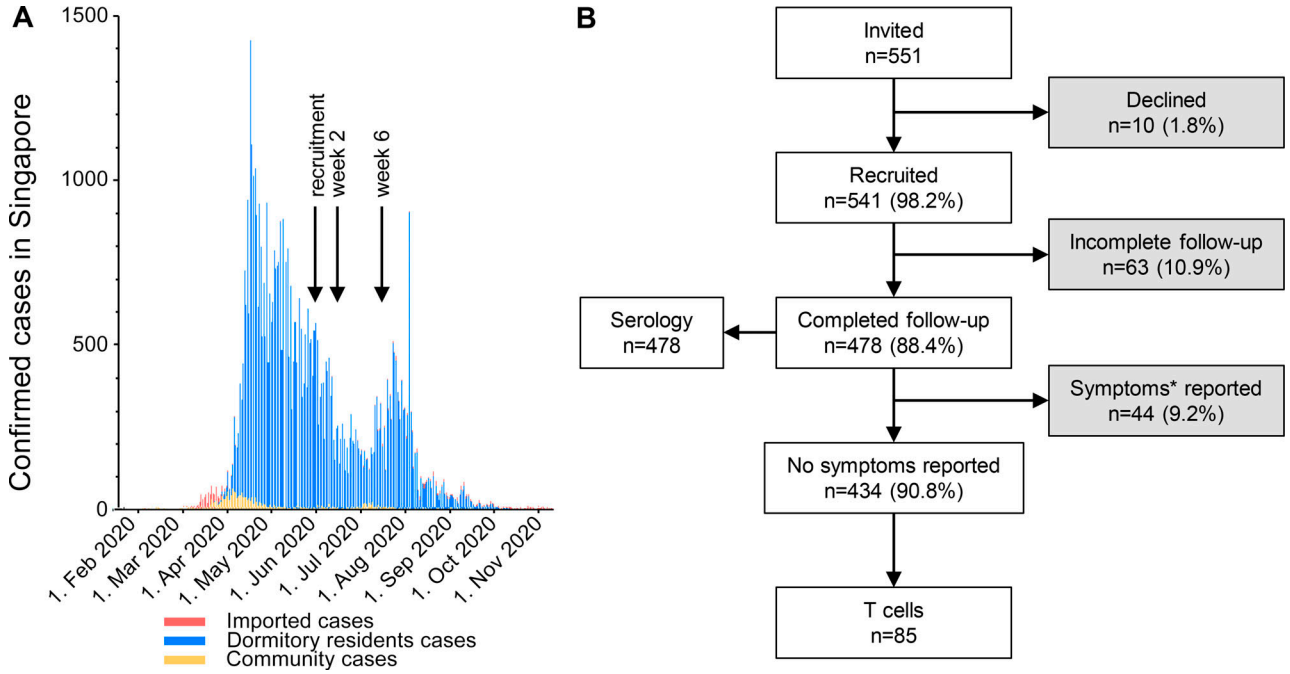

Figure 1. **Enrollment of SARS-CoV–exposed donors. (A)** Confirmed cases of SARS-CoV-2 infection in Singapore, divided into imported cases (red), cases among dormitory residents (blue), and all other community cases (yellow). Black arrows indicate the dates when blood samples were taken for the study. **(B)** Diagram showing the number of participants that were invited and recruited into the study. Participants who completed blood donations at recruitment and at 2- and 6-wk follow-up were tested for serology of anti-NP IgG (Abbott) and sVNT-nAb (GenScript). An additional blood sample was taken at week 6 from 85 asymptomatic participants with distinct serological profiles for the analysis of SARS-CoV-2–specific T cell responses. *, Symptoms: any of fever, cough, runny nose, sore throat, shortness of breath, fatigue, muscle ache, diarrhea, or anosmia.

assays (Fig. 3 A). We designed pools of 15-mer peptides covering the whole NP and M proteins and a selected peptide pool of 15-mers covering the most T cell immunogenic regions of S (Fig. 3 A and Fig. S2). As previously shown (Le Bert et al., 2020), we demonstrate by intracellular cytokine staining that IFN-γ was produced by CD4 and CD8 T cells reactive to peptide stimulation, both directly ex vivo and after expansion of short-term T cell lines restimulated with individual peptides (Fig. S3).

SARS-CoV-2–specific T cells are present in all asymptomatic seropositive individuals

Cells reactive to SARS-CoV-2 peptide pools were found in all anti-NP IgG–positive individuals regardless of the duration of antibody persistence (Fig. 3 B). Moreover, almost all anti-NP IgG–positive individuals had SARS-CoV-2–specific T cells reactive to at least three pools simultaneously, except for 2 individuals (of 24) who had lost anti-NP IgG (Fig. 3 B). In all groups with different serology profiles, the M peptide pool triggered the highest median frequency of specific T cells in comparison to the S pool and the two NP pools. Among the 13 persistently anti-NP IgG–negative participants, SARS-CoV-2–specific T cells were detected in four individuals (Fig. 3 B, right).

Frequency, multispecificity, and kinetics of SARS-CoV-2–specific cellular immunity in symptomatic and asymptomatic infection

Next, we compared the SARS-CoV-2–specific T cell response of the asymptomatic cohort with the response in a cohort of hospitalized COVID-19 patients with mild to severe symptoms (Table S1). Within the first 3 mo after viral exposure, the pattern of virus-specific T cells reactive to M, NP, and S peptides was similar between symptomatic COVID-19 patients and asymptomatically infected individuals, and both groups showed a higher frequency of M pool–reactive T cells compared with other pools (Fig. 4 A). Importantly, nearly all COVID-19 patients and asymptomatic individuals with serological evidence of infection had T cells recognizing at least three peptide pools (Fig. 4 B).

In contrast, the pattern of cross-reactive SARS-CoV-2–specific T cells in archived samples from SARS-CoV-2–unexposed individuals was different. The overall frequency was lower (10–100 spots per 10^6 peripheral blood mononuclear cells [PBMCs]), and where SARS-CoV-2 peptide–reactive T cells were present (20 of 51), they reacted mostly to a single peptide pool (Fig. 4, A and B). Only 1 of 51 unexposed individuals tested had T cells specific for three peptide pools.

To investigate whether virus-specific T cells undergo clonal reduction over time, we compared their strength and peptide pool specificity in individuals who seroconverted to anti-NP IgG positive within the previous 4 wk, individuals who seroconverted >6 wk previously, and in initially seropositive individuals who became anti-NP IgG negative. The frequency of SARS-CoV-2–specific T cells was higher in recent seroconverters (anti-NP IgG^+ <4 wk) than in those infected at an earlier time point (Fig. 4, C and D). Among those who became anti-NP IgG negative, the frequency of SARS-CoV-2–specific T cells was lower.

A decline in the frequency of SARS-CoV-2–specific T cells was also apparent from serial cross-sectional samples of COVID-19

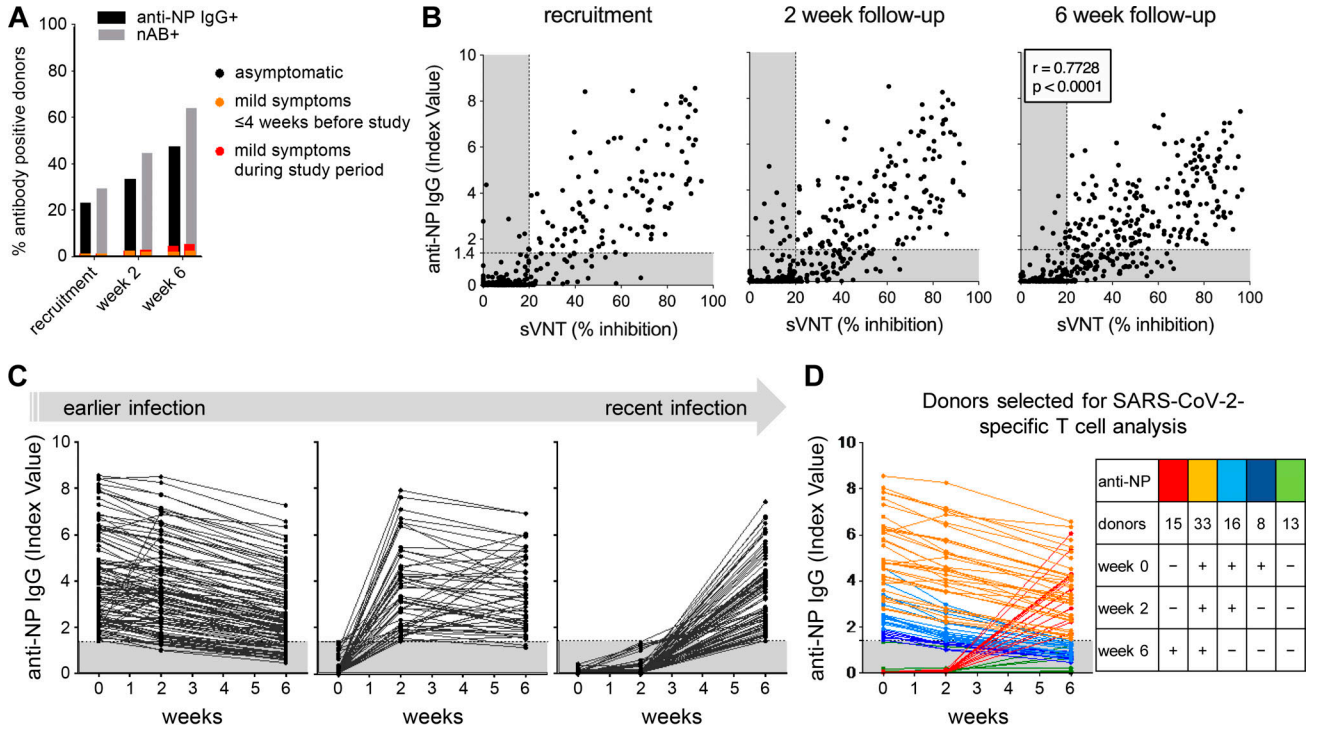

Figure 2. **SARS-CoV-2–specific antibody profile shows increasing infection prevalence among dormitory residents during the study period. (A)** Dormitory residents (n = 478) were tested longitudinally for serology. The percentage of donors positive for anti-NP IgG (black) and nAbs (gray) at recruitment and after 2 and 6 wk is shown; antibody-positive donors who experienced COVID-19 symptoms before (yellow) and during (red) the study are highlighted. **(B)** Dot plots show longitudinally the quantity of anti-NP IgG antibodies (y axis) and the percentage inhibition by virus neutralization antibodies (sVNT; x axis) in the serum of asymptomatic study participants (n = 434) at recruitment (left), after 2 wk (middle), and after 6 wk (right). The gray area marks the limit of assay detection. Spearman correlation. **(C)** Longitudinal anti-NP IgG levels of asymptomatic donors who were seropositive at recruitment (n = 106, left), who seroconverted at week 2 (n = 52, middle), and who seroconverted by week 6 (n = 77, right). **(D)** Anti-NP IgG serological profile of donors selected for SARS-CoV-2–specific T cell analysis at the 6-wk time point (n = 85). Donors with distinct antibody profiles are shown in different colors and are summarized in the table.

patients from the acute phase over a period of 7 mo after viral clearance (Fig. 4, E and F). Over time, some patients lost T cells specific to individual peptide pools, but M-specific T cells were maintained in all symptomatic and asymptomatic donors tested (Fig. 4, D and F).

Because there was a progressive reduction of SARS-CoV-2–specific T cells over time in all exposed individuals, we compared the kinetics of their decline between the asymptomatic and symptomatic cohorts. Among the recent seroconverters (<4 wk), the frequency of SARS-CoV-2–specific T cells was comparable with that in symptomatic COVID-19 patients tested during infection and within 1 mo after viral clearance (Fig. 4 G, left). However, the asymptomatic donors that had been exposed to SARS-CoV-2 ∼2–3 mo earlier (anti-NP$^+$ >6 wk earlier, first confirmed case 3 mo earlier) showed a reduced frequency of SARS-CoV-2–specific T cells compared with patients who recovered from symptomatic infection 2–3 mo earlier (Fig. 4 G, right). Thus, the total frequency of SARS-CoV-2–specific T cells declines faster after asymptomatic infection, but the result was significant only for M-reactive T cells (Fig. 4 H).

The pattern of cytokines induced by SARS-CoV-2–specific T cell activation in symptomatic and asymptomatic infection

To functionally define the antiviral cellular immune response present in asymptomatic and symptomatic SARS-CoV-2–infected individuals, we designed an assay in which the level of Th1 (IFN-γ, IL-2, and TNF-α), Th2 (IL-4), proinflammatory (IL-6, IL-1β, and IL-12p70), and regulatory (IL-10) cytokines was tested directly in whole blood after overnight stimulation with SARS-CoV-2 peptide pools (Fig. 5 A). Cytokines secreted during overnight stimulation can be produced either by the activation of virus-specific T cells directly, or indirectly by other cell types that respond to the activation of antigen-specific T cells (Fig. S4).

We used Uniform Manifold Approximation and Projection (UMAP) for unsupervised dimension reduction and clustering (Becht et al., 2018) of the secretomes of all peptide-stimulated samples after subtraction of cytokine levels present in corresponding DMSO controls (Fig. 5 B). This showed that secretion of cytokines classically produced by T cells, IFN-γ and IL-2, was overlapping. Moreover, IL-6 and IL-1β were cosecreted, as well as TNF-α and IL-10. IL-12p70 and IL-4 were undetectable in most samples. While stimulation of blood from unexposed donors did not induce any secretion of cytokines, the secretomes of SARS-CoV-2–infected donors differed between symptomatic and asymptomatic infection (Fig. 5 C). The majority of samples from asymptomatic donors cosecreted high levels of IFN-γ and IL-2, while the samples of symptomatic donors produced low levels of IFN-γ and IL-2. A subset of samples from symptomatic donors, enriched for those with severe disease, clustered with high levels of IL-6, TNF-α, IL-1β, and IL-10 secretion (Fig. 5 C).

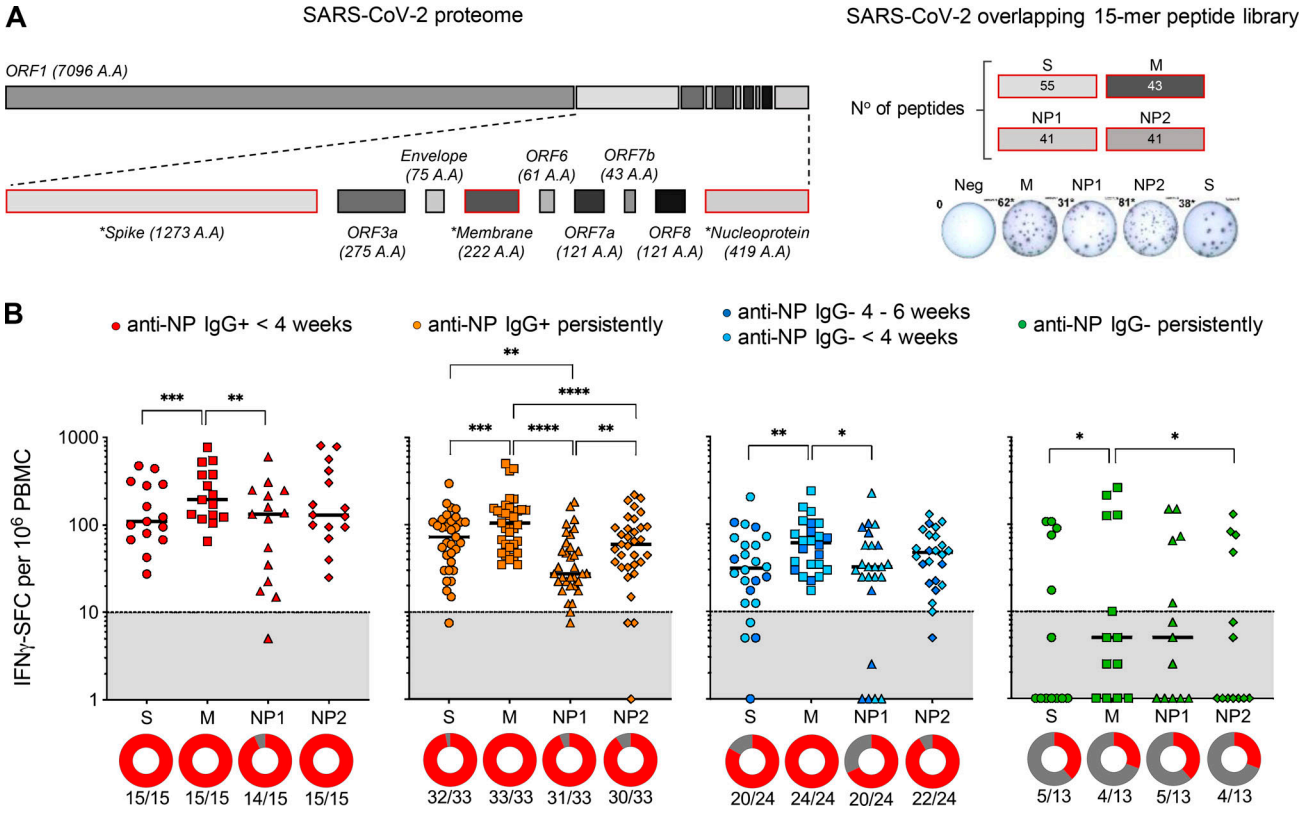

Figure 3. **Frequency of T cells specific for different SARS-CoV-2 proteins in asymptomatic donors with distinct serological profiles. (A)** SARS-CoV-2 proteome organization; analyzed proteins have a red outline and are marked by *. 15-mer peptides overlapping by 10 amino acids were split into pools covering NP1, NP2, and M and selected 15-mers covering the more T cell immunogenic regions of S. T cell reactivity was tested by ex vivo IFN-γ-ELISpot. **(B)** The frequency of IFN-γ SFCs reactive to the individual peptide pools is shown for the asymptomatic donors with distinct serological profiles (line = median). IFN-γ-SFC ≥10/10^6 PBMCs were considered positive (gray area is below limit of detection). Circles below represent the percentage of a positive response (red) to the individual peptide pools, and numbers of individuals tested are stated below the circles. Wilcoxon matched-pairs signed rank test. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

For the individuals with asymptomatic SARS-CoV-2 infection, we used the appearance, persistence, and decline of anti-NP IgG levels as a proxy for time since infection. This distinguishes patients with recent infection (<4 wk anti-NP IgG^+) from those who were infected at earlier time points and allowed us to compare them with symptomatic patients that cleared the virus within the last 4 wk. Strikingly, the cytokine profile between these two recently infected symptomatic and asymptomatic groups showed a nearly complete separation by UMAP (Fig. 5, D–F, left). Samples from recently infected asymptomatic individuals all cosecreted higher levels of IFN-γ (median, 292 pg/ml) and IL-2 (median, 251 pg/ml) than samples from recently infected symptomatic donors, with both mild (IFN-γ, 25 pg/ml; IL-2, 31 pg/ml) and severe (IFN-γ, 100 pg/ml; IL-2, 123 pg/ml) COVID-19. Moreover, they secreted higher levels of TNF-α (51 vs. 9 pg/ml), IL-6 (176 vs. 17 pg/ml), IL-1β (16 vs. 3 pg/ml), and IL-10 (9 vs. 3 pg/ml) than symptomatic patients with mild disease. In contrast, samples of patients who recovered after severe disease cosecreted very high levels of TNF-α (1,160 pg/ml), IL-6 (2,652 pg/ml), and IL-1β (1,530 pg/ml; Fig. 5 F). This difference in the profile of the secretomes was maintained when symptomatic and asymptomatic donors of similar age were compared (Fig. S5, A and B). Furthermore, deconvolution of the secretion profiles in response to the individual peptide pools covering the different SARS-CoV-2 proteins showed a significantly higher cytokine release in samples of asymptomatic individuals with all but the M pool (Fig. S5 C).

Next, we correlated the cytokine levels secreted by peptide stimulation of whole blood with the frequency of IFN-γ spot-forming cells (SFCs) detected by ELISpot assay in response to the same peptide pools (Fig. 6). The secretion levels of IFN-γ and IL-2 induced by peptide-stimulated samples correlated strongly (P < 0.0001) with the number of IFN-γ spots in both asymptomatic and symptomatic individuals, but higher levels of both cytokines in relation to the number of spots were detected in asymptomatic individuals.

In addition, levels of secreted proinflammatory (IL-6, TNF-α, and IL-1β) and regulatory (IL-10) cytokines correlated strongly with the number of IFN-γ spots in asymptomatic individuals (Fig. 6 A). However, in some symptomatic patients, high levels of IL-6, TNF-α, IL-1β, and IL-10 were produced but were uncorrelated with the number of spots detected in the ELISpot assays (Fig. 6 B).

Discussion

Despite their ability to efficiently control the infection, asymptomatic individuals who clear SARS-CoV-2 have been hypothesized

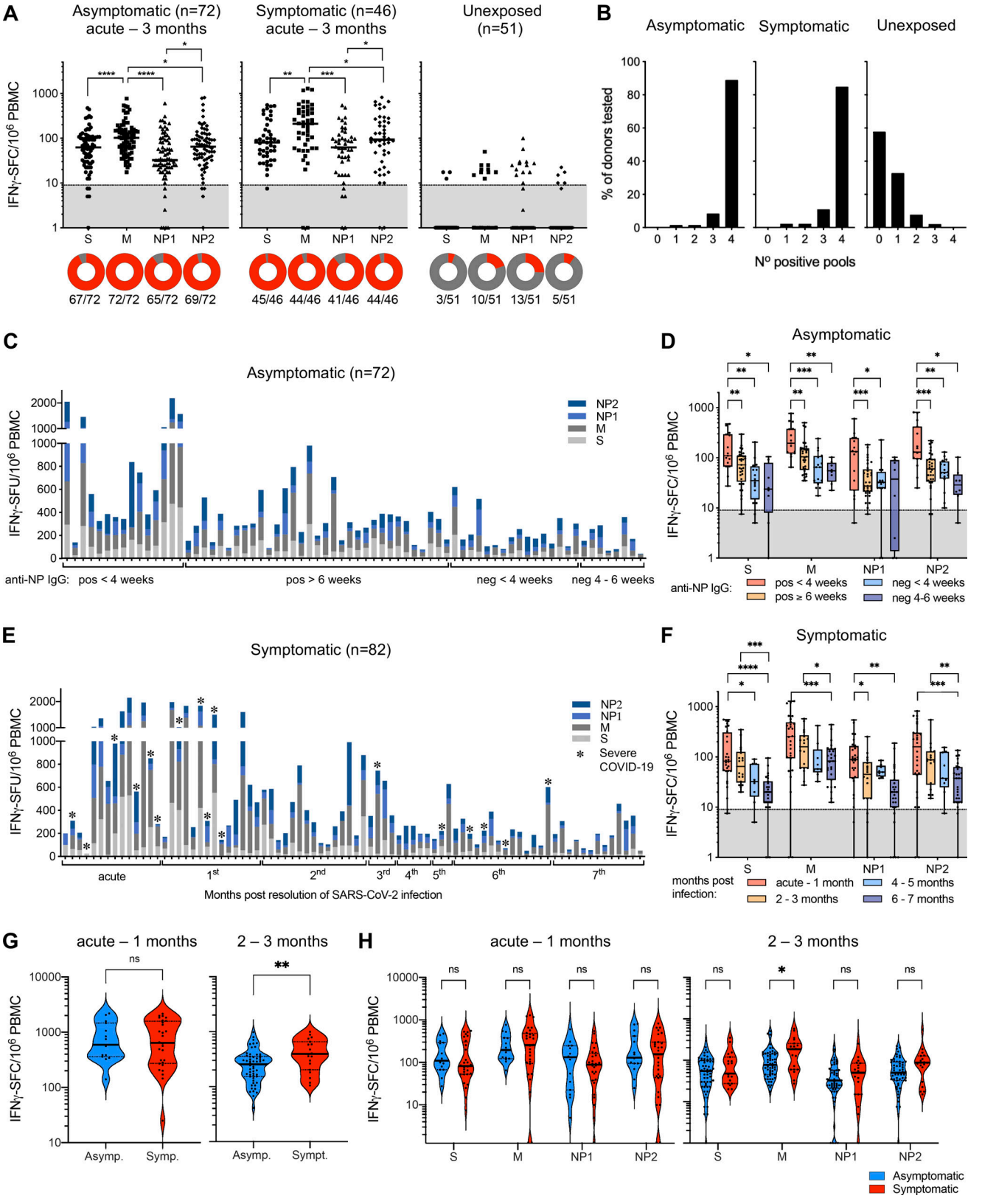

Figure 4. **Dynamics and hierarchy of SARS-CoV-2–specific T cells in asymptomatic and symptomatic infection. (A)** The frequency of IFN-γ SFCs reactive to the individual peptide pools is shown for asymptomatic SARS-CoV-2–infected donors who were serologically positive for anti-NP IgG at at least one time point during the study period (n = 72; left), for COVID-19 patients from acute to 3 mo after infection (n = 45; middle), and archived unexposed controls (n = 51; right; line = median). Circles below represent the frequency of a positive (IFN-γ-SFC ≥10/10^6 PBMCs) response (red) to the individual peptide pools. Repeated-measures one-way ANOVA with Greenhouse–Geisser correction, followed by Holm–Sidak's multiple comparisons test. **(B)** Bar graphs show the

percentage of donors reacting to the number of peptide pools tested. **(C)** Frequency of IFN-γ SFCs reactive to the individual peptide pools is shown for each donor of the asymptomatic cohort (n = 72). Donors are organized according to their serological status of anti-NP IgG antibodies. **(D)** The frequency of IFN-γ SFCs reactive to the individual peptide pools is shown in asymptomatic donors grouped by anti-NP IgG status (anti-NP IgG positive <4 wk: n = 15; anti-NP IgG positive >6 wk: n = 33; anti-NP IgG negative <4 wk: n = 16; anti-NP IgG negative 4–6 wk: n = 8). Unpaired t test. **(E)** Frequency of IFN-γ SFCs reactive to the individual peptide pools is shown for symptomatic COVID-19 patients organized by months after clearance of SARS-CoV-2 infection (n = 82). **(F)** The frequency of IFN-γ SFCs reactive to the individual peptide pools is shown in COVID-19 patients grouped by months after infection (acute to 1 mo: n = 28; 2–3 mo: n = 18; 4–5 mo: n = 9; 6–7 mo: n = 27). Unpaired t test. **(G and H)** Frequency of SARS-CoV-2 peptide–reactive cells in individuals with asymptomatic (blue) and symptomatic (red) infection during the acute phase until 1 mo after recovery (left; asymptomatic: n = 15; symptomatic: n = 28) and in convalescents 2–3 mo after infection (right; asymptomatic: n = 57; symptomatic: n = 19; G) and deconvoluted to the individual peptide pools (H). Mann–Whitney tests. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

to mount a reduced antiviral adaptive immune response (Long et al., 2020b). This hypothesis is supported mainly by measurement of SARS-CoV-2–specific antibodies (Atyeo et al., 2020; Long et al., 2020b; Henss et al., 2021) and B cell quantity (Woodruff et al., 2020).

Our study shows that the ability to mount a significant virus-specific T cell response is not necessarily associated with symptom severity. The overall magnitude of T cell responses against different structural proteins was similar in both asymptomatic individuals and COVID-19 patients. Moreover, the T cells induced by an asymptomatic infection appear to secrete higher quantities of IFN-γ and IL-2 and trigger a more coordinated production of proinflammatory and regulatory cytokines than T cells of symptomatic COVID-19 patients. Overall, we conclude that the asymptomatic SARS-CoV-2–infected individuals studied here raise an efficient and balanced antiviral cellular immunity that protects the host without causing any apparent pathology.

One of the principal characteristics of our study was the ability to select, through a 6-wk longitudinal serological study, asymptomatic individuals in whom the time of viral exposure could be reasonably estimated. The first confirmed SARS-CoV-2 infection in this dormitory was identified in mid-April 2020, so asymptomatic, seropositive individuals in our cohort were unlikely to have been infected >3 mo before sampling (performed in mid-July 2020; Fig. 1 A). More importantly, by evaluating the period of SARS-CoV-2 antibody seroconversion, we could separate individuals who were likely infected >6 wk before sampling from those who were likely infected within the last 4 wk.

The ability to estimate the time of infection allowed us to compare the magnitude and function of SARS-CoV-2–specific T cells present in asymptomatically infected individuals with those of COVID-19 patients at similar time points during and after infection. We demonstrated that all asymptomatic individuals capable of producing anti-SARS-CoV-2 antibodies also mount a T cell response, measured with IFN-γ ELISpot, that simultaneously targets different structural proteins of SARS-CoV-2 (NP, M, and S). In addition, we observed that, similarly to COVID-19–recovered patients, SARS-CoV-2–specific T cell frequency in asymptomatic individuals declined progressively over time. Surprisingly, more recently infected asymptomatic individuals possessed a SARS-CoV-2–specific T cell frequency of a magnitude indistinguishable from the one detected in COVID-19 patients (mild, moderate, and severe) tested during acute infection (SARS-CoV-2 PCR positive) or within 1 mo after viral clearance. This finding, even though analyzed with a single assay that might miss some fraction of SARS-CoV-2–specific T cells incapable of producing IFN-γ, further supports the accumulating evidence that the quantity of SARS-CoV-2–specific T cells is not proportional to disease severity during the early phase of infection (Rydyznski Moderbacher et al., 2020; Tan et al., 2021a). This is in contrast to the positive correlation between SARS-CoV-2–specific T cell frequency and presence/severity of symptoms reported by others. In those studies, however, SARS-CoV-2–specific T cell responses were evaluated several months after recovery (Peng et al., 2020; Reynolds et al., 2020; Sekine et al., 2020; Zuo et al., 2020 *Preprint*).

We further show that despite a similar magnitude of virus-specific T cells induced in asymptomatic and symptomatic infection, SARS-CoV-2–specific T cells seem to decline faster in asymptomatic individuals. SARS-CoV-2–specific T cell frequency detected 2–3 mo after asymptomatic infection was lower than that detected in COVID-19 patients at similar time after infection. A more extended study of the persistence of SARS-CoV-2–specific T cells in our asymptomatic cohort is needed to confirm such a trend. Nevertheless, at this moment our data suggest that the recently reported higher frequency of SARS-CoV-2–specific T cells in COVID-19–recovered patients in comparison to asymptomatic individuals 6 mo after recovery might not be caused by a higher set point present during the early phases of infection as suggested (Zuo et al., 2020 *Preprint*). An alternative explanation could be that SARS-CoV-2–specific T cells persist longer at a higher frequency in COVID-19–recovered patients, since viral antigen might persist more in symptomatic patients who usually have higher quantity of viral replication (Wang et al., 2020). The recent observation, that SARS-CoV-2–specific B cells progressively evolved after COVID-19 recovery due to the presence of SARS-CoV-2 antigen in the small bowel (Gaebler et al., 2021), makes such hypothesis even more plausible.

Finally, by analyzing the quantity of cytokines secreted in whole blood after pulsing with different SARS-CoV-2 peptide pools and correlating this quantity with the number of spots detected in our ELISpot analysis, we also show that asymptomatic SARS-CoV-2–infected individuals mount an efficient antiviral cellular immunity. We used this relatively simple approach for several reasons. In our study, we wanted to prioritize a direct ex vivo analysis of the T cell response in fresh blood rather than frozen PBMC, since freezing and thawing can reduce the detection of CD4 T cell responses (Ford et al., 2017) that represent the majority of virus-specific T cells in SARS-CoV-2

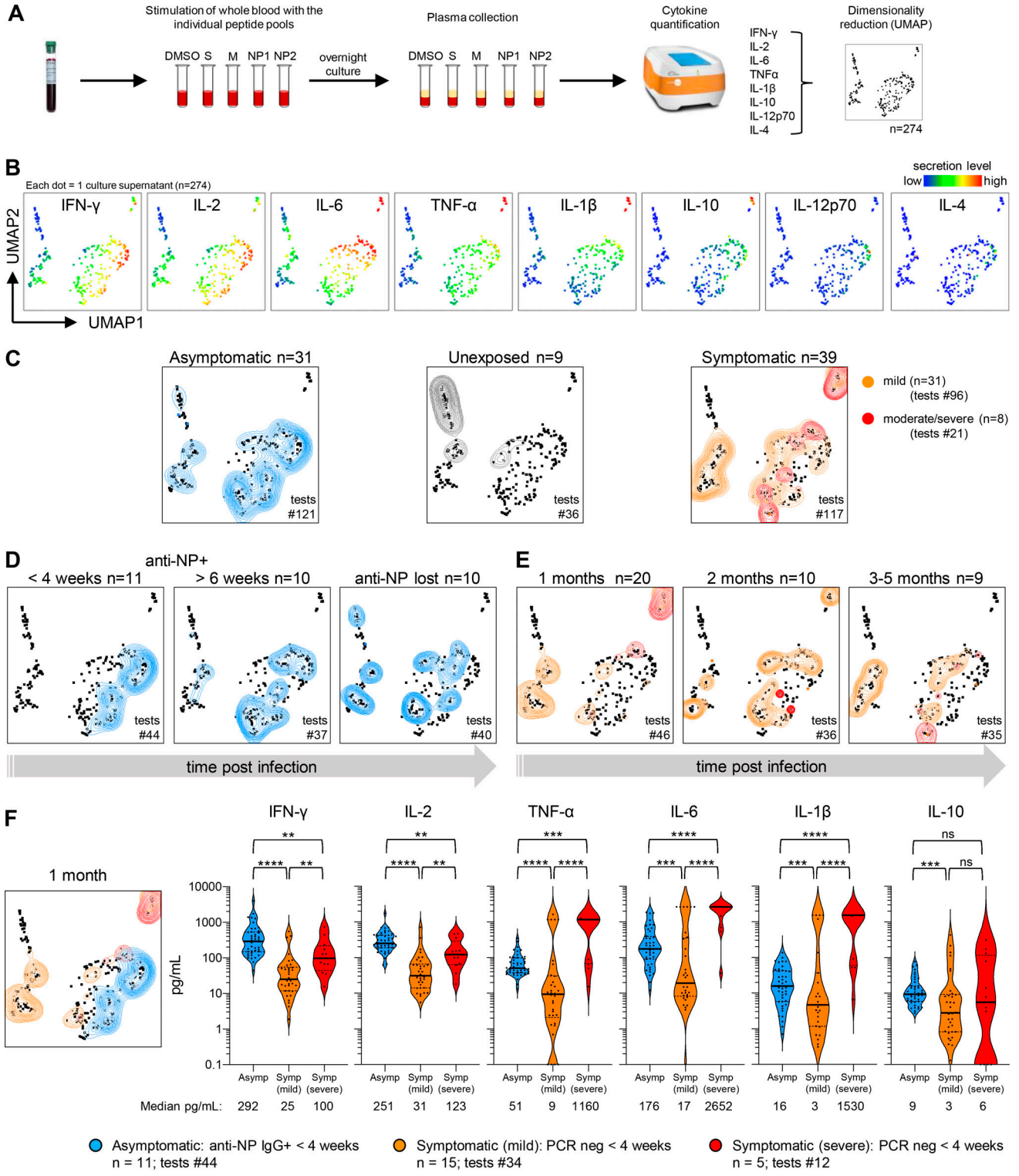

Figure 5. **Cytokine secretion profile of whole-blood cultures from asymptomatic and symptomatic convalescents stimulated with SARS-CoV-2 peptide pools. (A)** Schematic of whole-blood stimulation with SARS-CoV-2 peptide pools overnight and analysis of the cytokine secretion profile (after DMSO control subtraction) using unsupervised clustering algorithm UMAP. **(B)** UMAP plots generated with all analyzed blood culture supernatants (*n* = 274) with indicated cytokine secretion heatmaps. **(C)** Concatenated cytokine secretion profiles of peptide-pool stimulated whole blood from asymptomatic SARS-CoV-2–infected (left; blue), SARS-CoV-2–unexposed (middle; gray), and SARS-CoV-2 infected with mild (right; orange) and moderate to severe (right; red) symptoms overlaid on the global UMAP plot of all analyzed samples (black dots; each dot corresponds to one culture supernatant). Number of individuals tested are indicated above the quadrant (n); each individual was usually tested with four different peptide pools, and total tests performed are indicated within each quadrant (#). **(D)** The cytokine secretion profile of the samples from the asymptomatic cohort separated by donors who seroconverted to anti-NP IgG$^+$ <4 wk before (left), who were persistently seropositive (middle), and those who lost anti-NP IgG during the 6-wk study period (right). **(E)** The cytokine secretion

profile of the samples from the symptomatic cohort separated by donors who were in the first month (left), second month (middle), and third to fifth months (right) after SARS-CoV-2 infection. **(F)** The amount of indicated cytokines secreted upon whole blood stimulation with the peptide pools is compared between donors who had a recent (<4 wk ago) asymptomatic (blue; *n* = 11; tests # = 44) or mild (orange; *n* = 15; tests # = 34) or moderate to severe (red; *n* = 5; tests # = 12) symptomatic SARS-CoV-2 infection. Line = median concentration. Mann–Whitney tests. **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

infection (Grifoni et al., 2020a; Le Bert et al., 2020). We also wanted to analyze the global impact that T cell activation might have on other immune cells, particularly of the myeloid compartment, which is known to contribute substantially to the inflammatory reactions associated with SARS-CoV-2 infection (Silvin et al., 2020; Lee et al., 2020; Lucas et al., 2020). Clearly, this whole-blood cytokine assay has limitations, since it does not enable distinction of the cellular source of the different cytokines. However, it provides a holistic snapshot of virus-specific cellular immunity and can be performed on a large group of asymptomatic individuals tested in a limited time. The results obtained with unsupervised clustering were extremely clear in describing distinct cytokine secretion patterns in symptomatic versus asymptomatic individuals and revealed possible features of SARS-CoV-2–specific T cell responses that are worthy of more detailed characterization in future studies.

We observed a high secretion of TNF-α, IL-6, IL-1β, and IL-10 exclusively in the blood of a subset of patients who recently recovered from COVID-19, especially in patients with severe COVID-19. The high secretion of proinflammatory cytokines, however, was proportional neither to the quantity of IFN-γ and IL-2 detected in the same assay nor to the number of IFN-γ spots detected in the same patients analyzed in parallel with ELISpot. These findings seem to confirm that myeloid cells present in patients who recovered from symptomatic COVID-19 disease have been reprogrammed to promote inflammatory signals and are hyperresponsive to IFNs (Lee et al., 2020).

Secretion of the proinflammatory cytokines IL-6, TNF-α, and IL-1β was also detected in peptide-stimulated blood of asymptomatic individuals. Yet their quantity was directly proportional to the quantity of T cell cytokines (IL-2, IFN-γ). Surprisingly, the quantity of IL-2 and IFN-γ was much higher in asymptomatic individuals than in COVID-19–recovered patients. Moreover, the close correlation of IFN-γ spots and cytokine production detected in the same experiments strongly suggests that T cells of asymptomatic individuals are endowed with a higher IFN-γ and IL-2 production capacity than the T cells of COVID-19–recovered patients.

To our knowledge, these are the first data to show, through direct comparison, that SARS-CoV-2–specific cellular immune responses are more efficient in asymptomatic than symptomatic individuals. Previous results that support our findings: a low IFN-γ production of total (De Biasi et al., 2020) and SARS-CoV-2–specific (Rha et al., 2021) T cells has been reported in COVID-19 patients, while conversely, a high quantity of IFN-γ RNA was found in CD4 T cells of asymptomatic individuals (Zhao et al., 2020 *Preprint*).

Finally, we detected production of IL-10 proportional to T cell cytokines (IL-2 and IFN-γ) and to the numbers of spots activated by similar SARS-CoV-2 peptide activation only in asymptomatic

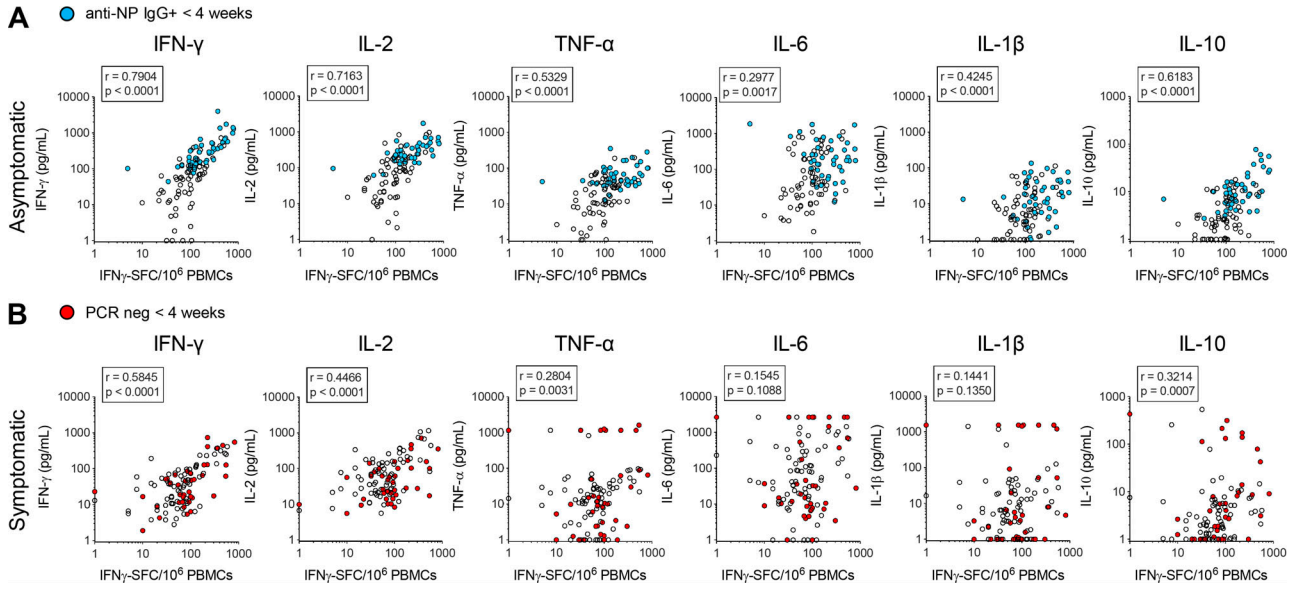

Figure 6. **Frequency and function of SARS-CoV-2 peptide pool–reactive cells. (A)** The frequency of IFN-γ SFCs reactive to the peptide pools analyzed by ELISpot assay (x axis) is plotted against the amount of the cytokines secreted following whole-blood stimulation with the identical peptide pools (y axis) of the asymptomatic cohort (*n* = 28; tests # = 109). Samples from donors with recent SARS-CoV-2 infection (<4 wk anti-NP IgG+) are highlighted in blue. Spearman correlation. **(B)** The frequency of IFN-γ–secreting cells (SFC) reactive to the peptide pools analyzed by ELISpot assay (x axis) is plotted against the amount of the cytokines secreted following whole-blood stimulation with the identical peptide pools (y axis) of the symptomatic cohort (*n* = 37; tests # = 109). Samples from donors with recent SARS-CoV-2 infection (<4 wk PCR neg) are highlighted in red. Spearman correlation.

individuals. Unfortunately, technical challenges (low quantity of IL-10 secretion by T cells; lack of fresh blood of asymptomatic subjects recently infected) has so far precluded the direct visualization of IL-10 production by T cells and not by other cell types (B cells and monocytes) known to secrete IL-10 (Couper et al., 2008). In conclusion, the overall picture of SARS-CoV-2–specific cellular immune responses detected by a combination of two different functional assays shows that patients with asymptomatic infection mount a coordinated and balanced activation of virus-specific T cells able to trigger production of high quantities of IFN-γ and IL-2 linked with secretion of IL-10. Animal models have already shown that the ability of T cells to secrete IFN-γ and IL-10 simultaneously led to an effective viral control without triggering pathological processes (Zhao et al., 2016; Sun et al., 2009). We suggest that this could be the functional signature of protective virus-specific cellular immune responses in asymptomatic SARS-CoV-2 infection. A detailed analysis at the single-cell level of the functional profile of SARS-CoV-2–specific T cells in symptomatically and asymptomatically infected individuals will be needed to formally demonstrate such hypothesis. One other limitation of our study is that the studied asymptomatic individuals are all male and mostly from India and Bangladesh. SARS-CoV-2–specific T cells present in asymptomatic female individuals and different ethnicities will need to be carefully analyzed to confirm that our findings can be generalized to different populations worldwide.

Materials and methods

Ethics statement

The study of migrant workers was approved by the Singapore Ministry of Health under Section 59A of the Infectious Diseases Act (2015); thus institutional review board approval was not required, and all participants gave verbal consent to participate. All COVID-19 patients provided written informed consent, and the study was approved by the Institutional Review Boards of the National University of Singapore (H-20-006), SingHealth (CIRB/F/2018/2387 and CIRB/F/2018/3045), and the National Healthcare Group Domain Specific Review Board (2012/00917).

Study population

The asymptomatic cohort comprised 541 men aged 19–59 yr (median 35 yr) recruited from randomly selected rooms within a SARS-CoV-2–affected dormitory housing >4,000 migrant workers largely from India and Bangladesh (~86%; Table S2) and followed up prospectively from May to July 2020 (Tan et al., 2021b). Detailed information about the measures to contain SARS-CoV-2 outbreaks in the dormitories can be found at the Singapore Ministry of Manpower website (https://www.mom.gov.sg/newsroom/press-releases/2020/1214-measures-to-contain-the-covid-19-outbreak-in-migrant-worker-dormitories). Note that at the time of the study, migrant workers were confined in the dormitories and received a monetary compensation provided by the Singapore government. Therefore, there was no economic incentive to report an asymptomatic state for fear of economic loss. In addition, thermometers and oximeters were distributed, and every migrant worker living in the dormitories checked and reported his temperature and oximeter readings twice a day to a medical team present at every dorm. Of the initially selected 541 men, 478 (88.4%) provided blood samples at recruitment and at 2 and 6 wk (Fig. 1). Participants also declared their health status at the time of blood draws, and their information was cross-checked with the medical post based in the dormitory. At the 6-wk follow up, a subset of 85 asymptomatic participants provided an additional blood sample for assessment of cell-mediated immune responses. Participants were not tested for infection by polymerase chain reaction (PCR) because at the time of the study, testing of asymptomatic individuals without clinical indication was not recommended.

For hospitalized symptomatic patients, blood samples were obtained from the PCR positivity phase until 193 d after PCR negativity from recovered COVID-19 patients ($n = 75$; $n = 16$ were sampled longitudinally; Table S1). All archived unexposed donor samples were collected before June 2019.

Antibody quantification

Sera were tested for anti-NP IgG antibodies (CMIA; Abbott Laboratories) on an Abbott Architect i2000SR automated instrument; a signal-to-cutoff ratio of ≥1.4 was defined as a positive result, following the manufacturer's recommendation. A second serum aliquot was tested with an sVNT that detects isotype-independent receptor-binding domain nAbs (cPass; GenScript; Tan et al., 2020). Based on the manufacturer's instructions, a virus inhibition threshold of ≥20% was considered a positive result.

SARS-CoV-2–specific T cell quantification

SARS-CoV-2–specific T cells were tested as described before (Le Bert et al., 2020). Briefly, PBMCs were isolated using Ficoll-Paque and directly tested by IFN-γ-ELISpot for reactivity to four SARS-CoV-2 peptide pools of 15-mers covering NP (NP-1, NP-2) and membrane (M) and one pool of 55 peptides covering the most immunogenic regions of S (Fig. S2). ELISpot plates (Millipore) were coated with human IFN-γ antibody (1-D1K; Mabtech) overnight at 4°C. 400,000 PBMCs were seeded per well and stimulated for 18 h with pools of SARS-CoV-2 peptides (2 µg/ml). For stimulation with peptide matrix pools or single peptides, a concentration of 5 µg/ml was used. Subsequently, the plates were developed with human biotinylated IFN-γ detection antibody (7-B6-1; Mabtech), followed by incubation with Streptavidin-AP (Mabtech) and KPL BCIP/NBT Phosphatase Substrate (SeraCare). To quantify positive peptide-specific responses, 2× mean spots of the unstimulated wells were subtracted from the peptide-stimulated wells, and the results were expressed as SFCs/10^6 PBMCs. We excluded the results if negative control wells had >30 SFCs/10^6 PBMCs or positive control wells (PMA/ionomycin) were negative.

Cell culture for T cell expansion

T cell lines were generated as follows: 20% of PBMCs were pulsed with 10 µg/ml of the overlapping SARS-CoV-2 peptides for 1 h at 37°C, washed, and cocultured with the remaining cells in AIM-V medium (Gibco, Thermo Fisher Scientific) supplemented with 2% AB human serum (Gibco, Thermo Fisher Scientific). T cell lines were cultured for 10 d in the presence of 20 U/ml of recombinant IL-2 (R&D Systems).

Flow cytometry

PBMCs or expanded T cell lines were stimulated for 5 h (or 7 h) at 37°C with or without SARS-CoV-2 peptide pools (2 µg/ml). After 1 h (or 3 h), 10 µg/ml brefeldin A (Sigma-Aldrich) and 1× monensin (BioLegend) were added. Cells were stained with the yellow LIVE/DEAD fixable dead cell stain kit (Invitrogen) and surface marker: anti-CD3 (SK7 or OKT3; BioLegend), anti-CD4 (SK3), anti-CD8 (SK1), anti-CD14 (TUK4; Miltenyi Biotec), anti-CD16 (3G8; BioLegend), anti-CD19 (SJ25-C1), and anti-CD56 (HCD56; BioLegend). Cells were subsequently fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences-PharMingen) and stained with anti–IFN-γ (25723; R&D Systems), anti–IL-2 (MQ1-17H12), anti–IL-6 (MQ2-13A5), anti–IL-10 (JES3-19F1), and anti–TNF-α (MAb11) antibodies and analyzed on a BD-LSR II FACS Scan. Data were analyzed by FlowJo (BD Biosciences). Antibodies were purchased from BD Biosciences-PharMingen unless otherwise stated.

Whole-blood culture with SARS-CoV-2 peptide pools

320 µl of whole blood drawn on the same day was mixed with 80 µl RPMI and stimulated with pools of SARS-CoV-2 peptides (M, NP1, NP2, or S; 2 µg/ml) or a DMSO control. After 15 h of culture, the culture supernatant (plasma) was collected and stored at −80°C until quantification of cytokines.

Cytokine quantification and analysis

Cytokine concentrations in the plasma were quantified using an Ella machine with microfluidic multiplex cartridges measuring IFN-γ, IL-2, TNF-α, IL-4, IL-6, IL-1β, IL-12p70, and IL-10, following the manufacturer's instructions (ProteinSimple). The level of cytokines present in the plasma of DMSO controls was subtracted from the corresponding peptide pool stimulated samples. Subsequently, concentrations of each cytokine in all culture supernatants were transformed using the logicle transformation function, and UMAP was run using 15 nearest neighbors (*nn*), *min_dist* of 0.2, and Euclidean distance (Becht et al., 2018). The results obtained from UMAP analyses were incorporated as additional parameters and converted to FCS files, which were then loaded into FlowJo to generate heatmaps of cytokine secretion on the reduced dimensions.

Statistical analysis

All tests are stated in the figure legends. P values (all two-tailed): *, <0.05; **, <0.01; ***, <0.001; ****, <0.0001.

Online supplemental material

Fig. S1 shows the kinetics of the SARS-CoV-2 nAb profile in the asymptomatic study cohort. Fig. S2 compares the frequency of T cells reactive to different pools of S peptides in COVID-19 convalescents. Fig. S3 demonstrates that $CD4^+$ and $CD8^+$ T cells are reactive to stimulation with SARS-CoV-2–specific peptides. Fig. S4 shows that cytokines can be produced by different immune cells following stimulation with SARS-CoV-2 peptide pools. Fig. S5 shows the cytokine secretion profile of whole-blood cultures from asymptomatic and symptomatic convalescents stimulated with SARS-CoV-2 peptide pools, separated by age and SARS-CoV-2 proteins. Table S1 shows the cohort of symptomatic COVID-19 patients. Table S2 compares the cohorts of SARS-CoV-2–exposed migrant works and symptomatic COVID-19 patients.

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Author contributions: N. Le Bert, A.T. Tan, and A. Bertoletti designed the experiments; H.E. Clapham and L.Y. Hsu conceived and contributed to study design for the migrant workers study; W.N. Chia, B.L. Lim, L-F. Wang, S.H. Lim, and W.Y. Wan performed antibody analysis; C.Y.L. Tham, K. Kunasegaran, J.M.E. Lim, and A. Chia performed all other experiments and analyzed data; J.M. Lim, L.W.L. Tan, N. Shankar, M. Zahari, Z.M. Tun, V. Kumar, and L.J. Sun contributed to design and implementation of the migrant workers study; N. Le Bert, C.-A. Dutertre, and A. Bertoletti analyzed and interpreted the data and prepared the figures; N. Le Bert and A. Bertoletti wrote the paper; Y-J. Tan, P.A. Tambyah, S. Kalimuddin, D. Lye, and J.G.H. Low recruited COVID-19 patients and provided all clinical data. A. Bertoletti designed and coordinated the study; C.C. Tam conceived and led the migrant workers study.

Disclosures: N. Le Bert and A.T. Tan reported a patent for a method to monitor SARS-CoV-2-specific T cells in biological samples pending. W.N. Chia reported a patent for a sublicense agreement with GenScript for the surrogate virus neutralization test pending (Duke-NUS). P. Tambyah reported grants from Arcturus, Roche, Shionogi, Sanofi-Pasteur, and Aj Biologics outside the submitted work. L. Wang reported a patent application on sVNT pending. A. Bertoletti reported personal fees from Oxford Immunotech and Qiagen outside the submitted work; in addition, A. Bertoletti had a patent for the use of peptide pools in whole blood for detection of SARS-CoV-2 T cells pending. C.C. Tam reported grants from Roche and personal fees from Verivax outside the submitted work. No other disclosures were reported.

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Supplemental material

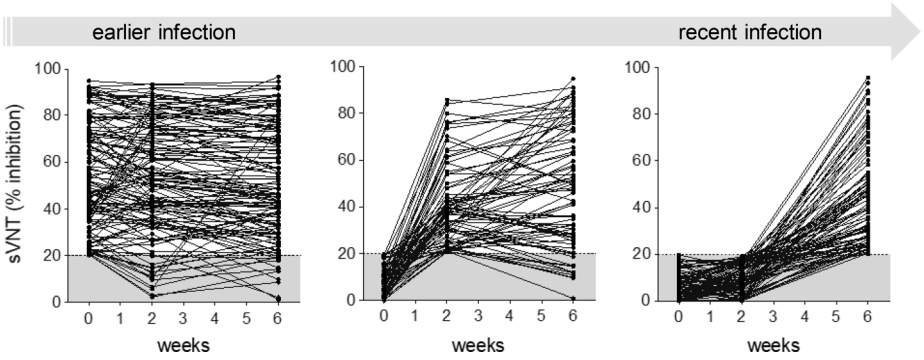

Figure S1. **Kinetics of SARS-CoV-2 nAb profile in the asymptomatic study cohort.** Longitudinal levels of nAbs measured as percentage inhibition by sVNT in asymptomatic donors who were seropositive at recruitment (n = 134, left), who seroconverted at week 2 (n = 81, middle), and who seroconverted by week 6 (n = 93, right). The gray area marks the limit of assay detection.

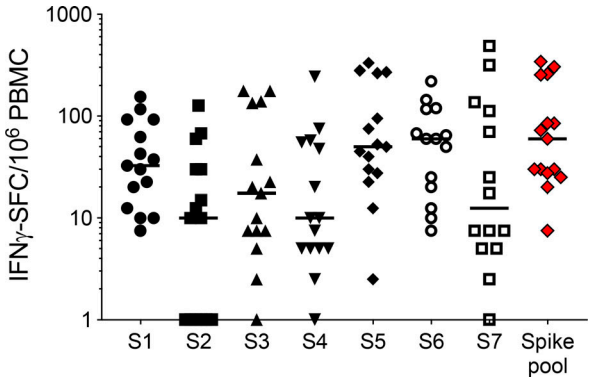

Figure S2. **T cells reactive to different pools of S peptides in COVID-19 convalescents.** Spike is a long protein with 1,276 amino acids; thus it requires 253 15-mer peptides overlapping by 10 amino acids to cover the whole protein, equaling 7 pools of ∼40 peptides. To reduce the number of peptide pools to test, we selected a single "S pool" comprising 55 peptides. For the selection, all sequences of published SARS-CoV-1 epitopes (http://www.iedb.org; positive assays only, T cells assays, host: human) were aligned with the library of S-SARS-CoV-2 15-mers. We selected the 15-mer peptides that cover the homologue sequence of the described SARS-CoV-1 epitope sequences. In addition, we added the 15-mer peptides that cover the predicted SARS-CoV-2 S epitopes published by Grifoni et al. (2020b). The 55 peptides cover 40.5% of the S protein. The frequency of reactive cells to the selected S pool (red) was compared with the seven pools of 15-mers overlapping by 10 amino acids covering together the entire S protein (S1–S7) in COVID-19 convalescents (n = 15).

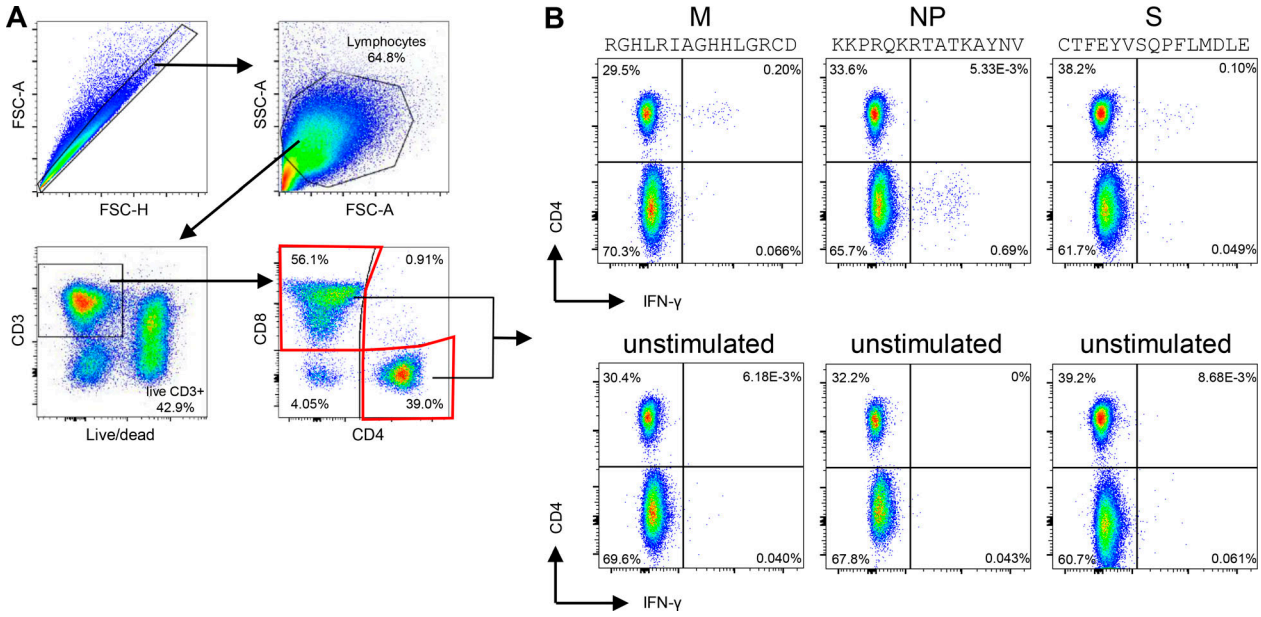

Figure S3. **CD4⁺ and CD8⁺ T cells are reactive to stimulation with SARS-CoV-2–specific peptides.** For asymptomatic donors (n = 3; <4 wk anti-NP IgG⁺), short-term T cell lines were generated by PBMC stimulation with the different peptide pools and a 10-d expansion protocol (Le Bert et al., 2020). A peptide pool-matrix strategy was applied that identified single T cell epitopes. Subsequently, the short-term T cell lines were restimulated for 5 h with the identified single peptides and analyzed by intracellular cytokine staining for IFN-γ. **(A)** The gating strategy to identify CD4⁺ and CD8⁺ T cells is shown. FSC, forward scatter; SSC, side scatter. **(B)** Dot plots show examples of CD4⁺ and CD8⁺ T cells producing IFN-γ in response to stimulation with three different peptides (upper panels) and the corresponding unstimulated controls (lower panels).

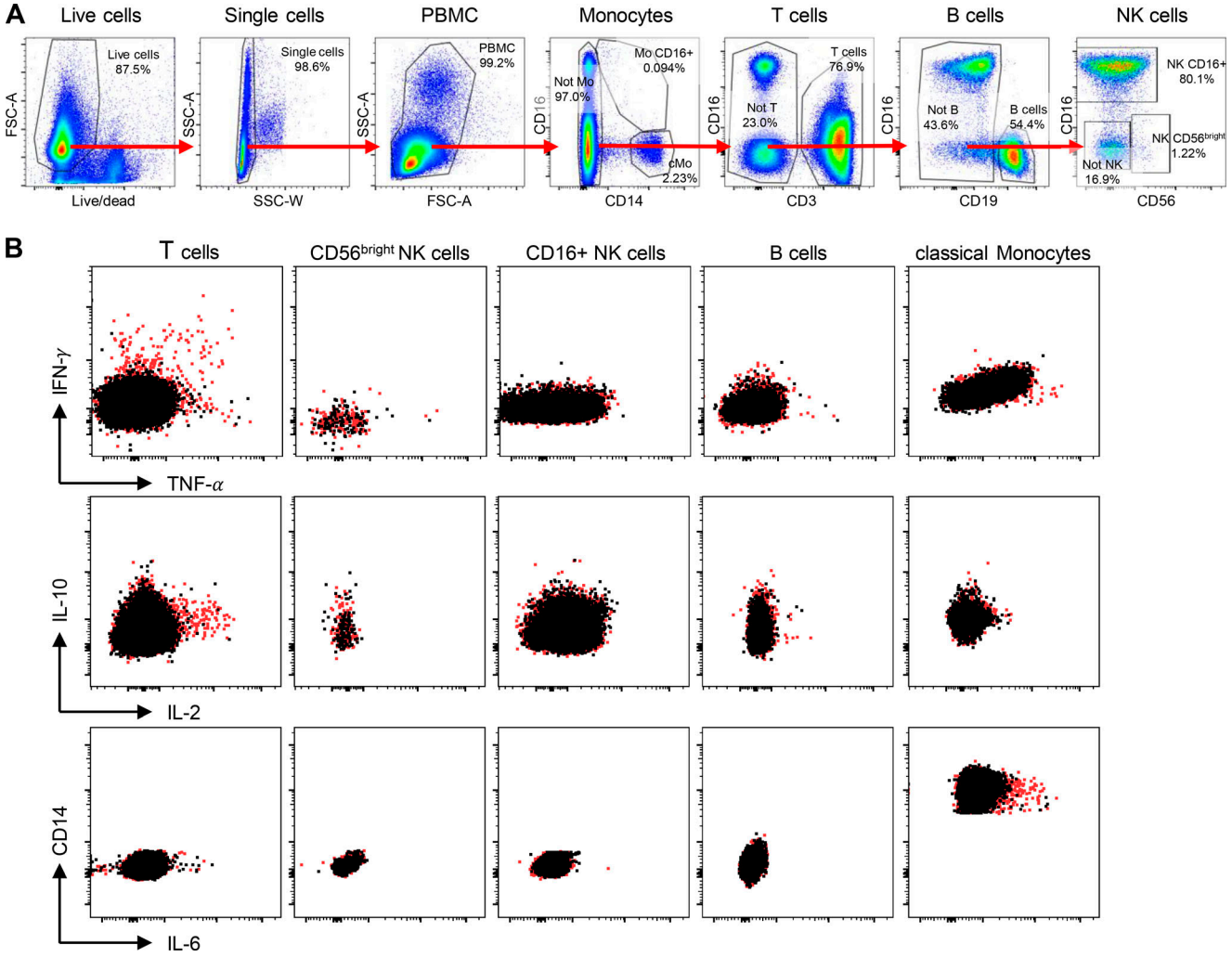

Figure S4. **Cytokines produced by immune cells following stimulation with SARS-CoV-2 peptide pools.** PBMCs (n = 3) were stimulated for 5 h (or 7 h for IL-6) with SARS-CoV-2 peptide pools and analyzed by intracellular cytokine staining. **(A)** The gating strategy to identify the different immune cell subsets is shown. FSC, forward scatter; SSC, side scatter. **(B)** Dot plots show examples (n = 1) of T cells producing IFN-γ, TNF-α, and IL-2 and monocytes producing IL-6 in response to stimulation with the M peptide pool (red) overlaid with the corresponding unstimulated controls (black).

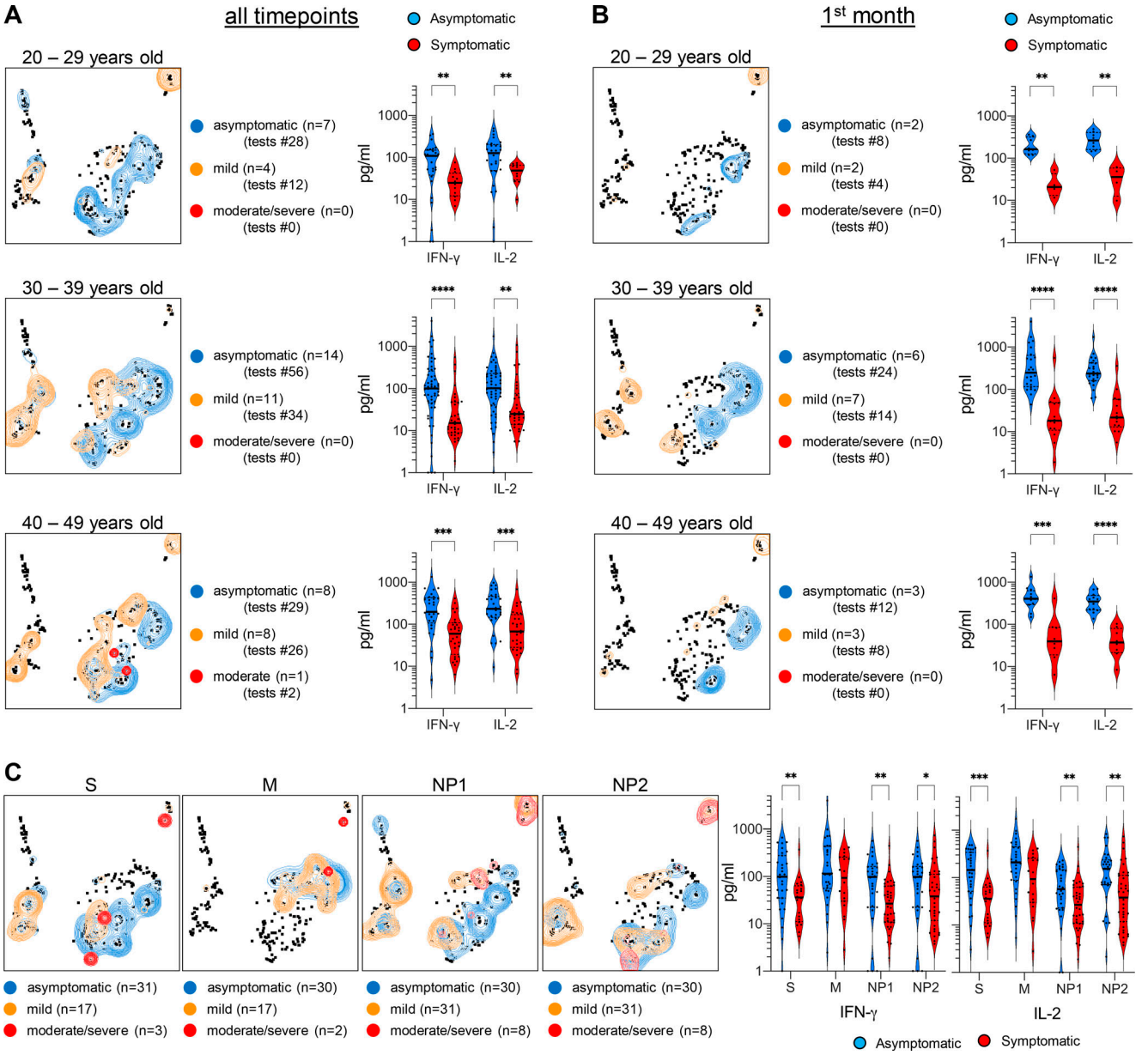

Figure S5. **Cytokine secretion profile of whole blood from asymptomatic and symptomatic convalescents stimulated with SARS-CoV-2 peptide pools, separated by age and SARS-CoV-2 proteins. (A)** UMAP plots comparing the cytokine secretion profiles of whole blood stimulated with SARS-CoV-2 peptide pools between asymptomatic (blue) and symptomatic (orange/red) SARS-CoV-2–infected individuals (all analyzed time points after infection). Donors of both groups were divided into different age groups (20–29 yr, top; 30–39 yr, middle; 40–49 yr, bottom). The violin plots show the quantity of IFN-γ and IL-2 detected in the culture supernatants. Number of individuals tested are indicated (n); each individual was usually tested with four different peptide pools, and total tests performed are indicated (#). **(B)** As in A, but only for the samples collected within 1 mo after infection. **(C)** UMAP plots comparing the cytokine secretion profiles of whole blood from asymptomatic (blue) and symptomatic (orange/red) SARS-CoV-2–infected individuals stimulated with the four different SARS-CoV-2 peptide pools shown individually. The violin plots show the quantity of IFN-γ (left) and IL-2 (right) detected in the different culture supernatants. Mann–Whitney tests. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

Provided online are two tables. Table S1 shows the cohort of symptomatic COVID-19 patients. Table S2 compares the cohorts of SARS-CoV-2–exposed migrant works and symptomatic COVID-19 patients.