EXHIBIT 1

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF COLORADO

Civil Action No. _____

Exhibit 1 of Complaint for Declaratory Judgment

**AFFIDAVIT OF DR. PETER A. MCCULLOUGH IN SUPPORT OF DECLARATORY
JUDGMENT COMPLAINT**

I, Doctor **Peter A. McCullough**, MD, MPH, being duly sworn, depose and state as follows:

1.   I make this affidavit in support of the above referenced Complaint as expert testimony in support thereof.

2.   The expert opinions expressed here are my own and arrived at from my persons, professional and educational experiences taken in context, where appropriate, by scientific data, publications, treatises, opinions, documents, reports and other information relevant to the subject matter.

Experience & Credentials

3.   I am competent to testify to the facts and matters set forth herein. A true and accurate copy of my *curriculum vitae* is attached hereto as **Exhibit A**.

4.   After receiving a bachelor's degree from Baylor University, I completed my medical degree as an Alpha Omega Alpha graduate from the University of Texas Southwestern Medical School in Dallas. I went on to complete my internal medicine residency at the University of Washington in Seattle, a cardiology fellowship including service as Chief Fellow at William Beaumont Hospital, and a master's degree in public health at the University of Michigan.

5.   I am board certified in internal medicine and cardiovascular disease and hold an additional certification in clinical lipidology, and previously echocardiography. I am on the active medical staff at Baylor University Medical Center and Baylor Jack and Jane Hamilton Heart and Vascular Hospital, in Dallas, Texas. I am also on staff at Baylor Heart and Vascular Institute, which promotes cardiovascular research and education. I practice internal medicine and clinical cardiology as well as teach and conduct research, and I am an active scholar in medicine with roles as an author, editorialist, and reviewer for dozens of major medical journals and textbooks. I am Professor of Medicine at Texas Christian University and the University of North Texas Health Sciences Center School of Medicine.

6.   I have led clinical, education, research, and program operations at major academic centers (Henry Ford Hospital, Oakland University William Beaumont School of Medicine) as well as academically oriented community health systems. I spearheaded the

clinical development of *in vitro* natriuretic peptide and neutrophil gelatinase associated lipocalin assays in diagnosis, prognosis, and management of heart and kidney disease now used worldwide. I also led the first clinical study demonstrating the relationship between severity of acute kidney injury and mortality after myocardial infarction. I have contributed to the understanding of the epidemiology of chronic heart and kidney disease through many manuscripts from the Kidney Early Evaluation Program Annual Data Report published in the American Journal of Kidney Disease and participated in clinical trial design and execution in cardiorenal applications of acute kidney injury, hypertension, acute coronary syndromes, heart failure, and chronic cardiorenal syndromes. I participated in event adjudication (involved attribution of cause of death) in trials of acute coronary syndromes, chronic kidney disease, heart failure, and data safety and monitoring of anti-diabetic agents, renal therapeutics, hematology products, and gastrointestinal treatments. I have served as the chairman or as a member of over twenty randomized trials of drugs, devices, and clinical strategies. Sponsors have included pharmaceutical manufacturers, biotechnology companies, and the National Institutes of Health.

7.   I frequently lecture and advise on internal medicine, nephrology, and cardiology for leading institutions worldwide. I am recognized by my peers for my work on the role of chronic kidney disease as a cardiovascular risk state. I have over 1,000 related scientific publications, including the "Interface between Renal Disease and Cardiovascular Illness" in Braunwald's Heart Disease Textbook. My works have appeared in the New England Journal of Medicine, Journal of the American Medical Association, and other top-tier journals worldwide. I am an associate editor of the American Journal of Cardiology and the American Journal of Kidney Diseases. I have testified before the U.S. Senate Committee on Homeland Security and Governmental Affairs, the U.S. Food and Drug Administration Cardiorenal Advisory Panel and its U.S. Congressional Oversight Committee, and the Texas Senate Committee on Health and Human Services.

8.   I am a Fellow of the American College of Cardiology, the American Heart Association, the American College of Physicians, the American College of Chest Physicians, the National Lipid Association, and the National Kidney Foundation. I am also a Diplomate of the American Board of Clinical Lipidology.

9.   In 2013, I was honored with the International Vicenza Award for Critical Care Nephrology for my contribution and dedication to the emerging problem of cardiorenal syndromes. I am the President of the Cardiorenal Society of America, an organization dedicated to bringing together cardiologists and nephrologists, and I engage in research, improved quality of care, and community outreach to patients with both heart and kidney disease.[1]

10.   I am the current President of the Cardiorenal Society of America, a professional organization dedicated to advancing research and clinical care for patients who have combined heart and kidney disease. I am the Editor-in-Chief of *Cardiorenal Medicine*, a

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[1] *See* http://www.cardiorenalsociety.org/.

primary research journal listed by the National Library of Medicine which is the only publication with a primary focus on research concerning patients with combined heart and kidney disease. Finally, I am the Editor-in-Chief of *Reviews in Cardiovascular Medicine*, a widely read journal that publishes reviews on contemporary topics in cardiology and is also listed by the National Library of Medicine.

11.   My appended *curriculum vitae* further demonstrates my academic and scientific achievements and provides a list of publications authored by me over the past thirty years.

12.   Since the outset of the pandemic, I have been a leader in the medical response to the COVID-19 disaster and have published "Pathophysiological Basis and Rationale for Early Outpatient Treatment of SARS-CoV-2 (COVID-19) Infection," the first synthesis of sequenced multidrug treatment of ambulatory patients infected with SARS-CoV-2 in the *American Journal of Medicine* and updated in *Reviews in Cardiovascular Medicine*.[2] I have forty-seven peer-reviewed publications on the COVID-19 infection cited in the National Library of Medicine. Through a window to public policymakers, I have contributed extensively on issues surrounding the COVID-19 crisis in a series of OPED's for *The Hill.* I testified on the SARS-CoV-2 outbreak in the U.S. Senate Committee on Homeland Security and Governmental Affairs on November 19, 2020. I testified on lessons learned from the pandemic response in the Texas Senate Committee on Health and Human Services on March 10, 2021, and on early treatment of COVID-19 for the Colorado General Assembly on March 31, 2021. Additionally, I testified in the New Hampshire Senate on legislation concerning the investigational COVID-19 vaccine on April 14, 2020. My expertise on the SARS-CoV-2 infection and COVID-19 syndrome, like that of infectious disease specialists, is approximately eighteen months old. I have formed my opinions based upon my direct clinical experience with acute and convalescent COVID-19 cases as well as on closely following the preprint and published literature on the outbreak. I have additionally, specifically reviewed all of the key published rare cases and reports concerning possible recurrence of SARS-CoV-2.

Opinion

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[2] McCullough PA, Kelly RJ, Ruocco G, Lerma E, Tumlin J, Wheelan KR, Katz N, Lepor NE, Vijay K, Carter H, Singh B, McCullough SP, Bhambi BK, Palazzuoli A, De Ferrari GM, Milligan GP, Safder T, Tecson KM, Wang DD, McKinnon JE, O'Neill WW, Zervos M, Risch HA. Pathophysiological Basis and Rationale for Early Outpatient Treatment of SARS-CoV-2 (COVID-19) Infection. Am J Med. 2021 Jan;134(1):16-22. doi: 10.1016/j.amjmed.2020.07.003. Epub 2020 Aug 7. PMID: 32771461; PMCID: PMC7410805 available at https://pubmed.ncbi.nlm.nih.gov/32771461/; McCullough PA, Alexander PE, Armstrong R, Arvinte C, Bain AF, Bartlett RP, Berkowitz RL, Berry AC, Borody TJ, Brewer JH, Brufsky AM, Clarke T, Derwand R, Eck A, Eck J, Eisner RA, Fareed GC, Farella A, Fonseca SNS, Geyer CE Jr, Gonnering RS, Graves KE, Gross KBV, Hazan S, Held KS, Hight HT, Immanuel S, Jacobs MM, Ladapo JA, Lee LH, Littell J, Lozano I, Mangat HS, Marble B, McKinnon JE, Merritt LD, Orient JM, Oskoui R, Pompan DC, Procter BC, Prodromos C, Rajter JC, Rajter JJ, Ram CVS, Rios SS, Risch HA, Robb MJA, Rutherford M, Scholz M, Singleton MM, Tumlin JA, Tyson BM, Urso RG, Victory K, Vliet EL, Wax CM, Wolkoff AG, Wooll V, Zelenko V. Multifaceted highly targeted sequential multidrug treatment of early ambulatory high-risk SARS-CoV-2 infection (COVID-19). Rev Cardiovasc Med. 2020 Dec 30;21(4):517-530. doi: 10.31083/j.rcm.2020.04.264. PMID: 33387997 available at https://pubmed.ncbi.nlm.nih.gov/33387997/.

13.   I have reviewed the Complaint for Declaratory Judgment which delineates the subject matter relating to proposed acts by the United States Department of Defense to compel Investigative New Drugs under the Emergency Use Authorization to the members of the Armed forces that have contracted and recovered from the Covid-19 virus.

14.   I am competent to opine on the medical aspects of these allegations based upon my above-referenced education and professional medical experience and the basis of my opinions are formed as a result of my education and experience.

15.   As a Medical Doctor and scientist in the biological health and treatment of human beings, I confirm and attest that:

a) the fatality rate of the Covid- 19 virus, or any variation thereof, including the so called "Delta" or "Lambda" (hereinafter "Virus") is .0046% across combined age groups in the United States;

b) the fatality rate associated with the Virus does not meet the definition of a "Pandemic" as declared by the Defendants World Health Organization, Centers for Disease Control and Prevention, The National Institutes of Health or any other governmental or non-governmental agency or entity acting with authority to make such a declaration;

c) there are widely available methods to reduce the risk of infection with SARS-CoV-2 including anti-infective oral and nasal sprays and washes, oral medications, and outpatient monoclonal antibodies, which are "approved" drugs by the Food and Drug Administration and highly effective in preventing and treating the Virus; (references: https://www.amjmed.com/article/S0002-9343(20)30673-2/fulltext; & https://rcm.imrpress.com/EN/10.31083/j.rcm.2020.04.264, & https://doi.org/10.23958/ijirms/vol06-i03/1100;

d) Of those people that have already contracted and recovered from the Virus there is no better protection against reinfection, including the foregoing named therapeutics.

e) In fact, more than thirty percent (+30%) of the global population have already had and recovered from the Virus due to its highly contagious and extremely low mortality rate.   Indeed, the Virus as an isolated infection is substantially less fatal than any year's normal flu or Corona Virus.

f) Multiple studies, including but not limited to: "Immunological memory to SARS-CoV-2 assessed for greater than six months after infection;" "SARS-CoV-2 infection induces long-lived bone marrow plasma cells in humans;" "Exposure to

SARS-CoV-2 generates T-cell memory in the absence of a detectable viral infection;" and "Protection afforded by the BNT162b2 and mRNA-1273 COVID-19 vaccines in fully vaccinated cohorts with and without prior infection;" have studied, documented and proven that long-term and usually lifetime protection is provided by antibodies developed naturally by people who have contracted flu and flu-family viruses, such as the Virus. Scientific and peer-reviewed articles cited are attached annexed as a part of this Sworn Affidavit as appendixes hereto.

g) Further, multiple studies, including but not limited to: "Necessity of COVID-19 vaccination in previously infected individuals;" "Comparison of kinetics of immune responses to SARS-CoV-2 proteins in individuals with varying severity of infection and following a single dose of the AZD1222;" and "Quantifying the risk of SARS-CoV-2 reinfection over time" have demonstrated that people who have been inoculated with any of the three Investigational New Drug, Emergency Use Only Covid 19 vaccines produced by Moderna, Pfizer and Johnson & Johnson, have had little, no or even negative effects on people who receive them after having developed natural immunity. Scientific and peer-reviewed articles cited are attached annexed as a part of this Sworn Affidavit as appendixes hereto.

h) There are many VAERS and other documented reports demonstrating that people who already had naturally developed immunity who later received one of the IND/EUA Covid 19 vaccines may have been injured or conditions exacerbated by the use of said vaccines after having natural immunity.

i) To put it concisely and bluntly, people who have the naturally created antibodies resulting from contracting and recovering from the Virus should not receive any inoculation against the virus or any family or variant thereof because it will do more harm than good.

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Peter A. McCullough, MD, MPH

State of Texas   §
   §
County of Dallas   §

The undersigned, being duly sworn, deposes and says:

I, Peter A. McCullough, MD, MPH, declare under the penalty of perjury of the laws of the United States of America, and state upon personal knowledge that:

I am an adult of sound mind, 58 years old, and declare that the information herein is true, correct and complete and that I have voluntarily affirmed this affidavit based upon my own personal knowledge, education, and experience, and under the penalty of perjury of the laws of the United States of America.

SUBSCRIBED AND SWORN TO BEFORE ME on the 16 day of August 2021, to certify which witness my hand and official seal.
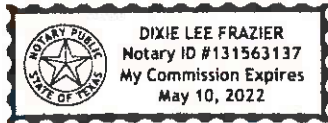
DIXIE LEE FRAZIER
Notary ID #131563137
My Commission Expires
May 10, 2022

Notary Public for the State of Texas

My Commission Expires: May 10, 2022

EXHIBIT 2

Necessity of COVID-19 vaccination in previously infected individuals

Nabin K. Shrestha,[1] Patrick C. Burke,[2] Amy S. Nowacki,[3] Paul Terpeluk,[4] Steven M. Gordon[1]

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Keywords: SARS-CoV-2; COVID-19; Incidence; Vaccines; Immunity;

Running Title: COVID-19 vaccination if already infected

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Summary: Cumulative incidence of COVID-19 was examined among 52238 employees in an American healthcare system. COVID-19 did not occur in anyone over the five months of the study among 2579 individuals previously infected with COVID-19, including 1359 who did not take the vaccine.

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ABSTRACT

Background. The purpose of this study was to evaluate the necessity of COVID-19 vaccination in persons previously infected with SARS-CoV-2.

Methods. Employees of the Cleveland Clinic Health System working in Ohio on Dec 16, 2020, the day COVID-19 vaccination was started, were included. Any subject who tested positive for SARS-CoV-2 at least 42 days earlier was considered previously infected. One was considered vaccinated 14 days after receipt of the second dose of a SARS-CoV-2 mRNA vaccine. The cumulative incidence of SARS-CoV-2 infection over the next five months, among previously infected subjects who received the vaccine, was compared with those of previously infected subjects who remained unvaccinated, previously uninfected subjects who received the vaccine, and previously uninfected subjects who remained unvaccinated.

Results. Among the 52238 included employees, 1359 (53%) of 2579 previously infected subjects remained unvaccinated, compared with 22777 (41%) of 49659 not previously infected. The cumulative incidence of SARS-CoV-2 infection remained almost zero among previously infected unvaccinated subjects, previously infected subjects who were vaccinated, and previously uninfected subjects who were vaccinated, compared with a steady increase in cumulative incidence among previously uninfected subjects who remained unvaccinated. Not one of the 1359 previously infected subjects who remained unvaccinated had a SARS-CoV-2 infection over the duration of the study. In a Cox proportional hazards regression model, after adjusting for the phase of the epidemic, vaccination was associated with a significantly lower risk of SARS-CoV-2 infection among those not previously infected (HR 0.031, 95% CI 0.015 to 0.061) but not among those previously infected (HR 0.313, 95% CI 0 to Infinity).

Conclusions. Individuals who have had SARS-CoV-2 infection are unlikely to benefit from COVID-19 vaccination, and vaccines can be safely prioritized to those who have not been infected before.

INTRODUCTION

The two FDA-approved (BNT162b2 mRNA [Pfizer-BioNTech] and mRNA-1273 [Moderna]) mRNA vaccines have been shown to be very efficacious in protecting against Severe Acute Respiratory Syndrome (SARS) – associated Coronavirus-2 (SARS-CoV-2) infection [1,2]. The effectiveness of the Pfizer-BioNTech vaccine in a real-world setting has also been shown to be comparable to the efficacy demonstrated in clinical trials [3,4]. Given these, there has been an understandable desire to vaccinate as many people as possible.

The ability to vaccinate a large part of the population is limited by the supply of vaccine. As of March 21, 2021, 78% of 447 million doses of the coronavirus disease 2019 (COVID-19) vaccines that had been deployed had gone to only ten countries [5]. The COVAX initiative was borne out of the recognition that equitable distribution of vaccines worldwide was essential for effective control of the COVID-19 pandemic. However, the reality is that there is great disparity in the availability of vaccines across countries. Countries with limited supplies of vaccine have to prioritize how their supply of vaccines will be allocated within their populations. Criteria used for such prioritization have included profession, age, and comorbid conditions. Data that inform prioritization criteria with help maximize the benefits of whatever vaccine is available.

Observational studies have found very low rates of reinfection among individuals with prior SARS-CoV-2 infection [6–8]. This brings up the question about whether it is necessary to vaccinate previously infected individuals. These studies notwithstanding, there remains a theoretical possibility that the vaccine may still provide some benefit in previously infected persons. A prior large observational study concluded that immunity from natural infection cannot be relied on to provide adequate protection and advocated for vaccination of previously infected individuals [9]. The CDC website recommends that persons previously infected with SARS-CoV-2 still get the vaccine [10]. Despite these recommendations, credible reports of previously infected persons getting COVID-19 are rare. The rationale often provided for getting the COVID-19 vaccine is that it is safer to get vaccinated than to get the disease. This is

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certainly true, but it is not an explanation for why people who have already had the disease need to be

vaccinated. A strong case for vaccinating previously infected persons can be made if it can be shown that

previously infected persons who are vaccinated have a lower incidence of COVID-19 than previously

infected persons who did not receive the vaccine.

The purpose of this study was to attempt to do just that, and thereby evaluate the necessity of the

COVID-19 vaccine in persons who were previously infected with SARS-CoV-2.

METHODS

Study design

This was a retrospective cohort study conducted at the Cleveland Clinic Health System in Ohio, USA. The study was approved by the Cleveland Clinic Institutional Review Board. A waiver of informed consent and waiver of HIPAA authorization were approved to allow access to personal health information by the research team, with the understanding that sharing or releasing identifiable data to anyone other than the study team was not permitted without additional IRB approval.

Setting

PCR testing for SARS-CoV-2 at Cleveland Clinic began on March 12, 2020, and a streamlined process dedicated to the testing of health care personnel (HCP) was begun shortly thereafter. All employees with a positive SARS-CoV-2 test were interviewed by Occupational Health, with date of onset of symptoms of COVID-19 being one of the questions asked. Vaccination for COVID-19 began at Cleveland Clinic on December 16, 2020. When initially started it was the Pfizer-BioNTech vaccine that was administered, until the Moderna vaccine became available, from which time employees received one or the other. All employees were scheduled to receive their second vaccine dose 28 days after the first one, regardless of which vaccine was given. The employee cohort was chosen for this study because of documentation of their COVID-19 vaccination and of any SARS-CoV-2 infection in the Occupational Health database.

Participants

All employees of the Cleveland Clinic Health System, working in Ohio, on Dec 16, 2020, were screened for inclusion in the study. Those who were in employment on December 16, 2020, were included.

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Variables

SARS-CoV-2 infection was defined as a positive nucleic acid amplification test. The date of

infection was taken to be the date of onset of symptoms when available, and the date of specimen

collection when not. A person was considered vaccinated 14 days after receipt of the second dose of the

vaccine (which would have been 42 days after receipt of the first dose of the vaccine for most subjects).

For the sake of consistency in the duration assumed for development of natural and vaccine immunity,

any person who tested positive for SARS-CoV-2 at least 42 days before the vaccine rollout date, was

considered previously infected. Other covariates collected were age, job location, job type (patient-facing

or non-patient facing), and job category. The job location variable could be one of the following:

Cleveland Clinic Main Campus, regional hospital (within Ohio), ambulatory center, administrative center,

or remote location. The job category was one of the following: professional staff, residents/fellows,

advance practice practitioners, nursing, pharmacy, clinical support, research, administration, and

administration support.

Outcome

The study outcome was time to SARS-CoV-2 infection, the latter defined as a positive nucleic

acid amplification test for SARS-CoV-2 on or after December 16, 2020. Time to SARS-CoV-2 infection

was calculated as number of days from December 16, 2020 (vaccine rollout date) to SARS-CoV-2

infection. Employees that had not developed a SARS-CoV-2 infection were censored at the end of the

study follow-up period (May 15, 2021). Those who received the Johnson & Johnson vaccine (81 subjects)

without having had a SARS-CoV-2 infection were censored on the day of receipt of the vaccine, and

those whose employment was terminated during the study period before they had SARS-CoV-2 infection

(2245 subjects) were censored on the date of termination of employment. The health system never had a

requirement for asymptomatic employee test screening. Most of the positive tests, therefore, would have

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been tests done to evaluate suspicious symptoms. A small proportion would have been tests done as part

of pre-operative or pre-procedural screening.

Statistical analysis

A Simon-Makuch hazard plot [11] was created to compare the cumulative incidence of SARS-

CoV-2 infection among previously infected subjects who were vaccinated, with those of previously

infected subjects who remained unvaccinated, previously uninfected subjects who were vaccinated, and

previously uninfected subjects who remained unvaccinated. Previous infection was treated as a time-

independent covariate (SARS-CoV-2 infection at least 42 days before Dec 16, 2020), and vaccination (14

days after receipt of the second dose of the vaccine) was treated as a time-dependent covariate (Figure 1).

Curves for the unvaccinated were based on data for those who did not receive the vaccine over the

duration of the study, and for those who did until the date they were considered vaccinated, from which

point onwards their data were recorded into the corresponding vaccinated set. A Cox proportional hazards

regression model was fitted with time to SARS-CoV-2 infection as the outcome variable against

vaccination (as a time-dependent covariate whose value changed on the date a subject was considered

vaccinated)[12]. Previous infection (as a time-independent covariate) and an interaction term for previous

infection and vaccination were included as covariates. The phase of the epidemic was adjusted for by

including the slope of the epidemic curve as a time-dependent covariate whose value changed

continuously with the slope of the epidemic curve. The analysis was performed by NKS and ASN using

the *survival* package and R version 4.0.5 [12–14].

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RESULTS

Of 52238 employees included in the study, 2579 (5%) were previously infected with SARS-CoV-2.

Baseline characteristics

Those previously infected with SARS-CoV-2 were significantly younger (mean ± SD age; 39 ± 13 vs. 42 ± 13, p<0.001), and included a significantly higher proportion with patient-facing jobs (65% vs. 51%, p<0.001). Table 1 shows the characteristics of subjects grouped by whether or not they were previously infected. A significantly lower proportion of those previously infected (47%, 1220 subjects) were vaccinated by the end of the study compared to 59% (29461) of those not previously infected (p<0.001). Of those vaccinated, 63% received the Moderna vaccine. Twelve percent of subjects with previous SARS-CoV-2 infection did not have a symptom onset date, suggesting they may possibly have been identified on pre-operative or pre-procedural screening, and may not have had symptomatic infection. When vaccination was begun, the epidemic in Ohio was at the peak of its third wave (Figure 2).

Cumulative incidence of COVID-19

Figure 3 is a Simon-Makuch plot showing that SARS-CoV-2 infections occurred almost exclusively in subjects who were not previously infected with SARS-CoV-2 and who remained unvaccinated. The cumulative incidence of SARS-CoV-2 infection among previously infected unvaccinated subjects did not differ from that of previously infected subjects who were vaccinated, and that of previously uninfected subjects who were vaccinated. For all three of these groups, the cumulative incidence of SARS-CoV-2 infection was much lower than that of subjects who were not previously infected and who remained unvaccinated. Of the 2154 SARS-CoV-2 infections during the study period, 2139 (99.3%) occurred among those not previously infected who remained unvaccinated or were waiting

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to get vaccinated, and15 (0.7%) occurred among those not previously infected who were vaccinated. Not one of the 2579 previously infected subjects had a SARS-CoV-2 infection, including 1359 who remained unvaccinated throughout the duration of the study.

Association of vaccination with occurrence of COVID-19

In a Cox proportional hazards regression model, after adjusting for the phase of the epidemic, vaccination was associated with a significantly lower risk of SARS-CoV-2 infection among those not previously infected (HR 0.031, 95% CI 0.015 – 0.061) but not among those previously infected (HR 0.313, 95% CI 0 – Infinity). The absence of events among those who were previously infected, whether they received the vaccine or not, precluded accurate or precise estimates for the latter effect size.

Duration of protection

This study was not specifically designed to determine the duration of protection afforded by natural infection, but for the previously infected subjects the median duration since prior infection was 143 days (IQR 76 – 179 days), and no one had SARS-CoV-2 infection over the following five months, suggesting that SARS-CoV-2 infection may provide protection against reinfection for 10 months or longer.

DISCUSSION

This study shows that subjects previously infected with SARS-CoV-2 are unlikely to get COVID-19 reinfection whether or not they receive the vaccine. This finding calls into question the necessity to vaccinate those who have already had SARS-CoV-2 infection.

It is reasonable to expect that immunity acquired by natural infection provides effective protection against future infection with SARS-CoV-2. Observational studies have indeed found very low rates of reinfection over the following months among survivors of COVID-19 [6–8]. Reports of true reinfections are extremely rare in the absence of emergence of new variants. When such reinfections occur, it would be purely speculative to suggest that a vaccine might have prevented them. Duration of protective immunity from natural infection is not known. However, the same also can be said about duration of protective immunity from vaccination. Uncertainty about the duration of protective immunity afforded by natural infection is not by itself a valid argument for vaccinating previously infected individuals. This study provides direct evidence that vaccination with the best available vaccines does not provide additional protection in previously infected individuals.

A prior study concluded that natural infection cannot be relied on to protect against COVID-19 [9]. That study was based on comparison of PCR-positivity rates during a second COVID-19 surge in Denmark between those who tested positive and negative during the first COVID-19 surge, and indirectly calculated that prior infection provided 80.5% protection against repeat infection, and that protection against those older than 65 years was only 47.1%. The study did not compare vaccinated and unvaccinated people, and it is therefore an assumption to consider that a vaccine would have provided better protection in that particular population. Furthermore, there was a gap of only seven weeks between the end of the first surge and the beginning of the second in that study. It is now well-known that a small number of people can continue to have positive PCR test results for several weeks to a few months after infection, one study finding that 5.3% remained positive at 90 days [15]. It is possible that some of the positives picked up in the early part of the second surge were not necessarily new infections but residual

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virus from the tail end of the first surge. Since the actual number of infections was small, a few such

misclassifications could change the rates substantially. Our study examined rates of SARS-CoV-2

infection in vaccinated and unvaccinated individuals and showed that those previously infected who did

not receive the vaccine did not have higher rates of SARS-CoV-2 infection than those previously infected

who did, thereby providing direct evidence that vaccination does not add protection to those who were

previously infected.

There are several strengths to our study. Its large sample size and follow-up of up to 5 months

provide us with an ample degree of confidence in its findings. A major strength of our study is that we

adjusted the analyses for the phase of the epidemic at all time points. The risk of acquisition of infection

is strongly influenced by the phase of the epidemic at any given time, and it is important to adjust for this

for accurate risk analyses. Given that was this a study among employees of a health system, and that the

health system had policies and procedures in recognition of the critical importance of keeping track of the

pandemic among its employees, we had an accurate accounting of who had COVID-19, when they were

diagnosed with COVID-19, who received a COVID-19 vaccine, and when they received it.

The study has its limitations. Because we did not have a policy of asymptomatic employee

screening, previously infected subjects who remained asymptomatic might have been misclassified as

previously uninfected. Given this limitation, one should be cautious about drawing conclusions about the

protective effect of prior asymptomatic SARS-CoV-2 infection. It should be noted though, that 12% of

the subjects classified as previously infected did not have a symptom onset date recorded, suggesting that

at least some of those classified as previously infected might have been asymptomatic infections. It is

reassuring that none of these possibly asymptomatically infected individuals developed COVID-19 during

the duration of the study. The study follow-up duration was short, being only five months, but this was

longer than published mRNA vaccine efficacy studies [1,2], and longer than the follow-up duration of the

largest published vaccine effectiveness studies to date [3,4]. Median freedom from reinfection (time from

initial infection until end of follow-up) in this study, for those previously infected, of almost 10 months, is

consistent with findings in an earlier study that immunoglobulin G (IgG) to the spike protein remained

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stable over more than six months after an episode of infection [16]. Our study included no children and few elderly subjects, and the majority would not have been immunosuppressed. Data governance policies in our institution precluded us from obtaining detailed clinical information on employees. While one cannot generalize this study's findings to assume that prior infection would provide adequate immunity in these groups, there is also no reason to expect a vaccine to provide additional protection in these same groups. Lastly, it is necessary to emphasize that these findings are based on the prevailing assortment of virus variants in the community during the study. It is not known how well these results will hold if or when some of the newer variants of concern become prominent. However, if prior infection does not afford protection against some of the newer variants of concern, there is little reason to suppose that the currently available vaccines would either. Vaccine breakthrough infections with variants have indeed been reported [17].

Our study's findings have important implications. Worldwide, COVID-19 vaccines are still in short supply. As of March 9, 2021, dozens of countries had not been able to administer a single dose of the vaccine [18]. As of May 17, 2021, only 17 countries had been able to reach ten percent or more of their populations with at least the first dose of vaccine [19]. Given such a scarcity of the vaccine, and the knowledge that vaccine does not provide additional protection to those previously infected, it would make most sense to limit vaccine administration to those who have not previously had the infection. In addition to profession, age, and comorbid conditions, previous infection should be an important consideration in deciding whom to prioritize to receive the vaccine. A practical and useful message would be to consider symptomatic COVID-19 to be as good as having received a vaccine, and that people who have had COVID-19 confirmed by a reliable laboratory test do not need the vaccine.

In conclusion, individuals who have laboratory-confirmed symptomatic SARS-CoV-2 infection are unlikely to benefit from COVID-19 vaccination, and vaccines can be safely prioritized to those who have not been infected before.

TRANSPARENCY DECLARATION

Conflict of Interest

Selection of "no competing interests" reflects that all authors have completed the ICMJE uniform disclosure form at www.icmje.org/coi_disclosure.pdf and declare: no support from any organization for the submitted work; no financial relationships with any organizations that might have an interest in the submitted work in the previous three years; no other relationships or activities that could appear to have influenced the submitted work.

Funding

None received.

Author contributions

NKS: Conceptualization, Methodology, Validation, Investigation, Data curation, Software, Formal analysis, Visualization, Writing- Original draft preparation, Writing- Reviewing and Editing, Supervision, Project administration.

ASN: Methodology, Formal analysis, Visualization, Validation, Writing- Reviewing and Editing.

PCB: Resources, Investigation, Validation, Writing- Reviewing and Editing.

PT: Resources, Writing- Reviewing and Editing.

SMG: Project administration, Resources, Writing- Reviewing and Editing.

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TABLES

Table 1. Study Subject Characteristics

Characteristic	Previously Infected (N = 2579)	Not Previously Infected (N = 49659)	*P* Value
Age, y, mean ± SD	39±13	42±13	<0.001
Patient-facing job	1676 (65)	25504 (51)	<0.001
Job location			<0.001
Cleveland Clinic Main Campus	1011 (39)	19595 (40)	
Regional hospitals	1096 (43)	16433 (33)	
Ambulatory centers	313 (12)	7767 (16)	
Administrative centers	138 (5)	4424 (9)	
Remote location	21 (<1)	1440 (3)	
Job category			<0.001
Professional staff	89 (4)	3775 (8)	
Residents and fellows	72 (3)	1669 (3)	
Advanced practice practitioners	154 (6)	2806 (6)	
Nursing	1142 (44)	13623 (27)	
Pharmacy	44 (2)	1274 (3)	
Research	328 (13)	6776 (14)	
Clinical support	111 (4)	3500 (7)	
Administration	614 (24)	15050(30)	
Administration support	25 (1)	1186 (2)	

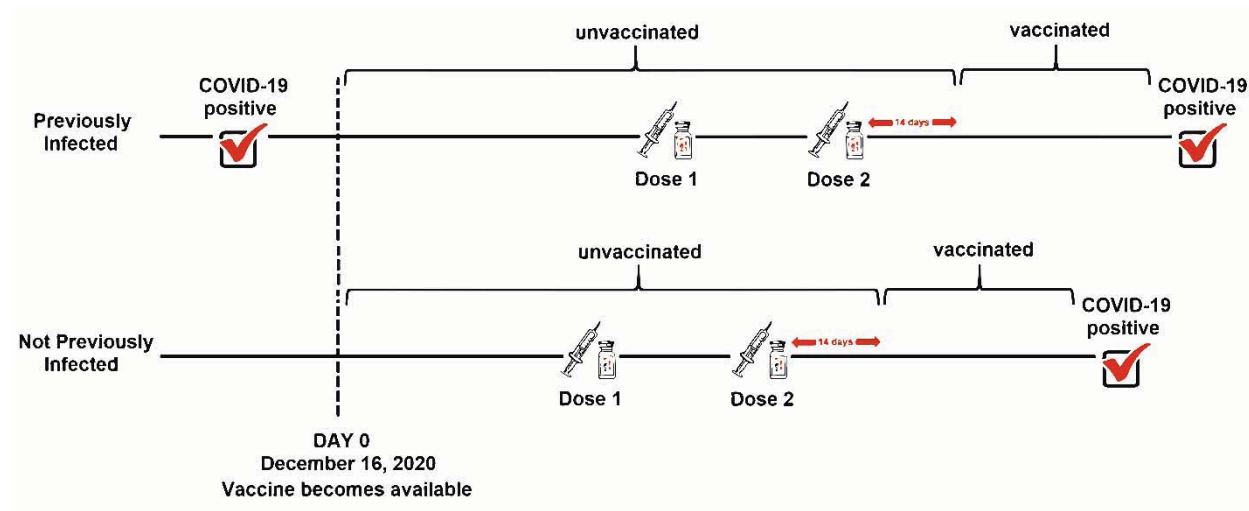Data are presented as no. (%) unless otherwise indicated

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FIGURES

Figure 1. Explanation of "previously infected" analyzed as a time-independent covariate and "vaccinated" treated as a time-dependent covariate.
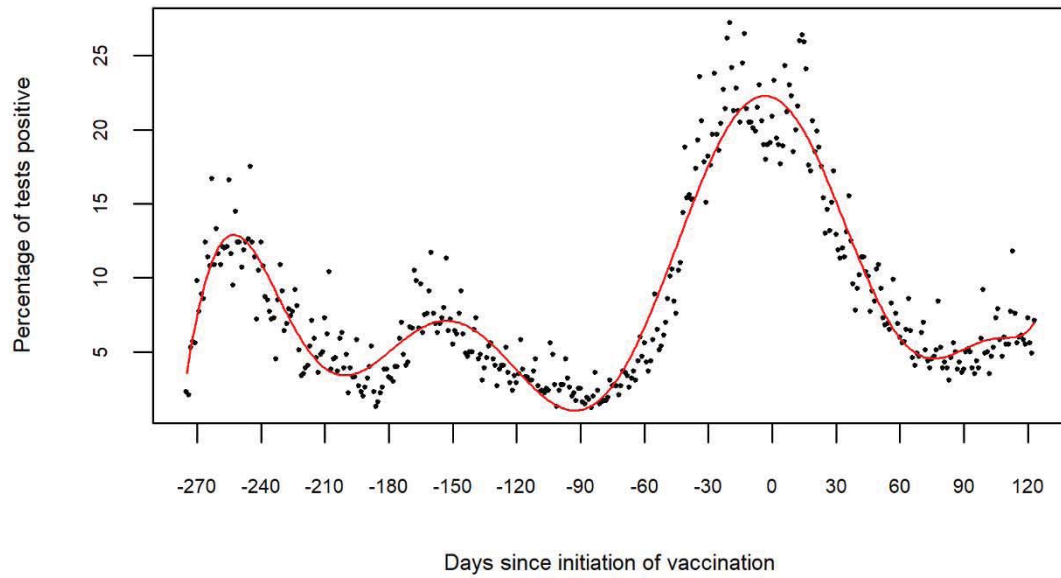
Figure 2. COVID-19 epidemic curve before and after vaccine rollout. Points on the scatter plot

represent the proportion of all COVID-19 PCR tests done at Cleveland Clinic that were positive on any

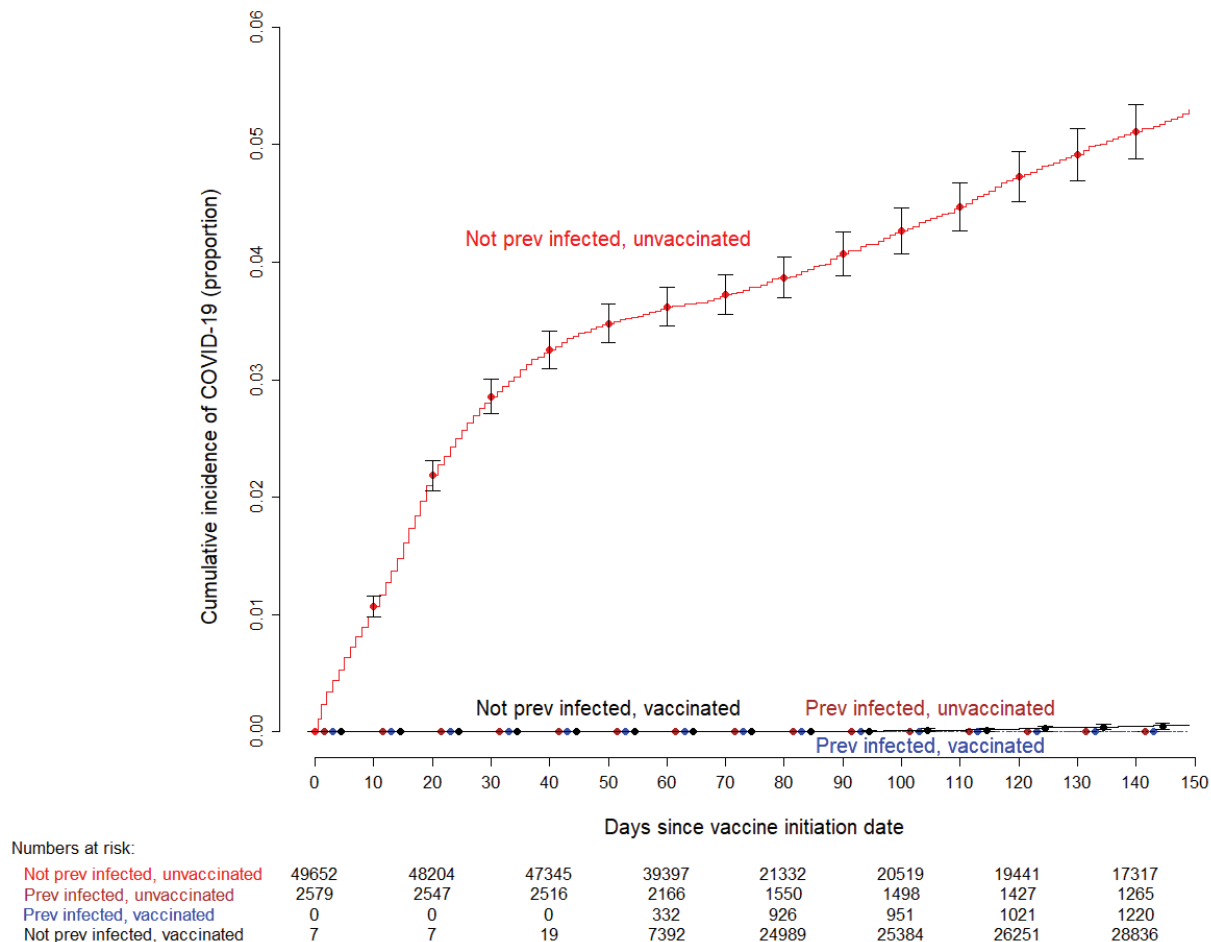given day. The colored line represents a fitted polynomial curve.

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Figure 3. Simon-Makuch plot showing the cumulative incidence of COVID-19 among subjects previously infected and not previously infected with COVID-19, who did and did not receive the vaccine. Curves for the unvaccinated are based on data for those who did not receive the vaccine during the duration of the study, and for those waiting to receive the vaccine. Day zero was Dec 16, 2020, the day vaccination was started in our institution. Error bars represent 95% confidence intervals. Seven subjects who had been vaccinated earlier as participants in clinical trials were considered vaccinated throughout the duration of the study. Twelve subjects who received their first dose in the first week of the vaccination campaign managed to get their second dose three weeks later, and were thus considered vaccinated earlier than 42 days since the start of the vaccination campaign.

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EXHIBIT 3

nature COMMUNICATIONS

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Exposure to SARS-CoV-2 generates T-cell memory in the absence of a detectable viral infection

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Jun He[4], Xinyue Mei [1], Yonghong Tang[4], Bijia Lin[1], Zhenjun Chen [5], James McCluskey [5], Ji Yang[1],
Alexandra J. Corbett [5] & Pixin Ran [1✉]

T-cell immunity is important for recovery from COVID-19 and provides heightened immunity for re-infection. However, little is known about the SARS-CoV-2-specific T-cell immunity in virus-exposed individuals. Here we report virus-specific CD4$^+$ and CD8$^+$ T-cell memory in recovered COVID-19 patients and close contacts. We also demonstrate the size and quality of the memory T-cell pool of COVID-19 patients are larger and better than those of close contacts. However, the proliferation capacity, size and quality of T-cell responses in close contacts are readily distinguishable from healthy donors, suggesting close contacts are able to gain T-cell immunity against SARS-CoV-2 despite lacking a detectable infection. Additionally, asymptomatic and symptomatic COVID-19 patients contain similar levels of SARS-CoV-2-specific T-cell memory. Overall, this study demonstrates the versatility and potential of memory T cells from COVID-19 patients and close contacts, which may be important for host protection.

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Since early 2020, SARS-CoV-2 has spread globally, triggering a pandemic that continues to cause devastating damage to public health and people's livelihoods. By the middle of November, the global COVID-19 cases have reached 50 million with the death toll exceeding a grim 1.2 million (John Hopkins University, USA). Although the mechanisms by which host immunity combats SARS-CoV-2 infection are far from being completely understood, significant knowledge in this area has been gained through the investigations of the association of COVID-19 clinical features and disease progression with host immune responses[1]. For example, our recent study established that the severity of COVID-19 inversely correlates with T-cell immunity of the host[2]. In the presence of adequate neutralizing antibodies, CD4^{+} and CD8^{+} T cells play a major role in the recovery of critical COVID-19 patients[2]. Other studies showed that in moderate and severe COVID-19 cases characterized by lymphopenia there was a drastic reduction in the numbers of both CD4^{+} and CD8^{+} T cells[3–5]. Although the reason for this reduction remains unknown, autopsy revealed extensive infiltration of T cells into the lungs[6]. Analysis of immune cells from bronchoalveolar lavage (BAL) fluid of COVID-19 patients demonstrated the presence of clonal expansion[7]. Moreover, virus-specific CD4^{+} T cell numbers were shown to be associated with the production of IgG that targets the receptor-binding domain (RBD) of SARS-CoV-2[8]. Notably, analyses of persistent COVID-19 cases showed that upon activation their T-cells appeared to lose polyfunctionality and cytotoxicity, trending towards an exhausted phenotype[9,10].

While most acute viral infections result in the development of protective immunity, available data suggest that long-term and robust-protective memory is not easily acquired for human coronavirus infections[11]. For example, one year after disease onset following MERS-CoV infection, the viral-specific IgG antibody became undetectable for some of the patients with mild symptoms[11–13]. The SARS-CoV-1 humoral response was relatively short-lived and memory B cells disappeared quickly after primary infection[14]. Recent mathematical modeling suggested a short duration (likely <2 years) of protective immunity is elicited after SARS-CoV-2 infection[15]. Furthermore, Long et al. have reported that the viral-specific IgG levels of SARS-CoV-2-infected individuals had an ~70% reduction during the early convalescent phase and a significant proportion of individuals (40% of asymptomatic patients and 12.9% of symptomatic patients) became IgG seronegative[16]. In contrast to the short-lived humoral response in SARS-CoV-1 survivors, the magnitude and frequency of specific CD8^{+} memory T cells, and to a lesser extent CD4^{+} memory T cells, persisted for 6–11 years, suggesting that T cells may confer long-term immunity[15]. Although it has been reported that SARS-CoV-2-specific CD4^{+} and CD8^{+} T cells were detected in 100 and 70% of convalescent COVID-19 patients, respectively[17], to date, it remains largely unclear how well the SARS-CoV-2 T cell memory is established and how the memory T cells respond upon re-exposure to viral antigens. Another important question that remains unresolved is whether close contacts, who had been confirmed to be negative in nucleic acid testing (NAT) and antibody screening, have gained any memory T cell immunity upon exposure to SARS-CoV-2.

In this study, we examined the proliferation and activation capability of the SARS-CoV-2 memory T cell pools of a large cohort of recovered COVID-19 patients, close contacts, and unexposed healthy individuals. Our results showed that the COVID-19 patients and close contacts developed SARS-CoV-2-specific T-cell immune memory. In addition, comparable levels of SARS-CoV-2-specific memory T cells were detected in the samples of asymptomatic and symptomatic COVID-19 patients.

Results

Proliferation capacity of memory T cells from recovered COVID-19 patients and close contacts. To assess the SARS-CoV-2-specific T-cell memory, human peripheral blood mononuclear cells (PBMCs) from 90 COVID-19 patients collected between 48–86 days after disease onset were stimulated in vitro for 10 days with peptide pools designed to target the spike glycoprotein (S), membrane glycoprotein (M), nucleocapsid (N), envelope glycoprotein (E) and ORF1ab region of RNA-dependent RNA polymerase (RdRp) of SARS-CoV-2. Our data showed that the memory CD4^{+} and CD8^{+} T cells of 94.44% and 83.33%, respectively, of the COVID-19 patients successfully underwent expansion (Fig. 1a–c). These results clearly indicate that most of the recovered COVID-19 patients have developed effective T cell memory pools against SARS-CoV-2.

Although the close contacts in our cohort were all negative in both nucleic acid test (NAT) and SARS-CoV-2 antibody screening, the possible exposure of these individuals to the virus may have led to the generation of T cell immunity even in the absence of a successful infection. To test this possibility, we performed a 10-day in vitro peptide stimulation assay for 69 close contacts from 45 family clusters. The results show that 57.97% (Fig. 1a–c) and 14.49% (Fig. 1b, c) of close contacts contained virus-specific memory CD4^{+} and CD8^{+} T-cells, respectively. Notably, all close contacts developed responses at lower frequencies than 4%, while 64 (71.11%) and 32 (35.56%) of the 90 COVID-19 patients developed marked responses at the frequencies of higher than 4% for IFNγ^{+}CD4^{+} T cells (Fig. 1a) and IFNγ^{+}CD8^{+} T cells (Fig. 1b), respectively. In comparison to the COVID-19 patients, a significantly lower proportion of close contacts responded ($p < 0.0001$ for CD4^{+}, Fig. 1a; $p < 0.0001$ for CD8^{+}, Fig. 1b).

In order to investigate whether the observed expanded T cells may have originated from pre-existing cross-reactive T cells specific for common cold coronaviruses from previous infections, we tested blood samples of 63 healthy donors collected before September of 2019. Following a 10-day in vitro peptide expansion only 3.17% of the healthy donors contained detectable levels of virus-specific memory CD4^{+} and CD8^{+} T cells, respectively (Fig. 1a–c), suggesting that cross-reactive T cells derived from exposure to other human coronaviruses do exist but are at a significantly lower frequency than those observed in close contacts.

The major differences between the proportion of COVID-19 patients and healthy donors ($p < 0.0001$ for CD4^{+}, Fig. 1a; $p < 0.0001$ for CD8^{+}, Fig. 1b), or between close contacts and healthy donors ($p < 0.0001$ for CD4^{+}, Fig. 1a; $p = 0.0157$ for CD8^{+}, Fig. 1b) with memory T-cells capable of proliferating in response to SARS-CoV2 peptides emphasize that exposure to SARS-CoV-2 can facilitate the establishment of the T memory immunity not only in COVID-19 patients, but also in some close contacts even in the absence of a successful infection. In addition, differences between COVID-19 patients and close contacts were observed in the frequency of double-positive (IFNγ^{+} TNF^{+}) CD4^{+} T cells ($p < 0.0001$ for CD4^{+}, Supplementary Fig. 1a, $p < 0.0001$ for CD8^{+}, Supplementary Fig. 1b), although CD4^{+}, but not CD8^{+} cells producing both cytokines were significantly higher in close contacts than healthy controls (Supplementary Fig. 1a, b).

Ex vivo analyses of SARS-CoV-2-specific memory T cells from COVID-19 patients and close contacts. Next, we measured the sizes of virus-specific memory pools for CD4^{+} and CD8^{+} T cells from 89 COVID-19 patients (1 COVID-19 sample was used up), 69 close contacts and 30 healthy donors by using an overnight "ex vivo" peptide stimulation assay. Our results demonstrated that a significant proportion of COVID-19 patients contained
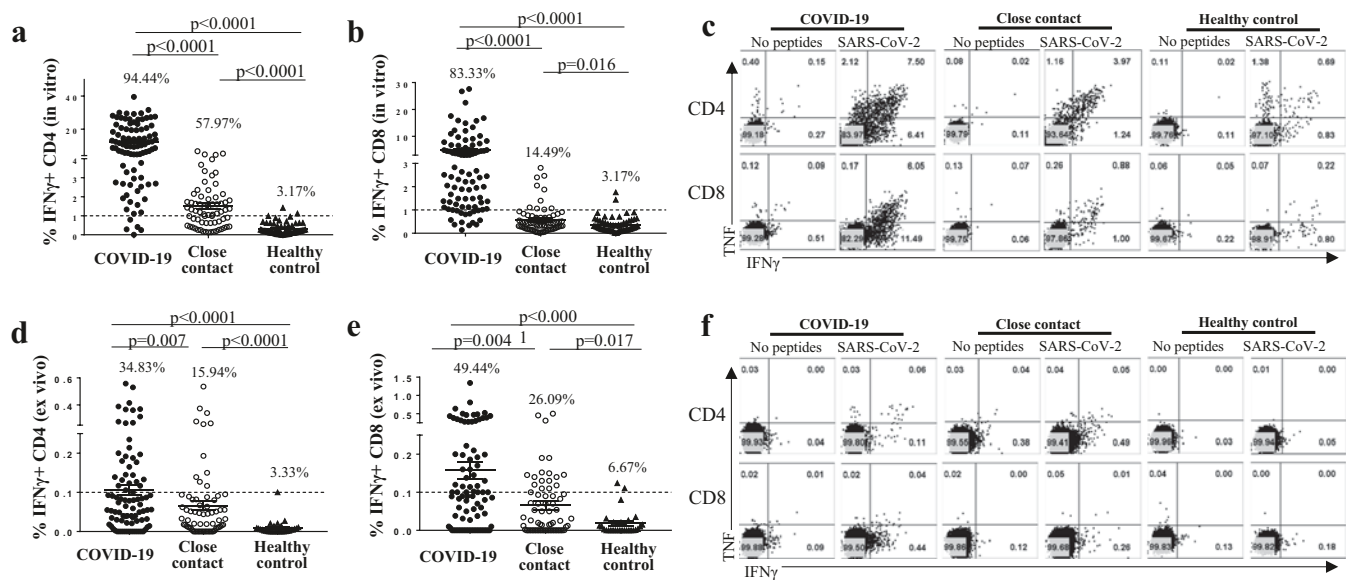
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Fig. 1 Memory T cells specific to SARS-2 were detected and can proliferate in vitro in COVID-19 patients and in close contacts. Donor PBMCs were stimulated with 15-mer peptide pools (overlapping by 11 amino acids) encompassing the entire spike (S), nucleocapsid (N), membrane (M), and envelope (E) proteins for 10 days (in vitro expansion, **a–c**) or overnight (ex vivo, **d–f**) in the presence of 10 U/ml rIL-2, IFNγ, and TNF expressing cells were enumerated by intracellular cytokine staining. Ninety COVID-19 patients (closed circle), their 69 close contacts (open circle), and 63 unexposed healthy donors (closed triangle) were assayed in vitro. For ex vivo experiments, the samples from the above cohort except for one from the COVID-19 group because of cells used up, and 30 of the 63 unexposed healthy donors were assayed. Graphs show the frequency of IFNγ expressing cells in (**a**) CD4$^+$ and (**b**) CD8$^+$ T cells after in vitro expansion and overnight stimulation and in (**d**) CD4$^+$ and (**e**) CD8$^+$ T cells after overnight stimulation. Dashed line is the cut off determined by the background staining (no peptide) for the healthy control group. The cut off threshold used for the overnight stimulation experiments was based on all negative controls (95% CI). The percentages shown are the frequency above this cut off. **c**, **f** Representative dot plots showing IFNγ and TNF expression in T cells after expansion (**c**) or overnight stimulation (**f**). **a**, **b**, **d**, **e** Error bars indicate mean frequencies of IFNγ$^+$ T cells ± SEM; Percentage shown on top of the plots indicates the frequencies of samples above the cutoff. The student t test was performed with two-sided p values as indicated. No peptides: no peptide stimulation control. SARS-CoV-2: with stimulation by SARS-CoV-2 overlapping peptide pools.

virus-specific T cells (34.83% for CD4$^+$, Fig. 1d; 49.44% for CD8$^+$, Fig. 1e; and cut off = 0.1%) at 48–86 days after disease onset. In addition, SARS-CoV-2-specific T cells were also detected in close contacts (15.94% for CD4$^+$, Fig. 1d and 26.09% for CD8$^+$, Fig. 1e). Significant differences were seen between the sizes of T cell memory pools of COVID-19 patients and close contacts (p = 0.007 for CD4$^+$, Fig. 1d and p = 0.004 for CD8$^+$, Fig. 1e). In contrast, in the case of the healthy donors, we found that only 1/30 (3.33%) and 2/30 (6.67%) of the samples contained cross-reactive memory CD4$^+$ and CD8$^+$ T cells, respectively (Fig. 1d, e), suggesting that the cross-reactive T-cell immunity only exists in a small number of unexposed healthy donors. Interestingly, comparing the frequency of double-positive (IFNγ$^+$ TNF$^+$) CD4$^+$ and CD8$^+$ T cells within individuals, these were higher in both COVID-19 patients and close contacts than in healthy controls (Supplementary Fig. 1c, d).

IFNγ-producing SARS-CoV-2-specific memory T cells are detectable in close contacts of infected individuals. To evaluate the quality of SARS-CoV-2-specific memory T cells, we measured the MFI of IFNγ by intracellular staining in the memory T cells from COVID-19 patients and close contacts. To increase the robustness of this experiment, we included an internal control where all of the samples were also assessed for the production of IFNγ following stimulation with CMV peptide pools spanning the pp65 protein. From the comparison between the MFI values of the different samples, it is clear that; (i) CMV peptides induced similar levels of IFNγ production by CD4$^+$ and CD8$^+$ T cells in the samples from COVID-19 patients and close contacts (Fig. 2a, c, e), (ii) the expression levels of IFNγ in CMV-specific T cells were 2-3 times higher than those of SARS-CoV-2-specific CD4$^+$ (Fig. 2c)

or CD8$^+$ T cells (Fig. 2e); (iii) SARS-CoV-2 peptides induced higher levels of IFNγ production in both CD4$^+$ (Fig. 2b, c) and CD8$^+$ (Fig. 2d, e) T cells from patients infected with COVID-19 compared with close contacts, the MFIs being twice as high in CD4$^+$ T cells from the infected group. Collectively, these results indicate that the activation capability of SARS-CoV-2-specific memory T cells from close contacts is lower than that in the COVID-19 patients, despite both groups having similar pre-existing immunity to CMV.

Memory T-cell immunity is detectable in both symptomatic and asymptomatic patients with COVID-19 infection. PBMCs from 72 symptomatic and 18 asymptomatic COVID-19 patients were used in the overnight ex vivo and 10-day in vitro expansion assays to evaluate the sizes, qualities and proliferation capacities of the memory T cell pools. Data in Fig. 3a, d show that following overnight stimulation by peptide pools, 4/18 (22.22%) and 7/18 (38.89%) of the samples from the asymptomatic patients with COVID-19 developed detectable numbers of SARS-CoV-2 specific IFNγ-producing CD4$^+$ T cells and CD8$^+$ T cells, respectively. For the symptomatic COVID-19 patients, 27/71 (35.23%) and 36/71 (50.70%) of the samples also developed virus-specific specific CD4$^+$ T cells and CD8$^+$ T cells, respectively (Fig. 3a, d). There was no significant difference in the sizes of the SARS-CoV-2-specific memory T-cell pools between the symptomatic and asymptomatic COVID-19 patients (p = 0.58 for CD4$^+$ and p = 0.66 for CD8$^+$, Fig. 3a, d). Meanwhile, the ex vivo analysis showed that the MFI of IFNγ staining of the memory T cells (SARS-CoV-2-specific) from the asymptomatic and symptomatic patients were 1536.37 ± 165.28 and 1182.18 ± 219.92 for CD4$^+$ (Fig. 3b) and 636.54 ± 56.25 and 578.47 ± 102.37 for CD8$^+$
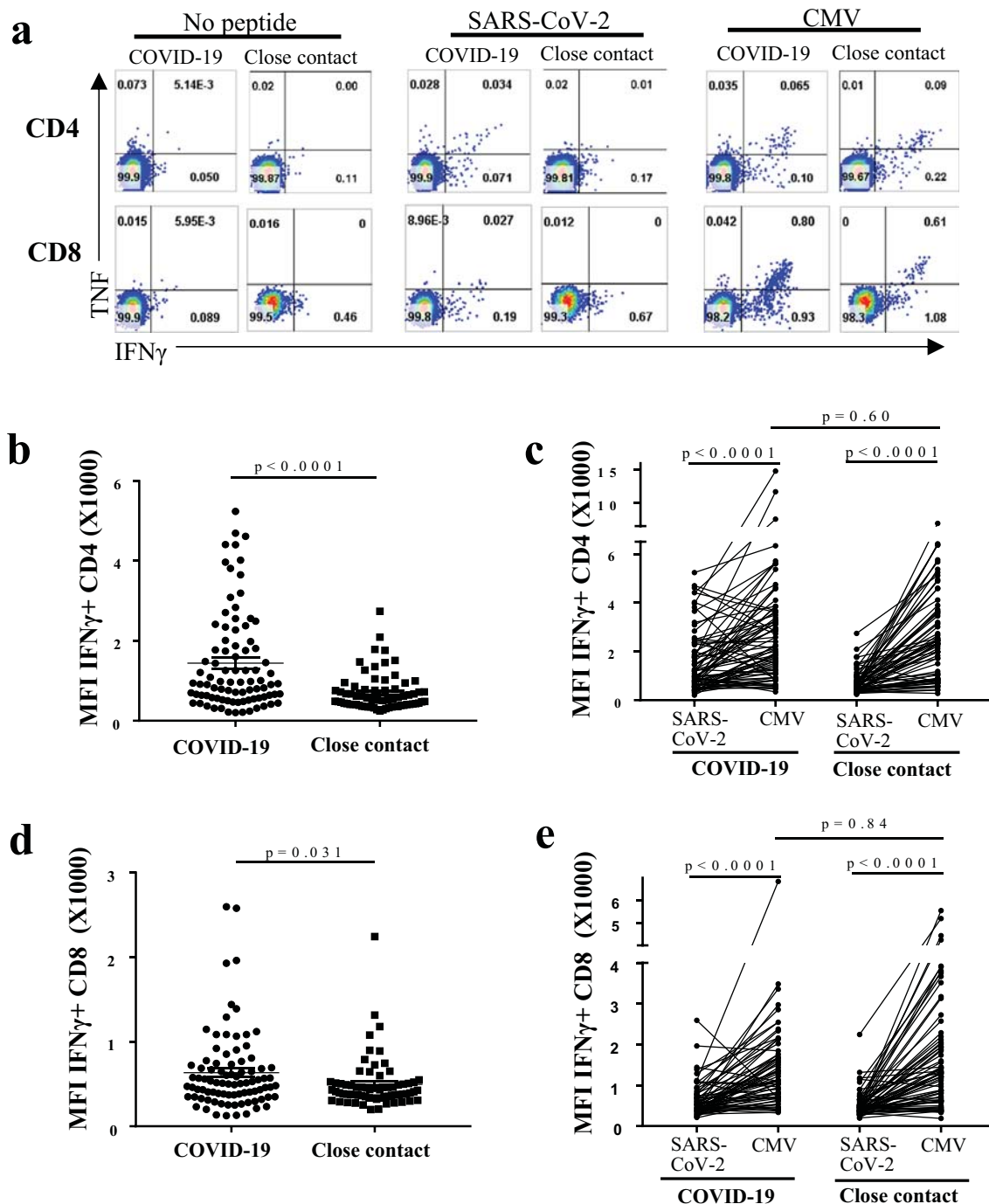
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Fig. 2 Functional analysis of SARS-CoV-2 specific memory T cells in Covid-19 patients and close contacts. Donor PBMCs were stimulated with SARS-CoV-2 or CMV 15-mer peptide pools overnight in the presence of 10 U/ml rIL-2, IFNγ and TNF expressing cells were enumerated by intracellular cytokine staining. **a** Representative FACS plots showing the expression of IFNγ and TNF in CD4+ and CD8+ T cells with or without SARS-CoV-2 or CMV peptide stimulation overnight, as indicated. **b, d** Mean Fluorescence Intensity (MFI) of IFNγ staining for (**b**) CD4+and (**d**) CD8+ T cells from COVID-19 patients (close circle, $n = 89$) and their close contacts (closed square, $n = 69$) after overnight stimulation with SARS-CoV-2 peptide pool. **c, e** Paired analyses of MFI for IFNγ of CD4+ (**c**) and CD8+ (**e**) T cells after overnight stimulation with SARS-CoV-2 or CMV peptide pools for COVID-19 patients ($n = 79$) and close contacts ($n = 69$). Each symbol represents a data point from one individual. **b–e** Error bars represent mean ± SEM. The student t test was performed with two-sided p values as indicated. No peptides: no peptide stimulation control. SARS-CoV-2: with stimulation by SARS-CoV-2 overlapping peptide pools. CMV: with stimulation by CMV overlapping peptide pools.

(Fig. 3e), respectively. Thus, there was no significant difference in the qualities of the memory T cells between the asymptomatic and symptomatic patients ($p = 0.39$ for CD4+ and $p = 0.44$ for CD8+, Fig. 3b, e).

In vitro peptide stimulation and expansion showed that 88.89% and 72.22% of CD8+ T cells from the symptomatic and asymptomatic patients, respectively, proliferated to detectable levels (Fig. 3f). For the CD4+ T cells, 97.22% and 83.33% of the samples from the symptomatic and asymptomatic patients, respectively, proliferated to levels above 1% (Fig. 3c). This indicates a slightly reduced proliferation capacity in SARS-CoV-2-specific T-cell immunity of asymptomatic patients ($p < 0.0001$, Fig. 3c).
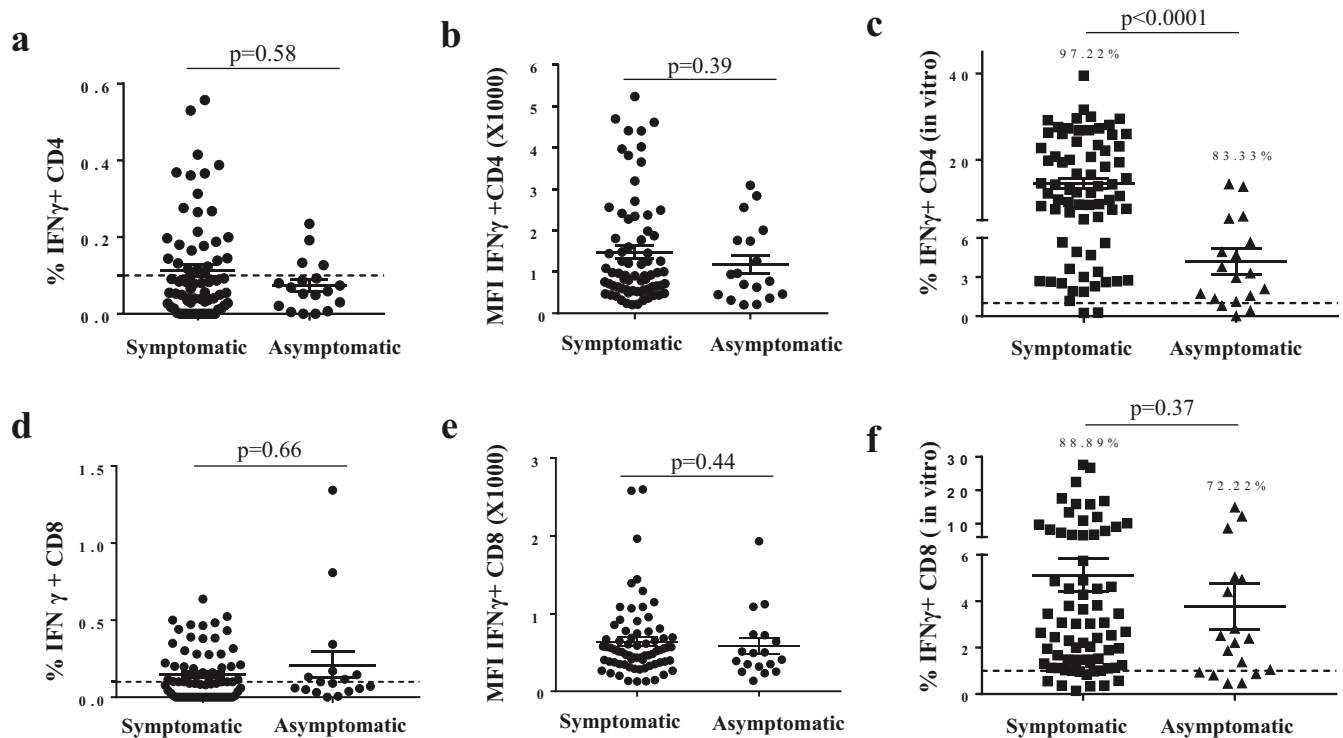
Fig. 3 Comparisons of the T cell memory and in vitro expansion of SARS-CoV-2 specific T cells between symptomatic and asymptomatic COVID-19 patients. a, **d** Frequencies of IFNγ expressing cells in CD4+ (**a**) and CD8+ (**d**) T cells in recovered symptomatic ($n = 71$) and asymptomatic ($n = 18$) COVID-19 patients ex vivo. **b**, **e** MFI of IFNγ staining in CD4+ (**b**) and CD8+ (**e**) T cells from symptomatic and asymptomatic COVID-19 patients. **c**, **f** Frequencies of IFNγ expressing (**c**) CD4+ and (**f**) CD8+ T cells after in vitro expansion in symptomatic ($n = 72$) and asymptomatic ($n = 18$) COVID-19 patients. Percentages shown are the frequencies above the cut off (1%, which was the upper limit observed in no-peptide control stimulations). Error bars represent mean ± SEM. The student's t test was performed with two-sided p values indicated.

SARS-CoV-2-specific T cells are stably maintained 48–86 days after onset of symptoms. We then examined if there was any correlation between the magnitude of the T cell responses (measured by an in vitro expansion assay) and the timespan between 48 and 86 days after symptom onset and found no relationship between the levels of SARS-CoV-2-specific T cells (CD4+ and CD8+) and the timespan within this period ($R^2 = 0.025$, $p = 0.14$ for CD4+, Supplementary Fig. 2a, and $R^2 = 0.005$, $p = 0.52$ for CD8+, Supplementary Fig. 2c). Meanwhile, our data also showed that there was no association between the levels of memory T cells measured by an ex vivo assay and the timespan between 48–86 days after disease onset ($R^2 = 0.064$, $p = 0.021$ for CD4+, Supplementary Fig. 2b and $R^2 = 0.066$, $p = 0.019$ for CD8+, Supplementary Fig. 2d). Together, our in vitro and ex vivo data suggest that CD4+ T memory and CD8+ T memory may have contracted to a stable plateau by the times these samples were collected. Furthermore, we also did not see any difference between severe COVID-19 and moderate COVID-19 patients in the proportion of SARS-Co-V2-specific IFNγ-producing CD4+ or CD8+ T cells expanded in vitro ($p = 0.71$ for CD4+, Supplementary Fig. 2e, $p = 0.48$ for CD8+, Supplementary Fig. 2f).

Memory CD4+ T-cell responses correlate with IgG titers against N protein and S RBD of SARS-CoV-2. The neutralizing antibody response in MERS-CoV-2 infection was previously shown to be dependent on the CD4+ T cell response[13]. To determine if this is also true for SARS-CoV infection, we performed correlation analyses between IgG titers (anti N and anti-RBD, Supplementary Table 1) and magnitude of memory T cells measured by in vitro and ex vivo assays. The sensitivity and accuracy of assays for IgG measurements were verified as shown

in Supplementary Table 2. Following in vitro expansion the virus-specific memory CD4+ T cell pool correlated with the titers of IgG against the S RBD region ($R^2 = 0.51$, $p < 0.0001$, Supplementary Fig. 3a) and the N protein ($R^2 = 0.48$, $p < 0.0001$, Supplementary Fig. 3b), whereas no apparent correlation between CD8+ T cells and IgG titers was observed ($R^2 = 0.28$, $p < 0.0001$, anti-S RBD IgG, Supplementary Fig. 3c and R2 = 0.28, $p < 0.0001$, anti-N IgG, Supplementary Fig. 3d). In the ex vivo assay, no correlation was found between either the virus-specific CD4+ T cells and IgG titres ($R^2 = 0.01$, $p = 0.27$ anti-S RBD IgG, Supplementary Fig. 3e and $R^2 = 0.01$, $p = 0.29$, anti-N IgG, Supplementary Fig. 3f) or the virus-specific CD8+ T cells and IgG titres ($R^2 = 0.03$, $p = 0.10$, anti-S RBD, Supplementary Fig. 3g and $R^2 = 0.03$, $p = 0.10$, anti-N IgG, Supplementary Fig. 3h), indicating that, due to the low numbers of specific T cells that can be detected ex vivo in the memory phase, expansion of T cells in vitro to increase their numbers may be necessary to observe these correlations.

Discussion
COVID-19 patients display a wide range of clinical phenotypes, including severe, moderate, mild, and asymptomatic cases, likely determined by a mix of host genetic factors, and the dose and route of infection. Individuals also exhibit a wide variation in cellular and humoral immune responses during the primary viral infection, with some patients displaying balanced viral-specific B cell and T cell immunity, whereas others rely either on a higher level of activation of neutralizing antibodies or on a stronger T cell response to fight off the virus[2]. In rare cases, individuals who suffer severe and long-lasting symptoms show highly imbalanced

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cellular and humoral immune responses whereby the levels of SARS-CoV-2 specific T-cell or antibody immunity are very low[2].

Close contacts, who are SARS-CoV-2-exposed, are often both NAT negative and antibody negative, indicating that SARS-CoV-2 failed to establish a successful infection within these individuals, presumably due to their exposure to limited numbers of viral particles or a short time of exposure. However, our analysis of the samples from 69 of these close contacts showed the presence of SARS-CoV-2 specific memory T-cell immunity. A similar observation was reported during the MERS epidemic where high-risk individuals (e.g., camel workers) who were NAT negative and antibody negative also developed significant levels of MERS-CoV specific memory T cells[13]. In addition, although in agreement with Sekine et al.[18], we found that some polyfunctional T cells were detectable in close contacts, cells producing both IFNγ and TNF appear largely specific for infected patients rather than for close contacts and healthy donors, suggesting that for COVID-19 patients, the occurrence of stronger antigen stimulation and greater inflammation during viral infection led to an enhanced polyfunctional T-cell response.

Our ex vivo stimulation analyses demonstrated that the pool sizes and quality of the SARS-CoV-2-specific CD4+ and CD8+ T memory cells from close contacts were around half of those from COVID-19 patients. Similarly, our in vitro expansion experiments showed that the SARS-CoV-2-specific CD4+ memory T cells of 57.97% and 94.44% of close contacts and COVID-19 patients, respectively, were able to proliferate. However, a more remarkable difference between the CD8+ proliferation frequencies of the two sample groups was observed, such that the SARS-CoV-2-specific CD8+ memory T cells of 14.49% and 83.33% of close contacts and COVID-19 patients, respectively, underwent proliferation. Theoretically, the initial activation of SARS-CoV-2-specific CD8+ and formation of CD8+ T memory are achieved through the endogenous pathway which processes viral antigens produced within the virus-infected host cells[19]. Presumably, without in situ replication of SARS-CoV-2, there are insufficient viral antigens within the host cells of close contacts to induce a robust CD8+ response resulting in CD8+ T memory in the majority of individuals. By contrast, the formation of CD4+ T memory does not rely on endogenous viral replication but involves endocytosis and/or phagocytosis of exogenous viral antigens, which are mostly derived from non-replicative viral particles or soluble viral proteins[19]. Thus, CD4+ T cell memory may be more easily achieved in uninfected exposed individuals.

Initially, we observed that SARS-CoV-2-specific memory CD4+ and CD8+ secreted low levels of IFNγ and only a small proportion of the T cells from COVID-19 patients gained multifunctionality (IFNγ and TNF dual expression). To vigorously validate this finding, we analysed the CMV-specific memory T cells in the same PBMC samples. Evidently, the levels of IFNγ and TNF expression and the numbers of CMV-specific CD4+ and CD8+ memory T cells were all significantly greater than those of the corresponding SARS-CoV-2-specific memory T cells (Fig. 2a), ruling out the possibility that SARS-CoV-2 infection inhibits the function of T cells of the host. Recent epidemiological data show that between 18 and 62% of SARS-CoV-2 infections are asymptomatic[20–23]. Therefore, determining how well protective immunity is established in asymptomatic COVID-19 patients will provide valuable information for understanding herd immunity and the design of strategies to combat secondary infections by the virus. To this end, we compared the T-memory immunity levels between asymptomatic and symptomatic COVID-19 patients and showed that the sizes and quality of their memory pools are comparable. Only the in vitro expansion capacity of memory CD4+ from asymptomatic COVID-19 patients was significantly lower. Since our data showed the magnitude of in vitro expansion of CD4+ memory T cells is

correlated to the IgG titers of anti-RBD and anti-N, it is possible that the antibody production of asymptomatic individuals is lower than that of symptomatic individuals. This observation is consistent with the findings that there is a rapid decay of anti-SARS-CoV-2 antibodies and IgG antibodies in asymptomatic patients[24].

In agreement with recent reports[17,25], our data also demonstrated the presence of cross-reactive memory CD4+ and CD8+ T cells, which target various surface proteins of SARS-CoV-2, in unexposed healthy donors. However, the failure of these cross-reactive memory CD4+ and CD8+ to expand in vitro suggests they have limited potential to function as part of a protective immune response against SARS-CoV-2. It is noteworthy that the SARS-CoV-2-reactive T cells detected in the unexposed healthy donors in our study were lower than those detected by Grifoni et al.[17] and Braun et al.[26], but were consistent with those reported by Peng et al.[27] and Zhou et al.[28]. Assumably, due to the use of different methodologies in assessing SARS-CoV-2-specific T-cell responses, it is difficult to directly reconcile the cell-number data between different studies. Thus, a thorough investigation is needed to determine whether the cross-reactive T memory can provide any protective immunity and exert an influence on the outcomes of COVID-19 disease.

In summary, by examining a substantial number of clinical samples, we determined the SARS-CoV-2-specific memory T-cell immunity in COVID-19 patients with various clinical symptoms. Despite some subtle differences, most patients developed measurable amounts of SARS-CoV-2-specific CD4+ and CD8+ memory T cells which were stably maintained between 48–86 days after convalescence. Importantly, our discovery of the presence of significant levels of SARS-CoV-2-specific memory T-cell immunity in a group of individuals (close contacts) who were exposed to but not infected by the virus highlights some unique characteristics in the dynamic interactions between SARS-CoV-2 and its human host. Although cross-reactive memory T cells were present in healthy donors who had never been exposed to SARS-CoV-2, their role in host protection needs to be thoroughly investigated as they were hardly able to proliferate. Together, our analyses add important information on the landscape of immune responses of a range of individuals in response to the primary SARS-CoV-2 encounter during the first wave of the pandemic.

Methods

COVID-19 patients, close contacts, and healthy donors. For this study, we recruited 90 COVID-19 patients and 69 close contacts. All of the COVID-19 patients (NAT+) had stayed in the hospital and then recovered. The medical data collected from the COVID-19 patients included symptoms at disease onset and records of physical examinations, laboratory tests and imaging. Asymptomatic COVID-19 patients were defined using strict criteria: they were negative for any signs of cough, fever, sore throat, runny nose or computed tomography (CT) image changes in the lungs. A blood sample was taken from each of the patients in the period between d48 and d86 after disease onset or returning a NAT+ result.

Close contacts were identified from family members or friends who had stayed with a SARS-CoV-2 infected individual(s) at the time from 5 days before their disease onset to hospitalization. They were classified as a close contact only if they also were within a close distance (<1.5 m) of a COVID-19 individual(s) in a confined space for >1 h or were living together with a known case for >24 h. Other important criteria were that they were NAT- and negative for SARS-CoV-2-specific antibodies (IgG and IgM) against S RBD and/or N and virus neutralization tests. For this study, a blood sample was taken from each of the close contacts at the time d48 and d86 after exposure to a known COVID-19+ individual.

Blood samples of 63 healthy donors were obtained from a local blood donation center in September 2019 (before the start of the COVID-19 pandemic) for unrelated studies. These donors were considered healthy as they had no known history of any significant systemic diseases. As the blood samples from healthy donors were frozen for a longer period of time compared to those from patients and close contacts, we assessed whether prolonged freezing had any effect on assay outcomes by comparing the CMV-specific T-cell responses (which would be expected to be the same) of close contacts and healthy donors (HC) in a control experiment. We found that there is no significant difference in the frequencies of CMV-specific CD4+ and CD8+ between the two groups of samples (CD4+: $p = 0.32$ and CD8+: $p = 0.37$).

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This study is approved by the Ethics Commission of the First Affiliated Hospital of Guangzhou Medical University (No.2020-51). The signed consent forms from all the participants were obtained.

Peptide pool design and preparation. SARS-CoV-2-specific peptides were designed and synthesized as follows. The protein sequences were derived from the SARS-CoV-2 reference (GenBank: MN908947.3). Four hundred and forty-seven 15-mer SARS-CoV-2 epitopes (overlapping by 11 amino acids) spanning the entire antigen region of spike (S), nucleocapsid (N), membrane (M), and envelope (E) proteins were generated with an online peptide generator (Peptide 2.0), and were synthesized by GL Biochem Corporation (Shanghai) with a purity of over 80%. One hundred and ten 18-mer peptides (overlapping by 10 amino acids) encompassing the ORF1ab region of RNA-dependent RNA polymerase (RdRP) were synthesized by GL Biochem Corporation (Shanghai). Each peptide was dissolved in DMSO, and was then pooled, with each at a concentration of 45 μM to form a stock.

PBMC isolation and ex vivo stimulation. PBMCs were isolated from heparinized whole blood by density-gradient sedimentation using Ficoll-Paque according to the manufacturer's instructions (GE Healthcare, 17-1440-02). 1×10^6 PBMCs were cultured in RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated FBS (Biological Industries, Israel Beit-Haemek), 100 U/ml penicillin (Gibco) and 0.1 mg/ml streptomycin (Gibco). The PBMCs were treated with the peptide pool containing 447 15-mer peptides and 110 18-mer peptides at 125 nM/each peptide in the presence of 10 U/ml rIL-2 and 1 μM GolgiPlug (BD Biosciences, San Diego, CA) overnight at 37 °C, 5% CO_2. The approach of using a large peptide pool to stimulate PBMCs was based on that developed by Chevalier M. F. et al.[29] and was validated for CMV peptides.

PBMC in vitro expansion culture and stimulation. For in vitro culturing and stimulation, 1×10^6 PBMCs were treated with the peptide pool (125 nM/each peptide), and incubated for 10 days. During this culturing, half of the medium was changed twice per week with fresh PRMI 1640 supplemented with 10% FBS and 10 U/ml rIL-2. The cells were subcultured when needed. The cells were then re-stimulated at day 10 with a medium containing the peptide pool (125 nM/each peptide) overnight before being stained for FACS analysis.

Flow cytometry. Cells harvested from the overnight or 10-day stimulation cultures were washed and incubated with Live/dead aqua V510 for 15 min on ice. Cells were then washed again and surface-stained for 30 min on ice with the following antibodies: anti-CD3-FITC (BioLegend, clone UCHT1, 1:200, Cat# 300406), anti-CD4-APC-Cy7 (BD Pharmingen™, clone RPA-T4, 1:200, Cat# 561839), anti-CD8-PerCPCy5.5 (BD Bioscience, clone RPA-T8, 1:200, Cat# 560662). After fixation and permeabilization with Cytofix and Perm (BD Bioscience, Cat# 554714) on ice for 15 min, intracellular staining (ICS) was performed on ice for 30 min with anti-TNF-PE-Cy7 (BD, clone MAb11, 1:200, Cat # 557647) and anti-IFNγ-APC (BD Pharmingen™, clone B27, 1:200, Cat# 554702). After the final wash, cells were resuspended in 200 μl FACS buffer. The samples were acquired using an FACSAria III instrument (BD Bioscience) and analyzed with FlowJo software (Treestar).

Detection of blood plasma IgG in COVID-19 patients and close contacts. The SARS-CoV-2-specific IgG in the blood plasma was detected with two ELISA kits targeting N protein and S protein RBD, separately (Guangzhou Darui, China), and one chemiluminescent immunoassay kit targeting N plus S protein (Shenzhen YHLO Biotech, China). The IgG levels specific to N plus S protein was also determined by using a lateral flow immunochromatographic assay kit (DIAG-REAT, Beijing, China). For immunochromatographic assays, the optical signal was quantified with a time-resolved immunochromatographic analyzer and was calculated according to established programmed standards. The cut off value for the assignment of positive samples was determined according to the manufacture's instructions. An individual was considered seropositive if a positive result was generated by all three assays.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All relevant data are available from the authors.

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Competing interests

The authors declare no competing interests.

Additional information

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EXHIBIT 4

REVIEW

WILEY

Quantifying the risk of SARS-CoV-2 reinfection over time

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Summary

Despite over 140 million SARS-CoV-2 infections worldwide since the beginning of the pandemic, relatively few confirmed cases of SARS-CoV-2 reinfection have been reported. While immunity from SARS-CoV-2 infection is probable, at least in the short term, few studies have quantified the reinfection risk. To our knowledge, this is the first systematic review to synthesise the evidence on the risk of SARS-CoV-2 reinfection over time. A standardised protocol was employed, based on Cochrane methodology. Electronic databases and preprint servers were searched from 1 January 2020 to 19 February 2021. Eleven large cohort studies were identified that estimated the risk of SARS-CoV-2 reinfection over time, including three that enrolled healthcare workers and two that enrolled residents and staff of elderly care homes. Across studies, the total number of PCR-positive or antibody-positive participants at baseline was 615,777, and the maximum duration of follow-up was more than 10 months in three studies. Reinfection was an uncommon event (absolute rate 0%–1.1%), with no study reporting an increase in the risk of reinfection over time. Only one study estimated the population-level risk of reinfection based on whole genome sequencing in a subset of patients; the estimated risk was low (0.1% [95% CI: 0.08–0.11%]) with no evidence of waning immunity for up to 7 months following primary infection. These data suggest that naturally acquired SARS-CoV-2 immunity does not wane for at least 10 months post-infection. However, the applicability of these studies to new variants or to vaccine-induced immunity remains uncertain.

KEYWORDS
COVID-19, SARS-CoV-2, reinfection

1 | INTRODUCTION

Following the emergence of a novel coronavirus (SARS-CoV-2) in China in December 2019 and the declaration by WHO of a public health emergency of international concern on 30 January 2020, countries worldwide have experienced epidemics of Covid-19. While much is yet unknown about the immune response following infection with SARS-CoV-2, evidence is emerging at a fast pace. The Health Information and Quality Authority (HIQA) of Ireland has conducted a series of rapid reviews on various public health topics relating to

SARS-CoV-2 infection. These reviews arose directly from questions posed by policy makers and expert clinicians supporting the National Public Health Emergency Team to inform the national response to the pandemic in Ireland.

Our team at HIQA previously concluded that SARS-CoV-2 infection produces detectable immune responses in most cases.[1] However, the extent to which previously infected people are immune to reinfection is uncertain. In the short term, protection against reinfection is probable, as few confirmed SARS-CoV-2 reinfections have been reported despite over 140 million infections worldwide since the beginning of the pandemic.[2]

The objective of this systematic review was to evaluate the risk and relative risk of SARS-CoV-2 reinfection over time, comparing previously infected individuals to those without evidence of prior infection. The review informed a range of policy questions relating to the duration of protective immunity (as in, prevention of reinfection) following SARS-CoV-2 infection.

2 | METHODS

A standardised protocol was employed[3] based on Cochrane methodology.[4] Electronic databases (PubMed, EMBASE and EuropePMC) were searched from 1 January 2020 to 19 February 2021 (Data S1). Table 1 outlines the Population, Outcome, Study design (POS) criteria for study selection.

Reinfection was defined as any reverse transcription polymerase chain reaction (RT-PCR) or antigen-confirmed SARS-CoV-2 infection in an individual with evidence of a prior SARS-CoV-2 infection. Evidence of prior infection included a previously documented immune response through antibody detection (seropositivity) and/or a prior SARS-CoV-2 diagnosis by RT-PCR or antigen testing followed by recovery (molecular or clinical evidence of viral clearance). No minimum time interval was defined between primary and secondary infections; however, cases within 90 days of initial infection were considered suggestive of prolonged viral shedding following the primary infection.

All potentially eligible papers, including preprints, were exported to Endnote x8.2 and screened for relevance by one reviewer. Following removal of irrelevant citations, two reviewers independently reviewed the full text of potentially relevant articles. For each included study, data on study design, participant demographics and relevant clinical and laboratory data were extracted by two reviewers. Quality appraisal was undertaken using the National Heart, Lung and Blood Institute (NIH) quality assessment tool for observational cohort studies.[6] The findings of the research question were synthesised narratively due to the heterogeneity of study designs and outcome data.

3 | RESULTS

The collective database search resulted in 1893 citations, with four citations retrieved from other sources (grey literature search). Following removal of duplicates, 1771 citations were screened for relevance. This resulted in 105 studies eligible for full text review (Figure 1), where a further 94 studies were excluded (Table S1).

Eleven studies were identified that met the inclusion criteria.[7–17] Five studies were conducted in the United Kingdom,[8,9,11,13,14] of which three enrolled healthcare workers[8,9,11] and two enrolled the

TABLE 1 Population outcome Study design criteria for systematic search

Population	Individuals (of any age) with evidence of prior SARS-CoV-2 infection, who subsequently recovered[a]
	Evidence of prior infection includes diagnosis by RT-PCR or antigen testing, or evidence of an immune response through antibody detection (seropositivity)
Outcomes	1. Risk of RT-PCR or antigen-confirmed SARS-CoV-2 reinfection over time 2. Relative risk of RT-PCR or antigen-confirmed SARS-CoV-2 reinfection, comparing populations with evidence of prior infection with populations with no prior evidence of infection, at specified time points 3. RT-PCR cycle threshold results, if reported 4. Whole genome sequencing results of reinfected cases comparing first and second infections, if reported
Types of studies	Include: Observational cohort studies (prospective or retrospective) Exclude: • Cohort studies that enrolled fewer than 100 participants unless the study reported comparative whole genome sequencing on all reinfection cases • Studies with durations of follow-up of less than 3 months • Animal studies

Abbreviation: RT-PCR, reverse transcription polymerase chain reaction.

[a]'Recovered' refers to molecular or clinical evidence of viral clearance following initial infection; definitions of recovery in primary studies were used. Common definitions include two consecutive negative respiratory RT-PCR tests 24 h apart and WHO clinical criteria of viral clearance (27 May 2020).[5]

FIGURE 1 PRISMA diagram of study selection
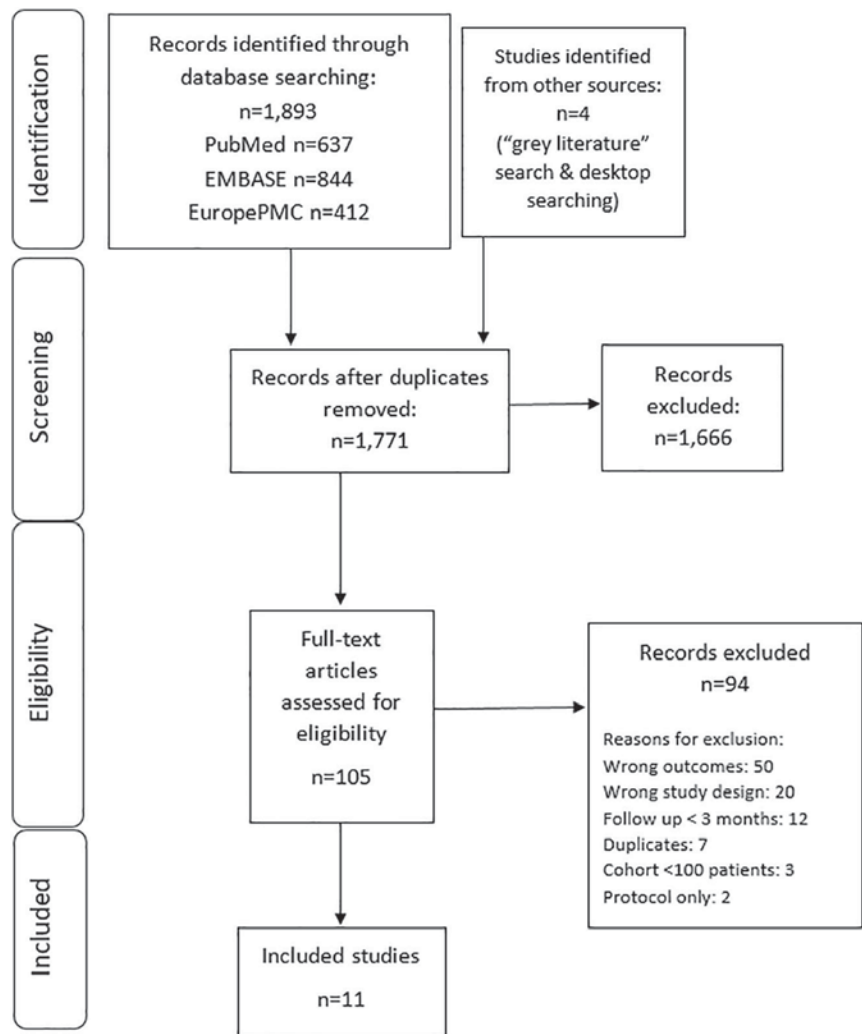
staff and residents of elderly care homes.[13,14] The remaining six studies were all general population studies, conducted in Austria,[16] Denmark,[17] Israel,[12] Qatar [7] and the United States.[10,15] Six studies were published as preprints at the time of submission.[7,8,10,12,14,15] Across studies, the total number of PCR- or antibody-positive participants at baseline was 615,777 (median: 8845; range: 88–378,606). The median follow-up of individuals within studies was 131 days (4.4 months; range of medians: 54–210 days), with a maximum follow-up of ≥300 days (10 months) in three studies.[12,14,16]

Studies reported a range of primary endpoints (Table 2 and Table S2). Studies either determined evidence of prior infection based on a history of RT-PCR confirmed infection ($n = 5$ studies),[10,12,15–17] documented antibody detection ($n = 4$ studies)[7,8,11,14] or a combination of both ($n = 2$ studies).[9,13] Three studies separately reported the relative risks of symptomatic reinfections and 'all' reinfections (symptomatic/asymptomatic),[8,11,15] one study reported symptomatic reinfections only[9] and the remaining studies did not differentiate between symptomatic and asymptomatic reinfections.[7,10,12–17] In addition to quantifying the absolute risks of SARS-CoV-2 reinfection, the risks compared with PCR-negative or antibody-negative cohorts at baseline were expressed by a number of different measures, such as relative risks, odds ratios, risk ratios and hazard ratios. Due to heterogeneity in outcome measures and populations, meta-analysis of data were not considered appropriate. The following sections narratively report the findings of included studies by population group (general population, healthcare workers, and residents and staff of care homes).

3.1 | General population studies

3.1.1 | Austria

In the study by Pilz et al.,[16] national SARS-CoV-2 infection data from the Austrian epidemiological reporting system were used to investigate potential reinfection events, with a maximum follow-up of 10 months. The primary outcome was the odds of PCR positivity in individuals who recovered from a confirmed SARS-CoV-2 infection during the first wave (22 February to 30 April 2020) compared with the odds of first infections in the remainder of the general population during the second wave (1 September to 30 November 2020). In total, 40 possible reinfections were recorded out of 14,840

TABLE 2 Summary of included studies and primary outcome results

First author; country; population	Participants[a] Follow-up	Author reported primary outcomes
Abu-Raddad 2021[7] (preprint); Qatar; General population	N = 43,044 antibody-positive at baseline **Median f/u:** 114 days (3.8 months) **Maximum f/u:** 242 days (8.1 months)	**Risk of reinfection (confirmed by WGS)[b]:** 0.10% (95% CI: 0.08%–0.11%) **Risk over time (any reinfection):** Incidence rate of reinfection by month of follow-up did not show any evidence of waning of immunity over seven months of follow-up
Hall 2021[8] (preprint); United Kingdom; HCWs	N = 6614 antibody-positive at baseline **Median f/u:** 202 days (6.7 months) **Maximum f/u:** 227 days (7.6 months)	**Adjusted odds ratio of reinfection comparing antibody or PCR-positive group with negative group** • 'Probable' reinfection[c]: aOR: 0.01 (95% CI 0.00–0.03) • All 'possible' and 'probable' reinfections: aOR: 0.17 (95% CI: 0.13–0.24) • Symptomatic reinfection: aOR: 0.08 (95% CI 0.05–0.13)
Hanrath 2020[9] United Kingdom; HCWs	N = 1038 PCR and/or antibody-positive at baseline **Median f/u:** 173 days (5.8 months) **Maximum f/u:** 229 days (7.6 months)	**Symptomatic reinfection:** A positive PCR test was returned in 0/1038 (0% [95% CI: 0–0.4) of those with previous infection, compared with 290/10,137 (2.9% [95% CI: 2.6–3.2) of those without ($p < 0.0001$ χ2 test)
Hansen 2021[17] Denmark; General population	N = 11,068 PCR positive at baseline **Median f/u:** 122 days (4.1 months) **Maximum f/u:** 295 days (9.8 months)	**Main analysis:** **aRR (any reinfection):** 0.20 (0.16–0.25). This represents 72 reinfections out of 1,346,920 person-days in PCR-positive group, compared with 16,819 new infections out of 62,151,056 person-days in PCR-negative group **Additional cohort analysis (that includes all infection periods):** aRR = 0.21 (0.18–0.25) by age group: • 0–34 years: aRR = 0.17 (0.13–0.23) • 35–49 years: aRR = 0.20 (0.14–0.28) • 50–64 years: aRR = 0.19 (0.13–0.27) • ≥65 years: aRR = 0.53 (0.37–0.75)
Harvey 2020[10] (preprint); United States; General population	N = 378,606 PCR positive at baseline **Median f/u:** 54 days (1.8 months) **Maximum f/u:** 92 days (3.1 months)	Ratio of positive NAAT results (comparing patients who had a positive antibody test at index vs. those without)[d]: 2.85 (95% CI: 2.73–2.97) at 0-30 days; 0.67 (95% CI: 0.6–0.74) at 31–60 days; 0.29 (95% CI: 0.24–0.35) at 60–90 days; 0.10 (95% CI: 0.05–0.19) at >90 days; note that NAAT positivity at <90 days is likely due to prolonged viral shedding
Jeffery-Smith 2021[13] United Kingdom; Staff &residents at care homes	N = 88 PCR and/or antibody-positive at baseline **Mean f/u:** 120 days (4 months) **Maximum f/u:** Unclear	**Relative risk (any reinfection):** 0.04 (95% CI: 0.005–0.27) This represents 1 reinfection out of 88 in seropositive group compared with 22/73 in seronegative group
Krutikov 2021[14] (preprint); United Kingdom; Staff & residents at care homes	N = 634 antibody-positive at baseline **Median f/u:** 79 days (2.6 months) **Maximum f/u:** 300 days (10 months)	**Relative adjusted hazard ratios (any reinfection):** Residents of care home: aHR = 0.15 (0.05–0.44)[e] Staff of care home: aHR = 0.39 (0.19–0.82)[e]
Lumley 2021[11] United Kingdom; HCWs	N = 1265 antibody-positive at baseline **Median f/u:** 139 days (4.6 months) **Maximum f/u:** 217 days (7.2 months)	**IRR[f](any reinfection):** 0.12 (95% CI: 0.03–0.47; $p = 0.002$); 2/1265 seropositive (both asymptomatic reinfections) and N = 223/11,364 seronegative had positive PCR. **Symptomatic reinfection:** Incidence was 0.60 per 10,000 days at risk in seronegative HCWs; there were no symptomatic infections in seropositive HCWs **Adjusted IRR[g]:** 0.11 (95% CI: 0.03–0.44; $p = 0.002$) (any reinfection)

TABLE 2 (Continued)

First author; country; population	Participants[a] Follow-up	Author reported primary outcomes
Perez 2021[12] (preprint); Israel; General population	N = 149,735 PCR positive at baseline **Median f/u:** 165 days (5.5 months) **Maximum f/u:** Approx. 325 days[h] (10.8 months)	**Overall reinfection risk:** 0.1% (any reinfection between Mar 2020 and Jan 2021) This represents 154 individuals who had two positive tests at least 100 days apart out of 149,735 individuals with a record of a prior positive PCR test
Pilz 2021[16] Austria; General population	N = 14,840 PCR positive at baseline **Median f/u:** 210 days (7 months) **Maximum f/u:** 300 days (10 months)	**Odds ratio:** 0.09 (95% CI: 0.07–0.13) (any reinfection) This represents 40 reinfections out of 14,840 individuals PCR positive in the first wave (0.27%) compared with 253,581 infections out of 8,885,640 (2.85%) in the remaining general population
Sheehan 2021[15] (preprint); United States; General population	N = 8845 PCR positive at baseline **Median f/u:** 131 days (4.4 months) **Maximum f/u:** 269 days (9 months)	Protective effectiveness (any reinfection): 78.5% (95% CI: 72.0%–83.5%)[i] Protective effectiveness against symptomatic infection: 83.1% (95% CI: 75.1%–88.5%)

Note: 'Any' reinfection—all reinfections, both symptomatic and asymptomatic. Numbers rounded to two decimal points. No cases were identified on the basis of antigen testing. The longest duration of follow-up was not stated in all studies or was provided only as an approximate estimate; when not stated, duration of follow-up was inferred from figures or tables within the study.

Abbreviations: aHR, adjusted hazard ratio; aOR, adjusted odds ratio (adjusted for week group); ARR, adjusted rate ratio; CI, confidence interval; f/u, follow-up; HCW, healthcare worker; IRR, incidence rate ratio; NAAT, nucleic acid amplification test; WGS, whole genome sequencing.

[a]In the baseline antibody and or PCR-positive group ('seropositive' or prior positive cohort).

[b]Based on cases with WGS confirming the first and second infections were from different viral strains (N = 16).

[c]'Possible' reinfection was defined as a participant with two PCR-positive samples ≥90 days apart with available genomic data, or an antibody-positive participant with a new positive PCR at least 4 weeks after the first antibody-positive result. A 'probable' case additionally required supportive quantitative serological data and or supportive viral genomic data from confirmatory samples.

[d]NAAT used as proxy; includes all symptomatic reinfections and prolonged viral shedding, comparing patients who had a positive antibody test at index versus those with a negative antibody.

[e]Multivariate analysis of risk of PCR-positive infection by baseline antibody status, stratified by LTCF and adjusted for sex and age.

[f]IRR is the relative incidence of subsequent positive SARS-CoV-2 PCR tests and symptomatic infections comparing antibody-positive and antibody-negative groups at baseline.

[g]After adjustment for age, gender and month of testing or calendar time as a continuous variable.

[h]The midpoint of a range of follow-up dates was taken (300–349 days).

[i]Authors report effectiveness with the following calculation: $1 - ([56/8845]/[4163/141480])$.

individuals with a history of prior infection during the first wave (0.27%), compared with 253,581 infections out of 8,885,640 individuals of the remaining general population (2.85%). This translated into an odds ratio of 0.09 (95% CI: 0.07–0.13).

3.1.2 | Denmark

In the study by Hansen et al.,[17] individual-level data were collected on patients who had been tested in Denmark in 2020 from the Danish Microbiology Database, with a maximum follow-up of 9.8 months. Infection rates were analysed during the second wave of the COVID-19 epidemic, from 1 September 2020 to 31 December 2020, comparing PCR-positive individuals with PCR-negative individuals during the first wave (March to May 2020). During the first wave (prior to June 2020), 533,381 people were tested, of whom 11,727 (2.2%) were PCR positive. Of these, 525,339 were eligible for follow-up in the second wave, of whom

11,068 (2.11%) had tested positive during the first wave. Among eligible PCR-positive individuals from the first wave, 72 (0.65%, 95% CI: 0.51%–0.82%) tested positive again during the second wave compared with 16,819 of 514,271 (3.27%, 95% CI: 3.22%–3.32%) who tested negative during the first wave. After adjusting for sex, age group and test frequency, the adjusted RR (aRR) of reinfection was 0.20 (95% CI: 0.16–0.25). Protection against repeat infection was estimated at 80.5% (95% CI: 75.4–84.5). In an alternative analysis, aRR by age category was reported. In individuals aged 65 years or more, the aRR was 0.53 (0.37–0.75), compared with 0.17, 0.20 and 0.19 in individuals aged 0–34 years, 35–49 years and 50–64 years, respectively.

3.1.3 | Israel

In the study by Perez et al.,[12] published as a preprint, preliminary reinfection rates within the members of a large healthcare provider

(Maccabi Healthcare Services) in Israel were reported, with a maximum follow-up of over 10 months. A total of 149,735 individuals had a recorded positive PCR test between March 2020 and January 2021. Among them, 154 members had two positive PCR tests at least 100 days apart and were included in this study. The reinfection rate was estimated at approximately 0.1%. In this cohort, 73 individuals (47.4%) had symptoms at both PCR-positive events.

3.1.4 | Qatar

In the study by Abu-Raddad et al., published as a preprint, 43,044 anti-SARS-CoV-2 nucleocapsid antibody-positive participants were followed for up to 8 months for evidence of reinfection.[7] This retrospective cohort was identified from a database that covers all serological testing for SARS-CoV-2 conducted in Qatar.

There was evidence of a decreasing trend in the incidence rate of reinfection with each additional month of follow-up from the first month (incidence rate: 0.97 per 10,000; 52 cases per 167,149 person-weeks) to the sixth month (zero cases per 19,148 person-weeks) (Mantel-Haenszel trend analysis p-value: <0.001), noting that early reinfection cases (i.e., within 3 months) were likely due to persistent viral shedding following the primary infection. There was an increase at ≥7 months; however, this was based on only one case of reinfection (out of 3094 person-weeks). Applying a confirmation rate obtained through viral genome sequencing in a subset of patients with supporting clinical evidence for reinfection, the risk of documented reinfection was 0.1% (95% CI: 0.08%–0.11%).

These reinfections were compared to a cohort of 149,923 antibody-negative individuals followed for a median of 17 weeks (range: 0–45.6 weeks). Risk of infection was estimated at 2.15% (95% CI: 2.08%–2.22%). The efficacy of natural infection in protecting against reinfection was estimated at 95.2% (95% CI: 94.1%–96.0%).

3.1.5 | United States

Two US studies were identified, both published as preprints. In the first, a retrospective database analysis of electronic health records was used to determine the risk of nucleic acid amplification technology (NAAT) test positivity, a proxy for reinfection, over a maximum follow-up of 3.1 months (Harvey et al.[10]). Of 3,257,478 unique patients with an index antibody test, 378,606 (11.6%) had a positive antibody result at baseline. The ratio of positive NAAT test results among patients who had a positive antibody test at index versus those with a negative antibody test at index declined from 2.85 (95% CI: 2.73–2.97) at 0–30 days; to 0.67 (95% CI: 0.6–0.74) at 31–60 days; to 0.29 (95% CI: 0.24–0.35) at 60–90 days and to 0.10 (95% CI: 0.05–0.19) at >90 days.

In the second, 150,325 patients were followed for a maximum of 10 months (Sheehan et al.[15]). In total, 56 reinfections were identified from the positive cohort of 8845 individuals, compared with 4163 infections from the negative cohort of 141,480 individuals. The protective effectiveness of prior infection against reinfection was

estimated at 78.5% (95% CI: 72.0–83.5) and 83.1% (95% CI: 75.1–88.5) against symptomatic reinfection.

3.2 | Healthcare workers

Three UK studies were identified that exclusively enrolled healthcare workers. In the first study, published as a preprint, 20,787 hospital staff were followed, of whom 32% ($n = 6614$) were assigned to the positive cohort (antibody or PCR positive) and 68% ($n = 14,173$) to the negative cohort (antibody negative, not previously known to be PCR or antibody positive) (Hall et al.[8]). In total, 1,339,078 days of follow-up data were analysed from the baseline positive cohort (maximum follow-up of 7.6 months). In total, 44 reinfections (2 probable and 42 possible) were detected in the baseline positive cohort (15 of which were symptomatic), compared with 318 new PCR-positive infections (249 of which were symptomatic) and 94 antibody seroconversions in the negative cohort. The adjusted odds ratio (aOR) was 0.17 for all reinfections ('possible' or 'probable'; 95% CI: 0.13–0.24). Restricting reinfections to probable reinfections only, participants in the positive cohort had a 99% lower odds of probable reinfection (aOR of 0.01, 95% CI: 0.00–0.03). Restricting reinfections to those who were symptomatic, investigators estimated that participants in the positive cohort had an aOR of 0.08 (95% CI 0.05–0.13).

In the second study, 1038 healthcare workers with evidence of previous infection (PCR and or antibody positive) and 10,137 without (negative antibody and PCR) were followed for a maximum of 7.6 months (Hanrath et al.[9]). A positive PCR test was returned in 0% (0/1038 [95% CI: 0%–0.4%]) of those with previous infection, compared to 2.9% (290/10,137 [95% CI: 2.6–3.2]) of those without ($p < 0.0001$, χ2 test).

In the third study, 12,541 UK healthcare workers were followed for up to 31 weeks to compare the incidence of SARS-CoV-2 infection in seropositive ($N = 1265$, including 88 who seroconverted during follow-up) versus seronegative ($N = 11,364$) groups at baseline (Lumley et al.[11]). A total of 223 anti-spike seronegative healthcare workers had a positive PCR test, 100 during screening while they were asymptomatic and 123 while symptomatic, whereas two anti-spike seropositive healthcare workers had a positive PCR test; both workers were asymptomatic when tested. Incidence varied by calendar time, reflecting the first (March through April) and second (October and November) waves of the pandemic in the United Kingdom and was consistently higher in seronegative healthcare workers. After adjustment for age, gender and month of testing or calendar time as a continuous variable, the incidence rate ratio in seropositive workers was 0.11 (95% CI: 0.03–0.44) compared with those who were seronegative at baseline.

3.3 | Residents and staff of elderly care homes

Two studies were identified that enrolled both residents and staff at UK care homes.[13,14]

In the first study (Jeffery-Smith et al.[13]), the risk of reinfection according to antibody seropositivity was investigated following outbreaks in two London care homes[13,18] over 4 months. The median age of residents was 84 and 85 in each care home.

In total, 88 individuals with evidence of prior infection were investigated for evidence of reinfection (antibody positive $N = 87$; PCR positive $N = 1$). The reinfection rate in this cohort was 1/88 (1.1%), and this reinfection event was observed in a staff member. By comparison, infection risk in the seronegative cohort was 30.1% (22/73, including four people diagnosed by seroconversion). The RR was estimated at 0.038 (95% CI: 0.005–0.273). The protection against reinfection after four months in seropositive group was estimated at 96.2% (95% CI: 72.7%–99.5%).

In the second study, published as a preprint, staff and residents in 100 long-term care facilities (LTCFs) in England were followed between October 2020 and February 2021 (Krutikov et al.[14]). In total, 2111 individuals were enrolled (682 residents and 1429 staff). The median age of residents was 86 years (IQR: 79–91) and 47 years for staff (IQR range: 34–56). Blood sampling was offered to all participants at three time points separated by 6–8 weeks intervals in June, August and October 2020. Samples were tested for IgG antibodies to nucleocapsid and spike protein. PCR testing for SARS-CoV-2 was undertaken weekly in staff and monthly in residents. The primary analysis estimated the adjusted hazard ratio (aHR) of a PCR-positive test by baseline antibody status (Cox regression adjusted for age and gender, and stratified by LTCF).

IgG antibodies to nucleocapsid were detected at baseline in 226 residents (33%) and 408 staff (29%). Staff and residents contributed 3749 and 1809 months of follow-up time, respectively. There were 93 PCR-positive tests in seronegative residents (0.054 per month at risk) compared with four in seropositive residents (0.007 per month at risk). There were 111 PCR-positive tests in seronegative staff (0.042 per month at risk) compared with 10 in seropositive staff (0.009 per month at risk). Controlling for the potential confounding effect of individual LTCFs, the relative aHRs for PCR-positive infection were 0.15 (95% CI: 0.05–0.44) and 0.39 (95% CI: 0.19–0.82) comparing seropositive versus seronegative residents and staff, respectively. Study authors concluded that the presence of IgG antibodies to nucleocapsid was associated with substantially reduced risk of reinfection in staff and residents for up to 10 months after primary infection, assuming that the earliest infections occurred in March 2020.

3.4 | Quality of included studies

The NIH quality assessment tools was used for appraisal of observational cohort studies.[6] Ten studies were considered of 'good' or 'fair' methodological quality (Table S3), with one study[10] that used a proxy measure for outcomes (NAAT test positivity) considered to be of poor quality.

Each of the 10 studies of 'good' ($n = 4$) or 'fair' ($n = 6$) methodological quality was considered large enough to adequately capture reinfection events in their respective populations. A number of studies was downgraded due to lack of controlling for confounders ($n = 7$ studies). In these studies, potential confounding variables were either not assessed or not measured appropriately, or the statistical analysis was not adequately described. As all studies were observational in nature, they cannot be used to demonstrate causality. Therefore, only associations between prior infection and reinfection risk can be measured. While estimates of the effectiveness of natural infection to prevent reinfection were reported in a number of studies, such measures cannot be reliably estimated on the basis of these data.

Six studies are currently published as preprints,[7,8,10,12,14,15] so have not yet been formally peer-reviewed, raising additional concerns about overall quality and the potential for results to change prior to formal publication.

4 | DISCUSSION

4.1 | Summary of findings

Eleven cohort studies estimated the risk or relative risk of SARS-CoV-2 reinfection in individuals who were either antibody-positive or who had a history of PCR-confirmed Covid-19 at baseline, compared with those who did not, for up to 10 months. Across studies, the total number of PCR- or antibody-positive participants at baseline was 615,777, with a maximum follow-up of over 10 months in three studies. Reinfection was a rare event (median PCR-confirmed reinfection rate: 0.27%, range: 0%–1.1%), with no study reporting an increase in the risk of reinfection over time.

Of the six general population studies, only one estimated the population-level risk of reinfection based on whole genome sequencing in a subset of patients with supporting evidence of reinfection.[7] The estimated risk was low (0.1% [95% CI: 0.08%–0.11%]) in this large cohort of 43,044 anti-SARS-CoV-2 nucleocapsid antibody-positive participants. Importantly, the incidence rate of reinfection by month did not show any evidence of waning of immunity over the seven months of follow-up. The remaining population-based studies (conducted in Austria, Denmark, Israel and the United States) also reported low absolute and relative risks of reinfection, and none reported an increased risk over time.

Only one study reported the relative risk of reinfection by age category, allowing comparisons across groups. In individuals aged 65 years or more, the aRR was 0.53 (0.37–0.75), compared with 0.17, 0.20 and 0.19 in individuals aged 0–34 years, 35–49 years and 50–64 years, respectively.[17] The lower protection in the over-65s group may be attributable to immunosenescence; however, little is known about this phenomenon in the context of COVID-19.

Two UK studies reported lower risks of reinfection in elderly individuals. Both studies enrolled residents of care homes (median age ≥84 years), a group that has been disproportionately affected by the COVID-19 pandemic, with high rates of infection and deaths among frail, elderly residents. In the first study, the relative risk of reinfection in staff and residents of two London care homes was very low

(RR = 0.038; 95% CI: 0.005–0.273), and the protection against reinfection after four months in seropositive group was estimated at 96.2% (95% CI: 72.7%–99.5%).[13] This relative risk was based on a single reinfection event in a seropositive staff member, indicating the relative risk in the elderly resident cohort is even lower. The second study reported higher relative rates of reinfection[14] in a sample of staff and residents (N = 2111) across 100 LTCFs in England. The study, conducted between October 2020 and February 2021, coincided with a period of high community prevalence of SARS-CoV-2 in the United Kingdom, associated with the rapid emergence of the B.1.1.7 variant.[19] The estimated aHR for reinfection was 0.15 (95% CI: 0.05–0.44) in residents and 0.39 (95% CI: 0.19–0.82) in staff. The higher relative rates of infection compared with the earlier UK study raises concerns regarding the impact of new variants on the protective immunity of natural infection. Nonetheless, only four cases of possible reinfection were identified in residents, and although all cases reported symptoms, none required hospital treatment. Taking into consideration that most residents were likely first infected during the first wave (up to 6 months prior), the risk of reinfection was substantially reduced in residents even in the context of high community transmission of the B.1.1.7 variant.

Three UK studies estimated the relative risk of reinfection specifically among healthcare workers.[8,9,11] The first study detected zero symptomatic infections in 1038 healthcare workers with evidence of a prior infection, compared with 290 in 10,137 without evidence of prior infection ($p < 0.0001$).[9] The second study detected two asymptomatic infections (and no symptomatic infections) out of 1265 seropositive individuals, compared with 223 infections (100 during screening while they were asymptomatic and 123 while symptomatic) out of 11,364 seronegative individuals.[11] After adjustment for age, gender and month of testing or calendar time, the incidence rate ratio in seropositive healthcare workers was 0.11 (95% CI: 0.03–0.44). The third study reported 44 reinfections in the baseline positive cohort of 6614 individuals (15 of which were symptomatic), compared with 318 new PCR-positive infections (249 of which were symptomatic) and 94 antibody seroconversions in the negative cohort of 14,173 individuals.[8] The aOR was 0.17 for all reinfections (95% CI: 0.13–0.24), and restricting reinfections to those who were symptomatic, the aOR was 0.08 (95% CI 0.05–0.13). This pattern of a lower relative risk of symptomatic reinfections in healthcare workers, compared with 'any' reinfection (symptomatic and asymptomatic), was also observed in the study by Sheehan et al. in general populations.[15] This finding suggests that not only is the risk of reinfection following natural infection low, when it does occur, it may represent a less severe form of disease.

4.2 | Strengths and limitations

To our knowledge, this is the first systematic review to quantify the risk of SARS-CoV-2 reinfection over time. All studies were considered large enough to adequately capture reinfection events in their respective populations. Results across studies consistently demonstrated a substantially lower risk of reinfection in previously infected individuals without a waning of the protective response over time. However, despite these strengths, there are a number of limitations associated with this review.

First, as the studies are observational in nature, the prevention of reinfection cannot be causally confirmed, although longitudinal associations can be estimated. Additional concerns relating to observational studies include the greater potential for bias. It is possible that antibody test results affected individual behaviour. Individuals with evidence of prior infection may have believed that they possessed immunity to SARS-CoV-2, resulting in a reduction in health-seeking behaviour and testing (outcome ascertainment bias). Conversely, these individuals may have increased their engagement in social behaviour, placing them at greater risk for infection. The overall direction of bias (whether over- or under-estimating reinfection) cannot be determined.

Second, studies included in this review could not determine whether past seroconversion, or current antibody levels, determine protection from infection. Furthermore, none could define which characteristics are associated with reinfection. For example, there is evidence to suggest immune responses are weaker following asymptomatic SARS-CoV-2 infections[20] and in immunocompromised patients,[21] which may increase susceptibility to repeat infection. Mucosal immunity and neutralising antibodies present in respiratory secretions may be more important for sterilising immunity than circulating IgG levels. The role of T-cell immunity was not assessed in any study; therefore, it is not possible to determine whether protection from reinfection is conferred through the measured antibodies or T-cell immunity. Future longitudinal serological cohorts may be able to determine protective correlates of immunity.

Third, only two studies undertook genomic sequencing of reinfected cases; consequently, the results of nine studies are only based on potential reinfections. The effect of this, however, is to overestimate the number of reinfections, thereby affirming the conclusion that reinfection is rare.

Fourth, due to the nature of a number of retrospective database analyses included in this review, many studies could not correlate symptomatic infections with protection against repeat infection or evaluate disease progression comparing first and second infections. This was true for studies that accessed large databases in Austria,[16] Denmark[17] and the United States.[10]

Finally, this review included a number of studies that were published as preprints (n = 6 studies[7,8,10,12,14,15]). While preprints have been pivotal to guide policy and practice throughout this pandemic, these studies have not yet been formally peer-reviewed raising concerns over the quality and accuracy of presented data.

4.3 | Generalisability of findings

There are a number of issues relating to the applicability and generalisability of the presented results. First, all but two studies preceded the widespread identification and spread of a number of new

viral strains of international concern (e.g., variant 202012/01 [also known as 501Y.V1/B.1.1.7] from the United Kingdom and 501Y.V2 [B.1.351] from South Africa, both identified in December 2020[22]). In the first study that extended beyond December 2020, reinfection events between March 2020 and January 2021 in Israel were recorded.[12] A higher number of reinfections was recorded in January 2021 compared with previous months. However, genomic sequencing was not reported and statistical analysis of the recorded data (e.g., controlling for confounders and significance testing) was not undertaken. In the second study, elderly care home staff and residents in the United Kingdom were followed between October 2020 and February 2021.[14] Sequencing data were not available for suspected reinfections, and study authors did not investigate the potential impact of new variants on the risk of reinfection. Nonetheless, the risk of reinfection was substantially reduced in elderly residents, most of whom were first infected up to 6 months previously. While these findings are reassuring, further research is needed on the role of natural immunity in populations that are experiencing the emergence and spread of new variants of concern.

Second, all presented data relate to unvaccinated cohorts as they preceded vaccine roll-out in 10 studies, and in the only study that was conducted during vaccine roll-out, all vaccinated individuals were excluded once 12 days had passed since their vaccination.[14] The applicability of the data to vaccinated populations is therefore unknown.

One preprint study (Lumley et al., 2021[23]), identified after our database search, reported reinfection rates among healthcare workers according to vaccination status and in relation to the B.1.1.7 variant. This study updates the 2020 study included in this review by the same authors[11] and presents data up to 28 February 2021. At this time point, 1456 of 13,109 participating healthcare workers had received two vaccine doses (Pfizer-BioNTech or Oxford-AstraZeneca). Compared to unvaccinated seronegative healthcare workers, natural immunity and two vaccination doses provided similar protection against symptomatic infection: no healthcare worker who had received two vaccine doses had a symptomatic infection, and incidence was 98% lower in seropositive healthcare workers (adjusted incidence rate ratio 0.02, 95% CI: <0.01–0.18). Two vaccine doses or seropositivity reduced the incidence of any PCR-positive result with or without symptoms by 90% (0.10, 95% CI: 0.02–0.38) and 85% (0.15 95% CI: 0.08–0.26) respectively. There was no evidence of differences in immunity induced by natural infection and vaccination for infections with the B.1.1.7 variant. These data suggest that both natural infection and vaccination both provide robust protection against SARS-CoV-2 infection, including against the B.1.1.7 variant. Future studies are expected to expand our understanding of the differences between natural and vaccine-acquired immunity and the impact of new variants.

Third, there is much uncertainty in relation to the risk of reinfection in younger and older age groups. Inconsistent data were identified relating to elderly populations, with one study reporting higher rates of reinfection compared with younger age groups[17] and two reporting low rates of reinfection in elderly residents of care homes (although these two studies did not compare risk across age groups).[13,14]

4.4 | Research in context and policy implications

This review was expected to inform a range of policy questions relating to the duration of protective immunity following infection with SARS-CoV-2, such as:

- How long can asymptomatic individuals who have recovered from a prior SARS-CoV-2 infection be exempted from restriction of movement policies if they become a close contact of a confirmed COVID-19 case?
- How long can asymptomatic individuals who have recovered from a prior SARS-CoV-2 infection be exempted from serial testing programmes?
- How long can asymptomatic patients who have recovered from a prior SARS-CoV-2 infection be exempted from the requirement for testing prior to scheduled admission to hospital?

This review identified a large body of evidence that indicates the duration of presumptive protective immunity may last for at up to 10 months post-infection. However, given the uncertainty that exists relating to reinfection potential with emerging variants, any policy changes may not be applicable to possible exposure to emerging immune escape variants of concern. In addition, policies should be kept under review and informed by the international evidence and national surveillance data. In light of the findings of this review, policy was updated in Ireland to extend the period of presumptive immunity from 3 months to 6 months; therefore, a person who is an asymptomatic contact of a case and has had a positive test result within the previous 6 months is exempt from restriction of movements and serial testing. A period of 6 months was selected over 10 months due to the ongoing uncertainties relating to new variants.

Increasingly, reinfection cases are being investigated on a country level and are reported on websites of national public health agencies (e.g., Czechia now report a national reinfection rate of 0.1%, or 1400 cases out of 1,225,000 infections[24]). Future longitudinal studies should focus on the following issues that were not addressed in the aforementioned studies, including:

- The durability of immunity beyond 10 months
- Immune correlates of protection
- Protective immunity in populations with comorbidities and the immunocompromised
- The impact of new variants on protective immunity

5 | CONCLUSIONS

Eleven large cohort studies were identified that estimated the risk of SARS-CoV-2 reinfection over time, including three that enrolled healthcare workers and two that enrolled elderly care home residents. All studies reported low relative SARS-CoV-2 reinfection rates in individuals with prior evidence of infection, compared with those

without, for up to 10 months. The relative risk of reinfection was low across studies, although there was some inconsistent evidence of a higher risk in older populations compared with younger populations. A limitation of this review was the uncertainty regarding the applicability of data to new variants of concern and to vaccinated populations.

CONFLICT OF INTEREST

No conflict of interest declared.

AUTHOR CONTRIBUTIONS

Eamon O Murchu: Investigation, formal analysis and writing-original draft. **Paula Byrne:** Investigation and writing-original draft. **Paul G. Carty:** Investigation and writing-original draft. **Cillian De Gascun:** Writing-reviewing and editing. **Mary /Keogan:** Writing-reviewing and editing. **Michelle O'Neill:** Supervision, writing-reviewing and editing. **Patricia Harrington:** Supervision, writing-reviewing and editing. **Máirín Ryan:** Supervision, writing-reviewing and editing. All authors attest they meet the ICMJE criteria for authorship.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

EXHIBIT 5

Comparison of kinetics of immune responses to SARS-CoV-2 proteins in individuals with varying severity of infection and following a single dose of the AZD1222

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NOTE: This preprint reports new research that has not been certified by peer review and should not be used to guide clinical practice.

Abstract

Background: While there have been many studies characterizing the IgG and IgA responses to different SARS-CoV-2 proteins in individuals with natural infection, the induction of IgG and IgA to different viral proteins in vaccinees have not been extensively studied. Therefore, we sought to investigate the antibody responses to SARS-CoV-2 following natural infection and following a single dose of AZD2221, in Sri Lankan individuals.

Methods: Using Luminex assays, we characterized the IgG and IgA responses in patients with varying severity of illness and following a single dose of the vaccine at 4 weeks and 12 weeks since onset of illness or following vaccination. Haemagglutination test (HAT) was used to assess the antibodies to the receptor binding domain of SARS-CoV-2 wild type (WT), B.1.1.7, B.1.351 and B.1.617.2 (VOCs) and surrogate neutralizing test to measure ACE2 receptor blocking antibodies.

Results: Those with mild illness and in vaccinees, the IgG responses to S1, S2, RBD and N protein increased from 4 weeks to 12 weeks, while it remained unchanged in those with moderate/severe illness. Those who had a febrile illness in 2017 and 2018 (controls) also gave IgG and IgA high responses to the S2 subunit. In the vaccinees, the most significant rise was seen for the IgG antibodies to the S2 subunit (p<0.0001). Vaccinees had several fold lower IgA antibodies to all the SARS-CoV-2 proteins tested than those with mild and moderate/severe illness at 4 weeks and 12 weeks. At 12 weeks the HAT titres were significantly lower to the B.1.1.7 in vaccinees and significantly lower in those with mild illness, and in vaccinees to B.1.351 and for B.1.617.2. No such difference was seen in those with moderate/severe illness.

Conclusions: Vaccinees had significantly less IgA to SARS-CoV-2, but comparable IgG responses to those with natural infection. However, following a single dose, vaccinees had reduced antibody levels to the variants of concern (VOC), which further declined with time, compared to natural infection.

Introduction

The COVID-19 pandemic due to SARS-CoV-2, continues to cause significant mortality and morbidity, and many countries are currently experiencing a worse situation, than at the beginning of the pandemic[1]. Emergence of SARS-CoV-2 variants of concern such as the B.1.1.7 (alpha) and more recently B.1.617.2 (delta) has led to exponential increase of the number of COVID-19 cases and deaths in many countries[1-3]. While the higher income countries have vaccinated a large proportion of their population, resulting in lower hospitalizations and deaths, many lower income and lower-middle income countries are grappling with the increase in the case loads, overburdening of health care resources and the inability to secure adequate doses of COVID-19 vaccines[4].

Although the duration of protection against re-infection from SARS-CoV-2 in not known, it has been shown that re-infection does occur, especially among older individuals, probably due to waning of immunity[5]. Re-infection has shown to occur particularly with certain variants such as P.1 (gamma) variant in Brazil despite a very high seroprevalence[6], and also with B.1.351 (beta) due to escape from natural and vaccine induced immunity[7]. Individuals who had experienced milder illness have shown to have reduced levels of neutralizing antibodies compared to those who had severe illness[8,9]. Apart from the presence of neutralizing antibodies to the receptor binding domain (RBD), antibodies specific to S2 and N protein of SARS-CoV-2 are also detected in patients who have recovered from COVID-19[10]. Although the usefulness of antibodies directed against S1, S2 and N protein in preventing re-infection are not known, although IgG and IgA specific to S1, S2 have been detected in breast milk of infected mothers

and therefore, possibly provide protection to the neonate[11]. Antibodies against the S2 subunit have been detected in unexposed individuals and S1, S2 and N protein specific memory B cell responses have been detected in those who were infected with SARS-CoV-2[12]. Children and adolescents who were unexposed to SARS-CoV-2 were shown to have a higher frequency of pre-existing IgG antibodies specific to S2, which were able to cross neutralize SARS-CoV-2[13]. The presence of high levels of cross-reactive antibodies to the S2 in children and adolescents have been speculated to reduce disease severity when infected with SARS-CoV-2[13,14]. Although many studies have investigated the role of SARS-CoV-2 specific IgG responses, virus specific IgA was detected during early illness and was shown to be able to neutralize the SARS-CoV-2 virus to a greater extent than virus specific IgG[15]. However, adults with severe illness had higher levels of SARS-CoV-2 specific IgA levels compared to adults with milder illness and children, and was shown to enhance neutrophil activation in vitro and thus release of inflammatory mediators[16]. Therefore, although virus specific IgA is an important component of mucosal immunity, its role in protection vs disease pathogenesis is not clear.

Currently there are several vaccines for COVID-19, which have shown to be safe and have high efficacy rates against the original Wuhan SARS-CoV-2 virus and variable efficacy against variants of concern[17-19]. However, due to non-availability of adequate quantity of vaccines and also in order to vaccinate as many individuals as fast as possible, some countries have increased the gap between the two doses of vaccine such as AZD2221 to 12 or 16 weeks[20]. While there have been many studies characterizing the IgG and IgA responses to different SARS-CoV-2 proteins in individuals with natural infection, the induction of IgG and IgA to different viral proteins in vaccinees have not been extensively studied. It was recently shown that the mRNA

vaccines induce high levels of both IgG and IgA antibodies against the spike protein[21]. However, there are limited data characterizing the IgG, IgA, ACE2-receptor blocking antibodies in individuals with varying severity of natural infection over time, in comparison to those who have received a single dose of the AZD2221 vaccine. Therefore in this study, we investigated the antibody responses in those with varying severity of natural infection and in those who received a single dose of the AZD2221 at 4 weeks and 12 weeks to the S1, S2, RBD and N proteins and also for SARS-CoV-2 variants of concern in a Sri Lankan population.

Methods

Patients

Patients confirmed with SARS-CoV2 infection based on the positive RT-PCR who were admitted to a COVID-19 treatment hospital, from the National Institute of Infectious Diseases (NIID), Sri Lanka. They were followed throughout their illness while they were in hospital and the severity grading was based on the worst severity while in hospital. Clinical disease severity was classified as mild, moderate and severe according to the WHO guidance on COVID-19 disease severity [22] . For this study we recruited two cohorts of patients. Serum samples from the patient cohort 1 (n=30) was used to determine the IgG and IgA antibody levels at 4 weeks since onset of illness, the ACE2 receptor blocking antibody levels and the antibodies to RBD by the HAT assay for the wild type (WT) and SARS-CoV-2 variants. The duration of illness was defined from the day of onset of symptoms and not the day of PCR positivity or admission to hospital. Based on the WHO COVID-19 disease classification, 15 patients had mild illness and 15 patients had moderate/severe illness[22]. As all the patients in the first cohort could not be traced at 12 weeks, in order to carry out the above assays, we recruited a second cohort of patients. Based on the WHO COVID-19 disease classification, 14 patients had mild illness and 6 patients had moderate/severe illness[22].

In order to compare the antibody responses following infection with one dose of the AZD1222 vaccine, we recruited 20 individuals 4 weeks following vaccination and 73 individuals, 12 weeks following vaccination. We also included serum samples from individuals who had a febrile illness in 2017 and early 2018. Ethical approval was received by the Ethics Review Committee

of Faculty of Medical Sciences, University of Sri Jayewardenepura. Informed written consent was obtained from patients.

<u>Haemagglutination test (HAT) to detect antibodies to the receptor binding domain (RBD)</u>

The HAT was carried out as previously described [23]. The B.1.1.7 (N501Y), B.1.351 (N501Y, E484K, K417N) and B.1.617.2 versions of the IH4-RBD reagent were produced as described [23], but included the relevant amino acid changes introduced by site directed mutagenesis. These variants were titrated in a control HAT with the monoclonal antibody EY-6A (to a conserved class 4 epitope[23,24]) and found to titrate identically with the original version so 100ng (50ul of 2ug/ml stock solution) was used for developing the HAT. The assays were carried out and interpreted as previously described [25]. The HAT titration was performed using 11 doubling dilutions of serum from 1:20 to 1:20,480, to determine presence of RBD-specific antibodies. The RBD-specific antibody titre for the serum sample was defined by the last well in which the complete absence of "teardrop" formation was observed.

<u>Surrogate neutralizing antibody test (sVNT) to detect NAbs</u>

The surrogate virus neutralization test (sVNT)[26], which measures the percentage of inhibition of binding of the RBD of the S protein to recombinant ACE2[26] (Genscript Biotech, USA) was carried out according the manufacturer's instructions as previously described by us[9]. Inhibition percentage $\geq 25\%$ in a sample was considered as positive for NAbs.

Results

The kinetics of SARS-CoV-2 specific IgG responses in those with natural infection

IgG responses to the S1, S2, RBD and N protein were measured in individuals with COVID-19 at 4 weeks and at 12 weeks since onset of illness and also in serum samples of 15 individuals who had a febrile illness in 2017 and early 2018. At 4 weeks since onset of illness, the highest magnitude of IgG antibody responses was seen for RBD in those with moderate/severe illness, whereas those with mild disease, had the highest responses to S2 (Figure 1A, table 1). Those who had a febrile illness in year 2017 and 2018 (controls), also had detectable antibody levels to S2, but not for other proteins. There was no difference in the antibody levels to S2 in those with mild illness compared to the controls (p=0.13), although those with milder disease had significantly higher antibody levels to S1 and RBD (p<0.0001) and N protein (p=0.0004), than the controls. In those who received a single dose of the AZD1222 vaccine, the IgG responses to the S1 and S2 components of the spike protein were similar, although the levels for the RBD was significantly higher (table 1). As expected, the IgG responses to the N protein was very low, but even lower than for the controls. The antibody levels to S1 (p=0.0002), S2 (p=0.01), RBD (p=0.002) and N (p<0.0001) proteins were significantly different between the three groups of individuals at 4 weeks (Figure 1A).

At 12 weeks since onset of illness, those with moderate/severe illness had the highest responses to N protein, whereas those with mild illness still had the highest responses to S2 (Figure 1B). At both time points for all proteins, those with moderate/severe disease had significantly higher antibody levels than those with milder illness (Table 1). The antibody responses to S1 (p=0.03),

S2 (p=0.04), RBD (p=0.02) and N protein (p=0.0002) were significantly different between the those with mild illness, moderate/severe disease and the vaccinees (Figure 1B). From 4 to 12 weeks, the S1 specific antibodies significantly rose in those with mild illness (p=0.004), while there was no significant change in the antibody levels to other proteins at 12 weeks (table 1). Patients who had moderate/severe illness sustained the same levels of antibodies for all four proteins from 4 weeks to 12 weeks. In the vaccinees, from 4 weeks to 12 weeks the IgG levels to S1 (p=0.008), S2 (p<0.0001) and RBD (p=0.003) had significantly increased (table 1).

The kinetics of SARS-CoV-2 specific IgA responses in those with natural infection

IgA responses to the S1, S2, RBD and N protein were measured in the above individuals with COVID-19 at 4 weeks and at 12 weeks since onset of illness and also in serum samples of 15 individuals who had a febrile illness in 2017 and early 2018. At 4 weeks and 12 weeks of illness, individuals with both mild and moderate/severe illness, had the highest levels of IgA antibodies to the RBD (Figure 1C and 1D). However, those with moderate/severe disease had significantly higher antibody responses to all four proteins when compared to those with mild illness at 4 weeks, but there was no difference at 12 weeks (table 1). Unlike what was observed with SARS-CoV-2 S2 specific IgG responses, those with mild illness had significantly higher IgA responses (p=0.02) but not to N protein (p=0.18) (Figure 2A). As expected, vaccinees had low responses to the N protein, with IgA levels similar to those seen in controls except for IgA to S1, which was higher in the vaccinees (p=0.003). Significant differences of IgA responses were seen in those with mild illness, moderate/severe illness and vaccinees for S1 (p=0.001), S2 (p=0.0003), RBD (p=0.0003) and N protein (p=0.04) (Figure 1C).

There was no difference in IgA levels to any of the proteins at 4 weeks compared to 12 weeks in patients with mild illness or with moderate/severe illness (table 1). However, significant differences were seen between the three groups to S1 (p=0.009), RBD (p=0.003) and N protein (p=0.02), but not for S2 (p=0.55) (Figure 1D).

ACE2 receptor blocking antibodies following natural infection and one dose of AZD1222

Due the lack of BSL-3 facilities to measure neutralizing antibodies, we used a surrogate test to measure the inhibition of binding of antibodies in patient sera to the ACE2 receptor. This was shown to be 100% specific in the Sri Lankan population, with none of the sera of individuals collected in 2017 and 2018, giving a positive response [9]. The ACE2 blocking antibodies were significantly higher in those with moderate to severe illness, when compared to those with mild illness at 4 weeks (p=0.03) and at 12 weeks (p=0.03) as reported previously[9] (Figure 2). In addition, the ACE2 receptor blocking antibodies significantly increased from 4 weeks to 12 weeks in those with moderate/severe illness (p=0.02) and in those with mild illness (p=0.03) (Figure 2). However, in those who received a single dose of the vaccine, the ACE2 blocking antibodies significantly reduced (p<0.0001) from levels at 4 weeks (median 77.32, IQR 60.05 to 90.77 % of inhibition) to 12 weeks (median 38.17, IQR 28.95 to 57.28 % of inhibition).

<u>Antibodies to the Receptor Binding Domain of the spike protein, including variants, measured by the Haemagglutination test (HAT)</u>

We previously evaluated the usefulness of the HAT assay in determining antibody responses to the RBD of the SARS-CoV-2, wild type (WT) virus, B.1.1.7 variant and the B.1.351 variants at 4 weeks following a single dose of the AZD1222 vaccine and had also evaluated this assay in naturally infected individuals in Sri Lanka [27]. In this study, we proceeded to investigate the differences in the antibody responses to the RBD in those with natural infection at 4- and 12-weeks following infection, and after a single dose of the AZD1222 vaccine. The antibody responses to the WT, B.1.1.7, B.1.351 and B.1.617.2 were measured.

In those with mild illness, at 4 weeks from onset of illness the median antibody titre to the WT was 160 (IQR 80 to 320), B.1.1.7 was 120 (IQR 70 to 320), B.1.351 was 10 (IQR, 0 to 80) and for B.1.617.2 it was 40 (IQR 20 to 80). At 12 weeks following the onset of illness, although there was a slight reduction in the antibody titres to the WT ($p=0.91$) and B.1.617.2 (0.61), this was not statistically significant (Figure 3A). In those with moderate/severe illness at 4 weeks from onset of illness the median antibody titre to the WT was 1280 (IQR 160 to 1280), B.1.1.7 was 640 (IQR 160 to 1280), B.1.351 was 40 (IQR, 0 to 160) and for B.1.617.2 it was 320 (IQR 80 to 1280) (Figure 3B). There was no significant difference between the antibody titres for the WT compared to B.1.1.7 ($p=0.12$), but clearly differed for B.1.315 ($p<0.0001$) and B.1.617.2 ($p=0.004$). Although the antibody titres for the WT and all the variants reduced from 4 to 12 weeks in those with moderate/severe illness, this was not statistically significant (Figure 3B).

At 4 weeks following a single dose of the vaccine, the median antibody titre to the WT was 80 (IQR 40 to 280), B.1.1.7 was 40 (IQR 20 to 160), B.1.351 was 20 (IQR, 0 to 70) and for B.1.617.2 it was 20 (IQR 0 to 70) (Figure 3C). At 12 weeks following a single dose of the vaccine, the antibody titre for WT was 80 (IQR 20 to 80), for B.1.1.7 it was 20 (IQR 0 to 80), for B.1.351 it was 20 (0 to 40) and for B.1.617.2 it was 0 (IQR 0 to 20). From 4 to 12 weeks, although there was no significance difference of the antibody titres of the RBD of the WT (p=0.05), B.1.351 (p=0.54) and B.617.2 (p=0.07), the antibody titres to B.1.1.7 significantly reduced (p=0.02) (Figure 3B). As previously described by us at 4 weeks following vaccination, the HAT titres were significantly lower to the B.1.1.7 (p=0.007), B.1.351 (<0.0001) and for B.1.617.2 (p<0.0001). However, there was no significance difference in antibody titres between B.1.351 and B.1.617.2 (p=0.43). At 12 weeks again the HAT titres were significantly lower to the B.1.1.7 (p<0.0001), B.1.351 (<0.0001) and for B.1.617.2 (p<0.0001) and no difference between antibody titres to B.1.351 and B.1.617.2.

Antibodies to the RBD were significantly different between those with mild illness, moderate/severe illness and with those with a single dose of the vaccine at 4 weeks (p=0.004) and at 12 weeks (p=0.02) (Figure 4A). This difference was also seen for the B.1.1.7 at 4 weeks between those with mild illness, moderate/severe illness and with those with a single dose of the vaccine at 4 weeks (p=0.0006) and at 12 weeks (p<0.0001) (Figure 4B) and for B.1.617.2 at 4 weeks (p=0.0002) and at 12 weeks (p=0.0004) (Figure 4C). However, there was no difference between the antibody titres to the B.1.351 between those with mild, moderate/severe illness and vaccinees at 4 weeks (p=0.36), but a significant difference was seen at 12 weeks (p=0.02) (Figure 4D).

Discussion

In this study we have investigated the kinetics of IgG and IgA responses to S1, S2, RBD and N protein, ACE2 receptor blocking antibodies and antibodies against SARS-CoV-2 variants, in individuals at 4 and 12 weeks following natural infection and in those who had a single dose of the AZD2221. Based on the Luminex assays, IgG and IgA levels to S1, S2, RBD and N, had increased from 4 weeks to 12 weeks in those with mild illness and in the vaccinees, although the increase was only significant in the vaccinees. In the vaccinees, the most significant rise was seen for the S2 subunit, while in those with mild illness the rise was seen for IgG antibodies for the RBD. In those with moderate/severe illness, while there was no change in the IgG responses from 4 to 12 weeks, responses to the N protein had increased although this was not significant. Therefore, the kinetics of antibody responses to S1, S2, RBD and N appears to vary based on the severity of natural infection and also appeared to be different in vaccinees. Interestingly, blood samples of those who had a febrile illness in 2017 and 2018 also showed IgG and IgA responses to the S2 subunit, suggesting the presence of S2 subunit cross-reactive antibodies, in these donors as previously seen in other studies[13,14]. Following a single dose of the AZD2221 vaccine, the antibodies against S2 appears to continue to rise from 4 to 12 weeks, possibly due to stimulation of pre-existing cross-reactive memory B cell responses to the S2 subunit[14].

SARS-CoV-2 specific IgA antibodies have shown to be generated during early illness and have potent neutralizing ability[15]. IgA antibodies to the RBD have shown to develop earlier than IgG and while some studies have shown that serum IgA does not associate with clinical disease

severity[15], in other studies, patients who developed severe disease were shown to have higher levels of virus specific IgA[28]. Serum IgA was shown to activate neutrophils, thereby leading to production of increased levels of inflammatory mediators possibly leading to disease pathogenesis[16]. We found that at 4 weeks of illness, those with moderate/severe illness had significantly higher serum IgA to S1, S2, RBD and N compared to those with mild illness, but these high levels of IgA declined and there was no differences between these two groups at 12 weeks since onset of illness. Vaccinees had several fold lower IgA antibodies to all the SARS-CoV-2 proteins tested than those with mild and moderate/severe illness at 4 weeks and 12 weeks. The importance of serum IgA in preventing re-infection is currently unknown and if those with lower IgA have reduced protection is currently unknown.

Although the IgG antibodies to S1, S2 and the RBD rose from 4 to 12 weeks in the vaccinees, the ACE2 receptor blocking antibodies, which were shown to correlate with neutralizing antibodies significantly decreased[26]. The HAT assay, which also measures antibodies to the RBD and has shown to correlate well with the ACE2 receptor blocking assay and with neutralizing antibodies[23,27], also showed that the RBD binding antibodies decreased from 4 to 12 weeks in the vaccinees. This suggests that although antibodies to RBD, S1 and S2 have increased in vaccinees from 4 to 12 weeks, they might not be neutralizing antibodies, possibly through targeting other epitopes in these regions.

Apart from assessing antibodies to the RBD to the wild type, we assessed the antibodies to three other VOCs, B.1.1.7, B.1.351 and B.1.617.2. At 4 weeks following vaccination, the vaccinees

had similar levels of antibodies to the RBD of WT as those with mild illness, the levels were significantly less for B.1.1.7 and for B.1.617.2. The antibody levels among vaccinees were similar to B.1.351 and B.1.617.2, showing equal reduction compared to antibody binding to the RBD of the WT. Following vaccination, these levels further declined at 12 weeks to VOCs but not to the WT, showing that a single dose of the AZD2221 was likely to offer less protection against VOCs. In fact, it has been shown that one dose of AZD2221 is only 33% effective in preventing symptomatic disease with B.1.617.2, 3 weeks following the first dose[29]. The efficacy of a single dose against B.1.617.2 is likely to decline further by 12 weeks, as the antibodies to RBD further waned. However, the efficacy of two doses of AZD2221 against hospitalization has been shown to be 92%, while for Pfizer-BioNTech was 96%[30]. Therefore, in countries which have outbreaks due to VOCs, especially B.1.617.2, it would be prudent to encourage second doses to increase efficacy as recommended. Interestingly, although those with mild or moderate/severe illness also had a marked reduction in antibodies to the RBD of B.1.351, they had significantly higher levels of antibodies to the RBD of B.617.2 at 4 weeks compared to B.1.351. However, by 12 weeks the antibody levels to both B.1.351 and B.1.617.2 were similar. Therefore, B.1.617.2 had less immune evasion than B.1.351 in those who were naturally infected, at least during early convalescence.

In summary, we have investigated the kinetics and differences in IgG and IgA antibody responses to the S1, S2, RBD and N in those with varying severity of infection and vaccinees who received a single dose of AZD2221, which showed that vaccinees had significantly less IgA to SARS-CoV-2, but comparable IgG responses those with natural infection. However, following

a single dose, vaccinees had reduced antibody levels to the VOCs, which further declined with time.

Acknowledgement

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Tables

	4 weeks Median (IQR)	12 weeks Median (IQR)	P value
Mild infection (IgG) S1 S2 RBD N	 734 (483 to 1071) 3503 (1656 to 5795) 539 (840 to 2960) 2094 (1554 to 4787)	 1336 (24 to 4714) 3579 (106.8 to 9912) 2952 (38.7 to 7516) 2694 (51 to 7547)	 0.59 0.68 0.59 0.84
Mild infection (IgA) S1 S2 RBD N	 152 (79 to 490) 354 (219 to 561.5) 656.5 (303 to 1616) 207.5 (78 to 468)	 192 (19 to 422.1) 380.2 (165.6 to 869) 770.5 (180.3 to 1520) 276.3 (165.5 to 496.5)	 0.69 0.71 0.98 0.31
Moderate/severe infection (IgG) S1 S2 RBD N	 4776 (1395 to 7833) 6869 (2001 to 11,131) 7486 (2784 to	 5064 (2744 to 6038) 8931 (7262 to 9607) 7829 (5083 to 8553) 9538 (8810 to	 0.96 0.85 0.67 0.31

	10,218)	10,844)	
	5831 (3123 to 9383)		
Moderate/severe infection (IgA)			
S1	1043 (220 to 1784)	391.8 (132.8 to 2021)	0.52
S2	934 (399 to 3679)	1378 (153.9 to 2269)	0.73
RBD	3375 (1192 to 5401)	1837 (506.1 to 4802)	0.38
N	661 (211.5 to 6165)	273 (75.9 to 596.1)	0.18
Vaccinated IgG			
S1	2215 (1223 to 3870)	3639 (2190 to 5617)	**0.008**
S2	1625 (1063 to 4329)	6460 (4143 to 9594)	**<0.0001**
RBD	4393 (2355 to 6131)	6209 (4481 to 8367)	**0.003**
Vaccinated IgA			
S1	76.5 (38.2 to 166.5)	36 (23 to 92)	**0.04**
S2	203.3 (101.3 to 310.9)	324.5 (143 to 788)	**0.02**
RBD	327.5 (183 to 612.8)	221 (116 to 437)	0.11

Table 1: Antibody responses to S1, S2, RBD and N protein of the SARS-CoV-2 in those with varying severity of illness and in those following a single dose of the AZD2221. MFI indicates the median fluorescence intensity.

Figure Legends

Figure 1: IgG and IgA antibody levels to S1, S2, RBD and N protein of SARS-CoV-2 in individuals following natural infection and following a single dose of the AZD1222 vaccine. Serum IgG antibodies to S1, S2, RBD and N protein were measured by Luminex assays at 4 weeks in those with mild illness (n=15), moderate/severe illness (n=15), vaccinees (n=20) and controls (n=19) (A) and again at 12 weeks in those with mild illness (n=14), moderate/severe illness (n=6), vaccinees (n=73) (B). IgA antibodies were also measured in the above groups at 4 weeks (C) and at 12 weeks (D). The Kruskal-Wallis test was used to determine the difference between the antibody levels between the three different groups (two-tailed). The lines indicate the median and the interquartile range.

Figure 2: ACE2 receptor blocking antibodies in patients with varying severity of illness and following a single dose of the AZD1222 vaccine. ACE receptor blocking antibodies were measured by the surrogate virus neutralizing test following natural infection at 4 weeks in those with mild illness (n=14) and moderate/severe illness (n=15) and at 12 weeks in those with mild

(n=14) and moderate/severe illness (n=6). Antibodies were also measured at 4 weeks (n=20) and

12 weeks (n=73) in vaccinees following a single dose of AZD2221. Mann-Whitney test (two

tailed) was used to determine the differences between antibody levels as 4 weeks and 12 weeks.

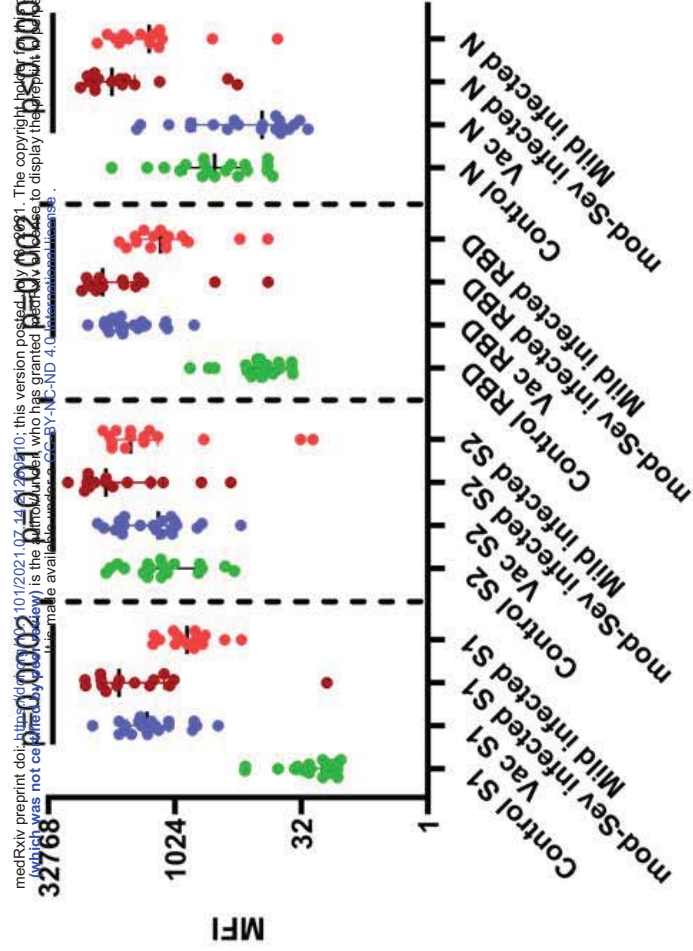The lines indicate the median and the interquartile range.

Figure 3: Comparison of antibody titres to RBD of the SARS-CoV-2 using the HAT assay in those with varying severity of infection and in vaccinees. Antibody titres were measured individuals with mild illness (n=14) to the WT, B.1.1.7, B.1.351 and B.1.617.2 at 4 weeks and 12 weeks since onset of illness (A), in those with moderate/severe illness at 4 weeks (n=15) and 12 weeks (n=6) since onset of illness (B) and in those who received one dose of AZD1222 vaccine at 4 weeks (n=16) and 12 weeks (n=73) following the vaccine (C). The difference between antibody titres to WT, B.1.1.7, B.1.351 and B.1.617.2 was determined using the Wilcoxon paired t test (two tailed) and the differences between antibody titres at 4 weeks and 12 weeks was determined using the Mann-Whitney test (two tailed). The lines indicate the median and the interquartile range.

Figure 4: Comparison of antibody titres to the RBD of the SARS-CoV-2 using the HAT assay for the wild type and for variants. Antibody titres were measured in patients with mild illness (n=14), moderate/severe illness (n=15) from 4 weeks since onset of illness and in those who received one dose of AZD1222 vaccine at 4 weeks (n=16), and again at 12 weeks in those who developed mild illness (n=14), moderate/severe illness (n=6) and in those who received 1
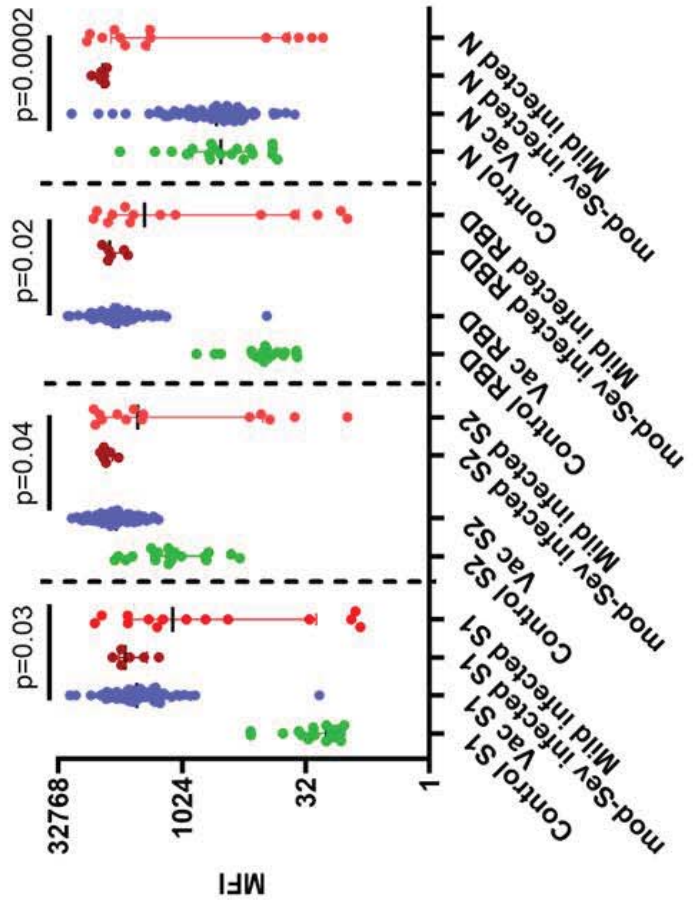
dose of AZD1222 vaccine (n=73), for the WT (A), B.1.1.7 (B), B.1.617.2 (C) and B.1.351 (D).

The Kruskal-Wallis test was used to determine the difference between the antibody levels

between the three different groups (two-tailed). The lines indicate the median and the
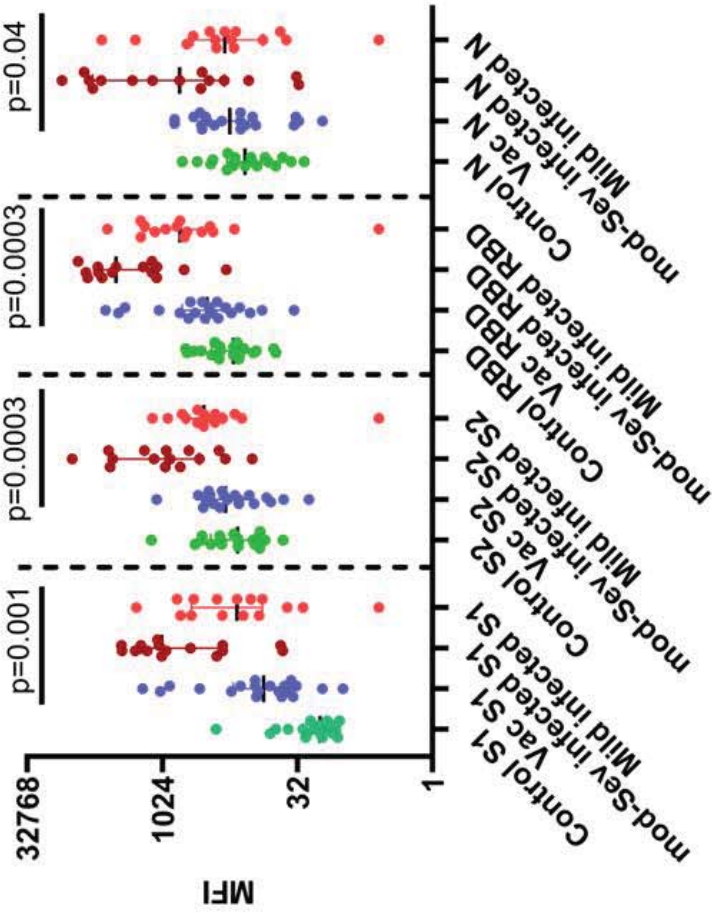
interquartile range.

EXHIBIT 6

nature

Accelerated Article Preview

SARS-CoV-2 infection induces long-lived bone marrow plasma cells in humans

Jackson S. Turner, Wooseob Kim, Elizaveta Kalaidina, Charles W. Goss, Adriana M. Rauseo, Aaron J. Schmitz, Lena Hansen, Alem Haile, Michael K. Klebert, Iskra Pusic, Jane A. O'Halloran, Rachel M. Presti & Ali H. Ellebedy

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Article

SARS-CoV-2 infection induces long-lived bone marrow plasma cells in humans

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Long-lived bone marrow plasma cells (BMPCs) are a persistent and essential source of protective antibodies[1–7]. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) convalescent individuals have a significantly lower risk of reinfection[8–10]. Nonetheless, it has been reported that anti-SARS-CoV-2 serum antibodies experience rapid decay in the first few months after infection, raising concerns that long-lived BMPCs may not be generated and humoral immunity against this virus may be short-lived[11–13]. Here we demonstrate that in patients who experienced mild infections (n=77), serum anti-SARS-CoV-2 spike (S) antibodies decline rapidly in the first 4 months after infection and then more gradually over the following 7 months, remaining detectable at least 11 months after infection. Anti-S antibody titers correlated with the frequency of S-specific BMPCs obtained from bone marrow aspirates of 18 SARS-CoV-2 convalescent patients 7 to 8 months after infection. S-specific BMPCs were not detected in aspirates from 11 healthy subjects with no history of SARS-CoV-2 infection. We demonstrate that S-binding BMPCs are quiescent, indicating that they are part of a long-lived compartment. Consistently, circulating resting memory B cells directed against the S protein were detected in the convalescent individuals. Overall, we show that SARS-CoV-2 infection induces a robust antigen-specific, long-lived humoral immune response in humans.

Reinfections by seasonal coronaviruses occur 6-12 months after the previous infection, indicating that protective immunity against these viruses may be short-lived[14,15]. Early reports documenting rapidly declining antibody titers in convalescent SARS-CoV-2 patients in the first several months after infection suggested that protective immunity against SARS-CoV-2 may be similarly transient[11–13]. It was also suggested that SARS-CoV-2 infection may fail to elicit a functional germinal center response, which would interfere with the generation of long-lived plasma cells[3–5,7,16]. Later reports analyzing samples collected approximately 4 to 6 months after infection indicate that SARS-CoV-2 antibody titers decline more slowly[8,17–21]. Durable serum antibody titers are maintained by long-lived plasma cells, non-replicating, antigen-specific plasma cells that are detected in bone marrow long after the disappearance of the antigen[1–7]. We sought to determine whether they were detectable in SARS-CoV-2 convalescent patients approximately 7 months after infection.

Biphasic decay of anti-S antibody titers

Blood samples were collected approximately 1 month after onset of symptoms from seventy-seven SARS-CoV-2 convalescent volunteers (49% female, 51% male, median age 49), the majority of whom had experienced mild illness (7.8% hospitalized, Extended Data Tables 1 and 2). Follow-up blood samples were collected three times at approximately 3-month intervals. Twelve convalescent participants received either the BNT162b2 or the mRNA-1273 SARS-CoV-2 vaccine between the last two timepoints; these post-vaccination samples were not included in our analyses. Additionally, bone marrow aspirates were collected from eighteen of the participants 7 to 8 months after infection and from eleven healthy volunteers with no history of SARS-CoV-2 infection or vaccination. Follow-up bone marrow aspirates were collected from five of the eighteen and one additional convalescent donor approximately 11 months after infection. (Fig. 1a, Extended Data Tables 3 and 4). We first performed a longitudinal analysis of circulating anti-SARS-CoV-2 serum antibodies. While anti-SARS-CoV-2 spike (S) IgG antibodies were undetectable in blood from controls, 74 of 77 convalescent participants had detectable serum titers approximately 1 month after onset of symptoms. Between 1- and 4-months post symptom onset, overall anti-S IgG titers decreased from a mean of 6.3 to 5.7 (mean difference 0.59±0.06, $P<0.001$). However, in the interval between 4- and 11-months post symptom onset, the decay rate slowed, and mean titers declined from 5.7 to 5.3 (mean difference 0.44±0.10, $P<0.001$, Fig. 1a).

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Article

In contrast to the anti-S antibody titers, IgG titers against the 2019/2020 inactivated seasonal influenza virus vaccine were detected in all control and SARS-CoV-2 convalescent participants and declined much more gradually, if at all over the course of the study, with mean titers decreasing from 8.0 to 7.9 (mean difference 0.16±0.06, *P*=0.042) and 7.9 to 7.8 (mean difference 0.02±0.08, *P*=0.997) across the 1-to-4- and 4-to-11-month intervals post symptom onset, respectively (Fig. 1b).

Induction of S-binding long-lived BMPCs

The relatively rapid early decline in anti-S IgG followed by slower decay is consistent with a transition of serum antibodies from being secreted by short-lived plasmablasts to a smaller but more persistent population of long-lived plasma cells generated later in the immune response. The majority of this latter population resides in bone marrow[1–6]. To investigate whether SARS-CoV-2 convalescent patients developed a virus specific long-lived BMPC compartment, we examined their bone marrow aspirates obtained approximately 7 and 11 months after infection for anti-SARS-CoV-2 S-specific BMPCs. We magnetically enriched BMPCs from the aspirates and then quantified the frequencies of those secreting IgG and IgA directed against the 2019/2020 influenza virus vaccine, tetanus/diphtheria vaccine, and SARS-CoV-2 S protein by ELISpot (Fig. 2a). Frequencies of influenza and tetanus/diphtheria vaccine specific BMPCs were comparable between control and convalescent participants. IgG- and IgA-secreting S-specific BMPCs were detected in 15 and 9 of the 19 convalescent participants, respectively, but not in any of the 11 control participants (Fig. 2b). Importantly, none of the convalescent patients had detectable S-specific antibody secreting cells in blood at the time of bone marrow sampling, indicating that the detected BMPCs represent bone marrow-resident cells and not contamination from circulating plasmablasts. Frequencies of anti-S IgG BMPCs were stable among the five participants sampled a second time approximately 4m later, and anti-S IgA BMPC frequencies were stable in four of the five, with one decreasing below the limit of detection (Fig. 2c). Consistent with their stable BMPC frequencies, anti-S IgG titers in the five participants remained consistent between 7- and 11-months post symptom onset. IgG titers measured against the receptor binding domain (RBD) of S, a primary target of neutralizing antibodies, were detected in four of the five convalescent patients and were also stable between 7- and 11-months post symptom onset (Fig. 2d). Frequencies of anti-S IgG BMPCs showed a modest but significant correlation with circulating anti-S IgG titers 7-8 months post symptom onset in convalescent participants, consistent with long-term maintenance of antibody levels by these cells. In accordance with previous reports[22–24], frequencies of influenza vaccine-specific IgG BMPCs and antibody titers exhibited a strong and significant correlation (Fig. 2e). Nine of the aspirates from controls and twelve of the eighteen collected 7m post symptom onset yielded a sufficient number of BMPCs for additional analysis by flow cytometry. We stained these samples intracellularly with fluorescently labeled S and influenza virus hemagglutinin (HA) probes to identify and characterize antigen specific BMPCs. As controls, we also intracellularly stained PBMC from healthy volunteers 1 week after SARS-CoV-2 or seasonal influenza virus vaccination (Fig. 3a, Extended Data Fig. 1a-c). Consistent with the ELISpot data, low frequencies of S-binding BMPCs were detected in ten of the twelve convalescent specimens analyzed, but not in any of the nine control specimens (Fig. 3b). While both recently generated circulating plasmablasts and S- and HA-binding BMPCs expressed Blimp1, BMPCs were differentiated by the lack of expression of Ki-67, indicating a quiescent state, as well as higher levels of CD38 (Fig. 3c).

Robust S-binding memory B cell response

Memory B cells (MBCs) form the second arm of humoral immune memory. Upon antigen re-exposure, MBCs rapidly expand and differentiate into antibody-secreting plasmablasts. We examined the frequency of SARS-CoV-2 specific circulating MBCs in convalescent patients as well as in the healthy controls. We stained peripheral blood mononuclear cells with fluorescently labeled S probes and determined the frequency of S-binding MBCs among isotype-switched IgD^{lo} CD20^{+} MBCs by flow cytometry. For comparison, we co-stained the cells with fluorescently labeled influenza virus hemagglutinin (HA) probes (Fig. 4a). S-binding MBCs were identified in convalescent patients in the first sample collected approximately 1 month after onset of symptoms, with comparable frequencies to influenza HA-binding memory B cells (Fig. 4b). S-binding memory B cells were maintained for at least 7m post symptom onset and were present at significantly higher frequencies compared to healthy controls, comparable to frequencies of influenza HA-binding memory B cells identified in both groups (Fig. 4c).

Discussion

This study sought to determine whether SARS-CoV-2 infection induces antigen-specific long-lived BMPCs in humans. We detected SARS-CoV-2 S-specific BMPCs in aspirates from 15 of 19 convalescent patients, and in none from the 11 control participants. Frequencies of anti-S IgG BMPCs modestly correlated with serum IgG titers 7-8 months after infection. Phenotypic analysis by flow cytometry demonstrated that S-binding BMPCs were quiescent, and their frequencies were largely consistent in five paired aspirates collected 7- and 11-months post symptom onset. Importantly, we detected no S-binding cells among plasmablasts in blood samples collected at the same time as the bone marrow aspirates by ELISpot or flow cytometry in any of the convalescent or control samples. Altogether, these data indicate mild SARS-CoV-2 infection elicits a long-lived BMPC response. Additionally, we showed that S-binding MBCs in blood of convalescent patients are present at similar frequencies to those directed against influenza virus HA. Overall, our results are consistent with SARS-CoV-2 infection eliciting a canonical T-dependent B cell response, in which an early transient burst of extrafollicular plasmablasts generates a wave of serum antibodies that decline relatively quickly. This is followed by more stably maintained serum antibody levels that are supported by long-lived BMPCs.

While this overall trend captures the serum antibody dynamics of the majority of participants, we observed that in three participants, anti-S serum antibody titers increased between 4- and 7-months post symptom onset after having initially declined between 1 and 4 months. This could be stochastic noise, could represent increased net binding affinity as early plasmablast-derived antibodies are replaced by those from affinity-matured BMPCs, or could represent increases in antibody concentration from reencounter with the virus (although none of the participants in our cohort tested positive a second time). While anti-S IgG titers in the convalescent cohort were relatively stable in the interval between 4- and 11-months post symptom onset, they did measurably decrease, in contrast to anti-influenza virus vaccine titers. While this could represent an intrinsically less durable anti-S BMPC response compared to that against influenza virus, the largely stable frequencies of anti-S BMPCs measured in the same individuals 7- and 11-months post symptom onset argue against this possibility. It is possible that the decline reflects a final waning of early plasmablast-derived antibodies. It is also possible that the lack of decline in influenza titers was due to boosting through exposure to influenza antigens from infection or vaccination. Our data suggest that SARS-CoV-2 infection induces a germinal center response in humans because long-lived BMPCs are thought to be predominantly germinal center-derived[7]. This is consistent with a report demonstrating increased levels of somatic hypermutation in MBCs targeting the receptor binding domain of the S protein in SARS-CoV-2 convalescent patients at 6 months compared to 1 month after infection[20].

To our knowledge, the current study provides the first direct evidence for induction of antigen specific BMPCs after a viral infection in humans.

However, we do acknowledge several limitations. Although we detected anti-S IgG antibodies in serum at least 7 months after infection in all 19 of the convalescent donors from whom we obtained bone marrow aspirates, we failed to detect S-specific BMPCs in four donors. Serum anti-S antibody titers in those four donors were low, suggesting that S-specific BMPCs may potentially be present at very low frequencies that are below our limit of detection. Another limitation is that we do not know the fraction of the S-binding BMPCs detected in our study that encodes neutralizing antibodies. SARS-CoV-2 S protein is the main target of neutralizing antibodies[17,25–30] and correlation between serum anti-S IgG binding and neutralization titers has been documented[17,31]. Further studies will be required to determine the epitopes targeted by BMPCs and MBCs as well as their clonal relatedness. Finally, while our data document a robust induction of long-lived BMPCs after SARS-CoV-2 infection, it is critical to note that our convalescent patients mostly experienced mild infections. Our data are consistent with a report showing that individuals who recovered rapidly from symptomatic SARS-CoV-2 infection generated a robust humoral immune response[32]. Therefore, it is possible that more severe SARS-CoV-2 infections could lead to a different outcome with respect to long-lived BMPC frequencies due to dysregulated humoral immune responses. This, however, has not been the case in survivors of the 2014 West African Ebola virus outbreak in whom severe viral infection induced long-lasting antigen-specific serum IgG antibodies[33].

Long-lived BMPCs provide the host with a persistent source of preformed protective antibodies and are therefore needed to maintain durable immune protection. However, longevity of serum anti-S IgG antibodies is not the only determinant of how durable immune-mediated protection will be. Indeed, isotype-switched MBCs can rapidly differentiate into antibody secreting cells upon pathogen reexposure, offering a second line of defense[34]. Encouragingly, the frequency of S-binding circulating MBCs 7 months after infection was similar compared to those directed against contemporary influenza HA antigens. Overall, our data provide strong evidence that SARS-CoV-2 infection in humans robustly establishes the two arms of humoral immune memory: long-lived BMPC and MBCs. These findings provide an immunogenicity benchmark for SARS-CoV-2 vaccines and a foundation for assessing the durability of primary humoral immune responses induced after viral infections in humans.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41586-021-03647-4.

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Article
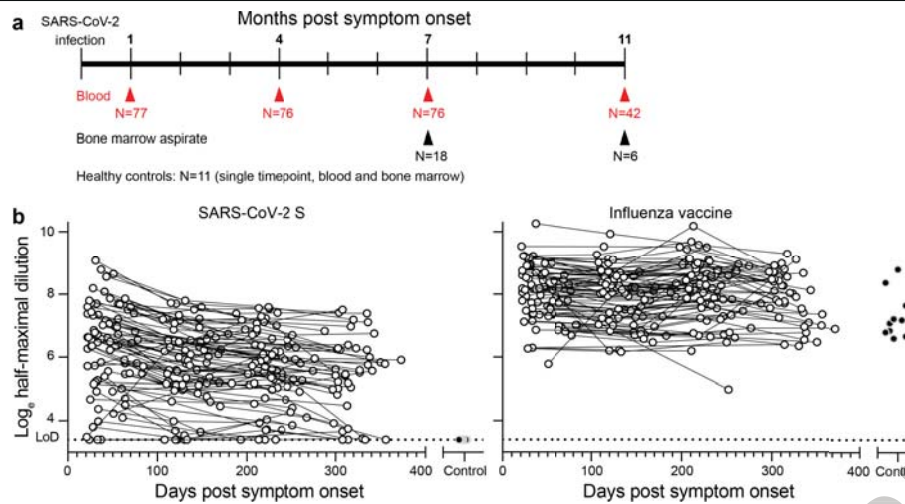
Fig. 1 | SARS-CoV-2 infection elicits durable serum anti-spike antibody titers. a, Study design. Seventy-seven SARS-CoV-2 convalescent patients with mild disease (ages 21–69) were enrolled and blood was collected approximately 1 month, 4 months, 7 months, and 11 months post onset of symptoms. Bone marrow aspirates were collected from eighteen of the participants 7 to 8 months after infection and from eleven healthy volunteers (ages 23–60) with no history of SARS-CoV-2 infection. Follow-up bone marrow aspirates were collected from five of the eighteen convalescent donors and one additional donor approximately 11 months after infection. **b**, Blood IgG titers against S (*left*) and influenza virus vaccine (*right*) measured by ELISA in convalescent patients (white circles) at the indicated time post onset of symptoms and controls (black circles). Dotted line indicates limit of detection. Means and pairwise differences at each timepoint were estimated using a linear mixed model analysis.
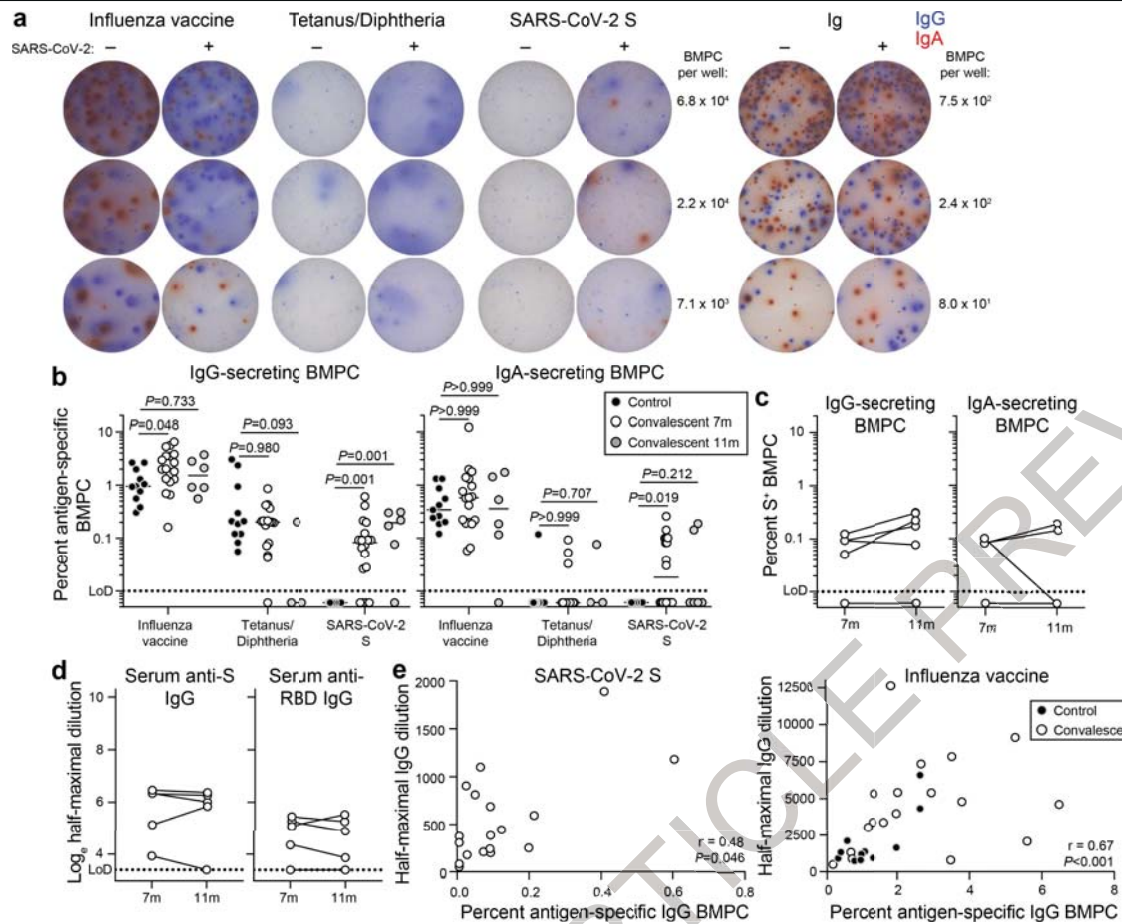
Fig. 2 | SARS-CoV-2 infection elicits S-binding long-lived BMPCs.
a, Representative images of ELISpot wells coated with the indicated antigens or anti-Ig and developed in blue and red for IgG and IgA, respectively after incubation of magnetically enriched BMPC from convalescent and control participants. **b**, Frequencies of BMPC secreting IgG (*left*) or IgA (*right*) antibodies specific for the indicated antigens, indicated as percentages of total IgG- or IgA-secreting BMPC in control (black circles) or convalescent participants 7m (white circles) or 11m (grey circles) post symptom onset. Horizontal lines indicate medians. *P*-values from two-sided Kruskal-Wallis tests with Dunn's correction for multiple comparisons between control and convalescent participants. Each symbol represents one sample

(n=18 convalescent, 11 control). **c**, Paired frequencies of BMPC secreting IgG (*left*) and IgA (*right*) specific for S from convalescent participants 7m and 11m post symptom onset. **d**, Paired anti-S (*left*) and anti-RBD (*right*) IgG serum antibody titers from convalescent participants 7m and 11m post symptom onset. Data in panels (c) and (d, *left*) are also shown in (b) and Fig. 1b, respectively. Each symbol represents one sample (n=5). **e**, Frequencies of IgG BMPC specific for S (*left*) and influenza virus vaccine (*right*) plotted against respective IgG titers in paired blood samples from control (black circles) or convalescent participants 7m post symptom onset (white circles). *P*- and *r*-values from two-sided Spearman's correlations. Each symbol represents one sample (n=18 convalescent, 11 control).

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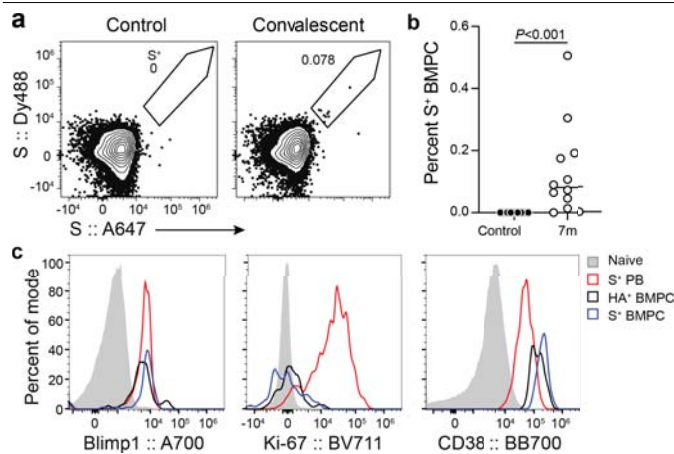
Fig. 3 | SARS-CoV-2 BMPCs are quiescent and distinct from circulating PBs.
a, Representative plots of intracellular S staining in CD20^{lo} CD38^{+} IgD^{lo} CD19$^{+/lo}$
CD3^{-} live singlet BMPCs (gating in Extended Data Fig. 1a) from control (*left*) and
convalescent (*right*) magnetically enriched BMPC 7 months after symptom
onset. **b**, Frequencies of S-binding BMPCs in total BMPC from control (black
circles) or convalescent participants 7m post symptom onset (white circles).
Horizontal lines indicate medians. *P*-value from two-sided Mann-Whitney
U-test. Each symbol represents one sample (*n*=12 convalescent, 9 control).
c, Histographs of Blimp1 (*left*), Ki-67 (*center*), and CD38 (*right*) staining on
S^{+} (blue) and HA^{+} (black) BMPC from magnetically enriched BMPC 7m post
symptom onset and S^{+} plasmablasts (red), and naïve B cells (grey) from healthy
donor PBMC 1 week after SARS-CoV-2 S immunization.
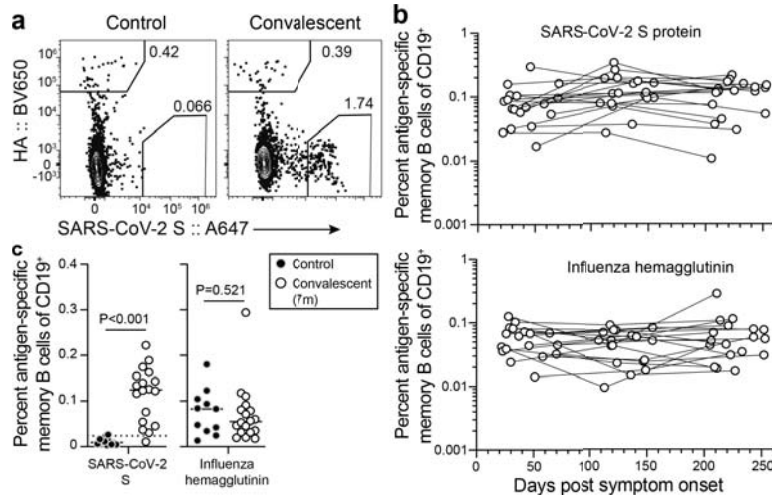
Fig. 4 | SARS-CoV-2 infection elicits a robust memory B cell response.
a, Representative plots of surface influenza virus hemagglutinin (HA) and S staining on CD20$^+$ CD38$^{lo/int}$ IgD^{lo} CD19$^+$ CD3$^-$ live singlet memory B cells (gating in Extended Data Fig. 1d) from control (*left*) and convalescent (*right*) PBMCs 7 months after symptom onset. **b**, Kinetics of S- (*top*) and HA- (*bottom*) binding memory B cells in PBMCs from convalescent patients collected at the indicated days post onset of symptoms. Data from the 7-month timepoint are also shown in (c). **c**, Frequencies of S- (left) and HA- (right) binding memory B cells in PBMC from control (black circles) and convalescent (white circles) participants 7 months after symptom onset. The dotted line in the S plot indicates limit of sensitivity, defined as the median + 2×SD of the controls. Each symbol represents one sample (*n*=18 convalescent, 11 control). Horizontal lines indicate medians. *P*-values from two-sided Mann-Whitney U-tests.

Article

Methods

Sample collection, preparation, and storage

All studies were approved by the Institutional Review Board of Washington University in St. Louis. Written consent was obtained from all participants. Seventy-seven participants who had recovered from SARS-CoV-2 infection and eleven controls without SARS-CoV-2 infection history were enrolled (Extended Data Tables 1 and 3). Blood samples were collected in EDTA tubes and peripheral blood mononuclear cells (PBMCs) were enriched by density gradient centrifugation over Ficoll 1077 (GE) or Lymphopure (BioLegend), remaining red blood cells were lysed with ammonium chloride lysis buffer, and cells were immediately used or cryopreserved in 10% dimethylsulfoxide in FBS. Approximately 30 mL bone marrow aspirates were collected in EDTA tubes from the iliac crest of eighteen convalescent participants and the controls. Bone marrow mononuclear cells were enriched by density gradient centrifugation over Ficoll 1077, remaining red blood cells were lysed with ammonium chloride buffer (Lonza) and washed with PBS supplemented with 2% FBS and 2 mM EDTA. Bone marrow plasma cells were enriched from bone marrow mononuclear cells using CD138 Positive Selection Kit II (Stemcell) and immediately used for ELISpot or cryopreserved in 10% dimethylsufoxide in FBS for flow cytometric analysis.

Antigens

Recombinant soluble spike protein (S) and its receptor binding domain (RBD) derived from SARS-CoV-2 was expressed as previously described[35]. Briefly, mammalian cell codon-optimized nucleotide sequences coding for the soluble version of S (GenBank: MN908947.3, amino acids 1-1213) including a C-terminal thrombin cleavage site, T4 foldon trimerization domain, and hexahistidine tag cloned into mammalian expression vector pCAGGS. The S protein sequence was modified to remove the polybasic cleavage site (RRAR to A) and two stabilizing mutations were introduced (K986P and V987P, wild type numbering). RBD, along with the signal peptide (amino acids 1-14) plus a hexahistidine tag were cloned into mammalian expression vector pCAGGS. Recombinant proteins were produced in Expi293F cells (ThermoFisher) by transfection with purified DNA using the ExpiFectamine 293 Transfection Kit (ThermoFisher). Supernatants from transfected cells were harvested 3 (for S) or 4 (for RBD) days post-transfection, and recombinant proteins were purified using Ni-NTA agarose (ThermoFisher), then buffer exchanged into phosphate buffered saline (PBS) and concentrated using Amicon Ultra-cel centrifugal filters (EMD Millipore). For flow cytometry staining, recombinant S was labeled with Alexa Fluor 647- or DyLight 488-NHS ester (Thermo Fisher); excess Alexa Fluor 647 and DyLight 488 were removed using 7-kDa and 40-kDa Zeba desalting columns, respectively (Pierce). Recombinant HA from A/Michigan/45/2015 (a.a. 18-529, Immune Technology) was labeled with DyLight 405-NHS ester (Thermo Fisher); excess DyLight 405 was removed using 7-kDa Zeba desalting columns. Recombinant HA from A/Brisbane/02/2018 (a.a.18–529) and B/Colorado/06/2017 (a.a. 18–546) (both Immune Technology) were biotinylated using the EZ-Link Micro NHS-PEG4-Biotinylation Kit (Thermo Fisher); excess biotin was removed using 7-kDa Zeba desalting columns.

ELISpot

Plates were coated with Flucelvax Quadrivalent 2019/2020 seasonal influenza virus vaccine (Sequiris), tetanus/diphtheria vaccine (Grifols), recombinant S, or anti-human Ig. Direct *ex-vivo* ELISpot was performed to determine the number of total, vaccine-binding, or recombinant S-binding IgG- and IgA-secreting cells present in BMPC and PBMC samples using IgG/IgA double-color ELISpot Kits (Cellular Technologies, Ltd.) according to the manufacturer's instructions. ELISpot plates were analyzed using an ELISpot counter (Cellular Technologies Ltd.).

ELISA

Assays were performed in 96-well plates (MaxiSorp; Thermo) coated with 100 μL of Flucelvax 2019/2020 or recombinant S in PBS, and plates were incubated at 4 °C overnight. Plates were then blocked with 10% FBS and 0.05% Tween20 in PBS. Serum or plasma were serially diluted in blocking buffer and added to the plates. Plates were incubated for 90 min at room temperature and then washed 3 times with 0.05% Tween-20 in PBS. Goat anti-human IgG-HRP (Jackson ImmunoResearch, 1:2,500) was diluted in blocking buffer before adding to wells and incubating for 60 min at room temperature. Plates were washed 3 times with 0.05% Tween20 in PBS, and then washed 3 times with PBS before the addition of o-Phenylenediamine dihydrochloride peroxidase substrate (Sigma-Aldrich). Reactions were stopped by the addition of 1 M HCl. Optical density measurements were taken at 490 nm. The half-maximal binding dilution for each serum or plasma sample was calculated using nonlinear regression (Graphpad Prism v8). The limit of detection was defined as 1:30.

Statistics

Spearman's correlation coefficients were estimated to assess the relationship between 7-month anti-S and anti-influenza virus vaccine IgG titers and frequencies of BMPCs secreting IgG specific for S and influenza virus vaccine, respectively. Means and pairwise differences of antibody titers at each timepoint were estimated using a linear mixed model analysis with a first order autoregressive covariance structure. Time since symptom onset was treated as a categorical fixed effect for the four different sample time points spaced approximately 3 months apart. *P*-values were adjusted for multiple comparisons using Tukey's method. All analyses were conducted using SAS 9.4 (SAS Institute Inc, Cary, NC, USA) and Prism 8.4 (Graphpad), and *P*-values < 0.05 were considered significant.

Flow cytometry

Staining for flow cytometry analysis was performed using cryo-preserved magnetically enriched BMPC and cryo-preserved PBMC. For BMPC staining, cells were stained for 30 min on ice with CD45-A532 (HI30, Thermo, 1:50), CD38-BB700 (HIT2, BD Horizon, 1:500), CD19-PE (HIB19, 1:200), CXCR5-PE-Dazzle 594 (J252D4, 1:50), CD71-PE-Cy7 (CY1G4, 1:400), CD20-APC-Fire750 (2H7, 1:400), CD3-APC-Fire810 (SK7, 1:50), and Zombie Aqua (all BioLegend) diluted in Brilliant Staining buffer (BD Horizon). Cells were washed twice with 2% FBS and 2 mM EDTA in PBS (P2), fixed for 1h using the True Nuclear permeabilization kit (BioLegend), washed twice with perm/wash buffer, stained for 1h with DyLight 405-conjugated recombinant HA from A/Michigan/45/2015, DyLight 488- and Alexa 647-conjugated S, Ki-67-BV711 (Ki-67, 1:200, BioLegend), and Blimp1-A700 (646702, 1:50, R&D), washed twice with perm/wash buffer, and resuspended in P2. For memory B cell staining, PBMC were stained for 30 min on ice with biotinylated recombinant HAs diluted in P2, washed twice, then stained for 30 min on ice with Alexa 647-conjugated S, IgA-FITC (M24A, Millipore, 1:500), IgG-BV480 (goat polyclonal, Jackson ImmunoResearch, 1:100), IgD-SB702 (IA6-2, Thermo, 1:50), CD38-BB700 (HIT2, BD Horizon, 1:500), CD20-Pacific Blue (2H7, 1:400), CD4-BV570 (OKT4, 1:50), CD24-BV605 (ML5, 1:100), streptavidin-BV650, CD19-BV750 (HIB19, 1:100), CD71-PE (CY1G4, 1:400), CXCR5-PE-Dazzle 594 (J252D4, 1:50), CD27-PE-Cy7 (O323, 1:200), IgM-APC-Fire750 (MHM-88, 1:100), CD3-APC-Fire810 (SK7, 1:500), and Zombie NIR (all BioLegend) diluted in Brilliant Staining buffer (BD Horizon), and washed twice with P2. Cells were acquired on an Aurora using SpectroFlo v2.2 (Cytek). Flow cytometry data were analyzed using FlowJo v10 (Treestar). In each experiment, PBMC were included from convalescent and control participants.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability statement

Relevant data are available from the corresponding author upon reasonable request.

35.   Stadlbauer, D. *et al*. SARS-CoV-2 Seroconversion in Humans: A Detailed Protocol for a Serological Assay, Antigen Production, and Test Setup. *Curr. Protoc. Microbiol*. **57**, (2020).

The WU353, WU367, and 368 studies were reviewed and approved by the Washington University Institutional Review Board (approval nos. 202003186, 202009100, and 202012081, respectively).

Article

Extended Data Fig. 1 | Flow cytometry identification of SARS-CoV-2 elicited plasma cells and memory B cells. a, d, Flow cytometry gating strategies for BMPC in magnetically enriched BMPC and plasmablasts in PBMC (a) and isotype-switched memory B cells and plasmablasts in PBMC (d). **b**, Representative plots of intracellular S and influenza virus hemagglutinin (HA) staining in BMPC from control (*left*) and convalescent (*right*) samples 7 months after symptom onset. **c**, Representative plots of intracellular S staining in plasmablasts in PBMC 1 week after seasonal influenza virus or SARS-CoV-2 vaccination.

Extended Data Table 1 | SARS-CoV-2 convalescent patient demographics

	Total N=77 N (%)	Bone marrow biopsy N=19 N (%)
Age (median [range])	49 (21-69)	52 (30-69)
Sex		
Female	38 (49.4)	7 (36.8)
Male	39 (50.6)	12 (63.2)
Race		
White	70 (90.9)	18 (94.7)
Black	1 (1.3)	0 (0)
Asian	4 (5.2)	0 (0)
Other	2 (2.6)	1 (5.3)
Comorbidities		
Asthma	13 (16.9)	3 (15.8)
Lung disease	0 (0)	0 (0)
Heart disease	3 (3.9)	0 (0)
Hypertension	13 (16.9)	6 (31.6)
Diabetes mellitus	3 (3.9)	3 (15.8)
Cancer	10 (13)	3 (15.8)
Autoimmune disease	4 (5.2)	2 (10.5)
Hyperlipidemia	8 (10.4)	2 (10.5)
Hypothyroidism	5 (6.5)	3 (15.8)
Gastroesophageal reflux disease	5 (6.5)	2 (10.5)
Other	26 (33.8)	10 (52.6)
Solid organ transplant	1 (1.3)	1 (5.3)
Obesity	1 (1.3)	0 (0)

Article

Extended Data Table 2 | SARS-CoV-2 convalescent patient symptoms

	Total N=77 N (%)	Bone marrow biopsy N=19 N (%)
First symptom		
Cough	12 (15.6)	3 (15.8)
Diarrhea	1 (1.3)	0 (0)
Dyspnea	2 (2.6)	1 (5.3)
Fatigue	7 (9.1)	0 (0)
Fever	22 (28.6)	9 (47.4)
Headache	8 (10.4)	2 (10.5)
Loss of taste	3 (3.9)	2 (10.5)
Malaise	4 (5.2)	1 (5.3)
Myalgias	9 (11.7)	0 (0)
Nasal congestion	2 (2.6)	0 (0)
Nausea	1 (1.3)	0 (0)
Night sweats	1 (1.3)	0 (0)
Sore throat	5 (6.5)	1 (5.3)
Symptom present during disease		
Fever	65 (84.4)	17 (89.5)
Cough	54 (70.1)	14 (73.7)
Dyspnea	31 (40.3)	11 (57.9)
Nausea	19 (24.7)	4 (21.1)
Vomiting	9 (11.7)	3 (15.8)
Diarrhea	39 (50.6)	10 (52.6)
Headaches	47 (61)	12 (63.2)
Loss of taste	42 (54.5)	11 (57.9)
Loss of smell	42 (54.5)	10 (52.6)
Fatigue	38 (49.4)	7 (36.8)
Malaise	6 (7.8)	1 (5.3)
Myalgias or body aches	34 (44.2)	8 (42.1)
Sore throat	12 (15.6)	1 (5.3)
Chills	25 (32.5)	6 (31.6)
Nasal congestion	6 (7.8)	0 (0)
Other	32 (41.6)	7 (36.8)
Duration of symptoms in days (median [range])	14 (1-43)	13 (6-30)
Days from symptom onset to positive SARS-CoV-2 PCR test (median [range])	6 (0-36)	6 (1-31)
Days from symptom onset to 1-month blood sample collection (median [range])	41 (21-84)	34 (22-71)
Hospitalization	6 (7.8)	1 (5.3)
COVID medications		
Hydroxychloroquine	2 (2.6)	0 (0)
Chloroquine	1 (1.3)	0 (0)
Azithromycin	14 (18.2)	6 (31.6)
Lopinavir/ritonavir	0 (0)	0 (0)
Remdesivir	0 (0)	0 (0)
Convalescent plasma	0 (0)	0 (0)
None	61 (79.2)	12 (63.2)
Other	2 (2.6)	1 (5.3)

Extended Data Table 3 | SARS-CoV-2 convalescent patient symptoms and follow up samples (months 4–11)

	Month 4		Month 7		Month 11	
	Total N= 76 N (%)	Bone marrow biopsy N=19 N (%)	Total N= 76 N (%)	Bone marrow biopsy N=18 N (%)	Total N= 42 N (%)	Bone marrow biopsy N=12 N (%)
Days from positive SARS-CoV-2 PCR test to follow up visit (median [range])	125 (102-192)	117 (105-150)	222 (191-275)	213 (200-247)	308 (283-369)	303 (283-325)
Days from symptom onset to blood sample collection (median [range])	131 (106-193)	124 (108-155)	227 (194-277)	222 (205-253)	314 (288-373)	309 (297-343)
Any symptom present at follow up visit	25 (32.9)	8 (42.1)	33 (43)	10 (55.6)	20 (47.6)	6 (50)
Fever	0 (0)	0 (0)	2 (2.6)	0 (0)	1 (2.4)	0 (0)
Cough	1 (1.3)	1 (5.3)	0 (0)	0 (0)	1 (2.4)	0 (0)
Dyspnea	7 (9.2)	2 (10.5)	6 (7.9)	3 (16.7)	6 (14.3)	3 (25)
Nausea	1 (1.3)	0 (0)	1 (1.3)	0 (0)	0 (0)	0 (0)
Vomiting	1 (1.3)	1 (5.3)	0 (0)	0 (0)	0 (0)	0 (0)
Diarrhea	2 (2.6)	1 (5.3)	1 (1.3)	0 (0)	0 (0)	0 (0)
Headaches	1 (1.3)	0 (0)	3 (3.9)	0 (0)	2 (4.8)	0 (0)
Loss or altered taste	8 (10.5)	0 (0)	9 (11.8)	1 (5.6)	5 (11.9)	1 (8.3)
Loss or altered smell	13 (17.1)	2 (10.5)	12 (15.8)	2 (11.1)	8 (19)	2 (16.7)
Fatigue	9 (11.8)	4 (21.1)	13 (17.1)	5 (27.8)	8 (19)	3 (25)
Forgetfulness/brain fog	8 (10.5)	6 (31.6)	12 (15.8)	6 (33.3)	10 (23.8)	4 (33.3)
Hair loss	5 (6.6)	1 (5.3)	3 (3.9)	1 (5.6)	2 (4.8)	0 (0)
Other	7 (9.2)	3 (15.8)	12 (15.8)	1 (5.6)	10 (23.8)	1 (8.3)
Joint pain	3 (3.9)	1 (5.3)	7 (9.2)	1 (5.3)	3 (7.1)	0 (0)

Article

Extended Data Table 4 | Healthy control demographics

Variable	Total N= 11 N (%)
Age (median [range])	38 (23-53)
Sex	
Female	4 (36.4)
Male	7 (63.6)
Race	
White	8 (72.7)
Black	1 (9.1)
Asian	1 (9.1)

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Corresponding author(s):	Ali H. Ellebedy
Last updated by author(s):	May 8, 2021

Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed	
☐	☒	The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒	A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☒	☐	A description of all covariates tested
☐	☒	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒	For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☐	☒	Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	Flow cytometry data were acquired using SpectroFlo software v2.2.
Data analysis	Flow cytometry data were analyzed using FlowJo v10 and Prism v8 ELISA and ELISpot data were analyzed using Prism v8 and SAS 9.4

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

All relevant data are available from the corresponding author upon reasonable request.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	No statistical methods were used to determine sample size. 77 convalescent patients and 11 control participants were enrolled based on recruitment; these numbers provided sufficient power to determine differences in SARS-CoV-2 responses between the groups.
Data exclusions	No data were excluded
Replication	Samples were collected from 77 convalescent patients and 11 control participants. ELISA for each participant at each timepoint was performed once with two technical replicates. ELISpot and flow cytometry experiments were performed once for each sample at each timepoint.
Randomization	Different experimental groups were not assigned.
Blinding	No blinding was done in this study; subjective measurements were not made.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology
☒	☐ Animals and other organisms
☐	☒ Human research participants
☒	☐ Clinical data

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☐	☒ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	IgG-HRP (goat polyclonal, Jackson ImmuoResearch 109-035-088), IgG-BV480 (goat polyclonal, Jackson Immunoresearch 109-685-098), IgD-SB702 (IA6-2, Thermo 67-9868-42), IgA-FITC (M24A, Millipore CBL114F), CD45-A532 (HI30, Thermo 58-0459-42), CD38-BB700 (HIT2, BD Horizon 566445), Blimp1-A700 (646702, R&D IC36081N), CD20-Pacific Blue (2H7, 302320), CD4-BV570 (OKT4, 317445), CD24-BV605 (ML5, 311124), streptavidin-BV650 (405232), Ki-67-BV711 (Ki-67, 350516), CD19-BV750 (HIB19, 302262), CD19-PE (HIB19, 302254), CD71-PE (CY1G4, 334106), CXCR5-PE-Dazzle 594 (J252D4, 356928), CD27-PE-Cy7 (O323, 302838), CD71-PE-Cy7 (CY1G4, 334112), CD20-APC-Fire750 (2H7, 302358), IgM-APC-Fire750 (MHM-88, 314546), CD3-APC-Fire810 (SK7, 344858); all Biolegend.
Validation	Commercial antibodies were validated by their respective manufacturers per their associated data sheets and titrated in the lab for their respective assay (ELISA or flow cytometry) by serial dilution

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)	Expi293F (Thermo)
Authentication	The cell line was not authenticated
Mycoplasma contamination	Cell lines were not tested for mycoplasma contamination. Growth rates were consistent with manufacturer's published data.
Commonly misidentified lines (See ICLAC register)	No commonly misidentified cell lines were used

Human research participants

Policy information about studies involving human research participants

Population characteristics	77 SARS-CoV-2 convalescent study participants were recruited, ages 21-69, 49.4% female, 50.6% male 11 healthy control participants with no history of SARS-CoV-2 infection were recruited, ages 23-53, 36.4% female, 63.6% male
Recruitment	Study participants were recruited from the St. Louis metropolitan area by the Washington University Clinical Trials Unit. Potential self-selection and recruiting biases are unlikely to affect the parameters we measured.
Ethics oversight	The study was approved by the Washington University IRB

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:

☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☒ All plots are contour plots with outliers or pseudocolor plots.

☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation	Peripheral blood and bone marrow mononuclear cells were isolated from EDTA anticoagulated blood and bone marrow aspirates, respectively using density gradient centrifugation, and remaining RBCs were lysed with ammonium chloride lysis buffer. Bone marrow plasma cells were magnetically enriched from bone marrow mononuclear cells and immediately used for ELISpot or cryopreserved in 10% dimethylsufoxide in FBS for flow cytometric analysis. PBMCs were immediately used or cryopreserved in 10% DMSO in FBS.
Instrument	Cytek Aurora
Software	Flow cytometry data were acquired using Cytek SpectroFlo software, and analyzed using FlowJo (Treestar) v10.
Cell population abundance	Cells were not sorted
Gating strategy	Gating strategies are shown in extended data figure

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.

EXHIBIT 7

Immunological memory to SARS-CoV-2 assessed for greater than six months after infection

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ABSTRACT

Understanding immune memory to SARS-CoV-2 is critical for improving diagnostics and vaccines, and for assessing the likely future course of the pandemic. We analyzed multiple compartments of circulating immune memory to SARS-CoV-2 in 185 COVID-19 cases, including 41 cases at $\geq$ 6 months post-infection. Spike IgG was relatively stable over 6+ months. Spike-specific memory B cells were more abundant at 6 months than at 1 month. SARS-CoV-2-specific CD4$^+$ T cells and CD8$^+$ T cells declined with a half-life of 3-5 months. By studying antibody, memory B cell, CD4$^+$ T cell, and CD8$^+$ T cell memory to SARS-CoV-2 in an integrated manner, we observed that each component of SARS-CoV-2 immune memory exhibited distinct kinetics.

INTRODUCTION

Coronavirus disease 2019 (COVID-19), caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is a serious disease that has resulted in widespread global morbidity and mortality. Humans make SARS-CoV-2-specific antibodies, CD4+ T cells, and CD8+ T cells in response to SARS-CoV-2 infection (*1–4*). Studies of acute and convalescent COVID-19 patients have observed that T cell responses are associated with lessened disease (*5–7*), suggesting that SARS-CoV-2-specific CD4+ T cell and CD8+ T cell responses may be important for control and resolution of primary SARS-CoV-2 infection. Ineffective innate immunity has been strongly associated with a lack of control of primary SARS-CoV-2 infection and a high risk of fatal COVID-19 (*8–12*), accompanied by innate cell immunopathology (*13–18*). Neutralizing antibodies have generally not correlated with lessened COVID-19 disease severity (*5*, *19*, *20*), which was also observed for Middle Eastern respiratory syndrome (MERS), caused by infection with the human coronavirus MERS-CoV (*21*). Instead, neutralizing antibodies are associated with protective immunity against secondary (2°) infection with SARS-CoV-2 or SARS-CoV in non-human primates (*3*, *22–25*). Additionally, human subjects with detectable neutralizing antibodies were protected from 2° COVID-19 in a ship outbreak (*26*). Passive transfer of neutralizing antibodies in advance of infection (mimicking the conditions of 2° infection) effectively limits upper respiratory tract (URT) infection, lower respiratory tract (lung) infection, and symptomatic disease in animal models (*27–29*). Passive transfer of neutralizing antibodies provided after initiation of infection in humans have had more limited effects on COVID-19 (*30*, *31*), consistent with a substantial role for T cells in control and clearance of an ongoing SARS-CoV-2 infection. Thus, studying antibody, memory B cell, CD4+ T cell, and CD8+ T cell memory to SARS-CoV-2 in an integrated manner is likely important for understanding the durability of protective immunity against COVID-19 generated by primary SARS-CoV-2 infection (*1*, *19*, *32*).

While sterilizing immunity against viruses can only be accomplished by high-titer neutralizing antibodies, successful protection against clinical disease or death can be accomplished by several other adaptive immune memory scenarios. Possible mechanisms of immunological protection can vary based on the relative kinetics of the immune memory responses and infection. For example, clinical hepatitis after hepatitis B virus (HBV) infection is prevented by vaccine-elicited immune memory even in the absence of circulating antibodies, because of the relatively slow course of HBV disease (*33*, *34*). The relatively slow course of severe COVID-19 in humans (median 19 days post-symptom onset (PSO) for fatal cases (*35*)) suggests that protective immunity against symptomatic or severe 2° COVID-19 may very well involve memory compartments such as circulating memory T cells and memory B cells (which can take several days to reactivate and generate recall T cell responses and/or anamnestic antibody responses) (*19*, *21*, *32*).

Immune memory, from either primary infection or immunization, is the source of protective immunity from a subsequent infection (*36–38*). Thus, COVID-19 vaccine development is closely tied to the topic of immunological memory (*1*, *3*). Despite intensive study, the kinetics, duration, and evolution of immune memory in humans to infection or immunization are not in general predictable based on the initial effector phase, and immune responses at short time points after resolution of infection are not very predictive of long-term memory (*39–41*). Thus, assessing responses over an interval of six months or more is usually required to ascertain the durability of immune memory.

A thorough understanding of immune memory to SARS-CoV-2 requires evaluation of its various components, including B cells, CD8+ T cells, and CD4+ T cells, as these different cell types may have immune memory kinetics relatively independent of each other. Understanding the complexities of immune memory to SARS-CoV-2 is key to gain insights into the likelihood of durability of protective immunity against re-infection with SARS-CoV-2 and 2° COVID-19 disease. In the current study, we assessed immune memory of all three branches of adaptive immunity (CD4+ T cell, CD8+ T cell, and

humoral immunity) in a cross-sectional study of 185 recovered COVID-19 cases, extending out to greater than six months post-infection. The findings have implications for immunity against 2° COVID-19, and thus the potential future course of the pandemic (*42*, *43*).

COVID-19 cohort

185 individuals with COVID-19 were recruited for this study. Subjects (43% male, 57% female) represented a range of asymptomatic, mild, moderate, and severe COVID-19 cases (Table S1), and were recruited from multiple sites throughout the United States. The majority of subjects were from California or New York. The majority of subjects had a mild case of COVID-19, not requiring hospitalization. 92% of subjects were never hospitalized for COVID-19; 7% of subjects were hospitalized, some of whom required intensive care unit (ICU) care (Table S1; hospitalization requirement not reported for 1 subject), consistent with the COVID-19 disease severity distribution in the USA. The majority of subjects (97%) reported symptomatic disease (Table S1; not reported for 1 subject). The ages of the subjects ranged from 19 to 81 years old (Table S1). Most subjects provided a blood sample at a single time point, between 6 days (d) post-symptom onset (PSO) and 240d PSO (Table S1), with 41 samples at $\geq$ six months PSO (d178 or longer). Thirty-eight subjects provided longitudinal blood samples over a duration of several months (2-4 time points. Table S1).

SARS-CoV-2 circulating antibodies over time

The vast majority of SARS-CoV-2 infected individuals seroconvert, at least for a duration of months (*1*, *2*, *4*, *20*, *44–46*). These estimates range from 91-99% in large studies (*20*, *46*). Durability assessments of circulating antibody titers in Figure 1 were based on data $\geq$ 20d PSO, using curve fits modeling a continuous decay, one-phased decay, or two-phased decay, with the best fitting model shown in blue. Negative and positive controls were used to standardize each assay and normalize across experiments. SARS-CoV-2 spike IgG endpoint ELISA titers in plasma were measured for all subjects of this cohort (**Fig. 1A-B**). Spike receptor binding domain (RBD) IgG was also measured (**Fig. 1C-D**), as RBD is the target of the vast majority of neutralizing antibodies against SARS-CoV-2 (*4*, *28*, *47*, *48*). SARS-CoV-2 pseudovirus (PSV) neutralizing antibody titers were measured in all subjects, as the functional complement of the antibody binding assays (**Fig. 1E-F**). Nucleocapsid (N) IgG endpoint ELISA titers were also measured for all subjects (**Fig. 1G-H**), as nucleocapsid is a common antigen in commercial SARS-CoV-2 serological test kits.

SARS-CoV-2 spike IgG titers were nearly stable from d20-d240 PSO, when assessing all COVID-19 subjects by cross-sectional analysis (half-life $t_{1/2}$ = 140d, **Fig. 1A**). Spike IgG titers were heterogenous among subjects (range 5 to 73,071; 575 median), as has been widely observed (*20*, *48*). This gave a wide confidence interval for the spike IgG $t_{1/2}$ (95% CI: 89 to 329d). While the antibody responses likely have underlying bi-phasic decay kinetics, the best fit curve was a linear decay, probably related to heterogeneity between individuals. SARS-CoV-2 nucleocapsid IgG kinetics were similar to spike IgG over 8 months ($t_{1/2}$ 67d, 95% CI: 49-105d. **Fig. 1G**). As a complementary approach, using paired samples from the subset of subjects who donated at two or more time points, the calculated spike IgG titer average $t_{1/2}$ was 100d, (95% CI: 64-220d, **Fig. 1B**) and the nucleocapsid IgG titer average $t_{1/2}$ was 67d, (95% CI: 54-88d, **Fig. 1H**). The percentage of subjects seropositive for spike IgG at 1 month PSO (d20-50) was 98% (54/55). The percentage of subjects seropositive for spike IgG at 6 to 8 months PSO (d >178) was 90% (36/40).

Cross-sectional analysis SARS-CoV-2 RBD IgG titers from d20-d240 PSO gave an estimated $t_{1/2}$ of 83d, 95% CI 62-127d (**Fig. 1C**). As a complementary approach, we again used paired samples, which gave an average $t_{1/2}$ of 68d, 95% CI: 57-85d (**Fig. 1D**). The percentage of subjects seropositive for RBD IgG at 6 to 8 months PSO was 88% (35/40). Thus, the RBD IgG titer maintenance largely matched that of

spike IgG. SARS-CoV-2 PSV neutralization titers in the full cohort largely matched the results of SARS-CoV-2 RBD IgG ELISA binding titers (**Fig.1E-F**). A one-phase decay model was the best fit (P=0.015, F test. Initial decay $t_{1/2}$ 27d, followed by an extended plateau phase. **Fig. 1E**), while a linear decay gave an estimated $t_{1/2}$ of 114d. Paired timepoints analysis of the PSV neutralization titers gave an estimated $t_{1/2}$ 87d, (95% CI: 68-123d, **Fig. 1F**). The percentage of subjects seropositive for SARS-CoV-2 neutralizing antibodies (titer $\geq$ 20) at 6 to 8 months PSO was 90% (36/40). Notably, even low levels of circulating neutralizing antibody titers ($\geq$ 1:20) were associated with a substantial degree of protection against COVID-19 in non-human primates (*24, 49*). Thus, modest levels of circulating SARS-CoV-2 neutralizing antibodies are of biological interest in humans.

SARS-CoV-2 spike IgA (**Fig. 1I-J**) and RBD IgA (**Fig.1K-L**) titers were also assessed. Paired timepoints analysis of spike IgA titers yielded an estimated $t_{1/2}$ of 214d, 95% CI 126-703d (**Fig. 1J**). Cross-sectional analysis of spike IgA fit a shohrt one-phase decay model with an extended plateau phase (initial $t_{1/2}$ of 11d, **Fig. 1I**). Circulating RBD IgA had an estimated $t_{1/2}$ of 27d, 95% CI 15-58d, decaying by ~90d in a majority of COVID-19 cases to levels indistinguishable from uninfected controls (**Fig. 1K**), consistent with observations 3 months PSO (*46, 50*). By paired sample analysis, long-lasting RBD IgA was made in some subjects, but often near the limit of sensitivity (LOS) (**Fig. 1L**).

SARS-CoV-2 memory B cells

To identify SARS-CoV-2-specific memory B cells, fluorescently labeled multimerized probes were used to detect B cells specific to spike, RBD, and nucleocapsid (**Fig 2A,** Fig. S1). Antigen-binding memory B cells (defined as IgD^{-} and/or CD27^{+}) were further distinguished according to surface immunoglobulin (Ig) isotypes: IgM, IgG or IgA (**Fig. 2B,** Fig. S1).

Spike-specific memory B cells in SARS-CoV-2 unexposed donors were rare (median 0.0078%. **Fig 2A, 2C.**). Cross-sectional analysis revealed that frequencies of SARS-CoV-2 spike-specific memory B cells increased over the first ~150d PSO and then plateaued (Pseudo-first order model for best fit curve. R^2 = 0.14. Better fit than second order polynomial model by Akaike's Information Criterion. **Fig 2C,** Fig. S2A). Spike-specific memory B cell frequencies increased from the first time-point (d36-d163) to the second time-point (d111-d240) in paired samples from 24 of 36 longitudinally tracked donors (**Fig 2D**).

RBD-specific memory B cells displayed similar kinetics to spike-specific memory B cells. As expected, RBD-specific memory B cells were undetectable in SARS-CoV-2 unexposed subjects (**Fig. 2E.** Fig. S2C). RBD-specific memory B cells appeared as early as 16d PSO, and the frequency steadily increased in the following 4-5 months (**Fig. 2E.** Fig. S2B-C). 29 of 36 longitudinally tracked individuals had higher frequencies of RBD-specific memory B cells at the later time point (**Fig. 2F**), again showing an increase in SARS-CoV-2 specific memory B cells several months post-infection. ~10-30% of spike-specific memory B cells from SARS-CoV-2 convalescent donors were specific for the RBD domain (**Fig. 2A,** Fig. S2B).

SARS-CoV-2 nucleocapsid-specific memory B cells were also detected after SARS-CoV-2 infection (**Fig. 2A**). Similar to spike- and RBD-specific memory B cells, nucleocapsid-specific memory B cell frequency steadily increased during the first ~5 months PSO (**Fig. 2G, 2H,** Fig. S2D). Antibody affinity maturation could potentially explain the increased frequencies of SARS-CoV-2-specific memory B cells detected by the antigen probes. However, geometric mean fluorescent intensity (MFI) of probe binding was stable over time (Fig. S2I-J), not supporting an affinity maturation explanation for the increased memory B cell frequencies.

Representation of Ig isotypes among the SARS-CoV-2 spike-specific memory B cell population shifted with time (**Fig. 2I-2O**). During the earliest phase of memory (20-60d PSO), IgM[+] and IgG[+] isotypes were similarly represented (**Fig. 2O**), but the IgM[+] memory B cells gradually disappeared (**Fig. 2M, 2N, 2O**), and IgG[+] spike-specific memory B cells then dominated by 6 months PSO (**Fig. 2O**). IgA[+]

spike-specific memory B cells were detected as a small fraction of the total spike-specific memory B cells (~5%, **Fig. 2O**). IgG$^+$ spike-specific memory B cell frequency increased while IgA$^+$ was low and stable over the 8 month period (**Fig. 2I-2L**). Similar patterns of increasing IgG$^+$ memory, short-lived IgM$^+$ memory, and stable IgA$^+$ memory were observed for RBD- and nucleocapsid-specific memory B cells over the 8 month period (**Fig. 2O-2Q, Fig. S2E-S2H**).

There is limited knowledge of memory B cell kinetics following primary acute viral infection in humans. We are not aware of other cross-sectional or longitudinal analyses of antigen-specific memory B cells covering a 6+ month window after an acute infection by flow cytometry, except for four individuals with Ebola (*51*) and two individuals studied after yellow fever virus immunization (*52*), and also excepting influenza vaccines, for which people have repeated exposures and complex immune history. In the yellow fever study, short-lived IgM$^+$ memory and longer-lasting isotype-switched memory B cells were observed in the two individuals. Overall, based on the observations here, development of B cell memory to SARS-CoV-2 appeared to be robust and likely long-lasting.

SARS-CoV-2 memory CD8$^+$ T cells

SARS-CoV-2 memory CD8$^+$ T cells in 155 subjects were identified using a series of 23 peptide pools covering the entirety of the SARS-CoV-2 ORFeome (*2*, *5*). The most commonly recognized ORFs were spike (S), membrane (M), nucleocapsid (N), and ORF3a (CD69$^+$ CD137$^+$, **Fig. 3A** and Fig. S3A-B), consistent with our previous study (*2*). The percentage of subjects with detectable circulating SARS-CoV-2 memory CD8$^+$ T cells at 1 month PSO (d20-50) was 61% (30/49, **Fig. 3B**). The proportion of subjects positive for SARS-CoV-2 memory CD8$^+$ T cells at $\geq$ 6 months PSO was 50% (9/18). SARS-CoV-2 memory CD8$^+$ T cells declined with an apparent $t_{1/2}$ of 166d in the full cohort (**Fig. 3B**) and $t_{1/2}$ 139d among 24 paired samples (**Fig. 3C**). Spike-specific memory CD8$^+$ T cells exhibited similar kinetics to the overall SARS-CoV-2-specific memory CD8$^+$ T cells ($t_{1/2}$ 271d for the full cohort and 164d among paired samples, **Fig. 3D-E**, respectively). Phenotypic markers indicated that the majority of SARS-CoV-2-specific memory CD8$^+$ T cells were T$_{EMRA}$ (*53*), with small populations of T$_{CM}$ and T$_{EM}$ (**Fig. 3F**). In the context of influenza, CD8$^+$ T$_{EMRA}$ were associated with protection against severe disease in humans (*54*). The memory CD8$^+$ T cell half-lives observed herein were comparable to the 123d $t_{1/2}$ observed for memory CD8$^+$ T cells within 1-2 years after yellow fever immunization (*55*). Overall, the decay of circulating SARS-CoV-2-specific CD8$^+$ T cell is consistent with what has been reported for another acute virus.

SARS-CoV-2 memory CD4$^+$ T cells

SARS-CoV-2 memory CD4$^+$ T cells in 155 subjects were identified using the same series of 23 peptide pools covering the SARS-CoV-2 ORFeome (*2*, *5*). The most commonly recognized ORFs were spike, M, N, ORF3a, and nsp3 (CD137$^+$ OX40$^+$, **Fig. 4A** and Fig. S4A-B), consistent with our previous study (*2*). Circulating SARS-CoV-2 memory CD4$^+$ T cell responses were quite robust (**Fig. 4B**). Approximately one third (35%, 17/49) of COVID-19 cases at 1 month PSO had > 1.0% SARS-CoV-2-specific CD4$^+$ T cells. SARS-CoV-2 memory CD4$^+$ T cells declined over the 6 month time frame of this study with an apparent $t_{1/2}$ of 96d in the full cohort (**Fig. 4B**) and $t_{1/2}$ 64d among paired samples (**Fig. 4C**). The percentage of subjects with detectable circulating SARS-CoV-2 memory CD4$^+$ T cells at 1 month PSO (d20-50) was 94% (46/49, **Fig. 4B**). The proportion of subjects positive for SARS-CoV-2 memory CD4$^+$ T cells at $\geq$ 6 months PSO was 89% (16/18). Spike-specific and M-specific memory CD4$^+$ T cells exhibited similar kinetics to the overall SARS-CoV-2-specific memory CD4$^+$ T cells (whole cohort $t_{1/2}$ 150d and 174d, respectively. **Fig. 4D-E**, and Fig. S4D). A plurality of the SARS-CoV-2 memory CD4$^+$ T cells present at $\geq$ 6 months PSO were T$_{CM}$ (**Fig. 4F**).

T follicular helpers (T$_{FH}$) are the specialized subset of CD4$^+$ T cells required for B cell help (*56*), and, therefore, critical for the generation of neutralizing antibodies and long-lived humoral immunity in

most contexts. Thus, we examined circulating T_{FH} (cT_{FH}) memory $CD4^+$ T cells, with particular interest in spike-specific memory cT_{FH} cells due to the importance of antibody responses against spike. Memory cT_{FH} cells specific for predicted epitopes across the remainder of the SARS-CoV-2 genome were also measured, using the MP_R megapool ([2]). Memory cT_{FH} cells specific for SARS-CoV-2 spike and MP_R were detected in the majority of COVID-19 cases at early time points (16/17 & 17/17. **Fig. 4G-H**, and Fig. S5A-C). cT_{FH} memory appeared to be stable, with 100% of subjects positive for spike cT_{FH} and 92% positive for MP_R cT_{FH} memory at 6 months PSO (**Fig. 4G-H**).

Recently activated cT_{FH} cells are PD-1^{hi} ([56]). Consistent with conversion to resting memory cT_{FH} cells, the percentage of PD-1^{hi} SARS-CoV-2-specific memory cT_{FH} dropped over time (**Fig. 4I**). CCR6$^+$ SARS-CoV-2-specific cT_{FH} cells have been associated with reduced COVID-19 disease severity ([5]) and have been reported to be a major fraction of spike-specific cT_{FH} cells ([5, 57]). Here we confirmed that a significant fraction of both spike-specific and MP_R cT_{FH} were CCR6$^+$. We also observed significant increases in the fraction of CCR6$^+$ cT_{FH} memory over time ($P < 0.001$ and $P < 0.01$ compared to bulk cT_{FH} at $\geq$ 6 months PSO. **Fig. 4J**). Overall, substantial cT_{FH} memory was observed after SARS-CoV-2 infection, with durability $\geq$ 6 months PSO.

Immune memory relationships

Additional features of immune memory to SARS-CoV-2 were considered, including relationships between the compartments of immune memory. Immune memory was examined for associations between magnitude of memory and disease severity. Circulating antibody titers of severe COVID-19 cases trended higher, consistent with other studies (Fig. S6A). No distinction was observed in B and T cell memory between hospitalized and non-hospitalized COVID-19 cases (Fig. S6B-F), though interpretations are limited by the relatively low number of severe cases in this cohort. The influence of gender on immune memory was also assessed. Overall, males had higher spike IgG (ANCOVA p=0.00019, **Fig. 5A**) and nucleocapsid and RBD IgG (**Fig. S7A-D**). Higher spike IgG in males was also observed in another convalescent cohort ([48]). In contrast, no differences were observed in SARS-CoV-2 memory B cell frequencies or T cells between males and females (**Fig. S7E-I**). In sum, the heterogeneity in immune memory to SARS-CoV-2 was not primarily attributable to gender or COVID-19 disease severity.

Very few published data sets compare antigen-specific antibody, B cell, $CD8^+$ T cell, and $CD4^+$ T cell memory to an acute viral infection in the same individuals. To our knowledge, this is the largest study of its kind, for any acute infection. We examined relationships between immune memory compartments to gain insights into the interrelationships between immune memory types and better interpret the totality of immune memory to SARS-CoV-2. We focused on RBD IgG, RBD IgA, RBD memory B cells, total SARS-CoV-2-specific $CD8^+$ T cells, and total SARS-CoV-2-specific $CD4^+$ T cells, due to their putative potential roles in protective immunity. The majority (59%) of COVID-19 cases were positive for all five of these immune memory compartments at 1-2 months PSO (**Fig. 5B**), with the incomplete responses largely reflecting individuals with no detectable $CD8^+$ T cell memory and/or poor RBD IgA responses (**Fig. 5C**). By 5+ months after COVID-19, the proportion of individuals positive for all five of these immune memory compartments had dropped to 40%; nevertheless, 96% of individuals were still positive for at least three out of five SARS-CoV-2 immune memory responses (**Fig. 5B**). Immune memory at 5+ months PSO represented different contributions by immune memory compartments in different individuals (**Fig. 5C**), again demonstrating heterogeneity of immune memory, with increasing heterogeneity in the population over time.

Interrelationships between the components of memory were examined by assessing ratios over time. The ratio of SARS-CoV-2 $CD4^+$ and $CD8^+$ T cell memory was largely stable over time (**Fig. 5D**, Fig. S8A). Given that serological measurements are the simplest measurements of immune memory at a

population scale, we examined how well such measurements may serve as surrogate markers of other components of SARS-CoV-2 immune memory over time. The relationship between circulating RBD IgG and RBD-specific memory B cells changed ~20-fold over the time range studied (R=0.60, **Fig. 5D,** Fig. S8B). The changing relationship between circulating RBD IgA and RBD-specific memory B cells was even larger, with a 40-fold shift (R=0.62, **Fig. 5D,** Fig. S8C). The relationship between RBD IgG and SARS-CoV-2 CD4$^+$ T cell memory was relatively flat over the time range studied (**Fig. 5D**); however, variation spanned a ~1000-fold range and thus predictive power of circulating RBD IgG for assessing T cell memory was poor due to heterogeneity between individuals (R=0.02, Fig. S8D-E). In aggregate, while heterogeneity of immune responses is a defining feature of COVID-19, immune memory to SARS-CoV-2 develops in almost all subjects, with complex relationships between the individual immune memory compartments.

Conclusion

In this study, we aimed to fill a gap in our basic understanding of immune memory after COVID-19. This required simultaneous measurement of circulating antibodies, memory B cells, CD8$^+$ T cells, and CD4$^+$ T cells specific for SARS-CoV-2, in a group of subjects with a full range of disease and distributed from short time points PSO out to $\geq$ 8 months PSO. To our knowledge, this is the first study of its kind, incorporating antigen-specific antibody, memory B cell, CD8$^+$ T cell, and CD4$^+$ T cell measurements, out past 6 months post-infection. By studying these multiple compartments of adaptive immunity in an integrated manner, we observed that each component of SARS-CoV-2 immune memory exhibited distinct kinetics.

The spike IgG titers were durable, with modest declines in titers at 6 to 8 months PSO at the population level. RBD IgG and SARS-CoV-2 PSV neutralizing antibody titers were potentially similarly stable, consistent with the RBD domain of spike being the dominant neutralizing antibody target. However, due to the nature of only having data at two time points, the paired sample longitudinal data set could not distinguish between models of a continuous log-linear decay and a bi-phasic decay with a slower half-life later. It is well recognized that the magnitude of the antibody response against SARS-CoV-2 is highly heterogenous between individuals. We observed that heterogenous initial antibody responses did not collapse into a homogeneous circulating antibody memory. That heterogeneity is thus a central feature of immune memory to this virus. For antibodies, the responses spanned a ~200-fold range. Additionally, the heterogeneity showed that long-term longitudinal studies will be required to precisely define antibody kinetics to SARS-CoV-2. Nevertheless, at 5+ months PSO, almost all individuals were positive for SARS-CoV-2 spike and RBD IgG.

Notably, memory B cells specific for spike or RBD were detected in almost all COVID-19 cases, with no apparent half-life at 5+ months post-infection. B cell memory to some other infections has been observed to be long-lived, including 60+ years after smallpox vaccination (*58*), or 90+ years after infection with influenza (*59*), another respiratory virus like SARS-CoV-2. The memory T cell half-lives observed over 6+ months PSO in this cohort (~166-271d for CD8$^+$ and ~96-174d for CD4$^+$ T cells) were comparable to the 123d $t_{1/2}$ observed for memory CD8$^+$ T cells soon after yellow fever immunization (*55*). Notably, the durability of a fraction of the yellow fever virus-specific memory CD8$^+$ T cells possessed an estimated $t_{1/2}$ of 485d by deuterium labeling (*55*). Using different approaches, the long-term durability of memory CD4$^+$ T cells to smallpox, over a period of many years, was an estimated $t_{1/2}$ of ~10 years (*58*, *60*), which is also consistent with recent detection of SARS-CoV T cells 17 years after the initial infection (*61*). These data suggest that T cell memory might reach a more stable plateau, or slower decay phase, later than the first 6 months post-infection.

While immune memory is the source of long-term protective immunity, direct conclusions about protective immunity cannot be made on the basis of quantifying SARS-CoV-2 circulating antibodies,

memory B cells, CD8$^+$ T cells, and CD4$^+$ T cells, because mechanisms of protective immunity against SARS-CoV-2 or COVID-19 are not defined in humans. Nevertheless, some reasonable interpretations can be made. Antibodies are the only component of immune memory that can provide truly sterilizing immunity. Immunization studies in non-human primates have indicated that circulating neutralization titers of ~200 may provide sterilizing immunity against a relatively high dose URT challenge (*62*), and neutralizing titers of ~3,400 may provide sterilizing immunity against a very high dose URT challenge (*63*), although direct comparisons are not possible because the neutralizing antibody assays have not been standardized (*3*). Conclusions are also constrained by the limited overall amount of data on the topic of protective immunity to SARS-CoV-2, though progress in this field has been exceptionally rapid by any standard.

Beyond sterilizing immunity, confining SARS-CoV-2 to the URT and oral cavity would minimize COVID-19 disease severity to 'common cold' or asymptomatic disease. This outcome is the primary goal of current COVID-19 vaccine clinical trials (*3, 64*). Such an outcome could potentially be mediated by a mixture of memory CD4$^+$ T cells, memory CD8$^+$ T cells, and memory B cells specific for RBD producing anamnestic neutralizing antibodies, based on mechanisms of action demonstrated in mouse models of other viral infections (*65–67*). In human COVID-19 infections, SARS-CoV-2-specific CD4$^+$ T cells and CD8$^+$ T cells are associated with lessened COVID-19 disease severity of an ongoing SARS-CoV-2 infection (*5*), and rapid seroconversion was associated with significantly reduced viral loads in acute disease over 14 days (*30*). Both of those associations are consistent with the hypothesis that SARS-CoV-2 memory T cells and B cells would be capable of substantially limiting SARS-CoV-2 dissemination and/or cumulative viral load, resulting in substantially reduced COVID-19 disease severity. The likelihood of such outcomes is also closely tied to the kinetics of the infection, as memory B and T cell responses can take 3-5 days to successfully respond to an infection. As noted above, given the relatively slow course of severe COVID-19 in humans, a large window of time is available for resting immune memory compartments to potentially contribute in meaningful ways to protective immunity against pneumonia or severe or fatal 2° COVID-19. The presence of sub-sterilizing neutralizing antibody titers at the time of SARS-CoV-2 exposure would blunt the size of the initial infection, and may provide an added contribution to limiting COVID-19 severity, based on observations of protective immunity for other human respiratory viral infections (*38, 68–70*) and observations of SARS-CoV-2 vaccines in non-human primates (*49, 63, 71*).

This study has limitations. Longitudinal data for each subject, with at least 3 time points per subject, would be required to distinguish between linear, one-phase with plateau, and two-phase decay best fit models for more precise understanding of long-term kinetics of SARS-CoV-2 antibodies. Nevertheless, the current cross-sectional data describe well the dynamics of SARS-CoV-2 memory B cells, CD8$^+$ T cell, and CD4$^+$ T cell over 6 months PSO. Additionally, circulating memory was assessed here; it is possible that local URT immune memory is a minimal, moderate, or large component of immune memory after a primary infection with SARS-CoV-2. This remains to be determined.

When considering potential connections between immune memory and protective immunity, it is key to consider the available epidemiological data. Individual case reports demonstrate that reinfections with SARS-CoV-2 are occurring (*72, 73*). What is currently lacking is an epidemiological framework for quantifying how rare or common such reinfection events are. Thus, interpretations of current events are very constrained. There is a high degree of heterogeneity in the magnitude of adaptive immune responses to this novel coronavirus. That heterogeneity was observed in this study to be carried on into the immune memory phase to SARS-CoV-2. As a result of the immune response heterogeneity, as observed in the cohort here, it may be expected that at least a fraction of the SARS-CoV-2-infected population with particularly low immune memory would be susceptible to re-infection relatively quickly. The source of heterogeneity in immune memory to SARS-CoV-2 is unknown and worth further examination. It is possible that some of that heterogeneity is a result of low cumulative viral load

or initial inoculum, essentially resulting in a very minor or transient infection that barely triggered an adaptive immune response in some individuals. Nevertheless, immune memory consisting of at least three immunological compartments was measurable in ~90% of subjects $\geq$ 5 months PSO, indicating that durable immunity against 2° COVID-19 disease is a possibility in most individuals.

METHODS

Human Subjects

The Institutional Review Boards of the University of California, San Diego (UCSD; 200236X) and the La Jolla Institute for Immunology (LJI; VD-214) approved the protocols used for blood collection for subjects with COVID-19 who donated at all sites other than Mt. Sinai. The Icahn School of Medicine at Mt. Sinai IRB approved the samples collected at this institution in New York City (IRB-16-00791). All human subjects were assessed for medical decision-making capacity using a standardized, approved assessment, and voluntarily gave informed consent prior to being enrolled in the study. Study inclusion criteria included a diagnosis of COVID-19 or suspected COVID-19, age of 18 years or greater, willingness and ability to provide informed consent. Although not a strict inclusion criterion, evidence of positive PCR-based testing for SARS-CoV-2 was requested from subjects prior to participation. 143 cases were confirmed SARS-CoV-2 positive by PCR-based testing (Table S1). Two subjects tested negative by SARS-CoV-2 PCR (Table S1). The remainder were not tested or did not have test results available for review (Table S1). Subjects who had a medical history and/or symptoms consistent with COVID-19, but lacked positive PCR-based testing for SARS-CoV-2 and subsequently had negative laboratory-based serologic testing for SARS-CoV-2 were then excluded; i.e., all COVID-19 cases in this study were confirmed cases by SARS-CoV-2 PCR or SARS-CoV-2 serodiagnostics, or both. Adults of all races, ethnicities, ages, and genders were eligible to participate. Study exclusion criteria included lack of willingness to participate, lack of ability to provide informed consent, or a medical contraindication to blood donation (e.g. severe anemia). Subject samples at LJI were obtained from individuals in California and at least seven other states.

Blood collection and processing methods at LJI were performed as previously described (*5*). Briefly, whole blood was collected via phlebotomy in acid citrate dextrose (ACD) serum separator tubes (SST), or ethylenediaminetetraacetic acid (EDTA) tubes and processed for peripheral blood mononuclear cells (PBMC), serum, and plasma isolation. Most donors were screened for symptoms prior to scheduling blood draws, and had to be symptom-free and approximately 3-4 weeks out from symptom onset at the time of the initial blood draw at UCSD or LJI, respectively. Samples were coded, and then de-identified prior to analysis. Other efforts to maintain the confidentiality of participants included the labeling samples with coded identification numbers. An overview of the characteristics of subjects with COVID-19 is provided in Table S1.

COVID-19 disease severity was scored from 0 to 10 using a numerical scoring system based on the NIH ordinal scale (*5*, *74*). A categorical descriptor was applied based on this scoring system: "asymptomatic" for a score of 1, "mild" for a score of 2-3, "moderate" for a score of 4-5, and "severe" for a score of 6 or more. Subjects with a numerical score of 4 or higher required hospitalization (including admission for observation) for management of COVID-19. The days PSO was determined based on the difference between the date of the blood collection and the date of first reported symptoms consistent with COVID-19. For asymptomatic subjects, the day from first positive SARS-CoV-2 PCR-based testing was used in place of the date of first reported COVID-19 symptoms.

SARS-CoV-2 ELISAs

SARS-CoV-2 ELISAs were performed as previously described (*2*, *5*, *75*). Briefly, Corning 96-well half area plates (ThermoFisher 3690) were coated with 1µg/mL of antigen overnight at 4°C. Antigens included recombinant SARS-CoV-2 RBD protein, recombinant spike protein (*5*), and recombinant nucleocapsid protein (GenScript Z03488). The following day, plates were blocked with 3% milk in phosphate buffered saline (PBS) containing 0.05% Tween-20 for 1.5 hours at room temperature. Plasma was heat inactivated at 56°C for 30-60 minutes. Plasma was diluted in 1% milk containing 0.05% Tween-20 in PBS starting at

a 1:3 dilution followed by serial dilutions by 3 and incubated for 1.5 hours at room temperature. Plates were washed 5 times with 0.05% PBS-Tween-20. Secondary antibodies were diluted in 1% milk containing 0.05% Tween-20 in PBS. For IgG, anti-human IgG peroxidase antibody produced in goat (Sigma A6029) was used at a 1:5,000 dilution. For IgA, anti-human IgA horseradish peroxidase antibody (Hybridoma Reagent Laboratory HP6123-HRP) was used at a 1:1,000 dilution. The HP6123 monoclonal anti-IgA was used because of its CDC and WHO validated specificity for human IgA1 and IgA2 and lack of crossreactivity with non-IgA isotypes (76).

Endpoint titers were plotted for each sample, using background subtracted data. A positive control standard was created by pooling plasma from 6 convalescent COVID-19 donors to normalize between experiments. The limit of detection (LOD) was defined as 1:3 for IgG, 1:10 for IgA. Limit of sensitivity (LOS) for SARS-CoV-2 infected individuals was established based on uninfected subjects, using plasma from normal healthy donors never exposed to SARS-CoV-2. For cross-sectional analyses, modeling for the best fit curve (e.g., one phase decay versus simple linear regression) was performed using GraphPad Prism 8.0. Best curve fit was defined by an extra sum-of-squares F Test, selecting the simpler model unless $P < 0.05$ (77). To calculate the $t_{1/2}$, $\log_2$ transformed data was utilized. Using the best fit curve, either a one phase decay non-linear fit or a simple linear regression (-1/slope) was utilized. Pearson R was calculated for correlation. For longitudinal samples, a simple linear regression was performed, with $t_{1/2}$ calculated from $\log_2$ transformed data for each pair. For gender analyses, modeling and $t_{1/2}$ was performed similar to cross-sectional analyses; ANCOVA (VassarStats or GraphPad Prism 8.4) was then performed between male and female data sets.

Neutralizing antibody assays

The pseudovirus neutralizing antibody assay was performed as previously described (5). Briefly, Vero cells were seeded in 96 well plates to produce a monolayer at the time of infection. Pre-titrated amounts of rVSV-SARS-Cov-2 (phCMV3-SARS-CoV-2 spike SARS-CoV-2-pseduotyped VSV-ΔG-GFP were generated by transfecting 293T cells) were incubated with serially diluted human plasma at 37°C for 1 hour before addition to confluent Vero monolayers in 96-well plates. Cells were incubated for 12-16 hours at 37°C in 5% CO_2. Cells were then fixed in 4% paraformaldehyde, stained with 1ug/mL Hoechst, and imaged using a CellInsight CX5 imager to quantify total number of cells expressing GFP. Infection was normalized to the average number of cells infected with rVSV-SARS-CoV-2 incubated with normal human plasma. The limit of detection (LOD) was established as < 1:20 based on plasma samples from a series of unexposed control subjects. Data are presented as the relative infection for each concentration of sera. Neutralization IC50 titers were calculated using One-Site Fit LogIC50 regression in GraphPad Prism 8.0.

Detection of antigen-specific memory B cells

To detect SARS-CoV-2 specific B cells, biotinylated protein antigens were individually multimerized with fluorescently labeled streptavidin at 4°C for one hour. Full-length SARS-CoV-2 spike (2P-stabilized, double Strep-tagged) and RBD were generated in-house. Biotinylation was performed using biotin-protein ligase standard reaction kit (Avidity, Cat# Bir500A) following the manufacturers standard protocol and dialyzed over-night against PBS. Biotinylated spike was mixed with streptavidin BV421 (BioLegend, Cat# 405225) and streptavidin Alexa Fluor 647 (Thermo Fisher Scientific, Cat# S21374) at 20:1 ratio (~6:1 molar ratio). Biotinylated RBD was mixed with streptavidin PECy7 (BioLegend, Cat# 405206) at 2.2:1 ratio (~4:1 molar ratio). Biotinylated SARS-CoV-2 full length nucleocapsid (Avi- and His-tagged; Sino Biological, Cat# 40588-V27B-B) was multimerized using streptavidin PE (BioLegend, Cat# 405204) and streptavidin BV711 (BioLegend, Cat# 405241) at 5.5:1 ratio (~6:1 molar ratio). Streptavidin PECy5.5 (Thermo Fisher Scientific, Cat# SA1018) was used as a decoy probe to gate out SARS-CoV-2

non-specific streptavidin-binding B cells. The antigen probes prepared individually as above were then mixed in Brilliant Buffer (BD Bioscience, Cat# 566349) containing 5μM free d-biotin (Avidity, Cat# Bir500A). Free d-biotin ensured minimal cross-reactivity of antigen probes. ~10^7 previously frozen PBMC samples were prepared in U-bottom 96-well plates and stained with 50μL antigen probe cocktail containing 100ng spike per probe (total 200ng), 27.5ng RBD, 40ng nucleocapsid per probe (total 80ng) and 20ng streptavidin PECy5.5 at 4°C for one hour to ensure maximal staining quality before surface staining with antibodies as listed in Table S2 was performed in Brilliant Buffer at 4°C for 30min. Dead cells were stained using LIVE/DEAD Fixable Blue Stain Kit (Thermo Fisher Scientific, Cat# L34962) in DPBS at 4°C for 30min. ~80% of antigen-specific memory (IgD$^-$ and/or CD27$^+$) B cells detected using this method were IgM$^+$, IgG$^+$, or IgM$^-$ IgG$^-$ IgA$^+$, which were comparable to non-specific memory B cells. Based on these observations, we concluded that the antigen probes did not significantly impact the quality of surface immunoglobulin staining. Stained PBMC samples were acquired on Cytek Aurora and analyzed using FlowJo10.7.1 (BD Bioscience). Frequency of antigen-specific memory B cells were expressed as a percentage of total B cells (CD19$^+$ CD20$^+$ CD38$^{int/-}$, CD3$^-$, CD14$^-$, CD16$^-$, CD56$^-$, LIVE/DEAD$^-$, lymphocytes), or as numbers per 10^6 PBMC (LIVE/DEAD$^-$ cells). LOD was set based on median + 2×SD of [1 / (number of total B cells recorded)] or median + 2×SD of [10^6 / (number of PBMC recorded)]. LOS was set as the median + 2×SD of the results in unexposed donors. Phenotype analysis of antigen-specific B cells was performed only in subjects with at least 10 cells detected in the respective antigen-specific memory B cell gate. In each experiment, PBMC from a known positive control (COVID-19 convalescent subject) and unexposed subjects were included to ensure consistent sensitivity and specificity of the assay. For each data set, second order polynomial, simple linear regression, and pseudo-first order kinetic models were considered. The model with a lower Akaike's Information Criterion value was determined to be better-fit and visualized.

Activation induced markers (AIM) T cell assay

Antigen-specific CD4$^+$ T cells were measured as a percentage of AIM$^+$ (OX40$^+$CD137$^+$) CD4$^+$ T and (CD69$^+$CD137$^+$) CD8$^+$ T cells after stimulation of PBMCs with overlapping peptide pools spanning the entire ORFeome, as previously described (2). Cells were cultured for 24 hours in the presence of SARS-CoV-2 specific MPs [1 μg/mL] or 5 μg/mL phytohemagglutinin (PHA, Roche) in 96-wells U-bottom plates at 1x10^6 PBMCs per well. A stimulation with an equimolar amount of DMSO was performed as negative control, PHA, and stimulation with a combined CD4 and CD8 cytomegalovirus MP (CMV, 1 μg/mL) were included as positive controls. Any sample with low PHA signal was excluded as a quality control. Antigen-specific CD4$^+$ and CD8$^+$ T cells were measured as background (DMSO) subtracted data, with a minimal DMSO level set to 0.005%. All positive ORFs (> 0.02% for CD4s, > 0.05% for CD8s) were then aggregated into a combined sum of SARS-CoV-2-specific CD4$^+$ or CD8$^+$ T cells. The threshold for positivity for antigen-specific CD4$^+$ T cell responses (0.03%) and antigen-specific CD8$^+$ T cell responses (0.12%) has been calculated using the median two-fold standard deviation of all negative controls measured (>150). The antibody panel utilized in the (OX40$^+$CD137$^+$) CD4$^+$ T and (CD69$^+$CD137$^+$) CD8$^+$ T cells AIM staining is shown in Table S2.

For surface CD40L$^+$ OX40$^+$ CD4$^+$ T cell AIM assays, experiments were performed as previously described (5), with the following modifications. Cells were cultured in complete RPMI containing 5% Human AB Serum (Gemini Bioproducts), 2Me, PenStrep, NaPy, and NE-AA. Prior to addition of peptide MPs, cells were blocked at 37C for 15 minutes with 0.5ug/mL anti-CD40 mAb (Miltenyi Biotec).

COMPETING INTERESTS

A.S. is a consultant for Gritstone, Flow Pharma, Merck, Epitogenesis, Gilead and Avalia. S.C. is a consultant for Avalia. LJI has filed for patent protection for various aspects of T cell epitope and vaccine design work. Mount Sinai has licensed serological assays to commercial entities and has filed for patent protection for serological assays. D.S., F.A., V.S. and F.K. are listed as inventors on the pending patent application (F.K., V.S.), and Newcastle disease virus (NDV)-based SARS-CoV-2 vaccines that name F.K. as inventor. All other authors declare no conflict of interest.

FIGURE LEGENDS

Figure 1. SARS-CoV-2 circulating antibodies over time. (A) Cross-sectional spike IgG from COVID-19 subject plasma samples (n=228). Linear decay preferred model for best fit curve, $t_{1/2}$ = 140d, 95% CI: 89-329 days. R = -0.20, p=0.003. **(B)** Longitudinal spike IgG (n=50), average $t_{1/2}$ 100d, 95% CI: 64-220d **(C)** Cross-sectional RBD IgG. Linear decay preferred model for best fit curve, $t_{1/2}$ = 83d, 95% CI: 62 to 127d. R = -0.34, p<0.0001. **(D)** Longitudinal RBD IgG, average $t_{1/2}$ of 68d, 95% CI: 57-86d **(E)** Cross-sectional PSV neutralizing titers. One-phase decay (blue line) preferred model for best fit curve, $t_{1/2}$ = 27d, 95%: CI: 11 to 153d. R = -0.27, p <0.0001. Linear fit shown as black line. **(F)** Longitudinal PSV neutralizing titers of SARS-CoV-2 infected subjects, average $t_{1/2}$ 87d, 95% CI: 68-123d **(G)** Cross-sectional nucleocapsid IgG. Linear decay preferred model for best fit curve, $t_{1/2}$ = 67d, 95% CI: 49-105d. R = -0.32, p<0.0001. **(H)** Longitudinal nucleocapsid IgG, average $t_{1/2}$ was 67d, 95% CI: 54-88d. **(I)** Cross-sectional Spike IgA titers. One-phase decay (blue line) preferred model for best fit curve, $t_{1/2}$ = 11d, 95%: CI: 5 to 25d. R = -0.14, p=0.04. Linear fit shown as black line. **(J)** Longitudinal Spike IgA, $t_{1/2}$ = 214d, 95% CI 126-703d. **(K)** Cross-sectional RBD IgA. One phase decay (blue line) preferred model for best fit curve, $t_{1/2}$ = 27d, 95% CI: 15 to 58d. R = -0.40, p<0.0001. Linear fit shown in black. **(L)** Longitudinal RBD IgA, average $t_{1/2}$ was 72d, 95% CI: 55-104d. For cross-sectional analyses, SARS-CoV-2 infected subjects (white circles, n=238) and unexposed subjects (gray circles, n=51). For longitudinal samples, SARS-CoV-2 subjects (n=50). The dotted black line indicates limit of detection (LOD). The dotted green line indicates limit of sensitivity (LOS) above uninfected controls. Unexposed = gray, COVID subjects = white. Thick blue line represents best fit curve. When two fit curves are shown, the thin black line represents the alternative fit curve.

Figure 2. Kinetics of SARS-CoV-2 memory B cell responses. (A) Example plots showing staining patterns of SARS-CoV-2 antigen probes on memory B cells (See Fig S1 for gating). One unexposed donor and three convalescent COVID-19 subjects are shown. Numbers indicate percentages. **(B)** Gating strategies to define IgM+, IgG+, or IgA+ SARS-CoV-2 spike-specific memory B cells. The same gating strategies were used for RBD- or nucleocapsid-specific B cells. **(C)** Cross-sectional analysis of frequency (% of CD19+ CD20+ B cells) of SARS-CoV-2 S-specific total (IgG+, IgM+, or IgA+) memory B cells. Pseudo-first order kinetic model for best fit curve (R^2 = 0.14). **(D)** Longitudinal analysis of SARS-CoV-2 spike-specific memory B cells. **(E)** Cross-sectional analysis of SARS-CoV-2 RBD-specific total (IgG+, IgM+, or IgA+) memory B cells. Second order polynomial model for best fit curve (R^2 = 0.21). **(F)** Longitudinal analysis of SARS-CoV-2 RBD-specific memory B cells. **(G)** Cross-sectional analysis of SARS-CoV-2 nucleocapsid-specific total (IgG+, IgM+, or IgA+) memory B cells. Pseudo-first order kinetic model for best fit curve (R^2 = 0.19). **(H)** Longitudinal analysis of IgG+ SARS-CoV-2 spike-specific memory B cells. **(I)** Cross-sectional analysis of SARS-CoV-2 spike-specific IgG+ memory B cells. Pseudo-first order kinetic model for best fit curve (R^2 = 0.24). **(J)** Longitudinal analysis of SARS-CoV-2 spike-specific IgG+ memory B cells. **(K)** Cross-sectional analysis of SARS-CoV-2 spike-specific IgA+ memory B cells. Second order polynomial model for best fit curve (R^2 = 0.10). **(L)** Longitudinal analysis of SARS-CoV-2 spike-specific IgA+ memory B cells. **(M)** Cross-sectional analysis of SARS-CoV-2 spike-specific IgM+ memory B cells. Second order polynomial model for best fit curve (R^2 = 0.17). **(N)** Longitudinal analysis of SARS-CoV-2 spike-specific IgM+ memory B cells. **(O)** Fraction of SARS-CoV-2 antigen-specific memory B cells that belong to indicated Ig isotypes at 1-8 months PSO. **(P)** Cross-sectional analysis of SARS-CoV-2 RBD-specific IgG+ memory B cells. Second order polynomial model for best fit curve (R^2 = 0.27). **(Q)** Cross-sectional analysis of SARS-CoV-2 nucleocapsid-specific IgG+ memory B cells. Second order polynomial model for best fit curve (R^2 = 0.26). n = 20 unexposed subjects (gray circles) and n = 180 COVID-19 subjects (n = 217 data points, white circles) for cross-sectional analysis. n = 36 COVID-19 subjects (n =

73 data points, white circles) for longitudinal analysis. The dotted black line indicates limit of detection (LOD). The dotted green line indicates limit of sensitivity (LOS).

Figure 3. SARS-CoV-2 memory CD8$^+$ T cells. (A) Representative flow cytometry plots of SARS-CoV-2-specific CD8$^+$ T cells (CD69$^+$ CD137$^+$, See Fig S3 for gating) after overnight stimulation with S, N, M, ORF3a, or nsp3 peptide pools, compared to negative control (DMSO). **(B)** Cross-sectional analysis of frequency (% of CD8$^+$ T cells) of total SARS-CoV-2-specific CD8$^+$ T cells. **(C)** Longitudinal analysis of total SARS-CoV-2-specific CD8$^+$ T cells in paired samples from the same subjects. **(D)** Cross-sectional analysis of spike-specific CD8$^+$ T cells. **(E)** Longitudinal analysis of spike-specific CD8$^+$ T cells in paired samples from the same subjects. **(F)** Distribution of T_{CM}, T_{EM}, and T_{EMRA} among total SARS-CoV-2-specific CD8$^+$ T cells. n = 155 COVID-19 subject samples (white circles) for cross-sectional analysis. n = 30 COVID-19 subjects (white circles) for longitudinal analysis. The dotted black line indicates limit of detection (LOD).

Figure 4. SARS-CoV-2 memory CD4$^+$ T cells. (A) Representative flow cytometry plots of SARS-CoV-2-specific CD4$^+$ T cells (CD137$^+$ OX40$^+$, See Fig S4 for gating) after overnight stimulation with S, N, M, ORF3a, or nsp3 peptide pools, compared to negative control (DMSO). **(B)** Cross-sectional analysis of frequency (% of CD4$^+$ T cells) of total SARS-CoV-2-specific CD4$^+$ T cells. **(C)** Longitudinal analysis of total SARS-CoV-2-specific CD4$^+$ T cells in paired samples from the same subjects. **(D)** Cross-sectional analysis of spike-specific CD4$^+$ T cells. **(E)** Longitudinal analysis of spike-specific CD4$^+$ T cells in paired samples from the same subjects. **(F)** Distribution of T_{CM}, T_{EM}, and T_{EMRA} among total SARS-CoV-2-specific CD4$^+$ T cells. (**G**, **H**) Quantitation of SARS-CoV-2-specific T_{FH} cells (surface CD40L$^+$ OX40$^+$, as % of CD4$^+$ T cells. See Fig S5 for gating) after overnight stimulation with **(G)** spike (S) or **(H)** MP_R peptide pools. (**I**) PD-1^{hi} SARS-CoV-2-specific T_{FH} at 1-2 months (mo) and 6 mo PSO. (**J**) CCR6$^+$ SARS-CoV-2-specific T_{FH} in comparison to bulk cT_{FH} cells in blood.
For A-F, n = 155 COVID-19 subject samples (white circles) for cross-sectional analysis. n = 30 COVID-19 subjects (white circles) for longitudinal analysis. The dotted black line indicates limit of detection (LOD). For G-J, n = 34 COVID-19 subject samples (white circles), n = 21 COVID-19 subjects at 1-2 mo, n = 13 COVID-19 subjects at 6 mo. The dotted black line indicates limit of detection (LOD).* p<0.05, **p<0.01, *** p<0.001, **** p<0.0001.

Figure 5. Immune memory relationships. (A) Relationship between gender and spike IgG titers over time. Males: One phase decay preferred model, $t_{1/2}$ = 23d, 95% CI: 7-224d, R = -0.26, p=0.0057. Females: linear decay preferred model, $t_{1/2}$ = 159d, 95% CI 88-847d, R = -0.18, p=0.05. **(B)** Immune memory to SARS-CoV-2 during the early phase (1-2 mo, black line), medium phase (3-4 mo, red line), or late phase (5+ mo, blue line). For each individual, a score of 1 was assigned for each response above LOS in terms of RBD-specific IgG, RBD-specific IgA, RBD-specific memory B cells, SARS-CoV-2 specific CD4$^+$ T cells, and SARS-CoV-2-specific CD8$^+$ T cells, giving a maximum total of 5 components of SARS-CoV-2 immune memory. Only COVID-19 convalescent subjects with all five immunological parameters tested were included in the analysis. n = 83 (1-2 mo), n = 53 (3-4 mo), n = 28 (5+ mo). **(C)** Percentage dot plots showing frequencies (normalized to 100%) of subjects with indicated immune memory components as described in (B) during the early (1-2 mo) or late (5+ mo) phase. "G", RBD-specific IgG. "B", RBD-specific memory B cells. "4", SARS-CoV-2 specific CD4$^+$ T cells. "8", SARS-CoV-2 specific CD8$^+$ T cells. "A", RBD-specific IgA. **(D)** Relationships between immune memory compartments in COVID-19 subjects over time, as ratios (full curves and data shown in Fig. S8). AU = arbitrary units, scaled from Fig. S8. "B:IgA", RBD-specific memory B cell ratio to RBD IgA antibodies. "B:IgG", RBD-specific memory B cell ratio to RBD IgG antibodies. "B:CD4", RBD-specific memory B cell ratio to SARS-CoV-2-specific CD4$^+$

T cells. "CD4:CD8", SARS-CoV-2-specific CD4$^+$ T cells ratio to SARS-CoV-2-specific CD8$^+$ T cells. "CD4:IgG", SARS-CoV-2-specific CD4$^+$ T cells ratio to RBD IgG antibodies.

SUPPLEMENTARY MATERIALS

Table S1. Participant characteristics

	COVID-19 (n = 185)
Age (years)	19-81 [Median = 40, IQR = 19.5]
Gender	
Male (%)	43% (79/185)
Female (%)	57% (106/185)
Race	
African American or Black (%)	3% (5/185)
Alaskan Native or American Indian (%)	1% (1/185)
Asian (%)	8% (14/185)
Native Hawaiian or Pacific Islander (%)	0% (0/185)
Multiracial (%)	1% (2/185)
Other (%)	1% (1/185)
Unknown (%)	10% (19/185)
White (%)	77% (143/185)
Ethnicity	
Hispanic or Latino (%)	15% (27/185)
Non-Hispanic (%)	80% (148/185)
Unknown (%)	5% (10/185)
Hospitalization status	
Never hospitalized (%)	92% (171/185)
Hospitalized (%)	7% (13/185)
Unknown if hospitalized (%)	1% (1/185)
Sample Collection Dates	March-October 2020
SARS-CoV-2 PCR Positivity	
Positive	77% (143/185)
Negative	1% (2/185)
Not performed	20% (37/185)
Unknown	2% (3/185)
Peak Disease Severity	
Asymptomatic (score 1)	2% (4/185)
Mild (non-hospitalized. Score 2-3)	90% (167/185)
Moderate (hospitalized. Score 4-5)	3% (6/185)
Severe (hospitalized. Score 6+)	4% (7/185)
Unknown	1% (1/185)
Days Post Symptom Onset at Collection; n = 233	6-240 (Median 90.5, IQR 99)
Blood Collection Frequency	
Multiple Time Point Donors (2-4 times)	21% (38/185)
Single Time Point Donors	79% (147/185)

Table S2. Memory B cell flow cytometry panel.

Reagents	SOURCE	IDENTIFIER
Mouse anti-human CD62L BV615 (clone SK11)	BD Bioscience	Cat# 565219
Mouse anti-human CD19 BUV563 (clone SJ25C1)	BD Bioscience	Cat# 612916
Mouse anti-human FCRL5 (CD307e) BUV615 (clone 509F6)	BD Bioscience	Cat# 751131
Mouse anti-human CD95 BUV737 (clone DX2)	BD Bioscience	Cat# 612790
Mouse anti-human CCR6 BUV805 (clone 11A9)	BD Biosicnece	Cat# 749361
Mouse anti-human CD138 BV480 (clone MI15)	BD Bioscience	Cat# 566140
Mouse anti-human IgD BV510 (clone IA6-2)	BioLegend	Cat# 348220
Mouse anti-human IgM BV570 (clone MHM-88)	BioLegend	Cat# 314517
Mouse anti-human CD24 BV605 (clone ML5)	BioLegend	Cat# 311124
Mouse anti-human CD20 BV650 (clone 2H7)	BioLegend	Cat# 302336
Rat anti-human CXCR5 BV750 (clone RF8B2)	BD Bioscience	Cat# 747111
Mouse anti-human CD71 BV786 (clone M-A712)	BD Bioscience	Cat# 563768
Mouse anti-human CD27 BB515 (clone M-T271)	BD Bioscience	Cat# 564642
Mouse anti-human IgA Vio Bright FITC (clone IS11-8E10)	Miltenyi Biotec	Cat# 130-113-480
Mouse anti-human CD3 PerCP (clone SK7)	BioLegend	Cat# 344814
Mouse anti-human CD14 PerCP (clone 63D3)	BioLegend	Cat# 367152
Mouse anti-human CD16 PerCP (clone 3G8)	BioLegend	Cat# 302030
Mouse anti-human CD56 PerCP (clone HCD56)	BioLegend	Cat# 318342
Rat anti-human IgG PerCP/Cyanine5.5 (clone M1310G05)	BioLegend	Cat# 410710
Mouse anti-human CD85j PE/Dazzle 594	BioLegend	Cat# 333716
Mouse anti-human CD11c PE/Cyanine5 (clone 3.9)	BioLegend	Cat# 301610
Mouse anti-human CD21 Alexa Fluor 700 (clone Bu32)	BioLegend	Cat# 354918

Table S3. Antibodies utilized in the CD8$^+$ and CD4$^+$ T cell activation induced markers (AIM) assays

Membrane Antibody	Fluorochrome	Clone/vendor/catalog	Dilution
CD45RA	BV421	HI100/BioLegend/304130	1:50
CD14	BUV563	M5E2/BD/741360	1:100
CD19	BUV805	HIB19/BD/742007	1:100
Live/Dead	ef506/Aqua	Thermo Fisher/65-0866-18	1:200
CD8	BV650	RPA-T8/BioLegend/301042	1:50
CD4	BV605	RPA-T4/BD/562658	1:25
CCR7	FITC	G043H7/BioLegend/353216	1:50
CD69	PE	FN50/BD/555531	1:10
OX40	PE-Cy7	Ber-ACT35/BioLegend/350012	1:50
CD137	APC	4B4-1/BioLegend/309810	1:25
CD3	AF700	UCHT1/Thermo Fisher/56-0038-42	1:25

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. SARS-CoV-2 memory B cells. (**A**) Gating strategies to define spike-, RBD-, or nucleocapsid-specific memory B cells.

Figure S2. Kinetics of memory B cell responses.   (**A**) Cross-sectional analysis showing SARS-CoV-2 Spike-specific memory B cell numbers per 10^6 PBMC. Second order polynomial model for best fit curve (R^2 = 0.14). (**B**) Percentage of Spike-specific B cells that are specific to RBD. Simple linear regression (R^2 = 0.024) (**C**) Cross-sectional analysis showing SARS-CoV-2 RBD-specific memory B cell numbers per 10^6 PBMC. Second order polynomial model for best fit curve (R^2 = 0.15). (**D**) Cross-sectional analysis showing SARS-CoV-2 nucleocapsid-specific memory B cell numbers per 10^6 PBMC. Second order polynomial model for best fit curve (R^2 = 0.14). (**E**) Cross-sectional analysis of frequency (% of $CD19^+$ $CD20^+$ B cells) of SARS-CoV-2 RBD-specific IgA^+ memory B cells. Second order polynomial model for best fit curve (R^2 = 0.036). (**F**) Cross-sectional analysis of frequency (% of $CD19^+$ $CD20^+$ B cells) of SARS-CoV-2 RBD-specific IgM^+ memory B cells. Second order polynomial model for best fit curve (R^2 = 0.034). (**G**) Cross-sectional analysis of frequency (% of $CD19^+$ $CD20^+$ B cells) of SARS-CoV-2 nucleocapsid-specific IgA^+ memory B cells. Second order polynomial model for best fit curve (R^2 = 0.0031). (**H**) Cross-sectional analysis of frequency (% of $CD19^+$ $CD20^+$ B cells) of SARS-CoV-2 nucleocapsid-specific IgM^+ memory B cells. Second order polynomial model for best fit curve (R^2 = 0.029). (**I**) Cross-sectional analysis of geometric mean fluorescence intensity of spike, RBD and nucleocapsid probes on S-, RBD- and nucleocapsid-specific memory B cells, respectively. Data shown are simple linear-regression lines for individual probes. (**J**) Cross-sectional analysis of geometric mean fluorescence intensity of spike, RBD and nucleocapsid probes on S-, RBD- and nucleocapsid-specific memory B cells, respectively, normalized to a positive control sample. Data shown are simple linear-regression lines for individual antigen.

Figure S3. SARS-CoV-2 circulating memory $CD8^+$ T cells. (A) Gating strategies to define SARS-CoV-2-specific $CD8^+$ T cells by AIM assay, using individual SARS-CoV-2 ORF peptide pools. **(B)** Representative examples of flow cytometry plots of SARS-CoV-2-specific $CD8^+$ T cells ($CD69^+$ $CD137^+$, after overnight stimulation with S, M, N, ORF3a, or nsp3 peptide pools, compared to negative control (DMSO) from three COVID-19 subjects and one uninfected control. **(C)** Cross-sectional analysis of total SARS-CoV-2-specific $CD4^+$ T cells, as per Figure 3, but graphing stimulation index (SI). n = 155 COVID-19 subject samples (clear circles) for cross-sectional analysis. n = 30 COVID-19 subjects (white circles) for longitudinal analysis.

Figure S4. SARS-CoV-2 circulating memory $CD4^+$ T cells. (A) Gating strategies to define SARS-CoV-2-specific $CD4^+$ T cells by AIM assay, using individual SARS-CoV-2 ORF peptide pools. **(B)** Representative examples of flow cytometry plots of SARS-CoV-2-specific $CD4^+$ T cells ($OX40^+$ $CD137^+$, after overnight stimulation with S, M, N, ORF3a, or nsp3 peptide pools, compared to negative control (DMSO). From three COVID-19 subjects and one uninfected control. **(C)** Cross-sectional analysis of total SARS-CoV-2-specific $CD4^+$ T cells, as per Figure 4, but graphing stimulation index (SI). **(D)** Cross-sectional analysis of M-specific $CD4^+$ T cells. **(E)** Longitudinal analysis of M-specific $CD4^+$ T cells in paired samples from the same subjects. n = 155 COVID-19 subject samples (white circles) for cross-sectional analysis. n = 30 COVID-19 subjects (white circles) for longitudinal analysis.

Figure S5. SARS-CoV-2 memory T_{FH} cells. (A) Gating strategies to define SARS-CoV-2-specific $CD4^+$ T cells by AIM assay, using S and MP_R peptide pools. **(B)** Representative examples of flow cytometry plots

of SARS-CoV-2-specific CD4^{+} T cells. Surface CD40L^{+} OX40^{+}, after overnight stimulation with S and MP_R peptide pools, compared to negative control (DMSO) from a representative COVID-19 subject and an uninfected control. **(C, D)** SARS-CoV-2-specific CD4^{+} T cells based on surface CD40L^{+} OX40^{+}, gated as in A, after overnight stimulation with S or MP_R peptide pools. n = 34 COVID-19 subject samples (white circles), n = 21 at 1-2 mo, n = 13 at 6 mo. The dotted black line indicates LOD.* p<0.05, **p<0.01, *** p<0.001, **** p<0.0001.

Figure S6. Immune memory and disease severity. (A) Spike IgG, as per Figure 1. Symbol colors represent disease severity (white: asymptomatic, gray: mild, black: moderate, red: severe). **(B)** Cross-sectional analysis of SARS-CoV-2 spike-specific total (IgG[+], IgA[+], or IgA[+]) memory B cells, as per Figure 2C, color coded based on subject COVID-19 disease severity. **(C)** Cross-sectional analysis of SARS-CoV-2 RBD-specific total (IgG[+], IgA[+], or IgA[+]) memory B cells, as per Figure 2E, color coded based on subject COVID-19 disease severity. **(D)** Cross-sectional analysis of SARS-CoV-2 nucleocapsid-specific total (IgG[+], IgA[+], or IgA[+]) memory B cells, as per Figure 2F, color coded based on subject COVID-19 disease severity. **(E)** Cross-sectional analysis of SARS-CoV-2-specific CD8^{+} T cells, as per Figure 3B, color coded based on subject COVID-19 disease severity. **(F)** Cross-sectional analysis of SARS-CoV-2-specific CD4^{+} T cells, as per Figure 4B, color coded based on subject COVID-19 disease severity.

Figure S7. Immune memory and gender. Cross-sectional analyses of SARS-CoV-2 serologies by male and female gender. **(A)** Nucleocapsid IgG titers. Males: Linear decay preferred model, $t_{1/2}$ = 69d, 95% CI: 42-209d, R = -0.28, p=0.0035. Females: linear decay preferred model, $t_{1/2}$ = 64d, 95% CI: 47-104d, R = -0.41, p<0.0001. **(B)** RBD IgG titers. Males: One phase decay preferred model, $t_{1/2}$ = 24d, 95% CI 10-122d, R = -0.38, p<0.0001. Females: linear decay preferred model, $t_{1/2}$ = 94d,95% CI: 64-179d R = -0.34, p=0.0.0002. **(C)** RBD IgA titers. Males: One phase decay preferred model, $t_{1/2}$ = 15d,95% CI 8-30d, R = -0.45, p<0.0001. Females: linear decay preferred model, $t_{1/2}$ = 92d, 95% CI: 60-195d, R = -0.32, p=0.0004. **(D)** Pseudovirus neutralizing titers. Males: One phase decay preferred model, $t_{1/2}$ = 16d, 95% CI: 7-49d, R = -0.35, p=0.0022. Females: linear decay preferred model, $t_{1/2}$ = 169d, 95% CI: 96-710d, R = -0.25, p=0.0069. **(E)** Spike IgA titers. Males: One phase decay preferred model, $t_{1/2}$ = 8d, 95% CI 4-13d, R = -0.22, p=0.019. Females: linear decay preferred model, $t_{1/2}$ = 337d, 95% CI 116-370d: R = -0.056, p=0.54. **(F)** Cross-sectional analysis of frequency (% of CD19^{+} CD20^{+} B cells) of SARS-CoV-2 spike-specific memory B cells (IgG^{+}, IgA^{+}, or IgM^{+}), as per Figure 2C, color coded based on subject gender Pseudo-first order kinetic model for best fit curves. R^2 = 0.27 (females), R^2 = 0.057 (males). No significant difference between males and females. p = 0.10 by One-way ANCOVA. **(G)** Cross-sectional analysis of SARS-CoV-2 RBD-specific total (IgG[+], IgA[+], or IgA[+]) memory B cells, as per Figure 2E, color coded based on subject gender. Second order polynomial model for best fit curves. R^2 = 0.37 (females) and R^2 = 0.12 (males). No significant difference between males and females. p = 0.24 by one-way ANCOVA. **(H)** Cross-sectional analysis of SARS-CoV-2 nucleocapsid-specific total (IgG[+], IgA[+], or IgA[+]) memory B cells, as per Figure 2F, color coded based on subject gender. Second order polynomial model for best fit curves. R^2 = 0.28 (females), R^2 = 0.16 (males). No significant difference between males and females. p = 0.45 by one-way ANCOVA. **(I)**. No significant difference between males and females. p = 0.16 by one-way ANCOVA. **(J)** No significant difference between males and females. p = 0.24 by one-way ANCOVA.

Figure S8. Immune memory relationships. (A) The ratio of SARS-CoV-2 specific CD4^{+} T cell frequency relative to SARS-CoV-2 specific CD8^{+} T cell frequency (best-fit simple linear regression line, R^2 = 0.02932). Two data points are outside the axis limits. **(B)** The ratio of RBD-specific memory B cell frequency (percentage) relative to RBD-specific IgG (pseudo-first order kinetic model, R^2 = 0.3659).

Three data points are outside the axis limits **(C)** The ratio of RBD-specific memory B cell frequency (percentage) relative to RBD IgA antibodies (pseudo-first order kinetic model, R^2 = 0.3804). Two data points are outside the axis limits. **(D)** The ratio of SARS-CoV-2 specific CD4$^+$ T cell frequency relative to RBD IgG antibodies (best-fit simple linear regression line, R^2 = 0.0003891). Two data points are outside the axis limits. **(E)** The ratio of RBD-specific memory B cell frequency (percentage) relative to total SARS-CoV-2 specific CD4$^+$ T cell frequency (best-fit simple linear regression line, R^2 = 0.2351). One data point is outside the axis limits. For Figure 5: The ratio of RBD-specific memory B cell frequency (percentage) relative to RBD IgA antibodies (orange curve; best-fit second order polynomial curve transformed by ×10^5), RBD IgG antibodies (magenta; best-fit simple linear regression line transformed by ×10^5) and total SARS-CoV-2 specific CD4$^+$ T cell frequency (blue; best-fit simple linear regression line transformed by ×10^2), or the ratio of SARS-CoV-2 specific CD4$^+$ T cell frequency relative to SARS-CoV-2 specific CD8$^+$ T cell frequency (till; best-fit simple linear regression line) and RBD IgG antibodies (black; best-fit simple linear regression line transformed by ×10^3).

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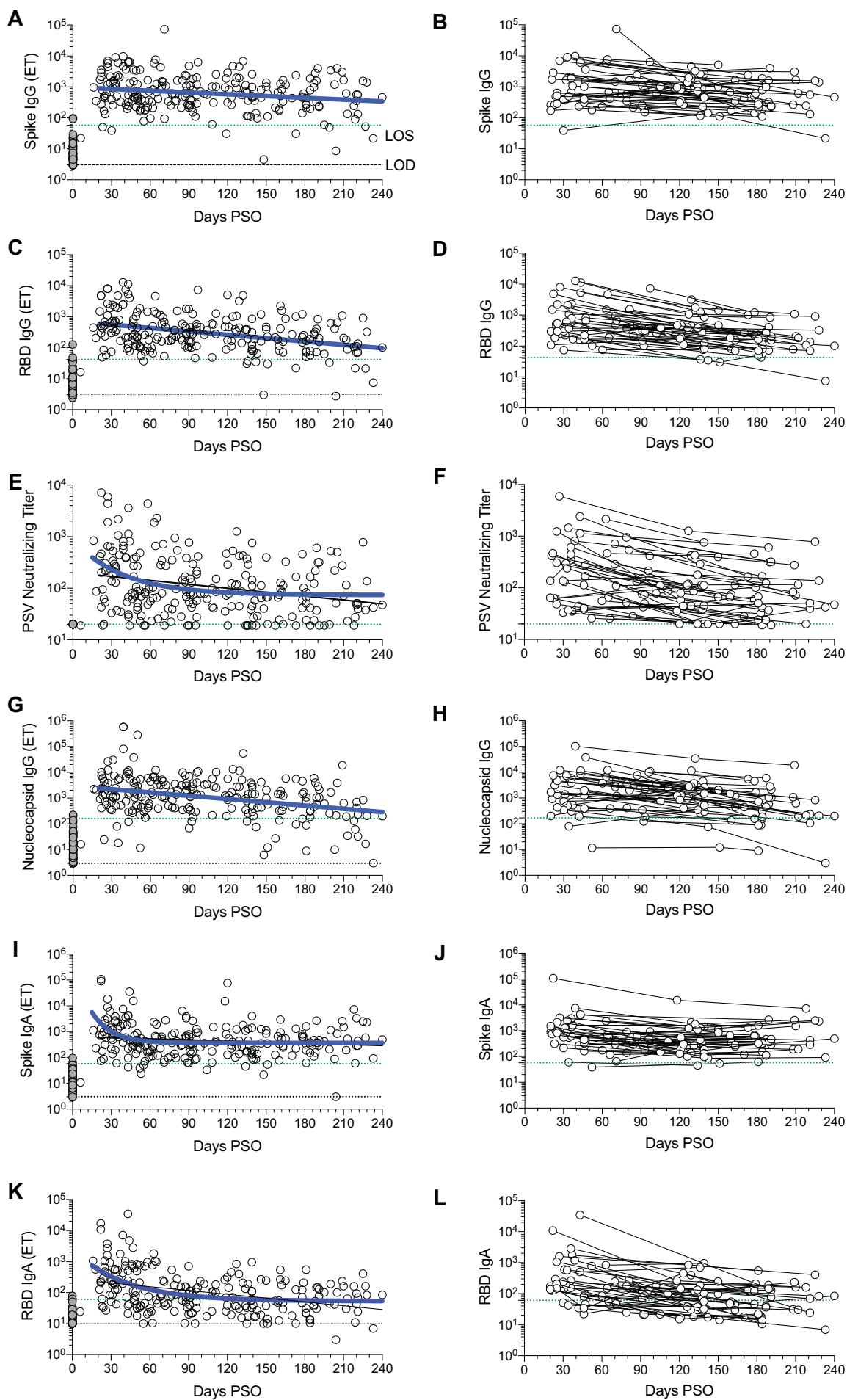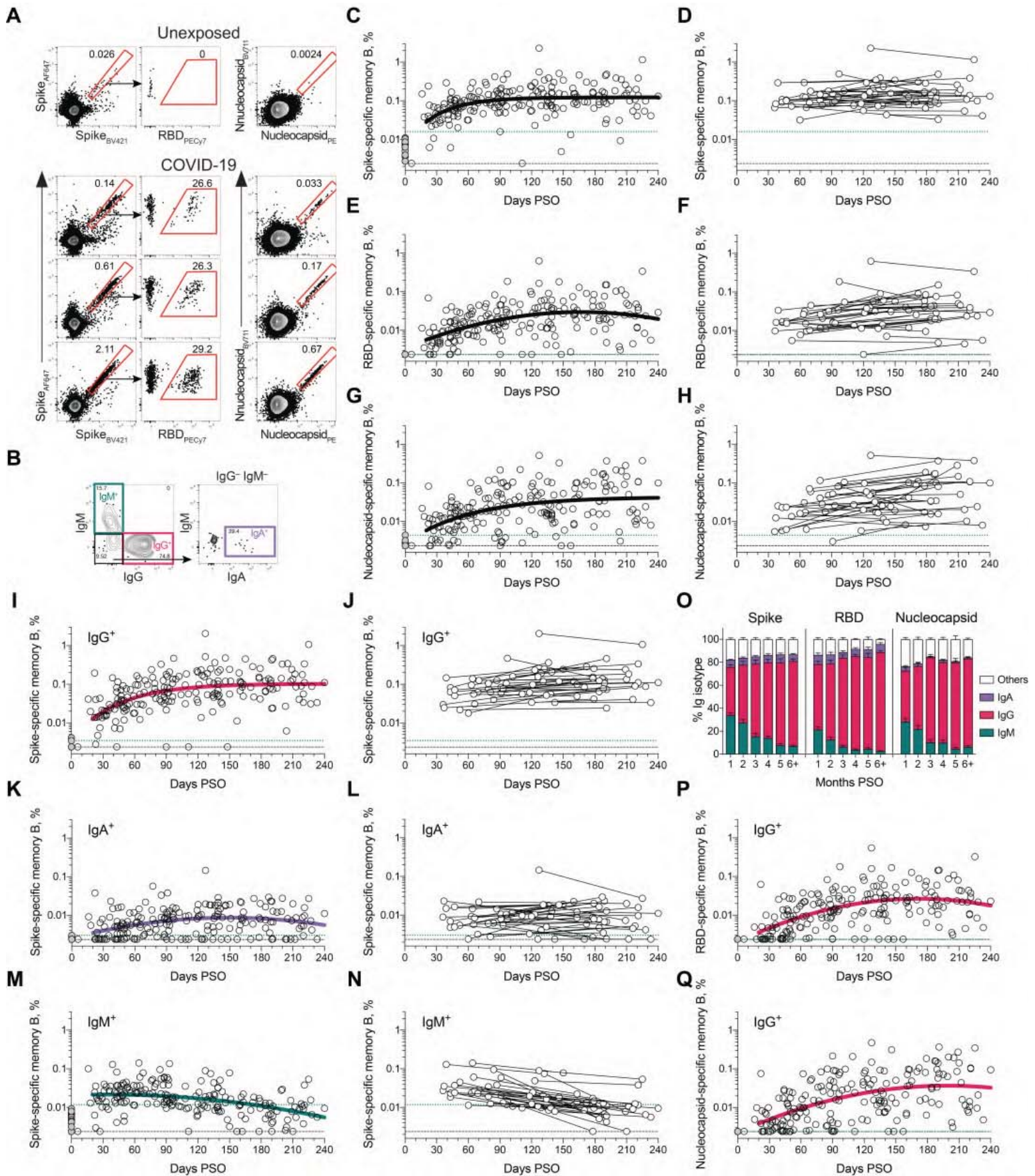
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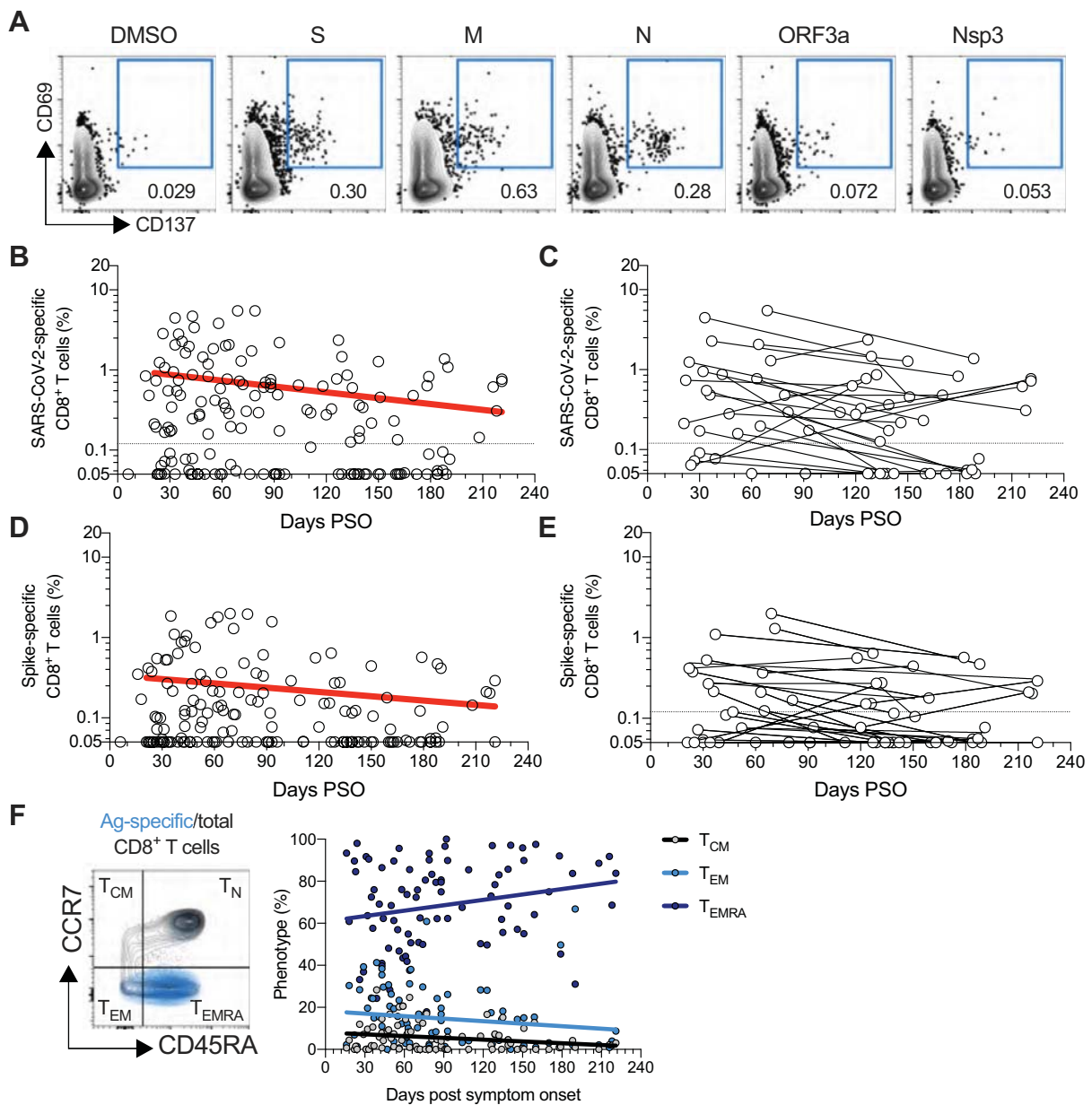
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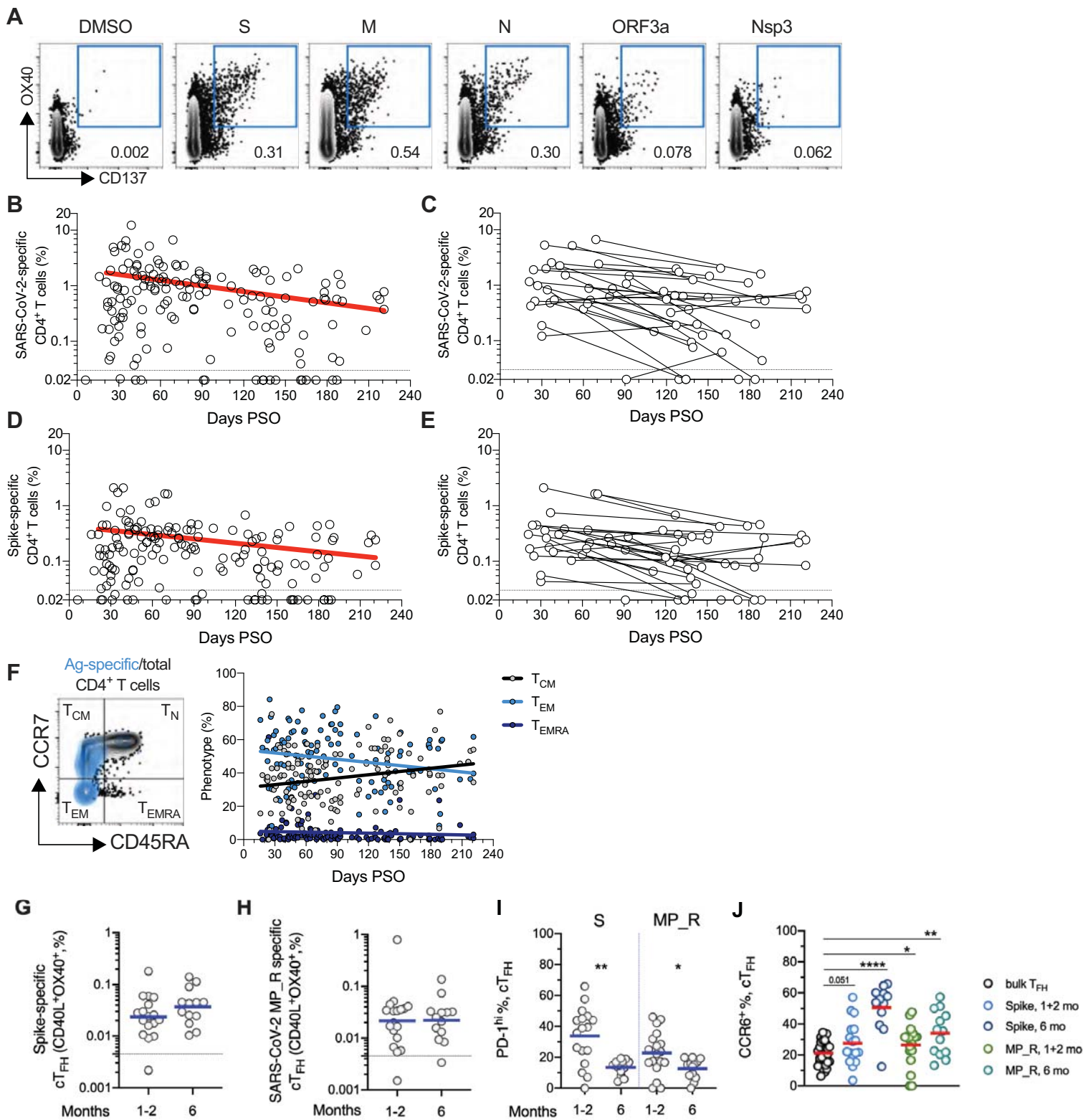
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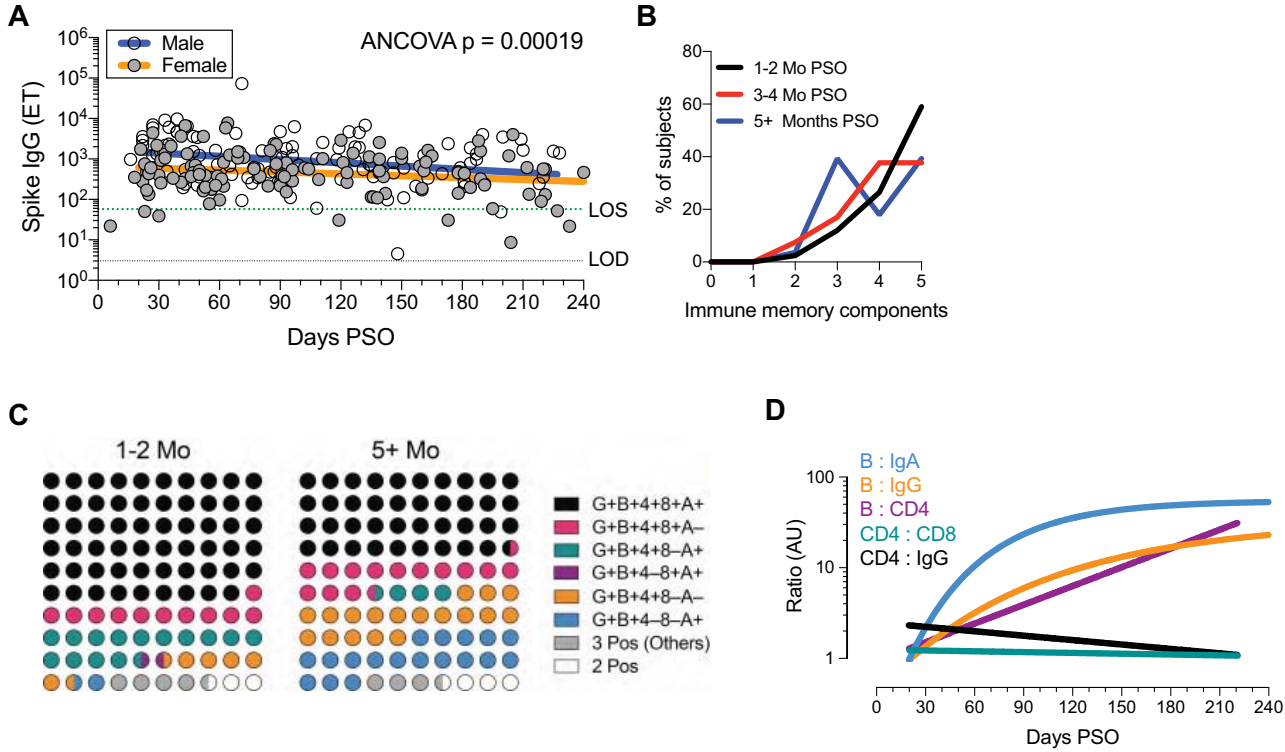
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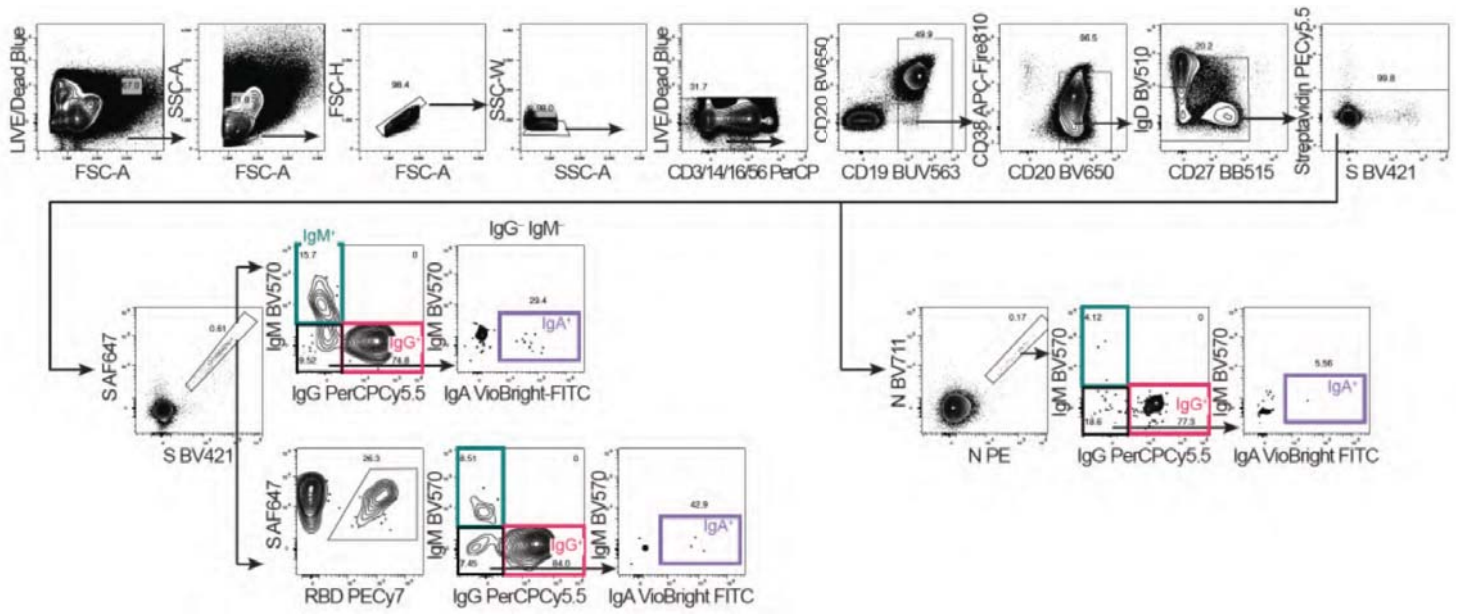
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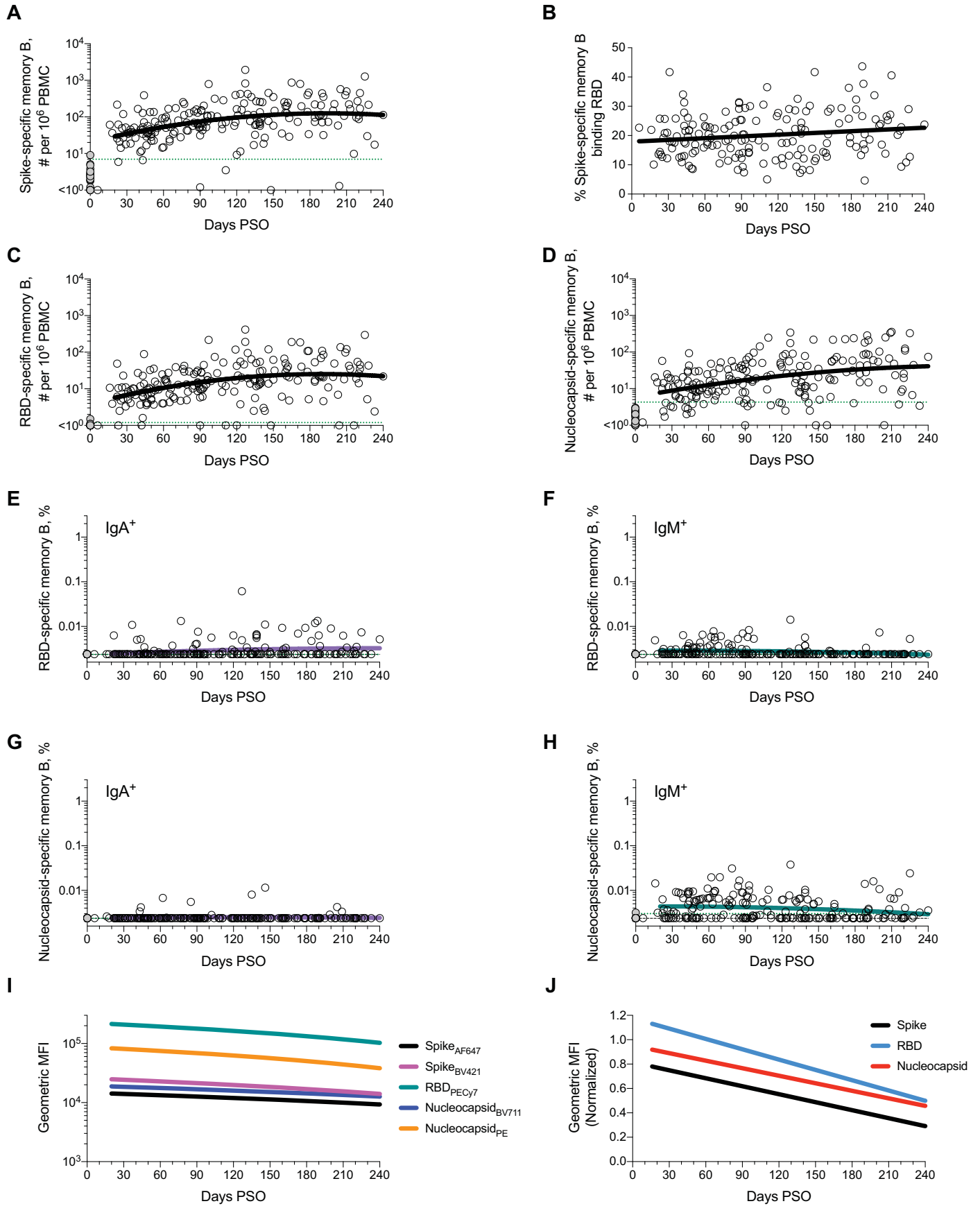
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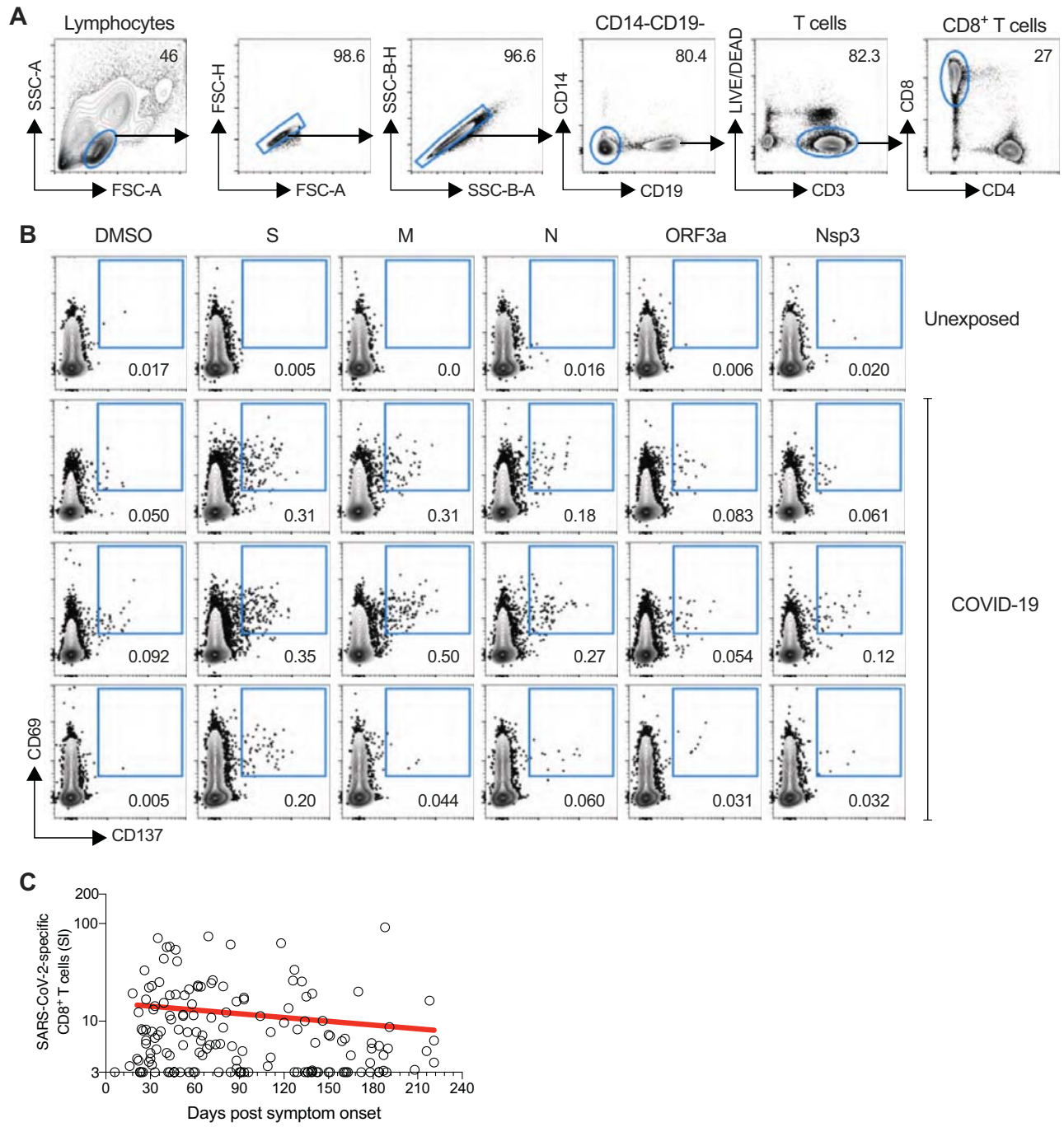
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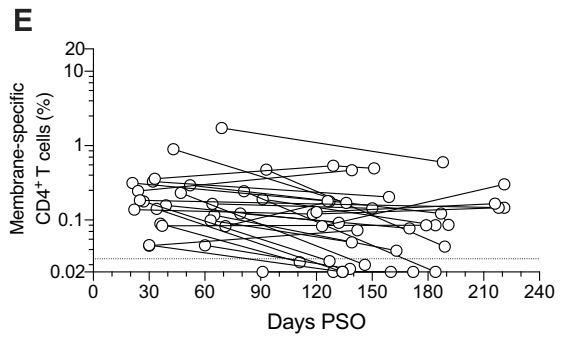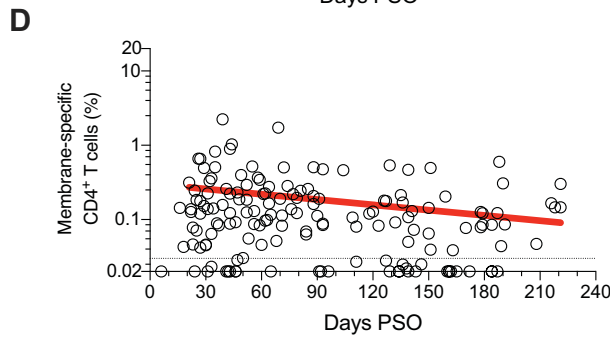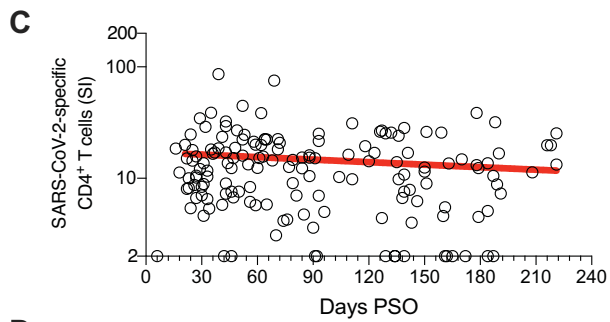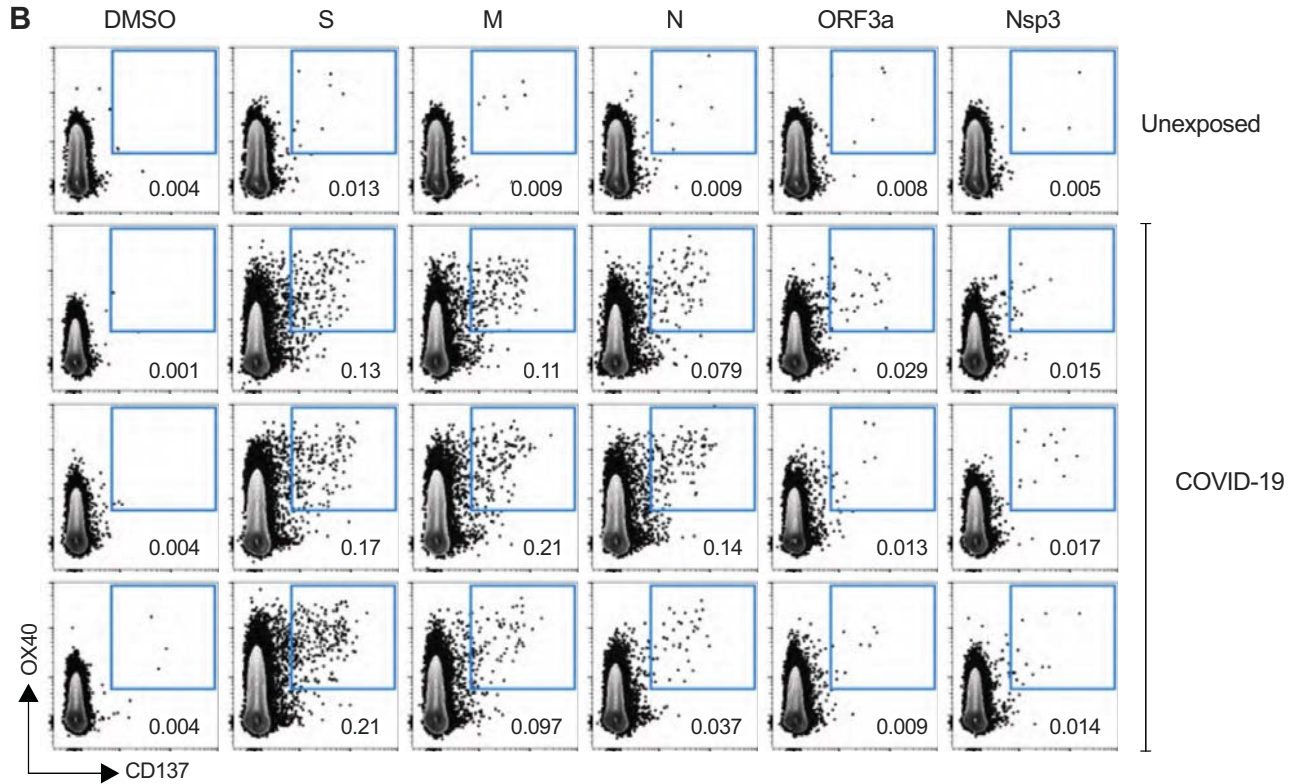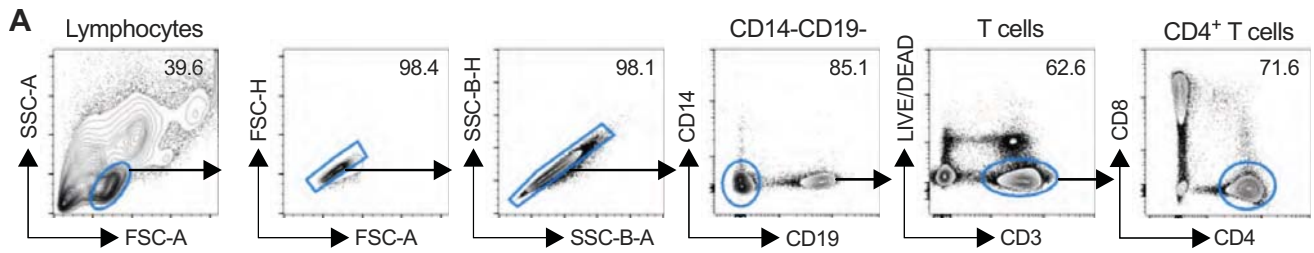
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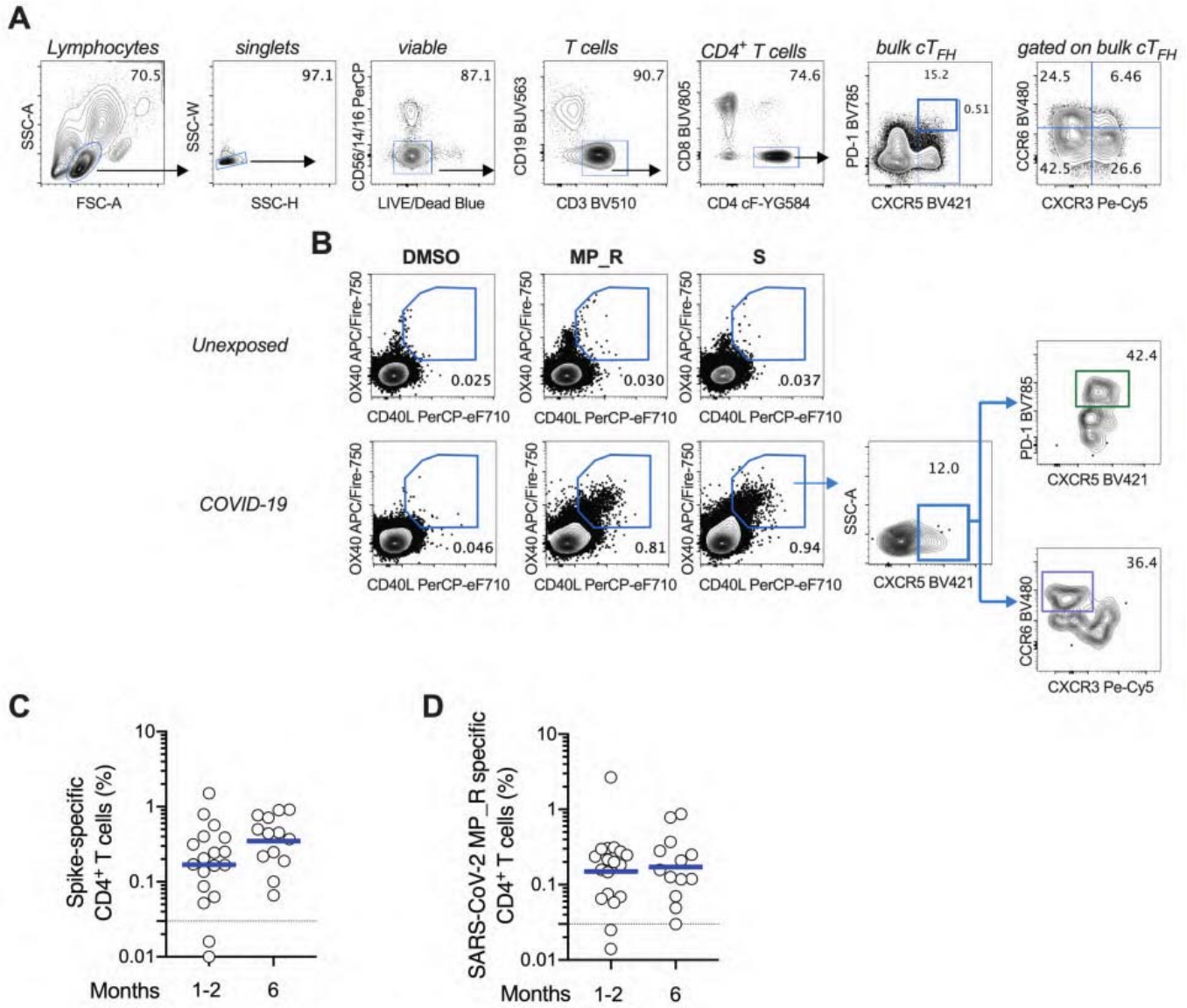
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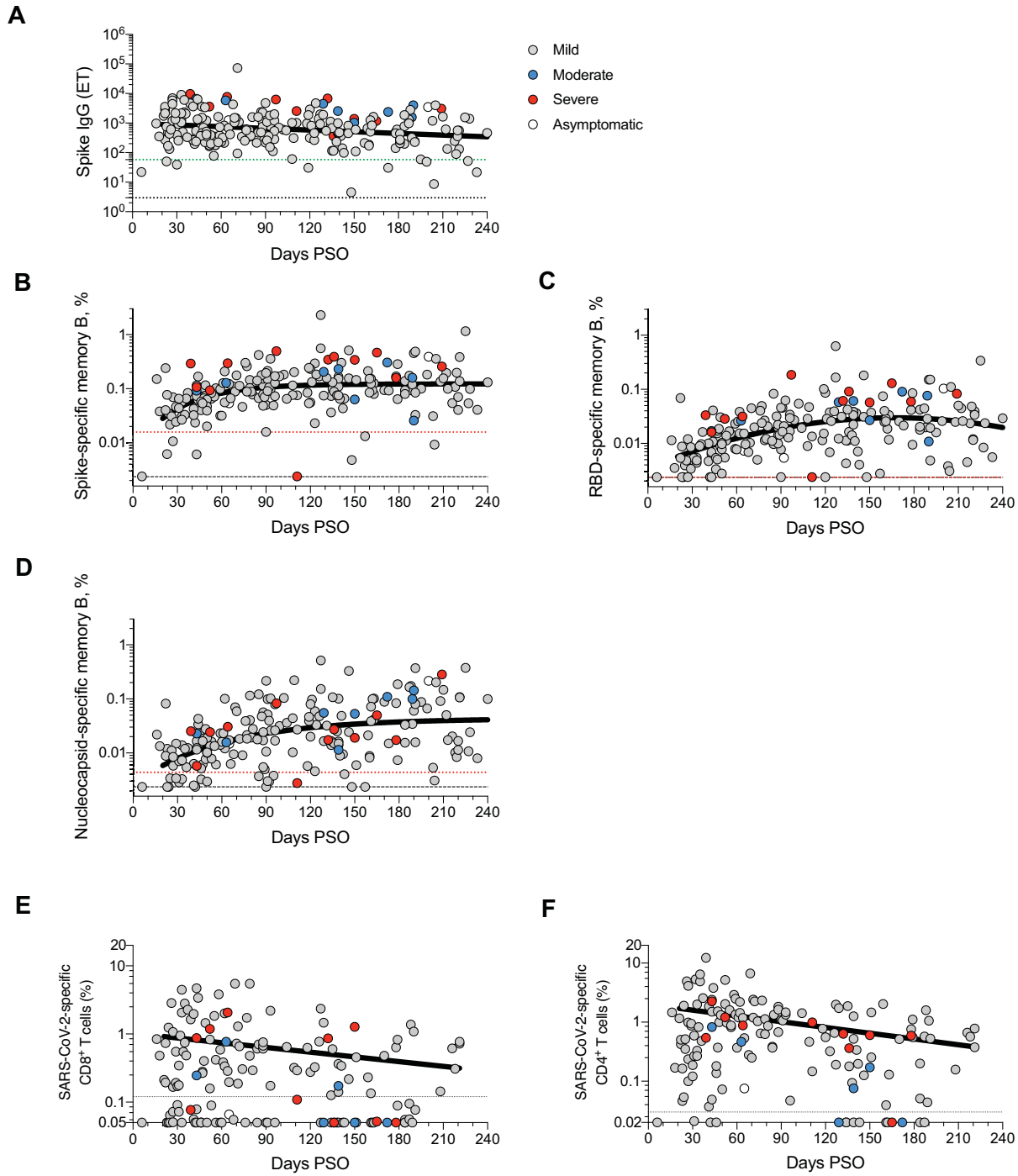
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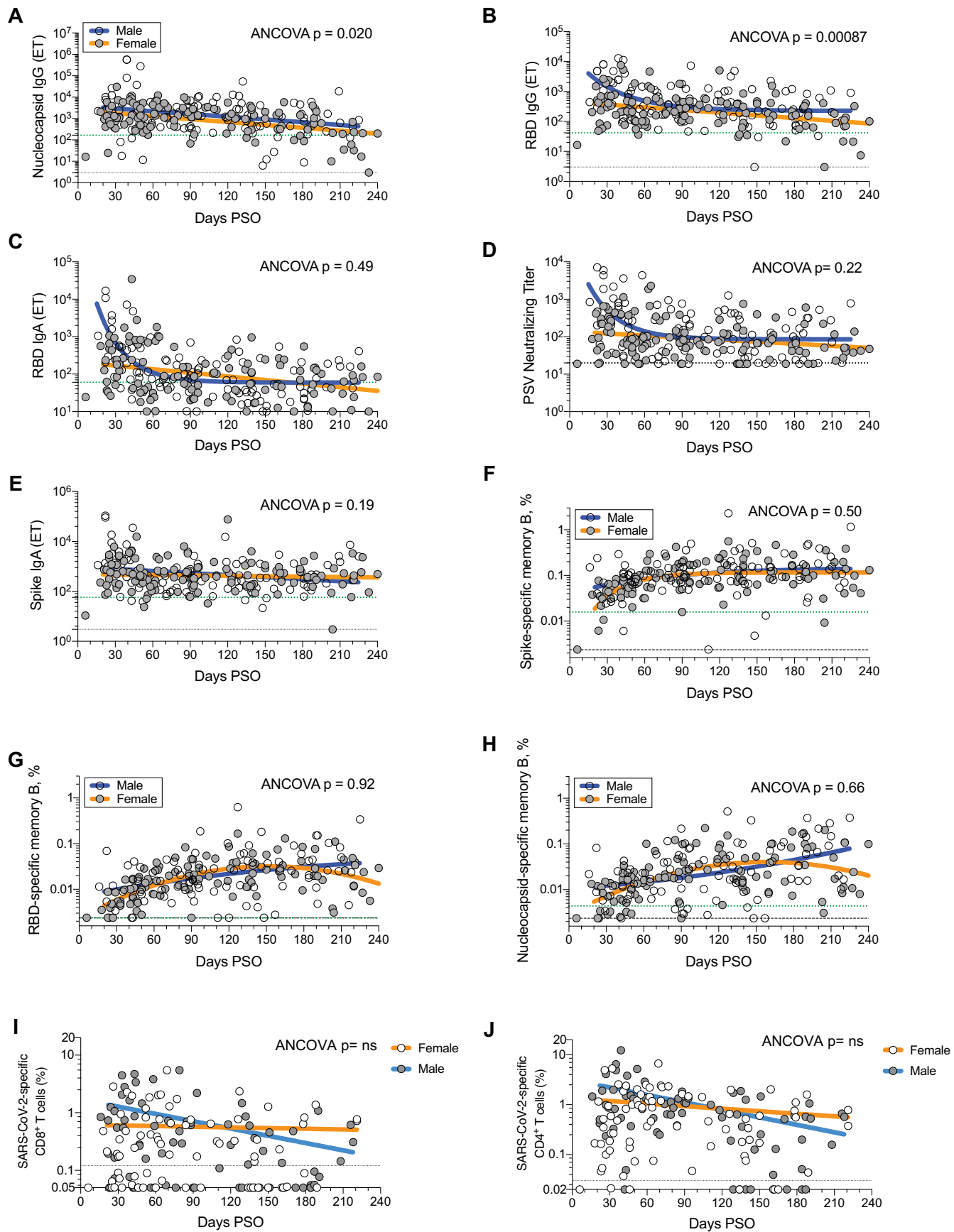
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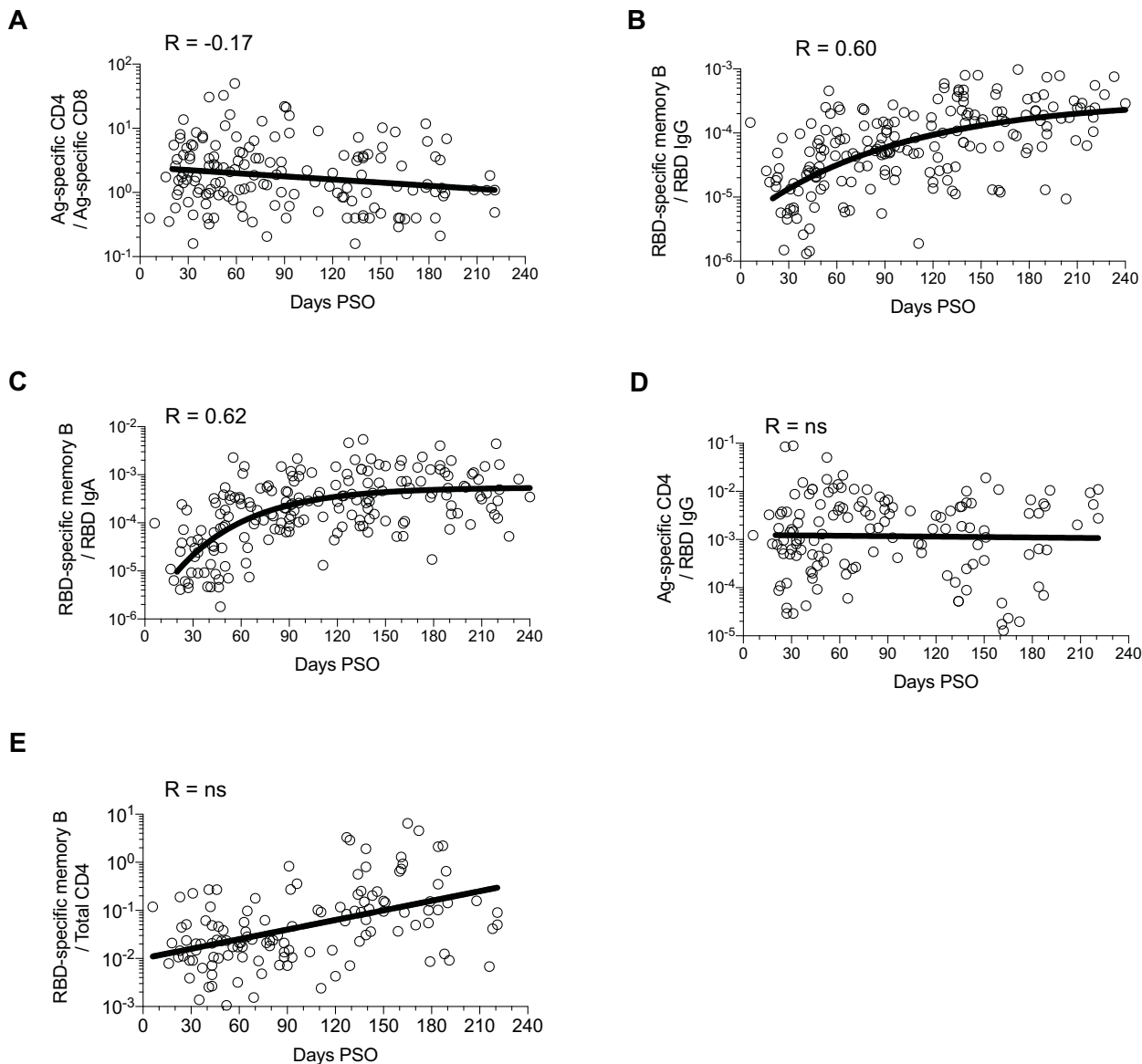
EXHIBIT 8

Protection afforded by the BNT162b2 and mRNA-1273 COVID-19 vaccines in fully vaccinated cohorts with and without prior infection

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Abstract

Effect of prior SARS-CoV-2 infection on vaccine protection remains poorly understood. Here, we investigated whether persons vaccinated after a prior infection have better protection against future infection than those vaccinated without prior infection. Effect of prior infection was assessed in Qatar's population, where the Alpha (B.1.1.7) and Beta (B.1.351) variants dominate incidence, using two national retrospective, matched-cohort studies, one for the BNT162b2 (Pfizer-BioNTech) vaccine, and one for the mRNA-1273 (Moderna) vaccine. Incidence rates of infection among BNT162b2-vaccinated persons, with and without prior infection, were estimated, respectively, at 1.66 (95% CI: 1.26-2.18) and 11.02 (95% CI: 9.90-12.26) per 10,000 person-weeks. The incidence rate ratio was 0.15 (95% CI: 0.11-0.20). Analogous incidence rates among mRNA-1273-vaccinated persons were estimated at 1.55 (95% CI: 0.86-2.80) and 1.83 (95% CI: 1.07-3.16) per 10,000 person-weeks. The incidence rate ratio was 0.85 (95% CI: 0.34-2.05). Prior infection enhanced protection of those BNT162b2-vaccinated, but not those mRNA-1273-vaccinated. These findings may have implications for dosing, interval between doses, and potential need for booster vaccination.

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Main text

Effect of prior acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection on vaccine protection against acquisition of infection remains poorly understood[1-3]. Qatar launched Coronavirus Disease 2019 (COVID-19) immunization in December 21, 2020, first using the BNT162b2[4] (Pfizer-BioNTech) vaccine and subsequently adding the mRNA-1273[5] (Moderna) vaccine[6,7]. As vaccination was scaled up following the FDA-approved protocol, the country experienced two back-to-back SARS-CoV-2 waves from January-June, 2021, which were dominated by the Alpha[8] (B.1.1.7) and Beta[8] (B.1.351) variants[6,7,9-11] (Methods). This provided an opportunity to assess whether persons vaccinated after a prior SARS-CoV-2 infection have better protection against future infection than those vaccinated without prior infection.

Leveraging the national, federated databases that have captured all SARS-CoV-2 vaccinations and PCR testing since the epidemic onset (Methods), we investigated this question using two retrospective, matched-cohort studies. We compared incidence of documented SARS-CoV-2 infection in the national cohort of individuals who completed ≥14 days after the second BNT162b2 vaccine dose, but who had experienced a prior PCR-confirmed infection, with incidence among individuals who completed ≥14 days after the second BNT162b2 dose, but who had not experienced a prior infection, between December 21, 2020-June 6, 2021 (Figure 1). The same comparison was made for the mRNA-1273 vaccine (Figure 2). Cohorts were matched in a 1:1 ratio by sex, 5-year age group, nationality, and calendar week of the first vaccine dose, to control for differences in exposure risk[12,13] and variant exposure[6,7,9-11]. Reporting of the study followed the STROBE guidelines (Supplementary Table 1).

Figures 1-2 show the process for identifying infections in these cohorts, and Table 1 presents their demographic characteristics. Using the Kaplan–Meier estimator[14], cumulative infection

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incidence among BNT162b2-vaccinated persons, with and without prior infection, was estimated at 0.14% (95% CI: 0.11-0.19%) and 0.93% (95% CI: 0.83-1.04%), respectively, after 63 days of follow-up (Figure 1). Incidence rates of infection were estimated, respectively, at 1.66 (95% CI: 1.26-2.18) and 11.02 (95% CI: 9.90-12.26) per 10,000 person-weeks. The incidence rate ratio was estimated at 0.15 (95% CI: 0.11-0.20).

Cumulative infection incidence among mRNA-1273-vaccinated persons, with and without prior infection, was estimated at 0.06% (95% CI: 0.03-0.12%) and 0.08% (95% CI: 0.04-0.15%), respectively, after 63 days of follow-up (Figure 1). Incidence rates were estimated, respectively, at 1.55 (95% CI: 0.86-2.80) and 1.83 (95% CI: 1.07-3.16) per 10,000 person-weeks. The incidence rate ratio was estimated at 0.85 (95% CI: 0.34-2.05).

Infection incidence was low in these cohorts during a time of intense incidence in Qatar[6,7,15], indicating that both vaccines were highly effective against the Alpha and Beta variants[6,7], which dominated incidence[9] (Methods). Still, prior infection of those BNT162b2-vaccinated further enhanced protection and reduced the incidence rate by 85% (6.6-fold) compared to those without prior infection. No evidence for such an effect was found for those mRNA-1273-vaccinated.

These findings are perhaps explained by the observed differences in effectiveness of these two vaccines against the Alpha and Beta variants, estimated in Qatar at 89.5% (95% CI: 85.9-92.3%) and 75.0% (95% CI: 70.5-78.9%) for BNT162b2, respectively[6], and at 100% (95% CI: 91.8-100.0%) and 96.4% (95% CI: 91.9-98.7%) for mRNA-1273, respectively[7].

The differences in effectiveness could have risen for a variety of reasons, such as differences in dosing, interval between doses, or the biology of both vaccines and their mechanisms of action. The dose of each of these two vaccines differed—it was 30-μg per dose for BNT162b2[4] and 100 μg per dose for mRNA-1273[5]. This may have resulted in a more activated immune response for

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the mRNA-1273 vaccine than the BNT162b2 vaccine, and made the existence of prior immunity due to natural infection of no additional benefit for the mRNA-1273 vaccine. The interval between doses also differed and was one week longer for mRNA-1273[5]. Evidence suggests that a longer dose interval could be associated with improved protection after receiving the second dose[16].

Limitations include identifying prior infection based on a record of a PCR-positive result, thereby missing those who may have been infected, but were unaware of their infection, or who did not seek testing by PCR to document the infection. Misclassification of prior infection status could lead to underestimation of the effect size of prior infection on vaccine protection. Depletion of the cohorts with prior infection due to COVID-19 mortality at time of the prior infection may have biased these cohorts toward healthier individuals with stronger immune responses. However, COVID-19 mortality has been low in Qatar's predominantly young and working-age population[12,17], and no evidence for such bias was found in the mRNA-1273 vaccine results, where the incidence rate was similar for those with and without prior infection.

We assessed risk of only documented infections, but other infections may have occurred and gone undocumented, perhaps because of minimal/mild or no symptoms. Our cohorts predominantly included working-age adults; therefore, results may not necessarily be generalizable to other population groups, such as children or the elderly. Matching was done for age, sex, nationality, and calendar week of the first vaccine dose, and could not be done for other factors, such as comorbidities or additional socio-demographic factors, as these were not available to study investigators. However, matching by age and sex may have served as a proxy given that co-morbidities are associated with older age and may be different between women and

men. Matching by nationality may have also captured some of the occupational risk given the distribution of the labor force in Qatar[18-20].

Imperfect assay sensitivity and specificity of PCR or antibody testing could have affected current or prior infection ascertainment. However, all PCR and serological testing was performed with extensively used, investigated, and validated commercial platforms with essentially 100% sensitivity and specificity (Methods). Unlike blinded, randomized clinical trials, the investigated observational cohorts were neither blinded nor randomized.

Our results demonstrate low infection incidence among those vaccinated with BNT162b2 or mRNA-1273, but among those vaccinated with BNT162b2, protection against infection was further enhanced and infection incidence was further reduced by prior infection. In contrast, those vaccinated with mRNA-1273 were as well protected as those who received the vaccine after a prior infection. These findings may have implications for the potential need of a booster vaccination.

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Author contributions

LJA conceived and co-designed the study, led the statistical analyses, and co-wrote the first draft of the article. HC co-designed the study, performed the statistical analyses, and co-wrote the first draft of the article. All authors contributed to data collection and acquisition, database development, discussion and interpretation of the results, and to the writing of the manuscript. All authors have read and approved the final manuscript.

Competing interests

Dr. Butt has received institutional grant funding from Gilead Sciences unrelated to the work presented in this paper. Otherwise, we declare no competing interests.

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Figure 1. The process for identifying SARS-CoV-2 infections in the national cohort of individuals who completed ≥14 days after the second BNT162b2 vaccine dose and who had experienced a PCR-confirmed infection before the first dose, compared with the process for identifying SARS-CoV-2 infections in the national cohort of individuals who completed ≥14 days after the second BNT162b2 vaccine dose, but who had experienced no PCR-confirmed infection before the first dose. Cohorts were matched in a 1:1 ratio by sex, 5-year age group, nationality, and calendar week of the first vaccine dose. Total follow-up time among BNT162b2-vaccinated persons, with and without prior infection, was 308,086.0 and 305,891.9 person-weeks, respectively.

1,188,549 vaccinated individuals between December 21 and June 6, 2021

254,133 individuals were excluded because they did not have two vaccine doses

260,611 individuals were excluded because they were not vaccinated with the BNT162b2 vaccine

102,426 individuals were excluded because they did not complete 14 days after the second vaccine dose

571,379 individuals who completed ≥14 days after the second BNT162b2 vaccine dose eligible for inclusion

519,339 individuals with no prior PCR-confirmed infection before the first vaccine dose

52,040 individuals with a prior PCR-confirmed infection before the first vaccine dose

15 individuals were excluded because they died before the start of the follow-up

8,364 individuals were excluded because they had a PCR-positive swab before the start of the follow-up

6,991 individuals were excluded because they had an antibody-positive test before the first vaccine dose

1 individual was excluded because the person died before the start of the follow-up

503,969 individuals eligible for inclusion in the analysis

52,039 individuals eligible for inclusion in the analysis

51,486 vaccinated individuals with no prior infection could be matched by 5-year age group, sex, nationality, and calendar week of first vaccine dose to vaccinated individuals with a prior PCR-confirmed infection, and were thus followed up from 14 days after the second vaccine dose up to *infection* with SARS-CoV-2, all-cause mortality, or end-of-study censoring

51,486 vaccinated individuals with a prior PCR-confirmed infection could be matched by 5-year age group, sex, nationality, and calendar week of first vaccine dose to vaccinated individuals with no prior infection, and were thus followed up from 14 days after the second vaccine dose up to *reinfection* with SARS-CoV-2, all-cause mortality, or end-of-study censoring

337 individuals infected with SARS-CoV-2

0 individuals died during follow-up

51,149 individuals followed until end-of-study

51 individuals reinfected with SARS-CoV-2

0 individuals died during follow-up

51,435 individuals followed until end-of-study

Abbreviations: PCR, polymerase chain reaction.

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Figure 2. The process for identifying SARS-CoV-2 infections in the national cohort of individuals who completed ≥14 days after the second mRNA-1273 vaccine dose and who had experienced a PCR-confirmed infection before the first dose, compared with the process for identifying SARS-CoV-2 infections in the national cohort of individuals who completed ≥14 days after the second mRNA-1273 vaccine dose, but who had experienced no PCR-confirmed infection before the first dose. Cohorts were matched in a 1:1 ratio by sex, 5-year age group, nationality, and calendar week of the first vaccine dose. Total follow-up time among mRNA-1273-vaccinated persons, with and without prior infection, was 70,729.9 and 70,872 person-weeks, respectively.

1,188,549 vaccinated individuals between December 21 and June 6, 2021

254,133 individuals were excluded because they did not have two vaccine doses

673,980 individuals were excluded because they were not vaccinated with the mRNA-1273 vaccine

7,081 individuals were excluded because they did not complete 14 days after the second vaccine dose

253,355 individuals who completed ≥14 days after the second mRNA-1273 vaccine dose eligible for inclusion

229,064 individuals with no prior PCR-confirmed infection before the first vaccine dose

24,291 individuals with a prior PCR-confirmed infection before the first vaccine dose

3,143 individuals were excluded because they had an antibody-positive test before the first vaccine dose

1 individual was excluded because the person died before the start of the follow-up

7 individuals were excluded because they died before the start of the follow-up

3,523 individuals were excluded because they had a PCR-positive swab before the start of the follow-up

222,398 individuals eligible for inclusion in the analysis

24,290 individuals eligible for inclusion in the analysis

24,052 vaccinated individuals with no prior infection could be matched by 5-year age group, sex, nationality, and calendar week of first vaccine dose to vaccinated individuals with a prior PCR-confirmed infection, and were thus followed up from 14 days after the second vaccine dose up to *infection* with SARS-CoV-2, all-cause mortality, or end-of-study censoring

24,052 vaccinated individuals with a prior PCR-confirmed infection could be matched by 5-year age group, sex, nationality, and calendar week of first vaccine dose to vaccinated individuals with no prior infection, and were thus followed up from 14 days after the second vaccine dose up to *reinfection* with SARS-CoV-2, all-cause mortality, or end-of-study censoring

13 individuals infected with SARS-CoV-2

0 individuals died during follow-up

24,039 individuals followed until end-of-study

11 individuals reinfected with SARS-CoV-2

0 individuals died during follow-up

24,041 individuals followed until end-of-study

Abbreviations: PCR, polymerase chain reaction.

**Table 1. Demographic characteristics of matched cohorts that received the BNT162b2 and mRNA-1273 vaccines.**

Characteristics	Vaccination with the BNT162b2 vaccine			Vaccination with the mRNA-1273 vaccine		
	Individuals with a prior PCR-confirmed infection	Individuals with no prior PCR-confirmed infection	p-value	Individuals with a prior PCR-confirmed infection	Individuals with no prior PCR-confirmed infection	p-value
Median age (IQR) — years	39 (32-48)	39 (32-48)	0.972	40 (33-47)	40 (33-47)	0.869
Age group — no. (%)						
<20 years	1,573 (3.1)	1,573 (3.1)	1.000	194 (0.8)	194 (0.8)	1.000
20-29 years	7,282 (14.1)	7,282 (14.1)		3,481 (14.5)	3,481 (14.5)	
30-39 years	18,027 (35.0)	18,027 (35.0)		8,216 (34.2)	8,216 (34.2)	
40-49 years	13,593 (26.4)	13,593 (26.4)		7,972 (33.1)	7,972 (33.1)	
50-59 years	7,468 (14.5)	7,468 (14.5)		3,368 (14.0)	3,368 (14.0)	
60-69 years	2,830 (5.5)	2,830 (5.5)		704 (2.9)	704 (2.9)	
70+ years	713 (1.4)	713 (1.4)		117 (0.5)	117 (0.5)	
Sex						
Male	36,970 (71.8)	36,970 (71.8)	1.000	18,697 (77.7)	18,697 (77.7)	1.000
Female	14,516 (28.2)	14,516 (28.2)		5,355 (22.3)	5,355 (22.3)	
Nationality†						
Bangladeshi	3,728 (7.2)	3,728 (7.2)	1.000	2,066 (8.6)	2,066 (8.6)	1.000
Egyptian	3,470 (6.7)	3,470 (6.7)		1,748 (7.3)	1,748 (7.3)	
Filipino	4,792 (9.3)	4,792 (9.3)		2,435 (10.1)	2,435 (10.1)	
Indian	13,033 (25.3)	13,033 (25.3)		8,180 (34.0)	8,180 (34.0)	
Nepalese	4,570 (8.9)	4,570 (8.9)		2,730 (11.4)	2,730 (11.4)	
Pakistani	1,892 (3.7)	1,892 (3.7)		1,105 (4.6)	1,105 (4.6)	
Qatari	9,700 (18.8)	9,700 (18.8)		1,047 (4.4)	1,047 (4.4)	
Sri Lankan	1,490 (2.9)	1,490 (2.9)		1,114 (4.6)	1,114 (4.6)	
Sudanese	1,259 (2.5)	1,259 (2.5)		481 (2.0)	481 (2.0)	
Other nationalities	7,552 (14.7)‡	7,552 (14.7)‡		3,146 (13.1)	3,146 (13.1)	

*Cohorts were matched in a 1:1 ratio by sex, 5-year age group, nationality, and calendar week of first vaccine dose.

†Nationalities were chosen to represent the most numerous groups in the population of Qatar.

‡ Individuals who received the BNT162b2 vaccine in Qatar comprised 96 other nationalities, while those who received the mRNA-1273 vaccine represented 78 other nationalities.

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Figure 3. Kaplan-Meier curves showing the cumulative incidence of documented SARS-CoV-2 infection in the national cohort of individuals who completed ≥14 days after the second vaccine dose and who had a prior PCR-confirmed infection, compared to the cumulative incidence of documented SARS-CoV-2 infection in the matched national cohort of individuals who completed ≥14 days after the second vaccine dose, but without prior PCR-confirmed infection. The curves compare vaccination with A) the BNT162b2 (Pfizer-BioNTech) vaccine and B) the mRNA-1273 vaccine. Cohorts were matched in a 1:1 ratio by sex, 5-year age group, nationality, and calendar week of the first vaccine dose. The curves for a longer time of follow up for only the BNT162b2 vaccine are in Supplementary Figure 1. Vaccination with BNT162b2 started few weeks before vaccination with mRNA-1273.

A — Vaccination with BNT162b2
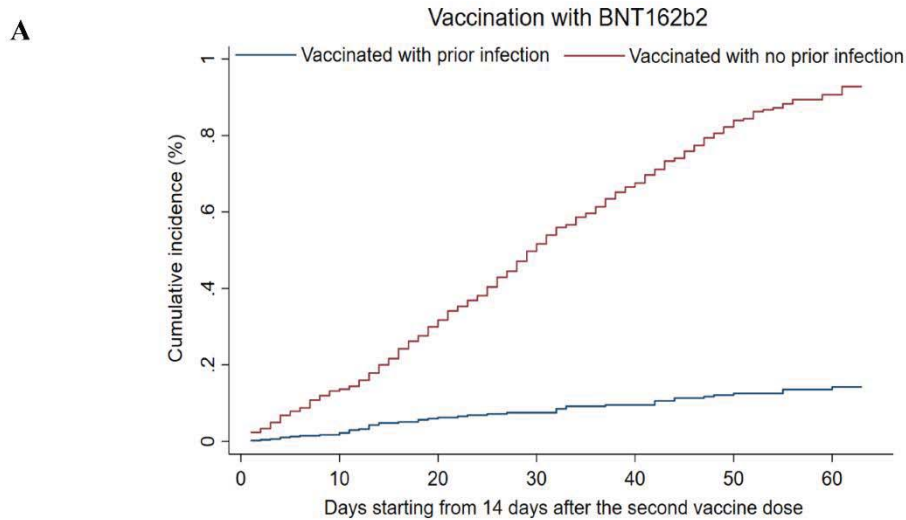
No. at risk							
Vaccinated with prior infection	51,486	40,461	34,010	30,485	28,666	23,158	15,134
Vaccinated with no prior infection	51,486	40,623	33,873	30,361	28,434	22,888	14,820
Cumulative No. of events							
Vaccinated with prior infection	0	14	29	33	39	47	50
Vaccinated with no prior infection	0	79	144	207	254	297	310
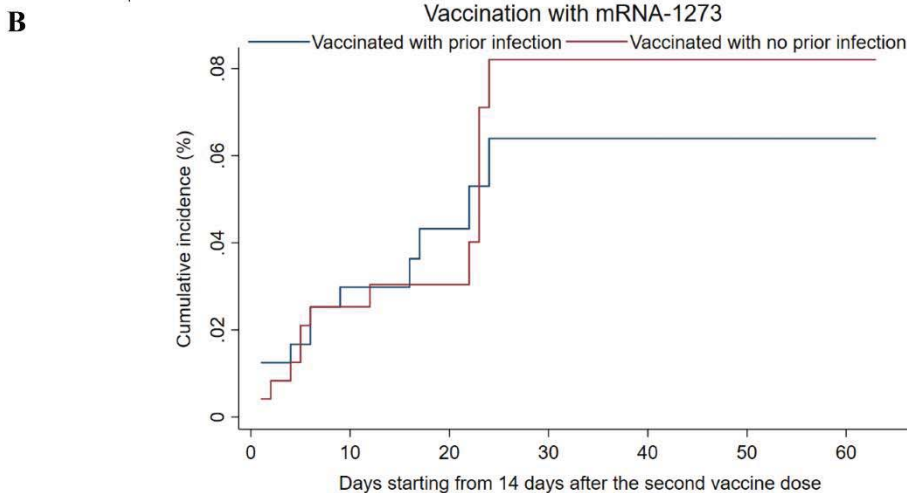
B — Vaccination with mRNA-1273

No. at risk							
Vaccinated with prior infection	24,052	21,094	11,830	4,903	363	173	33
Vaccinated with no prior infection	24,052	21,219	11,866	4,989	366	169	40
Cumulative No. of events							
Vaccinated with prior infection	0	7	9	11	11	11	11
Vaccinated with no prior infection	0	7	8	13	13	13	13

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Methods

Data sources and study design

Analyses were conducted using the centralized, integrated, and standardized national severe
acute respiratory syndrome coronavirus 2 (SARS-CoV-2) databases compiled at Hamad Medical
Corporation (HMC), the main public healthcare provider and the nationally designated provider
for all Coronavirus Disease 2019 (COVID-19) healthcare needs. Through a nation-wide digital
health information platform, these databases have captured all SARS-CoV-2-related data along
with related-demographic details with no missing information since the start of the epidemic,
including all records of polymerase chain reaction (PCR) testing, antibody testing, COVID-19
hospitalizations, vaccinations, infection severity classification per World Health Organization
(WHO) guidelines[21] (performed by trained medical personnel through individual chart reviews),
and COVID-19 deaths, also assessed per WHO guidelines[22]. Every PCR test conducted in Qatar,
regardless of location (outpatient clinic, drive-thru, or hospital, etc.), is classified on the basis of
symptoms and the reason for testing (clinical symptoms, contact tracing, random testing
campaigns (surveys), individual requests, routine healthcare testing, pre-travel, and port of
entry). Qatar has unique demographics by sex and nationality, since expatriates from over 150
countries comprise 89% of the population[12,23].

The nature of circulating SARS-CoV-2 virus was informed by weekly rounds of viral genome
sequencing and multiplex, quantitative, reverse-transcription PCR (RT-qPCR) variant
screening[24] of randomly collected clinical samples[6,7,9-11], as well as by the results of deep
sequencing of wastewater samples[9]. The weekly rounds of viral genome sequencing from
January 1-May 19, 2021 identified Beta (n=623; 50.9%), Alpha (n=193; 15.8%), Delta (n=43;
3.5%), and wild-type/undetermined variants (n=366; 29.9%) in 1,225 randomly collected, PCR-

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positive specimens[9,10]. Meanwhile, the weekly rounds of multiplex RT-qPCR variant screening from March 23-May 10, 2021 identified Beta-like (n=2,605; 66.4%), Alpha-like (n=970; 24.7%), and "other" variants (n=349; 8.9%) in 3,924 randomly collected PCR-positive specimens[9,11]. Sanger sequencing of the receptor binding domain of SARS-CoV-2 spike protein on 109 "other" specimens confirmed that 103 were Delta-like, 3 were B.1-like, and 3 were undetermined[9,11].

All records of PCR testing in Qatar were examined in this study. Every individual that met the inclusion criteria in the national database, that is being vaccinated with BNT162b2 or mRNA-1273 and completing ≥14 days after the second vaccine dose, for each of these cohort studies, was classified based on infection status (with or without PCR-positive swab before the start of the study). Individuals were matched based on infection status on a 1:1 ratio by sex, 5-year age group, nationality (>75 nationality groups), and calendar week of first vaccine dose to control for differences in exposure risk[12,13] and variant exposure[6,7,9-11]. Only matched samples were included in the analysis.

Further background on Qatar's epidemic, such as on reinfections[25,26], national seroprevalence surveys[12,18-20], PCR surveys[12], and other epidemiological studies can be found in previous publications on this epidemic[6,7,12,13,27-34].

Laboratory methods

Nasopharyngeal and/or oropharyngeal swabs (Huachenyang Technology, China) were collected for PCR testing and placed in Universal Transport Medium (UTM). Aliquots of UTM were: extracted on a QIAsymphony platform (QIAGEN, USA) and tested with real-time reverse-transcription PCR (RT-qPCR) using TaqPath™ COVID-19 Combo Kits (100% sensitivity and specificity[35]; Thermo Fisher Scientific, USA) on an ABI 7500 FAST (ThermoFisher, USA); extracted using a custom protocol[36] on a Hamilton Microlab STAR (Hamilton, USA) and tested

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using AccuPower SARS-CoV-2 Real-Time RT-PCR Kits (100% sensitivity and specificity[37]; Bioneer, Korea) on an ABI 7500 FAST; or loaded directly into a Roche cobas® 6800 system and assayed with a cobas® SARS-CoV-2 Test (95% sensitivity, 100% specificity[38]; Roche, Switzerland). The first assay targets the viral S, N, and ORF1ab regions. The second targets the viral RdRp and E-gene regions, and the third targets the ORF1ab and E-gene regions.

Antibodies against SARS-CoV-2 in serological samples were detected using a Roche Elecsys® Anti-SARS-CoV-2 assay (99.5% sensitivity[39], 99.8% specificity[39,40]; Roche, Switzerland), an electrochemiluminescence immunoassay that uses a recombinant protein representing the nucleocapsid (N) antigen for antibody binding. Results were interpreted according to the manufacturer's instructions (reactive: optical density (proxy for antibody titer[41]) cutoff index ≥1.0 vs. non-reactive: optical density cutoff index <1.0).

All PCR tests were conducted at the Hamad Medical Corporation Central Laboratory or Sidra Medicine Laboratory, following standardized protocols.

Statistical analysis

Descriptive statistics (frequency distributions and measures of central tendency) were used to characterize study samples. Significant associations were determined using two-sided p-values. The Kaplan–Meier estimator method[14] was used to estimate the cumulative risk of documented infection. Cumulative risk was defined as the proportion of individuals identified with an infection during the study period among all eligible individuals in each cohort.

Incidence rates of documented infection in each cohort were calculated by dividing the number of infection cases identified during the study by the number of person-weeks contributed by all eligible individuals in the cohort. Incidence rates and corresponding 95% CIs were estimated

using a Poisson log-likelihood regression model with the STATA 17.0[42] *stptime* command.

Follow-up person-time was calculated from the day each person completed 14 days after the

second vaccine dose up to the infection swab, all-cause death, or end-of-study censoring (June 6,

2021). The incidence rate ratio and corresponding 95% CI were calculated using the exact

method.

Statistical analyses were conducted in STATA/SE version 17.0[42].

Ethical approvals

The study was approved by the Hamad Medical Corporation and Weill Cornell Medicine-Qatar

Institutional Review Boards with waiver of informed consent.

Data availability

The dataset of this study is a property of the Qatar Ministry of Public Health that was provided to

the researchers through a restricted-access agreement that prevents sharing the dataset with a

third party or publicly. Future access to this dataset can be considered through a direct

application for data access to Her Excellency the Minister of Public Health

(https://www.moph.gov.qa/english/Pages/default.aspx). Aggregate data are available within the

manuscript and its Supplementary information.

Supplementary Material

Supplementary Table 1. STROBE checklist for cohort studies.

	Item No	Recommendation	Main Text page no
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	3
Objectives	3	State specific objectives, including any prespecified hypotheses	3
Methods			
Study design	4	Present key elements of study design early in the paper	3
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	15-18
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	15-17
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	16
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	16
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	15-16
Bias	9	Describe any efforts to address potential sources of bias	16
Study size	10	Explain how the study size was arrived at	16 & Figures 1-2
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	16-17
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	16-17
		(*b*) Describe any methods used to examine subgroups and interactions	NA
		(*c*) Explain how missing data were addressed	NA, see p.15
		(*d*) If applicable, explain how loss to follow-up was addressed	NA
		(*e*) Describe any sensitivity analyses	NA
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	Figures 1-2
		(b) Give reasons for non-participation at each stage	
		(c) Consider use of a flow diagram	
Descriptive data	14	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	Table 1
		(b) Indicate number of participants with missing data for each variable of interest	NA, see p.15
		(c) Summarise follow-up time (eg, average and total amount)	Table 1
Outcome data	15	Report numbers of outcome events or summary measures over time	3-4, Figure 3, and Supplementary Figure 1
Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	3-4, Figure 3, and Supplementary Figure 1
		(b) Report category boundaries when continuous variables were categorized	16
		(c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	NA
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	NA
Discussion			
Key results	18	Summarise key results with reference to study objectives	4-5
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	5
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	5-6
Generalisability	21	Discuss the generalisability (external validity) of the study results	5
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	Acknowledgements

Abbreviations: NA: not applicable;

Supplementary Figure 1. Kaplan-Meier curves showing the cumulative incidence of documented SARS-CoV-2 infection in the national cohort of individuals who completed ≥14 days after the second vaccine dose and who had a prior PCR-confirmed infection, compared to the cumulative incidence of documented SARS-CoV-2 infection in the matched national cohort of individuals who completed ≥14 days after the second vaccine dose, but without prior PCR-confirmed infection. The curves compare vaccination with A) the BNT162b2 (Pfizer-BioNTech) vaccine and B) the mRNA-1273 vaccine. Cohorts were matched in a 1:1 ratio by sex, 5-year age group, nationality, and calendar week of the first vaccine dose. The cumulative infection incidence among the BNT162b2-vaccinated persons, with and without prior infection, was estimated at 0.16% (95% CI: 0.11-0.23%) and 1.45% (95% CI: 1.20-1.76%), respectively, after 132 days of follow-up.

No. at risk

Vaccinated with prior infection	51,486	40,461	34,010	30,485	28,666	23,158	15,134	8,647	5,162	3,477	2,180	1,130	280	17
Vaccinated with no prior infection	51,486	40,623	33,873	30,361	28,434	22,888	14,820	8,380	5,050	3,393	2,116	1,088	289	27

Cumulative No. of events

Vaccinated with prior infection	0	14	29	33	39	47	50	50	50	51	51	51	51	51
Vaccinated with no prior infection	0	79	144	207	254	297	310	322	327	330	335	337	337	337

No. at risk

Vaccinated with prior infection	24,052	21,094	11,830	4,903	363	173	33
Vaccinated with no prior infection	24,052	21,219	11,866	4,989	366	169	40

Cumulative No. of events

Vaccinated with prior infection	0	7	9	11	11	11	11
Vaccinated with no prior infection	0	7	8	13	13	13	13

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