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Cutting Edge

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Cutting Edge: SARS-CoV-2 Infection Induces Robust Germinal Center Activity in the Human Tonsil

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Understanding the generation of immunity to SARS-CoV-2 in lymphoid tissues draining the site of infection has implications for immunity to SARS-CoV-2. We performed tonsil biopsies under local anesthesia in 19 subjects who had recovered from SARS-CoV-2 infection 24–225 d previously. The biopsies yielded >3 million cells for flow cytometric analysis in 17 subjects. Total and SARS-CoV-2 spike-specific germinal center B cells, and T follicular helper cells, were readily detectable in human tonsils early after SARS-CoV-2 infection, as assessed by flow cytometry. Responses were higher in samples within 2 mo of infection but still detectable in some subjects out to 7 mo following infection. We conclude the tonsils are a secondary lymphoid organ that develop germinal center responses to SARS-CoV-2 infection and could play a role in the long-term development of immunity. ***The Journal of Immunology*****, 2022, 208: 2267–2271.**

Infection from SARS-CoV-2 generates immune memory in the form of neutralizing Abs (a strong correlate of protective immunity [1]) and memory T and B cells. Understanding how adaptive immune responses to SARS-CoV-2 are generated is critical for informing approaches to control the COVID-19 pandemic. Effective humoral immunity to viral infection is primarily generated through interactions between B and T follicular helper (Tfh) cells in the germinal centers (GCs) of lymphoid tissues. The GC reaction drives the accumulation of somatic mutations in Ab genes, selecting high-affinity B cell clones and potent serum Ab responses. Although highly relevant to the generation of high-quality and durable immunity to SARS-CoV-2, this has not been well studied in subjects recovering from SARS-CoV-2 infection.

Ab and memory T cell responses in the blood wane relatively rapidly after acute SARS-CoV-2 infection before approaching stable maintenance (2–4). However, we and others find that SARS-CoV-2–specific memory B cells (MBCs) gradually accumulate in the circulation, approaching 1% of total MBCs several months postinfection (3, 5). SARS-CoV-2–specific MBCs might provide a mechanism to reduce the severity of reinfection (6). Ig genes in the blood-resident SARS-CoV-2–specific MBC population have been reported to continue to accumulate somatic mutations over months, with isolated monoclonal human Abs demonstrating affinity maturation (4, 7, 8). This suggests that continued GC activity in lymphoid tissues may be occurring, leading to a continuous maturation of the humoral response to SARS-CoV-2.

SARS-CoV-2 is acquired through the respiratory tract and is associated with multiple upper respiratory symptoms. The palatine tonsils are a specialized pharyngeal lymphoid organ that responds to upper respiratory tract infections, accessible to biopsy under local anesthetic. Tonsils may therefore likely comprise a lymphoid organ responding to SARS-CoV-2 infection. Tonsillectomy samples from children and adolescents are commonly studied, but such enlarged or inflamed samples may not recapitulate the normal tonsillar immunity. To our knowledge normal tonsils have not been previously studied outside of tonsillectomies for clinical reasons to assess lymphoid tissue immunity to respiratory pathogens. We recruited subjects convalescent from SARS-CoV-2 infection to undergo a tonsil biopsy to study GC B and Tfh cells.

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The online version of this article contains supplemental material.

Abbreviations used in this article: GC, germinal center; MBC, memory B cell; S^+, spike-specific; Tfh, T follicular helper.

Materials and Methods

Subjects and tonsil biopsies

We recruited 19 adults with confirmed prior SARS-CoV-2 infection (24–225 d after symptom onset) to donate blood and a tonsil biopsy (Supplemental Table I). Key exclusion criteria included elevated prothrombin time, thrombocytopenia, anticoagulation (with the exception of low-dose aspirin), prior tonsillectomy, current throat infection, or a hyperreactive gag reflex. Of 24 subjects screened, 5 failed due to a hyperreactive gag reflex. Subjects provided written informed consent and the studies were approved by the University of Melbourne (approval 2056689) and the Alfred and Epworth hospitals in Melbourne (approvals 180/20 and 406/20). Topical 10% lidocaine (10 mg/100 μl) was applied directly to the tonsils to provide local anesthesia and reduce the gag reflex. A small 3- to 5-mm incisional biopsy was taken after grasping the tonsillar lymphoid tissue with Blakesley forceps. As controls, we also analyzed mononuclear cells from the tonsils of healthy children or adolescents with enlarged tonsils and sleep apnea undergoing tonsillectomy prior to the COVID-19 pandemic ($n = 6$, Supplemental Table II).

Spike-specific B cells in PBMCs

Recombinant SARS-CoV-2 spike fluorescent probes were used to identify spike-specific B cells as previously described (9). For PBMCs, thawed cells were stained with Aqua viability dye (Thermo Fisher Scientific) and then surface stained with an S probe, CD19 ECD (J3-119) (Beckman Coulter), CD20 Alexa Fluor 700 (2H7), IgM BUV395 (G20-127), IgD PE-Cy7 (IA6-2), IgG BV786 (G18-145), streptavidin BV510 (BD Biosciences), CD14 BV510 (M5E2), CD3 BV510 (OKT3), CD8a BV510 (RPA-T8), CD16 BV510 (3G8), and CD10 BV510 (HI10a) (BioLegend). Cells were washed twice with PBS containing 1% FCS and fixed with 1% formaldehyde (Polysciences). Single MBCs were sorted and recombined H chain Ig gene sequences recovered as previously described (9).

Flow cytometry of tonsil biopsies

For the tonsil biopsies, single-cell suspensions were generated by passage through 70-μm filters within 2–6 h of biopsy. Fresh tonsil cell suspensions and donor-matched PBMCs and frozen mononuclear cells from healthy tonsils were stained with Aqua viability dye (Thermo Fisher Scientific), followed by surface staining with S probe, PD-1 BV421 (EH12.2H7; BioLegend), IgD AF488 (polyclonal; Southern-Biotech), CD3 AF700 (SP34-2; BD), CD4 BV605 (L200; BD), IgG BV786 (G18-145; BD Biosciences), CD20 allophycocyanin-Cy7 (2H7; BioLegend), CXCR5 PE-Cy7 (MU5UBEE; Life Technologies), CD14 BV510 (M5E2; BioLegend), CD8a BV510 (RPA-T8; BioLegend), CD16 BV510 (3G8; BioLegend), CD10 BV510 (HI10a; BioLegend), and streptavidin BV510 (BD Biosciences). Cells were washed and permeabilized with a transcription factor buffer set (BD Biosciences) prior to BCL6 AF647 (IG191E/A8; BioLegend) and Ki-67 BUV395 (B56; BD Biosciences) staining. Cells were washed twice and resuspended in PBS containing 1% FCS. Samples were acquired on a BD LSRFortessa using BD FACSDiva.

Results and Discussion

Spike-specific MBCs in blood maintained over time

We first studied blood spike-specific (S^+) MBCs and Ab somatic hypermutation to confirm extended generation of S^+ MBCs and changes in Ab genes in our cohort of SARS-CoV-2 convalescent subjects. S^+ MBCs in PBMCs were identified by flow cytometry as shown in Fig. 1A. We previously found that SARS-CoV-2 S^+ MBC frequencies accumulate in blood during the course of 150 d in a cohort of individuals recovered from mild or moderate SARS-CoV-2 infection (3), shown in gray in Fig. 2A. We now extend our longitudinal blood sampling from a subset of these subjects who underwent tonsil biopsies and found that S^+ MBCs in blood are stably maintained within the circulation at high frequencies out to at least day 225 postinfection, shown in red in Fig. 2A. The extended maintenance of MBCs at high levels is suggestive of robust lymphoid GC reactions.

GC interactions between B and Tfh cells result in the selection of mutated Ab genes with improved Ag affinity. To study this directly, we analyzed Ig genes expressed by circulating S^+ MBCs from three individuals at both 36–52 and 115–122 d

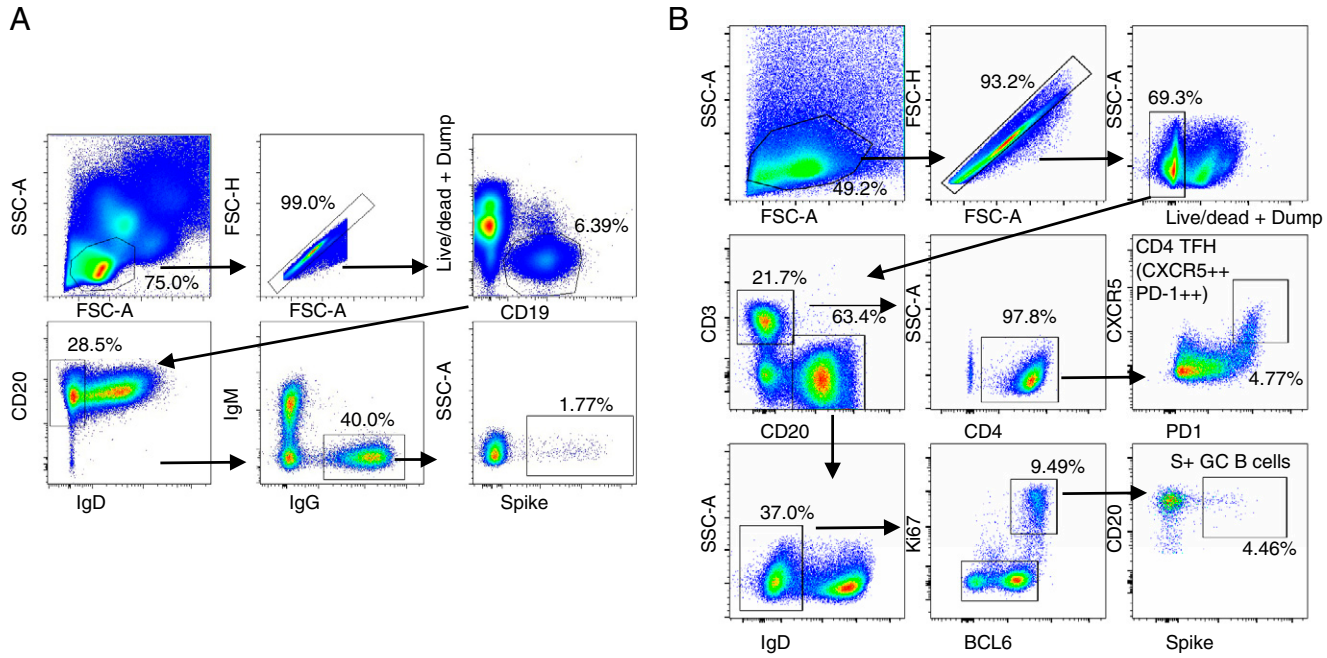

FIGURE 1. Gating strategy for resolving spike-specific B cells and Tfh cells. (**A**) Lymphocytes (forward scatter area [FSC-A] versus side scatter area [SSC-A]) in PBMCs were excluded for doublets (FSC-A versus FSH height [FSC-H]), and live, dump$^-$ ($CD14^-CD3^-CD8a^-CD16^-CD10^-$streptavidin$^-$) $CD19^+$ B cells were gated. Memory B cells ($CD20^+IgD^-IgG^+$) were assessed for binding to a SARS-CoV-2 spike (S) probe. (**B**) Tonsil lymphocytes (FSC-A versus SSC-A) were excluded for doublets (FSC-A versus FSC-H), and live, dump$^-$ ($CD14^-CD8a^-CD16^-CD10^-$streptavidin$^-$) $CD20^+IgD^-$ B cells were gated. Germinal center B cells ($Ki\text{-}67^+BCL6^+$) were assessed for binding to a SARS-CoV-2 spike (S) probe. Tfh cells were gated as $CD3^+CD4^+CXCR5^{++}PD\text{-}1^{++}$. Experiments were performed once.

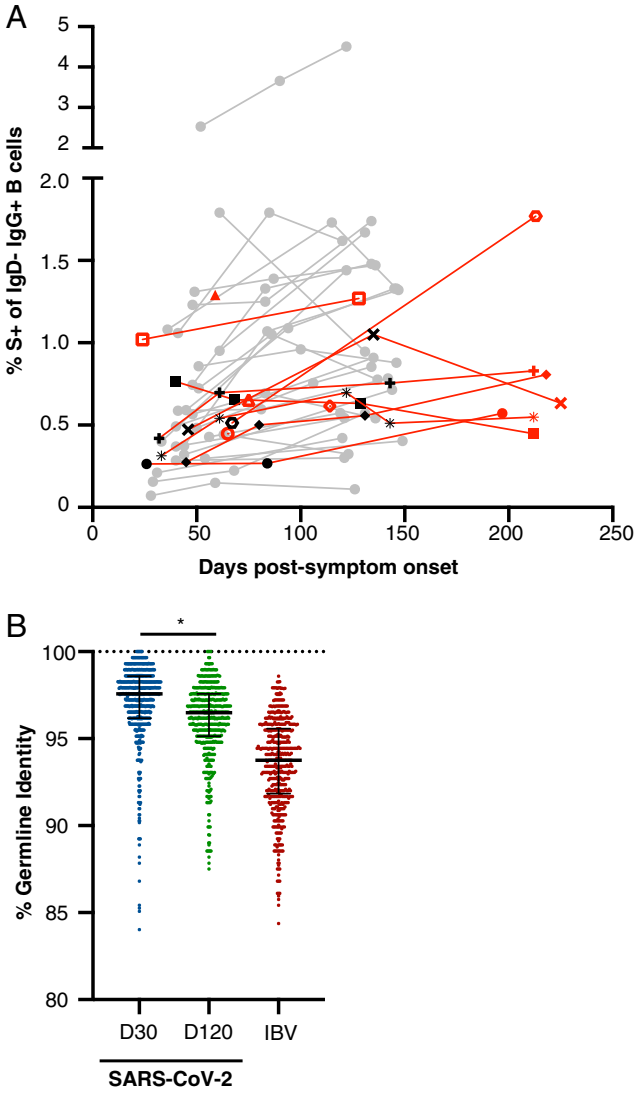

FIGURE 2. Longitudinal assessment of circulating memory B cells among individuals recovered from SARS-CoV-2 infection. (**A**) Frequencies of spike-specific memory B cells as a proportion of $CD19^+CD20^+IgD^-IgG^+$ B cells in PBMC samples were assessed longitudinally ($n = 38$ participants). Red indicates PBMCs contemporaneously sampled during tonsil biopsies; black indicates sampling in tonsil donors prior to biopsies; and gray indicates longitudinal assessment of PBMCs in individuals without tonsil biopsies. (**B**) Somatic mutation loads of spike-specific Ig genes sequenced from memory B cells isolated from three matched participants at day 30 (595 H chain sequences) or 120 (513 sequences) after symptom onset. For comparison, memory B cells specific for the hemagglutinin of influenza B virus ($n = 3$; 519 sequences) were used. Experiments were performed once. Bar represents median value. $^*p < 0.0001$, Mann–Whitney *U* test.

following infection. Sequencing of Ig genes expressed by circulating S^+ MBCs from three individuals at both 36–52 and 115–122 d following infection showed increased somatic mutation over time (Fig. 2B), consistent with other reports (4, 7). This is suggestive of enduring GC activity, although the polyclonal diversity of B cell responses to spike did not permit analysis at a clonal level.

Immune responses in tonsils following SARS-CoV-2 infection

We assessed whether persistent GC responses could be observed in a secondary lymphoid organ of the upper respiratory mucosa, the tonsils. Biopsy samples were collected from the tonsils of $n = 19$ convalescent individuals (8 female, 11 male) at time points ranging from 16 to 225 d after symptom onset (Supplemental Table I). One subject had longitudinal biopsies of the left and right tonsils at separate time points postinfection. The biopsy was generally well tolerated with pain reported in 8 of 20 (45%) procedures, graded as severe (grade 3) on two occasions, and resolved in all participants. There were no serious adverse events, infections, or bleeding complications, suggesting that this general procedure could be used in other human immunology studies.

The fresh tonsil biopsies were dissociated, with two samples yielding <0.5 million cells (not further analyzed), and the remainder yielding 3–11 million cells. Five samples were used for assay development and validation, and 13 samples (from 12 individuals) were assessed for the frequency of S^+ GC B cells and Tfh cells by flow cytometry (Fig. 1B). In two participants with tonsil specimens collected at 24 and 59 d after symptom onset, a S^+ GC B cell population representing a remarkable ~5% of all GC B cells was present (Fig. 3A, 3B). The S^+ GC B cell population was variable across our subjects and generally declined out to 7 mo postinfection, evidenced both in one individual for whom two longitudinal biopsies were collected (Fig. 3A, 3B) and across the cohort (Fig. 3B). To demonstrate spike probe specificity, we show that S^+ GC B cells are rare or absent in pre-pandemic tonsils surgically collected from individuals with enlarged tonsils causing breathing difficulties or sleep apnea (subject details in Supplemental Table II) (Fig. 3B).

GC Tfh cells were also readily detected in the tonsils of this SARS-CoV-2 convalescent cohort and declined during 7 mo across the cohort (Fig. 3C). Total GC B cells were readily observed in most tonsil biopsy samples, with generally lower levels at 7 mo postinfection (Fig. 3D). Frequencies of both GC Tfh and B cells were higher in "control' tonsils obtained by tonsillectomy (Fig. 3C, 3D) compared to biopsy of convalescent individuals. The high frequencies of Tfh cells are consistent with other reports of tonsillectomy samples (10, 11) and are potentially related to the chronic inflammation often observed in tonsillectomy samples obtained from younger cohorts. Future studies investigating basal levels of GC Tfh or B cells could be better informed with biopsy samples from healthy individuals and/or autopsy samples from people dying of unrelated conditions.

Our data demonstrate that samples of tonsils can be safely obtained post mild-to-moderate SARS-CoV-2 infection, and that the tonsil represents a site of GC reactions to SARS-CoV-2. Prominent populations of S^+ GC B cells were observed in many of the tonsil biopsy specimens, particularly when sampled within the first months postinfection.

The extent to which tonsil GC responses contribute to Ab responses in the plasma is unclear. We and others have observed important relationships between circulating Tfh cell and Ab responses to SARS-CoV-2 infection (9, 12), but direct analyses of at lymphoid sites will yield important insights. However, this initial study of 12 subjects is largely cross-sectional and limited to individuals with mild-to-moderate SARS-CoV-2 infection. Expanding into larger, more diverse cohorts will clarify the contribution of tonsil immunity to systemic Ab responses, as well as any impacts from age or severity of illness.

Although SARS-CoV-2 infection is typically an acute respiratory infection, studies have described persistence of SARS-CoV-2 Ag in

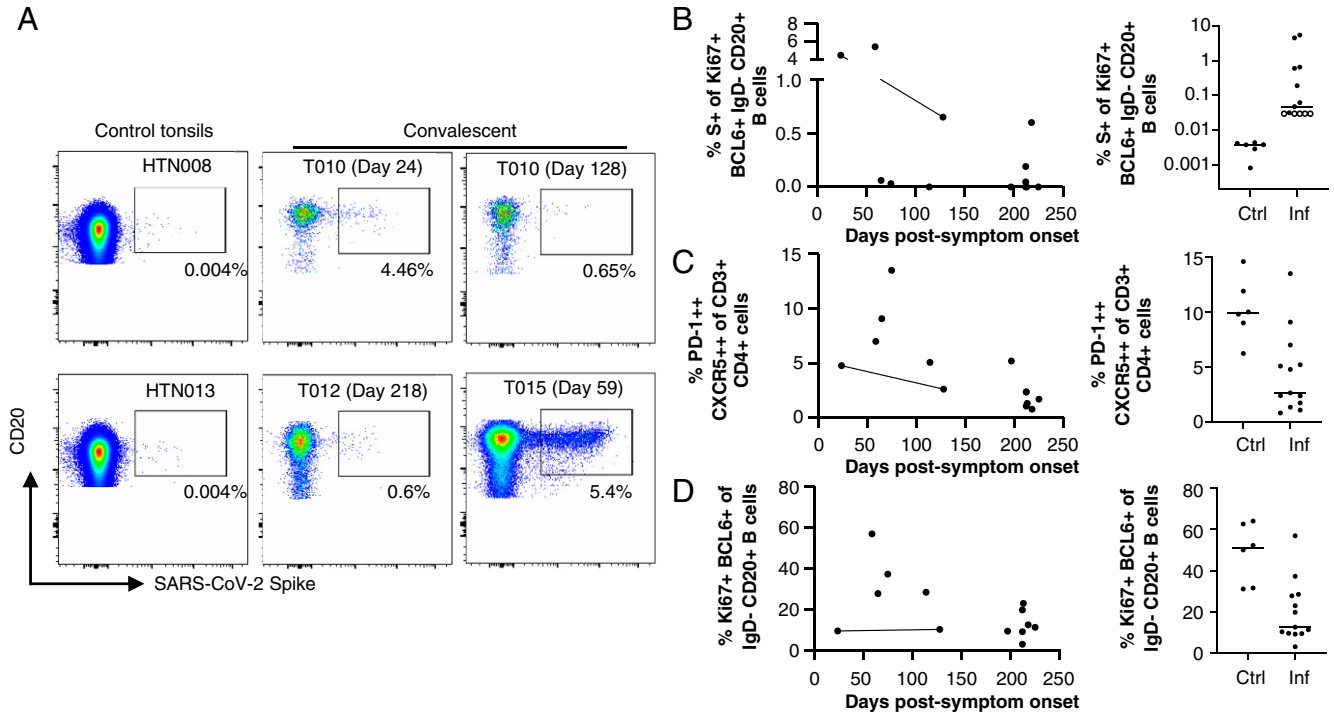

FIGURE 3. Germinal center responses in tonsils of individuals recovered from mild or moderate SARS-CoV-2 infection. (**A**) Representative plots of spike-specific germinal center B cells (S^+ Ki-$67^+BCL6^+IgD^-CD20^+$) from tonsils of SARS-CoV-2 convalescent individuals or control tonsils obtained prior to the COVID-19 pandemic. (**B**–**D**) Frequencies of (B) spike-specific germinal center B cells (S^+ Ki-$67^+BCL6^+IgD^-CD20^+$) (open circles denote tonsil samples with S^+ events below the limit of detection), (C) Tfh cells (PD-$1^{++}CXCR5^{++}CD3^+CD4^+$), and (D) total germinal center B cells (Ki-$67^+BCL6^+IgD^-CD20^+$). Experiments were performed once (n = 13 tonsil biopsies from n = 12 individuals with SARS-CoV-2 and n = 6 tonsillectomy samples obtained prior to COVID-19).

the nasopharynx and gut for some months (4, 13). We speculate that persistent Ag may be one factor that maintains GC responses and maturation of the B cell response in lymphoid tissues described herein, and by others in response to both infection and vaccination (14, 15). Spike-specific bone marrow–derived plasma cells can also persist for many months following SARS-CoV-2 infection (16). Tonsillar responses following infection are broadly similar to those observed by fine needle biopsy of the axillary lymph nodes of subjects receiving SARS-CoV-2 vaccines (15). The gradual decline in GC B cell and GC Tfh cell responses we observed over time is consistent with the generalized decay in serological Ab levels and the contraction of memory T cell populations (2, 3), although identifying baseline levels of GC B cells and GC Tfh cells in healthy tonsils across an age spectrum is an important area of further study. In summary, we provide the first description of immune responses to SARS-CoV-2 in tonsils, highlighting a methodology for sampling lymphoid immunity in humans and illuminating a potential link in the development of immunity against severe reinfection with SARS-CoV-2.

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Disclosures

The authors have no financial conflicts of interest.

References

1. Khoury, D. S., D. Cromer, A. Reynaldi, T. E. Schlub, A. K. Wheatley, J. A. Juno, K. Subbarao, S. J. Kent, J. A. Triccas, and M. P. Davenport. 2021. Neutralizing antibody levels are highly predictive of immune protection from symptomatic SARS-CoV-2 infection. *Nat. Med.* 27: 1205–1211.
2. Dan, J. M., J. Mateus, Y. Kato, K. M. Hastie, E. D. Yu, C. E. Faliti, A. Grifoni, S. I. Ramirez, S. Haupt, A. Frazier, et al. 2021. Immunological memory to SARS-CoV-2 assessed for up to 8 months after infection. *Science* 371: eabf4063.
3. Wheatley, A. K., J. A. Juno, J. J. Wang, K. J. Selva, A. Reynaldi, H. X. Tan, W. S. Lee, K. M. Wragg, H. G. Kelly, R. Esterbauer, et al. 2021. Evolution of immune responses to SARS-CoV-2 in mild-moderate COVID-19. *Nat. Commun.* 12: 1162.
4. Gaebler, C., Z. Wang, J. C. C. Lorenzi, F. Muecksch, S. Finkin, M. Tokuyama, A. Cho, M. Jankovic, D. Schaefer-Babajew, T. Y. Oliveira, et al. 2021. Evolution of antibody immunity to SARS-CoV-2. *Nature* 591: 639–644.
5. Rodda, L. B., J. Netland, L. Shehata, K. B. Pruner, P. A. Morawski, C. D. Thouvenel, K. K. Takehara, J. Eggenberger, E. A. Hemann, H. R. Waterman, et al. 2021. Functional SARS-CoV-2-specific immune memory persists after mild COVID-19. *Cell* 184: 169–183.e17.
6. Cromer, D., J. A. Juno, D. Khoury, A. Reynaldi, A. K. Wheatley, S. J. Kent, and M. P. Davenport. 2021. Prospects for durable immune control of SARS-CoV-2 and prevention of reinfection. *Nat. Rev. Immunol.* 21: 395–404.
7. Wang, Z., F. Muecksch, D. Schaefer-Babajew, S. Finkin, C. Viant, C. Gaebler, H. H. Hoffmann, C. O. Barnes, M. Cipolla, V. Ramos, et al. 2021. Naturally enhanced neutralizing breadth against SARS-CoV-2 one year after infection. *Nature* 595: 426–431.
8. Muecksch, F., Y. Weisblum, C. O. Barnes, F. Schmidt, D. Schaefer-Babajew, Z. Wang, J. C. C Lorenzi, A. I. Flyak, A. T. DeLaitsch, K. E. Huey-Tubman, et al. 2021. Affinity maturation of SARS-CoV-2 neutralizing antibodies confers potency, breadth, and resilience to viral escape mutations. *Immunity* 54: 1853–1868.e7.
9. Juno, J. A., H. X. Tan, W. S. Lee, A. Reynaldi, H. G. Kelly, K. Wragg, R. Esterbauer, H. E. Kent, C. J. Batten, F. L. Mordant, et al. 2020. Humoral and circulating follicular helper T cell responses in recovered patients with COVID-19. *Nat. Med.* 26: 1428–1434.
10. Heit, A., F. Schmitz, S. Gerdts, B. Flach, M. S. Moore, J. A. Perkins, H. S. Robins, A. Aderem, P. Spearman, G. D. Tomaras, et al. 2017. Vaccination establishes clonal relatives of germinal center T cells in the blood of humans. *J. Exp. Med.* 214: 2139–2152.
11. Kim, S. T., J. Y. Choi, B. Lainez, V. P. Schulz, D. E. Karas, E. D. Baum, J. Setlur, P. G. Gallagher, and J. Craft. 2018. Human extrafollicular $CD4^+$ Th cells help memory B cells produce Igs. *J. Immunol.* 201: 1359–1372.
12. Koutsakos, M., L. C. Rowntree, L. Hensen, B. Y. Chua, C. E. van de Sandt, J. R. Habel, W. Zhang, X. Jia, L. Kedzierski, T. M. Ashhurst, et al. 2021.

Integrated immune dynamics define correlates of COVID-19 severity and antibody responses. *Cell Rep. Med.* 2: 100208.
13. Cheung, C. C. L., D. Goh, X. Lim, T. Z. Tien, J. C. T. Lim, J. N. Lee, B. Tan, Z. E. A. Tay, W. Y. Wan, E. X. Chen, et al. 2022. Residual SARS-CoV-2 viral antigens detected in GI and hepatic tissues from five recovered patients with COVID-19. *Gut* 71: 226–229.
14. Poon, M. M. L., K. Rybkina, Y. Kato, M. Kubota, R. Matsumoto, N. I. Bloom, Z. Zhang, K. M. Hastie, A. Grifoni, D. Weiskopf, et al. 2021. SARS-CoV-2 infection generates tissue-localized immunological memory in humans. *Sci. Immunol.* 6: eabl9105.
15. Turner, J. S., J. A. O'Halloran, E. Kalaidina, W. Kim, A. J. Schmitz, J. Q. Zhou, T. Lei, M. Thapa, R. E. Chen, J. B. Case, et al. 2021. SARS-CoV-2 mRNA vaccines induce persistent human germinal centre responses. *Nature* 596: 109–113.
16. Turner, J. S., W. Kim, E. Kalaidina, C. W. Goss, A. M. Rauseo, A. J. Schmitz, L. Hansen, A. Haile, M. K. Klebert, I. Pusic, et al. 2021. SARS-CoV-2 infection induces long-lived bone marrow plasma cells in humans. *Nature* 595: 421–425.