Cross-reactive SARS-CoV-2 epitope targeted across donors informs immunogen design

Graphical abstract

Highlights

- Broad sarbecovirus reactivity exists in the human RBD-directed antibody repertoire
- Broadly cross-neutralizing antibodies predominantly target two conserved epitopes
- A rationally designed immunogen focuses responses to one conserved epitope
- Immune-focused murine antibody responses confer improved Omicron neutralization

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In brief
Hauser et al. characterize human antibodies elicited by SARS-CoV-2 vaccination or infection that bind broadly to different sarbecovirus receptor-binding domains. Broadly cross-neutralizing antibodies target two conserved epitopes. Structure-guided immunogen design focuses murine antibody responses to one such epitope in the context of pre-existing immunity, conferring improved Omicron neutralization.
Cross-reactive SARS-CoV-2 epitope targeted across donors informs immunogen design

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https://doi.org/10.1016/j.xcrm.2022.100834

SUMMARY

The emergence of the antigenically distinct and highly transmissible Omicron variant highlights the possibility of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) immune escape due to viral evolution. This continued evolution, along with the possible introduction of new sarbecoviruses from zoonotic reservoirs, may evade host immunity elicited by current SARS-CoV-2 vaccines. Identifying cross-reactive antibodies and defining their epitope(s) can provide templates for rational immunogen design strategies for next-generation vaccines. Here, we characterize the receptor-binding-domain-directed, cross-reactive humoral repertoire across 10 human vaccinated donors. We identify cross-reactive antibodies from diverse gene rearrangements targeting two conserved receptor-binding domain epitopes. An engineered immunogen enriches antibody responses to one of these conserved epitopes in mice with pre-existing SARS-CoV-2 immunity; elicited responses neutralize SARS-CoV-2, variants, and related sarbecoviruses. These data show how immune focusing to a conserved epitope targeted by human cross-reactive antibodies may guide pan-sarbecovirus vaccine development, providing a template for identifying such epitopes and translating to immunogen design.

INTRODUCTION

As severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) continues to evolve because of population immunity, antigenically distinct variants such as Omicron may emerge.1 This antigenic difference increases the likelihood that there will be re-infections or breakthrough infections following vaccination.1,14 Furthermore, surveillance efforts have identified related sarbecoviruses in animal reservoirs that use the human ACE2 receptor for viral entry, suggesting possible future pandemics.3–12 Consequently, there is a need for cross-reactive therapeutic antibodies or next-generation vaccines that protect against SARS-CoV-2 variants and novel sarbecoviruses with pandemic potential. While many SARS-CoV-2 therapeutic monoclonal antibodies developed early in the pandemic had reduced potency against Omicron, boosting with mRNA vaccines appeared to improve serum neutralization against this and other variants.4,13–15 Identifying infection- or vaccine-elicited cross-reactive antibodies can inform immunogen design efforts that focus responses to conserved epitopes.

Here, we analyzed 10 donors from a previous cohort that had serum neutralization against SARS-CoV-2 variant pseudoviruses4 to characterize cross-reactive memory B cells to additional variants and pre-emergent sarbecoviruses (Table S1). All donors had at least three documented exposures to SARS-CoV-2: 5 received two SARS-CoV-2 vaccines and had at least one SARS-CoV-2 infection, and 5 had three mRNA vaccinations and no known prior SARS-CoV-2 infection. Serum from each donor neutralized wild-type SARS-CoV-2, Delta, and Omicron, and SARS-CoV-1 pseudoviruses4 (Table S1). Neutralization was significantly reduced against Omicron and SARS-CoV-1 relative to wild-type SARS-CoV-2; neutralization of SARS-CoV-1 was also significantly reduced relative to Delta.

RESULTS

Cross-reactive memory B cell repertoire

We characterized the cross-reactive memory B cell repertoire targeting wild-type SARS-CoV-2, Omicron, and related sarbecoviruses SARS-CoV-1 and SHC014; these represent genetically diverse coronavirus clade members that use human ACE2 for viral entry.16 We isolated quadruple cross-reactive class-switched (swIg) B cells targeting the wild-type SARS-CoV-2, Omicron, SARS-CoV-1, and SHC014 receptor-binding domains
We found that of swIg B cells, a median of 0.3% (interquartile range [IQR] 0.2%–0.8%) bound both wild-type SARS-CoV-2 and Omicron RBDs (Figure S2 A). Of those cross-reactive B cells, a median of \( C_{24}^{35}\% \) (IQR 27%–43%) cross-reacted with both SARS-CoV-1 and SHC014 RBDs (Figure S2 B).

We obtained paired heavy- and light-chain sequences for 228 wild-type SARS-CoV-2+/Omicron+/SARS-CoV-1+/SHC0 14+ RBD quadruple cross-reactive class-switched B cells from 10 donors. While 33 different IGHV genes were used across 57 different alleles (Figures 1 A and S2C), IGHV3-30 was the most frequently used gene, consistent with observations from other RBD-directed B cells from convalescent patients with SARS-CoV-2.\(^{17–19}\) However, the distribution of IGHV gene usage varied from wild-type SARS-CoV-2 RBD-specific memory B cells as reported previously, with less frequent usage of several genes including IGHV3-53, 1–46, 3–23, 3–9, and 1–69.\(^{17–19}\) This suggests that a majority of wild-type SARS-CoV-2 RBD-specific B cell receptors may target non-conserved epitopes. IGKV1-39, 1–33, and 1–5 were the most prevalent genes (Figures 1 B and S2D). V-gene usage did not vary significantly between donors who were vaccinated and infected versus donors who were vaccinated and boosted. The median V-gene germline identity was \( C_{24}^{\sim} 16\) amino acids (IQR 14–20), and the median CDRL3 length was \( C_{24}^{\sim} 10\) amino acids (IQR 9–10) (Figure 1 D).

### Mapping cross-reactive antibody epitopes

We next selected 29 paired heavy and light chains that reflected the genetic diversity of the 228 sequenced B cell receptors (BCRs) for recombinant expression as immunoglobulin G (IgG) for further biochemical characterization. Recombinant antibodies were first tested in an ELISA for binding to wild-type SARS-CoV-2, Omicron, SARS-CoV-1, and SHC014 RBDs (Figure S2B).

We down-selected 19 antibodies from 5 donors (Table S2) based on expression levels for epitope mapping by cross-competition ELISA using the wild-type SARS-CoV-2 RBD (Figure 2B). We included control antibodies with structurally defined epitopes: B38 (class 1), P2B-2F6 (class 2), S309 (class 3), CR3022 (class 4), and ADI-55688 (precursor to broadly neutralizing therapeutic antibody ADG-2).\(^{16,20–24}\) While several antibodies competed with ACE2 receptor-binding motif (RBM)-directed antibodies B38, P2B-2F6, or ADI-55688, most cross-reactive antibodies mapped to two distinct epitopes. One class competed with CR3022 (class 4), while the other class showed no competition with any control antibodies in ELISA. To define the targeted epitope, we chose...
representative antibody D2G2 and assayed for competition using biolayer interferometry (BLI) with an additional panel of structurally characterized Fabs: two from mice that represent distinct RBD epitopes, Ab20 and Ab16, and four from human donors targeting minimally overlapping RBM and non-RBM epitopes on the RBD, G32A4, C98C7, G32R7, and G32Q4 (Figure 2C). Only G32R7 competed with D2G2, indicating that the other class of broadly cross-reactive antibodies targets a conserved class 3 epitope that minimally competes with S309, strongly suggesting that the epitope is across that face of the RBD (Figure 2D). Of the 11 class 3-directed antibodies characterized, 8 used IGHJ4; only 2 of the 8 class 4-directed antibodies used IGHJ4 (Table S2). There were no other clear patterns of antibody sequence features associated with either of the conserved epitopes, suggesting multiple evolutionary pathways that converge on these conserved epitopes. Broadly reactive antibodies have been previously shown to target both class 3 and 4 epitopes; the lack of class 1 and 2 antibodies characterized here indicates that broadly reactive antibodies targeting these epitopes are less represented in the human memory repertoire.
Cross-reactive antibody neutralization

We next performed pseudovirus neutralization assays on the 19 antibodies down-selected for cross-competition against wild-type SARS-CoV-2, SARS-CoV-1, WIV1, RaTG13, SHC014, and the SARS-CoV-2 variants Delta and Omicron. Representative antibodies targeting the two conserved epitopes based on competition were broadly neutralizing with IC50s ranging from \( \leq 1 \) to 200 \( \mu \text{g/mL} \) in a pattern that roughly correlated with antibody affinity (Figures 3A and 3B). Seven antibodies targeting the class 4 conserved epitope and 3 antibodies targeting the class 3 conserved epitope neutralized at least 4 of the 7 pseudoviruses tested (though none of the class 3 antibodies neutralized RaTG13). These data indicate that cross-reactive antibodies elicited by vaccination or infection that target both sites are broadly neutralizing antibodies. We also tested antibodies against authentic wild-type SARS-CoV-2 and Omicron and found preserved neutralization (Figure S3).

An engineered immunogen for epitope targeting

Based on these observations across human donors, we used a structure-guided approach to design an immunogen to focus antibody responses to the class 4 epitope. Using glycan engineering, we developed a hyperglycosylated immunogen that selectively exposed this epitope while occluding all other epitopes (Figures 4A, S4A, and S4B); this builds upon our previous hyperglycosylated immunogens to direct immune responses to conserved epitopes.25 This class 4-focusing immunogen has 11 engineered putative N-linked glycosylation sites; 9 are novel and two are native to SARS-CoV-2. We made trimeric versions of this immunogen using a non-immunogenic, hyperglycosylated GCN4 trimerization tag to increase valency and for subsequent testing in vivo25 (Figures S4C–S4F). Two murine cohorts were primed with the SARS-CoV-2 spike to approximate pre-existing vaccine or infection-elicited immunity to SARS-CoV-2 followed by boosting with trimeric wild-type SARS-CoV-2 RBD (“WT cohort”) or the hyperglycosylated immunogen (“HG cohort”) (Figure 4B).

Assessing immunogenicity and focusing

We assayed sera binding 14 days after the second boost against coronavirus RBDs by ELISA. Both cohorts had similar reactivity to all sarbecovirus RBDs tested, but the WT cohort showed a significant drop off in reactivity against the Omicron RBD versus the SARS-CoV-2 RBD, while the HG cohort did not (Figures S4G–S4H). To assess immune focusing to the class 4 epitope, we assayed competition of the murine class 4 antibody Ab16, which competes with CR3022 but has broad neutralization activity across SARS-CoV-2 variants and other sarbecoviruses.25 Ab16 competes with CR3022 and the class 4 donor antibodies (Figures 4C and S4F), and the Ab16 footprint was previously structurally characterized;25 Ab16 was also used to confirm the conformational integrity of the HG immunogen (Figure S4A). The HG cohort had significant increase in serum competition with Ab16, with a mean serum competition of \( \sim 2.5 \times \) relative to WT (Figure 4D); this indicates that boosting with the HG immunogen immune focuses to the class 4 epitope.

Immune focusing improves cross-neutralization

We assessed serum pseudovirus neutralization of WT SARS-CoV-2, Delta and Omicron variants, and related sarbecoviruses RaTG13, SARS-CoV-1, WIV1, and SHC014. Sera from all cohorts had broad
neutralization with NT50s ranging from \(10^{-3}\)–10 against WT SARS-CoV-2 to \(10^{-2}\)–10 against SARS-CoV-1 and WIV1 (Figure 4E). Neutralization of related sarbecoviruses RaTG13, SARS-CoV-1, WIV1, and SHC014 showed no significant differences between the WT and HG cohorts (Figure 4E). This is consistent with the fact that there is no significant decrease in the HG cohort between neutralization of WT SARS-CoV-2 and all other coronaviruses tested, while there are significant decreases in the WT cohort (Figures S4I–S4J). Because serum antibody responses from the HG cohort significantly competed with Ab16 (class 4 epitope), relative to the WT cohort (Figure 4D), this suggests that the observed immune focusing elicited by this first-generation immunogen may also neutralize future antigenically distinct SARS-CoV-2 variants. While these data demonstrate a proof-of-principle that focusing SARS-CoV-2 serum antibody responses to a broadly neutralizing epitope can improve breadth, the lack of improved serum neutralization across other sarbecoviruses (Figures S4I–S4J) indicates that further iterative design cycles are necessary.

**DISCUSSION**

A continued understanding of the nature of the broadly cross-reactive repertoire elicited by SARS-CoV-2 infection and vaccination is necessary given that additional SARS-CoV-2 variants and other related sarbecoviruses will emerge. Characterizing human cross-reactive antibodies may help identify candidate therapeutic monoclonal antibodies that limit the likelihood of viral escape. Currently available monoclonal antibodies were largely selected based on potent WT SARS-CoV-2 neutralization, but Omicron evades neutralization by many of them, particularly those that target epitopes in the RBM (class 1 and 2 epitopes). Emerging SARS-CoV-2 variants or other sarbecoviruses may still escape even broadly cross-reactive antibodies, but identifying multiple antibodies with distinct, non-overlapping epitopes may help maintain overall therapeutic efficacy.

Eliciting broadly cross-reactive antibody responses via vaccination, in combination with developing broadly protective therapeutic antibodies, may help reduce the likelihood that ongoing SARS-CoV-2 evolution and potential emerging sarbecoviruses will escape both existing protective immunity and available treatments. Indeed, broadly cross-reactive antibody responses have already been identified in patients vaccinated against SARS-CoV-2 following prior SARS-CoV-1 infection, indicating that it should be possible to elicit these responses in humans. Additionally, while boosting with WT SARS-CoV-2 protects against Omicron, protection may vary as other variants evolve; this underscores the need for a pan-sarbecovirus vaccine. 13–15
Efforts to focus the immune response to protective epitopes have extended to several different viruses, including influenza, HIV, respiratory syncytial virus (RSV), and SARS-CoV-2.25,36–38 Here, we used an immunogen design strategy to re-direct pre-existing antibody responses toward conserved epitopes by engineering putative N-linked glycosylation sites to "mask" undesirable epitopes.25,38 Immune re-focusing is particularly important in the context of immune imprinting to influence a memory-recall response that might otherwise be biased toward less conserved epitopes; such re-focusing can improve serum neutralization and protection. These immune imprinting and biased antibody responses were observed for SARS-CoV-229–41 and parallel observations from influenza.40–44 Consequently, how to focus humoral immune responses toward broadly protective epitopes must be evaluated in context of pre-existing immunity. Our results show that human subjects generate cross-reactive antibodies targeting conserved epitopes and that these antibodies are genetically diverse. In mice, selectively boosting responses to one of these conserved epitopes using an engineered immunogen improved Omicron variant neutralization in the context of pre-existing immunity. Further experiments will be required to evaluate the impact of this immune focusing on protection, but these data provide proof of principle for an approach to pan-sarbecovirus vaccine design by demonstrating that prior knowledge of the exact variant or emerging sarbecovirus may not be required to elicit a broadly neutralizing antibody response.

LIMITATIONS OF THE STUDY

This study aims to provide proof-of-principle that focusing SARS-CoV-2 serum antibody responses to a conserved epitope in the context of SARS-CoV-2 spike imprinting can improve breadth. Further experiments, including in vivo protection studies, is necessary to establish whether this change is clinically meaningful. Additionally, RBD-directed antibodies represent only one possible class of broadly neutralizing antibodies. Antibodies targeting other conserved regions on the N-terminal domain or elsewhere on the spike glycoprotein could also confer protection, but these antibodies were not isolated in our sorting strategy. However, we note that most potently neutralizing monoclonal antibodies are RBD directed, making additional RBD-directed antibodies promising candidates to achieve broad neutralization.16,45–47

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Human donor samples
  - Mice

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STAR METHOD DETAILS

- Immunogen and coronavirus protein expression and purification
- Probe generation
- Flow cytometry
- B cell receptor sequencing
- IgG expression and purification
- Serum and recombinant IgG ELISAs
- Competition ELISAs
- Competition biolayer interferometry
- Immunizations
- Pseudovirus neutralization assay
- Live virus neutralization assay

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xcrm.2022.100834.

ACKNOWLEDGMENTS

We thank Dr. Jason McLellan from the University of Texas, Austin, for the spike plasmid. We thank Nir Hacohen and Michael Farzan for the kind gift of the ACE2-expressing 293T cells. We also thank Mark C. Poznansky and David J. Gregory from the Vaccine and Immunotherapy Center at Massachusetts General Hospital in Boston, MA, for developing the human donor sample protocol. We acknowledge funding from NIH R01s AI146779 (A.G.S.) and AI124378, AI137057, and AI153098 (D.L.), a Massachusetts Consortium on Pathogenesis Readiness (MassCPR) grant (A.G.S.), and training grants NIGMS T32 GM007753 and T32 GM144273 (B.M.H.); T32 AI007245 (J.F.); F31 AI138368 (M.S.); and F30 AI160908 (B.M.H.). A.B.B. is supported by the National Institute on Drug Abuse (NIDA) Avenir New Innovator Award DP2DA040254 and the MGH Transformative Scholars Program as well as funding from the Charles H. Hood Foundation (A.B.B.). This independent research was supported by the Gilead Sciences Research Scholars Program in HIV (A.B.B.). The BSL3 Laboratory where authentic virus work was performed is supported by Harvard CFAR (P30 AI060354) and MassCPR funding.

AUTHOR CONTRIBUTIONS

Conceptualization, B.M.H.; methodology, B.M.H., J.F., M.S., W.F.G.B., and A.G.S.; investigation, B.M.H., J.F., M.S., L.R., K.J.S., M.L.S., Y.C., J.B., I.W.W., A.C., M.V., M.R.C., T.K., and W.F.G.B.; writing – original draft, A.G.S.; writing – review and editing, all authors; funding acquisition, B.M.H., I.W.W., A.C., M.V., M.R.C., T.K., and W.F.G.B.; and A.G.S.

DECLARATION OF INTERESTS

B.M.H. and A.G.S. have filed a provisional patent for the described immunogens.

Received: April 14, 2022
Revised: September 7, 2022
Accepted: November 8, 2022
Published: November 15, 2022

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| CR3022              | Produced in house | N/A |
| S309                | Produced in house | N/A |
| P2B-2F6             | Produced in house | N/A |
| B38                 | Produced in house | N/A |
| ADI-55688           | Produced in house | N/A |
| Ab16                | Produced in house | N/A |
| HRP-conjugated rabbit anti-mouse IgG antibody | Abcam | CAT#ab97046; RRID: AB_10680920 |
| HRP-conjugated goat anti-mouse IgG, human/bovine/horse SP ads antibody | Southern Biotech | CAT#1013-05 |
| anti-human CD19-PE-Cy7 | BioLegend | CAT#302216; RRID: AB_314246 |
| anti-human CD3-APC-Cy7 | BioLegend | CAT#317342; RRID:AB_2563410 |
| anti-human IgG-BV711 | BD Biosciences | CAT#564219; RRID: AB_2740459 |
| anti-human IgD-PerCp-Cy5.5 | BioLegend | CAT#348208; RRID: AB_10641706 |
| anti-human IgM-BV605 | BioLegend | CAT#314524; RRID: AB_2562374 |
| anti-human CD27-PE Dazzle | BioLegend | CAT#356422; RRID: AB_2564101 |
| anti-SARS-CoV-2 Nucleocapsid antibody | BioLegend | CAT#946102; RRID: AB_2892515 |
| anti-mouse IgG1 PE-Cy7 | BioLegend | CAT#406614; RRID: AB_2562002 |
| **Bacterial and virus strains** |        |            |
| Sarbecovirus pseudotyped viruses | Produced in house | N/A |
| US-WA1/2020 ancestral variant of SARS-CoV-2 | BEI Resources | Cat#NR-52281 |
| Omicron BA.1 variant isolate | MassCPR Virus Repository | |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| Monomeric and trimeric SARS-CoV-2 receptor binding domains | Produced in house | N/A |
| Monomeric SARS-CoV-1, WIV1, SHC014 and RaTG13 receptor binding domains | Produced in house | N/A |
| SARS-CoV-2 two-proline stabilized spike ectodomain | Produced in house | N/A |
| Hyperglycosylated (HG) SARS-CoV-2 trimer | This paper | N/A |
| Pierce HRV 3C protease | ThermoScientific | CAT#88946 |
| Sigma Adjuvant System | SigmaAldrich | CAT#S6322 |
| Streptavidin-PE conjugate | Invitrogen | CAT#12-4317-87 |
| Aqua Live/Dead amine-reactive dye | ThermoFisher | CAT#L34957 |
| Calcein, AM, cell-permeant dye | ThermoFisher | CAT#C3100MP |
| Streptavidin-BV421 conjugate | BioLegend | CAT#405225 |
| Streptavidin-BV650 conjugate | BioLegend | CAT#405232 |
| Streptavidin-APC conjugate | BioLegend | CAT#405207 |
| RNaseOUT | ThermoFisher | CAT#10777019 |
| TALON Metal Affinity Resin | Takara | CAT#635652 |
| Pierce Protein G Agarose | ThermoFisher | CAT#20399 |
| 1-Step ABTS substrate | ThermoFisher | Prod#37615 |

(Continued on next page)
**RESOURCE AVAILABILITY**

**Lead contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Aaron G. Schmidt (aschmidt@crysat.crystal.harvard.edu).

**Materials availability**

All unique/stable reagents generated in this study will be made available on request, but we may require a payment and/or a completed materials transfer agreement if there is potential for commercial application. For non-commercial use, all unique/stable reagents generated in this study are available from the lead contact with a completed materials transfer agreement.

**Data and code availability**

- B cell receptor sequences have been deposited in Genbank. Accession numbers are listed in Table S4. B cell receptor sequences are also available from Mendeley Data at https://doi.org/10.17632/4wmr6xb29g.1.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Human donor samples**

Human peripheral blood mononuclear cells were isolated for cell sorting from samples previously published4; samples were obtained with approval from the Partners Institutional Review Board (protocol 2020P002274). All measurements relating to donor samples were obtained from separate samples, rather than repeated measurements of the same samples. Table S1 contains all available data about these 10 donors; all other demographic data are unavailable.
Mice
All immunizations were performed using female C57BL/6 mice (Charles River Laboratories, strain 027) aged 6–10 weeks. Immunization experiments were conducted with institutional IACUC approval (MGH protocol 2014N000252).

Cell lines
Expi293F cells (Thermo Fisher Cat#A14527; RRID: CVCL_D615) cells were cultured in accordance with the manufacturer’s instructions. Human ACE2 expressing HEK 293T cells were a gift from Nir Hacohen and Michael Farzan and were cultured in Dulbecco’s Modified Eagle Medium (ThermoFisher) with 2% fetal bovine serum (Peak Serum FBS) and 1% penicillin-streptomycin at 10,000 U/mL (Gibco). A549-Ace2 cells (BEI Resources, Cat # NR-53821) were cultured in in D10+ media (DMEM (Corning) supplemented with HEPES (Corning), 1X Penicillin 100 IU/mL/Streptomycin 100 µg/mL (Corning), 1X Glutamine (Glutamax, ThermoFisher Scientific), and 10% Fetal Bovine serum (FBS; Sigma).

METHOD DETAILS

Immunogen and coronavirus protein expression and purification
Coronavirus receptor binding domains (RBDs) were based on the following Genbank sequences: SARS-CoV-2 RBD (Genbank MN975262.1), SARS-CoV-1 RBD (Genbank ABD72970.1, WV1 RBD (Genbank AGZ48828.1), RaTG13 RBD (Genbank QHR63300.2), SHC014 RBD (Genbank QJE50589.1). All constructs were purchased as gblocks following codon optimization using Integrated DNA Technologies. Genes were cloned into pVRC and sequence confirmed via Genewiz. Monomeric constructs used for ELISA coating and single B cell sorting included C-terminal HRV 3C-cleavable 8xHis and Avi tags, and trimeric constructs included a C-terminal HRV 3C-cleavable 8xHis tag and a hyperglycosylated GCN4 tag with two engineered C-terminal cystines, the lastermost tag being derived from a previously published hyperglycosylated GCN4 tag. The spike plasmid was provided courtesy of Dr. Jason McLellan at the University of Texas, Austin, containing C-terminal HRV 3C cleavable 6xHis and 2xStrep II tags and a non-cleavable foldon trimerization domain.

Proteins were expressed in Expi293F cells (ThermoFisher) after transfection using Expifectamine per manufacturer’s protocol. After 7 days, transfections were harvested and centrifuged for clarification. Immobilized metal-affinity chromatography using Cobalt-TALON resin (Takara) was performed via the 8xHis tag. Proteins were eluted with 350 mM imidazole, concentrated, and passed over a size exclusion column (Superdex 200 Increase 10/300 GL, GE Healthcare) in PBS (Corning). Affinity tags were cleaved by HRV 3C protease (ThermoScientific) and purified protein was isolated by orthogonal purification using Cobalt-TALON resin to remove the His-tagged HRV 3C protease, cleaved tag, and uncleaved protein.

Probe generation
Site-specific biotinylation of Avi-tagged coronavirus RBDs was performed using a BirA biotin-protein ligase reaction kit (Avidity) according to manufacturer’s protocol. Proteins were re-purified using the size exclusion column (Superdex 200 Increase 10/300 GL, GE Healthcare) in PBS (Corning). Streptavidin conjugates were added to a final conjugated probe concentration of 0.1 mg/mL with a final molar ratio of probe to streptavidin valency of 1:1. Probes were incubated at 4°C for at least one hour prior to being used to label cells.

Flow cytometry
Human peripheral blood mononuclear cells for cell sorting were stained with the following coronavirus RBD probes at a final concentration of 25 nM each: wild-type SARS-CoV-2-PE (streptavidin-PE from Invitrogen), Omicron-APC (streptavidin-APC from BioLegend), SARS-CoV-1-BV650 (streptavidin-BV650 from BioLegend), SHC014-BV421 (streptavidin-BV421 from BioLegend); labelling occurred at 4 °C for 30 minutes. A mixture of anti-human antibodies at 25 nM was then added: anti-human CD19-PE-Cy7 (BioLegend), anti-human CD3-APC-Cy7 (BioLegend), anti-human IgG-BV711 (BD Biosciences), anti-human IgD-PerCp-Cy5.5 (BioLegend), anti-human IgM-BV605 (BioLegend), anti-human CD27-PE Dazzle (BioLegend); labelling occurred for 30 minutes at 4 °C. Cells were then washed twice with PBS and labelled with near-IR live/dead stain (ThermoFisher) for 15 minutes at 4 °C. Cells were then washed twice with PBS prior to flow cytometry. Flow cytometry was run on a BD FACSaria Fusion cytometer (BD Biosciences), using FlowJo (version 10) for data analysis.

B cell receptor sequencing
96-well plates were prepared with 4 µL of lysis buffer (0.5X PBS, 10 mM DTT, 4 units of ThermoFisher RNaseOUT). Cells were sorted and centrifuged at 3000 RCF for 1 minute and stored at –80 °C. After later thawing, a reverse transcriptase reaction was performed by adding 20 µL of SuperScript IV VILO MasterMix (ThermoFisher). Previously published primers were used to perform two cycles of PCR with Herculase II Fusion DNA polymerase (Agilent) before submission to Genewiz for Sanger sequencing of variable heavy and light chains. Sequences were analyzed using IMGT High V-Quest.

IgG expression and purification
Variable heavy and light chain genes were synthesized as gBlocks from Integrated DNA Technologies and cloned into pVRC plasmids containing appropriate constant domains. Fabs and IgGs were purified and transfected using the same protocol for cell sorting.
immunogens and coating proteins. The heavy chain plasmid included a HRV 3C-cleavable 8xHis tag, and the IgG heavy chain vector included HRV 3C-cleavable 8xHis tags.

**Serum and recombinant IgG ELISAs**

Serum ELISAs were done using Corning clear flat-bottom 96-well high binding microplates. Plates were coated with protein at a concentration of 2.5 μg/mL (in PBS) at a working volume of 100 μL, then incubated overnight (>8 hours) at 4°C. Following incubation, coating solution was decanted, and all wells were blocked with 150 μL of 1% BSA in PBS with 1% Tween for 120 minutes at ambient temperature. After blocking, sera diluted 40-fold or IgGs at a specified concentration were prepared as primary antibody solution, then serially diluted 5-fold in tubes. For serum ELISAs, CR3022 IgG was also serially diluted (5-fold) from a 5 μg/mL starting concentration, to serve as a control curve. 40 μL of primary antibody solution was then added to each well, and plates were incubated at ambient temperature for 90 minutes. After incubation, plates were washed three times in PBS/Tween solution.

The secondary antibody solution consisted of Abcam HRP-conjugated rabbit anti-mouse IgG antibody (for mouse serum ELISAs) or Abcam HRP-conjugated goat anti-human IgG antibody (for human IgG ELISAs) diluted 1:20,000 in PBS. 150 μL of the resulting solution was added to each well before incubating for one hour at ambient temperature. Plates were again washed three times with PBS/Tween solution. Following manufacturer (ThermoFisher) protocol, 150 μL of 1xABTS development solution was added to each well for color development, which was arrested after 30 minutes with 100 μL of 0.1% sodium azide. Plates were read using a SpectraMaxD3 plate reader (Molecular Devices) for absorbance at 405 nm.

**Competition ELISAs**

Competition ELISAs were performed as previously described. Corning clear flat-bottom 96-well high binding microplates were coated with wild-type SARS-CoV-2 RBD at 2.5 μg/mL (in PBS) overnight (>8 hours) at 4°C. Coating solution was removed, and plates were blocked with PBS-Tween with 4% BSA for 120 minutes. Relevant IgGs were coated onto the plate at a concentration of 0.2 mg/mL using a working volume of 25 μL. An equivalent volume of biotinylated IgG was then added at a concentration of 0.2 μg/mL and incubated for 90 minutes at ambient temperature. Primary solution was decanted and plates were washed 3x with PBS/Tween buffer solution. 150 μL of HRP-conjugated streptavidin (ThermoFisher) was then incubated at a concentration of 1:5000 for secondary incubation of 1 hour. After this point, the protocol was identical to the serum ELISA method for steps beyond secondary incubation. A no-competition control was used for comparison to assess binding loss from competition, and percent loss was computed relative to this control; graphs of data including negative percent binding loss represent the loss as 0%.

**Competition biolayer interferometry**

Antibody competition with D2G2 (a representative of the group of antibodies that does not target a class 4 epitope) was assessed via biolayer interferometry (BLI). SARS-CoV-2 RBD at 8 μM was complexed with at least a 5-molar excess of Fab for 30 minutes. Binding of the complex to D2G2 Fab was measured using a BLItz (ForteBio). Ni-NTA sensors (Sartorius) were used with D2G2 immobilized and complexes as the analytes. Absorbance was recorded and used to qualitatively assess whether D2G2 was able to bind to the complex. All reagents were diluted in 1X Kinetics Buffer (ForteBio).

Antibody competition with Ab16 (a class 4 antibody) was also assessed via BLI. SARS-CoV-2 RBD at 8 μM was complexed with at least a 5-molar excess Ab16 Fab for 30 minutes, and then binding of the complex to the B3E3, B8E8, and D2G2 Fabs was measured using a BLItz (ForteBio) and compared to binding of SARS-CoV-2 RBD at 8 μM without Ab16 to qualitatively determine whether each Fab was able to bind the SARS-CoV-2 RBD:Ab16 complex. Fab2G sensors were used with B3E3, B8E8, or D2G2 immobilized and SARS-CoV-2 RBD:Ab16 complexes or SARS-CoV-2 RBD as the analytes. All reagents were diluted in 1X Kinetics Buffer (ForteBio).

**Immunizations**

Female C57BL/6 mice (Charles River Laboratories) aged 6–10 weeks were used for all immunizations. Mice were immunized by the intraperitoneal route, introducing 20 μL of protein adjuvanted with 50% w/v Sigma adjuvant per 100 μL of inoculum. Priming occurred on day –21, boosts occurred at days 0 and 21, and serum was collected on day 35 from all cohorts. Two separate replicates of the immunization experiments were performed, the first with N = 5 mice per cohort and the second with N = 10 mice per cohort. All immunizations were approved by institutional IACUC (MGH protocol 2014N000252).

**Pseudovirus neutralization assay**

Monoclonal IgGs and mouse sera were assayed against wild-type SARS-CoV-2, Omicron variant SARS-CoV-2, Delta variant SARS-CoV-2, SARS-CoV-1, WIV1, RaTG13, and SHC014 pseudotyped lentiviral particles expressing spike proteins described previously. Lentiviral particles were generated via transient transfection of 293T cells, with viral supernatant titers assessed via flow cytometry of 293T-ACE2 cells and the HIV-1 p24CA antigen capture assay (Leidos Biomedical Research, Inc.). All assays were performed in a 384-well format (plates from Greiner) using a Tecan Fluent Automated Workstation.

Mouse serum samples started at an initial 1:3 dilution followed by six subsequent serial 3-fold dilutions. Monoclonal IgGs started at an initial specified concentration and then were subsequently diluted 3-fold as well. Each plate well contained 20 μL of sera and 20 μL of pseudovirus (125 infectious units); this mixture was incubated for 1 hour at room temperature. 10,000 293T-ACE2 cells in 20 μL of media containing 15 μg/mL polybrene was then added to each well and incubated at 37°C for an additional 60–72 hours.
Assay buffers described previously \(^57\) were used to perform cell lysis, and luciferase expression was then measured with a SpectroMax L luminometer (Molecular Devices). Background luminescence from cells-only sample wells was calculated and subtracted from each sample well. Neutralization percentage at a given serum or monoclonal IgG concentration was then calculated by dividing by the luminescence of wells containing only virus and cells. GraphPad Prism (version 9) was used to fit nonlinear regressions to the data and used to calculate IC50 values for all serum samples with neutralization values greater than or equal to 80% at maximum concentration. NT50 values were then calculated by taking the reciprocal of the IC50 values.

**Live virus neutralization assay**

The live virus neutralization assays were carried out in the Biosafety Level 3 laboratory of the Ragon Institute of MGH, MIT, and Harvard.

A549-Ace2 cells (BEI Resources, Cat # NR-53821) were cultured in D10+ media (DMEM (Corning) supplemented with HEPES (Corning), 1X Penicillin 100 IU/mL/Streptomycin 100 μg/mL (Corning), 1X Glutamine (Glutamax, ThermoFisher Scientific), and 10% Fetal Bovine serum (FBS; Sigma). The cells were detached using Trypsin-EDTA (Fisher Scientific) and seeded at 40,000 cells per well in 96-well plates 16–20 hours before infection. 4 hours before the infection, the cell culture supernatant was removed and 75 μL of D2+ media was added (2% FBS instead of 10%).

The US-WA1/2020 ancestral variant of SARS-CoV-2 was obtained from BEI Resources (Cat # NR-52281). The Omicron BA.1 variant isolate was obtained from the MassCPR variant repository. In brief, the variant had previously been isolated in the Ragon Institute BSL3 by rescue on Vero-E6 cells (ATCC) from primary clinical specimens, and it was then contributed to the MassCPR variant repository. The sequence of all viral stocks was confirmed by whole genome sequencing to ensure that no additional mutation arose during virus expansion. In-house prepared viral stocks (US-WA1/2020 Passage 3 and omicron BA.1 Passage 1) were diluted in D+ media to 32,000 pfu/mL to achieve a multiplicity of infection of 0.01 when added to the cells in the 96-well plates.

The antibody stocks (1 mg/mL) were diluted in D+ media (DMEM supplemented with HEPES, 1X Penicillin 100 IU/mL/Streptomycin 100 mg/mL and 1X Glutamine) to 2x desired final concentration by 3-fold serial dilutions. Equal volumes of viral stock and antibody dilution solutions were mixed and incubated for 1 hour at 37°C and 5% CO₂. After the incubation, for each condition, 25 μL of virus-antibody solutions (or no antibody control) were added to triplicate wells in 96-well plates. The plates were then spininfected at 2,000 x g for 30 minutes at 37°C followed by a 48-hour incubation at 37°C and 5% CO₂.

After 48 hours, the cell culture supernatant was discarded, the cells were washed with PBS (Corning) then harvested using TrypLE (Life Technologies) and D10+ media before being transferred to a V-bottom 96-well plate for flow cytometry staining. Cells were washed in PBS, then stained with live/dead fixable blue stain (Thermo Fisher) for 30 minutes at 4°C. Cells were then washed in flow cytometry buffer (2% FBS in PBS) and fixed using 4% paraformaldehyde (Santa Cruz) for 30 minutes at 4°C. The fixed cells were removed from the BSL3 laboratory and prepared for intracellular staining using Perm/Wash buffer (BD Biosciences). The permeabilized cells were stained with mouse anti-SARS-CoV-2 Nucleocapsid antibody (Biolegend) for 30 minutes at 4°C, then washed and stained with secondary antibody labelled with PE-Cy7 (Biolegend) for 30 minutes at 4°C. Finally, the cells were washed and re-suspended in flow cytometry buffer. Flow cytometry was performed on a BD Symphony (BD Biosciences).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

All FCS files generated from flow cytometry were analyzed using FlowJo software (version 10). GraphPad Prism (version 9) was used to fit asymmetric 4PL curves to the neutralization data which were used to calculate IC50 and NT50 values. GraphPad Prism was also used to perform other statistical analyses: the Kruskal-Wallis non-parametric ANOVA with post hoc analysis using Dunn’s test for multiple comparisons was used to compare multiple populations, and the Mann-Whitney U test was used to compare two populations without consideration of paired samples. A p value < 0.05 was considered to be statistically significant.