The landscape of antibody binding to SARS-CoV-2
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Abstract
The search for potential antibody-based diagnostics, vaccines, and therapeutics for pandemic severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has focused almost exclusively on the spike (S) and nucleocapsid (N) proteins1–8. Coronavirus membrane (M), orf3a, and orf8 proteins are also humoral immunogens in other coronaviruses (CoVs)8–11 but remain largely uninvestigated for SARS-CoV-2. Here we show that SARS-CoV-2 infection induces robust antibody responses to epitopes throughout the SARS-CoV-2 proteome, particularly in M, in which one epitope achieved near-perfect diagnostic accuracy. We map 79 B cell epitopes throughout the SARS-CoV-2 proteome and demonstrate that anti-SARS-CoV-2 antibodies appear to bind homologous peptide sequences in the 6 known human CoVs. Our results demonstrate previously unknown, highly reactive B cell epitopes throughout the full proteome of SARS-CoV-2 and other CoV proteins, especially M, which should be considered in diagnostic, vaccine, and therapeutic development.

Introduction
Antibodies mediate protection from coronaviruses (CoVs) including SARS-CoV-21–8, severe acute respiratory syndrome coronavirus (SARS-CoV)8,12–15 and Middle Eastern respiratory syndrome coronavirus (MERS-CoV)8,16–19. All CoVs encode 4 main structural proteins, spike (S), envelope (E), membrane (M), and nucleocapsid (N), as well as multiple non-structural proteins and accessory proteins20. In SARS-CoV-2, anti-S and anti-N antibodies have received the most attention to date1–8, including in serology-based diagnostic tests1–5 and vaccine candidates6–8. However, anti-S antibodies have been linked to antibody dependent enhancement for SARS-CoV-2 and other CoVs7,21–26, and prior reports observed that not all individuals infected with SARS-CoV-2 produce detectable antibodies against S or N1–5, indicating a need for
expanded antibody-based options. Much less is known about antibody responses to other SARS-
CoV-2 proteins, though data from other CoVs suggest they may be important. Antibodies against
SARS-CoV M can be more potent than antibodies against SARS-CoV S9–11, and some
experimental SARS-CoV and MERS-CoV vaccines elicit responses to M, E, and orf88.
Additionally, previous work has demonstrated humoral cross-reactivity between CoV57,14,27–30
and suggested it could be protective24,30, although full-proteome cross-reactivity has not been
investigated. We designed a peptide microarray tiling the proteome of SARS-CoV-2 and 8 other
human and animal CoVs in order to assess antibody epitope specificity and potential cross-
reactivity with other CoVs, and we used this microarray to profile IgG antibody responses in 40
COVID-19 convalescent patients and 20 SARS-CoV-2-naïve controls.

**CoV reactivity in uninfected controls**

Greater than 90% of adult humans are seropositive for the “common cold” CoVs (CCCoVs:
HCoV-HKU1, HCoV-OC43, HCoV-NL63, and HCoV-229E)31,32, but it is unknown how these
pre-existing antibodies might affect reactivity to SARS-CoV-2 or other CoVs. We measured IgG
reactivity in sera from 20 SARS-CoV-2-naïve control subjects to CoV linear peptides,
considering reactivity that was ≥3 standard deviations above the mean for the log₂-quantile
normalized array data to be indicative of antibody binding. All sera exhibited binding in known
epitopes of at least 1 of the control non-CoV strains (poliovirus vaccine and rhinovirus; **Fig. 1**,
Extended data 1, Extended data 2) and were collected in Wisconsin, USA, where exposure to
SARS-CoV or MERS-CoV was extremely unlikely. We found that at least one epitope in
structural or accessory proteins had binding in 100% of controls for HCoV-HKU1, 85% of
controls for HCoV-OC43, 65% for HCoV-NL63, and 55% for HCoV-229E (**Fig. 2**, Extended
data 2). Apparent cross-reactive binding was observed in 45% of controls for MERS-CoV, 50%
for SARS-CoV, and 50% for SARS-CoV-2.

**SARS-CoV-2 proteome humoral profiling**

We aimed to map the full extent of binding of antibodies induced by SARS-CoV-2 infection and
to rank the identified epitopes in terms of likelihood of importance and immunodominance. We
deﬁned epitope recognition as antibody binding to contiguous peptides in which the average
log₂-normalized intensity for patients was at least 2-fold greater than for controls with t-test
statistics yielding adjusted p-values <0.1. We chose these criteria, rather than the 3 standard
development cut-off, in order to ensure that binding detected would be greater than background
binding seen in controls (2-fold greater) and to remove regions of binding that were not at least
weakly signiﬁcantly different from controls (adjusted p<0.1). These criteria identiﬁed 79 B cell
epitopes (**Fig. 3**, Extended data 3) in S, M, N, orf1ab, orf3a, orf6, and orf8. We ranked these
epitopes by minimum adjusted p-value for any 16-mer in the epitope in order to determine the
greatest likelihood of difference from controls as a proxy for immunodominance. The highest-
ranking epitope occurred in the N-terminus of M (1-M-24). Patient sera showed high-magnitude
reactivity (up to 6.7 fluorescence intensity units) in other epitopes in S, M, N, and orf3a, with
lower-magnitude reactivity (<3.3 fluorescence intensity units) epitopes in other proteins. The
epitopes with the greatest reactivity in S occurred in the fusion peptide (residues 788-806), with
less reactivity in the receptor binding domain (residues 319-541)6 (**Fig. 3**). Four detected
epitopes (553-S-26, 624-S-23, 807-S-26, and 1140-S-25) have previously been shown to be
potently neutralizing33–35, and all 4 of these ranked within the top 10 epitopes. Forty-two of our
detected epitopes (including 1-M-24, 553-S-26, 624-S-23, 807-S-26, and 1140-S-25; Extended
The highest specificity (100%) and sensitivity (98%), determined by linear discriminant analysis leave-one-out cross-validation, for any individual peptide was observed for a 16-mer within the 1-M-24 epitope: ITVEELKLLEQWNLV (Extended data 4). Fifteen additional individual peptides in M, S, and N had 100% measured specificity and at least 80% sensitivity.

Combinations of 1-M-24 with 1 of 5 other epitopes (384-N-33, 807-S-26, 6057-orf1ab-17, 227-N-17, 4451-orf1b-16) yielded an area under the curve receiver operating characteristic of 1 (Extended data 5) based on linear discriminant analysis leave-one-out cross-validation.

**Human, animal CoV cross-reactivity**

We defined cross-reactivity as binding by antibodies in COVID-19 convalescent sera to non-SARS-CoV-2 peptides at an average log2-normalized intensity at least 2-fold greater than in controls with t-test statistics yielding adjusted p-values <0.1. Antibodies in COVID-19-convalescent sera appeared to be cross-reactive with homologous epitopes in S, M, N, orf1ab, orf3, orf6, and orf8 in other CoVs (Fig. 4, Extended data 6, Extended data 7, Extended data 8). The greatest number of cross-reactive epitopes (70) were in the RaTG13 bat betacoronavirus (β-CoV), the closest known relative of SARS-CoV-2 (96% nucleotide identity)\(^{39,40}\), then the pangolin CoV (51 epitopes, 85% nucleotide identity with SARS-CoV-2)\(^{41}\), then SARS-CoV (40 epitopes, 78% identity\(^{38}\)). One region, corresponding to SARS-CoV-2 epitope 807-S-26, was cross-reactive across all CoVs, and one, corresponding to SARS-CoV-2 epitope 1140-S-25, was cross-reactive across all β-CoVs (Fig. 4). Epitope 807-S-26 includes the CoV S fusion peptide, and 1140-S-25 is immediately adjacent to the heptad repeat region 2, both of which are involved in membrane fusion\(^{42}\).

**Discussion**

M proteins are the most abundant proteins in CoV virions\(^{20}\). The N-terminus of M is known in other CoVs to be a small, glycosylated ectodomain that protrudes outside the virion and interacts with S, N, and E\(^{20}\), while the rest of M resides within the viral particle. Full-length SARS-CoV M has been shown to induce protective antibodies\(^{11,43}\), and patterns of antibodies binding to SARS-CoV M are similar to those we found in SARS-CoV-2\(^{26}\). SARS-CoV anti-M antibodies can synergize with anti-S and anti-N antibodies\(^{11,43}\), and M has been used in protective SARS-CoV and MERS-CoV vaccines\(^{8}\). However, the mechanism of protection of anti-M antibodies remains unknown, and this protein remains largely understudied and underutilized as an antigen.

Other groups have not previously identified the high magnitude binding we observed in M, though that may be due to using earlier sample timepoints or different techniques or algorithms\(^{44,45}\). Our results, in concert with prior knowledge of anti-SARS-CoV antibodies, strongly suggest that M, particularly the 1-M-24 epitope, as well as other novel epitopes that we identified should be investigated further as potential targets in SARS-CoV-2 diagnostics, vaccines, and therapeutics.

We also found that antibodies produced in response to SARS-CoV-2 infection appeared to cross-react with homologous epitopes throughout the proteomes of other human and non-human CoVs. Hundreds of CoVs have been discovered in bats and other species\(^{24,39-41,46,47}\), making future spillovers inevitable. The broad cross-reactivity we observed in some homologous peptide
sequences may help guide the development of pan-CoV vaccines\textsuperscript{18}, especially given that antibodies binding to 807-S-26 and 1140-S-25, epitope motifs cross-reactive across all CoVs and all $\beta$-CoVs, respectively, are known to be potently neutralizing\textsuperscript{33,34}. We cannot determine whether the increased IgG binding to CCCoVs in COVID-19 convalescent sera is due to newly developed cross-reactive antibodies or the stimulation of a memory response against the original CCCoV antigens. However, cross-reactivity of anti-SARS-CoV-2 antibodies with SARS-CoV or MERS-CoV is likely real, since our population was very unlikely to have been exposed to those viruses. A more stringent assessment of cross-reactivity as well as functional investigations into these cross-reactive antibodies will be vital in determining their capacity for cross-protection. Further, our methods efficiently detect antibody binding to linear epitopes\textsuperscript{48}, but their sensitivity for detecting parts of conformational epitopes is unknown, and additional analyses will be required to determine whether epitopes identified induce neutralizing or otherwise protective antibodies.

Many questions remain regarding the biology and immunology of SARS-CoV-2. Our extensive profiling of epitope-level resolution antibody reactivity in COVID-19 convalescent subjects provides new epitopes that could serve as important targets in the development of improved diagnostics, vaccines, and therapeutics against SARS-CoV-2 and dangerous human CoVs that may emerge in the future.
Figures

**Figure 1.** Patients and control subjects show reactivity to a poliovirus control. Sera from 20 control subjects collected before 2019 were assayed for IgG binding to the full proteome of human poliovirus 1 on a peptide microarray. Binding was measured as reactivity that was >3 standard deviations above the mean for the log$_2$-quantile normalized array data. Patients and controls alike showed reactivity to a well-documented linear poliovirus epitope (start position 613 [IEDB.org]; orange shading in line plot).

**Figure 2.** Control sera show reactivity frequently to CCCoVs and rarely to SARS-CoV, MERS-CoV, and SARS-CoV-2. Sera from 20 control subjects collected before 2019 were assayed for IgG binding to the full proteomes of 9 CoVs on a peptide microarray. Viral proteins are shown aligned to the SARS-CoV-2 proteome with each virus having an individual panel; SARS-CoV-2 amino acid (aa) position is represented on the x-axis. Binding was measured as
reactivity that was >3 standard deviations above the mean for the log₂-quantile normalized array data.

**Figure 3. Anti-SARS-CoV-2 antibodies bind throughout the viral proteome.** Sera from 40 COVID-19 convalescent subjects were assayed for IgG binding to the full SARS-CoV-2 proteome on a peptide microarray. B cell epitopes were defined as peptides in which patients’ average log₂-normalized intensity (black lines in line plots) is 2-fold greater than controls’ (gray lines in line plots) and t-test statistics yield adjusted p-values < 0.1; epitopes are identified by orange shading in the line plots.
Figure 4. Anti-SARS-CoV-2 antibodies cross-react with other CoVs. Sera from 40 COVID-19 convalescent patients were assayed for IgG binding to 9 CoVs on a peptide microarray; averages for all 40 are shown. Viral proteins are aligned to the SARS-CoV-2 proteome; SARS-CoV-2 amino acid (aa) position is represented on the x-axis. Regions cross-reactive across all β-CoVs (*) or cross-reactive for SARS-CoV or MERS-CoV (#) are indicated. Gray shading indicates gaps due to alignment or lacking homologous proteins. Cross-reactive binding is defined as peptides in which patients’ average log2-normalized intensity is 2-fold greater than controls’ and t-test statistics yield adjusted p-values < 0.1.

Extended data

Extended data 1. All 40 COVID-19 convalescent patients and all 20 naïve controls reacted to known epitopes in at least one control virus (rhinovirus and poliovirus strains).

Extended data 2. Percentages of the 40 COVID-19 convalescent patients and 20 naïve controls reacted to known epitopes in at least one control virus (rhinovirus and poliovirus strains).

Extended data 3. B cell epitopes in the SARS-CoV-2 proteome identified by antibody binding in 40 COVID-19 convalescent patients compared to 20 naïve controls.
Extended data 4. Specificity and sensitivity for past SARS-CoV-2 infection in 40 COVID-19 convalescent patients compared to 20 naïve controls of individual 16-mer peptides comprising epitopes throughout the full SARS-CoV-2 proteome.

Extended data 5. Epitopes paired with the 1-M-24 epitope obtained an area under the receiver operating characteristic curve (AUC-ROC) of 1.0 for SARS-CoV-2 infection in 40 COVID-19 convalescent patients and 20 naïve controls using leave-one-out cross validation with linear discriminant analysis.

Extended data 6. Alignment of epitopes in human and animal CoVs for which antibodies in sera from 40 COVID-19 convalescent patients showed apparent cross-reactive binding. Alignments were performed in Geneious Prime 2020.1.2 (Auckland, New Zealand).

Extended data 7. Cross-reactive binding of antibodies against other CoVs in 40 COVID-19 convalescent patients compared to 20 naïve controls.

Extended data 8. Cross-reactive binding of antibodies in 40 COVID-19 convalescent patients compared to 20 naïve controls in protein motifs in other CoVs aligned to SARS-CoV-2.

Extended data 9. B cell epitopes in the SARS-CoV-2 proteome identified by antibody binding in 40 COVID-19 convalescent patients compared to 20 naïve controls were differentiated using a cut-off of at least a 2-fold greater magnitude reactivity in patients vs controls and t-test statistics yielding adjusted p-values <0.1. Degrees of freedom, fold change, and standard deviation for each peptide are given.

Methods

Peptide microarray design and synthesis
Viral protein sequences were selected and submitted to Nimble Therapeutics (Madison, WI, USA) for development into a peptide microarray. Sequences represented include proteomes of all seven coronaviruses known to infect humans, proteomes of closely related coronaviruses found in bats and pangolins, and spike proteins from other coronaviruses (accession numbers and replicates per peptide shown in Supplementary Table 1). A number of proteins were included as controls, including poliovirus, seven strains of human rhinovirus, and human cytomegalovirus 65kDa phosphoprotein. We chose these controls given that we expect most human adults will have antibody reactivity to at least one of these proteins and proteomes. Accession numbers used to represent each viral protein are listed in the supplemental material (accession numbers and replicates per peptide shown in Supplementary Table 1). All proteins were tiled as 16 amino acid peptides overlapping by 15 amino acids. All unique peptides were tiled in a lawn of thousands of copies, with each unique peptide represented in at least 3 and up to 5 replicates (Supplementary Table 1). The peptide sequences were synthesized in situ with a Nimble Therapeutics Maskless Array Synthesizer (MAS) by light-directed solid-phase peptide synthesis using an amino-functionalized support (Geiner Bio-One) coupled with a 6-aminohexanoic acid linker and amino acid derivatives carrying a photosensitive 2-(2-nitrophenyl) propylxycarbonyl (NPPOC) protection group (Orgentis Chemicals). Unique peptides were synthesized in random
positions on the array to minimize impact of positional bias. Each array consists of twelve
subarrays, where each subarray can process one sample and each subarray contains up to
389,000 unique peptide sequences.

**Supplementary Table 1. Proteins represented on the peptide microarray**

<table>
<thead>
<tr>
<th>Protein(s)</th>
<th>GenBank accession number(s)</th>
<th>Number of replicates of each unique peptide</th>
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<tbody>
<tr>
<td><strong>Coronavirus proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severe acute respiratory syndrome coronavirus 2 proteome</td>
<td>NC_045512.2</td>
<td>4-5</td>
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<tr>
<td>Severe acute respiratory syndrome coronavirus proteome</td>
<td>NC_004718.3</td>
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<td>Middle Eastern respiratory syndrome coronavirus proteome</td>
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<td>NC_006577.2</td>
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<td>Human coronavirus OC43 proteome</td>
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<td>Human coronavirus 229E proteome</td>
<td>NC_002645.1</td>
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</tr>
<tr>
<td>Human coronavirus NL63 proteome</td>
<td>NC_005831.2</td>
<td>3</td>
</tr>
<tr>
<td>Bat coronavirus (RaTG13 isolate) proteome</td>
<td>MN996532.1</td>
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</tr>
<tr>
<td>Pangolin coronavirus proteome</td>
<td>MT072864.1</td>
<td>3</td>
</tr>
<tr>
<td><strong>Control proteins</strong></td>
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<td></td>
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<tr>
<td>Human rhinovirus A1 polyprotein</td>
<td>NC_038311.1</td>
<td>3</td>
</tr>
<tr>
<td>Human rhinovirus A7 polyprotein</td>
<td>DQ473503.1</td>
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</tr>
<tr>
<td>Human rhinovirus A16 polyprotein</td>
<td>L24917.1</td>
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</tr>
<tr>
<td>Human rhinovirus A36 polyprotein</td>
<td>JX074050.1</td>
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Human subjects

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of the University of Wisconsin-Madison. Clinical data and sera from subjects infected with SARS-CoV-2 were obtained from the University of Wisconsin (UW) COVID-19 Convalescent Biobank and from control subjects (sera collected prior to 2019) from the UW Rheumatology Biobank. All subjects were 18 years of age or older at the time of recruitment and provided informed consent. COVID-19 convalescent subjects had a positive SARS-COV-2 PCR test at UW Health with sera collected 5-6 weeks after self-reported COVID-19 symptom resolution except blood was collected for one subject after 9 weeks. Age, sex, medications, and medical problems were abstracted from UW Health’s electronic medical record (EMR). Race and ethnicity were self-reported. Hospitalization and intubation for COVID-19 and smoking status at the time of blood collection (controls) or COVID-19 were obtained by EMR abstraction and self-report and were in complete agreement. Two thirds of COVID-19 convalescent subjects and all controls had a primary care appointment at UW Health within 2 years of the blood draw as an indicator of the completeness of the medical information. Subjects were considered to have an immunocompromising condition if they met any of the following criteria: immunosuppressing medications, systemic inflammatory or autoimmune disease, cancer not in remission, uncontrolled diabetes (secondary manifestations or hemoglobin A1c ≥7.0%), or congenital or acquired immunodeficiency. Control and COVID-19 subjects were similar in regard to demographics and health (Supplementary Table 2). No subjects were current smokers.

Supplementary Table 2. Characteristics of COVID-19 Convalescent and Control Subjects

<table>
<thead>
<tr>
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<th>COVID-19 (n=40)</th>
<th>Control (n=20)</th>
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<td>Age, median (IQR) years</td>
<td>51 (19-83)</td>
<td>55 (22-83)</td>
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<tr>
<td>Sex, number female (%)</td>
<td>17 (42.5)</td>
<td>11 (55.0)</td>
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<tr>
<td>Race, number (%)</td>
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<td>Black</td>
<td>Asian</td>
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<tr>
<td></td>
<td>34 (85.0)</td>
<td>3 (7.5)</td>
<td>3 (7.5)</td>
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<tr>
<td></td>
<td>18 (90.0)</td>
<td>1 (5.0)</td>
<td>1 (5.0)</td>
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<th>Ethnicity, number Hispanic (%)</th>
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<th>1 (5.0)</th>
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<td>Charlson comorbidity score, median (IQR)</td>
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<td>2 (0.5, 4)</td>
</tr>
<tr>
<td>Immunocompromised, number (%)</td>
<td>9 (22.5)</td>
<td>7 (35.0)</td>
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<table>
<thead>
<tr>
<th>COVID-19 disease severity, number (%)</th>
<th>Hospitalized and intubated</th>
<th>Hospitalized without intubation</th>
<th>Not hospitalized</th>
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<tbody>
<tr>
<td></td>
<td>8 (20.0)</td>
<td>7 (17.5)</td>
<td>25 (62.5)</td>
</tr>
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</table>

**Peptide array sample binding**

Samples were diluted 1:100 in binding buffer (0.01M Tris-Cl, pH 7.4, 1% alkali-soluble casein, 0.05% Tween-20) and bound to arrays overnight at 4°C. After sample binding, the arrays were washed 3× in wash buffer (1× TBS, 0.05% Tween-20), 10 min per wash. Primary sample binding was detected via Alexa Fluor® 647-conjugated goat anti-human IgG secondary antibody (Jackson ImmunoResearch). The secondary antibody was diluted 1:10,000 (final concentration 0.1 ng/µl) in secondary binding buffer (1x TBS, 1% alkali-soluble casein, 0.05% Tween-20). Arrays were incubated with secondary antibody for 3 h at room temperature, then washed 3× in wash buffer (10 min per wash), washed for 30 sec in reagent-grade water, and then dried by spinning in a microcentrifuge equipped with an array holder. Fluorescent signal of the secondary antibody was detected by scanning at 635 nm at 2 µm resolution using an Innopsys 910AL.
microarray scanner. Scanned array images were analyzed with proprietary Nimble Therapeutics software to extract fluorescence intensity values for each peptide.

**Peptide microarray findings validation**

We included sequences on the array of viruses which we expected all adult humans to be likely to have been exposed to as positive controls: one poliovirus strain (measuring vaccine exposure), and seven rhinovirus strains. Any subject whose sera did not react to at least one positive control would be considered a failed run and removed from the analysis. All subjects in this analysis reacted to epitopes in at least one control strain (Fig. 1, Extended data 1, Extended data 2).

**Peptide microarray data analysis and data availability**

The raw fluorescence signal intensity values were log$_2$ transformed. Clusters of fluorescence intensity of statistically unlikely magnitude, indicating array defects, were identified and removed. Local and large area spatial corrections were applied, and the median transformed intensity of the peptide replicates was determined. The resulting median data was cross-normalized using quantile normalization. All peptide microarray datasets and code used in these analyses can be downloaded from https://github.com/Ong-Research/Ong_UW_Adult_Covid-19.git.

**Statistical analysis**

Statistical analyses were performed in R (v 4.0.2) using in-house scripts. For each peptide, a p-value from a two-sided $t$-test with unequal variance between sets of patient and control responses were calculated and adjusted using the Benjamini-Hochberg (BH) algorithm. To determine whether the peptide was in an epitope (in SARS-CoV-2 proteins) or cross-reactive for anti-SARS-CoV-2 antibodies (in non-SARS-CoV-2 proteins), we used an adjusted $p$-value cutoff of $<0.1$ (based on multiple hypothesis testing correction for all 119,487 unique sequences on the array) and a fold-change of greater than or equal to 2 and grouped consecutive peptides as a represented epitope. Linear discriminant analysis leave-one-out cross validation was used to determine specificity and sensitivity on each peptide and from each epitope using the average signal of the component peptides.

To identify cross reactive epitopes, we used each SARS-CoV-2 epitope sequence as a query, searched the database of proteins from the sequences in the peptide array using blastp (-word-size 2, num-targets 4000) to find homologous sequences in the bat, pangolin, and other human CoV strains, then determined whether the average log$_2$-normalized intensity for these sequences in patients was at least 2-fold greater than in controls with $t$-test statistics yielding adjusted $p$-values $<0.1$. Each blast hit was then mapped back to the corresponding probe ranges.

The clinical and demographic characteristics of convalescent subjects were compared to those of the controls using $\chi^2$ tests for categorical variables and Wilcoxon rank-sum tests for non-normally distributed continuous measures.

Heatmaps were created using the gridtext$^{50}$ and complexheatmap$^{51}$ packages in R. Alignments for heatmaps were created using MUSCLE$^{52}$. 

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(Additional text not shown)
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Author contributions


References:


