New-onset IgG autoantibodies in hospitalized patients with COVID-19

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COVID-19 is associated with a wide range of clinical manifestations, including autoimmune features and autoantibody production. Here we develop three protein arrays to measure IgG autoantibodies associated with connective tissue diseases, anti-cytokine antibodies, and antiviral antibody responses in serum from 147 hospitalized COVID-19 patients. Autoantibodies are identified in approximately 50% of patients but in less than 15% of healthy controls. When present, autoantibodies largely target autoantigens associated with rare disorders such as myositis, systemic sclerosis and overlap syndromes. A subset of autoantibodies targeting traditional autoantigens or cytokines develop de novo following SARS-CoV-2 infection. Autoantibodies track with longitudinal development of IgG antibodies recognizing SARS-CoV-2 structural proteins and a subset of non-structural proteins, but not proteins from influenza, seasonal coronaviruses or other pathogenic viruses. We conclude that SARS-CoV-2 causes development of new-onset IgG autoantibodies in a significant proportion of hospitalized COVID-19 patients and are positively correlated with immune responses to SARS-CoV-2 proteins.

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Coronavirus Disease 2019 (COVID-19), caused by Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) infection, is associated with many different clinical features that are commonly found in autoimmune diseases, including arthralgias, myalgias, fatigue, sicca, and rashes. Less common manifestations of autoimmunity have also been observed in COVID-19 patients, including thrombosis, myositis, myocarditis, arthritis, encephalitis, and vasculitis. These clinical observations, and the increasing proportion of “recovered” patients with persistent post-COVID-19 symptoms (so-called “long haulers”, or “long COVID”) suggest that inflammation in response to SARS-CoV-2 infection promotes tissue damage in the acute phase and potentially some of the long-term sequelae.

Autoantibodies, a hallmark of most but not all autoimmune disorders, have been described in COVID-19 patients. In the earliest report, approximately half of hospitalized patients at an academic hospital in Greece had high levels of serum autoantibodies, often associated with clinical findings such as rashes, thrombosis, and vasculitis. Serum anti-nuclear antibodies (ANA) were detectable in approximately one-third of patients. Woodward et al. reported that 23 of 48 (44%) critically-ill COVID-19 patients have positive ANA tests. Zuo described an even higher prevalence of thrombogenic autoantibodies, reporting that up to 52% of hospitalized COVID-19 patients have anti- phospholipid antibodies. They further showed that autoantibodies have the capacity to cause clots in mouse models.

We characterized 51 cross-sectional COVID-19 serum or plasma samples from patients who provided samples within seven days of hospitalization (Fig. 1). As expected, prototype reference samples from patients with classifiable autoimmune diseases were strongly positive for autoantibodies, recognizing 25 of the 53 arrayed proteins (Fig. 1, bottom left panel, and Supplementary Fig. 3). Serum from only four HC recognized a single autoantigen each (signal recognition particle 54, SRP 54; Smith/ribonuclear protein, Sm/RNP; guanosine nucleotide-binding protein alpha subunit, GNAL, a candidate autoantigen in autoimmune hypophysitis; and Ku 70/80, respectively, Fig. 1, middle panel). HC06 and HC30 each had high MFI anti-thyroperoxidase (TPO) and Proteinase 3 (PR3) antibodies, as these antibodies are associated with autoimmune vasculitis. Only one individual tested positive for PR3 antibodies (Supplementary Fig. 2). The levels of positivity in these clinical-grade assays are in line with those of the other antibodies and/or antiphospholipid antibodies.

Results

Anti-nuclear antibodies (ANA) are produced by one in four hospitalized COVID-19 patients. To determine if hospitalized patients with COVID-19 produce autoantibodies against prototypical autoantigens associated with systemic autoimmunity, we measured ANA using an indirect immunofluorescence assay in one of our cohorts (University of Pennsylvania). We found that ten out of 73 patients (14%) were positive at a dilution of 1:160, six were positive at 1:320, three were positive at 1:640, and one was positive with greater than 1:1280 (Supplementary Fig. 1a). ANA positive samples were further diluted at 1:320, 1:640, and 1:1280 to determine titers. A variety of ANA patterns were observed including diffuse, speckled, and nucleolar (Supplementary Fig. 1b, c and Supplementary Table 1). Three patients exhibited cytoplasmic staining but were negative for nuclear staining at 1:160. Given the finding of positive and weakly positive ANAs, we measured dsDNA antibodies. Only one individual out of 73 tested was positive for dsDNA antibodies at a dilution of 1:270, and this individual also was ANA positive with a speckled pattern (Supplementary Fig. 2). Since several patients who were severely or critically ill had thromboembolic and vascular events, we also analyzed the same 73 patients for Myeloperoxidase (MPO) and Proteinase 3 (PR3) antibodies, as these antibodies are associated with autoimmune vasculitis. Only one individual tested positive for PR3 antibodies (Supplementary Fig. 2). The levels of positivity in these clinical-grade assays are in line with those of the other antibodies and/or antiphospholipid antibodies.

We assembled three different custom bead-based protein arrays. Protein microarrays identify autoantibody targets in hospitalized COVID-19 patients. To systematically and simultaneously measure a large number of different autoantibodies in serum or plasma derived from patients acutely infected with SARS-CoV-2, we constructed a 53-plex COVID-19 Autoantigen Array (Fig. 1, left half of the panel). The array comprised well-characterized autoantigens (Supplementary Table 2) across multiple rheumatologic diseases (Supplementary Fig. 3). Included were prominent autoantigens targeted in systemic sclerosis (scleroderma, SSc, left panel; myositis and overlap syndromes, second panel); systemic lupus erythematosus (SLE), Sjögren’s syndrome, and mixed connective tissue disease (MCTD, third panel); gastrointestinal and endocrine autoimmune disorders (fourth panel); chromatin-associated antigens (fifth panel); and miscellaneous antigens, including proteins targeted in vasculitis or in which autoantibodies are postulated to be directly pathogenic (sixth panel).

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with COVID-19 had autoantibodies recognizing at least one traditional CTD autoantigen (Fig. 1, top panel). Using a stringent 5 SD cut-off, serum antibodies from eleven COVID-19 patients identified a single antigen, thirteen recognized 2-3 antigens, and one subject (Subject UP40) recognized nine different autoantigens. Ribosomal P proteins (P0, P1, and P2) were most prominently targeted (10 of 50 patients, 20%), but were not found in any of the HC. Similar results were observed in 48 Kaiser subjects analyzed using an earlier-generation 26-plex autoantigen microarray, identifying overlapping RNA-containing autoantigen complexes including RPP14 Th/To, the Ro/La particle, the U1-small nuclear ribonucleoprotein particle (U1-snRNP), thyroid antigens, and chromatin proteins as targets in hospitalized COVID-19 patients, but in none of the HC (Supplementary Fig. 4).

Rare antigens are seen in patients with autoimmune myositis (MDA5, Mi-2, and tRNA synthetases such as PL-7 and Jo-1), and candidate autoantigens in autoimmune myocarditis (troponin

**Fig. 1** High prevalence of autoantibodies in hospitalized COVID-19 patients. **a** Heatmap depicting serum IgG antibodies discovered using a 53-plex bead-based protein array containing the indicated autoantigens (x-axis). Autoantigens are grouped based on disease (scleroderma, myositis, and overlap syndromes such as mixed connective tissue disease (MCTD), SLE/Sjögren’s, gastrointestinal and endocrine disorders), DNA-associated antigens, and antigens associated with tissue inflammation or stress responses. COVID-19 patients (top panel), HC (n = 31, middle panel), and 8 prototype autoimmune disorders (bottom panel) are shown. Colors indicate autoantibodies whose MFI measurements are >5 SD (red) or <5 SD (black) above the average MFI for HC. MFIs <3000 were excluded. **b** Heatmap using a 41-plex array of cytokines, chemokines, growth factors, and receptors. The same samples in Panel A were also analyzed for anti-cytokine antibodies (ACA). Cytokines are grouped on the x-axis by category (interferons, interleukins, and other cytokines/growth factors/receptors). Prototype samples from patients with immunodeficiency disorders include three patients with Autoimmune Polyendocrine Syndrome Type 1 (APS-1), one patient with Pulmonary Alveolar Proteinosis (PAP), and three patients with Atypical Mycobacterial Infections (AMI). Colors indicate autoantibodies whose MFI measurements are >5 SD (red) or <5 SD (black) above the average MFI for HC. MFIs <3000 were excluded. Source data are provided as a Source Data file.
and MYH6, Fig. 2a), were observed in individual patients, as were rare SSc autoantigens (Th/To (RPP25), fibrillarin, and the U11/U12 snRNP, Fig. 2b). A subset of autoantibodies (e.g., antibodies that bind the complement inhibitor C1q, thrombosis-associated antibodies that target beta 2 glycoprotein 1 (β2-GP1), and vasculitis-associated antigens such as bactericidal permeability inducing protein (BPI)) that have been implicated in pathogenic inflammation in target organs, were also found in individual patients (Fig. 2c)⁴–⁶,¹⁴–¹⁶. Relatively common autoantigens such as Scl-70, CENP A/B, and Sm/RNP were infrequent. Thyroid autoantibodies were also commonly observed (12/147 subjects across our entire study, 8.2%, using cutoffs of 3000 MFI and 5 SD above HC). Thyroid dysfunction, which is relatively common in the general population, has been reported in COVID-19 patients⁴,⁵. In all cases where samples from more than one time point were available, anti-TPO and anti-thyroglobulin (TG) were already present at high MFI levels in the baseline sample. Taken together, these findings reveal that hospitalized patients with COVID-19 produce an increased frequency of autoantibodies, but that there is a substantial inter-individual variation in which autoantigens are targeted.

**Secreted proteins are common autoantigens in hospitalized COVID-19 patients.** In a pair of elegant studies, Bastard⁵ and Wang¹⁷ independently identified anti-cytokine antibodies (ACA) in patients with severe COVID-19. Both groups showed that a subset of ACA prevents binding of soluble factors to their cognate

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**Fig. 2** Serum autoantibodies in COVID-19 patients recognize antigens targeted in rare connective tissue diseases, and antigens associated with pathogenicity. Boxplots of twelve antigens corresponding to Fig. 1. **a** Antigens associated with autoimmune myositis and myocarditis (MDA5, troponin 1, MYH6 (alpha-myosin), PL-7, Jo-1, and Mi-2). **b** RNA-containing antigens associated with systemic sclerosis (RPP25 Th/To, Fibrillarin, and U11/U12). **c** Antigens that may be pathogenic (C1q and β2-GP1) and associated with vasculitis (BPI). MFI data in **a**–**c** are represented as boxplots where the middle line is the median, the lower and upper hinges correspond to the first and third quartiles, the upper whisker extends from the hinge to the largest value, and the lower whisker extends from the hinge to the smallest value. Individual MFI values are displayed as dots. MFI is shown on the y-axis. Subjects are shown on the x-axis (n = 51 unpaired UPenn and Marburg COVID-19 patients and n = 31 HC). Source data are provided as a Source Data file.
cell surface receptors and have been postulated to play a pathogenic role by thwarting protective immune responses to COVID-19. We created a 41-plex array comprising secreted proteins and cell surface receptors, modeled on arrays we and others have used previously to characterize "secretome" antibodies in immunodeficiency disorders, SLE, and systemic sclerosis patients (Supplementary Table 3). We observed even more striking results with the secretome array, which revealed that serum antibodies in 41/51 (80%) of hospitalized COVID-19 patients recognized at least one secreted or cell surface autoantigen (Fig. 1, the upper right half of panel), while only 2/31 (6%) HC subjects recognized a single antigen (interferon-gamma, IFN-γ in one and CD74 in the other, Fig. 1, middle right half of the panel). Interestingly, the IFN-γ+ HC subject (HC27) also had serum antibodies specific for Sm (a subunit of the U1-snRNP, using 5 SD cutoff) and for both Ro60 and La (using a 3 SD cutoff), suggesting this "healthy" subject is in preclinical evolution towards developing SLE, a disease in which we have previously described multiple different ACA including anti-IFN-α and anti-B cell-activating factor (BAFF) 

Interferons, particularly the Type I interferon IFN-α2, were targeted in multiple COVID patients at frequencies (n = 23 across all interferons, 45%) higher than recently published findings from other groups 

Many interleukins were also prominently identified as autoantibody targets in this screen (e.g., interleukins 1, 6, 10, 15, 17A, 22, and 31), as were cytokines with well-characterized functions such as leukemia inhibitory factor (LIF), the chemotactic chemokine macrophage inflammatory protein-1 alpha (MIP-1α), and vascular endothelial growth factor-B (VEGF-B). Several striking reactivities were observed in individual COVID-19 patients, including IL-12p70 (Subject UP47); the SARS-CoV-2 receptor angiotsin-converting enzyme-2 (ACE-2, Subject UMR19); granulocyte-macrophage colony-stimulating factor which is the causative autoantibody target in PAP (GM-CSF, Subject UP25); oncostatin-M (OSM, Subject UP40); and soluble receptor activator of nuclear factor kappa B (sRANK-ligand, Subject UP19). Subject UP17 was revealed to be carrying a tumor necrosis factor-alpha (TNF-α) inhibitor at the time of SARS-CoV-2 infection, explaining the high MFI reactivity to TNF (Fig. 1). MFI for all antigens except IL-12p70 were very high (>10,000) in individual patients. Autoantibodies against all interleukins, cytokines, and ACE-2 identified in the initial screen were also observed using a 5 SD cutoff in a second COVID-19 cohort (n = 98 longitudinal samples from 48 different patients, see Fig. 3, Supplementary Figs. 5 and 6), with few exceptions (e.g., IL-1α, although IL-1β was targeted using a 3 SD cutoff; IL-31, which met a 3 SD cutoff; and GM-CSF).

A subset of autoantibodies is triggered by SARS-CoV-2 infection. To determine if autoantibodies targeting traditional autoantigens or cytokines were generated de novo (versus existed prior to infection), we analyzed 48 hospitalized COVID-19 patients (Stanford University, University of Pennsylvania, and Marburg University) in whom samples were available at two or more different time points. Twenty-four patients had an available sample from the day of hospitalization or (day 0). The interval between the collection of the second sample ranged from 2–58 days (mean interval = 15.8 days). Two subjects (UP70 and UP71) also had a third sample drawn 14–21 days post ICU admission. To reduce batch effects, all samples at all time points were analyzed on the 53-plex COVID-19 Autoantigen Array in the same instrument run (Supplementary Fig. 5 top panel) together with HC (Supplementary Fig. 5, middle panel, n = 16) and serum samples from prototype autoimmune diseases served as positive controls (Supplementary Fig. 5, bottom panel, n = 8). As with the unpaired samples described in Fig. 1, autoantibodies from patients with paired samples had high MFI in individual patients. Some patients have again identified whose serum recognized a large number of autoantigens (Supplementary Figs. 5 and 6). Twenty-five (52%) of hospitalized COVID-19 patients had autoantibodies against at least one autoantigen. Serum autoantibodies recognized two or more antigens (range 2–7 antigens) in seven patients (15%) (Fig. 3a, and Supplementary Fig. 6). The longitudinal analysis identified prominent increases in autoantibodies at the second available time point (Fig. 3b). In 9 individual patients (19%), autoantibody measurements were above the average for HC at the earliest available time point and MFI increased by at least 50%, exceeding the 5 SD and 3000 MFI cutoff at the later time point (Fig. 3b, e.g., MDA5, subject UP50; BPI, subject UP52; Supplementary Fig. 6). Some autoantibodies were at or below the average for HC at the first time point and increased over time (e.g., histones and histone H3, subject UP65; and β2GPI, subjects UP65 and UP52), suggesting these autoantibodies were directly triggered by SARS-CoV-2 infection. Others were already elevated at the first time point and did not have large increases in MFI over time (n = 22, 45%) (Fig. 3c and Supplementary Fig. 6). In a small number of cases, autoantibody MFI levels decreased below the SD and MFI cutoffs over time (n = 5, 10%), suggesting that their development might be transient (e.g., PL-7, subject UP70, Fig. 3b). Anti-TPO and anti-Scl-70 (Fig. 3c) remained elevated at high levels in all seropositive subjects throughout the time of measurement, suggesting that these autoantibodies were already present at hospitalization and likely represent preclinical (asymptomatic), unreported, or undiagnosed autoimmunity. To further evaluate the potential evolution of autoantibodies, we performed ANA testing on 21 individuals with paired samples. Eight individuals (38%) had positive or weak positive ANA reactivity. Among these 8 individuals, ANAs were present at both time points in three, changed in the intensity of staining in two, and were positive at only one of the two time points in the final three (Supplementary Fig. 1b and Supplementary Table 1). Taken together, these data indicate that autoantibody levels change over time in individual COVID-19 patients, consistent with their production and, in some cases, transience during acute illness.

We next examined whether IgG ACA is triggered by SARS-CoV-2 infection. Paired samples from the same 48 subjects described above were used to probe the 41-plex cytokine array, again in a single, batched run. As observed with unpaired samples (Fig. 1), 28 of 48 (58%) of COVID-19 patients had at least one ACA (Supplementary Fig. 6). Of these twenty-eight, sera from fifteen patients recognized one cytokine, five recognized two cytokines, and eight recognized three or more cytokines (range 3–12 antigens). Interferons, IL-17, and RANK-L were the most common targets, and interferons, IL-17, and IL-22 were new targets in some patients (Fig. 3d). In addition to Subject UMR19 (Fig. 1), a second patient with high MFI ACE-2 autoantibodies was also identified (Subject UMR12, Fig. 3d). Increased MFI was observed for one or more autoantibodies at later time points in 12 patients (24%). Several were present at MFI levels near or below the average for HC at baseline and were induced to high MFI levels at the second time point (e.g., anti-IFN-α, subject UMR07; anti-IFN-γ, subjects UP63 and UP65; and anti-IL-22, subjects
UP54, UP63, UP65, and UMR10, Fig. 3d). In many patients, ACA MFI levels were significantly elevated at the first time point and decreased at the later time point, suggesting that some ACA were pre-existing and/or developed transiently following SARS-CoV-2 infection. Subject SU09 had very high MFI levels of anti-TNF-α at both time points, attributed to anti-TNF therapy. We conclude that antibodies against cytokines, chemokines, growth factors, and receptors are common in hospitalized COVID-19 patients.

Many are triggered in response to SARS-CoV-2 infection, even at later time points distant from the time of infection (e.g., anti-IL-22, subject UMR10, day 29, Fig. 3d).

To further evaluate the change in autoantibodies targeting traditional autoantigens or cytokines over time, we performed a targeted analysis of 21 of the 48 patients who had paired autoantibody data specifically at D0 and D7 of hospitalization (Supplementary Fig. 7). Almost all patients (18/21, 86%) had demonstrable changes in the number of antibodies, defined at varying thresholds of sensitivity (>3 SD vs. 3–5 SD vs. >5 SD) between D0 and D7 (Supplementary Fig. 7a). When combining the number of autoantibodies targeting traditional autoantigens or cytokines (Supplementary Fig. 7b), there is a trend towards increased numbers both of autoantibodies and ACAs per subject.
over time. Higher numbers of individuals with more autoantibodies and ACAs at D7 compared to D0 at the 3–5 SD threshold were observed (Supplementary Fig. 7b and 7c), but the difference in medians between D0 and D7 was not statistically significant. Nevertheless, these data clearly show that there is an ongoing evolution in both the numbers and levels of autoantibodies and ACAs with time in hospitalized COVID-19 patients.

We next sought to quantify how much more frequently autoantibodies targeting traditional autoantigens and cytokines are observed in hospitalized COVID-19 patients than in HC. We conducted 2-sample tests for equality of proportions comparing the number of ANA or ACA+ COVID-19 patient subjects and HC. Any subject with an MFI value that was >5 SD above the mean MFI of the HC and >3000 for at least one of the 94 antigens (combining traditional autoantigens and cytokines) was considered ANA or ACA+. Autoantibodies were found to be significantly more common in the unpaired patient subjects than in HC (p = 4 × 10^-11 using a one-tailed, 2-sample test for equality of proportions). Forty-five of 51 (88%) of the paired hospitalized patients with COVID-19 were ANA or ACA+ compared to 5 of 31 (16%) HC. Autoantibodies were also found to be significantly more common in the paired patient subjects than in HC (p = 2 × 10^-5 using a one-tailed, 2-sample test for equality of proportions). Twenty-eight of 48 (58%) of the paired hospitalized patients with COVID-19 were ANA or ACA+ compared to 0 of 16 HC.

**Broad anti-viral immune responses target internal viral proteins in hospitalized COVID-19 patients.** We have used protein arrays for epitope mapping and to measure antibody responses in influenza vaccines22 and in a nonhuman primate human immunodeficiency virus (HIV) vaccine study.23 We used a similar approach here to characterize anti-viral responses following SARS-CoV-2 infection. We created a 28-plex COVID-19 viral array that included structural and surface proteins from SARS-CoV-2 as well as eight commercially available recombinant non-structural proteins localized to the interior of the virus (Supplementary Table 4). As an initial validation, we compared array-based detection and measurement using a clinical-grade ELISA (R = 0.81, Spearman’s, p < 0.0001 for anti-SARS-CoV-2 nucleocapsid; R = 0.60, Spearman’s, p < 0.0001 for anti-SARS-CoV-2 RBD, Supplementary Fig. 8a and 8b, respectively). By studying the anti-viral antibody (AVA) response, we hoped to understand if certain viral antigens might correlate with the development of autoimmune responses. We hypothesized that poorly controlled SARS-CoV-2 infection leads to the development of serum antibodies that recognize not just structural proteins such as the SARS-CoV-2 spike protein, but also nonstructural proteins, and that a subset of these viral proteins might correlate with the development of autoimmunity. Proteins from related coronaviruses were also included to explore whether pre-existing antibodies to seasonal coronaviruses might correlate negatively or positively with disease severity, and with autoimmunity.

Figure 4 depicts a heatmap representation of IgG reactivity based on MFI (Fig. 4a, left panel) and calculation of SD above average MFI for HC (Fig. 4b, right panel). As expected, nearly all patients had broad immune responses to viral structural proteins (first seven antigens on left, Fig. 4a, b). Twelve patients had low MFI levels at the earliest time point (almost all were day 0, defined as a collection within the first 24–72 h of hospitalization but developed high MFI IgG SARS-CoV-2 antibodies when tested at later time points, consistent with previously published findings in the setting of acute illness24 (Fig. 4a). Other subjects (e.g., subject UP50) already had broad AVA responses at day 0, suggesting the subject had been infected for a significant period of time prior to hospital admission.

IgG antibody levels against non-structural SARS-CoV-2 proteins were significantly elevated in a majority of patients (n = 35, 73%), particularly papain-like protease (PLPro, n = 13 patients), open reading frame proteins (Orf 8, n = 14 patients and Orf 3a, n = 18 patients), and nonstructural proteins (NSP 1, n = 20 patients, and NSP 9 n = 31 patients, but not NSP 7 (n = 0), NSP8 (n = 1) or 3C-like protease (n = 3)). The number of targeted non-structural proteins increased over time in 20 of 49 (40%) patients when compared with the earliest available time point, and were absent (n = 14), did not change (n = 7), or decreased (n = 8) in the remaining patients. Of the eight patients who showed a decrease in the number of targeted non-structural SARS-CoV-2 antigens over time, all but one decreased by a single antigen, and three were patients who had samples collected at an interval of 37 days, making it likely that the immune responses were transient. A majority of patients had linked antibody responses in which multiple non-structural antigens were targeted in the same subject. In rare patients (e.g., subject UP65, see SARS-CoV-2 protein PLPro, Figs. 4a and 4b), the initial immune response was focused on an internal protein (or was pre-existing) and later evolved to target spike and other SARS-CoV-2 surface or structural proteins. We conclude that antibody responses in hospitalized COVID-19 patients are not limited to structural proteins, that linked responses to multiple non-structural proteins are observed over time, and that NSP9 is the most commonly recognized internal SARS-CoV-2 protein of those tested on the array.

**New-onset IgG autoantibodies are temporally associated with anti-SARS-CoV-2 IgG responses.** We next identified a subgroup of patients (n = 12) whose anti-SARS-CoV-2 antibody responses suggested that they had been infected at a time point that was proximate to hospitalization and capture of the first sample. Our analysis identified patients without an anti-viral response at the first time point suggesting the sample was collected close to the time of infection. Selection criteria for patients who were early in their anti-viral responses included (i) the first available sample was within three days of hospitalization; (ii) anti-spike 1 IgG levels were <3000 MFI at baseline; (iii) anti-RBD IgG levels were <20,000 MFI at baseline; and (iv) at least a 2-fold increase in MFI for IgG against both S1 and RBD was observed at the next available time point. We then studied these patients to further determine if new IgG autoantibodies appeared at the second time point, providing evidence that SARS-CoV-2 directly triggers the development of autoantibodies.

We compared IgG reactivities at both time points for all twelve subjects who met the above criteria on COVID-19 autoantigen arrays (Fig. 5a, left panel) and cytokine arrays (Fig. 5b, middle panel) with anti-viral responses using the virus array (Fig. 5c, right panel). Four of twelve patients were found to have at least one newly induced autoantibody at the later time point (white boxes). Two of these four patients had two or more new autoantibodies (Subjects UP52, n = 5 antigens; and subject UP65, n = 10 antigens). β2GPI, histones, and the 54 kD component of the myositis autoantigen signal recognition particle (SRP 54) were the most common antigens identified (n = 2 subjects each). Given the small sample size, no clear correlations were identified between individual autoantibodies and IgG response to a specific viral protein (Fig. 5d and Supplementary Fig. 9).

Although 4 patients developed one or more new-onset autoantibodies using these criteria, the number of patients with longitudinal samples meeting these criteria was small (12 patients total), preventing us from drawing conclusions about the prevalence of this phenomenon. To further explore this finding, we performed a secondary analysis of the paired samples’ data to...
**Fig. 4 Measurement of anti-viral IgG responses using a COVID-19 viral array.** **a** Heatmap depicting IgG antibodies using a 28-plex bead-based protein array. Viral protein antigens are grouped based on sixteen proteins from SARS-CoV-2 (left panel), other coronaviruses (middle panel), and other viruses (right panel), labeled on the x-axis. Most recombinant viral proteins were engineered to include a 6X-His-tag, which was used to validate conjugation to beads using an anti-epitope monoclonal antibody (bottom of the panel). The same COVID-19 patients from Fig. 3 (see Supplementary Figures 9 and 10) were analyzed (top panel, \( n = 94 \)) longitudinal COVID-19 samples, including paired samples from 44 subjects and 2 subjects who had 3 available time points each, subjects UP70 and UP71. HC (\( n = 16 \), middle panel). Two patient sample pairs (UP63 and UMR20) were excluded from analysis due to technical failure on the viral array assay. Colors correspond to the MFI values shown at right. **b** Heatmap depicting statistically significant anti-viral IgG responses. Colors indicate IgG antibodies whose MFI measurements are >5 SD (red) or <5 SD (black) above the average MFI for HC samples collected prior to the COVID-19 pandemic. Source data are provided as a Source Data file.
identify those autoantibodies against traditional autoantigen or autoantibodies against cytokines that met all four of the following criteria: (1) MFI was at or below the mean for HC at first time point; (2) MFI increased by at least 2-fold at second time point; (3) MFI increased by at least 3 SD above the mean for HC at second time point; and (4) MFI was at least 3000 at the second time point. Although similar to the analysis we performed to obtain the data shown in Fig. 5, this new analysis separated out those patients whose earliest time point also may include strong anti-viral responses. This new analysis shows that even after a strong anti-viral response has occurred, 23% of patients developed at least one new autoantibody later in the hospitalization.

Finally, we correlated autoantibodies targeting traditional autoantigens or cytokines with anti-viral IgG responses using array data from the cohort described in Fig. 3, focusing on Penn and Marburg samples which had been collected at time points as proximate to the day of hospitalization as possible. We compared patients who had one or more autoantibodies \( (n = 15 \text{ first time point}, n = 13 \text{ second time point}) \) with patients who had no autoantibodies \( (n = 21 \text{ first time point}, n = 23 \text{ second time point}) \). Anti-SARS-CoV-1 RBD correlated positively with the autoantibody-positive group \( (p = 0.002 \text{ at the second time point using one-tailed Wilcoxon rank-sum test}, p = 0.044 \text{ using Bonferroni correction for each time point}) \). NSP1 \( (p = 0.03 \text{ at second time point}, p = 0.08 \text{ at first time point}) \) and ME \( (p = 0.04 \text{ at first time point}, p = 0.06 \text{ at the second time point}) \) trended positively when correlating with autoantibodies but were not statistically significant after Bonferroni correction. An identical analysis was performed on ACA+ vs. ACA- patients, showing no correlations with IgG responses to any viral proteins, including influenza, SARS-CoV-1, and seasonal coronaviruses (OC43 RBD, 229E-FL-GCN4, NL63 RBD, and HKU1 RBD).

**Discussion**

We have used a multiplexed, bead-based platform to identify circulating antibodies in hospitalized patients with COVID-19 and have generated integrated results from three different protein microarrays to discover COVID-19 associated autoantigens and link them to anti-viral responses. Our studies have led to several important findings that provide further insights into COVID-19 pathogenesis. First, we found that approximately half of hospitalized COVID-19 patients develop serum autoantibodies against one or more antigens on our array even though only a quarter of all patients are ANA+. Increased levels of autoantibodies are not simply a reflection of hypergammaglobulinemia because they are produced out of proportion to total IgG serum concentration. In
most individuals, only a small number of autoantigens are targeted, which is more consistent with a sporadic loss of self-tolerance than a global increase in autoantibody production. Second, the autoantibodies we discovered are found in relatively rare connective tissue diseases that are not typically measured in clinical labs, and some are predicted to be pathogenic. Third, a surprisingly large number of ACA were identified, far more than just the interferon autoantibodies described recently. Fourth, antibodies recognizing nonstructural SARS-CoV-2 proteins were identified that correlate positively with autoantibodies. Finally, and perhaps most importantly, some autoantibodies are newly triggered by SARS-CoV-2 infection, suggesting that severe COVID-19 can break tolerance to self.

Approximately 60–80% of all hospitalized COVID-19 patients in our study had at least one ACA, with a greater number of different ACA specificities generated in individual patients than observed for traditional autoantigens. Two recent studies demonstrated that IFN-α and IFN-α-blocking ACA are found in patients with severe COVID-19. Anti-IFN antibodies with blocking activity were absent in all 663 tested patients with mild COVID-19, strongly linking the presence of anti-IFN to disease pathogenesis and severity. Another study reported that type I interferon (IFN) deficiency could be a hallmark of severe COVID-19, while other investigators pointed towards an untuned anti-viral immune response due to delayed-type I/II interferon expression. Bastard identified blocking ACA for additional cytokines including IL-6, IL-22, and IL-12p70. ACA without blocking activity still may be biologically important, for example by potentiating receptor binding or prolonging cytokine production. A subset of array-identiﬁed ACA in SLE patients from NIH were recently identiﬁed contribute to the formation of inﬂammatory immune complexes in situ, particularly at endothelial surfaces. For example, neutrophil extracellular traps (NETs) have been implicated in COVID-19 patients with vasculitis. Antineutrophil Cytoplasmic Antibody (ANCA) associated vasculitis (AAV) has been strongly associated with neutrophil activation and generation of pro-inﬂammatory NETs containing nucleic acids, histones, and inﬂammatory peptides. While we did not observe elevated levels of MPO or PR3, we identiﬁed high MFI anti-BPI antibodies in 6% of COVID-19 patients. The detection of autoantibodies to BPI and core as well as linker histones raises the possibility that NETs contribute to the generation of autoantibodies in severe COVID-19, a possibility that is in line with the neutrophilia that accompanies severe acute disease. Disseminated microvascular coagulopathy and microvascular injury in lung and skin from COVID-19 patients correlate with fibrin deposition and thrombus formation. SARS-CoV-2 membrane proteins including the spike protein (but not SARS-CoV-2 RNA) colocalize with activated complement in ACE-2+ microvascular endothelia of COVID-19 lung tissue and normal-appearing skin. Magro and colleagues hypothesize that spike protein on the surface of circulating pseudovirions binds to endothelial ACE-2 (whose gene is interferon-inducible), providing a nidus for activation of complement and formation of microthrombi. Anti-C1q (a SLE autoantigen), anti-β2GPI (which is thrombogenic), anti-BPI, and anti-ACE-2 (if non-blocking) that were discovered in our screen would be predicted to exacerbate these pathogenic processes.

Severe infection may also result in an “all-hands-on-deck” immune response that results in loss of tolerance due to the presence of pro-inflammatory mediators that may lessen the requirement for T cell help. Some patients with severe acute COVID-19 appear to mount extracellular B cell responses that are characterized by expanded B cells and plasmablasts, loss of germinal centers, and loss of expression of Bcl-6. Antibody repertoire analysis from hospitalized COVID-19 patients during acute disease include massive clones with low levels of somatic mutation (SHM) and elongated CDR3 sequences which can be associated with polyclonality and are reminiscent of immune responses seen in acute Ebola and salmonella infection. It has been suggested that these responses resemble SLE flares in which autoreactive B cells are also activated via an extrafollicular, TLR7-dependent pathway. Although SARS-CoV-2 genomic RNA could itself serve as a costimulatory TLR7 ligand, many of the autoantigens we have identiﬁed also bind to structural RNAs such as the U1-snRNA (found in Sm/RNP complexes), 75 RNA (a component of SRP), and tRNAs (e.g., 12, 1 PL-7, and PL-12) which might activate dendritic cells in a TLR7-dependent manner.

One of the most important unanswered questions raised by our studies is why specific molecules are targeted in hospitalized COVID-19 patients. For newly triggered ACA, the most likely explanation is that they arise as a consequence of severe disease along with high levels of viremia, tissue injury, and elevated local levels of pro-inflammatory cytokines and chemokines. However, it is also possible that the presence of ACAs could affect the regulation of self-reactive lymphocytes by altering the half-lives of the receptor interactions of the target molecules. For traditional autoantigens, one possibility is that viral proteins or the SARS-
CoV-2 RNA genome and self-molecules physically interact, and that the initial immune response to the viral protein in a highly inflammatory microenvironment expands to include self-proteins through linked recognition and intermolecular epitope spreading. Another possibility is molecular mimicry in which one or more viral proteins or epitopes cross-react with self-proteins leading to loss of tolerance and development of autoimmunity. Experiments to explore these mechanisms are ongoing.

The vast majority of studies on SARS-CoV-2 proteins have focused on viral structural proteins to develop efficient and accurate diagnostic assays, and to identify specific epitopes on surface proteins for the development of vaccines and therapeutic monoclonal antibodies. These proteins are also the major focus of the immune response in most infected individuals. Here, we developed a multiplexed viral protein array that enables simultaneous measurement of antibody responses against 28 different proteins from 13 different viruses. We determined that non-structural proteins are recognized by antibodies in a large proportion of hospitalized COVID-19 patients, suggesting that B cell responses expand over time to involve additional viral molecules. IgG antibody levels against NSP1 and ME correlated positively with the presence of at least one autoantibody. We hypothesize that prolonged inability to eradicate and clear virus expands the adaptive immune response to target non-structural viral proteins, some of which might physically interact or cross-react with autoantigens in the context of an intense local or systemic inflammatory environment, exceeding a threshold for breaking tolerance to self. In contrast, patients who rapidly mount neutralizing antibody responses to the viral spike protein about “intraviral epitope spreading” and may be less likely to develop autoantibodies. Why anti-SARS-CoV-1 RBD IgG responses associate with autoantibody-positive patients is unclear. Future longitudinal studies are needed to determine whether broad B cell responses play any direct pathogenic role in patients with prolonged hospital courses or in patients with long-term sequelae of COVID-19 infection; to correlate anti-viral responses with autoantibodies targeting traditional autoantigens or cytokines over time using much larger COVID-19 cohorts including patients who are asymptomatic or have a mild disease; and to explore whether specific SARS-CoV-2 proteins might cross-react with autoantigens discovered in our screens.

Many studies of hospitalized COVID-19 patients, including our study, suffer from important limitations. First, confounding variables exist including heterogeneous demographics, medications at hospitalization, individualized treatment approaches, and, in some cases, unknown history of pre-existing medical or autoimmune conditions. Second, “Day 0” is not day 0 of infection but instead refers to a time point most proximate to hospitalization. Our viral array results (Figs. 4 and 5) confirm that the time between initial infection and sample acquisition was heterogeneous, potentially confounding interpretation of autoantibody results. Third, not all antigens (e.g., lipids, hydrophobic proteins, and carbohydrates) are compatible with our screening methodology, and as a result we have certainly missed some reactivities. Fourth, we did not include patients who were asymptomatic, had mild COVID-19, were vaccinated for SARS-CoV-2, had other severe viral illnesses, or were children. Finally, our analysis was limited to hospitalized patients during acute illness, with follow-up times of days rather than months or years.

Although beyond the scope of these studies, our data generate many more questions that need to be addressed in the coming years – questions that can only be answered by generating large cohorts of prospectively enrolled subjects with new-onset viral syndromes, including patients with COVID-19, respiratory illnesses which resemble COVID-19, and subjects enrolled in COVID-19 vaccine trials. Are autoantibodies targeting traditional autoantigens or cytokines specific to COVID-19, or is their presence shared more broadly in patients with influenza and other severe acute illnesses? Are autoantibodies found in convalescent serum used to treat patients with severe COVID-19? Do any of these autoantibodies underly some of the signs and symptoms observed in “long COVID”, do they lead to classifiable autoimmune disease, and can they be used as predictive markers or identify subsets of patients who would benefit from targeted immunotherapies?

Our studies have begun to quantify the impact of SARS-CoV-2 on autoimmunity, identifying which antigens and specific autoimmune diseases to surveil in patients who have been infected, and contributing to our mechanistic understanding of COVID-19 pathogenesis. These studies provide a starting point for large-scale epidemiology studies to determine the extent of autoimmunity that results from SARS-CoV-2 infection, and its long-term impacts on the health care system and the economy. While the COVID-19 pandemic is leaving a wake of destruction as it progresses, it also provides an unprecedented opportunity to understand how exposure to a new virus could potentially break tolerance to self, potentially giving rise to autoimmunity and other chronic, immune-mediated, diseases.

**Methods**

**Serum and plasma samples.** Hospitalized COVID-19 patients. Serum or plasma samples were obtained following protocols approved by local institutional review boards (IRB) from 147 unique hospitalized subjects (n = 99 unpaired; n = 98 paired longitudinal samples from 48 distinct subjects). Samples were obtained from four centers in three distinct geographic areas: Northern California (Kaiser Permanent Health Care System, n = 48 unpaired samples from hospitalized subjects, IRB# 55718) collected in March and April 2020; and Stanford Occupational Health Clinic, 20 paired samples from 10 unique hospitalized subjects IRB# 55689) collected between April and June 2020; Philadelphia, Pennsylvania (University of Pennsylvania, n = 50 unpaired; and 44 paired samples from 21 unique hospitalized subjects, IRB # 808542) obtained between April and June 2020; and Marburg, Germany (Philips University Marburg, 1 unpaired; and 34 paired samples from 17 unique hospitalized subjects collected between April and June 2020, IRB# 57/20). Clinical characteristics of the cohorts can be found in Supplementary Tables 5–7 and in Supplementary Figs. 10 and 11.

**Healthy Controls.** Serum and plasma samples from anonymous healthy controls (HC, n = 41) were obtained prior to the COVID-19 pandemic from Stanford Blood Bank and Stanford Hospital and Clinics.

**Bead-based antigen array content.** We created three different custom, bead-based antigen arrays modeled on similar arrays that we previously used to study autoimmune and immunodeficiency disorders, and for characterizing vaccine responses. Antigens were selected based on published datasets; literature searches that have implicated specific antigens in COVID-19; potential for mechanistic contribution to COVID-19 pathogenesis; and compatibility with bead-based platforms. A complete list of all antigens, vendors, and catalogue numbers can be found in Supplementary Tables 2–4. The “COVID-19 Autoantigen Array” included 53 commercial protein antigens associated with CTD (Supplementary Table 2). The “COVID-19 Cytokine Array” comprised 41 proteins including cytokines, chemokines, growth factors, acute phase proteins, and cell surface proteins (Supplementary Table 3). Specific “secretome” proteins included a subset of molecules identified in previous large screens in patients with systemic lupus erythematosus (SLE) (59-plex screen)33, systemic sclerosis (sclerodermia or SSc, 221-plex screen, manuscript in preparation)36, Autoimmune Polyclonidine Syndrome Type 1 (APS-1)35, Atypical Mycobacterial Infections (AMI)34, Immunosuppression Polyclonidinephropy X-linked (IPX)34, and Mycobacterial Infections (AMI)34, more recently in COVID-19. The “COVID-19 Viral Array” included 54 recombinant, purified SARS-CoV-2 proteins from commercial sources, or recombinant proteins produced in the labs of several of the authors (Peter Kim and Taia Wang, Supplementary Table 4)35. We also included proteins or protein fragments from SARS-CoV-1, Middle East Respiratory Syndrome (MERS), nonpathogenic coronaviruses (OC43, 229E, NL63, and HKU1), Hepatitis B, Mumps, Rubella, Rubeola, Ebola, and Influenza (hemaglutinin (HA) from A/California/07/2009 H1N1).

**Array construction.** Antibodies were coupled to carboxylated magnetic beads (MagPlates C, Luminex Corp.) such that each antigen was linked to beads with unique barcodes33,35,36. In brief, unless stated otherwise, 8 μg of each antigen or control antibody was diluted in phosphate-buffered saline (PBS) and transferred to 96-well plates. Diluted antigens and control antibodies were conjugated to 1 x 10^7

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carboxylated magnetic beads per ID. Beads were distributed into 96-well plates (Greiner BioOne), washed, and in suspension in phosphate buffer (0.1 M NaH2PO4, pH 7.4). Hep-2 cell plate washer (Biotek) was added to the well plate. The bead surface was activated by adding 100 µl of phosphate buffer containing 0.5 mg 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Pierce) and 0.5 mg N-hydroxysuccinimide (Pierce). After 20 min incubation on a shaker, beads were washed and resuspended in activation buffer (0.05% M NaOH, 0.1% NH4Cl, 0.25% Tween 20, 1 mM EthyleneDiamine TetraAcetic Acid, MES, pH 5.0). Dilutions of antigens and control antibodies were incubated with beads for 2 h at room temperature. Beads were washed three times in 100 µl PBS-Tween, re-suspended in 60 µl storage buffer (Blocking reagent for Enzyme-Linked Immunosorbent Assay, ELISA/RT) and 50 µl diluted in plates at 4 °C. The immobilization of some antigens and control antibodies on the correct bead IDs was confirmed by analysis using commercially available mouse monoclonal antibodies, or antibodies specific for epitope tags such as 6X-histidine. In addition, prototype human plasma samples derived from patients with autoimmune diseases were used with known reactants and patterns (e.g. ds-DNA, Sc-l, centromere, SSA, SSB, cardiolipin, whole histones, and RNP, all purchased from ImmunoVision; also from Stanford Autoimmune Diseases Biobank, and OMF); APS-1, IPEX, PAP, or AMI; prototype human plasma samples from healthy donors were tested under the same conditions (GEO) database. The accession code is provided in our data availability statement.

**Array proton**. Serum or plasma samples were first heat-inactivated at 56 °C for 1 h, then tested at 1:100 dilution in 0.05% PBS-Tween supplemented with 1% (w/v) bovine serum albumin (BSA) and transferred into 96-well plates in a randomized layout. The bead array was distributed into a 384-well plate (Greiner BioOne) by transfer of 5 µl bead array per well. 45 µl of the 1:100 diluted sera were transferred into 384-well plate containing the bead array. Samples were incubated for 60 min on a shaker at room temperature. Beads were washed with 3 x 60 µl PBS-Tween on a plate washer (EL406, Biotech) and 50 µl of 1:50 diluted R-phycocerythrin (R-PE) conjugated Fc-specific goat anti-human IgG Fab/Fc fragment 2 (Jackson ImmunoResearch, Cat # 109-116-098) was added to the 384-well plate for detection of bound human IgG. After incubation with the secondary antibody for 30 min, the plate was washed with 3 x 60 µl PBS-Tween and re-suspended in 60 µl PBS-Tween prior to analysis using a FlexMap3D system (Luminex Corp.) and Luminex xPONENT® version 4.2 software. A minimum of 100 events per bead ID were counted. Binding events were displayed as Mean Fluorescence Intensity (MFI). To ensure reproducibility and rigor, all samples were run in duplicate in each experiment. Most samples were analyzed twice using the same array run on different days, showing high concordance (mean Pearson correlation coefficient 0.94, standard deviation (SD) 0.09 for one representative dataset). Prototype autoimmune sera were also heat-inactivated and compared with untreated prototype autoantibody serum on the same arrays, with similar results.

**Anti-dsDNA ELISA.** Anti-DNA ELISA was performed on calf thymus DNA (Sigma, Cat # D3664) coated Immobilon (Immunol-1B) 96-well plates (Thermo Scientific, Cat # 3355) that were then blocked with 1% BSA (Sigma, Cat # A9796) in PBS (Corning, Cat # 21-0931-CV). Dilutions of patient and control plasma in 1:100 dilution buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 0.5% non-fat milk powder by weight) and incubated for 1 h at room temperature. Goat anti-human IgG-HPF (Cat # 109-035-008, Jackson ImmunoResearch Laboratories, West Grove, PA) was diluted 1:10,000 with sample dilution buffer and 50 µl of secondary antibody was added to each well. After washing 3 x (PBS-Tween), plates were incubated for 0.5 h at room temperature and washed again 3 x (PBS-Tween), 50 µl of TMB substrate (Cat # 555214, Fisher Scientific, Waltham, MA) was applied for the color development at room temperature for 10 mins and stopped with 50 µl of 250 mM hydrochloric acid. All samples were run in duplicate. Anti-SARS-CoV-2 Nucleocapsid Assays were run with a commercially produced kit (Cat # CV3002, LifeSensors, Malvern, PA) and performed according to the manufacturer’s instructions. Serum and plasma samples were diluted at 1:100 and plates were read at OD450 nm. The RBD and nucleocapsid ELISAs were repeated to confirm the results. ELISA plates were read at OD450 nm (CLARIOstar plate reader, BMG LABTECH, Cary, NC).

**Statistical analyses.** All data analysis and statistics were performed using R and various R packages. For normalization, average MFI values for “bare bead” IDs were subtracted from augmented MFI values for antigen conjugated bead IDs. The average MFI for each antigen was calculated using samples from healthy subjects known to be uninfected with SARS-CoV-2 (all obtained before December 2019). Antibodies were considered “positive” if MFI was ≥5 SD above the average MFI for HC for that antigen, and MFI was >3000 units, a threshold which is more stringent than commonly published in related literature. A less stringent 3 SD cutoff used in a Luminex assay to measure SARS-CoV-2 immunoglobulins in blood and saliva was also employed for comparison in some experiments. An example can be found in Supplementary Figs. 3 and 4. ELISA and antibody number data were visualized in GraphPad Prism v9.0.0 (86). Complete heatmaps v2.7.7 was used for all heatmaps. Deidentified array data are publicly available on the Gene Expression Omnibus (GEO) database. The accession code is provided in our data availability statement.

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

**Data availability.** All raw and normalized Luminex protein array data have been made publicly available in the Gene Expression Omnibus (GEO) database with the SuperSeries accession code GSE180743. All other data, such as data generated from the experiments described in the current manuscript, are available from the corresponding author upon request. Clinical data other than data already shown in Supplementary Figures 10 and 11 are not available, to remain compliant with HIPAA requirements. Source data are provided with this paper.

**Code availability.** Code used for data analysis and figure generation is available from the corresponding authors upon reasonable request.

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Author contributions


Competing interests

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Additional information

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