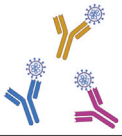

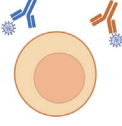
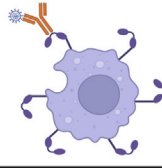
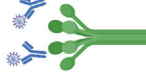



Article

Immune profiles to distinguish hospitalized versus ambulatory COVID-19 cases in older patients

COVID-19 High risk cohort (older, male, Black/Hispanic)		
	Ambulatory	Hospitalized
 SARS-CoV-2-specific antibody levels	=	
 S2 vs. S1 antibody concordance	No	Yes
 Virus neutralization	=	
 Spike-specific antibody dependent cellular phagocytosis (ADCP)	=	
 Complement binding to spike-specific antibodies	=	
 Serum IL-27	Low	High

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Highlights

Comparable SARS-CoV-2 Ab profiles of hospitalized and ambulatory cases

Concordance of Ab responses to spike S2 vs. S1 subunits only in hospitalized cases

High levels of serum IL-27 after convalescence in hospitalized patients

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Article

Immune profiles to distinguish hospitalized versus ambulatory COVID-19 cases in older patients

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SUMMARY

A fraction of patients with COVID-19 develops severe disease requiring hospitalization, while the majority, including high-risk individuals, experience mild symptoms. Severe disease has been associated with higher levels of antibodies and inflammatory cytokines but often among patients with diverse demographics and comorbidity status.

This study evaluated hospitalized vs. ambulatory patients with COVID-19 with demographic risk factors for severe COVID-19: median age of 63, >80% male, and >85% black and/or Hispanic. Sera were collected four to 243 days after symptom onset and evaluated for binding and functional antibodies as well as 48 cytokines and chemokines.

SARS-CoV-2-specific antibody levels and functions were similar in ambulatory and hospitalized patients. However, a strong correlation between anti-S2 antibody levels and the other antibody parameters, along with higher IL-27 levels, was observed in hospitalized but not ambulatory cases. These data indicate that antibodies against the relatively conserved S2 spike subunit and immunoregulatory cytokines such as IL-27 are potential immune determinants of COVID-19.

INTRODUCTION

The emergence and rapid spread of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in Wuhan, China, in December 2019 has led to a pandemic that continues to impact people worldwide. While research about this virus has progressed rapidly, leading to expeditious development of many types of diagnostic tests, treatments, and vaccines, important questions remain about the dynamic virus-host interactions that result in a wide range of disparate disease outcomes.

Coronavirus disease 2019 (COVID-19), the disease caused by SARS-CoV-2, can occur with different clinical manifestations. During the initial outbreak in China, most patients presented with mild to moderate symptoms which resolved without medical interventions, but ~15% of patients progressed rapidly to severe disease requiring hospitalization.^{1,2} Among the hospitalized, patients also require different levels of intervention. Around a third of hospitalized patients developed acute respiratory disease syndrome (ARDS) and require mechanical ventilation.³ Elderly patients and individuals with comorbidities, such as cardiovascular disease, diabetes mellitus, chronic lung disease, chronic kidney disease, obesity, hypertension, and cancer, are at higher risk for COVID-19 and have a much higher mortality rate than healthy younger adults.^{4,5} In addition, the overall COVID-19 case-fatality ratio is at least 2.4 times higher in men than in women.^{6–8}

Understanding the immune responses associated with disease severity and recovery is essential to develop and apply effective treatments against COVID-19. While high levels of binding antibodies (Abs), in particular IgA, and neutralizing Abs have been associated with disease severity,^{9–12} comparison of severe and mild cases needs to consider age, sex, race, and comorbidities, as racial and gender-based differences were apparent in the manifestation of COVID-19.¹³ Moreover, a study of infected patients from a wide range of age groups (1–102 years) demonstrated that anti-SARS-CoV-2 Ab levels varied depending on age.¹⁴ The levels in the 1- to 10-years-old group were around 3- to 4-fold higher than the 25- to 102-years-old groups. Another study comparing SARS-CoV-2-infected male and female patients from diverse ethnic groups showed no difference

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in Ab titers.¹⁵ However, male patients had higher plasma levels of innate immune cytokines and more robust induction of non-classical monocytes, while female patients developed significantly more robust T cell activation.¹⁵ IgG Fc glycome composition was also shown to predict disease severity but comparison was within a patient cohort that included both sexes and had an age range of 21–100 years old.¹⁶ Autoantibodies targeting type I interferons have been linked to severe COVID-19^{17–21}; however, age and comorbidities affect the prevalence of autoantibodies.²² A recent study further pointed to a higher prevalence of antineutrophil cytoplasmic antibodies during acute disease in severe COVID-19 cases and a correlation between the presence of autoantibodies with higher antiviral Ab and inflammatory responses.²³

In addition to Abs, cytokine responses in severe vs. mild COVID-19 cases have been evaluated. An association was shown between SARS-CoV-2 infection and marked elevation of several plasma cytokines including interferon gamma-induced protein 10 (IP-10), chemokine (C-X-C motif) ligand 16 (CXCL16), interleukin (IL)-1 β , IL-2R, IL-4, IL-6, IL-8, IL-10, IL-17, and IL-27.^{11,24–35} Several other studies provided evidence that type I interferon deficiency may lead to severe COVID-19, implying that disease severity may be due to impaired viral clearance and uncontrolled viral replication due to poor induction of early innate immunity.^{18,24,36–39} Another study revealed a positive association of the illness duration in severe cases with levels of IL-8 and soluble IL-2R α .⁴⁰ Furthermore, a longitudinal study on 40 hospitalized patients with COVID-19 found 22 cytokines that were correlated with disease severity.⁴¹ Yet, in a study looking at cytokine and leukocyte profile of patients with COVID-19 >60 vs. <60 years old, another set of cytokines/chemokines was found to correlate with older age, longer hospitalization, and a more severe form of the disease.⁴² Similar to the Ab studies, most analyses of cytokine responses to COVID-19 were conducted by comparison of patient cohorts with diverse demographics and risk factors.

Further investigations of Ab and cytokine responses to SARS-CoV-2 among racial/ethnic minority US populations at risk of developing severe COVID-19 are warranted. Indeed, a study among US veterans showed that Black and Hispanic individuals have experienced an excess burden of SARS-CoV-2 infection that was not entirely explained by underlying medical conditions or where they lived or received care.⁴³ Immune responses to vaccinations also are influenced by host genetic and demographic variables such as race, ethnicity, and sex, as demonstrated by induction of higher neutralizing Ab titers following rubella vaccination in individuals of African descent as compared to the European and Hispanic subjects, although the cytokine responses were comparable.⁴⁴ Contrastingly, stronger cytokine responses were seen in recipients of dengue trivalent and tetravalent vaccines who self-identified as Black African descent vs. White, although White subjects elicited more robust responses to virus challenge than Black subjects.⁴⁵

Here, we evaluated SARS-CoV-2-specific (spike, RBD, S1, S2, and nucleoprotein) Ab responses and conducted multiplex analysis of cytokines and chemokines in a cohort with risk factors for severe COVID-19. Of the 52 VA subjects from James J. Peters VA Medical Center (JJP VAMC), 81% were male, 79% were >50 years old (median of 63), and 85% belonged to US minority groups (Black and/or Hispanic). All were infected with SARS-CoV-2, as confirmed by diagnostic RT-PCR, during April–November 2020. Convalescent serum samples collected four to 243 days post disease onset were studied. We compared different categories of patients with COVID-19 within this VA cohort: ambulatory (n = 42) vs. hospitalized (n = 10) patients, COVID-19 patients with (n = 24) vs. without (n = 28) comorbidities. We also compared a subset of specimens (n = 20) from hospitalized vs. ambulatory cases that were matched based on sex, time post-infection, comorbidities, and spike-specific Ig levels. In addition, contemporaneous COVID-19-negative samples were studied. The data demonstrate that, in this cohort of older and mostly male VA patients, hospitalized and ambulatory patients had comparable binding and functional Abs, but diverged in their responses to the S2 spike subunit. Moreover, heightened levels of certain cytokines were detected and maintained in convalescent sera from hospitalized vs. ambulatory cases.

RESULTS

Ab responses to different SARS-CoV-2 antigens among hospitalized and ambulatory VA patients

Fifty-two COVID-19-convalescent (Table S1) and 21 COVID-19-negative serum samples were collected from a cohort of patients who received care at the JJP VAMC. Samples were collected between March and December 2020, during the first year of COVID-19 pandemic in New York City. COVID-19 diagnosis was confirmed by positive PCR test. The vast majority of the COVID-19-convalescent subjects was male (80%), Black/Hispanic (85%), and >50 years old (79%, median = 63, Q1-Q3 = 52–74), which are

representative demographics of the US Veterans population. The preponderance for male and older age in this cohort allowed for characterization of immune responses in the population known to be at risk for severe COVID-19. The patients with COVID-19 were categorized in two groups: hospitalized ($n = 10$) vs. ambulatory ($n = 42$). No difference in age and race/ethnicity was observed between the hospitalized vs. ambulatory patients (Figure 1A). However, all the hospitalized patients were male, while 10 of the 42 ambulatory patients were female. Analyses also considered the presence or absence of comorbidities known to be associated with more severe COVID-19, which included HIV, obesity, chronic obstructive pulmonary disease, diabetes, asthma, end stage renal disease, or pulmonary embolism.

Longitudinal specimens of the patients with COVID-19 were initially tested for SARS-CoV-2 nucleoprotein-specific IgG (Figure 1B). These patients with COVID-19 generated IgG responses against nucleoprotein, which peaked around day 75 post-infection. The responses declined subsequently, but the Abs were detected above cutoff up to 250 days post-infection in all except for two ambulatory patients. Samples from these two patients, collected at day 27 and day 214 post-infection, although negative for nucleoprotein-specific IgG (Figure 1B), were positive for spike-specific IgM and IgG, respectively (data not shown), and were both positive for total Ig against spike and nucleoprotein (Figure 1C). Of note, the anti-nucleoprotein IgG levels among hospitalized patients overlapped with those of ambulatory patients, and no difference was apparent in the peak levels and the decline rates between the two groups (Figure 1B). There was also no apparent clustering of patients with vs. without comorbidities (Figure 1B).

The specimens collected at the last time point from each subject were available for further investigation. The time points ranged from 4 to 243 days after symptom onset, with median of 178 and 188 days for hospitalized and ambulatory group, respectively (Figure 1A). To examine the relative levels of Abs induced by hospitalized vs. ambulatory patients against spike and its domains as compared to nucleoprotein, the serum samples were titrated for total Ig against spike, RBD, S1, S2, and nucleoprotein antigens (Figures 1C–1E and S2). Patients with COVID-19 produced highly variable levels of Abs against each of the five antigens tested, but all displayed Ig reactivity above cutoff (calculated as mean + three standard deviations (SD) of five COVID-19-negative specimens) (Figure 1C and S2A). The S2-specific Ab levels were relatively low (Figure S2A), similar to past reports on other cohorts of infected and vaccinated subjects.⁴⁶ There was a trend of higher median levels of Abs against all five antigens in the hospitalized group as compared to the ambulatory group, but the differences did not reach statistical significance and the individual Ab levels from the two groups essentially overlapped (Figures 1C and S2A), indicating that the two groups of patients could not be differentiated by their anti-SARS-CoV-2 Ab levels. Moreover, there was no difference in the Ab levels of patients with vs. without comorbidities (Figures 1D and S2B).

Because the sample collection times were over a wide range of days after disease onset, we searched for changes in total Ig levels against each of the five SARS-CoV-2 antigens over time after disease onset (Figures 1E and S2C). The Ig responses to spike did not decline over the 250 days post-onset (slope of 56.7 and $p = 0.71$) (Figure 1E). Ig levels against RBD and S1 also did not decrease over this period, while the S2- and nucleoprotein-specific Ig levels demonstrated a non-significant decline (Figure S2C). Interestingly, when the specimens were divided according to the disease severity, the S2-specific Ig levels overtime showed a trend of positive slope for the hospitalized patients and negative slope for the ambulatory patients (Figure 1F). This differential pattern was not seen with Abs against S1 or RBD (data not shown).

Neutralization activities against SARS-CoV-2 among hospitalized and ambulatory VA patients

COVID-19-convalescent sera were tested for neutralization activities against SARS-CoV-2 using a pseudovirus bearing SARS-CoV-2 spike protein (WA1 strain) as performed previously.^{46–48} COVID-19-negative sera ($n = 21$) were tested in parallel as control. Samples from all COVID-19-positive patients had neutralization activity reaching readily above 50%, while none of the COVID-19-negative sera did ($ID_{50} < 10$) (Figure 2). Neutralization titers of hospitalized and ambulatory patients did not differ, although the sample size of the hospitalized group was small ($n = 10$). The ID_{50} values ranged from 60 to 3313 for hospitalized patients (median of 194) and from 43 to 4402 for ambulatory patients (median of 162.5). Neutralization titers were also similar between groups with vs. without comorbidities associated with severe COVID-19 (Figure 2B). In addition, the neutralization titers showed no association with age (Figure 2C) and no decline with time (Figure 2D), demonstrating that these parameters do not influence SARS-CoV-2-neutralizing titers detected in sera of this VA cohort.

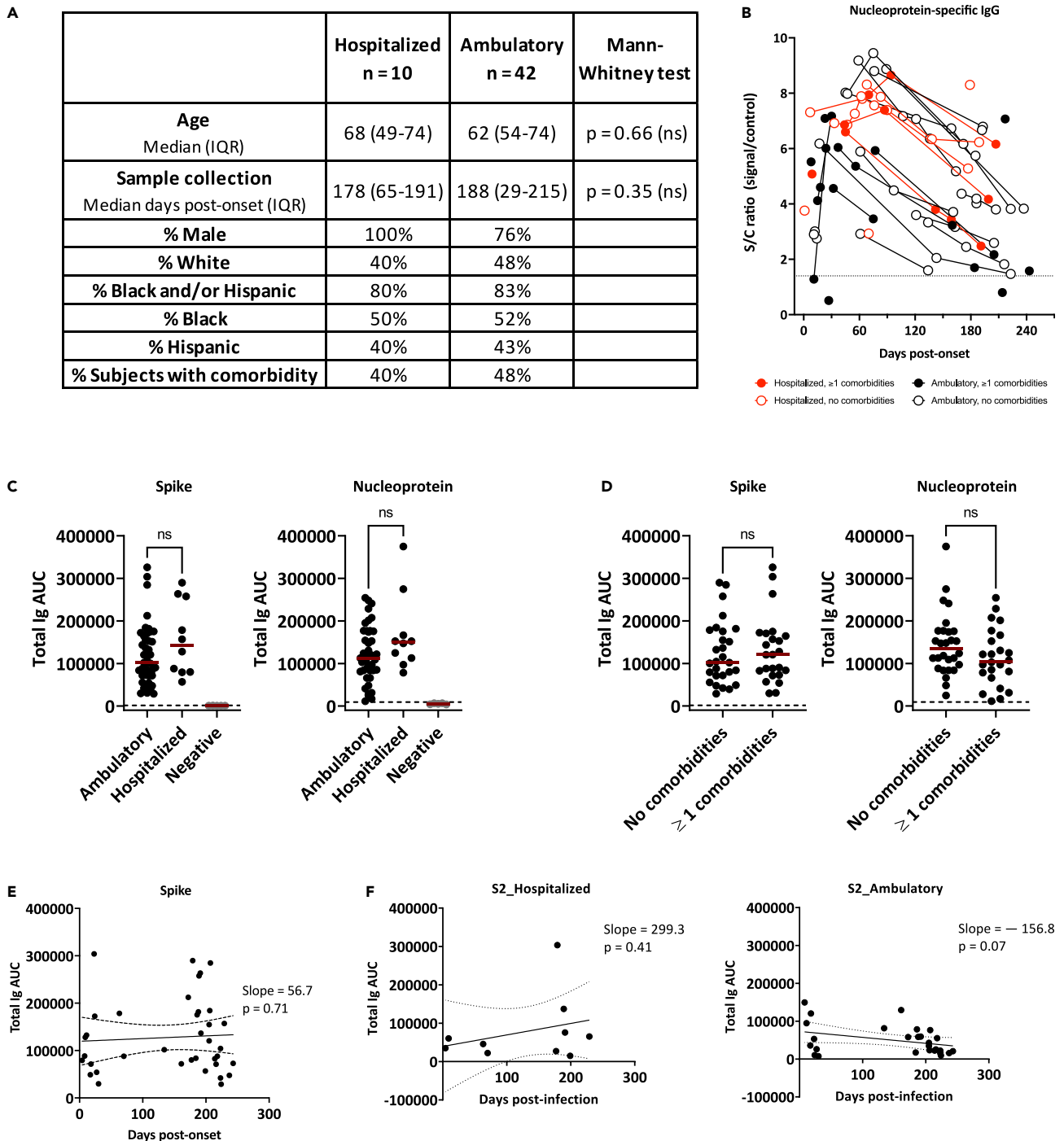


Figure 1. Total Ig antibody responses to SARS-CoV-2 among hospitalized and ambulatory patients in the VA cohort

(A) Demographic and clinical data of hospitalized (n = 10) vs. ambulatory (n = 42) patients.

(B) IgG responses to nucleoprotein in hospitalized and ambulatory patients with or without comorbidities. The dotted line represents the cut-off at 1.4.

(C) Total Ig levels against spike and nucleoprotein in hospitalized (n = 10) vs. ambulatory (n = 42) patients. AUC: area under the curve. The red line represents the median. The dotted line represents the cutoff, calculated as mean + 3SD of 5 COVID-19-negative specimens.

(D) Total Ig levels against spike and nucleoprotein between patients with (n = 24) vs. without (n = 28) comorbidities. The red line represents the median. The dotted line represents the cutoff, calculated as mean + 3SD of 5 COVID-19-negative specimens.

(E) Changes of spike-specific total Ig levels over time post-symptom onset. The dotted lines represent the linear regression 95% confidence bands.

(F) Changes of S2-specific total Ig levels over time post-symptom onset in hospitalized (n = 10, left) vs. ambulatory (n = 42, right) patients. The dotted lines represent the linear regression 95% confidence bands.

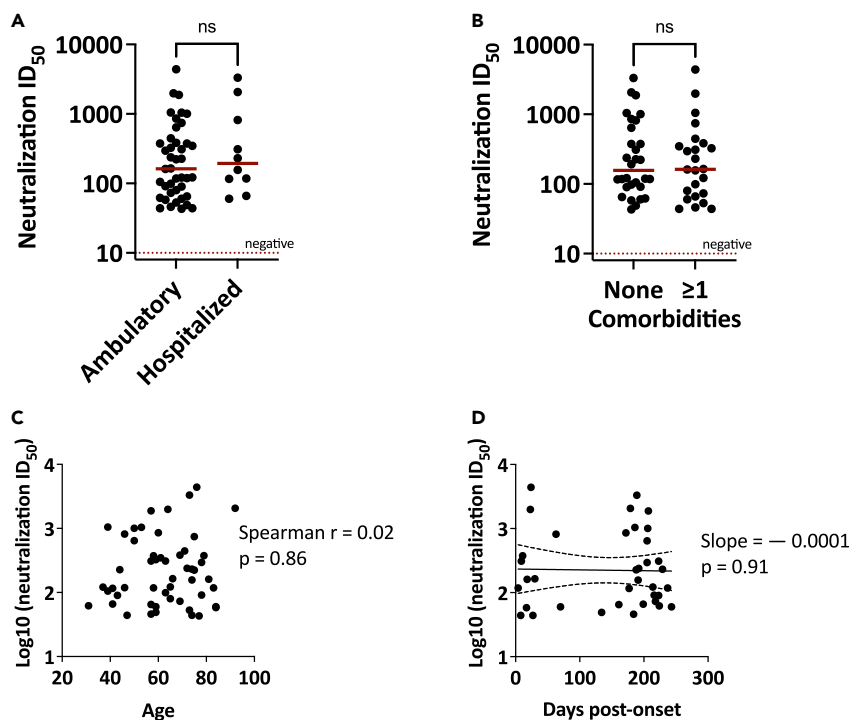


Figure 2. Neutralization activities against SARS-CoV-2 among hospitalized and ambulatory patients in the VA cohort

(A) Neutralization titers in sera of ambulatory (n = 42) vs. hospitalized (n = 10) patients. The red line represents the median. Negative is set at 10, the lowest reciprocal dilution.

(B) Neutralization titers in sera of patients with (n = 24) vs. without (n = 31) comorbidities. The red line represents the median. Negative is set at 10, the lowest reciprocal dilution.

(C) Spearman correlation between neutralization titers and age of patients.

(D) Changes in neutralization titers over time post-symptom onset. The dotted lines represent the linear regression 95% confidence bands.

Isotypes of serum Abs against SARS-CoV-2 spike produced by hospitalized and ambulatory patients

To understand the Fc properties of Abs raised against SARS-CoV-2 in patients presenting with distinct COVID-19 severity, we examined spike-specific Ig isotypes in a subset of hospitalized (n = 6) vs. ambulatory cases (n = 14) in which each hospitalized patient was matched to two or three ambulatory patients based on sex, time post-infection (early: <25 days post-infection vs. late: >130 days post-infection), comorbidities (none vs. at least one), and spike-specific total Ig levels (< half a log₁₀) (Table S2). Four COVID-19-negative plasma specimens were included to establish background levels. All the patients had IgM, IgG1, IgG3, and IgA1 spike-specific Abs. The levels of these isotypes and subtypes did not differ between the hospitalized and ambulatory groups (Figure 3). Similar results were observed with Ig isotypes against RBD (Figure S3). Some patients also mounted IgG2 and IgG4 Ab responses. The levels of these IgG subtypes were relatively low, and no difference was apparent between matched hospitalized and ambulatory patients. These data demonstrate that hospitalized and ambulatory patients display a comparable array of serum Ig isotypes against SARS-CoV-2 spike and RBD.

Fab-mediated neutralization and Fc-mediated activities of SARS-CoV-2-specific Abs among matched hospitalized and ambulatory VA patients

The matched set of hospitalized and ambulatory specimens was next tested for Ab activities against SARS-CoV-2. In addition to Fab-mediated neutralization, we examined Fc-mediated activities: spike-specific ADCP and spike- or RBD-complement binding (C1q and C3d) (Figure 4 and S4). Similar to the neutralization data shown in Figure 2 for all samples, neutralization levels between matched hospitalized (median ID₅₀ of 175) and ambulatory (median ID₅₀ of 142) specimens were not different (Figure 4A). While

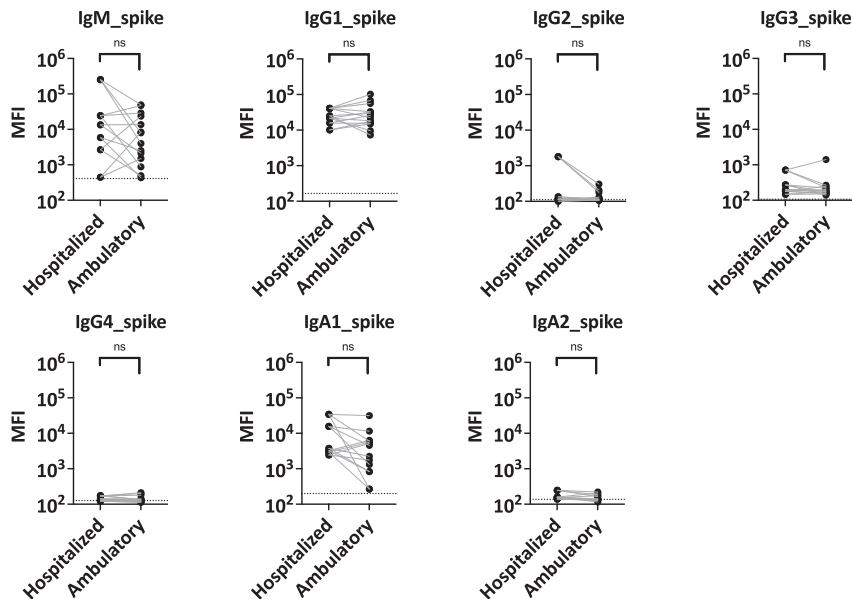


Figure 3. Antibody isotypes against SARS-CoV-2 in sera of matched hospitalized vs. ambulatory VA patients

The levels of spike-specific IgM, IgG1-4, IgA1 and IgA2 were measured in sera from matched hospitalized vs. ambulatory patients. The data are represented as mean fluorescent intensity (MFI) at a 1:200 serum dilution, each sample was tested in duplicate. The dotted line represents the cut-off. Statistical significance was calculated by a Wilcoxon matched-pairs test; each hospitalized patient was compared to 2 or 3 matched ambulatory patients.

certain hospitalized patients displayed higher ID₅₀ than their matched ambulatory counterparts, some others had lower ID₅₀ values. Fc-mediated spike-specific ADCP also showed no apparent trend, and the levels were indistinguishable between hospitalized and ambulatory groups (median AUC of 518 and 485, respectively) (Figure 4B). When complement binding and activation were examined, the capacity of spike- and RBD-specific Abs for C1q binding was found to be similar for both groups, and the C3d deposition levels also did not differ (Figures 4C, 4D, and S4).

Correlation matrix was compiled from both binding and functional Ab data of the hospitalized and matched ambulatory groups. A remarkable difference was noted: in the hospitalized group, a strong positive correlation was observed between the levels of S2-specific Ig and the other Ab parameters tested, whereas the correlation was weaker or absent in the ambulatory group (Figures 5A and 5B). Indeed, in the ambulatory group, poor correlation was apparent between the anti-S2 Ig levels and the Ig levels against the entire spike protein, S1, or RBD (Figure 5B). These results were confirmed using an S2 antigen from a different source and with additional 17 hospitalized specimens from a separate cohort (Figure S5 and Table S3). These results indicate a discordance of Ab responses against the relatively conserved S2 subunit vs. the rest of spike regions in the ambulatory cases but not the hospitalized cases.

Differences in cytokines responses among hospitalized and ambulatory patients

In addition to evaluating the Ab responses, we examined the cytokines profile of these convalescent patients with COVID-19. Fifty-two COVID-19-convalescent patient sera were tested for 48 cytokines and chemokines, many of which participate in induction and modulation of inflammatory responses (Figures 6, 7, and S6–S8). We compared patients with COVID-19 with different disease severity and asked whether the cytokines/chemokine responses were elevated in hospitalized patients compared to ambulatory patients (Figures 6, 7 and S6). The median modified Z scores showed an overall higher response in the hospitalized vs. ambulatory patients ($p = 0.03$), with higher median values observed in a constellation of cytokines/chemokines (e.g. IL-4, IL-7, IL-8, IL-13, IL-17A, IL-17E/IL-25, IL-18, IL-22, IL-27, MCP-3, M-CSF, MDC, MIG/CXCL9, MIP-1 α , MIP-1 β , PDGF-AA, and TNF- β) (Figure S6B). Correlation matrices of these cytokine/chemokine data further supported the notion of a stronger coordination of cytokine/chemokine

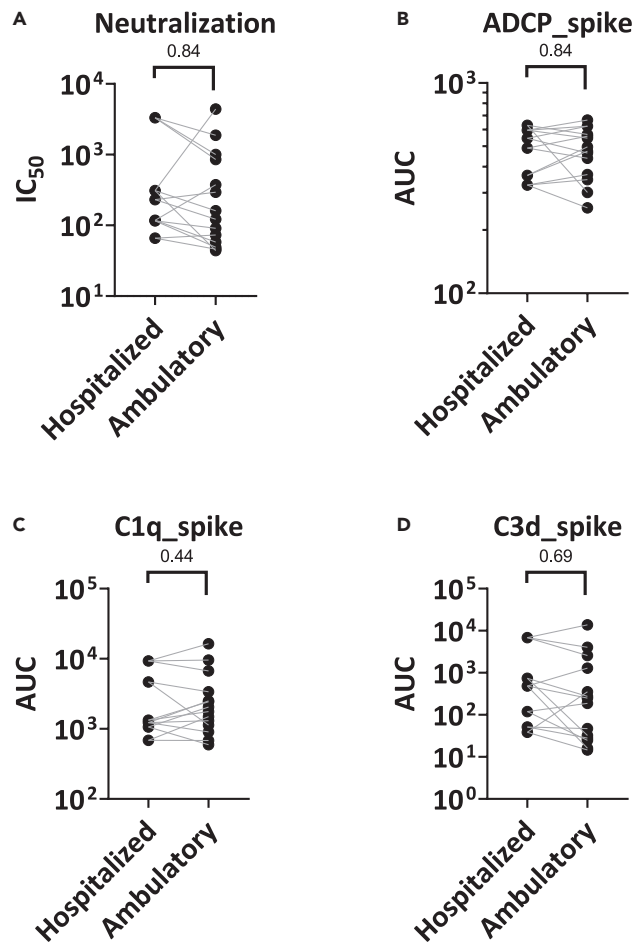


Figure 4. Fab-mediated neutralization and Fc-mediated activities of SARS-CoV-2-specific antibodies in sera from matched hospitalized and ambulatory VA patients

(A) Neutralization activities in matched hospitalized vs. ambulatory patients. IC₅₀: reciprocal serum titers yielding 50% neutralization.
 (B) Spike-specific ADCP activities in matched hospitalized vs. ambulatory patients.
 (C) C1q binding to spike-specific antibodies in matched hospitalized vs. ambulatory patients.
 (D) C3d binding to spike-specific antibodies in matched hospitalized vs. ambulatory patients. AUC: area under the titration curve. Statistical significance was calculated by a Wilcoxon matched-pairs test: six specimens from hospitalized patients were compared to the average of 2 or 3 matched specimens from ambulatory patients. ns: not significant ($p > 0.05$).

alterations among the hospitalized patients as compared to the ambulatory patients (Figures S7A and S7B). This observation was confirmed with an additional cohort of 17 hospitalized patients (Figure S7C).

Comparison was also performed on individual cytokines or chemokines, and two cytokines were found to be significantly higher in hospitalized vs. ambulatory patients: IL-4 and IL-27 (Figure 6A). Their levels did not significantly decline over 250 days post disease onset (slope = -0.0001 and -0.0005 , and $p > 0.05$, Figure S8). When we evaluated the subset of matched severe and mild specimens, only IL-27 remained significantly higher in hospitalized vs. ambulatory patients with p value of 0.03 (Figure 6B). Therefore, the higher IL-27 levels between the hospitalized vs. ambulatory groups were evident in the plots of all patients with COVID-19 (Figure 7A) and in the matched subset (Figure 7B). For comparison, two other cytokines were also plotted (Figures 7C–7F). IL-4 was higher in sera of hospitalized vs. all ambulatory patients (Figure 7C) but showed inconsistent patterns in the matched subsets (Figure 7D). FGF-2, on the other hand, was comparable in hospitalized patients and all or matched ambulatory patients (Figures 7E and 7F). Notably, the

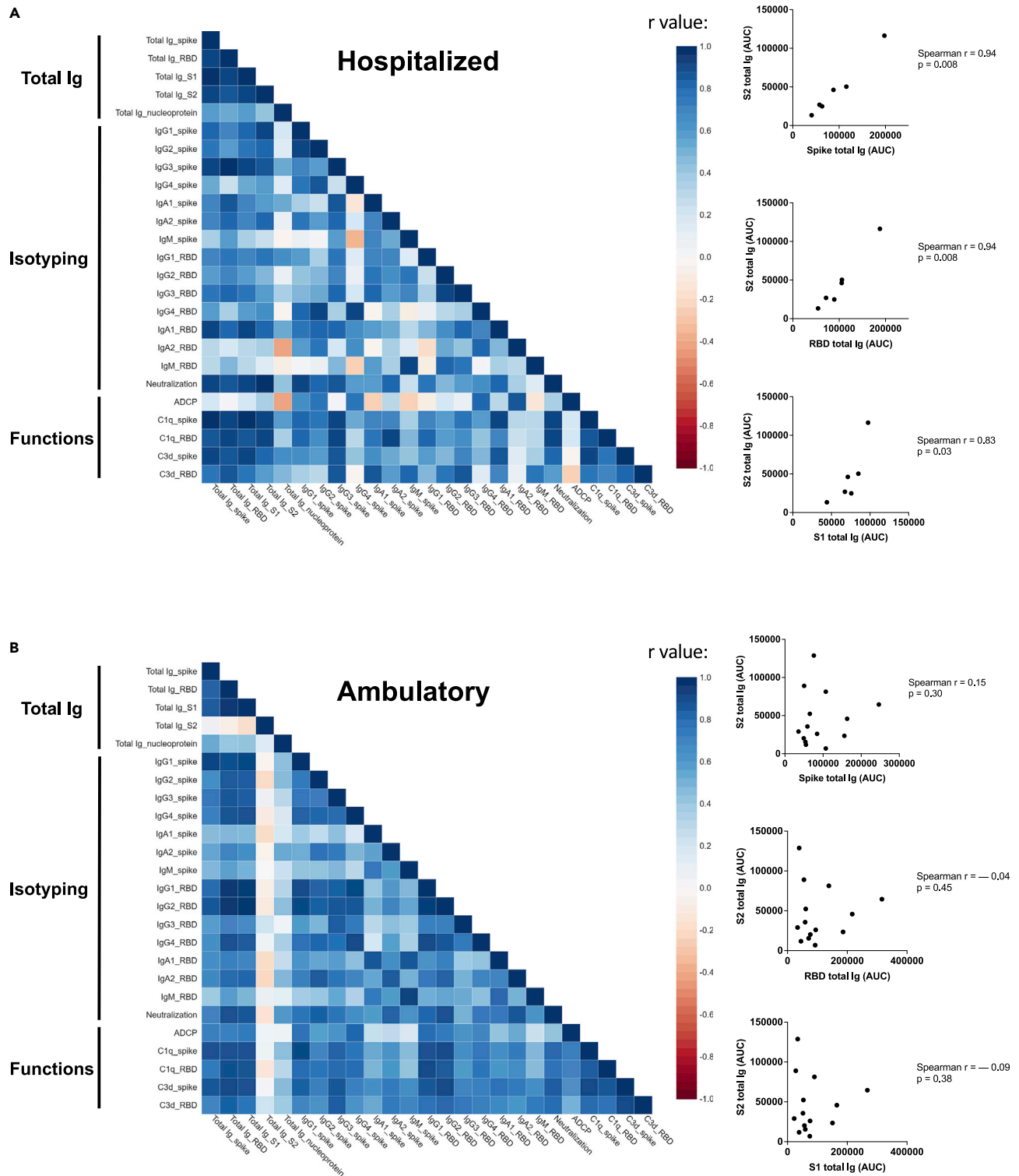


Figure 5. Correlations of binding and functional Ab levels in matched hospitalized and ambulatory VA patients

Spearman correlation matrix of serum Ab binding levels and functional activities were generated to compare matched (A) hospitalized (n = 6) vs. (B) ambulatory (n = 14) patients. The color code indicates the Spearman R value.

A

All Hospitalized vs. Ambulatory	
sCD40L	
EGF	
Eotaxin	
FGF-2	
FLT-3L	
Fractalkine	
G-CSF	
GM-CSF	
GRO α	
IFN- α 2	
IFN γ	
IL-1 α	
IL-1 β	
IL-1RA	
IL-2	
IL-3	
IL-4	0.002
IL-5	
IL-6	
IL-7	
IL-8	
IL-9	
IL-10	
IL-12p40	
IL-12p70	
IL-13	
IL-15	
IL-17A	
IL-17E/IL-25	
IL-17F	
IL-18	
IL-22	
IL-27	0.02
IP-10	
MCP-1	
MCP-3	
M-CSF	
MDC	
MIG/CXCL9	
MIP-1 α	
MIP-1 β	
PDGF-AA	
PDGF-AB/BB	
RANTES	
TGF α	
TNF α	
TNF β	
VEGF-A	

B

Matched Hospitalized vs. Ambulatory	
sCD40L	
EGF	
Eotaxin	
FGF-2	
FLT-3L	
Fractalkine	
G-CSF	
GM-CSF	
GRO α	
IFN- α 2	
IFN γ	
IL-1 α	
IL-1 β	
IL-1RA	
IL-2	
IL-3	
IL-4	
IL-5	
IL-6	
IL-7	
IL-8	
IL-9	
IL-10	
IL-12p40	
IL-12p70	
IL-13	
IL-15	
IL-17A	
IL-17E/IL-25	
IL-17F	
IL-18	
IL-22	
IL-27	0.03
IP-10	
MCP-1	
MCP-3	
M-CSF	
MDC	
MIG/CXCL9	
MIP-1 α	
MIP-1 β	
PDGF-AA	
PDGF-AB/BB	
RANTES	
TGF α	
TNF α	
TNF β	
VEGF-A	

Higher, p<0.0001
Higher, p<0.001
Higher, p<0.01
Higher, p<0.05
ns

Figure 6. Differential cytokine/chemokine responses among hospitalized vs. ambulatory VA patients

Statistically different responses in (A) hospitalized vs. ambulatory patients and (B) matched hospitalized vs. ambulatory patients were indicated by color codes. Statistical significance was calculated by unpaired (A) or paired (B) non-parametric t-test. Significance was similarly achieved with other tests (parametric t-test and non-parametric Mann Whitney Wilcoxon in R).

IL-27 levels correlated with IL-1 α , IL-3, IL-6, and TNF- α (Spearman $r = 0.61$ – 0.75 and $p = 0.01$ – 0.03) in the hospitalized patients, but not in the ambulatory patients (Figure S7).

DISCUSSION

This study examined serum Ab and cytokine/chemokine responses in SARS-CoV-2-infected patients with demographic risk factors for developing severe COVID-19, which included older age, male sex, and Black/Hispanic racial or ethnic background. Most patients (81% or 42/52), however, had mild disease requiring no hospitalization, and only 10 patients were hospitalized. Comparison of the hospitalized patients with ambulatory patients within the cohort revealed comparable virus-specific Ab responses in terms of binding levels, neutralization titers, and Fc-mediated activities of ADCP and complement deposition. However, the S2-specific Ig responses were distinguishing in that anti-S2 Ab levels tended to increase in hospitalized patients and decrease in ambulatory patients over time and correlated with all other Ab parameters tested more strongly among hospitalized patients than ambulatory patients. Furthermore, the overall cytokine responses in sera, which were collected from four days to >7 months post disease onset, were elevated in the hospitalized vs. ambulatory patients. The levels of IL-4 and IL-27 were notably higher in the hospitalized group and did not show apparent decline over the >7-month period. The greater levels of IL-27, but not IL-4, were apparent even when the hospitalized patients were matched to subsets of ambulatory patients by absence or presence of comorbidities, early or late sample collection time, and spike-specific total Ig levels. Nonetheless, the importance of S2-specific Abs and IL-27 in contributing to and/or resulting from severe COVID-19 remains unclear and warrants further investigation.

IL-27 is a heterodimeric IL-12 family cytokine, consisting of IL-12p35-related p28 and Epstein-Barr virus-induced 3 (EBI3) proteins.⁴⁹ Together with IL-12, IL-27 is an initiator of the polarization of CD4⁺ cells toward a Th1 phenotype.^{50,51} IL-27 also promotes IFN- γ production by CD8⁺ T cells and NKT cells.⁵² Myeloid cell populations, including macrophages, inflammatory monocytes, microglia, and dendritic cells (DCs) are the main sources of IL-27, which can be elicited by a range of microbial and immune stimuli.⁵³ IL-27 signaling in turn can induce the release of a range of pro-inflammatory cytokines such as IL-1, TNF- α , IL-18, and IL-12 from keratinocytes, mast cells, and monocytes.^{54,55} Indeed, our data showed that IL-27 levels correlated with those of IL-1 α and TNF- α and that, along with IL-27, IL-18 was elevated in hospitalized vs. ambulatory cases albeit without attaining statistical significance. In a study looking at cytokine and leukocyte profile of 44 SARS-CoV-2-infected patients that were separated in two groups based on age (> or <60 years old), IL-27, together with CXCL8, IL-10, IL-15, and TNF- α , was associated with older age, longer hospitalization, and more severe COVID-19 disease.⁴² On the contrary, in a Singapore cohort, no difference was observed in IL-4 and IL-27 levels between symptomatic and asymptomatic patients.⁵⁶ Rather, higher levels of MCP-1 and PDGF-BB were detected in patients with persistent symptoms vs. symptom-free patients.⁵⁶ Beyond COVID-19, the levels of IL-27 were found to increase in patients with a range of pulmonary inflammatory diseases including tuberculosis, asthma, influenza, acute lung injury, lung cancer, chronic obstructive pulmonary disease, acute lung injury, acute respiratory distress syndrome, and community-acquired pneumonia,^{57–62} indicating that an elevated IL-27 level is likely a common response to lung infections and injuries.

In contrast to Th1-promoting IL-27, IL-4 is a cytokine that induces differentiation of naive helper T cells to Th2 cells. Upon activation by IL-4, Th2 cells subsequently produce additional IL-4 in a positive feedback loop. IL-4 is produced primarily by mast cells, Th2 cells, eosinophils, and basophils.⁶³ IL-4, along with other Th2 cytokines, is involved in the airway inflammation observed in the lungs of patients with allergic asthma.⁶⁴ In our COVID-19 cohort, IL-4 levels were higher when the hospitalized cases were compared with all ambulatory patients but not with matched ambulatory patients. Conflicting results also have been published regarding IL-4, which was either associated with severe COVID-19 in a large scale review,²⁶ or was thought to be beneficial for the recovery of patients with COVID-19 in a small study comparing patients with faster or slower recovery.⁶⁵

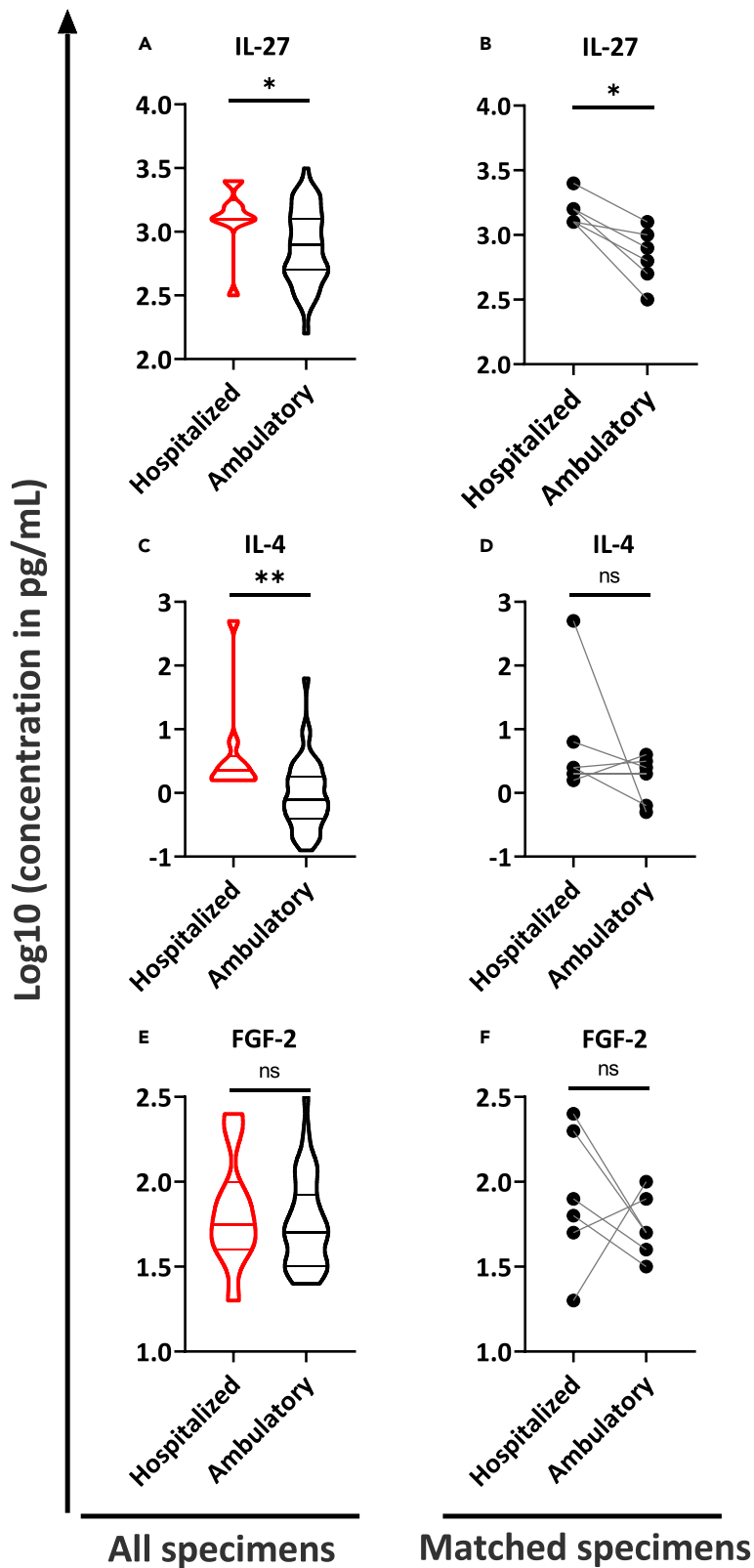


Figure 7. Cytokines responses among hospitalized and ambulatory VA patients

Data from three cytokines (IL-27, IL-4, and FGF-2) are presented as examples to show similarities and differences between hospitalized vs. all ambulatory patients (A, C, and E) or between matched hospitalized vs. ambulatory patients (B, D, and F).

Figure 7. Continued

F) Statistical significance was calculated in GraphPad Prism by unpaired (A, C, and E) or paired (B, D, and F) non-parametric t-test (*: $p < 0.05$, **: $p < 0.01$, ns: $p > 0.05$). Significance was similarly achieved with parametric t-test and non-parametric Mann Whitney Wilcoxon in R.

A constellation of elevated cytokines has been associated with severe cases of COVID-19. In this study, we observed higher median values for several cytokines in hospitalized vs. ambulatory groups, although only IL-27 was significantly elevated in hospitalized patients as compared to all and matched ambulatory patients. Of note, in the hospitalized patients but not in the ambulatory patients, the levels of IL-27 correlated with those of IL-1 α , IL-3, IL-6, and TNF- α , which were previously reported to be elevated and correlated with severe COVID-19.^{41,42,66,67} A study looking at serum levels of IL-6, IL-8, and TNF- α at the time of hospitalization also found that they were strong and independent predictors of patient survival in a cohort of hospitalized patients.⁶⁶ Yet, another study measuring IL-6 and IL-18 serum levels found IL-18 to correlate with other inflammatory markers and reflect disease severity.⁶⁸ A different study looking at 48 cytokines, chemokines, and growth factors showed significantly higher IL-12 levels in the asymptomatic and mild disease groups than in the moderate and severe disease groups, while IL-4 levels were comparable and IL-27 was not examined.⁶⁹ Nonetheless, these studies did not separate the individuals based on associated comorbidities, age, or racial/ethnic groups.

Limited information is available about cytokine levels and activities among US minority groups, in particular African Americans and Hispanics who have higher rates of SARS-CoV-2 infection, hospitalization, and death.^{43,70,71} African Americans are also more likely to have diabetes, hypertension, obesity, asthma, and heart disease, all of which are comorbidities associated with severe COVID-19. Moreover, little is known about cytokine levels in this population and in the context of associated comorbidities. The COVID-19-associated hospitalization rates are also higher among males than females (5.1 vs. 4.1 per 100,000 population).⁷⁰ Indeed, markers of brain and endothelial injury and inflammation were shown to be sex specifically regulated in SARS-CoV-2 infection.⁷² Nonetheless, scant information is available regarding the differences/similarities in cytokine levels between males and females, even though sex differences are likely to impact immune responses to SARS-CoV-2, as seen against other viruses; for example, pDC responses are sex hormone regulated and sex differences in cytokine and chemokine production and neutrophil recruitment during influenza virus infection.⁷³

In terms of Ab responses, the levels of anti-SARS-CoV-2 Abs, in particular IgA, and neutralization activities have been positively associated with COVID-19 severity (8–10). A longitudinal study of Italian patients presenting a wide range of clinical manifestations identified anti-S1 IgA as an indicator of COVID-19 severity.⁷⁴ Here, we performed Ig isotyping with the entire spike protein, precluding assessment of Ig isotypes against S1 and S2 subunits separately. In another study, more rapid induction of S2-reactive IgG during the first week of infection, along with IgG cross-reactivity with the common human beta coronaviruses (β -hCoVs), correlated with COVID-19 severity, implicating a biased early response toward S2 epitopes cross-reactive with hCoVs.⁷⁵ Comparison of spike proteins from SARS-CoV-2 (WA1) and hCoV strains shows varying levels of amino acid conservation across different spike regions. The S1 subunit and RBD exhibit only 25%–30% identity, while the S2 subunit has higher percent identity: 41%–42% vs. S2 of hCoV OC43 and HKU1 (β -hCoVs), and 34% vs. S2 of hCoV 229E and NL63 (α -hCoVs). In our study, a distinguishable feature of Ab responses was the stronger concordance of Ig levels against S2 with anti-S1 Ig, anti-RBD Ig, and the other tested Ab parameters in the severe vs. ambulatory cases. These results suggest that the Ab responses to the S1 and S2 regions of spike were upregulated synchronously in the patients who went on to have severe COVID-19, but not in patients with mild cases. The S2-specific Ig responses were relatively low in the two groups but tended to decline over time and disconnect from those of S1 in the ambulatory group. The S2 epitopes targeted by Abs from severe vs. mild cases in our study have not been determined, but Abs against certain sites in the more conserved S2 subunit may play a role in preventing or promoting progression to severe COVID-19. Neutralizing mAbs against S2 have been reported, such as 3A3, specific for a conserved epitopes in the hinge region between the heptad repeat 1 helix and the central helix⁷⁶ and B1 recognizing the beta-hairpin region,⁷⁷ although the prevalence of these Abs in patients with COVID-19 is unknown. Garrido et al. demonstrated a correlation of IgG targeting different immunodominant-conserved S2 regions with COVID-19 severity: Abs against heptad repeat 2 region and S2' fusion peptide region correlated with milder and more severe disease, respectively⁷⁸; the functional aspects of these anti-S2 Abs are yet to be evaluated. Further studies also are warranted to focus on the activities of pre-existing and SARS-CoV-2-induced S2-specific Abs, including neutralization, Fab affinity, Fc glycosylation, and

affinity for Fc receptors and complement, induced in patients with different disease severity and in individuals after COVID-19 vaccination.

Fc-mediated Ab functions also have been associated with COVID-19 severity. Abs-mediated Fc γ R1a and Fc γ R11a activation positively correlated with symptom severity in a cohort of ambulatory New York patients at 1–2 months post disease onset.⁷⁸ In addition, IgG Fc glycome composition was shown to predict disease severity, with patients with a poor disease outcome having, at diagnosis, IgG deficient in galactosylation and sialylation and more bis-GlcNAc structures (13). Since the Fc glycan composition influences Fc-mediated Ab functions, further investigation into this aspect of anti-SARS-CoV-2 Abs may reveal distinct Fc activities elicited in patients with COVID-19 and after vaccination. We previously observed higher complement-binding potency of SARS-CoV-2 spike-specific Abs elicited by vaccination vs. infection (35). In this study, comparable levels of complement binding activities were seen in spike-specific serum Abs from hospitalized vs. ambulatory patients with COVID-19. The ADCP activity was also similar, while other Fc activities such as antibody-dependent cellular cytotoxicity (ADCC) and binding affinity for the different activating and inhibitory Fc γ receptors have not yet been studied.

In summary, among the older and mostly male patients studied here, SARS-CoV-2-specific Ab levels and functional activities did not distinguish hospitalized and ambulatory COVID-19 cases. However, a discordant S2-specific Ab response was noted among the ambulatory patients. Moreover, higher levels of cytokines, notably IL-27, were induced and maintained in hospitalized vs. ambulatory cases. These data offer a pathway to pursue for a better understanding of the immune mechanisms that play a role in protection against vs. progression to severe COVID-19.

Limitations of study

We should note that this study was subject to several limitations. The sample sizes were relatively small. The hospitalized group included only survivors and received a plethora of therapeutic interventions including remdesivir, hydroxychloroquine, steroid, anti-cytokine or complement Abs, and convalescent plasma (Tables S1 and S3). Future study with larger sample sizes is needed to differentiate hospitalized patients requiring different levels of care and interventions and to match patient groups by race/ethnic groups and specific comorbidities. In addition, all samples were collected from our patients during the first wave of infection in New York City in March–December 2020, when the initial SARS-CoV-2 variant was predominant, and no vaccine or potent antiviral therapeutic interventions were available. It is unknown if the data presented herein can be extrapolated directly to responses against Delta, Omicron, and other variants emerging in the future. Efficacious vaccines and therapeutics now widely available to patients with COVID-19 would also have a significant impact on Ab and cytokine/chemokine responses to infection. The antiviral functional assays were also limited to neutralization, ADCP, and complement binding, without ADCC, and to the evaluation of the original SARS-CoV-2 variant using recombinant protein or pseudovirus. Lastly, our study is primarily cross-sectional with samples collected from different subjects over a wide range of time points post-infection. Although for the most part no change was apparent in the levels of Abs and cytokines from 4 days to 250 days post disease onset, the individual responses at the early and late time points are likely to differ and were not studied herein. The longitudinal samples tested for the initial nucleoprotein-IgG study were not available for the subsequent experiments.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.isci.2022.105608>.

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AUTHOR CONTRIBUTIONS

J.K., S.Z-P., C.U., and C.E.H. wrote and edited the manuscript. J.K., C.E.H., and S.Z-P. designed the exper-iments. J.K. and G.S.L. performed the experiments and collected the data. J.K., G.S.L., A.N., C.U., and C.E.H. analyzed the data. K.Y.O., F.A., and B.L. provided protocols, antigens, cells, and pseudovirus stocks. V.S., G.K., M.C.B., and C.G. provided banked human samples and metadata, J.C.B. and R.E-G. obtained specimens and clinical data. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

The Icahn School of Medicine at Mount Sinai has filed patent applications relating to SARS-CoV-2 serological assays (U.S. Provisional Application Number 63/051,858, which list Viviana Simon as co-inventor).

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-human Ig H and L-biotin	Abcam	Cat#ab97158
Mouse anti-human IgG1 Fc-biotin	Invitrogen	Cat#MH1515
Mouse mAb anti-human IgG2 Fc-biotin	Southern Biotech	Cat#9060-08
Mouse mAb anti-human IgG3 Hinge-biotin	Southern Biotech	Cat#9210-08
Mouse mAb anti-human IgG4 Fc-biotin	Southern Biotech	Cat#9200-08
Mouse mAb anti-human IgA1 Fc-biotin	Southern Biotech	Cat#9130-08
Mouse mAb anti-human IgA2 Fc-biotin	Southern Biotech	Cat#9140-08
Goat anti-human IgM-biotin (hIgG and hIgA absorbed)	Southern Biotech	Cat#2020-08
Anti-C1q-PE antibody	Santa Cruz	Cat#sc-53544 PE
Anti-C3d-biotin	Quidel	Cat#A702
Bacterial and virus strains		
SARS-CoV-2 pseudotyped particles WA1	Lee lab	Ikegame et al., 2021 ^{47,48} , Oguntuyo et al., 2020
Biological samples		
VA cohort (n = 73)		Table S1 and S3
PVI cohort (n = 17)		Table S1 and S3
Chemicals, peptides, and recombinant proteins		
Spike	Krammer lab	Amanat et al., 2020; Stadlbauer et al., 2020 ^{79,80}
RBD	Krammer lab	Amanat et al., 2020; Stadlbauer et al., 2020 ^{79,80}
S1	ProSci Inc, CA	Cat#97-087
S2	ProSci Inc, CA	Cat#97-079
Nucleoprotein	ProSci Inc, CA	Cat#97-085
S2	ProSci Inc, CA	Cat#11-184
C1q Component from Human Serum	Sigma	Cat#C1740
Complement Sera Human	Sigma	Cat#S1764
Experimental models: Cell lines		
THP-1 cells		
Vero-CCL81 cells		
Software and algorithms		
GraphPad Prism 9	GraphPad Software, San Diego, CA	
R version 4.1.0 and corplot package	The R Foundation for Statistical Computing	

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Catarina E. Hioe (catarina.hioe@mssm.edu, catarina.hioe@va.gov).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- 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 subjects

Fifty-two COVID-19-convalescent sera samples and 21 contemporaneous COVID-19-negative samples were collected at JJP VAMC under IRB#BAN-1604 and the JJP VAMC Quality Improvement project "Evaluation of the clinical significance of two COVID-19 serologic assays". The COVID-19-convalescent specimens were separated into hospitalized (n = 10) and ambulatory (n = 42) (Tables S1 and S2; Figure S1). The criteria for hospitalization, during the first wave of the pandemic, were: O₂ saturation at/or below 93% at rest and/or severe chronic obstructive pulmonary disease (COPD) or congestive heart failure (CHF). An additional set of 17 COVID-19-convalescent sera samples from hospitalized individuals were sourced from the Personalized Virology Initiative (PVI) cohort (IRB#16-00791 | STUDY#16-01215) (Table S3 and Figure S1) All participants of the PVI cohort and other research protocols provided written informed consent and agreed to future research and sample sharing. Samples were coded prior to processing, testing, and sharing. Before use, all sera were heat-inactivated (30 min at 56°C, for Ab binding and activities experiments) or treated with 1% Triton X-100 (30 min at room temperature, for the cytokine experiments).

Cell lines

- Vero-CCL81 cells were maintained in Dulbecco's modification of Eagle medium (DMEM, Gibco) containing 10% heat-inactivated fetal bovine serum (FBS, Corning), and 1X Penicillin/Streptomycin (Gibco). Cells were passaged one day prior to use.
- THP-1 cells were purchased from ATCC and maintained in RPMI 1640 medium (Lonza) containing 2 mM L-Glutamine (Sigma), 10% FBS (Corning), 10 mM HEPES (Gibco), 0.05 mM beta-mercaptoethanol (Sigma), and 1X Penicillin/Streptomycin (Gibco). After revival the cells were passaged three times before using them for the assay.

Recombinant proteins

SARS-CoV-2 spike (full-length external region, amino acids 1-1213) and RBD (amino acids 319-541) proteins were produced as described before.^{79,80} S1 (amino acids 16-685), S2 (amino acids 686-1213), and nucleoprotein (amino acids 1-419) antigens were purchased from ProSci Inc, CA (catalog #97-087, #97-079 and #97-085, respectively). Another stock of recombinant S2 protein was purchased from ProSci Inc, CA (catalog #11-184) for confirmation purposes, and data with the two S2 antigens correlated strongly (Spearman $r = 0.84$ and $p < 0.0001$, not shown). All antigens were of SARS-CoV-2 Wuhan-Hu-1 (WA1) strain.

METHOD DETAILS

Ab binding assay

The initial Ab evaluation was done on the Abbott Architect instrument using the Abbott SARS-CoV-2 IgG assay, a chemiluminescent microparticle immunoassay to detect IgG against the virus nucleoprotein. Subsequent Ab analyses were performed using the multiplex bead Luminex platform, in which recombinant SARS-CoV-2 spike, RBD, S1, S2, and nucleoprotein antigens were coupled to beads and experiments performed as follows.^{81,82} For total Ig responses, specimens were diluted 4-fold from 1:100 to 1:6,400 or 102,400 (plasma), reacted with antigen-coated beads, and treated sequentially with biotinylated anti-human total Ig antibodies and PE-streptavidin. The quantification was based on MFI values at the designated sample dilutions. Titration curves were plotted for each antigens tested, and the end-point titers were determined. The isotyping assays were performed at a single 1:200 dilution using human Ig isotype or subclasses antibodies and the MFI values were shown. For the C1q assay, beads with spike-Ab or RBD-Ab

complexes were incubated with C1q Component from Human Serum (Sigma, #C1740) for 1 h at room temperature, followed by an anti-C1q-PE antibody (Santa Cruz, #sc-53544 PE). For the C3d assay, Complement Sera Human (33.3%, Sigma, #S1764) was added to the beads for 1 h at 37°C, followed by a biotinylated monoclonal anti-C3d antibody (Quidel, #A702). The relative levels of C1q and C3d deposition were obtained as MFI, from which titration curves were plotted and areas-under the curves (AUC) were calculated.

COV2pp production and titration

SARS-CoV-2 pseudotyped particles (COV2pp) of WA1 strain were produced as described.^{46–48} Pseudoviruses were titrated on 20,000 Vero-CCL81 cells seeded 24 h before infection. At 18–22 h post-infection, the infected cells were washed and Renilla luciferase activity was measured with the Renilla-Glo Luciferase Assay System (Promega #E2720) on a Cytation3 (BioTek) instrument.

COV2pp neutralization

Virus was pre-incubated with diluted samples for 30 min. The virus-sample mix was then added to Vero-CCL81 cells seeded 24 h earlier and spinoculated. Infection was measured after 18–22 h by luciferase activity.

The percentage of neutralization was calculated as follows:

$$\text{Percentage of neutralization} = 100 - \left(\frac{\text{sample RLU} - \text{cell control RLU}}{\text{virus control RLU}} * 100 \right)$$

Serum ID₅₀ titers were calculated as the reciprocal sample dilution achieving 50% neutralization.

Antibody-dependent cellular phagocytosis (ADCP)

Assays to measure spike-specific ADCP were performed using a published protocol⁸³ with some modifications reported elsewhere.⁸² Briefly, FluoSpheres carboxylate-modified yellow-green fluorescent microspheres (Thermo Fisher, #F8823) were coupled with SARS-CoV-2 spike protein using the xMAP Antibody Coupling Kit (5 µg protein/~36.4 × 10⁹ beads, Luminex #40–50016). Spike-conjugated microspheres were incubated with diluted plasma for 2 h at 37°C in the dark. After washing and centrifugation (2,000 g, 10 min), the beads (~3 × 10⁸ beads, 10 µL/well) were incubated with THP-1 cells (0.25 × 10⁵ cells, 200 µL/well) for 16 h. The samples were analyzed on an Attune NxT flow cytometer (Thermo Fisher, #A24858). Data analysis was performed using FCS Express 7 Research Edition (De Novo Software). ADCP scores were calculated as follows:

$$\begin{aligned} & (\% \text{ microsphere positive cells}) \\ & * (\text{geometric mean fluorescent intensity of the microsphere positive cells}) \end{aligned}$$

Cytokine/chemokine assay

The samples were tested for 48 cytokines/chemokines by Eve Technologies Corporation, Canada (Human Cytokine/Chemokine 48-Plex Discovery Assay Array (HD48)). Data were presented as concentrations (pg/mL) or modified Z scores. The modified Z scores used to normalize the data were calculated as:

$$\text{Modified Z score} = \frac{0.6745 * (\text{Single data value} - \text{Median of the dataset})}{\text{Median absolute deviation of the dataset}}$$

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed as designated in the figure legends using GraphPad Prism 9 (GraphPad Software, San Diego, CA). Correlation matrices were generated using R version 4.1.0 (The R Foundation for Statistical Computing) and corplot package.