Serum Fc-Mediated Monocyte Phagocytosis Activity Is Stable for Several Months after SARS-CoV-2 Asymptomatic and Mildly Symptomatic Infection

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ABSTRACT We investigated the temporal profile of multiple components of the serological response after asymptomatic or mildly symptomatic SARS-CoV-2 infection, in a cohort of 67 previously SARS-CoV-2 naive young adults, up to 8.5 months after infection. We found a significant decrease of spike IgG and neutralization antibody titers from early (11 to 56 days) to late (4 to 8.5 months) time points postinfection. Over the study period, S1-specific IgG levels declined significantly faster than that of the S2-specific IgG. Further, serum antibodies from PCR-confirmed participants cross-recognized S2, but not S1, of the betacoronaviruses HKU1 and OC43, suggesting a greater degree of cross-reactivity of S2 among betacoronaviruses. Antibody-Dependent Natural Killer cell Activation (ADNKA) was detected at the early time point but significantly decreased at the late time point. Induction of serum Antibody-Dependent Monocyte Phagocytosis (ADMP) was detected in all the infected participants, and its levels remained stable over time. Additionally, a reduced percentage of participants had detectable neutralizing activity against the Beta (50%), Gamma (61 to 67%), and Delta (90 to 94%) variants, both early and late postinfection, compared to the ancestral strain (100%). Antibody binding to S1 and RBD of Beta, Gamma, Delta (1.7 to 2.3-fold decrease), and Omicron (10 to 16-fold decrease) variants was also significantly reduced compared to the ancestral SARS-CoV-2 strain. Overall, we found variable temporal profiles of specific components and functionality of the serological response to SARS-CoV-2 in young adults, which is characterized by lasting, but decreased, neutralizing activity and antibody binding to S1, stable ADMP activity, and relatively stable S2-specific IgG levels.

IMPORTANCE Adaptive immunity mediated by antibodies is important for controlling SARS-CoV-2 infection. While vaccines against COVID-19 are currently widely distributed, a high proportion of the global population is still unvaccinated. Therefore, understanding the dynamics and maintenance of the naive humoral immune response to SARS-CoV-2 is of great importance. In addition, long-term responses after asymptomatic infection are
not well-characterized, given the challenges in identifying such cases. Here, we investigated the longitudinal humoral profile in a well-characterized cohort of young adults with documented asymptomatic or mildly symptomatic SARS-CoV-2 infection. By analyzing samples collected preinfection, early after infection and during late convalescence, we found that, while neutralizing activity decreased over time, high levels of serum S2 IgG and Antibody-Dependent Monocyte Phagocytosis (ADMP) activity were maintained up to 8.5 months after infection. This suggests that a subset of antibodies with specific functions could contribute to long-term protection against SARS-CoV-2 in convalescent unvaccinated individuals.

**KEYWORDS** SARS-CoV-2, COVID-19, infection, antibodies, neutralization, Fc-mediated functions

The antibody response following viral infection promotes viral clearance through neutralization (1) and activation of phagocytic or cytotoxic cells (2, 3). Virus neutralization is a well-studied primary protective mechanism against SARS-CoV-2 infection (4). Antibody responses in individuals recovering from mild or asymptomatic SARS-CoV-2 infection are lower in magnitude than those with more severe disease (5–9). Longitudinal evaluation of antibody responses in SARS-CoV-2 infected individuals demonstrated rapidly declining neutralizing antibody titers over a period of several months (10–20), which might increase the risk of reinfection (19, 21–23).

The Fc-mediated functions of antibodies primarily facilitate clearance of virus and virus-infected cells by leukocytes expressing Fcγ receptors (FcγR), and can also modulate adaptive immune responses (3). The humoral response following moderate to severe SARS-CoV-2 infection can elicit antibodies capable of inducing antibody-dependent cellular phagocytosis (ADCP) and antibody-dependent cellular cytotoxicity (ADCC), as well as complement cascade protein C3b deposition (24, 25).

Despite overwhelming evidence that SARS-CoV-2 vaccines are safe and induce durable protection against illness and severe disease (26–31), to date nearly 32% of the total US population has not been fully vaccinated (32). A proportion of the unvaccinated population has likely already contracted the virus in the past 2 years. However, immunity induced by wild-type (ancestral or Wuhan-Hu-1[Wu] strain) SARS-CoV-2 infection may not be sufficient to protect from emerging variants of concern (VOC), as evidenced in particular by the Delta (B.1.617.2) and the Omicron (B.1.1.529) VOC (33–42). We previously reported that serum SARS-CoV-2 antibodies correlate with protection from subsequent infection in a longitudinal study of new United States Marine Corps (USMC) recruits (22). Understanding the durability and polyfunctionality of antibody responses in unvaccinated individuals who recovered from mild or asymptomatic SARS-CoV-2 infection will reveal correlates of protective immunity that have implications for rational vaccine design and establishment of community-wide preventative measures. The antibody dynamics in young adults, who are typically asymptomatic and a major source for spread of SARS-CoV-2, are of particular interest (43). In this study, we aimed to characterize the dynamics and persistence of SARS-CoV-2 specific IgG antibodies induced after infection, the functionality of serum antibodies with respect to neutralization and Fc-mediated activity, and cross-reactivity against VOC in young adults that experienced mild or asymptomatic SARS-CoV-2 infection.

**RESULTS**

**Cohort description.** Sixty-seven participants with previously PCR-confirmed SARS-CoV-2 infection were selected (see Materials and Methods). Preinfection serum samples were obtained 7 days or more before the first SARS-CoV-2 PCR+. Early postinfection samples were collected between 11 and 56 days (29 ± 13 days) after first PCR+, and late serum samples between 4.3 months (129 days) to 8.5 months (255 days) after first PCR+ (199 ± 27 days). One early and one late sample from each participant was included in the study. The average age at enrollment was 18.8 ± 1.5 years old, and 7.46% of the participants were females. The participants identified their race as either...
White (61.19%), Black (19.40%), Asian (4.48%), American Indian/Alaska Native (1.49%), Native Hawaiian/Other Pacific Islander (1.49%), other (2.99%), or non-specified (5.57%), and their ethnicity as Hispanic (25.37%), Non-Hispanic (47.76%), or non-specified (26.87%). Out of the 67 participants, 36 (53.73%) had asymptomatic infection during CHARM, and 31 (47.3%) presented at least one symptom (symptomatic). The symptoms reported were runny nose, sore throat, cough, shortness of breath, nausea or vomiting, diarrhea, abdominal pain, headache, muscle aches, fatigue, decreased ability to taste or smell, chills, subjective fever, or fever (temperature over 100.4 F measured at each visit). No participant required hospitalization.

**Dynamics of S IgG responses and neutralization after asymptomatic or mild infections.** To study the dynamics of the antibody responses, we first measured the levels of S-specific IgG in serum at early (11 to 56 days) and late (4 to 8.5 months) time points post-first PCR+. A significant reduction in the IgG titers was observed between the early (mean titer 2,244, 95% CI 1,706–2,951) (Fig. 1A) and late time point (mean titer 853, 95% CI 654–1,111) (Fig. 1A). Interestingly, all participants had detectable S IgG titers at both time points despite the overall decrease. We next analyzed if the IgG trend depends on the magnitude of the initial antibody response by grouping the participants into 4 groups according to their early titers: 150–450 (n = 12); 1,350 (n = 23); 4,050 (n = 20); 12,150 (n = 12). The decrease in titers followed similar trends for those with early titers of 1,350 or higher (Fig. 1B), while participants with low titers early after infection (150–450) showed no change. This relatively stable mean titer is partly due to 3/12 participants that showed an increase in titers, perhaps because of a subsequent infection, which might be more likely in individuals with lower antibody titers (22).
Next, we analyzed the dynamics of serum neutralization activity using live SARS-CoV-2 in 18 participants by randomly selecting 4 to 5 participants from each of the 4 early titer groups. A significant decrease in neutralization activity was found between early (mean half-maximal inhibitory dilution [ID50] of 356, 95% CI 203 to 627) and late (mean ID50 of 129, 95% CI 68 to 242) time points (Fig. 1C and D). Similar results were found using a pseudovirus (PV) neutralization assay (early: mean ID50 616, 95% CI 351 to 1084; late: mean ID50 118, 95% CI 72 to 194) (Fig. 1E and F). SARS-CoV-2 neutralization activity was detected for all participants at the early time points, and for all but 2 participants at the late time point. All participants showed detectable PV neutralization activity at both time points. SARS-CoV-2 and PV neutralization assays showed a highly significant correlation at both time points ($P < 0.0001$) (Fig. S1), indicating that the PV assay is an adequate surrogate for SARS-CoV-2 neutralizing activity.

We next evaluated if the dynamics of the antibody response are associated with the presence of symptoms during infection. We found a very similar profile between asymptomatic and symptomatic participants, with a decline of IgG titers in both groups (Fig. S2A). A trend toward lower neutralizing activity was found in asymptomatic compared to symptomatic participants (Fig. S2B and C). However, no significant differences were found between the 2 groups. Therefore, asymptomatic infections result in antibody responses with similar dynamics to mildly symptomatic infections.

**Anti-S2 IgG antibodies are stable up to 8 months after asymptomatic or mild infection.** We further characterized the temporal antibody profile by analyzing the levels of IgG antibodies against RBD, S1, S2, and N using a Luminex assay. From a subset of 28 participants, we included one preinfection sample, one early sample (11 to 56 days) and one late sample (4 to 8.5 months). We found that RBD, S1, S2, and N antibody levels were maintained over the preinfection baseline for all participants at both postinfection time points (Fig. 2A and B). Levels of S1 and N IgG antibodies showed a significant decrease between the early and the late postinfection time points, while RBD IgG showed a decrease which did not reach statistical significance ($P = 0.085$). Interestingly, levels of S2 IgG in this assay were stable over time, with similar levels at the 2 postinfection time points ($P = 0.510$).

We then analyzed the correlation between IgG levels specific to the 4 antigens and the levels of neutralization, at both early and late time points. RBD and S1-specific IgG showed highly significant levels of correlation with ID50 values obtained by SARS-CoV-2 neutralization (Fig. 2C) ($P < 0.0001$ for RBD and $S1$ IgG) and PV neutralization assays (Fig. 2D) ($P < 0.001$ and $P = 0.005$ for RBD and $S1$ IgG, respectively) at the late time point, but there was not a significant correlation at the early time point. N- and S2-specific IgG levels did not significantly correlate with ID50 values measured by SARS-CoV-2 or PV neutralization assays at either of the 2 time points (Fig. 2C and D). No differences between asymptomatic ($n = 14$) and symptomatic participants ($n = 14$) were found for any of the antigens (Fig. S3A).

**Cross-reactivity and preexisting immunity to seasonal betacoronaviruses.** We next hypothesized that the stability of S2 IgG antibody levels compared to S1 IgG is due to their higher level of conservation (Fig. 3A) and cross-reactivity with previously circulating seasonal betacoronaviruses, and the preexisting immunity in the population. The rationale behind this hypothesis is that the presence of memory B cells previously primed by cross-reactive epitopes of endemic betacoronaviruses might promote a longer-lived response against more conserved regions upon a recall exposure to SARS-CoV-2 (44). Therefore, we evaluated the levels of S1 and S2–specific IgG antibodies against OC43, HKU1, and SARS-CoV-2 in preinfection, early, and late postinfection sera from 66 participants for whom preinfection samples were available. SARS-CoV-2 infection induced IgG recognizing S2, but not S1, from OC43 and HKU1 (Fig. 3B and C). Similar to the observation using the Luminex assay, we found a significantly slower decline of SARS-CoV-2 S2 IgG levels than S1 IgG levels, as shown by the different slopes using simple linear regression models (Fig. 3D and E) ($P < 0.0001$, $t$ test). However, in this case, we found a significant decrease between the early and late time points in both S1 and S2 IgG (Fig. 3C). Possible explanations for the different results in S2 IgG...
FIG 2 RBD, S1, S2, N IgG specific responses using a Luminex assay. (A) Dynamics of RBD, S1, S2, and N IgG antibodies using preinfection, early (11 to 56 days) and late (4 to 8.5 months) time points in 28 participants. Mean ± SD are indicated. Comparisons were done using the Wilcoxon signed-rank test. (Continued on next page)
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maintenance are the use of different experimental platforms (enzyme-linked immunosorbent assay [ELISA] versus Luminex), and that a higher number of samples was included in this later assay, which could provide greater power for statistical comparisons.

A correlation analysis between preinfection levels of OC43 and HKU1 S1 and S2 IgG and postinfection levels of SARS-CoV-2 S1 and S2 IgG showed overall a weak positive association (Fig. 3F). Significant correlations were found between OC43 S2-IgG preinfection levels and SARS-CoV-2 S2 IgG at the early time point, and HKU1 S1-IgG preinfection levels and SARS-CoV-2 S2 IgG at later time points.

In conclusion, these data support higher cross-reactivity for S2- than for S1-specific IgG antibodies from seasonal betacoronaviruses and SARS-CoV-2. Modest levels of correlation between preinfection IgG antibodies recognizing seasonal betacoronaviruses OC43 and HKU1, and postinfection S2 SARS-CoV-2 IgG antibodies suggest a possible role for preexisting immunity in promoting induction and stability of S2 IgG responses.

Dynamics of Fc-mediated antibody functions. In addition to the neutralization activity, mostly mediated by antibodies targeting the S1 protein, IgG have functions that are mediated by their Fc regions. To investigate Fc-mediated antibody functions, we optimized Antibody-Dependent Monocyte Phagocytosis (ADMP) and Antibody-Dependent Natural Killer cell Activation (ADNKA) assays, performed using primary human immune cells and live SARS-CoV-2-infected cells, thus providing a biologically relevant output. To account for intrinsic inter-individual variability independent of SARS-CoV-2 infection, we included preinfection samples for each participant as a reference. Samples from the same 18 participants used in the neutralization assays were included in these analyses.

For the ADMP assay, Vero E6 cells infected with mNG-SARS-CoV-2 were incubated with sera, and then cocultured with primary monocytes (see Materials and Methods section). The percentage of mNG − monocytes, indicative of phagocytosis of infected cells, was recorded. Interestingly, we found stable ADMP activity, with no significant differences in the fold change percentage of phagocytosis (with respect to preinfection baseline) between early and late time points (Fig. 4A and B). Next, we analyzed the correlation between ADMP and the levels of RBD-, S1-, S2-, and N-specific IgG (Fig. 4C, D, E, and F, respectively). S2 IgG showed the highest positive correlation with the fold change ADMP, which was significant at the early time point (\( P = 0.024 \)) and nearly significant at the late time point (\( P = 0.084 \)) (Fig. 4E). S1 IgG showed a significant correlation at the late time point (\( P = 0.033 \)) but not at the early time point (\( P = 0.38 \)) (Fig. 4D). RBD and N IgG levels did not correlate significantly ADMP (Fig. 4C and F). To further explore the relationship between IgG levels and ADMP, we used linear mixed models (LMM) in which both times points were considered (Fig. 4G). Again, a significant association was found between S2 IgG and ADMP (\( P = 0.038 \)), and S1 IgG and ADMP (\( P = 0.0415 \)).

For the ADNKA assay, virus-infected Vero E6 cells were pre-incubated with sera, followed by coculture with human peripheral blood mononuclear cells (PBMC). We analyzed the ability of sera to induce activation of natural killer (NK) cells (CD3−CD56+) in the culture by measuring the activation markers CD107a, IFNγ, or TNF-α (See Materials and Methods section). Results are expressed as fold change percent of NK cells expressing CD107a + (Fig. 5A and B), IFNγ + (Fig. 5C and D) or TNF-α + (Fig. 5E and F) cells over preinfection baseline. All early postinfection sera had higher percentages of NK cells with expression of CD107a and TNF-α at the postinfection time point than at the preinfection time point, and all participants but one had increased levels of NK IFNγ + cells (Fig. 5A to C). A significant decrease over time was found for the 3 activation markers, and 3/18, 7/18, and 0/18 participants showed the same or lower percentage of CD107a +, IFNγ +, or TNF-α + NK cells than preinfection baseline at the late time point.

FIG 2 Legend (Continued)

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We did not find any significant correlations between the IgG levels against the 4 antigens tested in the Luminex assay and the ADNKA results (Fig. S4A to C). However, LMM including early and late time points, revealed a positive significant association of the fold change percentage of NK CD107a+ cells and levels of S2 IgG.

**FIG 3** Pre-existing immunity and cross-reactivity with seasonal coronaviruses. (A) Matrix showing protein percent identity of S1 and S2 subunits for SARS-CoV-2 and seasonal beta-coronaviruses (www.uniprot.org, Clustal Omega program; Uniprot accession number for S proteins: SARS-CoV-2: P0DTC2; OC43: A0A5B9MU5; HKU1: U3NAI2). (B) (C) Dynamics of HKU1, OC43, and SARS-CoV-2 S1 (B) and S2 (C) IgG antibodies by ELISA using preinfection (PRE), early (11 to 56 days) and late (4 to 8.5 months) samples in n = 66 participants. Mean ± SD are indicated. Comparisons were performed using the Wilcoxon signed-rank test, corrected for multiple comparisons using Bonferroni Method. (D) and (E) Dynamics of postinfection SARS-CoV-2 S1 (D) and S2 (E) IgG responses by ELISA using linear regression (lines and equations are shown). Dashed lines in (B), (C), (D), and (E) (SARS-CoV-2 plots) represent the OD492 threshold calculated based on the average of 8 negative controls plus three times their standard deviation for S1 and S2. (F) Correlation analysis (Spearman) of S1 and S2 IgG preinfection levels for seasonal betacoronaviruses and S1 and S2 SARS-CoV-2 IgG postinfection levels.
No other significant associations were found using LMM between ADNKA and antigen-specific IgG levels (Fig. S4D).

Overall, our data indicate the ADMP serum activity was stable at least for 4 to 8.5 months after infection and correlated with anti-S2 and anti-S1 IgG levels, while ADNKA significantly decreased between the early and late time points.

Reduced antibody binding and neutralization levels against VOC. Given the emergence of multiple VOC during 2021, we used the PV neutralization assays to assess if the neutralizing activity elicited upon asymptomatic or mildly symptomatic infection is affected against particular VOC. Specifically, we tested PV expressing the S protein from a Wuhan-related strain but containing the amino acid change D614G (Wu-D614G), Beta, Gamma, and Delta VOC. We found a significant decrease in the neutralizing activity against Beta, Gamma, and Delta PV with respect to Wu-D614G (Fig. 6A). At both early and late time points, the Beta VOC showed highest reduction of neutralizing activity, followed by Gamma and Delta. Interestingly, at the early time point after infection, we found a stronger decline in VOC PV neutralizing activity than at the late point (Fig. 6A). This phenomenon might be due to a different quality and breadth of the serum antibodies at later times after infection (45, 46). Despite this higher fold-decrease at the early time point, we found similar percentages of sera with

(P = 0.0003) (Fig. 5G). No other significant associations were found using LMM between ADNKA and antigen-specific IgG levels (Fig. S4D).
detectable neutralizing activity at both time points, with Beta demonstrating the lowest percentage (50%) (Fig. 6A).

Next, we performed additional assays to evaluate serum IgG binding to RBD and S1 from Wu-D614G, Beta, Gamma, and Delta VOC at 4 to 8.5 months postinfection. We evaluated the median fold-decrease in sera with a titer of at least 150 against Wu/Wu-D614G RBD ($n=54$) or S1 ($n=65$). A consistent reduction in IgG binding to Beta, Gamma, and Delta RBD (2.17, 2.33, and 1.71 median fold-decrease, respectively) was found when compared to Wu RBD (Fig. 6B). IgG binding to Beta, Gamma, and Delta S1 showed similar decrease levels (1.74, 2.21, and 2.34 median fold-decrease, respectively) when compared to Wu-D614G S1. Finally, we evaluated the serum IgG binding at the late time point to the Omicron VOC (Fig. 6C). This assay showed a strong AUC decrease for Omicron RBD (15.79 median fold-decrease) and S1 (9.82 median fold-decrease) compared to Wu/Wu-D614G.

DISCUSSION

Over the 2 years following the SARS-CoV-2 pandemic, numerous studies described the immune response to infection in cohorts that differ in multiple ways, including geographical distribution, severity of disease, and underlying health conditions. The development of an antibody response and its positive association with severity are well established (5–9). Age is known to be an important determinant of disease severity and magnitude of the humoral immune response (47–50). However, the dynamics of the functionality of the antibody responses, including Fc-mediated functions, particularly following asymptomatic infections, are not yet well understood. Here, we leveraged a well-characterized cohort, where participants were sampled before and after
infection in a controlled setting, to study the persistence and quality of the humoral immune response to asymptomatic or mildly symptomatic SARS-CoV-2 infection. We found that S-specific IgG titers and neutralizing activity declined over time, but positive S IgG titers and neutralizing activity were detected in virtually all late postinfection sera. We resolved the dynamics of IgG responses to individual SARS-CoV-2 proteins and noted more stable S2-specific IgG responses than S1-specific IgG responses. The S2 IgG response to SARS-CoV-2 infection was also cross-reactive with S2 from seasonal coronaviruses HKU1 and OC43. Fc-mediated antibody functions, including enhancement of monocyte phagocytosis and NK cell activation, were induced, but only ADMP remained stable. Finally, we observed significantly lower recognition of RBD/S1 proteins of VOC as well as neutralization activity.

**FIG 6** Serum neutralization activity and RBD/S1 IgG binding to VOC. (A) Serum neutralization activity in early (11 to 56 days) and late (4 to 8.5 months) sera using a PV assay (n = 18). ID50: half-maximal inhibitory dilution. (B) and (C) IgG binding to RBD and S1 from Wuhan-1-related strain and VOC. Median n-fold decrease (n-f) versus the Wu or Wu-D614G strains is indicated under VOC data and was calculated including only for those sera with Wu/Wu-D614G IgG titers ≥ 150. AUC: Area Under the Curve. Mean ± SD are indicated. Comparisons were performed using the Wilcoxon signed-rank test, corrected for multiple comparisons using Bonferroni Method. ***, P < 0.001; **, P < 0.01; *, P < 0.05.
One of the strengths of this study is the availability of preinfection baseline samples, which allowed us to analyze the influence of preexisting immunity to seasonal betacoronaviruses on the development of the specific humoral responses to SARS-CoV-2. The S2 subunit of seasonal and pandemic betacoronaviruses is more conserved than the S1 subunit (Fig. 3A) (51, 52). In agreement with this, we found induction of IgG antibodies recognizing OC43 and HKU1 S2, but not S1, after SARS-CoV-2 infection. Therefore, preexisting cross-reactive S2 responses may be boosted upon SARS-CoV-2 infection, contributing to the stability of the S2 IgG response. Although the correlation between preinfection serum IgG recognition of OC43 and HKU1, and SARS-CoV-2 IgG postinfection was modest, it might support a role for preexisting immunity in promoting induction of S2 IgG responses. Interestingly, an epitope-resolved analysis found 2 epitopes in S2 of seasonal and pandemic coronaviruses that are cross-recognized by antibodies from COVID-19 convalescent individuals (53). In a vaccination study, BNT162b2 immunization of SARS-CoV-2 naive individuals induced a robust S2-specific plasmablast response 7 days postvaccination, while RBD- and S1-specific cells were only increased 3 weeks later (54). The S2-specific B cell response displayed great clonal diversity, which likely represents a secondary recall response from memory B cells from previous exposures rather than a primary SARS-CoV-2 specific response. Interestingly, anti-S2 IgG responses may be a highly sensitive way of identifying asymptomatic SARS-CoV-2 infections (55). Preexisting antibody responses may, however, interfere with generation of neutralizing antibodies against de novo epitopes of SARS-CoV-2, skewing the humoral response toward epitopes shared between SARS-CoV-2 and endemic coronaviruses (56–58).

We further profiled the dynamics of antibody Fc-mediated functions, specifically ADMP and ADNKA. This is the first study using live SARS-CoV-2 virus-infected target cells and primary human immune cells as effector cells, in a biologically relevant setting. We found that the ADMP serum activity induced after SARS-CoV-2 infection remained stable over the 4 to 8.5 months we studied, while ADNKA displayed a marked decrease. A summary of the dynamic profiles of RBD, S1, S2, and N antibody levels and the serum functions is shown in Fig. 7. Interestingly, ADMP showed a significant correlation with S2 IgG antibody levels, which were maintained as well overtime. An association of S2 IgG antibodies was also found with ADNKA (NK CD107a+ cells). These correlations might be a consequence of unrelated parallel processes induced simultaneously after infection and do not necessarily imply that Fc-functions are mapped to IgG against specific antigens. However, this observation should be considered in further studies to clarify if ADCP or ADCC functions are preferentially linked with recognition of specific epitopes, as previously described for influenza virus or Ebola virus (3, 59–61). Ebola virus glycoprotein specific IgG monoclonal antibodies (MAbs) isolated from human survivors displayed a broad range of Fab-mediated and Fc-mediated functions, and the ones providing greatest protection from lethal infection were broadly poly-functional and were mapped to precise epitopes on the stalk region of the glycoprotein (60). In influenza virus infections, MAbs targeting conserved
hemagglutinin (HA) stalk regions require Fc-FcγR engagement to protect from lethal challenge, but MAbs directed against the hypervariable HA head are independent of FcγR engagement (59). Notably, ADCC was mapped to broadly-neutralizing HA stalk antibodies, which are also known to be boosted after infection with a different influenza strain (62). Interestingly, a previous study found that individuals with asymptomatic or paucisymptomatic SARS-CoV-2 infections developed FcγR-binding S2-specific antibodies, but not FcγR-binding S1 or RBD antibodies (63). A recent study reported that vaccine-induced S-specific antibodies continue to drive Fc-effector functions against Omicron, even after several months of vaccination (64).

We also found that neutralization of Beta, Delta, and Gamma VOCs was reduced compared to that from the ancestral strain, in line with previous reports (40, 65), and found a strong reduction of IgG binding to the Omicron variant, as was also seen by others (42, 66, 67). Our data shows that this pattern is maintained several months into convalescence after asymptomatic or mild symptomatic infection.

One of the limitations of this study is the low representation of females in this cohort (n = 5). We analyzed the sex-specific dynamics of IgG S titers and did not find a decrease in titers in females (Fig. S5A). No differences between males and females were observed in the levels of S1 and S2 IgG levels against seasonal coronaviruses or SARS-CoV-2 (Fig. S5B). However, due to the low numbers of females, confident conclusions in sex differences cannot be drawn from this analysis. Due to the characteristics of the cohort (mainly white, young adults), our data might not be representative of children or older adults, or other races/ethnicities. Another limitation is that we did not analyze memory T cell responses, which have been reported to be highly stable after natural infection (10, 68), and highly conserved across VOC (69–72).

Collectively, we describe the multifaceted dynamics of the humoral responses in mild symptomatic and asymptomatic SARS-CoV-2 infection in a cohort of young, otherwise healthy, US Marines up to 8.5 months following infection.

**MATERIALS AND METHODS**

**Cohort and data collection.** The COVID-19 Health Action Response for Marines (CHARM) study cohort (March-November 2020) has been previously described (22, 43, 73). A second phase of this study, named CHARM 2.0, was established for follow up of CHARM participants to assess long-term immunity and health effects of SARS-CoV-2 infection. At the initiation of CHARM 2.0, the recruits that participated in CHARM had graduated from basic training and began serving as Marines at various USMC locations. CHARM participants on active duty were identified and offered participation in CHARM 2.0. A study team traveled to military installations across the US and abroad where eligible Marines were on duty. Consenting participants completed a questionnaire that specified demographic data, as well as information associated with their medical history since their participation in the original CHARM study and underwent a series of clinical and laboratory evaluations. Blood samples were collected, and serum was isolated (serum separator tubes), and stored at −80°C until use. At the study visit, a questionnaire was completed. This study involves samples from the first 67 participants that were enrolled in the CHARM 2.0 study (February and March 2021), and (i) had at least 1 SARS-CoV-2 PCR+ test during their participation in CHARM, (ii) had a serum sample available later than 11 days post-first PCR+ at the CHARM study, and (iii) were not vaccinated against COVID-19 before sample collection. Serum was inactivated at 56°C for 1 h before use in the subsequent assays.

**ELISA.** Serum IgG SARS-CoV-2 specific antibodies were evaluated using an ELISA as previously described (22, 43). Plates were coated with 2 μg/mL of recombinant Histidine-tagged spike (S) protein (Table S1). After washing and blocking steps, 6 serial 1:3 serum dilutions starting at 1:50 were dispensed in the wells. At least 2 sera positive controls, 8 sera negative controls (collected before July 2019, BioChemed Services, Winchester, VA), and 4 blanks were included in every assay. Peroxidase-conjugated goat F(ab')2 Anti-Human IgG (Abcam) was used at 1:8,000 dilution, and plates were incubated for 1 h. The assay was developed using o-phenylenediamine, and the reaction stopped with 3 M HCl. Optical density at 492 nm (OD492) was measured using a microplate reader (SpectraMax M2, Molecular Devices). Each dilution was considered positive when its OD492 value was higher than the average of the negative controls, plus 3 times their standard deviation (SD) at the correspondent dilution, or higher than 0.15 OD492. Serum samples were considered positive for each assay when at least 2 consecutive dilutions were positive. For analysis of VOC IgG binding, plates were coated with RBD or S1 from the ancestral Wu (Wu-D614G for S1) strain, Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2), or Omicron (B.1.1.529, BA.1) (Table S1). Six serial dilutions of serum (3-fold starting at 1:50) were assayed following the same procedure described above. Area under the curve (AUC) values were calculated by plotting OD492 versus the dilution factor. ELISAs to evaluate IgG binding to S1 and S2 of SARS-CoV-2, HKU1 and OC43 (Table S1) were done at a 1:50 serum dilution, and OD492 was used for analysis.
Neutralization assays. Studies involving infectious SARS-CoV-2 were performed as previously described (73). Two-fold serial dilutions of heat-inactivated serum at an initial dilution of 1:20, were prepared in serum free media (Minimum Essential Medium containing 25 mM HEPES and 0.05 g/L Gentamicin sulfate) and incubated with an equal volume of a solution containing 200 PFU of SARS-CoV-2-mNG (mNeonGreen) (74) for 1 h at 37°C. Virus-serum mixtures were then added to Vero E6 monolayers in 96-well optical black plates (Thermo Scientific) and incubated at 37°C. Plates were read using the BioTek Cytation 7 plate reader (EX 485 nm, EM 528 nm) at 48 h postinfection. Following background signal correction, virus neutralization half-maximal inhibitory dilution (ID50) values were determined using a 4-parameter logistic regression.

PV neutralization assays. PV was produced using SARS-CoV-2 spike gene sequences obtained from the GSAID database (https://gisaid.org/). Wuhan-D614G, Beta, Delta, and Gamma spike gene sequences were codon optimized for human expression, with 19 amino acids removed from the C-terminus, synthesized by GenScript (Piscataway, NJ), and cloned into pcDNA3.1+ expression vectors. Lentivirus derived reporter and packaging plasmids were previously described (75), and generously made available through BEI resources (NIAID, NIH). PV was produced in HEK293T/17 cells (ATCC) and transfected with spike, ZsGreen reporter, and packaging plasmids using Lipofectamine 3000 reagent (Thermo Fisher Scientific). HEK293-ACE2 cell lines were developed using HEK293 cells (ATCC) transfected with an ACE2 expression vector (GeneCopoeia). Stable transfectants were selected using G418 (Invivogen), and subclones were selected based on maximal ACE2 expression. HEK293-ACE2 cells were cultured in DMEM containing 10% FBS and 1x Penicillin/Streptomycin, and supplemented with G418. Sample sera and controls were diluted 1:20 in DMEM 10% FBS, followed by 1:2 serial dilutions. PV was then added to the serum dilutions and the mixture was incubated at 37°C 30 min. HEK293-ACE2 cells were then added, and plates were incubated at 37°C for 48 h, after which time were treated with trypsin (Quality Biological) and re-suspended in PBS 10% FBS. Plates were read using a FACSDiva II equipped with an HTS running BD FACSDiva software (Version 8.0.1). Nonlinear regression analysis was then used to calculate ID50 by plotting percent positive cells as a function of log dilution.

Fc-mediated functional assays. Antibody-Dependent Monocyte Phagocytosis (ADMP) and Antibody-Dependent Natural Killer cell Activation (ADNKA) were optimized using SARS-CoV-2-infected Vero E6 cells (target) and human peripheral blood mononuclear cells (PBMC, effector) from healthy donors, with modification of the procedures described previously (70).

ADMP assays were performed with human monocytes (effector cells) purified from PBMC using CD14 microbeads (Miltenyi). Vero E6 cells were infected with SARS-CoV-2-mNG at a multiplicity of infection (MOI) 0.1 for 48 h. On day 2, target cells were harvested with trypsin-EDTA (0.25%) and pre-incubated with 1:20 diluted serum at 37°C for 90 min. Monocytes were added to the target cells at 4:1 ratio (effector:target) and cocultured for 4 h. Then, the cells were stained with CD14-PE (clone 63D3, BioLegend) and CD66b-APC (Clone G10F5, BioLegend) for 10 min. Cells were washed once with PBS and fixed with 4% paraformaldehyde twice, as per BS3 protocol. Cells were acquired in LSR Fortessa and analyzed with FlowJo software version 10.8 (Tree Star). Monocytes were identified as CD14+ CD66b+BV605 (clone 5.1H11, BioLegend). The percent phagocytosis by monocytes was calculated as frequency of mNG+ cells, as per BS3 protocol. A fold change in percent phagocytosis relative to prior infection was used to quantify ADMP induction in this study.

ADNKA was performed using PBMCs from healthy donors (effector cells) and Vero E6 cells infected with SARS-CoV-2-mNG at MOI of 0.1 for 48 h (target cells), harvested with trypsin-EDTA (0.25%) and pre-incubated with 1:20 diluted serum at 37°C for 90 min. PBMC were added to the Vero cells-serial mixture at a 4:1 ratio (effector:target). The cells were infected with PV at 37°C for 5 h in the presence of GolgiStop (0.7 μL/mL, BD Biosciences), Brefeldin-A (1:1000, Biolegend) and anti-CD107a-FITC (clone H4A3, BioLegend) antibody. After coculture, the cells were stained with live/dead Fixable Aqua (Thermo Fisher Scientific), anti-CD3–APC/Fire750 (clone SK7, BioLegend), and anti-CD56–BV605 (clone 5.1H11, BioLegend). Cells were washed with PBS and fixed with 4% paraformaldehyde twice, as per BS3 protocol. The fixed cells were moved to BSL2 for intracellular cytokine staining (ICS). For ICS, the cells were incubated 20 min with permeabilization buffer (BioLegend) and stained with antibody cocktail containing anti-IFNy-APC (Clone 45.B3, BioLegend) and anti-TNFα-b-V421 (Clone 2C7, BioLegend). Cells were washed and analyzed in LSR Fortessa with Flowjo software version 10.8 (Tree Star). NK cells were identified as CD3-CD56+ cells from the lymphocyte gate. NK cell activation was quantified as % CD56+ CD107a+, CD56-IFNy+ and/or CD56- TNFα+ among CD3- fraction of PBMCs (Fig. S7). A ≥ 1.8-fold change in percent of ADNKA for any serum relative to no serum was considered optimal activation of NK cells. Fold change in % of NK activated cells from prior infection was used to quantify ADNKA induction in this study.

Luminex xMAP-based multiplex assays. Evaluation of IgG antibody levels to S glycoprotein antigens, S1 subunit, S2 subunit, RBD, and to nucleocapsid (N) protein of SARS-CoV-2 was performed with the MILLIPLEX Multiplex Immunoassays SARS-CoV-2 Antigen Panel 1 IgG and SARS-CoV-2 Antigen Panel 1 IgA (Millipore), following manufacturer’s recommendations. Data were collected using the Luminex 100/200 System.

Statistics. Statistical analysis was performed with R studio (version 1.3.1093), R (version 4.0.2), and GraphPad Prism (versions 8.3.1 and 9.2.0). Simple linear regression was used to investigate the temporal trends of the immune parameters over time. Correlation analyses (Spearman) and linear mixed models (LMM, time point as fixed effect and subject identifier as random effect) were used to describe the association of immune parameters. Log_{10} transformed data were used for analyses when indicated. Pairwise comparisons were performed using the Wilcoxon signed-rank test, and correction for multiple hypothesis testing was done using the Bonferroni method. An alpha value of 0.05 was considered for all the analysis.
**Study approval.** This study was approved by the Naval Medical Research Center (NMRC) institutional review board (IRB), protocol numbers NMRC.2020.0006 (CHARM) and NMRC.2021.0004 (CHARM 2.0), in compliance with all applicable U.S. federal regulations governing the protection of human subjects. Research performed at Icahn School of Medicine at Mount Sinai (ISMMS) as part of this study was reviewed by the ISMMS Program for Protection of Human Subjects and the Naval Information Warfare Center Pacific (NIWC Pacific) Human Research Protection Program (HRPP) and received non-human subjects (NHS) determination. All participants provided written informed consent.

For ADMP and ADNKA assays, PBMCs were isolated from the buffy coats/blood of de-identified healthy donors obtained from the commercial source (Gulf Coast Regional Blood Center, Houston, TX), with IRB approval number of UTMB-10-221.

**Data availability.** De-identified data from this study is provided as a Supplemental Data set.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available only online.

**SUPPLEMENTAL FILE 1**, PDF file, 2.2 MB.

**SUPPLEMENTAL FILE 2**, XLSX file, 0.1 MB.

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S.V. performed serological assays, analyzed data, and wrote the manuscript; S.P., P.S., C.A.B., V.J. performed serological assays and analyzed data. A.S.-S., N.A.K., and A.S.M. performed serological assays. E.C., C.B., J.M., A.M., E.N., C.W.G., S.E.L., R.L., V.A.S., M.S. contributed to sample and data collection. N.M. and Y.B.X. contributed to sample preparation, assisted with serological assays, and data management. Y.G., C.K.P. contributed to data management and data analysis; M.G. contributed to project management; A.B. supervised data generation; S.C.S. supervised data generation and data analysis; A.G.L. supervised sample and data collection, and contributed to scientific discussions; I.R. supervised data generation, analyzed data, prepared figures, and wrote the manuscript. All the authors critically reviewed and edited the manuscript.

We declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. P.S., C.A.B., E.C., C.B., J.M., A.M., E.N., C.K.P., C.W.G., S.E.L., R.L., V.S., M.S., and A.G.L. are military Service members or U.S. Government employees. This work was prepared as part of their official duties. Title 17, U.S.C., §105 provides that copyright protection under this title is not available for any work of the U.S. Government. 355 Title 17, U.S.C., §101 defines a U.S. Government work as a work prepared by a military Service member or employee of the U.S. Government as part of that person’s official duties. The views expressed in the article are those of the authors and do not necessarily express the official policy and position of the US Navy, the Department of Defense, the US government, or the institutions affiliated with the authors.

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